Morphometric study of placental and umbilical cord vascularization in pigs derived from assisted reproductive technologies

Trabajo Fin de Máster (TFM) del Máster Biología y Tecnología de la Reproducción en Mamíferos

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DECLARACIÓN EXPLÍCITA FIRMADA DE LA ASUNCIÓN DE LA ORIGINALIDAD DEL TRABAJO

Úrsula Álvarez Martín con DNI 72894744Q, autora del Trabajo de Fin de Máster del título Máster Universitario en "Biología y Tecnología de la Reproducción en Mamíferos", declaro que el trabajo, en lo que a su autoría se refiere, es original y que todas las fuentes utilizadas para su elaboración han sido debidamente citadas.

En Murcia a 31 de agosto de 2022

Contents

Abstract

Animals born from *in-vitro* produced (IVP) embryos show abnormalities in the placenta and umbilical cord that may be related to the vascularization. Moreover, placental, and umbilical functions are known to affect fetal growth and pup weight both in animals and humans, but their long-term effects are still unknown. The aim of this study is to analyze the placental and umbilical vascular morphometry in pigs (n=19) born by means of artificial insemination (AI group) and after transfer of IVP embryos cultured in presence (RF-IVP group) or absence (C-IVP group) of reproductive fluids in order to assess the relationship between vascular parameters and the average daily gain from birth to 3.5 years age. Placenta and umbilical cord samples were collected after birth and fixed in 10% formaldehyde for paraffin embedding. Histological sections of 5 μm thickness were cut, stained with hematoxylin-eosin and photographed at 5x for vascular and morphometric analysis with ImageJ® and Slide Viewer®. Placental vascular morphometry was similar among groups, but the vascular area of small vessels (arterioles-venules and small vessels) was higher in the C-IVP group. Regarding the umbilical cord, the area (AI 43.1±12.8, IVP 56.6±14.9 mm^2), perimeter (AI 26.4±3.9, IVP 30.5±4.7 mm^2), diameter (AI 8.4±1.0, IVP 10.3±1.9 mm^2) and Wharton's gelatin area (AI 36.9±12.0, IVP 48.9±12.8 mm^2) were significantly higher in IVP-derived than AI-derived animals, whereas arterial and venous morphometric data were similar between groups (AI: 1.97±0.4 y 4.4±1.4 mm^2, IVP: 2.5±0.6 y 5.6±3.0 mm^2). Several placental vascular and morphometric parameters were correlated with growth of animals from birth up to 3.5 years age. In conclusion, long-term growth of IVP-produced animals seems to be related to placental and umbilical cord vascular development where the provision of reproductive fluids in IVP improves results resembling those *in-vivo*.

Keywords: *in-vitro* production, reproductive fluids, placenta, umbilical cord, vascularization, pigs.

Resumen

El uso de las técnicas de reproducción asistida (ARTs) es beneficioso para la producción animal. Sin embargo, las investigaciones sobre el uso de estas técnicas en diversas especies muestran la aparición de anomalías en la placenta y en el cordón umbilical que puede estar relacionadas con la vascularización de embriones producidos *in-vitro* (IVP). De hecho, se sabe que el funcionamiento placentario y umbilical afecta al crecimiento fetal y el peso de las crías tanto en animales como en humanos, pero sus efectos a largo plazo son aún desconocidos. El objetivo de este estudio es analizar la morfometría vascular placentaria y umbilical de embriones porcinos producidos *in-vivo* por inseminación artificial (AI) e *in-vitro* añadiendo (RF-IVP) o no (C-IVP) fluidos reproductivos al medio de cultivo; y estudiar la relación entre los parámetros vasculares y la ganancia media diaria de los cerdos durante su crecimiento. Las muestras de placenta y cordón umbilical se recogieron después del nacimiento y fueron fijadas en formaldehído al 10 % para su inclusión en parafina. Se cortaron secciones histológicas de 5 μm de grosor que fueron teñidas con hematoxilina-eosina y fotografiadas a 5x para el análisis vascular y morfométrico con ImageJ® y Slide Viewer®. Los resultados de la morfometría vascular placentaria fueron similares entre los tres grupos. Sin embargo, el área vascular en los vasos de pequeño calibre (arteriolas-vénulas y vasos pequeños) fue significativamente más alto en el grupo C-IVP respecto de los otros dos grupos. En cuanto al cordón umbilical, el área (AI 43.1±12.8, IVP 56.6±14.9 mm^2), perímetro (AI 26.4±3.9, IVP 30.5±4.7 mm), diámetro (AI 8.4±1.0, IVP 10.3±1.9 mm) y gelatina de Wharton (AI 36.9±12.0, IVP 48.9±12.8 mm^2) fueron significativamente más altos en los embriones producidos *in-vitro*, mientras que los parámetros morfométricos arterial y venoso fueron similares entre los grupos (AI: 1.97±0.4 y 4.4±1.4 mm^2, IVP: 2.5±0.6 y 5.6±3.0 mm^2). Estos hallazgos demuestran que el crecimiento de los cerdos puede estar relacionado con el desarrollo vascular de la placenta y del cordón umbilical, donde el aporte de fluidos reproductivos en las IVP mejora los resultados asemejándose a aquellos *in-vivo*.

Palabras clave: producción *in-vitro*, placenta, fluidos reproductivos, cordón umbilical, vascularización, cerdos.

1. Introduction

The assisted reproductive technologies (ARTs) have numerous major effects on placental and fetal growth and development when is compared to natural pregnancies, as well as offspring outcome in several species. Some of those effects have been described in both cloned embryo transfer and *in-vitro* fertilization, which impaired placental steroid metabolism, altered placental vascularization, placental/fetus ratio and abnormal expression of angiogenic factors were observed (Reynolds et al., 2014). In animal production, the use of ARTs implies high benefits such a high genetic potential and productivity for the livestock companies. The artificial insemination (AI) is the most used reproductive technique of sow breeding (Knox, 2016). However, the *in-vitro* production (IVP), embryo transfer and somatic cell nuclear transfer are still being investigated (Ao et al., 2017; Martinez et al., 2019; Valadão, 2020) due to their high incidence of polyspermy, high rate of neonatal mortality, low capacity for embryonic development and the low viable embryo culture (Ao et al., 2017; Valadão, 2020). Furthermore, the selection and handling of donor and recipient sows added to the embryo transfer procedure have an impact on pigs' production (Martinez et al., 2019).

