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The impact of *in vitro* embryo production on placental and umbilical cord vascularization is minimized by the addition of reproductive fluids

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ABSTRACT

Animals born from *in-vitro*-produced (IVP) embryos show changes in the placenta and umbilical cord vascularization. This study compares the placental and umbilical vascular morphometry in pigs (n = 19) born through artificial insemination (AI group) or after transfer of IVP embryos cultured with (RF-IVP group) or without (C-IVP group) reproductive fluids. The relationship between vascular parameters and animal growth during the first year of life was also analyzed. Samples were collected at birth, fixed, paraffin-embedded, cut in sections, stained, and photographed for vascular and morphometric analysis with ImageJ® and Slide Viewer®. The average daily weight gain was individually scored from birth to the first year of life. No differences were found in placental vascular morphometry among groups, except for the vascular area of small vessels (arterioles, venules, and small vessels) that was higher in the C-IVP group. Regarding the umbilical cord, the values for perimeter (AI: 26.40 ± 3.93 mm; IVP: 30.51 ± 4.74 mm), diameter (AI: 8.35 ± 1.01 mm; IVP: 10.26 ± 1.85 mm), area (AI: 43.18 ± 12.87; IVP: 56.61 ± 14.89 mm^2), and Wharton's jelly area (AI: 36.86 ± 12.04 mm^2; IVP 48.88 ± 12.80 mm^2) were higher in IVP-derived than AI-derived animals, whereas arterial and venous morphometric data were similar between groups. A correlation study showed that placental and umbilical cord vascular phenotypes affect the further growth of pigs. In conclusion, assisted reproductive technologies impact small caliber vessels in the placenta and morphometric parameters in the umbilical cord. The addition of reproductive fluids in IVP-embryo contributes to reduce the differences with *in vivo*-derived animals.

1. Introduction

In several species, assisted reproductive technologies (ART) have numerous major effects on offspring outcome, and in placental and fetal growth and development when compared to natural pregnancies [1]. In animal production, the use of ART involve various benefits such as high genetic potential and productivity for livestock companies being artificial insemination (AI) the mostly used ART for sow breeding [2] whereas the *in vitro* embryo production (IVP), embryo transfer and somatic cell nuclear transfer have not been fully applied to field conditions due to the high incidence of polyspermy, low capacity for embryonic development *in vitro*, and high rate of neonatal mortality in porcine species [3,4]. Furthermore, the selection and handling of donor and recipient sows for the embryo transfer programs have an impact on pigs' production [5].

Placental and umbilical cord alterations derived from the use of ART have already been described in cattle, sheep, mice [6,7] and humans [8], being reported diverse placental abnormalities such as placental autophagy [9], and differences in size and abnormal vascularization compared with individuals conceived without ART [8,10,11]. Specifically in pigs, fetal growth and mortality are strongly linked with the placental performance and umbilical cord

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development [12,13] and aberrant expression of the angiogenic factors PEG3 and LUM has been described in placentae obtained from IVP pigs [14]. However, this abnormal expression is mitigated by the use of reproductive fluids (oviductal and uterine) in the culture medium and IVP animals generated with these secretions show similar PEG3 and LUM expression than *in vivo* derived animals obtained by AI [14].

Piglet survival is not only conditioned by production parameters such as birth weight, litter size, farrowing period, birth order, environmental temperature, and nutritional or health status [15]. Other factors such as nutrition and oxygen transport, which are mediated by adequate placental and umbilical development, have immediate effects on the placental phenotype and, consequently, on the well-being of the offspring throughout adulthood [16,17]. However, the characteristics of placental and umbilical vascularization in ART-derived pigs and their influence on mid and postnatal growth are not well known. Thus, this work aimed to perform a morphometric study of the vascularization in the placenta and umbilical cord in pigs derived from different ART (AI and IVP with or without reproductive fluids in the culture medium); and to study the relationship between placental and umbilical cord parameters and animal's growth, assessed as the average daily weight gain (ADG).

2. Materials and methods

2.1. Ethics

All procedures used in this study were approved by the CEEA (Comité Ético de Experimentación Animal) from the University of Murcia (Spain) and authorized by "Dirección General de Agricultura, Ganadería, Pesca y Acuicultura" from Región de Murcia (project number A13170706).

2.2. Animals

Animals in the study (n = 26) were born using artificial insemination (AI group) or after transfer of *in vitro* derived embryos (IVP group) produced with (RF-IVP group) or without (C-IVP group) reproductive fluids in the embryo culture medium [18]. AI-derived animals were purebred Large White individuals, while the IVP animals were the result of crossing purebred Large White semen of the same boar as for the AI animals with oocytes from finishing gilts, resulting from mating Duroc or Pietrain boars with Large White x Landrace sows. All animals were handled following the current regulations on animal protection and pig farm management and their general health status was daily supervised by an experienced veterinarian. All pigs were accommodated in the Veterinary Teaching Farm, Veterinary Faculty, University of Murcia, Murcia (Spain), nourished under the same conditions and *ad libitum* water supply.

