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The seminal plasma of the boar is rich in cytokines, with significant individual and intra-ejaculate variation

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Running head: Cytokines in boar seminal plasma

Abstract

Problem: The boar, as human, sequentially ejaculates sperm-rich and sperm-poor fractions. Seminal plasma (SP) spermadhesins (PSP-I/PSP-II) induce a primary endometrial inflammatory response in female sows, similar to that elicited by semen deposition in other species, including human. However, the SP is also known to mitigate such response, making it transient to allow for embryo entry to a cleansed endometrium. Although cytokine-involvement has been claimed, the exploration of cytokines in different SP-fractions is scarce. This study determines Th1, Th2, Th17 and Th3 cytokine profiles in specific ejaculate SP-fractions from boars of proven fertility.

Methods: SP-samples from the sperm rich fraction (SRF) and the sperm-poor post-SRF fractions (post-SRF) of manually-collected ejaculates from eight boars (four ejaculates per boar) were analyzed by commercial multiplex bead assay kits (Milliplex MAP, Millipore USA) for interferon- γ , interferon gamma-induced protein 10, macrophage-derived chemokine, growth-regulated oncogene, granulocyte-macrophage colony-stimulating factor, monocyte chemoattractant protein-1, interleukins (IL)-6, IL-8, IL-10, IL-15, IL-17 and transforming growth factor (TGF)- β 1- β 3.

Results: Cytokine concentrations differed between the ejaculate fractions among boars, being highest in the post-SRF.

Conclusions: Boar SP is rich in Th1, Th2, Th17 and Th3 cytokines, with lowest concentrations in the sperm peak-containing fraction, indicating its main immune influence might reside in the larger, protein-rich sperm-poor post-SRF.

Keywords:

Ejaculate fractions, immunomodulatory molecules, seminal plasma peptides, pig.

Introduction

Seminal plasma (SP), the **heterogenous composite fluid where** spermatozoa are suspended during ejaculation, **is mainly composed by secretions of the male accessory sexual glands (in pigs; seminal vesicles, prostate and bulbo-urethral/urethral glands), but also contains caudae epididymides intraluminal fluid that accompanies the emitted spermatozoa.**¹ In pigs, the large (150-250 mL) ejaculate is expelled in **three** time-sequenced fractions, which differ in sperm numbers and SP quantity and composition. **The first fraction is the so-called watery pre-sperm fraction that precedes sperm emission (urethral/bulbourethral secretion). The following fraction is conspicuous, called sperm-rich fraction (SRF) because it contains most (above 80%) of spermatozoa suspended in cauda epididymal fluid and prostate secretion, with a minor portion of seminal vesicle secretion. Following this dense, 30-50 mL large fraction, the rest of the non-coagulating ejaculate is called post-SRF, containing much fewer spermatozoa in a larger (often 120-200 mL) amount of SP mainly derived from the seminal vesicles, although secretion from the prostate gland is rather constant during the entire ejaculate and thus also present in this fraction.**² A coagulating end-secretion, with typical tapioca-like gel balls closes the ejaculation cycle, these secretions being formed in the bulbourethral glands and aimed at coagulating the protein-rich last part of the ejaculate to plug the cervical canal.¹ Semen collection in pigs is routinely done using manual pressure on **the glans penis (the so-called gloved-hand method) and allows for separate collection of the various fractions during ejaculation. As such, the SRF is most often retrieved for preparation of artificial insemination (AI) semen doses and for other sperm processing technologies such as freezing and sex-sorting.**^{3,4} **The SP affects sperm function, demonstrated both *in vivo* and**

in vitro studies with a focus on SP-proteins^{5,6,7}. For instance, spermadhesins such as AWN-1 bind to the sperm plasmalemma and follow them to the oocyte, influencing the process of sperm capacitation and sperm-oocyte binding.⁸ The SP is also able to mitigate the initial inflammatory response elicited by semen deposition, restricting this to a cleansing transient state preparing the endometrium for the descending early embryos.⁹ Even the SP of specific fractions or portions within the SRF (the so-called sperm-peak fraction) seem to conspicuously affect the functionality and survival of ejaculated, processed (chilled or frozen-thawed spermatozoa), thus indicating the sequential ejaculation process has physiological implications, at least *in vivo*.¹⁰ In commercial enterprises of boar semen production for AI, boar ejaculates are now being collected using semi-automatic collecting devices which collect the entire ejaculate in one single flask, thus obtaining a non-physiological mixture of SP components. The impact of this practice is yet unknown.