Changes derived from the use of ARTs have already been observed in the placenta (Palmieri et al., 2008; Delle Piane et al., 2010). The placental autophagy (Toschi et al., 2016) and placental abnormalities such as differences in size and abnormal vascularization have been previously observed in animals (Fidanza et al., 2014; Yanaihara et al., 2018; Sacha et al., 2022). Specifically in pigs, it has been shown that fetal growth and mortality are highly associated with the placental performance and umbilical cord development (van Rens et al., 2005; Rootwelt et al., 2013). In fact, aberrant angiogenic factors in placentas of IVP derived pigs have been confirmed (París-Oller et al., 2021) but the use of reproductive fluids in the *in-vitro* culture seemed to resemble the placental genetic expression observed in AI animals. The aberrant placental expression of PEG3 gene and the angiogenic factor LUM in IVP animals generated with reproductive fluids (RF-IVP) were avoided, whereas IVP pigs produced without reproductive fluids (C-IVP) had an abnormal expression of PEG3 and LUM (París-Oller et al., 2021).

Birth weight, litter size, farrowing period, birth order, environmental temperature, nutritional state, health, gender, and mother and piglet behavior are some of the factors that affect piglets' pre-weaning survival (Panzardi et al., 2013). Moreover, other factors such as nutrition and oxygen transport that are mediated by placental development and adequate umbilical cord function have immediate and long-term effects on placental phenotype and affect the growth and wellbeing of offspring throughout adulthood (Sferruzzi-Perri & Camm, 2016). The performance of the placenta and umbilical cord influences fetal growth and offspring weight, but little is known about its effects in long-term ART-derived pigs. Its relationship with placental vascularization or whether there are placental vascular compensatory mechanisms in embryos derived from IVP, as in other species, is also not well known (Miles et al., 2004).

Hence, the detailed study of placental vessels development and umbilical cord performance would be useful to know the impact of IVP in placental vascularization and if there is a link the placental phenotype to long-term growth. In addition, the relationship between the placenta and umbilical cord performance and the pigs' growth needs to be investigated. Thus, the aim of this study was to perform the morphometric study of the vascularization in placenta and umbilical cord of pigs derived from AI and *in-vitro* production using reproductive fluids (RF-IVP) or not (C-IVP) in the culture medium, as well as to determine the relationship between these findings and the average gain daily of the pigs up to 3.5 years age.

2. Materials and methods

2.1. Animals

This study was performed in accordance with 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.

Same genetic line crossbred sows (commercial crossbreed Landrace x Large White) were used. All pigs were accommodated and nourish under the same conditions while water was supplied *ad libitum*.

Sows' estrous synchronization was performed after weaning and they were chosen as donors or recipients when showing heat signs 4 to 5 days after weaning. Estrous detection was conducted in two ways; showing a vasectomized boar to the sows to stimulate estrous expression and performing the back pressure test. Sows were considered in heat when they remain immobilized under back pressure. Sows were inseminated with same IVP boar semen after estrous beginning.

Three experimental groups were obtained from different embryo origin, one of the pigs born from *in-vivo* embryos (the latter born by artificial insemination; AI group) and two groups of *in-vitro*-produced (IVP) embryos that were produced with (RF-IVP group) and without (C-IVP group) reproductive fluids into the culture. IVP embryos were produced after *in-vitro* fertilization of *in-vitro* matured oocytes and further culture up to blastocysts stage in media supplemented with or without porcine oviductal fluid as described previously (París-Oller et al., 2021). All embryos (AI, RF-IVP and C-IVP) were produced with spermatozoa from the same boar.

2.2. In-vitro *production and embryo transfer*

Prepubertal crossbred gilts (Landrace x Large White) ovaries were collected at the slaughterhouse and carried to the laboratory in saline solution with 100 mg/mL kanamycin sulfate at 38.5°C. They were washed in 0.04% (w/v) cetrimide solution once and in saline solution twice. Cumulus cell-oocyte complexes (COCs) were obtained from 3-6 mm diameter antral follicles then washed in Dulbecco's PBS (DPBS) supplemented with 1 mg/mL polyvinyl alcohol (PVA) twice and in NCSU-37 (maturation medium) supplemented as previously described (Canovas et al., 2017) previously equilibrated for a minimum of 3 hours at 38.5°C under 5% CO_2 in air twice.50-55 COCs were cultured in 500 µL maturation medium with dibutyryl cAMP, eCG and hCG at 38.5°C under 5% CO_2 in air for 22h. After that they were washed twice and cultured in fresh maturation medium without hormones for 20-22 h (Funahashi et al., 1997).

Cumulus cells were partially detached mechanically after maturation and mature oocytes were split in two groups for *in-vitro* fertilization.

The C-IVP group contained 3 mg/mL bovine serum albumin and the RF-IVP group had 3 mg/mL BSA-FAF and 1% (v/v) porcine oviductal fluid (POF) from the late follicular phase of the estrous cycle (NaturARTs® POF-LF, EmbryoCloud, Murcia, Spain). Fertility-tested boar ejaculated spermatozoa was used. IVF sperm was selected with NaturARTs® PIG sperm swim up medium (EmbryoCloud, Murcia, Spain) supplemented with 0.5% BSA for C-IVP or 1% (v/v) POF from late follicular phase of the estrous cycle (NaturARTs® POF-LF, EmbryoCloud, Murcia, Spain) for RF-IVP group. Spermatozoa concentration and motility were tested, and sperm were diluted to get a concentration of $5–15\times10^3$ spermatozoa/mL. For *in-vitro* fertilization oocytes and spermatozoa were cultured during 20 h at 38.5°C under 5% CO_2.

NCSU-23 medium was used as embryo culture medium and it was supplemented as previously described (Canovas et al., 2017). Zygotes were cultured in NCSU23-A in the presence or absence of 1% (v/v) POF from the early luteal phase (NaturARTs® POF-EL, EmbryoCloud, Murcia, Spain) for the RF-IVP and C-IVP, respectively. Embryo culture was preferred at 38.5ºC under 5% CO_2 and 7% O_2.

After cleavage evaluation 2-4 cell embryos were washed and passed to NCSU23-B medium with or without 1% (v/v) of porcine uterine fluid (PUF) from mid luteal phase of the estrous cycle (NaturARTs® PUF-ML, EmbryoCloud, Murcia, Spain) for the RF-IVP and C-IVP, respectively. Embryos were cultured at 38.5 °C under 5% CO_2 and 7% O_2 until day 5-6. Blastocysts were transferred by single-site paralumbar laparo-endoscopy at day 7 post-*in-vitro* fertilization.