2.3. Tissues collection and average daily gain (ADG) calculation

The deliveries were assisted by qualified personnel to maintain the traceability of the samples. The sows were housed in separate cages at the farrowing to allow contact with the piglets and prevent crushing. At birth, placenta and umbilical cord were identified as previously described to have sample traceability [4,14]. Placenta samples were collected at 3–5 cm from the insertion of the umbilical cord and samples were immediately fixed in a 10% buffered formalin solution for histological study. After the expulsion, placenta weight (g) and placenta area (cm^2) were calculated by image analysis performed by tracing its contour on paper (ImageJ® 1.52a software, National Institute of Health, USA) [14]. Placenta efficiency (g/g), which indicates the grams of fetus produced per gram of placenta, was calculated as piglet birth weight/placental weight. Pigs were weighed with a digital scale at birth (0 days) and throughout their first year of life. Average daily gain (ADG) was calculated as (last weight-previous weight)/(last date-previous date). Placentae and umbilical cord samples were collected from same animals (n = 26). However, 7 placentae samples were not in good conditions for further morphological study and were discarded. Thus, analysis was performed from 19 placentae samples and 26 umbilical cords. Since birthweight, placenta weight and placenta efficiency are lower in pigs derived from ART [14] and birthweight is associated with umbilical cord diameter [19], in the current study pigs of both sexes, with similar birth weight, and close placental efficiency, area and weight, were selected from each group to avoid the impact of these parameters on further results (Table 1).

2.4. Histological analysis of placenta and umbilical cord

Placental and umbilical cord samples were fixed (10% formaldehyde) and routinely processed for paraffin embedding. Sections of 5 μm-thickness were stained with Hematoxylin-Eosin (H&E) for histological analysis. The placenta slides were photographed at 5x (Microscope Software ZEN 3.2 lite, Zeiss®) and the images were analyzed (ImageJ software. NIH®) to obtain the total analyzed placental area, blood vessel number, vessel area and total vascular area (Fig. 1). Two expert operators classified the blood vessels based on their size [20], wall and lumen characteristics: wide irregular lumen and thin wall for veins, and round lumen and thick wall for arteries [21]. Vessels were classified as capillary (1–500 μm^2), arteriole/venule (501–1000 μm^2), small artery/vein (1001–3000 μm^2), medium-sized artery/vein (3001–30000 μm^2), and large artery/vein (>30,000 μm^2). The vessels number in the placenta (arteries, veins, or capillaries), the vascular density (total number of arteries, veins, or capillaries respectively per 1 mm^2 placenta), vascular area (area occupied by arteries, veins or capillaries divided by the total placenta area analyzed), and the total vascular volume (mL) (percentage of vascular area multiplied by placenta weight in grams) were calculated.

Slides with the umbilical cord samples were digitalized at 0.172 pixel/μm resolution at 20x (Pannoramic MIDI II scanner3D Histech®) and analyzed by two expert operators (Virtual Microscope Software Slide Viewer 2.5 3D Histech®) to calculate the arteries area, vein area, Wharton's jelly (WJ) area and thickness; and umbilical cord area, perimeter, and diameter (Fig. 2). Umbilical cord diameter was measured by tracing a line between the outer-to-outer border from the long-axis view of the cord [22]. Umbilical blood vessels were categorized on their size and wall characteristics [23]. The pig umbilical cord has only one vein with higher calibre than arteries and tunica media has wider scattered muscular tissue fibres, whereas it has two smaller arteries with a concentric highly developed elastic tunica media. In the WJ, two zones were differentiated as perivascular and intermediate zone according to their connective tissue. The perivascular zone corresponds to the umbilical cord region surrounding the two umbilical cord arteries and one vein and has a typical mucous connective tissue, where stand-out fusiform cells widely separated, capillaries, arterioles, and venules. The intermediate zone corresponds to the regions between the perivascular jelly and the umbilical epithelium, which also presents mucous connective tissue but with rounded cells, small bundles of collagen fibrils and fewer capillaries [23].

Table 1
Placental parameters of pigs used in the current study: animals were born from artificial insemination (AI) or *in vitro* embryo production (IVP) with (RF-IVP) and without (C-IVP) reproductive fluids. Placenta efficiency: rate between animal weight birth in grams divided by placenta weight in grams. Placenta ratio: placenta weight divided by animal weight birth. Data are expressed as mean ± SD. No statistical differences were observed.

	AI	IVP	C-IVP	RF-IVP
Animals (n)	6	13	5	8
Birth weight (g)	1363.33 ± 226.07	1478.46 ± 274.76	1468.00 ± 173.55	1485.00 ± 334.79
Placenta weight (g)	158.33 ± 67.06	189.00 ± 64.37	150.0 ± 43.59	205.71 ± 2.12
Placenta area (cm^2)	1735.28 ± 289.50	1651.34 ± 295.49	1702.59 ± 313.65	1608.63 ± 301.85
Placenta efficiency (g/g)	9.60 ± 3.37	8.35 ± 2.79	10.26 ± 4.36	7.53 ± 5.36
Placenta ratio (g/g)	0.12 ± 0.03	0.13 ± 0.04	0.10 ± 0.05	0.14 ± 0.06

