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Impact of embryo transfer and *In vitro* production of embryos on phenotypical and molecular traits of offspring in the porcine species *(Sus scrofa)*

Impacto de la transferencia de embriones y la producción *In vitro* de embriones sobre los rasgos fenotípicos y moleculares de la descendencia en la especie porcina *(Sus scrofa)*

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Abbreviations list

ADWG	Average Daily Weight Gain
AGD	Ano-genital distance
AI	Artificial insemination
ALB	Albumin
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
ART	Assisted reproductive technologies
AS	Angelman Syndrome
AST	Aspartate aminotransferase
BGC	Blood glucose concentration
BSA	Bovine serum albumin
BW	Body weight
BWS	Beckwith-Wiedemann Syndrome
cAMP	Dibutyryl Cyclic Adenosine Monophosphate
CG	Cortical granules
CHCM	Cell hemoglobin concentration mean
CHr	Hemoglobin content of reticulocytes
CK	Creatine kinase
COCs	Cumulus cell-oocyte complexes
CREA	Creatinine
CRISPR	Clustered regularly interspaced short palindromic repeats (DNA)
CRL	Crown rump length
DOHaD	Developmental Origins of Health and Disease hypothesis
EC	Embryo culture
eCG	Equine chorionic gonadotrophin
EL	Early luteal phase (estrous cycle)
ET	Embryo transfer

FCS	Fetal calf serum
GGT	Gamma-glutamyl transferase
GLOB	Globulins
GV	Germinal vesicle (oocyte)
HB	Hemoglobin
hCG	Human chorionic gonadotrophin
HCT	Hematocrit
HDW	Hemoglobin concentration distribution width
hpi	Hours post insemination
ICM	Inner cell mass (embryo)
ICSI	Intracytoplasmic sperm injection
IETS	International Embryo Technology Society
IGR	Intrauterine growth restriction
im	Intramuscular injection (administration route)
iv	Intravenous injection (administration route)
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* produced (embryos)
LBW	Low birth weight
LF	Late follicular phase (estrous cycle)
LOS	Large Offspring Syndrome
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MCVr	Average size of reticulocytes
ML	Mid luteal phase (estrous cycle)
MPM	Mean platelet mass
MPV	Mean platelet volume

NCSU-37	North Carolina State University medium-37
NCSU-23	North Carolina State University mediun-23
OGTT	Oral glucose tolerance test
OHSS	Ovarian hyperstimulation syndrome
OPU	Ovum pick-up
PBS	Dulbecco's Phosphate buffered saline
PCDW	Platelet component distribution width
PCR	Polymerase chain reaction
PCT	Platelecrit
PDW	Platelet distribution width
pFF	Porcine follicular fluid
PGD	Preimplantation genetic diagnosis
PLT	Platelets
PMDW	Platelet mass distribution width
pOF	Porcine oviductal fluid
pUF	Porcine uterine fluid
PVA	Polyvinyl alcohol
PWS	Prader-Willi Syndrome
q-PCR	Quantitative real-time PCR
RBC	Red blood cells (concentration of erythrocytes)
RDW	Red blood cell distribution width
RF	Reproductive fluids
SCNT	Somatic cell nuclear transfer
SD	Standard deviation
SEM	Standard error mean
SRS	Silver-Russell Syndrome

TBIL	Total bilirubin
TP	Total protein
TRIGL	Triglycerides
UC	Umbilical cord
WBC	White blood cells
ZP	Zona pellucidae

Summary

Assisted reproductive technologies (ART) are a growing science globally. From humans to animals, either to solve infertility issues or to increase animal productivity, many techniques have been developed and applied. The use of ART in animals has a dual goal: on the one hand, all the ART were and are developed first in animals, serving as model to humans; and, on the other hand, the increase in the use of ART in livestock is also related to human population growth and the necessity of higher meat or milk production.

Indeed, animal models are widely used in science to study a wide variety of diseases in humans or to investigate the function of specific genes, among other applications. Of mammalian models mice are the most used species due to its easy maintenance, low cost and extensive genetic resources, among other positive traits. However, in the reproductive field, although the mouse has provided a lot of valuable data, it also has some limitations and, in some contexts, it may not be the best model to compare to human. Particularly, regarding the female cycle and anatomy, the use of livestock animals has been promoted as a valuable and useful complement to the information obtained in mice. In this sense, the pig has been of special interest to all biomedical fields, due to its genetic, physiological and anatomical similarities with humans, such as those related to its organ size, or to its circulatory and digestive systems. At the same time, the short lifespan as well as the short gestation length, make the pig more attractive than other species, such as cow, for most such studies.

As for the second goal, the use of ART in agriculture has become more relevant in terms of developing new procedures to achieve specific objectives at a lower cost. Those objectives, however, are not always reached at a high efficiency, or, in other cases, may generate undesired consequences. For example, artificial insemination (AI) is widely established on pig farms providing high productive yields, but at the same time it increases inbreeding within the same population. Embryo transfer (ET), on the other side, widely established in cattle, has presented limitations in the porcine species due to the surgical requirement or the high number of embryos needed to be transferred. The development of non-surgical ET, although it has brought great advantages as a

safer procedure or to minimize the risk of disease transmission as well as a lower derived cost, is still of very limited use in the pig industry.

The short and long term consequences of the use of ART in humans and animals are still poorly established. It is known that gametes and early embryos are in a critical stage of epigenetic reprogramming and are highly sensitive to the environmental conditions. Repercussions on the birth weight of ART-derived children and animals, increased frequency of cardiovascular diseases, or even diabetes, have been reported after the use of ART and represent a concern to the field. Thus, more studies on the short, medium and long term consequences of each technique should be performed.

Thus, for all the above mentioned reasons, in this thesis, we hypothesized that the ET technique per se, and in absence of any confounder factor, influences the mother reproductive issues and the phenotype of the offspring. Also, we propose that the source of protein in the culture media in which the embryos are produced is a factor influencing the outcomes of the IVP procedures and, also, the short- and long-term phenotypical traits of the offspring.

To test these hypotheses, we proposed the following objectives:

1. To decipher the impact of ET in isolation on reproductive parameters and on phenotypic traits of the offspring in the short term. This objective was accomplished by the tasks developed in Experiment 1.
2. To determine the impact of the protein source of the culture media on in vitro embryo development, pregnancy and parturition outcomes, and placental and umbilical cord molecular traits. This objective was accomplished by the tasks developed in Experiment 2.
3. To analyse, on the long-term, until month 6 of age, the phenotype of the offspring in terms of growth and haematological profile, and to determine glucose tolerance at day 45 of life. This objective was accomplished by the tasks developed in Experiment 3.

Crossbred sows (Landrace x Large White) with the same genetic line were used as donors (1-11 parities), and recipients (2-3 and 0-9 parities in experiments 1 and 2) respectively.

In experiment 1, embryos were collected from donor sows and non-surgically transferred to recipients (DeepBlue® ET catheter, Minitüb, Tiefenbach, Germany), with around 40 being the average number of *in vivo*-derived embryos transferred per recipient. Estrous synchrony of the recipients was between 0 and +24h with respect to the donors. Three of the 13 recipients became pregnant and the gestation length was within normal ranges. Three litters from sows artificially inseminated were used as control (AI group), resulting in 42 piglets derived by AI compared to 39 derived by ET. All piglets were weighed at birth in addition to days 3 and 15 of age where blood samples were also collected. In order to know if weight was conditioned by sex, separate analyses were conducted. Data showed that females derived from ET were significantly heavier than those derived from AI, although these differences disappeared on day 3 and 15 of age. No differences were found when average daily weight gain (ADWG) was studied. However, when haematological and biochemical parameters were analysed, females from ET showed significant differences ($p<0.05$) at day 3 in some: concentration of erythrocytes (RBC); hemoglobin (Hb); haematocrit (HTC); hemoglobin concentration distribution width (HDW), average size of reticulocytes (MCVr) and leucocytes (WBC). Most of these differences disappeared by day 15 of age except WBC and MCVr. On this day, significantly lower values were observed only in urea concentration. On the other hand, at day 3, males from ET showed significant differences ($p<0.05$) for WBC, platelets indices, alkaline phosphatase (ALP) and albumin (ALB), although this difference disappeared on day 15, finding instead at this day significant differences for gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST) and lipases ($p<0.05$).

Despite these findings, all the parameters analysed were within the established physiological range.

In experiment 2*, in vitro* production (IVP) of embryos was performed by using or not reproductive fluids (RF) (NaturARTs®, EmbryoCloud, Murcia, Spain), from different phases of the estrous cycle, as additives to the culture media. According to the supplementation of the medium, there were two different groups:

Control (C)-IVP (medium plus 3mg / ml BSA) and RF-IVP (medium plus 3mg / ml BSA and 1% (v/v) porcine RF). No differences were found on cleavage rate or blastocyst yield when embryos were analysed (p >0,05).

On day 5 or 6 (considering day 0 as IVF day) embryos were surgically transferred to recipients by using a paralumbar laparo-endoscopy single-site technique. The synchrony of the recipients was between -24 and -48h with respect to embryos. The percentage of females that became pregnant was 38,5% (5/13) and 36,4% (4/11) for RF-IVP and C-IVP respectively. All pregnancies came to term except one from the RF-IVP group due to an abortion on day 28 of gestation. Piglets derived from four different litters from AI were used as control (AI group). No statistically significant differences were found in gestation length between groups, and the total number of born piglets was 19 (11 males, 8 females), 30 (11 males, 19 females) and 59 (25 males, 34 females) for RF-IVP, C-IVP and AI groups respectively (p>0.05). One out of 19 animals from the RF-IVP group, as well as 2 out of 30 from C-IVP and 4 out of 59 from AI were born dead. On the other hand, when the litter size was analysed, significant differences were observed between RF-IVP vs AI and between C-IVP vs AI (p<0.05).

