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Original Research Article

Artificial insemination of all ejaculated sperm fractions accelerates embryo development and increases the uterine vascularity in the pig

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ABSTRACT

The semen of boar is characterized by ejaculation in well-differentiated fractions with specific concentration, composition, and volume. The 'sperm-rich fraction' (SRF), the most concentrated seminal fraction, is habitually collected in insemination centers to make artificial insemination (AI) doses. The absence of the other fractions in AI doses could alter the uterine reaction to AI and not trigger essential responses that could maximize fertility. Thus, there is an urge to ascertain the impact of different ejaculate fractions on the uterus after AI to optimize the semen doses. This work analyzed specific parameters related to fertility in pregnant artificially inseminated sows ($n = 15$) with ac-cumulative fractions of the semen of boars ($n = 6$): F1, composed of the sperm-rich fraction (SRF); F2, composed of F1 plus the intermediate fraction; F3, composed of F2 plus the post-SRF. Non-inseminated sows ($n = 5$) were included as control (C). The different types of seminal dose did not affect the number of ovulated follicles (CL; corpora lutea, $p > 0.05$) but did affect the embryo development ($p < 0.05$). The proportion of embryos in morula stages was significantly higher in AI-F1 sows (84.4%, $p < 0.05$). Morulas and blastocysts were balanced in AI-F2 or AI-F3 ($p > 0.05$). Independently of the type of seminal dose (F1, F2, or F3), we observed by immunohistochemistry that AI significantly increased uterine vascularization, although with some anatomical differences. The cranial region of the uterine horns was significantly more vascularized in AI-F1 or AI-F2 sows (26.7 ± 2.3 and 28.6 ± 2.0%, respectively), and AI-F3 showed significantly less vascularization at that point (17.8 ± 1.6%, $p < 0.05$). To summarize, the synergistic effect of all ejaculate fractions accelerates embryo development, at least during the preimplantation period, and increases the uterine reaction to AI in certain parts of the uterus.

1. Introduction

The most widely used assisted reproductive technique in pigs is artificial insemination (AI), which is applied in 99% of worldwide large-scale farms [1]. Boar semen is collected on farms or AI centers, and the doses are prepared by diluting in an extender, fixating the number of motile spermatozoa and reducing the seminal plasma (SP) content. These doses are then refrigerated at 17 °C and distributed to the farms. In this way, one ejaculate can inseminate more than one female, significantly increasing the farm's efficiency.

The boar's complete ejaculate is emitted in well-separated fractions during the ejaculation. The 'sperm-rich fraction' (SRF) contains most sperm and a low proportion of SP. The fraction called 'the post-SRF' is the largest in volume and has very few sperm [2]. The 'intermediate fraction' is emitted between these two fractions, with middle characteristics compared to SRF and post-SRF. Until a few years ago, the seminal doses were exclusively obtained with spermatozoa from the SRF. It seemed that the sperm from SRF were more resistant to refrigeration once mixed with the extender [3,4]. Moreover, the small proportion of the SP in the AI doses obtained with the SRF was considered beneficial for sperm preservation, maintaining the fertility of the preserved liquid semen [5].

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Luther and Waberski (2019) [6] observed that 10% porcine SP during prolonged storage for 144 h in a short-term extender produced a heterogeneous response between individuals. The motility of some boars remained above the admissible threshold for use, but in another group of animals, it showed a dramatic decrease. This decrease in motility was buffered only by the presence of residual amounts of SP from the SRF, which is related to the fact that highly diluted spermatozoa were more susceptible to the adverse effects of SP after prolonged exposure. The protective effect of limited concentrations of SP was described as the effect of SP-proteins differentially expressed among the ejaculate fractions, with different actions on the development and/or functionality of spermatozoa [7].

Spermatozoa from SRF show superior resistance to preservation both in refrigeration and freezing than the mean resistance of spermatozoa from the whole ejaculate [3,4]. The explanation for this phenomenon is that these spermatozoa are mainly exposed to the epididymal fluid and prostatic secretion, avoiding the supposedly detrimental protein-rich secretions of the vesicular and bulbourethral glands [8–10]. In contrast, other authors suggested that SP from SRF is unnecessary and may reduce the fertility potential of stored semen [11]. A recent study on the use of whole ejaculate in preparing AI doses showed that it did not adversely affect sperm preservation. Even the reproductive impact of using the complete ejaculate meant substantial economic savings by reducing the amount of extender in semen doses [12].