2.5. Statistical analyses

Data presented in the manuscript were analyzed firstly by embryo origin: *in vivo* (AI group) *vs. in vitro* (IVP group). A second analysis was done within IVP groups to explore the likely effect of using reproductive fluids during IVP (C-IVP group *vs.* RF-IVP group). A final comparison was done among the three experimental groups (AI, C-IVP and RF-IVP) to study whether the addition of reproduction fluids during IVP (RF-IVP group) might approximate the results to those observed in *in vivo*-derived animals (AI group). One-way ANOVA test and Tukey's test with the SPSS® v28 software (IBM, USA) were used to determine significant differences ($P < 0.05$) between experimental groups for each studied variable since the assumptions of homogeneity of variances and normality were met. The statistical units to evaluate the morphometric vascular parameters were the number of histological images analyzed. Repeated measures ANOVA test was carried out to study differences in ADG among groups. Pearson's correlation test ($P < 0.05$) was carried out to check the relationship between growth (ADG parameter) and placental and umbilical cord parameters. The correlation was interpreted as very high (1.00–0.90), high (0.89–0.70), moderate (0.69–0.50), low (0.49–0.30) positive or negative correlation; and negligible correlation (0.29–0.00) [24].

3. Results

Different vascular parameters were studied in a total of 19 placentae: 6 pigs born from AI-group and 13 pigs from IVP-group, 8 of them produced with reproductive fluids (RF-IVP group) and 5 without fluids (C-IVP group). No statistical differences were found between groups in placental morphometric parameters (Table 1).

3.1. Placental vascularization and vascular architecture

A high number of images (n = 220) was analyzed in each group allowing to study a placental area of 44.95 mm^2, 19.43 mm^2 and 45.86 mm^2 in the AI, C-IVP and RF-IVP groups, respectively (Table 2). From the total vessels analyzed in the AI group, 16.70% were arterial, 46.30% venous, and 36.90% capillary. These values were like those obtained in the IVP group, with 16.08% arteries, 48.10% veins, and 35.82% capillaries. However, the addition of reproductive fluids to the embryo culture media implied variations in the vascular proportions, since in the C-IVP group the 13.96% were arterial vessels, 53.26% venous and 32.76% capillaries compared to 17.47%, 44.70% and 37.82% in the RF-IVP group respectively. The vascular density was the only parameter that resulted significantly higher in C-IVP than RF-IVP and AI groups for

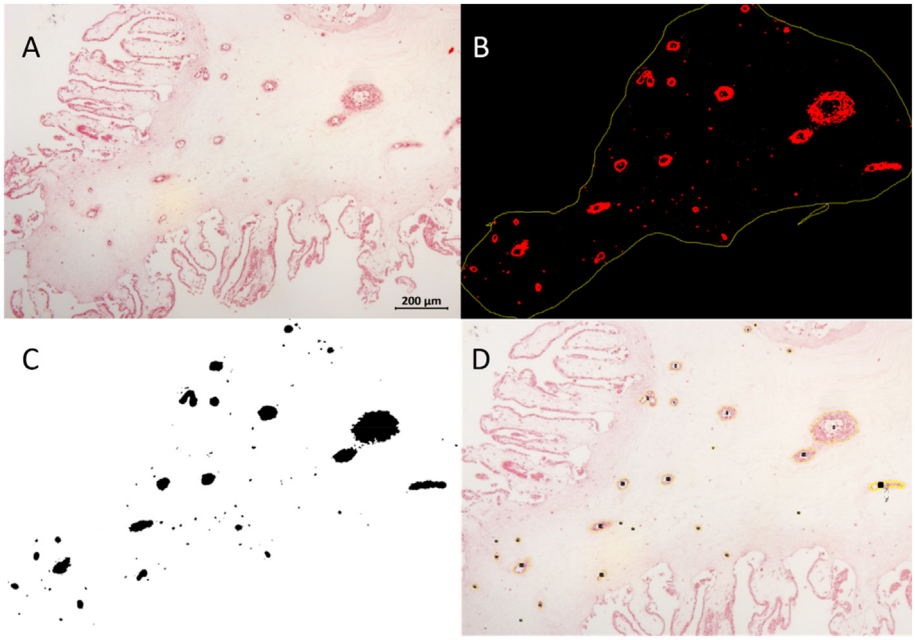

Fig. 1. Vascular analysis of placenta images. A. Placental area to analyze. B. Detected vessels (red) after selecting the fetal mesenchyme (green line) area and running the program macro. C. Detection and fill of the vessels to calculate the vascular area. D. Final identification of each vessel surrounded by a yellow line and numbered for further analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