On the day of farrowing, samples of placenta and umbilical cord were taken from all piglets with the aim of analyze the expression levels of selected genes. In addition, placental parameters such as placental weight (g), placental area (cm^2) and placental efficiency (g/g) were studied. The placental surface area was analysed by ImageJ 1.52a software (National Institute of Health, USA), subsequently multiplying the area by two. Piglets from C-IVP showed a significantly larger surface area compared to the RF-IVP and AI groups. In addition, placental efficiency, which is defined as the ratio of birth weight to placental weight and reflects grams of fetus produced per gram of placenta, was calculated, showing piglets from the RF-IVP group had lower placental efficiency compared to the other two groups. No morphoanomalies were found in the offspring.

When gene expression levels were analysed by quantitative real-time PCR (q-PCR), the results showed an over-expression for the *PEG3* and *LUM* genes in placental tissue from piglets derived from C-IVP compared to those derived by AI (p<0.05). However, no differences were found in the transcript levels of the remainder of the genes analysed either in the C-IVP or RF-IVP group (p >0,05).

In experiment 3, piglets from experiment 2 were weighed at birth using a digital hanging scale and measured from the frontal region of the skull to the rump, which is a growth parameter named crown rump length (CRL).

Furthermore, in males, the anogenital distance (AGD), which is a marker of reproductive ability, was also measured. The first differences in AGD appeared on day 9 between AI and C-IVP groups ($p<0.05$), differences that remained apparent until day 30 when, in addition, differences appeared between AI and RF-IVP groups ($p<0.05$). Some of these differences were observed on some following days until day 180 of age, where no significant differences were found between any group.

For birth weight significant differences ($p<0.05$) between piglets born from the AI group and those derived from IVP (RF-IVP, C-IVP) were found. However, when piglets were separated by sex/group, males from the C-IVP groups were significantly heavier and longer than those derived from AI. However, no differences were found on this day in the anogenital distance ($p>0.05$).

Females both from RF-IVP and C-IVP were significantly heavier than females from AI, although only females from the C-IVP group were significantly longer than those derived by AI. The phenotype of the offspring was analysed during the 6 months after birth (from day 0 to day 180 of age) by studying growth, haematological profile, and glucose tolerance. The results obtained throughout this time showed that body weight both in males and females from C-IVP remains the heaviest throughout the study, showing those animals from the RF-IVP group intermediate values between the C-IVP and AI groups. Similarly, when ADWG was analysed, it was higher for the C-IVP group followed by the RF-IVP and AI groups. Considering all the data, when ADWG was analysed there were no significant differences ($p>0.05$) between the different groups regarding ADWG. However, when analyzing the data day by day, differences were found between some groups ($p<0.05$). On the other hand, the distance from the crown to the rump (Crown rump length, CRL) was also higher for the C-IVP group followed by the RF-IVP and AI groups. This trend was also observed when analyzing the AGD.

As in Experiment 1, haematological parameters were measured in experiment 3. When data were analysed, despite some exceptions, animals from *in vitro*-derived embryos with RF as additives showed a more similar haematological profile (RBC, Hb, HTC, MCV, MCH, RDW, HDW, CHr, PLT and PCT) to those derived from AI than piglets from *in vitro*-derived embryos without fluids. However, despite these observations, all values were within the established physiological ranges.

At 45 days of age, an oral glucose tolerance test (OGTT) was performed. For that, the two males and two females of highest and lowest weight per litter were selected. Blood samples were collected from the auricular lateral vein before (time 0) and after glucose intake at the following times: 5, 10, 15, 20, 30, 45, 60, 90, 120 and 150 min. The results showed a similar tendency in response to external glucose intake between piglets from *in vitro*-derived embryos and AI, showing in both males and females a monophasic curve from time 0 to 150 min, the time at which basal glucose levels were recovered.

In summary, the research developed in this thesis showed that the growth as well as the haematological and biochemical profiles of piglets is not affected by ET during the first 15 days of age. Nonetheless, females showed higher weight at birth in ET than in AI and this fact should be kept in mind, as it has been shown in various studies that increased birth weight is one of the most common findings in ART-derived calves and it has been related to the LOS as well as to the BWS in humans, although it has not been described in pigs until now.

On the other hand, although reproductive yield is not affected by the protein source of the culture media, there is an influence on placental efficiency and some molecular traits in this transitory organ. Amongst the imprinted genes analysed in placenta and umbilical cord, *PEG3* was upregulated in placental tissues from C-IVP embryos vs AI, whilst the RF-IVP group showed intermediate values. It could be a consequence of the culture media used in this group, and the supplementation with RF to IVF and EC media could mitigate this effect.

Furthermore, there is an effect derived from the *in vitro* production of embryos on growth and some haematological parameters, which is mitigated by the addition of RF to the culture media. In addition, effects of fluid addition were dependent

on sex. The addition of RF to IVF and EC media could partially mitigate the effects of embryonic stress in the case of males, providing the offspring with a phenotype similar to those born through AI, while the benefits of adding RF are less pronounced in female embryos.

Resumen

Las técnicas de reproducción asistida (TRA) son una rama de la ciencia en crecimiento a nivel mundial. Muchas de estas técnicas se han desarrollado y aplicado tanto en humanos como animales, ya sea para resolver problemas de infertilidad o en el caso de animales para aumentar también el rendimiento productivo. El uso de TRA en animales tiene un doble objetivo: por un lado, todas las TRA han sido y son desarrolladas primero en animales sirviendo de modelo para la especie humana; y, por otro lado, debido al crecimiento demográfico de la población, su uso ha sido incrementado en animales de abasto con el objetivo de abastecer una mayor demanda de carne y leche por parte del consumidor.

De hecho, a nivel científico, el uso de modelos animales está ampliamente establecido para estudiar una amplia variedad de enfermedades en la especie humana o para investigar la función de genes específicos, entre otras aplicaciones. Dentro de los modelos de mamíferos, el ratón es la especie más utilizada debido a su fácil mantenimiento, bajo coste y amplios recursos genéticos, entre otros aspectos positivos. Sin embargo, en el campo reproductivo, aunque el ratón ha proporcionado una gran cantidad de datos de gran valor, también presenta algunas limitaciones y en algunos casos, no es el mejor modelo para establecer compara raciones con la especie humana. Teniendo en cuenta la anatomía y ciclo reproductor femenino, se ha fomentado el uso de animales de grandes animales como un complemento valioso y útil a la información obtenida en ratones. En este sentido, el cerdo ha sido de especial interés en todos los campos biomédicos dadas sus similitudes genéticas, fisiológicas y anatómicas con la especie humana, como es el parecido en el tamaño de sus órganos, o de su sistema circulatorio y digestivo. Al mismo tiempo, debido a su corta esperanza de vida, así como el breve periodo de

gestación, el cerdo se ha convertido en un modelo más atractivo en comparación con otras especies como la vaca para la mayoría de estos estudios.

Por otro lado, el uso de TRA en ganadería ha adquirido más relevancia en cuanto al desarrollo de nuevos procedimientos para lograr un mayor rendimiento productivo a un menor coste. Sin embargo, estos objetivos no siempre son alcanzados con una alta eficiencia, e incluso en otros casos, pueden dar lugar a consecuencias no deseadas. Por ejemplo, la inseminación artificial (IA) está ampliamente establecida en granjas porcinas al proporcionar altos rendimientos productivos. Sin embargo, al mismo tiempo, aumentan la endogamia dentro de una misma población. La transferencia embrionaria (TE), por otro lado, ampliamente establecida en la especie bovina, ha presentado algunas limitaciones en la especie porcina debido a la necesidad de llevar a cabo un procedimiento quirúrgico o a la gran cantidad de embriones necesarios en cada transferencia. Sin embargo, aunque el desarrollo de la TE no quirúrgica ha proporcionado grandes ventajas por ser un procedimiento más seguro o por minimizar el riesgo de transmisión de enfermedades, así como suponer un menor coste, su uso es aún muy limitado en la industria porcina.

Las consecuencias a corto y largo plazo derivadas del uso de TRA en humanos y animales aún están poco establecidas. Se sabe que tanto gametos como embriones tempranos se encuentran en una etapa crítica de reprogramación epigenética, siendo muy sensibles a las condiciones ambientales. Muchos estudios han demostrado algunos de los posibles efectos derivados de estas técnicas sobre el peso al nacimiento, tanto de niños como animales, o la mayor predisposición a desarrollar enfermedades cardiovasculares, o incluso diabetes, lo que ha puesto de manifiesto un cierto grado de preocupación en este campo. Sin embargo, a fin de conocer las posibles consecuencias derivadas a corto, medio y largo plazo, aún han de realizarse más estudios que proporcionen una mayor cantidad de información al respecto.

Por estas razones, en esta tesis planteamos la hipótesis de que la técnica de TE *per se*, y en ausencia de cualquier factor de confusión, influye en los resultados reproductivos de la madre, así como en el fenotipo de la descendencia. Por otro lado, proponemos que la fuente de proteína de los

medios de cultivo en los que son producidos los embriones puede influir tanto en los resultados obtenidos tras la producción *in vitro* de embriones como en los rasgos fenotípicos de la descendencia a corto y largo plazo.

Para comprobar estas hipótesis, se propusieron los siguientes objetivos:

1. Estudiar a corto plazo el impacto de la TE, de forma aislada, sobre los parámetros reproductivos y los rasgos fenotípicos de la descendencia. Este objetivo se logró mediante las tareas desarrolladas en el **Experimento 1**.
2. Determinar el impacto de la fuente proteica de los medios de cultivo sobre el desarrollo embrionario, las tasas de gestación y parto, así como los rasgos moleculares de cordón umbilical y placenta de los lechones. Este objetivo se logró mediante las tareas desarrolladas en el **Experimento 2**.
3. Analizar, a largo plazo (hasta el 6º mes de vida), el fenotipo de la descendencia en cuanto al crecimiento y perfil hematológico, así como determinar la tolerancia a la glucosa mediante la realización de una prueba a los lechones el día 45 de vida. Este objetivo se logró mediante las tareas desarrolladas en el **Experimento 3**.