After the semen dose deposition by AI, SP interacts with tissues from the female reproductive tract and induces molecular and cellular changes that increase the chances of conception and pregnancy [13,14]. SP promotes the release of cytokines that support the development of preimplantation embryos and induce the expression of angiogenesis at the uterine endometrium and implantation factors related to the attainment of the status of maternal immune tolerance to hemiallogeneic embryos [15].

The female genital tract response to the SP is specific and depends on the SP composition and balance of signaling factors. Uterine responses can either increase or decrease fecundability, improving or diminishing a male's chance of successful fertilization (reviewed by Ref. [15]). This work aimed to analyze the specific effect of the main factions of the boar ejaculate on uterine vascularization and embryonic development of pregnant sows after AI with different accumulative fractions of the ejaculate.

2. Material and methods

2.1. Reagents

Unless otherwise indicated, all chemicals were obtained from Sigma-Aldrich Química, S.A. (Madrid, Spain) or Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Ethics

All the procedures of this work were approved by the Ethical Committee of Animal Experimentation of the University of Murcia on June 1, 2020 (PID2019-106380RB-I00) and by the Animal Production Service of the Agriculture Department of the Region of Murcia (Spain) (A13230106) following the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63/UE on animal protection. Through the experiments, animals were handled carefully, avoiding any unnecessary stress. All the experiments were performed following relevant guidelines and regulations.

2.3. Animals

German Pietrain boars aged 30.8 ± 2.6 months were housed in individual pens (according to the European Commission Directive for Pig Welfare) with sawdust in a commercial boar stud (Sergal Gestió Ramadera, Lleida, Spain). An automatic climate system constantly controlled the temperature between 18 and 22 °C. Boars had a restricted regime of feeding according to their nutritional requirements. Water was available *ad libitum*. Crossbred sows Large White x Landrace (Danbred genetic) with similar body condition ~ 3 (on a scale from 1 to 5, where 1 was extremely thin and 5 was extremely fat). They were also selected at weaning with parity of 3.7 ± 0.6 (mean ± SD), weaning-to-estrus interval (WEI) of 3.6 ± 0.5 days (mean ± SD), and lactation length between 24 and 28 days. After weaning, sows were housed in individual gestation crates with free access to water and 4.0 kg feed/day. The estrus was detected from the day of weaning and once a day in the presence of a sexually mature boar.

2.4. Semen collection

Ejaculates from proven fertility boars were collected by the manual method. The semen collection was performed by an experienced technician identifying the different fractions of the ejaculate according to the color and consistency as in Luongo et al. (2022) [12]: i) the SRF, identified by the characteristic dense white color; ii) the intermediate fraction, identified by larger volume than SRF and moderated dense white color (composed of a few spermatozoa); iii) the post-sperm-rich fraction (post-SRF), characterized by a water-like liquid aspect (absence or a scarce number of spermatozoa). Samples composed of more than one fraction for the AI dose preparation were collected as accumulative fractions during ejaculation. The pre-sperm-rich fraction (pre-SRF) and the gel fraction were discarded. All samples from each boar met the minimum standards according to Gadea et al. (2004) [16] of sperm volume (>75 mL), concentration (>200 × 10^6 spermatozoa/mL), motility (>75% of motile spermatozoa and with progressivity of >2.5 using an arbitrary scale from 0 to 5), acrosome integrity (>90% intact acrosome), and normal morphology (>90%) to make AI doses.

2.5. Preparation of seminal doses and artificial insemination (AI)

Immediately after collecting the different accumulative fractions of each ejaculate, samples were processed to obtain AI doses. The sperm concentration was first determined in the samples using a calibrated sperm analyzer (Androvision® Minitüb, Tiefenbach, Germany), and then they were diluted in the AndroStar® Plus extender (Minitüb, Tiefenbach, Germany) up to the final volume of 60 mL and a concentration of 33 × 10^6 spermatozoa/mL. Once the doses were prepared, they were stored at 16 °C for up to 3 days. Sows were inseminated in individual crates by a post-cervical AI method with the combined catheter-cannula kit (Soft & Quick®, Tecno-Vet, S.L., Barcelona, Spain). For AI, the catheter was introduced to the uterine body by an experienced technician who followed the standard protocol for post-cervical AI in farms.