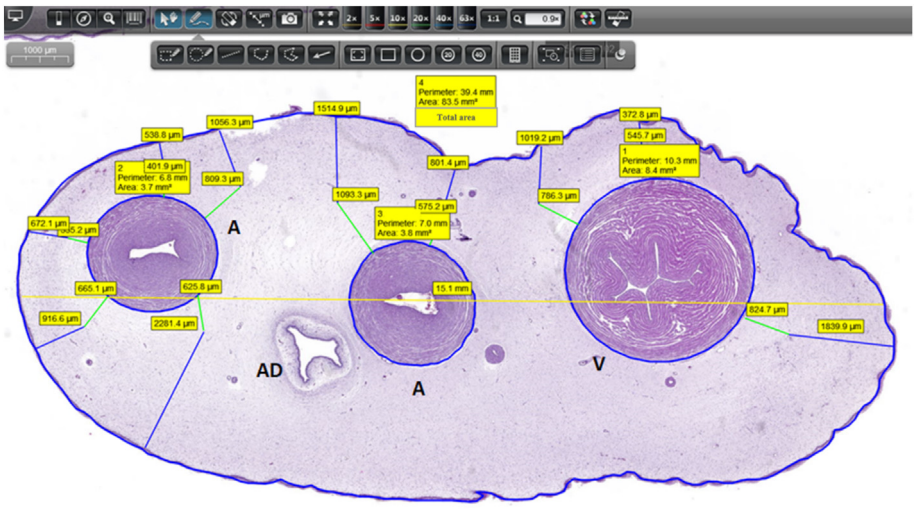

Fig. 2. Vascular and morphometric analysis from a digitalized slide of a pig umbilical cord. The thickness of the intermedia and perivascular zone in Wharton's jelly (WJ) was measured by blue and green lines respectively. The umbilical cord diameter measurement is indicated by the yellow line and the perimeter by the blue line surrounding it. Umbilical arteries (A), umbilical vein (V), and allantoid duct (AD) are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

venous part. Meanwhile, venous density was similar between AI and RF-IVP group. Regarding the vascular area, no significant differences were observed between groups even though C-IVP group showed an upward trend that mainly affected the venous area. The total vascular volume was similar between AI and IVP groups with values around 30 mL.

Focusing on the placental vascular architecture, the number of vessels in the AI and RF-IVP groups were similar regardless of their caliber (Fig. 3A). However, C-IVP group showed a downward trend in all vascular sizes. The vascular area (%) was similar in the three groups, highlighting significant differences ($P < 0.05$) in small caliber vessels (arteriole-venule and small vessels) of C-IVP group compared to the other groups (Fig. 3B). Vascular density resulted in significant differences related to arterioles/venules, small and medium vessels (Fig. 3C), especially related to the greater vascular density of C-IVP group.

3.2. Morphometric and vascular parameters of umbilical cord

Umbilical cords from 8 AI pigs and 18 IVP pigs (5 C-IVP and 13 RF-IVP) were analyzed (Table 3). The umbilical cord perimeter, diameter, and area were significantly higher in IVP than AI pigs ($P < 0.05$), being these differences mainly attributed to C-IVP group, since RF-IVP and AI group showed similar umbilical perimeter, diameter and area results. Within IVP animals, no differences were found between the *in vitro*-derived pigs produced with (RF-IVP) or without (C-IVP) reproductive fluids. Concerning umbilical vascular parameters, no differences were found for total vascular area

Table 2
Placenta vascular parameters in pigs born from artificial insemination (AI) or *in vitro* embryo production (IVP) with (RF-IVP) and without (C-IVP) reproductive fluids. Litters (n): number of litters from which the animals come. Placenta images analyzed (n): histological photographs analyzed (includes the entire section of the slide with 10–15 photos of each animal). Placenta area: average placenta area analyzed in all placenta sections. Total vessels: the amount of examined arteries, veins, or capillaries per placenta. Vascular density: total number of arteries, veins, or capillaries respectively per 1 mm^2 of the analyzed placenta. Vascular area (%): placenta area corresponding to vessels divided by the total placenta area analyzed expressed as a percentage. Arterial/Venous/Capillary area are the arteries, veins or capillaries divided by the total placenta area analyzed. Total vascular volume (mL): percentage of vascular area multiplied by placenta weight in grams. Data are expressed as mean ± SD.

	AI	IVP	C-IVP	RF-IVP
Animals (n)	6	13	5	8
Litters (n)	4	6	3	3
Placenta images analyzed (n)	72	148	55	93
Placenta area analyzed (mm^2)	44.95	65.30	19.43	45.86
Total vessels analyzed (n)	1910	3395	1346	2049
Arteries number	319	546	188	358
Veins number	886	1633	717	916
Capillaries number	705	1216	441	775
Vascular density				
Arterial density	7.69 ± 2.74	8.76 ± 2.41	9.95 ± 2.86	8.02 ± 1.91
Venous density	19.08 ± 4.57	27.69 ± 15.99	39.01 ± 22.26^{y}*	20.61 ± 2.50^{z}
Capillary density	16.22 ± 3.57	19.34 ± 7.48	19.61 ± 8.40	19.16 ± 7.45
Vascular area (%)	19.42 ± 8.06	20.62 ± 6.41	23.11 ± 6.21	19.07 ± 6.41
Arterial area	10.21 ± 4.75	11.64 ± 5.81	11.90 ± 4.27	11.47 ± 6.88
Venous area	8.69 ± 5.74	8.40 ± 6.52	10.60 ± 7.91	7.02 ± 5.60
Capillary area	0.42 ± 0.23	0.59 ± 0.21	0.61 ± 0.25	0.57 ± 0.20
Total vascular volume (mL)	28.62 ± 12.27	32.07 ± 5.96	28.70 ± 1.72	33.77 ± 6.73

^{y,z} denote significant differences between *in vitro* groups (C-IVP *vs.* RF-IVP) ($P < 0.05$).
* denote significant differences between *in vivo* group and any *in vitro* groups (AI *vs.* C-IVP or RF-IVP).