The pig SP is particularly rich in proteins (30-40 g/L, most of this contents present in the post-SRF^{11,12}) but also in peptides, ions, energy substrates, buffer substances, amino acids, lipids and hormones.^{5,13} These SP-proteins participate in key events related to fertilization, from participation in the sperm transport through the female genitalia, the establishment of the oviductal sperm reservoir, sperm capacitation and gamete interaction.¹⁴ Moreover, SP-proteins and peptides act as signals for the female immune system, modulating the maternal tolerance towards embryo and placental development, thus conditioning embryo development in eutherian mammals, including human.⁵ Among the latter are cytokines, secreted by activated immune cells and male/female genital epithelia, including that of male sexual accessory glands or of the vagina, cervix and uterus.^{15,16} Cytokine levels in SP have,

moreover, associated with poor sperm quality or functionality, and to sub-fertility in men with or without pathological processes in their genital tract.¹⁷⁻¹⁹

In the case of the boar, a species whose fractionated ejaculate sequence mimics that of human, the proteome of the ejaculate is rather well known.⁵ Yet, the peptidome is less screened.^{5,6} For instance, to the best of our knowledge, only few cytokines have been identified in boar SP, namely the transforming growth factor- β (TGF- β) 1 and 2, interferon- γ (IFN- γ) and interleukins (IL)-6 and IL-10.^{20,21} with clear quantitative variation between ejaculate fractions. In comparison, many other cytokines, e.g IL-6, IL-8, IL-10, IL-11, IL-23 and IFN- γ , have been found in human ejaculates, some of them displaying either positive or negative correlations with sperm quality and fertility.^{22,23} Whether the boar SP possess a similarly full battery of cytokines remains to be explored.

The purpose of the present study was, therefore, to identify and quantify a representative battery of measurable cytokines in the most conspicuous fractions of the boar SP, covering Th1, Th2, Th17 and Th3 –related cytokine responses.

Material and methods

Animals and ejaculates

All procedures involving animals were performed according to international guidelines and were approved by the Bioethics Committee of Murcia University (research code: 639/2012). Eight healthy, fertility-proven boars of different breeds or crossbreeds were used as semen providers. Boars were housed in individual pens in an AI center (AIM Iberica, Calasparra, Murcia, Spain), environmentally controlled (15-25 °C) with windows exposed to natural

daylight and supplementary light for a total of 16 h of light per day. Boars were provided with *ad libitum* access to water and were fed commercial feedstuff according to the nutritional requirements for adult boars subjected to regular ejaculate collection.²⁴ Thirty-two ejaculates (four per boar) were manually collected fraction-wise, namely separating the SRF and the following post-SRF, using the gloved-hand method. All collected ejaculates fulfilled the standards of quantity and sperm quality thresholds for the preparation of AI-semen doses (more than 200×10^6 spermatozoa/mL, 70% of them motile and 75% depicting with normal morphology).

Seminal plasma processing and storage

Aliquots of each ejaculated SRF and post-SRF were immediately transferred to 15 mL tubes and centrifuged twice (Rotofix 32A; Hettich Zentrifugen, Tuttlingen, Germany) at 1,500g for 10 min at room temperature (RT). The second supernatant was, after being examined by light microscopy to ensure it was sperm-free, transferred to 3 mL-cryotubes. The SP-samples were transported within 2 h of collection in insulated containers (15-17 °C) to the Andrology Laboratory at the Veterinary Teaching Hospital of the University of Murcia (Spain) and frozen (-80 °C) until isothermally-shipped for cytokine determination at the Department of Clinical & Experimental Medicine, Linköping University, Linköping (Sweden).

Measurement of cytokines in boar seminal plasma

The Luminex's xMAP® technology, a multiplexed microsphere-based flow cytometric assay, was used to examine the presence and relative concentration of a battery of cytokines and chemokines including IL-6, IL-8, the monocyte chemoattractant protein-1 (MCP-1) or the growth factor granulocyte macrophage colony-stimulating factor (GM-CSF) (associated

with a pro-inflammatory immune response); the anti-inflammatory/immune deviating, Th3-associated IL-10 and TGF- β 1, TGF- β 2 and TGF- β 3; the Th1-associated IFN- γ and interferon gamma-induced protein (IP-10/CXCL10); the Th2-associated macrophage-derived chemokine (MDC); the Th17-associated IL-17 and growth-regulated oncogene (GRO/CXCL1); as well as the inducer of NK cell proliferation and IL-15.