2.6. Collection of female genital tracts and corpora lutea (CL) identification

Female genital tracts were obtained at a local slaughterhouse and transported to the laboratory in a tempered container at 34–38 °C within 60 min of slaughter. Once in the laboratory, uterine tracts were washed twice in physiological salt solution (0.9% NaCl) supplemented with 0.1% antibiotic-kanamycin at 38.5 °C. The dissection of the tissues was carried out on a cooled surface. Once ovaries were dissected, the corpora lutea (CL) were identified as homogeneous and solid structures in the ovary. The CL counting was carried out in the ovaries, and the mean number of CL from the left and right ovaries and the total number of CL were registered per sow.

2.7. Collection of embryos

Uterine horns were dissected to remove the mesosalpinx, and the uterine lumen was flushed from the caudal part of the uterine horn to the

utero-tubal junction with 10 mL of phosphate-buffered saline (PBS) at 4 °C. The buffer infusion through the uterine lumen was performed with a catheter (24G BD Insyte™, 381212, Becton Dickinson Infusion Therapy Systems, Inc., Sandy, Utah, USA) adapted to a 10 mL syringe. The flushing medium was collected in a Petri dish, then inspected under the stereomicroscope for the localization of the embryos. Once obtained, the embryos were transferred to a new Petri dish with fresh PBS for washing and counted and classified according to the state of preimplantation development. Embryos were obtained from all AI-sows in the study.

2.8. Vascular study by immunohistochemistry and image analysis

After the ovaries and embryos were removed from the uterus, uterine tissue was cut from the female genital tract and histological sections were collected at three sites: i) cranial uterine horn, approximately 10–15 cm proximal to the oviduct; ii) mid-uterine horn, equidistant to the utero-tubal junction and uterine body; and iii) caudal uterine horn, 2–5 cm cranial to the uterine body. Full-thickness biopsies of 3–5 mm uterine tissue were collected using a sterile scalpel and tweezers and washed in PBS. The uterine sections were fixed in 10% formaldehyde and routinely processed for paraffin embedding, and sections of 5 μm-thickness were stained for histological evaluation. Immunochemistry for the specific detection of endothelial cells was performed using an anti-CD31 antibody. The technique started with a heat pretreatment for antigen unmasking in PT-link for 20 min at 97 °C with a commercial EDTA solution at pH 9.0. The histological sections were incubated with the primary antibody polyclonal rabbit anti-CD31 (1:250, AB28364, Abcam, Cambridge, UK) overnight at 4 °C. The rabbit linker (K800921-2, Agilent Dako, Santa Clara, CA, USA) enhanced the primary antibody signal and incubated for 10 min at 37 °C. The secondary antibody (Vector ImmPRESS Goat anti-Rabbit IgG, HRP) was then added, incubated for 20 min at 37 °C, and followed by 3,3 diaminobenzidine revealing (DAB, commercial kit solution from Agilent Dako). Finally, contrast staining with hematoxylin was performed, and the slide was mounted in a permanent medium. The slides were digitalized at 0.172 pixel/μm resolution at 20x magnification (Pannoramic MIDI II scanner3D Histech®, Budapest, Hungary) and photographed at 10x with a virtual microscope (Software Slide Viewer 2.5® 3D Histech). The images of the uterine endometrium were analyzed (ImageJ® software, NIH) using a macro specifically designed for antibody detection and calculating the area occupied by vessels, defined as vascular area (Fig. 1).

2.9. Experimental design

Fig. 2 graphically shows a scheme of the experimental design. The semen samples from independent boars (n = 6) were obtained to prepare homospermic AI doses according to Luongo et al. (2022) [12]: i) F1, composed by the SRF; ii) F2, composed of F1 plus the intermediate fraction; iii) F3, composed by F2 plus the post-SRF. Sows (n = 20) were randomly divided into four groups of five sows each. Samples composed of more than one fraction for the AI dose preparation were collected as accumulative fractions during ejaculation. Each group of sows was inseminated with one type of AI dose (AI-F1, AI-F2, or AI-F3) or non-inseminated (C, control) and sacrificed six days later. The experimental design was divided into two different experiments:

Experiment 1. Counting of CL in ovaries and evaluation of the state of preimplantation embryo development in pregnant sows from groups AI-F1, AI-F2, and AI-F3. This experiment aimed to ascertain if the fraction of the ejaculate can modulate the initial progression of embryo development and, thus, the potential fertility. CL were counted in both ovaries and the mean and total number of CL were registered per sow. Each embryo's development state was registered as the proportion of morulae and blastocysts from the total embryos collected per experimental group.