Para ello se emplearon cruces de cerdas (Landrace x Large White), de la misma línea genética, como donantes (1-11 partos) y receptoras (2-3 y 0-9 partos para los Experimentos 1 y 2 respectivamente).

En el **Experimento 1**, se obtuvieron embriones producidos *in vivo* a partir de cerdas donantes. Estos fueron transferidos de forma no quirúrgica a cerdas receptoras mediante el empleo de un catéter (DeepBlue® TE, Minitüb, Tiefenbach, Alemania), siendo alrededor de 40 el número de embriones transferidos por cada hembra. La sincronía de las cerdas receptoras respecto a las donantes fue de entre 0 y + 24 h. Tres de un total de 13 cerdas transferidas quedaron gestantes, encontrándose el periodo de gestación dentro del rango de normalidad. Por otro lado, 3 camadas procedentes de cerdas inseminadas artificialmente fueron usadas como grupo control (grupo IA), dando lugar a 42 lechones frente a los 39 procedentes de TE. Todos los lechones fueron pesados

al nacimiento, así como los días 3 y 15 de vida, donde, además, se tomaron muestras de sangre. Para saber si el peso podía estar condicionado por el sexo, todos los análisis se llevaron a cabo en machos y hembras por separado. Los datos mostraron que las hembras procedentes de TE eran significativamente más pesadas que aquellas procedentes de IA, aunque estas diferencias desaparecieron los días 3 y 15 de vida.

No se encontraron diferencias significativas cuando se analizó la ganancia media diaria (GMD). Sin embargo, al analizar los parámetros hematológicos y bioquímicos, las hembras procedentes de TE mostraron diferencias significativas ($p<0.05$) a día 3 en algunos de los parámetros analizados: concentración de eritrocitos (RBC); hemoglobina (Hb); hematocrito (HTC); distribución de Hb (HDW), tamaño promedio de reticulocitos (MCVr) y leucocitos (WBC). La mayoría de estas diferencias desaparecieron el día 15 de vida, excepto para WBC y MCVr. Sin embargo, cuando se analizó la bioquímica sanguínea, este día se observaron valores significativamente más bajos solo en la concentración de urea de las hembras del grupo TE. Por otro lado, en el día 3, los machos de TE mostraron diferencias significativas ($p<0.05$) en la concentración de leucocitos, índices plaquetarios, fosfatasa alcalina (ALP) y albúmina (ALB), aunque estas diferencias desaparecieron a día 15, encontrando en cambio diferencias significativas en la concentración de gamma-glutamil transferasa (GGT), aspartato aminotransferasa (AST) y lipasas ($p <0.05$) este día.

A pesar de estos hallazgos, todos los parámetros analizados estaban dentro del rango fisiológico establecido.

Para el **Experimento 2**, la producción *in vitro* (PIV) de embriones se realizó mediante la adición o no de fluidos reproductivos (FR) (NaturARTs®, EmbryoCloud, Murcia, España) como aditivos a los medios de cultivo, procedentes de diferentes fases del ciclo estral. Según la suplementación del medio, encontramos dos grupos diferentes: Control (C) -PIV (medio más 3 mg / ml de BSA) y FR-PIV (medio más 3 mg / ml de BSA y 1% (v / v) de fluido reproductivo porcino). No se encontraron diferencias en el porcentaje de cleavage o división embrionaria, ni en el porcentaje de embriones que alcanzaron el estadío de blastocisto ($p>0,05$).

El día 5 o 6, considerando el día 0 el día de fecundación *in vitro* (IVF), los embriones se transfirieron de forma quirúrgica a las hembras receptoras mediante el uso de una técnica de laparoendoscopía paralumbar de sitio único. La sincronía de las receptoras respecto a los embriones fue de entre -24 y -48h. El porcentaje de cerdas que quedaron gestantes fue de un 38,5% (5/13) y 36,4% (4/11) para los grupos FR-PIV y C-PIV respectivamente. Todas las gestaciones llegaron a término, excepto la de una de las cerdas del grupo FR-PIV que abortó el día 28 de gestación. Los lechones procedentes de cuatro camadas diferentes de IA fueron usados como control (grupo IA). No se encontraron diferencias estadísticamente significativas en la duración del periodo gestación entre los diferentes grupos. El número total de lechones nacidos fue de 19 (11 machos, 8 hembras), 30 (11 machos, 19 hembras) y 59 (25 machos, 34 hembras) para los grupos FR-PIV, C-PIV y IA respectivamente ($p> 0.05$). El número de lechones nacidos muertos fue de 1 de un total de 19 procedentes del grupo FR-PIV; 2 de 30 procedentes de C-PIV y 4 de 59 de IA. Por otro lado, cuando se analizó el tamaño de la camada, se observaron diferencias significativas entre el grupo IA y los otros dos grupos experimentales (FR-PIV y C-PIV), ($p<0.05$).

El día del parto, se tomaron muestras de placenta y cordón umbilical a todos los lechones con el objetivo de analizar los niveles de expresión génica de un determinado número de genes seleccionados. Además, se estudiaron parámetros placentarios como el peso (g) y área de la placenta (cm^2), y la eficiencia placentaria (g / g). La superficie de la placenta fue analizada mediante el software ImageJ 1.52a (Instituto Nacional de Salud, EE. UU.), y a continuación, se multiplicó el valor del obtenido por dos. Los lechones del grupo C-PIV mostraron un área de superficie significativamente mayor en comparación con los grupos FR-PIV y IA. Por otro lado, se calculó la eficiencia placentaria, definida como la relación entre el peso al nacimiento y el peso de la placenta. Esta relación refleja los gramos de feto producidos por gramo de placenta, mostrando así, que los lechones del grupo FR-PIV, tenían una menor eficiencia placentaria en comparación con los otros dos grupos. No se detectó ningún tipo de morfoanomalía en la descendencia obtenida.

Cuando se analizaron los niveles de expresión génica mediante PCR a tiempo real (q-PCR), los resultados mostraron una sobreexpresión para los

genes *PEG3* y *LUM* en el tejido placentario de los lechones del grupo C-PIV en comparación con los del grupo IA ($p<0,05$). Sin embargo, no se encontraron diferencias en los niveles de transcripción del resto de los genes analizados en los grupos C-PIV o FR-PIV ($p> 0,05$).

Para el **Experimento 3**, todos los lechones procedentes del Experimento 2 fueron pesados al nacer mediante el uso de una balanza digital. También se midieron desde la región frontal del cráneo hasta la grupa, que es un parámetro de crecimiento llamado longitud cráneo caudal (CRL).

Además, a todos los machos también se les midió la distancia anogenital (AGD), que es un marcador de capacidad reproductiva. Las primeras diferencias en este parámetro aparecieron el día 9 de vida entre los grupos IA y C-PIV ($p<0.05$). Estas diferencias permanecieron hasta el día 30, cuando, además, aparecieron también entre los grupos IA y FR-PIV ($p<0.05$). Algunas de ellas fueron encontradas en algunos de los días siguientes del estudio hasta el día 180, donde dejaron de observarse.

En cuanto al peso al nacimiento, se encontraron diferencias significativas ($p <0.05$) entre lechones nacidos mediante IA y aquellos producidos *in vitro* (FR-PIV, C-PIV). Sin embargo, cuando fueron separados por sexo, los machos de los grupos C-PIV fueron significativamente más pesados y de mayor longitud que los nacidos por IA. Sin embargo, no se encontraron diferencias en este día en la distancia anogenital ($p>0.05$).

Por otro lado, las hembras de los grupos FR-PIV y C-PIV fueron significativamente más pesadas que las hembras de IA. Por otro lado, solo las hembras del grupo C-PIV mostraron una longitud significativamente mayor que aquellas procedentes de IA.

El fenotipo de la descendencia se analizó durante los 6 meses posteriores al nacimiento (desde el día 0 hasta el día 180 de vida) mediante el estudio del crecimiento, el perfil hematológico y la tolerancia a la glucosa. Los resultados obtenidos a lo largo de este tiempo mostraron que el peso corporal, tanto en machos como en hembras del grupo C-PIV seguía siendo superior a lo largo del estudio, mostrando en cambio los lechones del grupo FR-PIV con unos valores situados entre los grupos C-PIV y IA. Del mismo modo, cuando se analizó la

GMD, ésta fue mayor en el grupo C-PIV seguido por los grupos FR-PIV y IA. Considerando todos los datos, cuando se analizó la GMD no se encontraron diferencias significativas ($p>0.05$) entre los diferentes grupos. Sin embargo, al analizar los datos día a día, se encontraron diferencias entre algunos ellos ($p <0.05$). Por otro lado, la distancia cráneo caudal (CRL) también fue mayor en el grupo C-PIV seguido por los grupos FR-PIV y IA. Esta tendencia también se observó al analizar la DAG.

Al igual que en el Experimento 1, los parámetros hematológicos se midieron también en el experimento 3. Cuando se analizaron los datos, a pesar de algunas excepciones, los animales procedentes de embriones producidos *in vitro* con FR como aditivos mostraron un perfil hematológico más similar (RBC, Hb, HTC, volumen corpuscular medio (MCV), hemoglobina corpuscular media (MCH), amplitud de distribución eritrocitaria (RDW), HDW, contenido de Hb reticulocitaria (CHr), plaquetas (PLT) y plaquetocrito (PCT)) a los nacidos mediante IA en comparación con los lechones procedentes de embriones producidos *in vitro* sin fluidos. Sin embargo, a pesar de estas observaciones, todos los valores estaban dentro de los rangos fisiológicos establecidos.