2.3. Pregnancy diagnosis, data and samples collection

Ultrasonography was used to diagnose pregnancy at 21-26 day after the beginning of estrous. At the farrowing, the sows were housed in separate cages to allow contact with the piglets and prevent crushing. Day of birth, length of gestation, farrowing rate, survival rate, and litter size were recorded. After birth, the piglets were weighed and measured with a digital hanging scale.

Placentas were identified as previously described (Ao et al., 2017) and the umbilical cords were labeled to have sample traceability (París-Oller et al., 2021). After the placental expulsion, its weight (g) and its area (cm^2) were calculated by image analysis performed on tracing its contour on paper (ImageJ® 1.52a software, National Institute of Health, USA). Placenta and umbilical cord samples were taken and immediately fixed in 10% buffered formalin solution for histological study.

Placenta efficiency (g/g), which indicates the grams of fetus produced per gram of placenta, was calculated as piglet birth weight / placental weight.

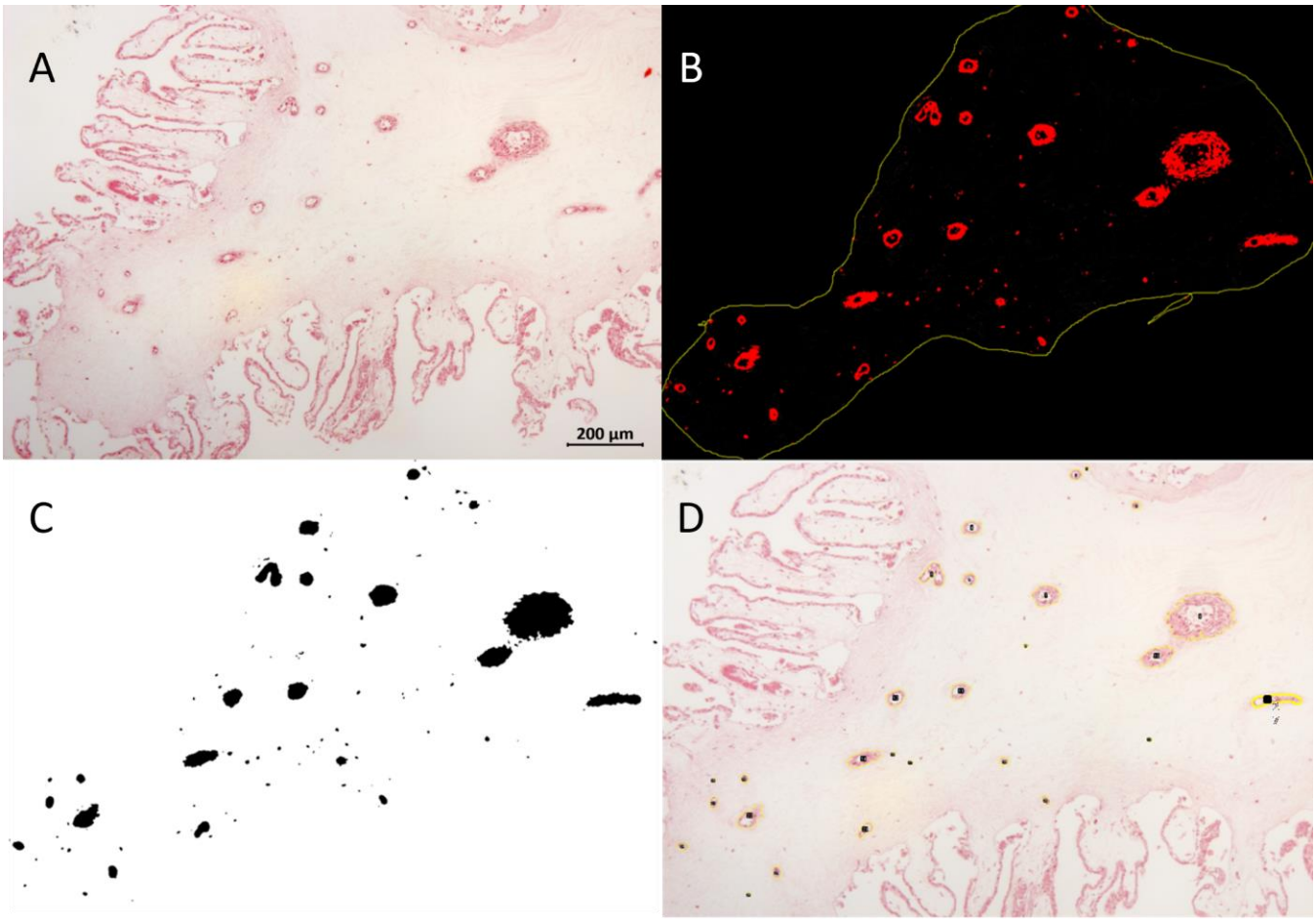
Pigs were weighed throughout their life (days 9, 15, 30, 45, 60, 90, 120, 150, 180, 365, 915, 1129 and 1283) and average gain daily (AGD) was quantified by the following formula:

$$AGD = (\text{last weight} - \text{previous weight}) / (\text{last date} - \text{previous date}).$$

2.4. Histological analysis

Fixed placental and umbilical cord samples were routinely processed for paraffin embedding, and sections of 5 µm thickness were stained with Hematoxylin-Eosin (H&E). The slides of placenta were photographed at 5x (Microscope Software ZEN 3.2 lite, Zeiss®) and the images were analyzed (ImageJ® software. NIH) to get the blood vessel number, vessel area, total vascular area and total analyzed placental area (Figure 1). The blood vessels were classified by two expert operators based on their size (Seidmann et al., 2017), wall and lumen characteristics: wide irregular lumen and thin wall for veins, and round lumen and thick wall for arteries (Gazquez & Blanco, 2004). The classification of the vessels was the following: 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 (>30000 μm^2).