Experiment 2. Determination of the vascular area in three different segments of the uterus in pregnant sows from groups AI-F1, AI-F2, and AI-F3. This experiment aimed to elucidate the influx of the accumulative seminal fractions on the uterine preparation for the initial phases of embryo development. Histological sections were collected at three sites: i) cranial uterine horn; ii) mid-uterine horn; and iii) caudal uterine horn. The slides were photographed with a virtual microscope and five to seven images of each histological section were captured. These images were digitalized, analyzed and the % of the vascular area was registered per picture (Fig. 1).

2.10. Statistical analysis

The statistical data analysis was carried out with the IBM SPSS v. 24 software (SPSS Inc. Chicago, IL, USA). The CL counting and vascular area data were analyzed by one-way ANOVA and the least significant difference multiple comparison post hoc Tukey test. Results were obtained as the mean ± standard error of the mean (SEM). The data on the state of embryo development were analyzed with the non-parametric Kruskal-Wallis test and shown as proportion ± standard error (SE). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Corpora lutea (CL) counting

In the study of the mean number of CL in ovaries as indicative of ovulated follicles, there were no statistical differences between groups

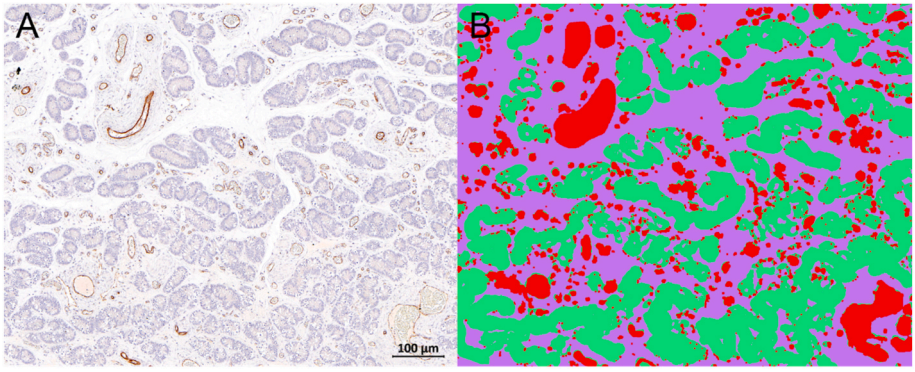

Fig. 1. Sample of the vascular study of the endometrium by image analysis. **A)** Histological uterine section image treated for immunohistochemistry for vascular endothelium evidencing with anti-CD31 (note the positive vascular lumens marked in brown). **B)** Resulting image after detection of the endothelium, in which the vascular lumen is filled and thickened to encompass the vascular wall. The total area occupied by blood vessels (red) is calculated and differentiated from the rest of the tissue (glands in green and connective tissue in purple) in each image. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

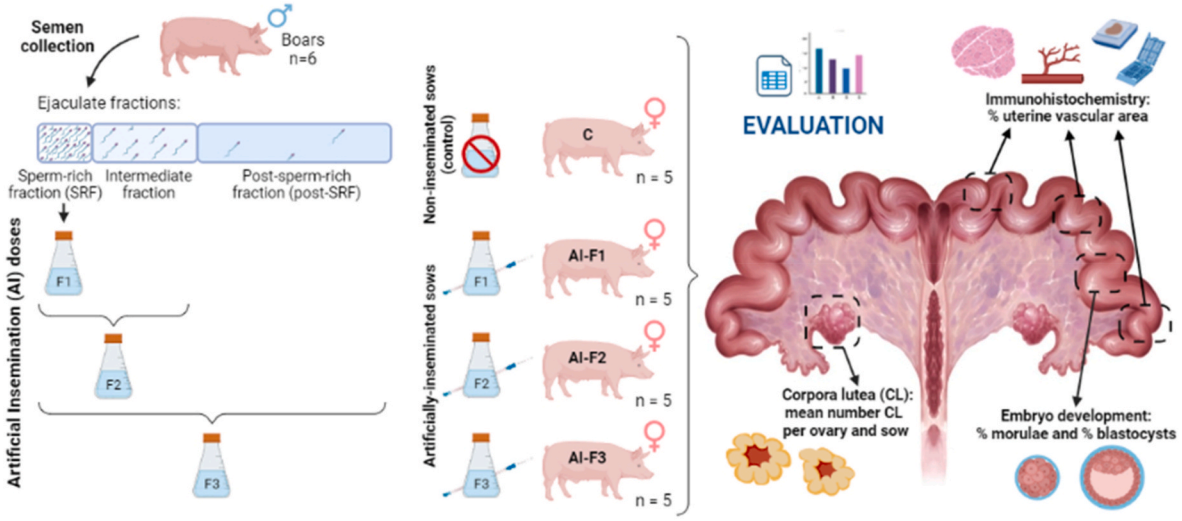

Fig. 2. Graphic scheme of the experimental design (created with BioRender.com).