A los 45 días de edad, se realizó una prueba oral de tolerancia a la glucosa (OGTT). Para ello, se seleccionaron los dos machos y las dos hembras de mayor y menor peso por camada y se tomaron muestras de sangre de la vena auricular lateral antes (tiempo 0) y después (tiempos 5, 10, 15, 20, 30, 45, 60, 90, 120 y 150 min), de una administración externa de glucosa por vía oral. Los resultados mostraron una tendencia similar en respuesta a la administración externa entre lechones procedentes de embriones producidos *in vitro* respecto a aquellos producidos mediante IA, mostrando tanto machos como hembras una curva monofásica desde el tiempo 0 a los 150 min, momento en que se recuperaron los niveles basales de glucosa.

En resumen, el trabajo desarrollado a lo largo de esta tesis muestra que el crecimiento, así como los perfiles hematológicos y bioquímicos de los lechones no se ven afectados por la TE durante los primeros 15 días de edad. Sin embargo, las hembras procedentes de este grupo mostraron un mayor peso al nacimiento respecto a aquellas procedentes de IA. Este hecho debe tenerse en cuenta, ya que, como se ha demostrado en varios estudios, el aumento de

peso al nacimiento es uno de los hallazgos más comunes en terneros procedentes de TRA y, además, ha sido relacionado con el Síndrome del ternero gigante (LOS), así como con el Síndrome de Beckwith-Wiedemann (BWS) en humanos, aunque no se ha descrito en cerdos hasta ahora.

Por otro lado, aunque el rendimiento reproductivo no se ve afectado por la fuente de proteínas de los medios de cultivo, hay una influencia en la eficiencia placentaria y algunos rasgos moleculares de este órgano transitorio. Entre los genes de impronta analizados en la placenta y el cordón umbilical, *PEG3* estaba sobreexpresado en tejidos placentarios de lechones del grupo C-PIV frente al grupo IA, mientras que el grupo FR-PIV mostró valores intermedios. Esto podría ser una consecuencia de los medios de cultivo empleados en este grupo, pudiendo mitigar este efecto la suplementación con FR en medios de IVF y cultivo embrionario

Además, existe un efecto derivado de la producción *in vitro* de embriones sobre el crecimiento y algunos parámetros hematológicos, que es mitigado mediante la adición de FR a los medios de cultivo, siendo estos efectos además dependientes del sexo. En definitiva, la adición de FR a los medios de IVF y EC podría mitigar parcialmente los efectos derivados del estrés embrionario en el caso de los machos, proporcionando a la descendencia un fenotipo similar a los nacidos a través de la IA. Sin embargo, los beneficios obtenidos tras agregar FR son menos pronunciados en los embriones femeninos.

Introduction

Growing evidence about the effects of assisted reproductive technologies (ART) on the short- and long-term health of the offspring have led to an increase in research in this field, not only in humans but also in other mammalian species (El Hajj & Haaf, 2013; Hargreave et al., 2019).

Nowadays, many studies describe the impact that stressful conditions, derived from gametes and embryo manipulation on the first week of preimplantation development, can have on the epigenetic reprogramming that occurs during this period (Canovas et al., 2017a; Menelaou et al., 2019; Ramos-Ibeas et al., 2019). From the ovarian hyperstimulation treatments to the embryo transfer (ET), each step in the ART represents a possible alteration in the epigenome with putative consequences in the transcriptome and physiology of the offspring (Hattori et al., 2019; Kindsfather et al., 2019; Van Montfoort et al., 2012). It is known that the use of these techniques for infertility treatments can affect the birth weight and developmental growth, evolving also to an increased risk for cardiovascular diseases, diabetes and obesity in adulthood (Feuer & Rinaudo, 2017), when compared to naturally conceived offspring.

While in human ART-derived offspring it is difficult to asses which technology is responsible for which effect (i.e. artificial insemination (AI), in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), ET, especially when some of these approaches are simultaneously applied on the same couple), or even distinguish the effect of the global process from the parental inherited alterations, animal models offer a simple way to decipher the isolated impact of one specific ART. Thus, large animals like the pig, represent an excellent model due to its similar anatomical and physiological characteristics with humans (Bellinger et al., 2006; Lorenzen et al., 2015). This situation creates the opportunity of its use as model of ART, since highly selected healthy male and female breeders are available in commercial farms and AI centers, thus limiting genetic variability.

For years, the ET technique has presented some limitations in porcine species since it required the collection and transfer of embryos surgically (Cameron *et al.*, 2004). But the development of new devices (DeepBlue® ET

catheter, Minitüb, Tiefenbach, Germany) has made it possible to cross the complex anatomy of the sow genital tract. Therefore, embryos can be deposited as deep as possible in one uterine horn of non-sedated recipient, minimizing the risk of disease transmission and uterine infections (E. A. Martinez et al., 2019). Nevertheless, to date, *in vivo* embryo collection by uterine flushing via transrectal palpation is still a challenge due to the length of the uterine horns in gilts and sows, something that does not occur in other species like cattle and horses (Yoshioka et al., 2020).

Currently, there are many studies (Wieczorek et al., 2015; Yoshioka et al., 2012a) that have focused on obtaining live piglets through the use of ET but, to our knowledge, little is known about the possible consequences on the health of the offspring. On the other hand, limitations in techniques such as *in vitro* fertilization (IVF) or embryo culture (EC), have resulted in practically no animals produced after embryo transfer (ET) of in vitro produced embryos. This fact, in turn, implies that there are no studies on live animals that were *in vitro* produced (PIV) in which their health, growth rate, productive indixes, reproductive capacity, etc. can be assessed, compared to those naturally produced.

Cánovas et al. (Canovas et al., 2017b) showed that the imitation of the physiological environment, by using reproductive fluids during *in vitro* fertilization and embryo culture in pig, improves embryo development such that they are more similar to those produced *in vivo*. However, the best proof of the success of an *in vitro* embryo production system is that obtained after transfer to recipient females, evaluating not only the percentages of pregnancies that are achieved but also the health of the offspring after birth. For this reason, it is necessary to analyze the implantation capacity and full-term develop in embryos obtained using reproductive fluids (RF). By doing this, it will be possible to find out if there are alterations in the epigenetic marks that can lead to diseases or alterations in adulthood.

Physiological parameters, including haematological and biochemical, are important indicators that provide information about the general state of health; however, little is known about these parameters in newborn piglets and young pigs since few studies (Casas-Díaz et al., 2015; Perri, 2015; Ventrella et al., 2017) have published reference intervals for consistent comparisons. Similarly,

anogenital distance, which is a marker of reproductive capacity, has been positively correlated with the size of the reproductive organs and therefore with seminal quality in rats and humans, (Eisenberg et al., 2011; Mendiola et al., 2011(Dean & Sharpe, 2013). However, there are far less data in the porcine species (Mack et al., 2014).

Taken into consideration all the above mentioned issues, this work was conducted , using the pig model, to shed light on the impact of some ART (namely ET, PIV and culture medum composition) on different phenotypical and molecular traits of the offspring until the age of 6 months.

Bibliographic review

1. The importance of improving the efficiency of reproductive biotechnologies in porcine species.

In recent years, Assisted Reproductive Technologies (ART) have become more important, not only in the human species, where more than 8 million children have been born worldwide through the use of these techniques (Adamson et al., 2018), but also in animal production. Due to continuous growth of the world population, there is an interest in improving production and reducing costs in farming for food (Day, 2000; Fowler et al., 2018; Sans & Combris, 2015). According to the latest report published by the Trade and Markets Division of FAO, poultry is the most consumed meat worldwide followed by pork and beef (FAO, 2019) . But in some regions, particularly in Asia, pork is one of the most traditional consumed meats, and the prediction is that these countries will continue to increase their consumption and demand (OECD/FAO, 2019).

To respond to such demands, the need to develop new technologies to produce high quality animals has risen. Producing large-scale pig embryos from gametes of animals of high genetic value, would help achieve that goal, by accelerating the production of pigs with highly selected genetics (Fowler et al., 2018).

The use of ART to increase meat production is still far from being widely established in the pig. The development and extended use of some specific technologies such as embryo transfer (ET), would not only provide greater genetic selection, but would also a decrease the risk of diseases transmission since it avoids the entry of new animals in breeding centers and farms, decrease transport costs, and minimize the effect on animal welfare during transportation (E. A. Martinez et al., 2019). However, ET requires a large number of viable embryos (ideally cryopreserved) ready to be transferred to the recipient mothers and, currently the technological procedures necessary to obtain these numbers of embryos are not yet available as will be explained in detail in the following sections.

Thus, more effective pig production would not only help to deliver the need for meat but would as well help other areas. Indeed, the pig has been suggested and largely used as an excellent human-study model (reviewed by Soom *et al.*, 2011), due to its similar anatomical, physiological and genetics characteristics (Bellinger et al., 2006; Lorenzen et al., 2015). This model plays an important role in disease research and it is useful in the biomedical field for different purposes such as the production of organs for xenotransplants (Ekser et al., 2009) or the production of proteins of special interest, such as insulin (Bersch et al., 1982).

In addition, the pig is a particularly suitable model in reproduction because it allows us to study and understand, in an acceptable period of time, what the long-term effects derived from the use of ART could be, since the approximate life expectancy of the swine species is 15 years, which is much more tractable relatie to the current human life-span. The most used model in the reproductive field is still the mouse as it has several positive aspects such as: a short gestation period; easy and low cost maintenance; the absence of underlying infertility or the ability to phenocopy the alterations observed in humans correctly (Overgaard et al., 2018; Vrooman & Bartolomei, 2017); and has a hemochorial placenta similar to humans (Chavatte-Palmer & Tarrade, 2016). However, this model also has some limitations for use in translational studies due to their small size, short lifespan, and different physiology (Clouard, et al., 2012; Beaujean et al., 2015). For these reasons, there is an increasing need of using other animals to bridge the gap between the mouse model and the human species (Zou et al., 2019), being the pig one of the closest species to humans (Roura et al., 2016).

The section below describes the current situation of different ART in pigs with specific considerations about their options to be included in the productive systems of the farms today or in the near future.

1.1. Main ART procedures in pig

Artificial insemination.

Currently, artificial insemination (AI) is the most successfully widely implemented technique in pig farms worldwide due to the good yield obtained and the large size of the litters. In Western Europe, for instance, more than 90% of the sows have been bred by AI for more than two decades (Maes et al., 2011).