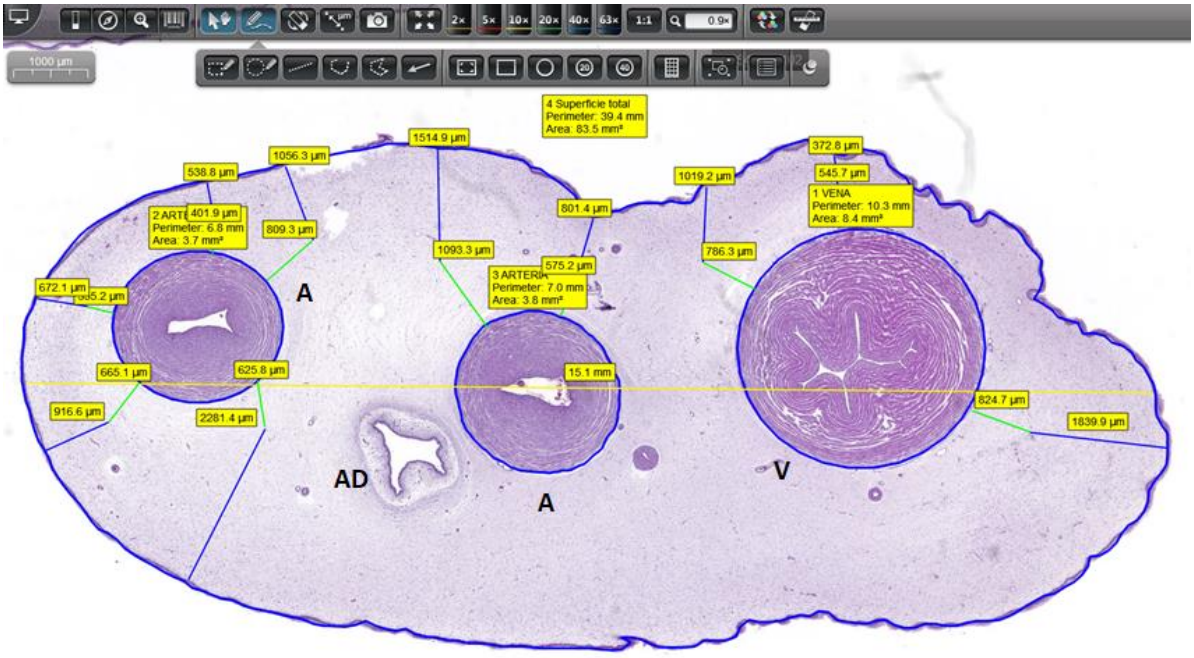
Figure 1. Analysis of blood vessels in the placenta image. *A. Placental area to analyze. B. Selection of the fetal mesenchyme area. After running macro, image showing the detected vessels (red). C. Detection and fill the vessels to calculate the vascular area. D. Final image with each vessel marked (surrounded yellow line) and labeled with a number in the late analysis.*



The umbilical cord slides were digitalized 0.172 pixel/µm resolution at 20x (Pannoramic MIDI II scanner3D Histech®, Budapest, Hungría) and analyzed by two expert operators (Virtual Microscope Software Slide Viewer 2.5® 3D Histech) to calculate the arteries area, vein area, Wharton Jelly (WJ) area and thickness, umbilical cord area, perimeter, and diameter (Figure 2). Umbilical cord diameter (Ghezzi et al., 2003) was measured tracing a line between the outer-to-outer border from the long-axis view of the cord (Figure 2).

The blood vessels were categorized on their size and wall characteristics (Jordan, 1919). Pig umbilical cord has only one vein, with a higher caliber than arteries, and tunica media has wider scattered muscular tissue fibers. While 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 its connective tissue. The perivascular zone has a typical mucous connective tissue, where stand out fusiform cells widely separated and capillaries, arterioles, and venules. The intermediate zone has also mucous connective tissue but with rounded cells, small bundles of collagen fibrils and less capillaries (Jordan, 1919).

Figure 2. Vascular and morphometric analysis in digitalized slide of umbilical cord. *The thickness of the intermedia and perivascular zone in Wharton's jelly was measured by blue and green lines* respectively. *Umbilical cord diameter measure is shown with yellow line. Arteries (A), vein (V) and allantoid duct (AD) are shown.*



2.5. Statistical analysis.

One-way ANOVA test and Tuckey's test with the SPSS® v28 software (IBM, USA) were used to determine significant differences ($p<0.05$) between both experimental groups for each parameters studied.

In addition, the correlation analysis with Pearson's test ($p< 0.05$) was carried out to check the relationship between the experimental groups and all the parameters studied. The data 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) (Mukaka, 2012).

3. Results

A total of 19 porcine placentas of animals derived from different embryo origin were studied: 6 animals born by *in-vivo* techniques (AI group) and 13 animals born by *in-vitro* techniques (8 produced with reproductive fluids, RF-IVP group; and 5 without fluids, C-IVP group).

The piglet birth weight, placenta weight, placenta efficiency and placenta ratio in the 3 groups were similar ($p> 0.05$) (Table 1). The placenta sections analyzed were 72, 55 and 93 in the AI, C-IVP and RF-IVP groups respectively. The placenta area analyzed ranged 19.4-45.9 mm^2 (Table 1).

The analyzed vessels varied from 1910 to 2049 (Table 2). Regarding the vascularization of the analyzed area, the vascular area was higher in C-IVP group. However, no statistical differences were found with the rest of the groups. Total vascular volume was higher in RF-IVP group while it was similar between the other two groups. Arteries, veins and capillaries parameters were similar among groups ($p>0.05$), nevertheless the % vascular area was higher in the C-IVP, especially in venous vascularization. Furthermore, the vascular number density was higher in the C-IVP group in capillary vascularization (Table 2).

Table 1. Placenta morphometric parameters analyzed from in-vivo (AI) and in-vitro (C-IVP and RF-IVP) pig embryo origin. *Placenta efficiency: rate between animal weight birth in grams divided by placenta weight in grams. Placenta ratio: placenta weight divided by animal weight birth. Placenta sections analyzed: number of placenta pictures analyzed. Placenta area analyzed: average of placenta area analyzed in all placenta sections.*

		Embryo origin			***In-vitro* embryo (IVP)**	
		***IN-VIVO* (AI)**	***IN-VITRO* (IVP)**		**C-IVP**	**RF-IVP**
Animals (N)		6	13		5	8
Sex	Female	3	8		4	4
	Male	3	5		1	4
Animal weight birth (g)		1363 ± 226.1	1478.5 ± 274,8		1468 ± 173.6	1485 ± 334.8
Placenta weight (g)		158.3 ± 67.1	189.0 ± 64.4 (n=10)		150.0 ± 43.6	205.7 ± 2.1
Placenta area (cm^2)		1735.3 ± 289.5	1651.3 ± 295.5 (n=11)		1702.6 ± 313.7	1608.6 ±301.9 (n=6)
Placenta efficiency (g/g)		9.6 ± 3,4	8.3 ± 2.8 (n=10)		10.3 ± 4.4 (n=3)	7.5 ± 5.4 (n=7)
Placenta ratio (g/g)		0.12 ± 0.03	0.13 ± 0.04 (n=10)		0.10 ± 0.05 (n=3)	0.14 ± 0.06 (n=7)
Placental sections analyzed (n)		72	148		55	93
Placenta area analyzed (mm^2)		44.9	65.3		19.4	45.9

Data are expressed as mean ± SD.