($p > 0.05$, Table 1). Sows inseminated with the different types of AI doses according to the seminal fraction(s) (AI-F1, AI-F2, or AI-F3) or non-inseminated (C) showed similar mean and the total number of CL in their ovaries. All the sows showed a mean number of CL in the left ovary between 10.6 ± 1.8 and 14.2 ± 1.2, between 10.2 ± 0.9 and 14.2 ± 0.4 in the right one, and a mean total number between 21.6 ± 1.9 and 28.4 ± 1.3.

3.2. Embryo development

The results of embryo development in pregnant sows from groups AI-F1, AI-F2, and AI-F3, according to the type of seminal dose inseminated (F1, F2, or F3), are shown in Fig. 3 as proportion (%) ± SE. In AI-F1 sows, embryo development was slower than in AI-F2 and -F3 sows since the highest proportion of them (84.4 ± 4.5%) developed to morula, and just 15.6 ± 4.5% reached the blastocyst stage ($p < 0.05$). In addition, AI-F2 and AI-F3 showed similar embryo development, producing a balanced proportion of morulae and blastocysts. A 44.3 ± 6.8% of the embryos in AI-F2 sows developed up to morula and 55.7 ± 6.7% to blastocyst ($p > 0.05$). Similarly, 47.8 ± 7.5% of embryos in AI-F3 sows developed up to morula and 52.2 ± 7.3% to blastocysts ($p > 0.05$).

3.3. Uterine vascularization

This study aimed to elucidate the effect of SP on the vascular irrigation of the uterine endometrium as indicative of the establishment of appropriate conditions for embryo implantation in this organ. A total of 8.93 mm^2 of uterine endometrium tissue were analyzed by 302 histological images. The interaction of the ejaculate with the uterus

Table 1
Mean number of corpora lutea (CL) per ovary and mean total number of CL per sow.

Group of sows	Sows (n)	Left ovary	Right ovary	Total CL
C	5	10.6 ± 1.8	13.0 ± 2.0	23.6 ± 2.3
AI-F1	5	14.2 ± 1.2	14.2 ± 0.4	28.4 ± 1.3
AI-F2	5	11.4 ± 1.1	10.2 ± 0.9	21.6 ± 1.9
AI-F3	5	11.2 ± 1.1	11.2 ± 1.2	22.4 ± 2.1

Sows (n = 20) inseminated with different artificial insemination (AI) doses from boars (n = 6) according to the seminal fraction(s) used: F1, composed of the sperm-rich fraction (SRF); F2, composed of F1 plus the intermediate fraction; F3, composed of F2 plus the post-SRF. Non-inseminated sows were considered as control (C). Results are expressed as the mean ± SEM. Data were compared by one-way ANOVA and Tukey post hoc test ($p < 0.05$).