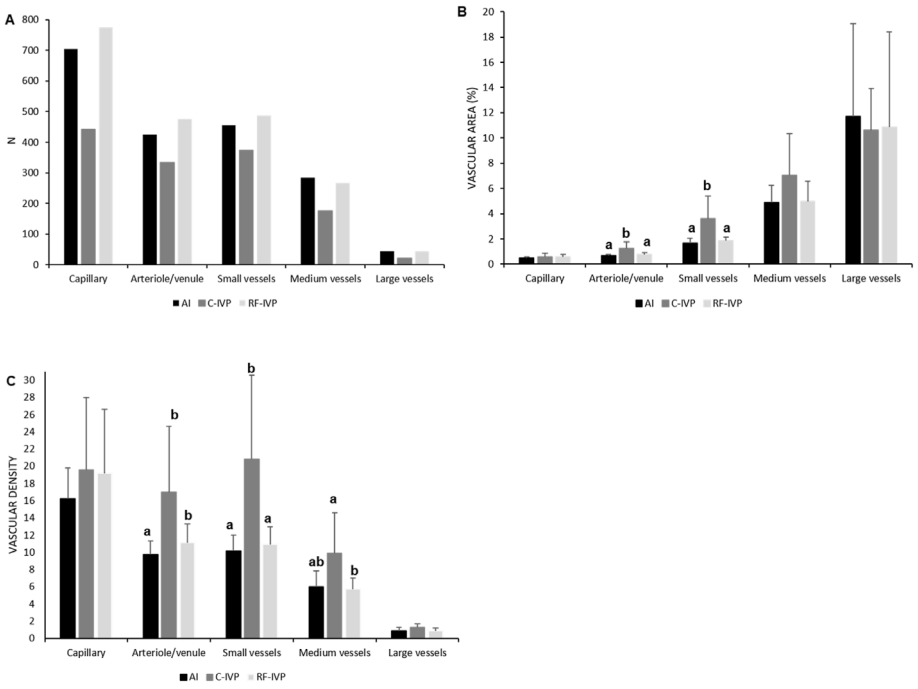

Fig. 3. Placental vascular architecture of pigs obtained through different assisted reproduction techniques: artificial insemination (AI), or *in vitro* embryo production with (RF-IVP) or without (C-IVP) reproductive fluids. A. Number of vessels in the placenta analyzed classified by size. B. Vascular area (%): placenta area corresponding to different size vessels divided by the total placenta area analyzed. C. Vascular density: total number of different size vessels per 1 mm^2 of analyzed placenta. [a,b] denote significant differences between groups ($p < 0.05$). Data are expressed as mean ± SD.

although it was observed a tendency for a higher arterial area in the umbilical cord of IVP pigs (2.45 ± 0.63 mm^2) than AI pigs (1.95 ± 0.42 mm^2) (P = 0.054). The ratios UC area and diameter/Weight birth were similar between groups.

The Wharton's jelly (WJ) area was significantly increased by 26% in IVP compared with AI pigs due to the significantly higher values of C-IVP group, but no differences were observed between C-IVP and RF-IVP groups (Table 4). As for the thickness recorded in perivascular and intermedia areas of WJ, the perivascular area was thicker in the C-IVP group than in the AI and RF-IVP groups, even though no differences were observed between AI and IVP pigs together.

3.3. Correlation between placenta and umbilical cord parameters and growth during the first year of life

ADG was significantly higher in *in vitro*-derived animals with the highest ADG in C-IVP on days 15 and 30, and the RF-IVP group on days 60 and 90 (Table 5). Correlation between ADG and placental and umbilical cord parameters showed an association of placental and umbilical cord vascular phenotype with animal growth during their first year of life (Table 6). In AI-derived animals, growth was strongly linked with placental vascular parameters, where the arterial area in the placenta showed a correlation with the pig's growth from a very early age and through the first year of life. The strong correlation in AI-derived animals observed at 1 year of age should be considered cautiously since this result might be overestimated due to the low number of animals at this age (n = 5). On the other side, morphometric parameters of umbilical cord showed a positive correlation with ADG at the mid-age of 5 months.

In IVP-derived pigs (Table 7), the link between growth and placental vascular phenotype seemed not to be as relevant as for AI animals and only the capillary area was positively and moderately correlated with ADG at 5 months of age. However, umbilical cord phenotype was strongly correlated with IVP piglet growth with almost all variables showing a moderate-high correlation at the

Table 3
Morphometric and vascular parameters of the umbilical cord (UC) in pigs born from artificial insemination (AI) or *in vitro* embryo production (IVP) with (RF-IVP) and without (C-IVP) reproductive fluids. Total vascular area (%): umbilical cord area corresponding to vessels divided by the umbilical cord area. Arterial area: mean area of the two umbilical cord arteries measured in mm^2. Venous area: area of the umbilical cord vein measured in mm^2. Data are expressed as mean ± SD.