Pre-coated magnetic beads (Cat#HCYTOMAG-60K-11 for human reactivity, Merck Millipore, Billerica, MA, USA) were used for the determination of IL-6, IL-8, MCP-1, IL-10, IFN- γ , IP-10/CXCL10, MDC, IL-17, GRO/CXCL1, GM-CSF, and IL-15; while for quantification of TGF- β 1, TGF- β 2 and TGF- β 3, a 3-plex kit (Cat#TGFB-64K-03 for pig, human, mouse, rat, non-human primate, canine, feline reactivity, Merck Millipore) were used, following the methods described by the manufacturers in 96-well multiscreen plates. A cytokine standard curve, comprising six standard points, was built for each cytokine with the highest standard point at 10,000 pg/mL and the lowest standard point at 3.2 pg/mL, while for TGF- β the highest point was 10,000 pg/mL and the lowest 9.8 pg/mL. Serum matrix (SM), provided in the kits, was used to mimic the composition of the seminal environment in the standard, control and blank measurements. Two controls provided in the kits, were added in singlets. Following sonication, bead solution was added to each well for incubation at 4 °C in the dark, for 18 h. After incubation, the plate was emptied using a multiscreen vacuum manifold (Merck Millipore), washed twice, detection antibody added, incubated at RT in darkness for 60 min, before streptavidin-phycoerythrin addition and further incubated for 30 min. After washing, the plates were run on a Luminex 200 TM (Luminexcorp, Austin, TX, USA) with xPONENT software version 3.1.7 (Luminex corp) for acquisition and Masterplex 2010 version 2.0.0.68 (Mirai Bio Group, San Francisco, CA, USA) for data analysis. The median fluorescent intensity was analysed using a 5-parameter logistic curve-fitting to

calculate the concentrations of the cytokines in the samples. The samples for TGF- β were acidified (pH <3) with 8 μ L of HCl, and processed as described above for the cytokines and chemokines, except that the samples were diluted 1:30 v/v with xxx before analyses.

The data of cytokine concentration are shown for each ejaculate fraction (SRF and post-SRF) as means ($\pm$ SEM), median and percentiles.

Statistical analysis

Statistical analyses were carried out using the SPSS Statistics version 19 (IBM SPSS Statistics, Chicago, IL, USA). Normal distribution of residuals was tested using Kolmogorov-Smirnov test. The variation in concentrations of cytokines among boar and ejaculate fractions was analysed using mixed models of ANOVA, including ejaculate or ejaculate-fraction (four ejaculates per boar) as random effect. A *P*-value <0.05 was considered to be statistically significant.

Results

A total of 14 measurable cytokines were identified in the SP of the two main boar ejaculate fractions. With the exception of TGF- β 3, only measurable in the SP of 4 boars, all other cytokines explored were quantifiable in the SP of all boars (Figures 1-3). While the cytokines representative of Th1, Th2, Th3 and Th17 responses showed measurable concentrations in the SP from the post-SRF, three of them, GRO/CXCL1, IL-8 and IL-15, did not show measurable concentrations in the SP from SRF (Figures 1 and 2). The measurable concentrations of all of them varied significantly (*P* < 0.001) between boars. For those

cytokines detectable in either fraction of the ejaculate, their concentrations differed, with the post-SRF invariably showing the highest ($P < 0.05$) concentrations (Figures 1-3).

Discussion

To the best of our knowledge, this is the first study measuring cytokines representative of the Th1, Th2, Th3 and Th17 response groupings in the SP of a major livestock species; the pig, far beyond the five known to date.^{20,21} Such large number of cytokines (e.g. 14) had only been identified so far in human and murine SP, which had been compared as whole ejaculate despite having dissimilar anatomy, physiology, ejaculate composition and type of ejaculate.^{25,26} In human, cytokine type and concentrations have been related to the normality or (in) fertility of the males examined. For instance, high concentrations of IFN- γ , IL-17, MCP-1, MDC, IL-6 and IL-8 had been measured in SP-samples from infertile men or having pathological processes in their genital tract.^{17-19,27,28} Some other SP-cytokines, such as IL-10, had shown contradictory results among studies. Huleihel et al. (1999) reported lower concentrations of IL-10 in infertile than fertile men, whereas Miller et al. (2002) reported opposite results.^{29,30} These conflicting results, together with the little current knowledge of the role played by SP-cytokines on male reproductive performance, calls for further studies. Comparatively, the concentrations of IP-10/CXCL10, GRO/CXCL1, MCP-1 and TGF- β 2 seemed higher in boar SP than in human SP.^{16,31,32} In contrast, the concentration of INF- γ , MDC, IL-8 and TGF- β 3 are apparently lower in boar SP than in human SP.^{16,19,23} Whether these differences simply represent species differences or whether they have a relationship to physiological events in the male or the female, remains to be determined.