The principle of this method is based on the selection of animals with a high genetic value in order to improve and maintain those features of interest throughout the generations (Knox, 2016).

Boar sperm cryopreservation, on the other side, allows the storage of valuable genetic material from animals of interest for years. If combined with AI, the potential for improving the quality of the offspring would increase largely; however, only 1% of all artificial inseminations performed use frozen-thawed semen (Yeste, 2015). This could be due to the low yields obtained, since both, fertility and the number of piglets per litter, are inferior compared with the results obtained after the use of AI with refrigerated semen (Bonet et al., 2014). In addition, the higher complexity of the insemination procedure when using cryopreserved samples contributes to their limited use among farmers.

Ovarian stimulation

Ovarian stimulation consists of exogenous hormone administration to increase the number of oocytes collected from one single animal or to synchronize ovulation. In gilt and sows, the treatment is based on the use of gonadotrophins, such as equine chorionic gonadotrophin (eCG) and human chorionic gonadotrophin (hCG).

The most common protocol used consists of the stimulation of follicular growth and estrous expression by an intramuscular injection of eCG, (1000-1500IU) 24 hours post-weaning, followed by hCG (750 IU) 24-72h post eCG, to induce ovulation. In this way, the number of ovulated oocytes increases.

During porcine embryo transfer, the synchronization between the stage of embryo development or donors and recipients is crucial, since several studies have shown that the highest pregnancy rates are obtained when recipients started estrus shortly after the donors (Hazeleger *et al.,* 2000; Angel *et al.*, 2014). Ziecik *et al.* (Ziecik et al., 2005) reported that, although the number of embryos obtained from prepuberal gilts was higher after the use of superovulation treatment, a large portion were degenerated. In addition, the percentage of hatched blastocysts after *in vitro* culture was also lower compared to non-superovulated animals. However, in a more recent study, Angel *et al.* (Angel *et al.*, 2014) found that the number of viable embryos obtained, in this case from sows, was not affected after superovulation treatment. However, in the porcine industry, weaning is used to synchronize estrous naturally between sows prior to

AI and, at present, superovulation treatments are only used in prepubertal gilts and sows for scientific purposes.

In vitro embryo production (IVP)

Three steps are involved in this procedure, which are *in vitro* maturation of oocytes (IVM), in vitro fertilization (IVF), or intracytoplasmic sperm injection (ICSI), and in vitro culture of embryos (EC).

According to the International Embryo Technology Society (IETS) in 2018 (IETS, 2018), there was no record on the use of porcine embryos. In 2017, the same institution reported that 43 fresh in vivo-derived embryos were transferred. In 2016, only 397 pig embryos were collected worldwide *in vivo* and none of them were produced *in vitro* (Perry, 2018). Although not all centers/institutions report their information, it is evident that porcine embryo collection is lower compared to the bovine species, where 935,000 embryos were obtained *in vivo* in 2016 and 2,600 were produced *in vitro* from oocytes obtained from ovaries collected in slaughterhouses (Perry, 2018). In addition, 2,000,000 bovine oocytes are obtained annually by Ovum Pick Up (OPU), a technique which consists in a needle connected to a vacuum system, allowing the aspiration of the ovarian follicles content through its visualization by an transvaginal ultrasound-guided probe (Galli, 2001).

In pig, this OPU technique is not of commercial interest since, as previously described, AI provides good productive yields. In addition, the low efficiency of *in vitro* fertilization (IVF) and subsequent embryo culture (EC), compared to AI (Kikuchi et al., 1999) or to the OPU efficiency in other species, like cattle, make this technique even less attractive in productive terms. However, recently, Yoshioka et al., (Yoshioka et al., 2020), reported that the collection of high genetic value oocytes could be performed in field conditions without the need for a surgical procedure. To achieve this, a small number of oocytes were collected by OPU and IVP was performed, later transferring the obtained embryos, resulting in the birth of piglets with the desired genetic value avoiding the risk of disease transmission. This is, thus, a field that needs to be further explored for future commercial applications.

In vitro oocyte maturation

This process involves both, nuclear and cytoplasmic maturation of the oocyte from prophase I (germinal vesicle, GV) to metaphase II (MII) stage.

To date, most oocytes are obtained from ovaries collected in slaughterhouse due to their low cost and viability (Grupen, 2014). For this, follicles with a size between 3 and 6 mm diameter are selected and the oocytes are removed by aspiration or dissection of the follicle (Pilar Coy & Romar, 2002). Different studies have shown that, depending on the follicular size, the competence of the oocytes to develop varies, thus underscoring the importance of careful follicle selection for IVM procedures(Kohata-Ono C, Wakai T, 2019).

Another factor to consider here is the age of the sows from which the oocytes are obtained. Most of the ovaries are from prepubertal gilts, and it is known that their oocytes have a lower developmental capacity after IVM and IVF than those obtained from adult sows. Furthermore, they are more susceptible to being penetrated by more than one sperm during IVF (Marchal et al., 2001), a process known as polyspermy that will be further explained in the next section.

After collection, oocytes are examined under a stereomicroscope and those that have a homogenous cytoplasm and that are surrounded by several layers of cumulus cells are selected (Pilar Coy & Romar, 2002). Cumulus cells contribute to cytoplasmic maturation by reducing the amount of fragmented DNA (Nagai, 2006), although it has been recently suggested that their removal 20 h after the start of in vitro maturation is beneficial (Ferré-Pujol et al., 2019).

Several criteria must be considered during the development of this step such as the oocyte quality being affected by the season, being significantly lower during summer compared to winter (Bertoldo et al., 2010).

On the other hand, the culture media used also plays an important role in outcomes, since although most oocytes are immature because most of the gilts slaughtered are prepubertal, if they are placed in the appropriate medium, they will be able to resume meiosis and reach the MII stage, at which point they can be fertilized (Yuan et al., 2017) and develop into an embryo. The most commonly used culture media are Tissue Culture Medium (TCM)-199, North Carolina State University (NCSU)-23, NCSU-37 media and modified Whitten's Medium (mWM) (Fowler et al., 2018).

Fetal calf serum (FCS) was used for years as an additive for the maturation media; however, it seemed to have a negative effect on oocytes, with a lowered ability for male pronuclear formation (Niwa, 1993). This led to the replacement of FCS with porcine follicular fluid (pFF). This additive has supported a high success

rate in the nuclear and cytoplasmic maturation (Romar et al., 2016), since it also protects the oocyte from oxidative stress (Tatemoto et al., 2004).

In vitro fertilization

Once the oocytes have matured to the MII stage, they can be used for IVF, a process that involves bringing the oocytes into contact with the sperm in a medium containing the necessary components for the final capacitation of the spermatozoa. When the oocyte is penetrated, two processes are triggered; on the one hand the resumption of meiosis and extrusion of the second polar body, and on the other hand the cortical reaction, which prevents the entry of more than one spermatozoa. Then, the zygote is formed and transferred to a culture medium with a composition and physical-chemical characteristics suitable for its development.

One of the most inherent problems with porcine IVF has been polyspermy, a phenomenon in which the oocyte is penetrated by more than one sperm (Hunter, 1991). The exposure to a large number of sperm during IVF in a culture dish could be the cause of the polyspermy (Abeydeera, 2002). However, reducing the amount of gamete cells in the dish does not solve the problem, as it also causes a decrease in the penetration rate (Abeydeera, 2002; Grupen, 2014), and even using oocytes matured *in vivo*, a low concentration of sperm in IVF fails to avoid polyspermy (Coy *et al.,* 1993).

As previously described, the cortical reaction tries to block the entry of more than one spermatozoa; thus it would be plausible that a failure during this event could be responsible for the entry of additional sperm. The cortical reaction involves the release of enzymes contained in the cortical granules (CG), which modify and eliminate sperm receptors around the oocyte, including those at the zona pellucida (Wang et al., 1998). However, in a study conducted by Wang et al., (1998), no differences were found in the release of CG between oocytes matured *in vivo* and *in vitro*, although they did find significant differences between the thickness and hardness of the zona pellucida, indicating that the cortical reaction might not be the only process involved in blocking polyspermy, and suggesting that an improvement in IVM culture media might be required.

Despite all these considerations, up to date, the method that has shown to reduce the incidence of polyspermy more drastically has been the incubation of oocytes before IVF in oviductal fluid (OF) collected from slaughtered adult sows

during the pre-ovulatory phase of the estrous cycle (Coy *et al.*, 2008). The incubation for 30 min caused a reduction of the number of sperm bound to ZP and an increase in the resistance of the zona pellucida to the sperm binding; consequently, the percentages of monospermic after IVF increased by 10 times compared with oocytes that had not been incubated in oviductal fluid (Coy *et al.*, 2008). This study therefore demonstrated that the imitation of the physiological microenvironment, placing the oocytes in the medium in which they would naturally find themselves after ovulation, is an effective strategy for improving the IVF results.

As for the sperm selection method, the use of swim up protocols instead of density gradients has been an important advance in terms of regulating polyspermy, in addition to allowing to achieve a final blastocyst yield of up to 40% when using a specific medium for porcine sperm (Embryocloud, Murcia, Spain) that includes oviductal fluid (OF) in its composition (Canovas et al., 2017b).

Intracytoplasmic sperm injection (ICSI) in pig could solve the problem of polyspermy. However, the procedure is costly, requiring expensive equipment and trained staff, making the routine use of this technique a challenge due also to the lower efficiency when compared with IVF. Nevertheless, the birth of piglets has been possible and reported (García-Vázquez et al., 2010; Katayama et al., 2007).

Embryo culture

After IVF, porcine embryos are cultured to a specific stage (morulae or blastocyst) depending on whether they are to be transferred to a recipient female or for other research purposes.