No statistical differences were found.

Table 2. Vascular data in the placenta of pigs obtained from in-vivo (AI) and in-vitro (C-IVP and RF-IVP) pig embryo origin. *Total vessels analyzed: amount of examined vessels per placenta. Vascular area (%): placenta area corresponding to vessels divided by the total placenta area analyzed expressed as percentage. Total vascular volume (mL): percentage of vascular area multiplied by placenta weight in grams. Vessels number (%): number of vessels (arteries, veins or capillaries) in the placenta, expressed as percentage. Arterial/Venous/Capillary: placenta area corresponding to arteries, veins or capillaries respectively divided by the total placenta area analyzed. Arterial/Venous/Capillary number density: total number of arteries, veins, or capillaries respectively per 1 mm^2 of placenta.*

	Embryo origin		***In-vitro* embryo (IVP)**	
	***IN-VIVO* (AI)**	***IN-VITRO* (IVP)**	**C-IVP**	**RF-IVP**
Animals (N)	6	13	5	8
Total vessels analyzed (N)	1910	3395	1346	2049
Vascular area (%)	19.4 ± 8,1	20.6 ± 6.4	23.1 ± 6.2	19.1 ± 6.4
Total vascular volume (mL)	28.6 ± 12.3	37.5 ± 18.1 (n=10)	28.7 ± 1.7 (n=3)	41.3 ± 20.9 (n=7)
Arteries				
Vessels number (%)	319 (16.7)	273 (15.8)	188 (14.0)	358 (17.5)
Arterial area (%)	10.2 ± 4.8	11.6 ± 5.8	11.90 ± 4.3	11.47 ± 6.9
Arterial number density	0.05 ± 0.01	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.02
Veins				
Vessels number (%)	886 (46.4)	817 (49.0)	717 (53.3)	916 (44.7)
Venous area (%)	8.7 ± 5.7	8.4 ± 6.5	10.6 ± 7.9	7.0 ± 5.6
Venous number density	0.15 ± 0.07	0.13 ± 0.06	0.14 ± 0.09	0.11 ± 0.03
Capillaries				
Vessels number (%)	705 (36.9)	608 (35.3)	441 (32,7)	775 (37,8)
Capillar area (%)	0.4 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.2
Capillar number density	0.12 ± 0.04	0.09 ± 0.04	0.88 ± 0.07	0.10 ± 0.02

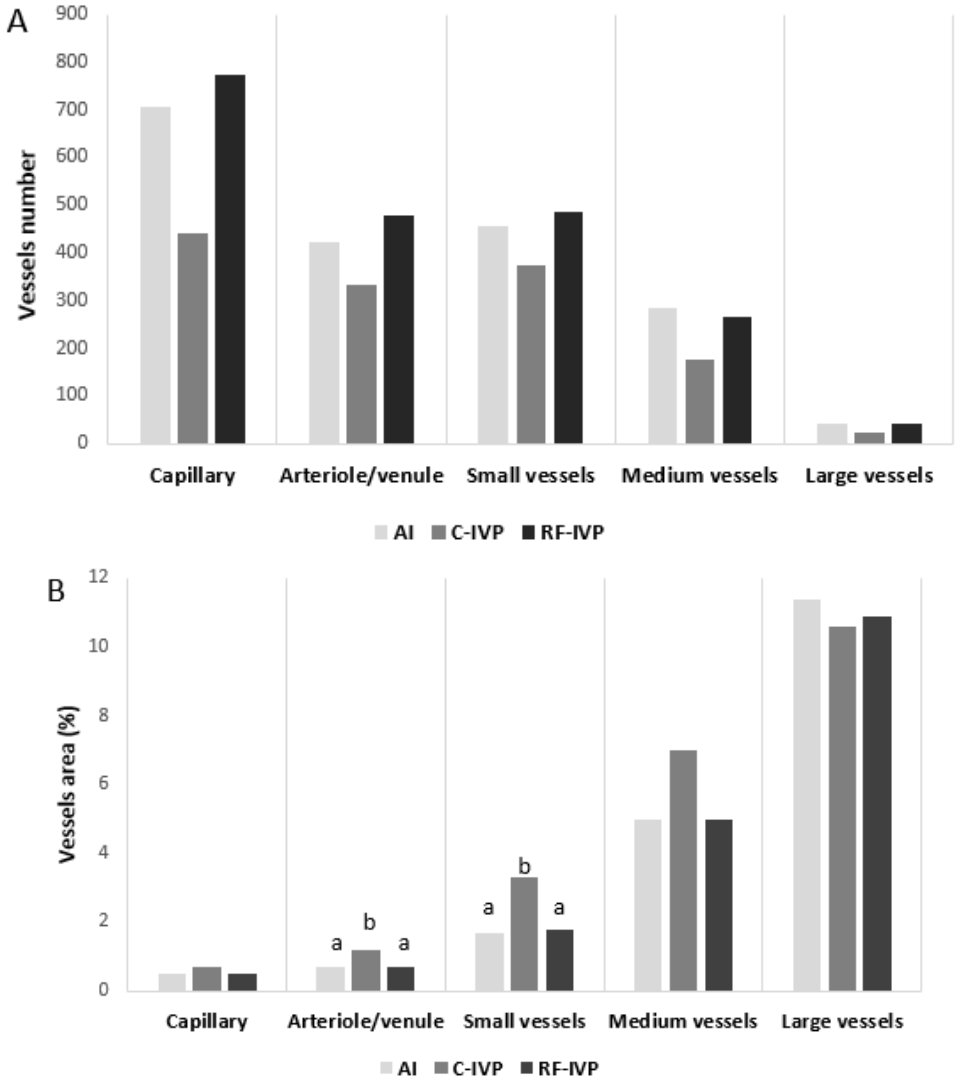
Data are expressed as mean ± SD.