	AI	IVP	C-IVP	RF-IVP
Animals (n)	8	18	5	13
UC perimeter (mm)	26.40 ± 3.93[a]	30.51 ± 4.74[b]	32.77 ± 5.32*	29.65 ± 4.39
UC diameter (mm)	8.35 ± 1.01[a]	10.26 ± 1.85[b]	11.08 ± 2.42*	9.94 ± 1.59
UC diameter/Weight birth ratio	7.31 ± 1.71	7.85 ± 1.97	7.59 ± 1.69	7.96 ± 2.11
UC area (mm^2)	43.18 ± 12.87[a]	56.61 ± 14.89[b]	63.90 ± 15.06*	53.79 ± 14.41
UC area/Weight birth ratio	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
Total vascular area (%)	15.33 ± 4.36	13.64 ± 5.04	10.82 ± 3.63	14.70 ± 5.20
Arterial area (mm^2)	1.95 ± 0.42	2.45 ± 0.63	2.68 ± 0.65	2.36 ± 0.66
Venous area (mm^2)	4.36 ± 1.36	5.60 ± 3.02	5.70 ± 1.80	5.57 ± 3.37

[a,b] denote significant differences between AI and IVP groups ($P < 0.05$).
* denote significant differences between *in vivo* group and any *in vitro* groups (AI *vs.* C-IVP or RF-IVP).

Table 4
Morphometric and vascular parameters of Wharton's jelly (WJ) in the umbilical cord of pigs born from artificial insemination (AI) or *in vitro* embryo production (IVP) with (RF-IVP) and without (C-IVP) reproductive fluids. WJ area is the area corresponding to the WJ tissue, calculated as the umbilical cord area minus the vessels area. Data are expressed as mean ± SD.

	AI	IVP	C-IVP	RF-IVP
Animals (n)	8	18	5	13
WJ area (mm^2)	36.86 ± 12.04[a]	48.88 ± 12.80[b]	56.66 ± 11.83*	45.86 ± 12.27
Perivascular WJ thickness (μm)	515.33 ± 177.46	551.77 ± 157.48	698.20 ± 196.05[y]*	495.42 ± 99.73[z]
Intermedia WJ thickness (μm)	1002.77 ± 299.20	1222.27 ± 350.56	1148.84 ± 306.83	1250.53 ± 373.64

[a,b] denote significant differences between AI and IVP groups (P < 0.05).
[y,z] denote significant differences between *in vitro* groups (C-IVP *vs.* RF-IVP) (P < 0.05).
* denote significant differences between *in vivo* group and any *in vitro* groups (AI *vs.* C-IVP or RF-IVP).

early age of 2–3 months (Table 7). Contrary to what was observed in AI-derived animals, data showed that the wider the umbilical cord and WJ measurement were, the lower the growth of IVP-derived pigs at the age of 2–3 months was. This correlation disappeared throughout the animal's life.

4. Discussion

The morphometric and vascular analysis of the placenta and umbilical cord has shown that the embryonic origin (*in vivo* or *in vitro*) influences vascular and morphometric parameters, and that the use of reproductive fluids in the *in vitro* protocols approximates the results to *in vivo* data. Moreover, the placental vascular phenotype impacts the growth of individuals through their first year of life. A large amount of placental tissue analyzed and the detailed vascular study allowed us to determine concise changes in vascularization, since most of available studies are based on analysis made from <1 mm^2 of placental tissue [25,26] whereas from 19 to 65 mm^2 were analyzed in the current study.

Placenta plays a critical role in maintaining and protecting fetal development, whose vascularization is affected by the embryonic origin [10]. Thus, abnormalities such as a decrease in arterial number, lumen size, and branching, have been extensively observed in humans born from IVP-embryos [27]. However, the study of placental vascularization in other species is much more limited [6] being mainly carried out in ruminants [6,10,28,29] while in pigs the effect of embryo origin and ART on placenta vascularization is barely known [4,14,25]. In our study, vascular architecture between AI and RF-IVP groups was similar, but the C-IVP group showed a higher venous density, and a higher vascular area for small-caliber vessels (arteriole/venule and small vessels). These results are in concordance with a previous study in humans reporting a higher number of small-caliber vessels in the ART-derived than in naturally conceived placenta [26]. In the current study, the differences observed between IVP groups might be explained by the use of reproductive fluids. We have described that reproductive fluids IVP favoured the expression of the angiogenic factors PEG3 and LUM in porcine placenta having AI and RF-IVP groups a similar angiogenic gene expression [14]. These results suggest that the inclusion of reproductive fluids in the *in vitro* protocols reproduces to some extent the *in vivo* environment leading to similar placental vascular architecture between *vivo* and *vitro* groups.

Regarding the umbilical cord, changes in umbilical insertion and the absence of one of the arteries have been reported in humans derived from ART with a higher risk of developing anomalies [8,30] although no umbilical changes in the macroscopic morphometry were shown and length and diameter were similar between *in vitro* fertilization and natural conception [8]. Nevertheless, the use of more aggressive and summative ART techniques, such as SCNT, resulted in a larger umbilical diameter in cloned cows [31]. In pigs, a previous study showed that an altered macroscopic appearance of the umbilical cord and a higher umbilical diameter/birth weight ratio resulted in a higher risk of mortality [4,13,19]. As expected, in our study this ratio was similar between groups since pigs with similar birth weights were used. However, the umbilical diameter was significantly higher in IVP pigs as it has been reported in cows cloned by SCNT [31]. The umbilical diameter in IVP-derived pigs showed a non-significant upward trend in the C-IVP whereas RF-IVP and AI groups were similar, therefore suggesting again an approximation to *in vivo* results when natural fluids are included in the *in vitro* protocols. Concerning umbilical vascular parameters, no differences were observed except for a tendency for a higher arterial area in IVP than in AI pigs. In contrast, other authors described smaller arteries [33], thrombosis and histological alterations in the umbilical arterial wall of ART-derived pigs born from SCNT embryos [32]. These differences might be related to the different embryonic origins and the cumulative effect of several

Table 5
Average daily gain (ADG) (gr) in pigs born from artificial insemination (AI) or *in vitro* embryo production (IVP) with (RF-IVP) and without (C-IVP) reproductive fluids during the first year of life. Data are expressed as mean ± SD. d (days), mo (month) and yr (year).