During the embryo culture process, the embryonic development capacity presents a low efficiency and, in addition, a low embryo quality (Kikuchi et al., 1999) when compared with *in vivo* derived embryos. This could be partly explained due to the high rates of polyspermy but also due to suboptimal culture conditions (Nagai, 2006).Nonetheless, it is possible to obtain viable embryos capable of reaching the blastocyst stage.

Several culture media are used, such as NCSU-23, Beltsville Embryo Culture Medium (BECM) (Dobrinsky et al., 1996), Porcine Zygote Medium (PZM), a protein-free chemically defined medium (Yoshioka et al., 2002) or porcine zygote medium 3 (PZM-3) plus 1.69mM arginine (MU1).

In Table 1, the average total cell number per blastocyst achieved, by different authors, according to the culture medium used is reported.

Table 1. *Mean number of cells reached by pig blastocysts grown in different culture media at day 6.*

Culture media	Mean number of cells per blastocyst (day 6)	Reference
BECM-3 + BSA-FAF supplemented with Fetal Bovine Serum (FBS)	170 (Day 8)	(Dobrinsky et al., 1996)
NCSU-23	25.3	(Machaty, 1998)
PZM-3 (PZM + 3 mg/ml of Bovine Serum Albumin (BSA))	41.1	(Yoshioka et al., 2002)
PZM-4 (PZM + 3 mg/ ml of polyvinyl alcohol)	38.4	(Yoshioka et al., 2002)
In vivo	57.6	(Yoshioka et al., 2002)
MU1+ 10 mM Glycine	55.9	(Redel et al., 2016)
NCSU-23 supplemented with Reproductive Fluids (RF)	82 (Day 7)	(Canovas et al., 2017b)

Yoshioka *et al*. (Yoshioka et al., 2002) reported that it was possible to achieve the blastocyst stage in a defined medium in the absence of a protein source. In addition, this could allow to homogenize the experiments performed in the laboratories reducing the risk of contamination avoiding the use of components such as bovine serum albumin (BSA) and eliminating the differences that could exist between different batches.

However, despite these advantages, the most used medium today is NCSU-23 (Fowler et al., 2018), which uses BSA as a protein source. Recently in a study by Canovas *et al* (Canovas et al., 2017b), it was shown that by using this medium supplemented with OF during the first two days of culture, and with uterine fluid (UF) from day 3 to 7, it was possible to achieve highest percentages of *in vitro* blastocysts referred so far in the porcine species (higher than 40%). Furthermore, this study shows that the imitation of the physiological environment

increases the number of embryos that develop *in vitro*, their development kinetics and the total cell number, being more similar to embryos produced *in vivo*.

Embryo transfer

If we look back, the first embryo transfer in a mammalian species was performed in rabbit in 1890 by Heape (Heape, 1890), but it was not until 1950 when the first embryo transfer was performed in pig by Kvasnickii (Kvasnitsky, 2001), resulting in one litter of four piglets.

Although the use of this technique allows the transfer of embryos with a high genetic value and a minimum risk of disease transmission and lower cost (E. A. Martinez et al., 2019), its use for commercial purposes is very limited, unlike other livestock species such as cattle (Perry, 2018). However, it is widely used in biomedical and biotechnology research, but few data are available and, in fact, according to the International Embryo Technology Society data (IETS), in 2018 there was no evidence of ET activity in swine (Viana, 2019).

On the other hand, it was estimated that in 2015, approximately 15.000 porcine embryos were transferred, most of them related to somatic cell nuclear transfer (SCNT, known also as cloning) and transgenesis (Callesen, *et al.,* 2019). In fact, in a recent study by Cho *et al.*(Cho et al., 2018), insulin-deficient piglets (*INS* Knockout Pigs) were produced with the aim of serving as diabetes research model by using the CRISPR/Cas9 and SCNT techniques followed by surgical embryo transfer.

For years, the main drawback derived from the use ET in pig has been the need to perform a surgical procedure for embryo collection and embryo transfer due to the complex anatomy of the reproductive system of the sow and the high number of embryos required (E. A. Martinez et al., 2014, 2015).

Conversely, the embryo collection by uterine flushing via transrectal, and the use of non-surgical embryo transfer has been widely used in other large species, such as cattle (Hasler, 2014), and horses (Valenzuela et al., 2018).

The first non-surgical embryo transfer in pig was performed by Polge and Day in 1968 (Polge & Day, 1968), but its use was not widely used due to the limited success. Later, the shift of surgical methods towards less-invasive procedures, such as endoscopy, facilitated embryo transfer (reviewed by Hazeleger and Kemp, 2001).

Other later trials on the use of these techniques were still far from optimal, with farrowing rates and litter sizes lower when compared to surgical procedures (R. D. Cameron et al., 1989; Wallenhorst & Holtz, 1999).

The first minimally-invasive procedure consisted of using the endoscope for the transfer of porcine embryos into the uterus, which was performed by Stein-Stefani and Holtz in 1987 (J. Stein-Stefani and W. Holtz, 1987). Unfortunately, the technique was not very successful, as only 2 recipients out of a total of 14 transferred became pregnant. Later, to optimize the endoscopic procedure, Besenfelder *et al.,* (Besenfelder et al., 1997) carried out embryo collection using this system and the subsequent transfer of embryos into the oviduct and uterus, resulted in the birth of live piglets.

A few years later, with the purpose of facilitating the commercial application of the non-surgical embryo transfer by avoiding the need for sedation, new instruments and devices were developed. The Swinlet®, a commercial catheter, was used by Ducro-Steverik *et al.,* (Ducro-Steverink et al., 2004) to perform a non-surgical embryo transfer depositing the embryos in the uterine body. The farrowing rate was 41%, with a litter size of 7,2 ± 2,8. In the same year, Martinez *et al*.(E. A. Martinez et al., 2004), developed a new catheter capable to cross the cervix of the sow to the deep of one of the uterine horns, allowing the transfer of morulae and blastocyst, resulting in 70.8% of farrowing rate and a litter size of 6,9 ± 0,7, similar to that obtained surgically, where the farrowing rate ranges from 60% to 80% and the litter size from 7 to 8 piglets (R. D. Cameron et al., 1989; Wallenhorst & Holtz, 1999).

Another aspect to be considered is the volume of medium entering in the uterus during embryo transfer. Several authors have reported that the use of a small volume of medium instead of a large volume, results in a higher pregnancy rate and litter size (Hazeleger & Kemp, 1994; E. A. Martinez et al., 2004). Also, Nakazama et al., (Nakazawa et al., 2008) reported that the use of a larger volume, (3.2-10 ml), resulted in a lower pregnancy rate due to embryo outflow from the uterine horns. Additionally, Yoshioka *et al.,* (Yoshioka *et al.,* 2012b) concluded that using 2,5ml or less may not affect pregnancy rate after non-surgical transfer of embryos produced *in vitro*.

Embryo cryopreservation

Gametes and embryo cryopreservation technology provides the possibility to store high value genetic material for a long period of time, preserving samples from selected animals or the conservation of endangered species (Comizzoli & Holt, 2019).

The difficulty to cryopreserve porcine embryos for later embryo transfer, and thus facilitate its industrialization, has been one of the limitations found due to the phenomenon of chilling injury (Polge et al., 1974; Pollard, J.W. and Leibo, 1994; Youngs, 2001). Therefore, most research has focused on studying the relationship between this sensitivity to hypothermia and cryosurvival with the high lipid content of porcine embryos (Dobrinsky et al., 2000).In other mammals like bovine species, these procedures are well established (Rodriguez-Martinez, 2012). Even in mouse or rabbit, embryos survived when they were frozen and stored in liquid nitrogen (Polge et al., 1974).

Vitrification is a method of cryopreservation that emerges as an alternative to the traditional slow freezing procedures due to the inefficiency in porcine species for the long-term storage of morulae and blastocysts (Gomis et al., 2013). However, despite the fact that live piglets have been born using this technique combined with subsequent surgical embryo transfer (Berthelot *et al.*, 2000), the embryo survival rates are still lower than those obtained in other species (Bartolac et al., 2018).

Cuello *et al.* (Cuello et al., 2005) showed an alternative by using, for the first time, a non-surgical embryo transfer method combined with vitrified/warmed blastocysts from slaughtered donors. Acceptable farrowing rates and litter size were achieved although pregnancy rate was lower than that obtained surgically. Some years later the same experiment was performed but, in this case, they evaluated the effectiveness of surgical and non-surgical procedures separately after the transfer of 30 and 30-40 vitrified embryos (morulae or unhatched blastocyst) respectively (E. A. Martinez et al., 2015). Finally, it was concluded that the results obtained after the non-surgical transfer of 40 vitrified embryos were higher than those obtained when transferring 30, and similar to those obtained surgically. This could offer an alternative to the pig industry by allowing greater genetic selection in order to obtain higher productive yields.

2. Short and long-term effects of ART on offspring

Nowadays, there are more and more studies that shed light on the possible effects derived from the use of ART, which can be caused by an adverse environment on critical periods of embryo development. From ovarian stimulation, IVM, IVF or ICSI, embryo culture, embryo transfer, and preimplantation genetic diagnosis (PGD), both gametes and/or embryos are submitted to a non-physiological microenvironment with different conditions and environmental stressors that could alter the normal embryo development with future consequences on the offspring.

Genomic imprinting is an epigenetic mechanism of gene regulation in which one allele, according to the parental origin, is silenced or expressed. This phenomenon is regulated by DNA methylation, acquired during gametogenesis (Monk et al., 2019). In mammals, imprinted genes are involved in the regulation of fetal growth and placental development (Lim & Ferguson-Smith, 2010). Hence, alterations on DNA methylation could lead to imprinting disorders, which may result in anomalous phenotypes. Considering that epigenetic reprogramming, which is very sensitive to these changes, occurs during the most critical period (from fertilization to implantation), normal development could be affected under stressful conditions with possible alterations at adulthood (D. J.P. Barker, 2007; Ventura-Juncá et al., 2015). This is supported by the Developmental Origins of Health and Disease (DOHaD) hypothesis, based on the influence of maternal environment on early development and its consequences in adult life (D. J. Barker, 1998).