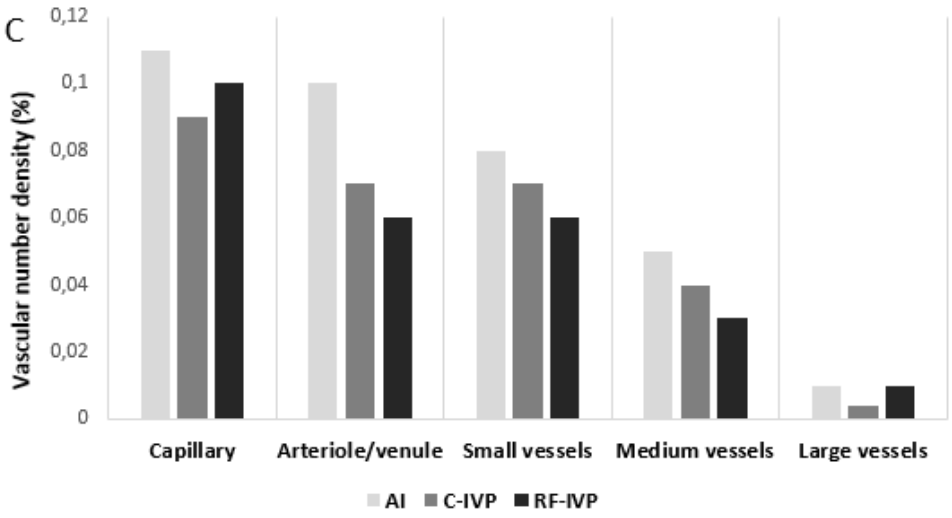
No statistical differences were found.

Focusing on the placental vascular architecture (Figure 3), the values of the number of vessels in the AI and RF-IVP groups were similar regardless of their caliber. However, C-IVP group showed a lower number of vessels in all sections ($p>0.05$).

The vessels area (%) and vessels number density (%) were 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.

***Figure 3**. **Placental vascular architecture of pigs obtained through different assisted reproduction techniques. A**. The vessels number in the placenta classified by size. **B**. The vessels area (%), which is the placenta area corresponding to different size vessels divided by the total placenta area analyzed. **C.** The vessels number density that is the total number of different size vessels per 1 mm^2 of placenta. Statistically significant differences are expressed with different letter a-b (p< 0,05).*





Correlation was measured between AGD and placental vascular and morphometric parameters (Table 3). Moderate positive and negative correlation was found at day 45 AGD regarding placenta weight and placenta efficiency respectively. The vascular area (%) was moderate negatively correlated with AGD at day 9. Very high negative correlation was found in AGD at day 1129 with venous area and moderate-high positive correlation in the capillary area were found at days 90 and 150 respectively. The correlation in vessel number density parameters (Table 3) was moderate negatively at days 15 and 45 for arterial number density, high negatively at day 1129 for venous number density and moderate negatively at day 120 for capillary.

Table 3. Correlation between the average gain daily (AGD) at different days and placenta morphometric parameters *(Pearson's linear correlation coefficient)**.** Vascular area (%), arterial area, venous area, capillary area, arterial number density, venous number density, capillary number density, total vascular volume, placenta weight and placenta efficiency.*

Age (days)	Vascular area (%)	Arterial area	Venous area	Capillary area	Arterial number density	Venous number density	Capillary number density	Total vascular volume	Placenta weight	Placenta efficiency
9	-0.544*	-0.433	-0.193	0.233	-0.144	-0.350	-0.250	-0.353	-0.012	0.122
15	0.154	0.295	-0.114	0.276	-0.547*	-0.039	-0.262	-0.003	-0.039	0.051
30	0.272	-0.060	0.364	-0.077	-0.038	0.049	-0.427	-0.010	-0.331	0.051
45	-0.365	-0.126	-0.277	-0.065	-0.522*	-0.226	-0.274	0.359	0.686**	-0.597*
60	0.119	0.036	0.105	-0.086	-0.216	0.046	-0.347	0.450	0.228	-0.462
90	-0.147	0.021	-0.190	0.605*	-0.320	-0.381	-0.349	0.314	0.437	-0.300
120	-0.008	-0.157	0.141	0,141	-0.293	-0.167	-0.506*	-0.135	0.054	-0.255
150	-0.010	0.256	-0.261	0.706**	-0.165	-0.127	0.340	-0.058	-0.019	0.335
180	0.206	0.157	0.092	0.113	0.199	0.090	0.076	0.173	-0.079	-0.146
365	-0.142	-0.160	0.042	-0.459	-0.122	0.094	-0.324	0.019	0.150	-0.339
915	-0.299	0.813	-0.832	0.800	-0.529	0.038	0.458	-0.627	0.350	0.280
1129	-0.799	0.379	-0.942**	0.546	-0.413	-0.816*	0.084	0.080	0.476	-0.041
1283	0.405	0.069	0.711	-0.317	0.576	-0.116	-0.081	0.115	-0.043	-0.038

** Significative correlation 0.01. *Significative correlation 0.05. Correlation interpretation: Very high correlation (1.00-0.90), high correlation (0.89-0.70), moderate correlation (0.69-0.50), low correlation (0.49-0.30) and negligible correlation (0.29-0.00).

The umbilical cord was studied in 8 *in-vivo* and 18 *in-vitro* pigs. Cord area, perimeter, and diameter (Table 4) were significantly higher in the *in-vitro* group ($p < 0.05$) due to the high values of C-IVP group mainly. Concerning vascular parameters, a smaller arterial area was found in the *in-vivo* group without statistical differences with the *in-vitro* group, where the values of C-IVP and RF-IVP were similar. The venous area with similar values between all groups were found.

Table 4. morphometric and vascular parameters of the umbilical cord (UC) in pigs derived from in-vivo (AI) and in-vitro (C-IVP and RF-IVP) pig embryo origin. *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. Total vascular area (%): umbilical cord area corresponding to vessels divided by the umbilical cord area. UC diameter: umbilical cord width in mm.*

	Embryo origin		***In-vitro* embryo (IVP)**	
	***IN-VIVO* (AI)**	***IN-VITRO* (IVP)**	**C-IVP**	**RF-IVP**
Animals (n)	8	18	5	13
UC area (mm^2)	43.1 ± 12.8^a	56.6 ± 14.9^b	63.9 ± 15.1	53.8 ± 14.4
UC perimeter (mm)	26.4 ± 3.9^a	30.5 ± 4.7^b	32.8 ± 5.3	29.6 ± 4.4
UC diameter (mm)	8.4 ± 1.0^a	10.3 ± 1.9^b	11.1 ± 2.4	9.9 ± 1.6
UC area/Weight birth ratio	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
UC diameter/Weight birth ratio	7.3 ± 1.7	7.9 ± 1.9	7.6 ± 1.7	8.0 ± 2.1
Arterial area (mm^2)	1.97 ± 0.4	2.5 ± 0.6	2.6 ± 0.6	2.3 ± 0.6
Venous area (mm^2)	4.4 ± 1.4	5.6 ± 3.0	5.7 ± 1.8	5.6 ± 3.4
Total vascular area (%)	15.3 ± 4.4	13.6 ± 5.0	10.8 ± 3.6	14.7 ± 5.2

Data is expressed as mean ± SD.