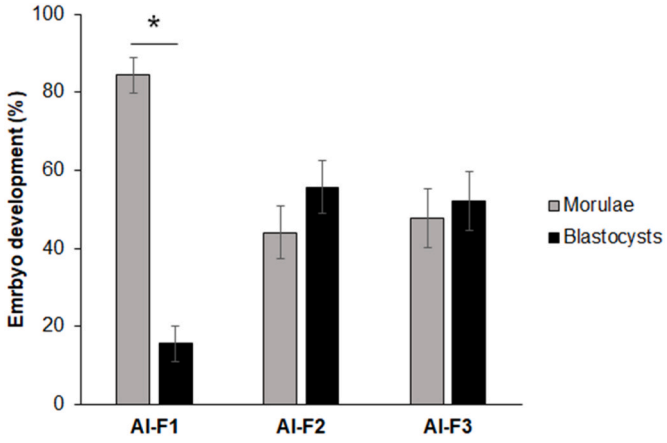

Fig. 3. Proportion of embryos in the morula and blastocyst stage of preimplantation development form the total collected from uterine horns of sows (n = 15) inseminated with different semen doses from boars (n = 6) according to the seminal fraction(s) used for artificial insemination (AI): F1, composed of the sperm-rich fraction (SRF); F2, composed of F1 plus the intermediate fraction; F3, composed of F2 plus the post-SRF. The results are shown as the percentage of embryos in each stage of development (morula or blastocyst). Data were compared using the non-parametric Kruskal-Wallis test for independent samples. Results are shown as proportion (%) ± SE. Asterisk (*) shows statistical differences ($p < 0.05$) between the two states of embryo development (morulas and blastocysts) within experimental group AI-F1, AI-F2, or AI-F3.

represented a significant increase of 5.1% in the vascular area of AI-sows compared to C-sows ($p < 0.05$, Fig. 4). C-sows had 20.0 ± 0.0 % uterine vascular area compared to the 24.2 ± 1.1% developed in AI-F1, 27.5 ± 1.0% in AI-F2, and 23.0 ± 1.0% in AI-F3. Sows from groups AI-F1, AI-F2, and AI-F3 did not show significant differences in uterine vascular area ($p > 0.05$). Thus, AI itself had a significant influence on uterine vascularization.

According to the specific anatomical region and type of seminal dose used for AI (Table 2), we observed that the cranial region of the uterine horns presented higher endometrial vascularization in AI-F1 or AI-F2 sows (26.7 ± 2.3 and 28.6 ± 2.0%, respectively; $p < 0.05$). However, AI-F3 sows showed significantly less vascularization at that point (17.8 ± 1.6%, $p < 0.05$). No differences in other anatomical regions of the uterus were detected when different seminal doses were applied ($p > 0.05$). Comparing the endometrial vascularization within the same type of AI dose, F1 produced a progressive and significantly higher uterine

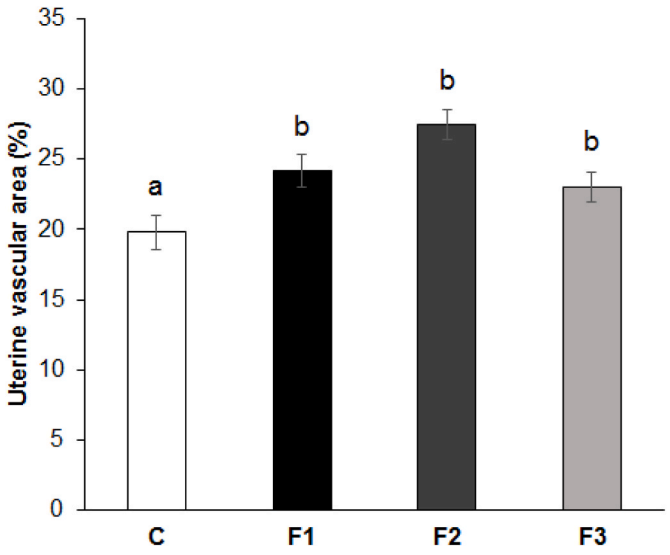

Fig. 4. Percentage of uterine vascularity in sows (n = 20) inseminated with different AI doses from boars (n = 6) according to the seminal fraction(s) used for artificial insemination (AI): F1, composed of the sperm-rich fraction (SRF); F2, composed of F1 plus the intermediate fraction; F3, composed of F2 plus the post-SRF. Non-inseminated sows were considered as control (C). The results are shown as the mean percentage (±SEM) of the vascular area in the different histological sections within the experimental group. Data were compared by one-way ANOVA and Tukey post hoc test. Different letters between experimental groups (a, b) indicate significant statistical differences ($p < 0.05$).

Table 2
Percentage of the vascular area in different anatomical regions of the uterus in artificially inseminated sows.

Group of sows	Sows (n)	Cranial horn (%)	Mid-uterine horn (%)	Caudal horn (%)
AI-F1	5	26.7 ± 2.3^{Aa}	24.4 ± 1.9^{Aab}	21.4 ± 1.5^{Ab}
AI-F2	5	28.6 ± 2.0^{Aa}	28.0 ± 1.7^{Aa}	25.6 ± 1.7^{Aa}
AI-F3	5	17.8 ± 1.6^{Ba}	26.8 ± 1.8^{Ab}	24.7 ± 1.5^{Ab}