It is assumed that each of the techniques involved in assisted reproduction, including the culture media used and manipulation of gametes and embryos, could be associated with lower implantation and pregnancy rates, fetoplacental anomalies, perinatal mortality, low birth weight (LBW), and a higher predisposition to suffer rare imprinting disorders, (Schieve *et al*., 2002; Jackson *et al.*, 2004; Chen *et al.*, 2015; Pisarska *et al.*, 2018; Valenzuela-Alcaraz *et al.*, 2018; Hattori *et al.*, 2019). However, it is difficult to differentiate whether such alterations are due to confunding factors underlying infertility, ART, or both (reviewed by Berntsen *et al.*, 2019).

Animal models represent a useful tool, since both fertile males and females can be used to avoid the confounding effects of infertility, allowing the study of the techniques separately (Vrooman and Bartolomei, 2017). Short and long-term effects of ART described in different species are summarized below.

2.1. Human

ART represents a helpful tool for couples with fertility problems, and the number of patients relying on assisted reproduction is increasing. Since the successful birth of Louis Brown in 1978, the number of children born through ART has reached 8 million worldwide (ESHRE, 2018), with IVF and ICSI being the most widely used techniques (Berntsen et al., 2019; Huang et al., 2020). These techniques require ovarian stimulation, oocyte collection, embryo culture and embryo transfer.

Even though most ART babies and children are healthy, there is an increased risk of adverse perinatal and long-term outcomes (Feuer *et al*., 2013; Jiang *et al.*, 2017) such as higher growth rate associated with higher incidence of cardiovascular risks or even pulmonary disfunction (Kleijkers et al., 2014). Thus, there is an increasing concern for both, the safety of use of these techniques and their potential effect on maternal and children health (da Silva et al., 2020).

Ovarian stimulation arises from the need of increasing IVF success for couples with fertility problems. However, the development of large numbers of oocytes in a non-physiological environment could lead to adverse effects on folliculogenesis, gametogenesis, embryo implantation, and adulthood (reviewed by Santos *et al*., 2010). In addition, ovarian hyperstimulation syndrome (OHSS) has been described after an external administration of gonadotropins (Kumar et al., 2011), also involving a risk to maternal health.

Furthermore, ovarian stimulation has been linked to multiple pregnancies, (Fauser *et al*., 2005), which are associated to a higher risk of hypertension and gestational diabetes, abnormal placentation, haemorrhages, preterm delivery, intrauterine growth restriction (IGR), low birth weight (LBW) and congenital malformation, among others developmental anomalies (De Geyter et al., 2018; Z. Jiang et al., 2017; Qin et al., 2015). Different strategies have been tried such as the implementation of single embryo transfer, in order to lower the rate of

multiple pregnancies (Zhu et al., 2016), thus leading to an increased use of cryopreservation methods of the surplus embryos. Consequently, this has allowed the transfer of frozen embryos reducing the ovarian stimulation cycles but also the prevalence of OHSS (Maheshwari et al., 2018; Roque et al., 2019).

Nonetheless, preterm delivery and LBW or small birth weight for gestational age are still more frequent in ART-derived than naturally conceived children (Castillo et al., 2019; Cavoretto et al., 2018), where it has also noted a correlation between LBW and the development of cardiovascular and metabolic diseases (David J.P. Barker & Bagby, 2005; Meister et al., 2018; Zandstra et al., 2020).

Ceelen *et al*., (Ceelen et al., 2009), reported that IVF children with LBW showed a faster growth rate later in late infancy compared with naturally conceived children. However, it was also affirmed that, although this phenomenon was associated with a higher blood pressure, it was independent of other factors such as birth weight, gestational age and body size, predisposing to a higher risk to develop cardiovascular diseases and type 2 diabetes. Curiously, this ‘‘catch-up’’ growth phenome, was also previously described in mice by Jimenez-Chillaron *et al.*, (Jimenez-Chillaron et al., 2006) later developing glucose intolerance in adult life.

Other ART-related placental anomalies, such as abnormal placentation and umbilical cord insertion, have been described in IVF/ICSI pregnancies (Pirtea et al., 2016; Vermey et al., 2018). Considering that the placenta provides the necessary oxygen and nutrients from the mother to the fetus, and imprinting genes plays an important role during placental, embryo, and fetal development (reviewed by Rhon-Calderon *et al.*, 2019), alterations in DNA methylation could lead to IGR, preeclampsia or placenta previa, with potential effects on the offspring health (Bloise et al., 2014).

For example, anomalies such as Angelman Syndrome (AS), Prader-Willi Syndrome (PWS), Beckwith-Wiedemann Syndrome (BWS), or Silver-Russell Syndrome (SRS) are related with different imprinting disorders (Lawrence & Moley, 2008). Besides that, BWS, characterized by an overgrowth and several malformations (Weksberg et al., 2010), shares some features with another syndrome described in cattle and sheep, called “Large Offspring Syndrome (LOS)” as will be explained in the next section.

In a recent study by Hattori *et al.*, (Hattori et al., 2019), focused on the correlation between a high incidence of imprinting disorders and with the use of ART, it was shown, by using genome-wide DNA methylation approaches, that imprinted defects were linked with a higher incidence of AS, PWS, BWS and SRS in ART offspring. Additionally, alterations in DNA methylation were significantly higher in PWS individual compared with those naturally conceived.

Apart from the differences found in growth parameters, cardiovascular or metabolic diseases, other anomalies have been associated with ART. It has been described a higher incidence of neurodevelopmental disorders, like mental retardation (Niederberger, 2013) in IVF children or reproductive alterations (Belva et al., 2017) and cancer increased risk (Hargreave et al., 2019). However, because most of the results are heterogeneous and the data are still limited, determining the cause of these anomalies remains a controversial issue.

2.2. Farm animals

Reproductive biotechnologies have been thoroughly used in farm animals to achieve a higher genetic selection and, therefore, increase the productive yields. Unlike the human species, where ART are used to treat infertility problems, in animals, they are also used to accelerate genetic improvement or with research purposes (Hyttel et al., 2000), allowing to delve into the molecular mechanisms that occur from the oocyte maturation to the embryo development.

Although mostly healthy offspring has been obtained, as previously described in human species, they are also prone to later health issues due to effects derived from ART procedures. Ovarian stimulation treatments are associated with a low fertilization rate in dairy cattle, which could be explained due to the negative effect on the oocyte metabolism, and therefore, with a lower embryo development capacity (Sartori *et al.,* 2010; Gad *et al.*, 2011; Bloise *et al.,* 2014).

Similarly, in porcine species, Ziecik *et al.*, (Ziecik et al., 2005) reported a poor embryo quality after the external administration of gonadotropins in prepuberal gilts. However, in a study by Angel et al. (Angel *et al.*, 2014) it was shown that oocyte maturation, fertilization rate and embryo quality were not

affected after ovarian stimulation, although, in this case, embryos were from sows instead of prepuberal gilts.

Differences between *in vivo* and *in vitro* produced embryos have been reported and might determine the embryo quality. For instance, in cattle there have been observed morphological differences like a darker cytoplasm in those embryos produced *in vitro* due to increased lipid accumulation (Abe et al., 2002; Fair et al., 2001), further reducing its cryopreservation capacity. In addition, the inner cell mass (ICM) in blastocysts is smaller under *in vitro* conditions compared with *in vivo* both in bovine and pig (Hyttel & Niemann, 1990; Van Soom et al., 1997).

In pig, due to the high incidence of polyspermy in this species, Han *et al.,* (Han et al., 1999) showed that, even though presumptive embryos from polyspermic fertilization can achieve blastocyst stage, the ICM is smaller compared with monospermic embryos, also resulting in a lower developmental capacity.

Given that ART procedures, as well as the culture media used, have been associated with epigenetic defects, there is a growing interest to optimize the culture conditions to assure their least possible impact. Cánovas *et al.,* (Canovas et al., 2017a), by using different culture conditions, found that methylation patterns and gene expression of embryos produced *in vitro,* with reproductive fluids as additives, were more similar to their *in vivo* counterparts, compared with those produced with BSA.

It is well documented that changes induced by exposure to suboptimal conditions, can be reflected in phenotypic defects. Indeed, in cattle and sheep it has been noted a higher incidence of embryo loss, higher pregnancy length, large body size and birth weight in ART-derived gestations. However, the most clear evidence is the large offspring syndrome (LOS), an imprinting disorder with similar features to BWS in human such as macrosomia, macroglossia or visceromegaly, the latter only described in cattle (Z. Chen et al., 2013; Y. Li et al., 2019). Other anomalies observed in IVP cattle due to an overgrowth in late gestation are a higher incidence of dystocia, hydrallantois, and neonatal mortality (Bertolini et al., 2002, 2004).

On the other hand, Bertolini *et al.,* (Bertolini et al., 2002) have associated this phenomenon as a placental compensatory mechanism triggered by a

developmental delay at the early stage of pregnancy in embryos and fetus. This was also affirmed by Miles *et al.,* (Miles et al., 2004, 2005). They reported that bovine placenta from *in vitro* derived embryos showed a lower vascularization at early pregnancy, which is later compensated with a higher number of blood vessels and therefore a higher vascular endothelial growth factor (VEGF) expression, which participates in the angiogenesis process. Similarly, some authors have correlated placental anomalies, with a higher incidence of postnatal mortality in lamb and calves cloned (Loi et al., 2006; Miglino et al., 2007).

More recently, Zheng *et al.,* 2017 found similar results with reduced vascularization in placentas from piglets derived of SCNT, however, unlike cattle and mice, cloned piglets showed a significantly LBW. Among the studies performed in cloned piglets, most of them agree on a LBW, litter size and different gene expression patterns compared with AI derived piglets (Hyttel et al., 2000; L. Jiang et al., 2007; Park et al., 2005). Interestingly, Jiang et al., 2007 (L. Jiang et al., 2007) also found significant differences in the organ weight, showing cloned piglets a lower weight in lung, and kidney at birth, but significant larger liver, spleen and kidney at 1 month old.