Statistically significant differences are expressed with different letter for embryo origin, a-b ($p < 0,05$).

Table 5. Correlation between the average gain daily (AGD) and umbilical cord (UC) morphometric and vascular parameters *(Pearson's linear correlation coefficient). Umbilical cord area, perimeter and diameter, umbilical cord arterial area, venous area and total vascular area (% area corresponding to vessels of the umbilical cord area).*

Age (days)	**UC AREA**	**UC PERIMETER**	**UC DIAMETER**	**ARTERIAL AREA**	**VENOUS AREA**	**TOTAL VASCULAR AREA**
9	0.355	0.352	0.406	0.194	0.225	-0.300
15	0.213	0.143	0.099	0.172	-0.133	-0.370
30	-0.120	-0.150	0.070	-0.136	-0.013	-0.021
45	-0.242	-0.281	-0.268	-0.202	0.069	0.081
60	-0.393	-0.411	-0.228	-0.126	-0.400	-0.123
90	-0.084	-0.094	0.092	-0.286	-0.103	-0.037
120	-0.368	-0.217	-0.356	-0.276	-0.275	-0.049
150	0.412	0.470*	0.339	0.316	0.266	0.038
180	-0.179	-0.019	-0.089	0.188	-0.287	0.071
365	-0.309	-0.240	-0.149	-0.323	-0.391	-0.201
915	0.542	0.445	0.351	0.443	0.007	-0.732*
1129	0.396	0.218	0.220	0.255	0.118	-0.670
1283	0.163	0.125	-0.035	-0.411	0.274	0.661

*Significative correlation 0.05 (bilateral).

The correlation in the umbilical cord vascular and morphometric parameters (Table 5) showed low positive relation of diameter and perimeter at day 150. Moreover, total vascular area was highly negative correlated with day 915 AGD.

The WJ study (Table 6) indicated significant differences between the *in-vivo* and *in-vitro* groups related to their area. The analysis of the thickness of each zone showed that thickness of perivascular and intermedia zone was similar in AI and IVP. However, when analyzing the *in-vitro* group in detail, the thickness of the perivascular zone was found to be significantly greater in C-IVP than in RF-IVP group ($p<0.05$). Moreover, this zone in the WJ was low positive correlated at day 9 (Table 7).

Table 6. Statistical differences of umbilical cord Wharton Jelly (WJ) morphometric parameters in in pigs derived from in-vivo (AI) and in-vitro (C-IVP and RF-IVP) pig embryo origin*. The thickness WJ perivascular zone is umbilical cord region surrounding the two umbilical cord arteries and vein. The thickness WJ intermedia zone corresponds to the regions between the perivascular jelly and the umbilical cord epithelium. WJ area is the area corresponding to the WJ tissue, calculated as the umbilical cord area minus vessels area.*

	Embryo origin			***In-vitro* embryo (IVP)**	
	***IN-VIVO* (AI)**	***IN-VITRO* (IVP)**		**C-IVP**	**RF-IVP**
Animals (N)	8	18		5	13
WJ area (mm^2)	36.9 ± 12.0^a	48.9 ± 12.8^b		56.7 ± 11.8	45.9 ± 12.3
Thickness WJ Perivascular (µm)	515.3 ± 177.5	551.8 ± 157.5		698.2 ± 196.0^a	495.4 ± 99.8^b
Thickness WJ Intermedia (µm)	1002.3 ± 299.2	1222.3 ± 350.6		1148.8 ± 306.8	1250.5 ± 373.6

Data is expressed as mean ± SD.

Statistically significant differences are expressed with different letter for embryo origin, a-b ($p< 0,05$).

Table 7. Correlation between the average gain daily (AGD) and umbilical cord Wharton's jelly (WJ) parameters *(Pearson's linear correlation coefficient). WJ perivascular (WJ region surrounding the two umbilical cord arteries and vein), WJ intermedia (jelly region corresponding from the perivascular part to the umbilical cord epithelium) and WJ area (umbilical cord area which corresponds to the WJ without the vessels).*

DAY	WJ PERIVASCULAR	WJ INTERMEDIA	WJ AREA
9	0.446*	0.074	0.394
15	0.336	-0.236	0.291
30	-0.335	0.212	-0.117
45	-0.092	0.084	-0.261
60	-0.345	-0.078	-0.357
90	-0.300	-0.093	-0.079
120	-0.073	-0.258	-0.342
150	0.251	0.267	0.381
180	-0.087	-0.247	-0.173
365	-0.076	-0.225	-0.255
915	0.619	0.231	0.659
1129	0.712	0.065	0.502
1283	-0.822	0.870	0.025

*Significative correlation 0.05.

4. Discussion

The placenta plays a critical role in maintaining and protecting the fetal development, which can be compromised by changes in the placental development of ART derived animals (Palmieri et al., 2008). The present work suggests that placenta vascular phenotype impacts the growing and AGD of individuals through their early and mid-term life. Moreover, this study shows that the embryonic origin influences the development of placental and umbilical vascularization in the pig, where the use of reproductive fluids in *in-vitro* procedures bring results near to *in-vivo* data.

Just like in humans, where birthweight and placenta weight are similar across *in-vitro* and *in-vivo* groups (Yanaihara et al., 2018), in our study, piglet birthweight, placenta weight, placenta area, and placenta efficiency were similar between groups.

However, some studies in pigs have reported lower birthweight, placenta weight, placenta area and placenta efficiency in pigs born from somatic cell nuclear transfer (Ao et al., 2017) suggesting that in pigs, placental parameters might be influenced by the *in-vitro* techniques used and a more detrimental effect with summation of different ART since somatic cell nuclear transfer is a more stressful process than conventional IVP. In bovine, birthweight and placentas from *in-vitro* derived animals were significant higher compared with *in-vivo* group, while placental efficiency was similar between groups (Miles et al., 2004). However, samples in Miles's study were taken before calf's birth and it is well-known that *in-vitro* derived cattle suffer overgrowth, in addition to the fact that both species' farrowing rates differ. In relation to the placental ratio, our findings showed no difference between groups while in mice both a higher ratio and no variations have been linked with different *in-vitro* fertilization protocols (Delle Piane et al., 2010). It must be also considered that in our study a lower number of animals were examined for this parameter so it would be dared to extract a binding conclusion form this observation.