Sows (n = 15) inseminated with different semen doses from boars (n = 6) according to the seminal fraction(s) used for artificial insemination (AI): F1, composed of the sperm-rich fraction (SRF); F2, composed of F1 plus the intermediate fraction; F3, composed of F2 plus the post-SRF. Results are expressed as the mean ± SEM. Data were compared by one-way ANOVA and Tukey post hoc test ($p < 0.05$). Different capital letters (A, B) denote statistical differences between groups of inseminated sows within the same anatomical region (same column; $p < 0.001$). Different lower-case letters (a, b) denote statistical differences between anatomical regions within the same group of inseminated sows (same row; $p < 0.05$).

vascularization in the ascendant direction from the caudal uterine horn to the cranial region (Table 2; $p < 0.05$). F2 did not produce a different percentage of vascularized area, and F3 produced a contrary effect to F1, with higher ($p < 0.05$) endometrial vascularization in the caudal part of the uterus (caudal uterine body and mid-uterine horns).

4. Discussion

The SP targets a series of uterine reactions essential for fertilization success. Besides initiating a controlled inflammatory response that affects several aspects of reproductive function, SP stimulates ovulation and induces an altered transcription in several tissues in the female tract that modulates embryo development [13]. SP also initiates the immune adaptation to promote the endometrial receptivity to implantation, placental development, and uterine vascular adaptation to placentation [17]. Depending on the SP composition and balance of signaling factors, female responses can either increase or decrease fecundability, improving or diminishing a male's chance of success. It is known that exposure of the female genital tract to SP triggers ovulation [18] both at coitus in natural mating or using seminal doses in AI. Moreover, porcine SP administered just before ovulation modulates CL development via recruitment and activation of ovarian macrophages [19], contributing to post-ovulatory tissue remodeling, formation of CL, and steroidogenic function of luteal cells [20]. AI is currently the primary method of assisted reproduction in pigs. However, preparing AI doses implies the dilution of the semen in extenders, significantly reducing the proportion of SP contained in AI doses. At this point, the effect of the SP concentration shortage on the female response is still unknown. Considering that the preparation of seminal doses with accumulative fractions of the ejaculate results in a different SP concentration [12], this study analyzed the impact of SP from these doses on CL formation, embryo development, and uterine vascularization. As expected, there were no differences between sows in the number of CLs formed, although we should consider that the time of CL formation can vary by barely a few hours. Adams et al. [17,21], studying ovulation-induced animals such as alpacas and llamas, demonstrated that an ovulation-inducing factor (OIF) identified as βNGF, present in the SP, is associated with a preovulatory surge in LH and that the infusion of SP produced ovulation in 29.3 ± 0.7 h after AI.

The different accumulative ejaculated fractions influenced the early embryo development, increasing the proportion of blastocyst obtained when SP was higher in the AI dose (F2 and F3 vs. F1). Other authors got similar results as the case of Martínez et al. (2019) [22], who observed that SP infusions before AI positively impacted the preimplantation embryo development and altered the expression of the endometrial genes and pathways potentially involved in embryo development.

The uterine vascularization was significantly lower in non-inseminated sows, indicating that the interaction between the ejaculate and the uterus could stimulate the formation of new vessels in the endometrium. These results reinforce the idea previously demonstrated by other authors with a macroscopic study of the endometrium [14] or the study of the proliferation of endothelial cells in the porcine endometrium [23].

Porcine SP elicits an endometrial response in sows characterized by the recruitment of inflammatory leukocytes and the induction of several pro-inflammatory cytokines and cyclo-oxygenase-2. The consequences are evident in the preimplantation and early pregnancy periods in which altered leukocyte populations and cytokines last for at least nine days [24]. Several studies demonstrated that SP has many components related to multiple effects on the female reproductive tract, which are essential for the conceptus and pregnancy [25]. Some of these components are related to angiogenesis as the transforming growth factor beta (TGFβ) [26], the vascular endothelial growth factor (VEGF, also referred to as VEGF-A) [27], and prostaglandins (PGs) [28]. We observed that the vascular area increased in the sows that had been inseminated. We hypothesize that the angiogenic factors in the SP from only one fraction of the ejaculate were sufficient to induce a response. This theory is supported by the work of Carlsson et al. (2016) [29], in which they observed an elevated concentration of angiogenic factor VEGF-A in prostasomes. These prostasomes are produced by the prostate and delivered to the SRF. However, VEGF-A was only analyzed in the preimplantation period, and its significance could be more related to sperm elimination and uterine inflammatory response than to the development of the pregnancy. Furthermore, Malysz-Cymborska et al. (2014) [30] showed that AI affected the mRNA and protein profiles of the VEGF system in the porcine oviduct. They observed that disrupting VEGF system expression may be crucial to many events occurring during the periovulatory period and consequently could lead to deprivation of VEGF-dependent factors necessary for proper fertilization, gamete transport, and embryo development. We should also consider that the SP-induced vascular bed development was apparent shortly before the time corresponding to maternal recognition of pregnancy in the pig. These data indicate that the porcine uterus can be sensitized shortly after SP exposure to evoke