A main purpose of our study was to detect and measure the cytokine IL-15 in SP. This cytokine is a member of the four- α helix bundle cytokine family, which acts as a T-cell stimulant and plays a pivotal role in cell-mediated immunity by activating T-cell proliferation and B-cell antibody production, and by promoting natural killer cell cytotoxicity.^{33,34} Moreover, IL-15 has been demonstrated to play a relevant role in uterine function, acting through IL-15 endometrium receptors during implantation and early placental development.³⁴ In addition, it has been suggested that IL-15 is involved in the regulation of metrial gland function during mouse pregnancy.³⁵

The origin and particularly the role played by the above measured SP-cytokines on the male or female reproductive tract are far from clear. Regarding their origin, previous studies suggested that cytokines as GRO/CXCL1 and IP-10/CXCL10 can be synthesized by somatic cells in the seminiferous tubules (Sertoli cells and myoid cells).³⁶ However, the bulk of SP-cytokines is mainly synthesized by the accessory sexual glands.³⁷⁻³⁹ The prostate in humans and the seminal vesicles in mice are the main source of the latent isoforms of TGF- β , while a comparative origin in the pig can only be indirectly related to the origin of the fluids in every fraction.^{37,38} In that respect, both prostate and seminal vesicle secretions are primary contributors. Since the SP of SRF comes mainly from the prostate and epididymides and less from seminal vesicles while the SP of the post-SRF comes mainly from seminal vesicles (with a testimonial contribution of bulbourethral glands), it may be speculated that the seminal vesicles could be the main origin for many cytokines.² However, it is also reasonable to hypothesize that the three isoforms of TGF- β , present in both ejaculate fractions, could be synthesized by both prostate and seminal vesicles, but that the acidic SRF fraction (owing to the acidic cauda epididymis fluid) might be most relevant for the activation of the cytokine in the boar.⁴⁰ It is less likely that extravasation from blood to the SP is as alternative major

mechanism, particularly considering the boar has a membranous type of penis where blood flow (dramatically increased in humans for instance) is not noticeably increased during ejaculation.⁴¹

Moreover, cytokines secreted by epididymal and accessory sexual glands can be released to the SP within or in relation to exosomes which, either of epididymal (epididymosomes) or prostatic (prostasomes) origin, depict immunomodulatory properties.^{42,43} The SP-cytokines, independently of their origin, interact with the epithelia of the female genitalia which they confront during semen deposition. Such interaction can occur via exposure of SP directly to the epithelium, or through their binding to the sperm surface (as in the case of TGF- β , which binds to the post-acrosome domain in human).⁴⁴ Several SP-components, apart from bacterial lipopolysaccharides (LPS), TGF- β , IL-8 etc., are able to induce epithelial production of cytokines. For instance TGF- β induces epithelial production of GM-CSF in mice and human.^{37,39} IFN- γ is, on the contrary, capable of inhibiting the TGF- β signaling of epithelial cells.⁴⁵ Considering all above information, the transient inflammatory response of the female genitalia caused by semen deposition that occurs in all mammals studied so far after seems to be dual, elicited by spermatozoa and SP proteins.⁵ Moreover, SP-cytokines and those produced by the responding female genital epithelium seem also to participate. However, this inflammation resumes, often within hours, and information is available indicating the presence of TGF- β elicits –via epithelial production of GM-CSF- a monocytic transformation into macrophages within the endometrial epithelium and its subyacent lamina propria.⁹ These macrophages would further migrate via the lymphatic system to other areas, modify the immune response, terminating the inflammatory primary response and initiate a state of immunological tolerance towards implantation and further embryo development.^{39,46,47} Initially, it was believed that the porcine SP had anti-inflammatory properties, decreasing the

immune response in the uterus, thereby facilitating the sperm transport in the female genital tract and the colonization of the utero-tubal junction.⁴⁸ However, the current results show that the boar SP contains cytokines with pro- and anti-inflammatory properties, which confirms the dual SP-effect above described for in human and mice, would also appear in sows. Such effect was partially demonstrated by Jalali et al.,(2014) who asserted that the SP produces an increase in Treg-immune cells and a decrease in expression of inflammatory cytokines in the sow endometrium.⁴⁹