The influence of embryo origin on the placental vascularization has been already described (Fidanza et al., 2014). Placental vascularization abnormalities, including a decrease in arterial number, lumen size, and branching, have been extensively observed in humans born from IVP-embryos (Riesche & Bartolomei, 2018) but studies on IVP pigs are very limited (Ao et al., 2017). Considering placental vascular architecture, the vessels percentage, density, and area were similar in the AI and RF-IVP groups, which could be an indication that addition of reproductive fluids to the culture media during early IVP-embryos development would aid in simulating *in-vivo* conditions (Canovas et al., 2017; López Albors et al., 2017). A reduction in the vessels number in C-IVP group compared to the AI and RF-IVP groups is contrary to that found in humans, where a high number of vessels in the ART embryos has been described (Abdel-Hamid et al., 2017). However, it must be considered that the number of placental villous sections analyzed in that study was vastly smaller with only 0.20-0.25 mm^2 placenta villous analyzed, whereas in current study data were obtained from 19.4-45.9 mm^2 placenta.

Furthermore, it is relevant that the percentage of vessels area for the small caliber vessels (arteriole/venule and small vessels) was significantly higher in AI and RF-IVP groups suggesting that adding reproductive fluids to the culture medium could help to mimic the *in-vivo* environment and to reduce the *in-vitro* stress that early embryo finds during culture. Despite not being significant differences among groups in the vessels number density, this parameter was lower for the IVP groups like what was observed in sheep obtained from *in-vitro* fertilization (Grazul-Bilska et al., 2014).

Alterations in the umbilical cord have also been reported in humans derived from ART that observed higher risk of developing anomalies (Delbaere et al., 2007). In porcine, a study showed that pig's birthweight was associated with umbilical cord diameter, where a higher umbilical cord diameter-birthweight ratio resulted in a higher risk of mortality (Fordyce et al., 2021). In the present study, the umbilical cord diameter-birthweight ratio was similar between groups even though umbilical cord diameter and umbilical cord area were significantly higher in the IVP pigs. These findings agree with what was observed in cloned cattle that had higher umbilical cord diameter than the AI counterparts (Kohan-Ghadr et al., 2008). IVP pigs' umbilical cord area and perimeter, as well as the WJ area, were larger than *in-vivo* parameters. Furthermore, the size of the umbilical cord vessels was similar between the *in-vitro* and *in-vivo* groups implying that the variations in umbilical cord size would be caused by WJ development. It should be observed that the C-IVP group's perivascular WJ thickness was greater than that of the RF-IVP group, suggesting that different effects may occur when embryo are produced with reproductive fluids since the latter are close to *in-vivo* data. This fact is so relevant since the perivascular region contains most mesenchymal stem cells, which are cells that can differentiate in a variety of tissues, making them extremely useful in the treatment of autoimmune, cardiovascular, hematological, hepatic, and neurodegenerative diseases (Davies et al., 2017). Regarding the umbilical vessels, it has been reported in pigs that ART-derived embryos may have defective umbilical cords with smaller arteries (Park et al., 2009), which possibly conditioning the growth data and showing abnormalities. However, in our study *in-vivo* and *in-vitro* groups had similar artery size, this could be related to the different embryo origin.

While in our study embryos were obtained from IVP, in Park's study embryos were obtained by SCNT, a much more invasive and stressful ART.

Aberrant placental vascularization and altered placentation and its impact on fetal growth and birth weight have been demonstrated in several species (Reynolds et al., 2014). Additionally, damaged umbilical cords have a deleterious impact on these variables. The effectiveness of the placenta and umbilical cord is known to be impacted by ARTs. However, no studies on its long-term consequences in pigs are currently available. Our findings showed a correlation between placental morphometric variables and weaning. The placental vascularization may be involved in the postnatal growth of pigs since there was a negative correlation with the first's weeks of growth. In addition, the correlation was found during fattening period with capillary parameters and negative correlation was found at 3 years of age with venous parameters. Umbilical cord parameters such as diameter and umbilical cord diameter birthweight ratio have impact on the animals weight at 150 days (Fordyce et al., 2021). In agreement with these results, our data showed that umbilical cord perimeter, diameter and WJ perivascular thickness were correlated with pig's AGD.

5. Conclusions

The ARTs seem affect the size of small caliber vessels (arteriole/venule and small caliber), despite the fact that the IVP pigs have similar placental morphometric parameters to *in-vivo* animals. Thus, changes in vessels area small-sized vessels may contribute to impaired placental vascularization in pigs produced from ART. Furthermore, *in-vitro* production appears to increase the umbilical cord area, perimeter, diameter and perivascular WJ area of the pigs whereas the size of vessels was not affected. Specifically, the perivascular zone is that contains mesenchymal cells, hence modifications to this region may have an impact on these cells. Moreover, the addition of reproductive fluids to IVP seems to improve the results by mimicking the *in-vivo* conditions. Finally, it seems that there is a link between placental vascularization and umbilical cord morphometry and pig growth, because the data over time has allowed us to observe changes in their growth.

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8. Declaración de actividad

Yo, Úrsula Álvarez Martín, declaro que he participado en la toma de imágenes de las muestras de placenta y cordón, el análisis de las mismas, así como en el análisis de los resultados, su interpretación y la posterior redacción del presente trabajo.

INFORME ANTIPLAGIO

Dña. Raquel Romar Andrés y Dña. Ester Párraga Ros, TUTORAS de la estudiante Dña. Úrsula Álvarez Martín que presenta el Trabajo Fin de Máster titulado "**Morphometric study of placental and umbilical cord vascularization in pigs derived from assisted reproductive technologies**", en el curso académico 2021-2022.

EXPONEN QUE,

Tras examinar el mencionado TFM a través de la herramienta antiplagio TURNITIN, considerando el porcentaje de similitud detectado por el programa (17 %) y las citas incluidas en el mismo, no se han detectado indicadores de plagio, sin que pueda ser totalmente descartado en base a las limitaciones inherentes a dicho sistema.

En Murcia, a 30 de Agosto de 2022

UNIVERSIDAD DE
MURCIA

RAQUEL ROMAR ANDRÉS, PROFESORA TITULAR DEL DEPARTAMENTO DE FISIOLOGÍA DE LA UNIVERSIDAD DE MURCIA

ESTER PÁRRAGA ROS, PROFESORA ASOCIADA DEL DEPARTAMENTO DE ANATOMIA Y ANATOMÍA PATOLÓGICA COMPARADAS

INFORMAN

Que el trabajo Fin de Máster titulado "**Morphometric study of placental and umbilical cord vascularization in pigs derived from assisted reproductive technologies**" presentado por Dña. Úrsula Álvarez Martín ha sido realizado bajo nuestra supervisión y cumple los requisitos necesarios para su defensa.

En Murcia a 30 de Agosto de 2022.