prolonged effects on PG synthesis and angiogenesis in the endometrium, persisting over of the preceptive phase. Thus, SP can affect uterine receptivity and embryo-maternal interactions in pigs through locally direct and/or indirect mechanisms [31].

Another relevant aspect observed in this work was the percentage of vascular area in the different regions of the uterus and how it varied according to the ejaculate fraction used. Close to the oviduct, F1 and F2 produced the highest percentage of endometrial vascularization, while F3 induced it in the central uterine region. The physiological explanation for these results is complex as there are few bibliographical references concerning this fact. Gardela et al. (2022) [32] observed that semen components (spermatozoa and/or SP) act differently on specific segments of the genital tract. Their results confirmed changes in the expression of cell proliferation and differentiation-related transcripts in response to both sperm and SP. The spermatozoa from the SRF (first 10 mL of SRF and the remainder of the SRF portion) are mainly exposed to epididymal fluids and prostate secretion, and neither one is especially rich in proteins [33]. The post-SRF is the ejaculate fraction that proportionally contains the largest amount of SP and, in turn, is the most protein-rich [34], so it can potentially elicit distinct gene expression changes during sperm transport [35]. In this work, although the ejaculate was diluted, all the semen fractions and SP content are inseminated when F3 AI is used. At the same time, AI with F1 consisted of only the sperm-peak fraction of the ejaculated spermatozoa surrounded by a diluted distinct fraction of the SP, essentially composed of epididymal cauda fluid and prostate secretion. Therefore, up- or down-regulated genes for angiogenesis in different sections of the uterus could be affected. However, the physiological relevance of the differences in this work concerning the % of the vascular area in the different regions of the uterus remains to be clarified. There is a homogeneous implantation of the embryos along the uterine horns and not concentrated in areas with more vascularization. Anderson and Parker (1976) [36] did not observe any relationship between the vascular arrangement or blood supply to particular segments of the uterine horn and embryonic or placental development during the first 30 days of pregnancy. Controversial data were found concerning fetal weight since some authors described that the fetal weight was greater in fetuses located near the utero-tubal and cervical ends of the uterine horn during the late stages of pregnancy [37]. However, other authors found no consistent relationship between uterine vasculature and fetal weight [38]. Álvarez-Rodríguez et al. (2020) [35] studied the porcine distal and proximal endometrium after copulation and observed increased gene expression of vascular factors. They observed that genes related to angiogenesis and vascular remodeling (poFUT1, NTN4) were, among others, overrepresented, with distal and proximal uterine segments exhibiting the highest number of differential expression of genes (DEGs).

In accordance with the results of Luongo et al. (2022) [12], using the entire ejaculate or SRF when elaborating the AI doses did not modify the reproductive performance. This fact could indicate that the SP effect on the female uterus could be oriented to trigger the inflammatory response rather than impacting on pregnancy rates. More studies are needed to explain the significance of the increase in uterine vascularization after insemination.

5. Conclusions

Preparing AI doses with the whole ejaculate increases the output of developing blastocysts in pregnant sows. The vascular area and the location of new vessels in the endometrium of pregnant sows also increase after insemination, and they are modified by the fraction of the ejaculate used in the preparation of the AI doses.

Declaration of competing interest

The authors declare not to have had any financial or other relationship with people, organizations, institutions, or companies that inappropriately influenced this work.

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CRediT authorship contribution statement

Santa María Toledo-Guardiola: Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Ester Párraga-Ros:** Writing – review & editing, Writing – original draft, Software, Methodology. **Juan Seva:** Writing – review & editing, Software, Methodology. **Chiara Luongo:** Writing – review & editing, Methodology. **Francisco A. García-Vázquez:** Writing – review & editing, Methodology. **C. Soriano-Úbeda:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Investigation, Formal analysis, Data curation. **C. Matás:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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