The **current** study also provides clear evidence of differences among boars in both presence and concentration of some cytokines. Differences in concentration were expected since it is well known that SP-composition differs quantitatively and qualitatively among boars, probably **genomically-driven**.^{50,51} It is particularly **interesting** that the cytokine TGF- β 3 was not measurable in SP-samples of four of the eight boars analyzed and that the concentrations clearly differed between porcine and human.³⁹ **Noteworthy**, these four boars also showed low concentrations of TGF- β 1. **Considering the three isoforms of TGF- β have impact on fertility through mediation of maternal immune tolerance after implantation, this finding initiates the question: are these levels related to male (or female) fertility after insemination?** ^{20,52} **It could, for instance**, be speculated that boars with absence of TGF- β 3 together with low concentrations of TGF- β 1 in their SP may have low reproductive performance. This would be challenged in further studies, **pertaining novel preliminary findings (Barranco et al., unpublished data)** that the SP of high-fertility boars showed consistent amounts of TGF- β over successive ejaculates, compared to highly variable inter-ejaculate concentrations in boars with significantly lower fertility. Further studies of a larger number of males with clear differences in fertility after AI are obviously needed.

An peculiarity of the ejaculate of boar (and of man) is that it provides two fractions with significant differences in sperm number and proportion of SP that are objectively distinguishable during the traditional manual collection of ejaculates. With the exception of the three isoforms of TGF- β , the concentrations of the other measurable cytokines differed between fractions. The post-SRF, which contains fewer spermatozoa bathing in larger volumes of SP, showed the highest cytokine concentrations in all boars examined. Moreover, some cytokines, specifically GRO/CXCL1, IL-8 and IL-15 could only be detected in the post-SRF fraction. For semen handling purposes (AI, semen freezing etc) manual collection of the SRF, containing most spermatozoa in a relatively low quantity of SP, has been customary. However, there is an increasing use of semi-automatic devices for entire-ejaculate collection, a practice that lower labor costs for the AI-enterprises.⁵³ This change not only implies the sperm suspension is greatly extended into a non-physiologically mixed-SP, deviating from the SP-composition encountered *in vivo* or when manual fractionated semen collection is done. Whether the different distribution and amount of cytokines influences the reproductive performance of boar ejaculates after AI should be explored. In addition, considering that the specific types and amounts of SP cytokines seem to contribute to the endometrial preparation for embryo implantation, we could hypothesize that avoiding the use of specific fractions may have an implication on resulting fertility rates.¹⁶

In conclusion, boar seminal plasma is rich in cytokines, whose concentrations differ among boars and also between the two main ejaculate fractions, showing the post-sperm rich fraction (post-SRF) had the highest concentrations. Further studies to elucidate the role of these cytokines on the reproductive performance of boar ejaculates are needed.

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Figure legends:

Figure 1. Box-whisker plot showing the variation in cytokines and chemokines produced by T helper cells mediating type 1 response (Interferon γ [INF- γ] and Interferon gamma-induced protein 10 [IP-10/CXCL10]); type 2 response (Macrophage-derived chemokine [MDC]) and type 17 response (Interleukin-17 [IL-17] and Growth-regulated oncogene [GRO/CXCL1]) in the seminal plasma from the sperm-rich fraction (SRF) and the post-sperm fraction, (POST-SRF) of 32 ejaculates from 8 boars (4 per boar). The boxes enclose the 25th and 75th percentiles; the line is the median; and the whiskers extend to the 5th and 95th percentiles. Data are the means $\pm$ SEM of 4 semen samples per boar.

Figure 2. Box-whisker plot showing the variation in pro-inflammatory cytokines and chemokines (Interleukin [IL]-6,-8,-15, Granulocyte-macrophage colony-stimulating factor [GM-CSF] and Monocyte chemoattractant protein-1 [MCP-1]) in the seminal plasma from the sperm-rich fraction (SRF) and the post-sperm fraction, (POST-SRF) of 32 ejaculates from 8 boars (4 per boar). The boxes enclose the 25th and 75th percentiles; the line is the median; and the whiskers extend to the 5th and 95th percentiles. Data are the means $\pm$ SEM of 4 semen samples per boar.

Figure 3. Box-whisker plot showing the variation in cytokines and chemokines produced by T helper cells type 3 response (anti-inflammatory/immunotolerance-related Interleukin [IL]-10 and Transforming growth factor [TGF] β 1, β 2, β 3) in the seminal plasma from the sperm-rich fraction (SRF) and the post-sperm fraction, (POST-SRF) of 32 ejaculates from 8 boars (4 per boar). The boxes enclose the 25th and 75th percentiles; the line is the median; and the whiskers extend to the 5th and 95th percentiles. Data are the means $\pm$ SEM of 4 semen samples per boar.