ADG	AI	IVP	C-IVP	RF-IVP
9 d	160.83 ± 68.98	216.43 ± 79.94	248.66 ± 60.26	196.28 ± 87.59
15 d	97.14 ± 33.89[a]	205.48 ± 95.06[b]	277.66 ± 32.57[y]*	160.39 ± 94.01[z]
30 d	140.16 ± 28.79[a]	198.11 ± 48.81[b]	225.69 ± 40.04*	180.86 ± 47.79
1.5 mo	151.57 ± 27.41	168.81 ± 57.60	134.12 ± 63.27	190.50 ± 44.74
2 mo	342.19 ± 41.26[a]	493.91 ± 90.10[b]	445.33 ± 66.57	524.27 ± 92.98*
3 mo	381.67 ± 323.38[a]	714.66 ± 151.41[b]	647.06 ± 120.62	756.90 ± 160.25*
4 mo	764.70 ± 328.39	962.27 ± 346.20	880 ± 364.08	1013.69 ± 349.09
5 mo	1088.89 ± 189.54	1221.34 ± 485.72	1066.67 ± 241.91	1298.67 ± 570.22
6 mo	833.33 ± 264.57	521.04 ± 818.11	528.57 ± 396.41	514.76 ± 1101.27
1 yr	567.10 ± 5.58	592.98 ± 139.84	489.93 ± 68.26	631.62 ± 142.64

[a,b] denote significant differences between AI and IVP groups (P < 0.05).
[y,z] denote significant differences between *in vitro* groups (C-IVP *vs.* RF-IVP) (P < 0.05).
* denote significant differences between *in vivo* group and any *in vitro* groups (AI *vs.* C-IVP or RF-IVP).

Table 6
Pearson's correlation between growth rate (calculated as the average daily gain [ADG]) and placental and umbilical cord vascular parameters during the first year of life in pigs born from **artificial insemination (AI)**. d (days), mo (month) and yr (year).

ADG	Placenta							Umbilical cord				
	Arterial density	Venous density	Capillary density	Vascular area	Arterial area	Venous area	Capillary area	Diameter	Area	Arterial area	WJ area	Perivascular WJ thickness
9 d	−0.034	−0.885	−0.858	−0.878	**−0.975***	−0.424	0.896	0.521	0.564	0.004	0.528	0.565
15 d	0.763	−0.004	−0.115	0.035	−0.171	0.435	−0.032	−0.728	−0.728	−0.232	−0.657	−0.538
30 d	0.738	−0.522	−0.666	−0.189	−0.468	0.435	0.447	−0.713	−0.783	−0.487	−0.728	−0.708
1.5 mo	−0.883	−0.145	0.063	−0.632	−0.386	**−0.955***	0.257	−0.320	−0.535	0.322	−0.571	−0.126
2 mo	0.854	0.307	0.172	0.367	0.172	0.645	−0.354	−0.749	−0.585	0.069	−0.521	−0.483
3 mo	0.004	−0.914	−0.899	−0.855	**−0.969***	−0.373	0.915	0.005	−0.349	**−0.896***	−0.304	−0.179
4 mo	−0.130	−0.931	−0.891	−0.916	**−0.993****	−0.489	0.945	−0.308	−0.463	−0.075	−0.494	−0.208
5 mo	−0.814	−0.869	0.040	−0.968	−0.806	−0.979	0.951	**0.966****	**0.973****	−0.167	**0.966****	**0.900***
6 mo	0.172	−0.821	−0.884	−0.645	−0.882	−0.241	0.689	0.246	0.200	−0.301	0.270	0.527
1 yr	$-1.000^{n/a}$	$-1.000^{n/a}$	$1.000^{n/a}$	$-1.000^{n/a}$	$-1.000^{n/a}$	$-1.000^{n/a}$	$1.000^{n/a}$	0.435	0.722	0.492	0.679	0.659

*P < 0.05. **P < 0.01. $^{n/a}$ overestimated value, n very low (n = 5).

ART when SCNT is used thus leading to greater changes a posteriori. Therefore, the changes in the umbilical diameter, perimeter and area in the present study suggest an involvement of the larger size of the arteries and the rest of the umbilical components that are not vessels. Indeed, the WJ area was higher in IVP-group, specifically the thickness of the perivascular portion in the C-IVP group. Results between AI and RF-IVP groups were similar demonstrating again the beneficial role of natural fluids in IVP protocols to approximate the results to those obtained in naturally conceived pregnancies. These findings are particularly relevant for further research since the perivascular WJ contains most mesenchymal stem cells with the potential to differentiate in a variety of tissues making them extremely useful in the treatment of autoimmune, cardiovascular, haematological, hepatic, and neurodegenerative diseases [33] and they have been recently used as an alternative source of stem cells in transgenic pigs [34].

The clear link between the placenta and umbilical cord parameters with neonatal abnormalities and birth weight has been reported in different species [4,28,35]. However, studies about the likely relationship between placenta and umbilical cord phenotype with long-term growth in pigs are not so numerous, much less in IVP-derived animals. We have previously observed that IVP-derived piglets (C-IVP and RF-IVP) had equal rate of live-born piglets/born piglets, litter size, and number of piglets alive at 3 and 15 days of age. Moreover, weight birth of male piglets was similar between AI and IVP groups. Only birth weight of female piglets was higher in IVP than AI group [18]. To our knowledge, this is the first study showing a correlation between umbilical cord parameters and body weight in IVP-derived pigs. Even with a discrete number of animals, it has been demonstrated that both placental vascularization and umbilical cord morphometry are correlated negatively with early, mid, and long-term growth being the role of the umbilical cord particularly relevant in IVP-derived pigs. Our results are coincident with a previous study showing the strong association between the umbilical cord diameter with pre-weaning mortality in AI-derived pigs as well as the link between the umbilical cord diameter and umbilical cord diameter/birth weight ratio with pig's weight at 5 months [19]. However, it must be considered that some confusing effects derived from genetic lines and insemination might be affecting the results. Despite all pigs in present study (AI and IVP) were conceived from the same boar, genetic differences from the maternal line (crossbreed animals in IVP groups) could be affecting some of these results. Also, it cannot be ruled out the possibility that the effect of crossbreeding may be compounded by the effect of epigenetic marks resulting from the *in vitro* fertilization and culture of the embryos in IVP groups [36], and that a cumulative effect may have been produced thus affecting the growth differences observed between groups. Even it must be considered that in this study animals from AI group were inseminated but animals from IVP-group were not. So the likely effect of seminal plasma on further expression of vascular growth factors at the genital tract [37] cannot be ruled out. Therefore, the participation of different "confusing effect" factors should be considered and further studies performed.

In conclusion, the detailed study on the vascular categorization performed by size and type of vessel (artery, vein, and capillary) in a wide area of placenta tissue has revealed a different vascularization of porcine ART-derived placenta. These modifications in

Table 7
Pearson's correlation between growth rate (calculated as the average daily gain [ADG]) and placental and umbilical cord vascular parameters during the first year of life in pigs born from *in vitro* produced-embryos (IVP). d (days), mo (month) and yr (year).

ADG	Placenta			Umbilical cord					
	Arterial density	Vascular area	Capillary area	Perimeter	Diameter	Area	Venous area	WJ area	Intermedia WJ thickness
9 d	−0.053	**−0.617***	−0.070	0.190	0.290	0.220	0.149	0.473	0.276
15 d	**−0.578***	0.053	0.153	0.154	0.092	0.258	−0.131	0.465	0.356
30 d	0.077	0.249	−0.476	−0.335	−0.147	−0.279	−0.105	−0.311	−0.277
1.5 mo	−0.478	−0.400	−0.195	−0.321	−0.295	−0.205	0.073	−0.084	−0.214
2 mo	−0.098	−0.084	−0.480	**−0.764****	**−0.615****	**−0.736****	**−0.636****	−0.395	**−0.700****
3 mo	−0.265	−0.130	0.279	−0.187	−0.059	−0.125	−0.270	**−0.484***	−0.123
4 mo	−0.252	0.139	−0.215	−0.349	−0.547*	−0.464	−0.406	−0.014	−0.419
5 mo	−0.086	−0.003	**0.695***	0.352	0.123	0.238	0.217	0.108	0.179
6 mo	0.169	0.357	0.121	−0.015	−0.062	−0.176	−0.279	−0.134	−0.176
1 yr	−0.110	−0.193	−0.476	−0.378	−0.265	−0.463	−0.467	−0.146	−0.412

*P < 0.05. **P < 0.01.

vascularization are attenuated when natural fluids are used in the IVP protocols approximating the results to those observed *in vivo* as we have already described in other phenotypic parameters [38]. So, enrichment of IVP culture media with reproductive fluids contribute to reduce phenotypical [39]differences between in vitro-derived and artificial insemination-derived piglets. Moreover, the umbilical cord perimeter, diameter area, and WJ area were bigger in IVP-derived pigs. Finally, there is a correlation between placental vascularization and umbilical cord morphometry with pig body weight through their first year of life.

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Ethical approval

This study was approved by the University of Murcia CEEA (Comité Ético de Experimentación Animal). Animal experiments were performed after approval of the "Dirección General de Agricultura, Ganadería, Pesca y Acuicultura" – Región de Murcia-nr. A13170706.

Informed consent

No informed consent is required for this study.

CRediT authorship contribution statement

Ester Párraga-Ros: Conceptualization, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Úrsula Álvarez-Martín:** Data curation, Investigation, Writing – original draft. **Juan Seva:** Resources, Writing – review & editing. **Pilar Coy:** Resources, Writing – review & editing, Funding acquisition. **Raquel Romar:** Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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