Even though numerous studies in cattle have enabled insights into the impact derived from ART, it is still necessary to progress in the porcine species, where the information is almost inexistent.

2.3. Mice.

To date, the use of the mouse model has been widely established to analyze the long-term effects of ART, in part due to the short generational intervals, easy maintenance or low costs derived (reviewd by Soom *et al.*, 2011).

It is known that suboptimal conditions during ART procedures in mice are also associated with epigenetic alterations which had led to fetal and placental anomalies and postnatal alterations as it has been described in other mammalian species (Duranthon & Chavatte-Palmer, 2018).

In addition, in absence of hormonal treatment, and even a minimal *in vitro* manipulation, mice have shown placental morphoanomalies and imprinting defects (de Waal et al., 2015a). In fact, previously, Feuer *et al.* (Feuer et al., 2014) reported that, even under the best known culture conditions, in absence of

superovulation and ET, both IVF and EC could lead to a lower implantation rate. Interestingly, they also noted that mice manifested sexually dimorphic adult phenotypes like metabolic disorder.

Previously, Bloise *et al.* (Bloise et al., 2012a) reported that, even though the placenta was apparently normal, some imprinting genes related to glucose and amino-acid transport showed a lower expression in IVP mice compared with those *in vivo* derived. Donjacour *et al.* (Donjacour et al., 2014) found that outbred IVP mice, under suboptimal culture conditions, showed different growth pattern, significant glucose intolerance besides an hypertrophy of the left heart compared with control. However, Slc38a4 imprinting gene, which is involved in amino acid transport, was down-regulated in adipose tissue in IVP females but not in males.

On the other hand, Fernández-Gonzalez *et al.* (Fernández-Gonzalez et al., 2004) reported that *in vivo* derived embryos from superovulated mice and then cultured with fetal calf serum (FCS) as supplement, showed behavioral defects such as anxiety or short memory trace compared with those derived from a media without FCS.

Finally, it is important to point that mice have been, until now, the only species in which transgenerational effects of in vitro culture could have been studied (Calle et al., 2012), precisely for the ease by which they can be raised in laboratories and their short gestational length. If similar studies could be carried out in pigs in the next future, their interest for the scientific community and the society in general would be, with no doubt, notable.

Given the increasing interest in the use of animal models for the study of ART procedures, it will be possible in few years to better understand the mechanisms involved in the described anomalies and to improve the outcomes derived from them, then allowing their future use in animal production.

For these reasons, studies that shed light on long-term effects derived from these techniques are today more necessary than ever before. To improve and develop new procedures and culture media that minimize, as far as possible, the potential adverse effects derived from ART, continue to be one of the biggest challenges of the reproductive biology field. Thus, the present study aims to contribute one more step further down this exciting road.

Hypothesis and objectives

As explained in the previous section, the porcine industry would benefit from the full development of reproductive technologies and therefore, more research is needed to reach this goal. In addition, very little is known about the impact of some of these technologies on the phenotype of the offspring, and this is an important point to consider for the industry sector. Thus, in the present study, we aimed to improve embryo transfer and *in vitro* production in the porcine model.

We hypothesized that:

1. The embryo transfer technique per se, and in absence of any confounder factor, influences the mother reproductive issues and the phenotype of the offspring.
2. The source of protein in the culture media where the embryos are produced is a factor influencing the outcomes of the IVP procedures and, also, the short- and long-term phenotypical traits of the offspring.

To confirm our hypotheses, we proposed the following objectives:

4. To decipher the isolated impact of the embryo transfer technique on reproductive issues and on phenotypical traits of the offspring at short term. This objective was accomplished by the tasks developed in Experiment 1.
5. To determine the impact of the protein source of the culture media on the in vitro embryo development, the pregnancy and parturition outcomes, and the placental and umbilical cord molecular traits. This objective was accomplished by the tasks developed in Experiment 2.
6. To analyse, at long-term, until month 6 of age, the phenotype of the offspring in terms of growth and haematological profile, and to determine glucose tolerance at day 45 of life. This objective was accomplished by the tasks developed in Experiment 3.

Material and methods

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma-Aldrich Chemical S.A. (Madrid, Spain).

1. Ethics.

The experimental work performed in this study was submitted to evaluation by the CEEA (Comité Ético de experimentación Animal) from University of Murcia. After approval, authorization from “Dirección General de Agricultura, Ganadería, Pesca y Acuicultura” – Región de Murcia- nr A13170706 was given to perform the experiments with animals.

2. Animals.

Experiments were performed in two different pig farms of the same company (Cefu S.A., Murcia, Spain). Crossbred sows (Landrace x Large White) with the same genetic line were used as donors (1-11 parities), and as recipients (2-3 and 0-9 parities in experiments 1 and 2) respectively.

All animals were housed and fed under the same conditions and water was provided *ad libitum*.

Weaning was used for estrous synchronization, and sows that showed signs of being clearly in heat 4-5 days after weaning were used as donors or recipients.

Estrous detection was performed by exposing a mature vasectomized boar to stimulate the estrous expression of the sow and applying the back-pressure test. Those sows that remained immobilized under such pressure were considered in heat.

All donors were artificially inseminated twice, 0 and +24 hours after the onset of estrus, with semen from a boar of the same breed and proven fertility.

For Experiment 1, estrous synchrony of the recipients was between 0 and +24h regarding the donors. A total of thirty-two and thirteen sows were used as

donors and recipients respectively, although 7 donors of the total were eliminated because of the number of embryos collected was insufficient or zero.

Six litters of *Sus scrofa* were obtained, being 78 the total live piglets born (38 females and 40 males):

- AI group (n= 41): Piglets were obtained by AI. This group was the control of our experiment.
- ET group (n= 37): Piglets were obtained from embryos produced *in vivo*, subsequently transferred by non-surgical embryo transfer.

On the other hand, for Experiment 2, the synchrony of the recipients was between -24 and -48h regarding the embryos (Angel *et al.*, 2014), being twenty-three the total gilts or sows used. Animals that were inseminated in the same farm under the same condition were used as control (AI group).

The 12 litters of *Sus scrofa* obtained in experiment 2 were used for Experiment 3. In total, 105 live piglets (58 females and 47 males) were born, allocated in the different groups as follows:

AI Group (n = 57): Piglets were obtained by AI. This group was the control of our experiment.

C-IVP (n = 29): Piglets were obtained from females that became pregnant after the transfer of embryos produced *in vitro* using culture media with BSA as the only protein source.

- RF-IVP group (n = 19): Piglets were obtained from females that had become pregnant after the transfer of embryos produced *in vitro* using reproductive fluids as additives in the culture media

3. *In vivo* embryo collection (Experiment 1).

Considering day 0 as the onset of estrous, on days 6-8, donors were sedated by administration of ketamine (10 mg/kg, Imalgene® 1000, im), medetomidine (0.02 mg/kg, Domtor®, im) and midazolam (0.2 mg/kg, Dormicum®, im). Then, sows were euthanized with an overdose of sodium pentobarbital (200 mg/ml, Eutanax®, iv) and immediately after, a midventral laparotomy was performed to excise the uterus.

Embryos were collected by flushing from the uterine bifurcation to the tip of each uterine horn with 60 ml of a modified Tyrode´s lactate-Hepes-polyvinil alcohol (TL-HEPES-PVA) medium at 38,5ºC. This medium was composed of 114 mM NaCl, 3.2 mM KCl, 2 mM NaHCO3, 0.34 mM KH2PO4, 10 mM Na-lactate, 0.5 mM MgCl2·6H2O, 2 mM CaCl2·2H2O, 10 mM Hepes, 0.2 mM Na-pyruvate, 12 mM sorbitol, 0.1% (w/v) polyvinylalcohol, 25 µg/ml gentamicin, and 65 µg/ml potassium penicillin G (Funahashi et al., 2000). Embryo's development stage and quality were evaluated under a stereomicroscope and morulae, unhatched and/or hatched blastocyst were used. Embryos were washed in the same medium and remained in a culture dish maintained at 38,5ºC until transferred.

The period of time between uterus removal in donors and the non-surgical embryo transfer in recipients was between 60 to 90 minutes.

4. Non-surgical embryo transfer (Experiment 1).

The perineal area of the recipients was carefully washed with soap and water and dried gently. A latex glove was placed in the tail to avoid cross contamination of the area.

Nonsurgical ET catheters (DeepBlue® ET catheter, Minitüb, Tiefenbach, Germany) were used to transfer the embryos collected from donors (6-7 days after the onset of estrous, as above indicated). Intrauterine insertion of the catheter was performed as previously described by Angel et al. (Angel *et al.*, 2014). Briefly, *in vivo* derived embryos were loaded in a 1 ml syringe using the following sequency of aspiration: 0,1ml of TL-HEPES-PVA medium, 0, 1 ml air, 0,1ml of TL-HEPES-PVA medium with embryos, 0,1 ml air, and finally 0,1ml TL-HEPES-PVA medium. Then, the syringe was attached to the catheter and the embryos were gently introduced by pushing the syringe plunger. An additional 0.5 ml of TL-HEPES-PVA medium were used for washing the catheter. Finally, the catheter was removed and re-washed with the same medium on a culture dish to verify no embryos remained inside.

The number of embryos per transfer and sow varied between 39 and 99. After the transfer, a dose of amoxicillin (20mg/kg body weight) was injected intramuscularly to each recipient (Clamoxyl LA®; Pfizer, Madrid, Spain).

5. *In vitro* embryo production (Experiment 2).

Porcine follicular, oviductal and uterine fluids for the different steps of the IVP were provided by EmbryoCloud, (NaturARTs®, Murcia, Spain).

5.1. Oocyte collection and in vitro maturation.

Ovaries from prepubertal crossbred gilts (Landrace x Large white) were obtained at the slaughterhouse and transported at 38,5ºC to the laboratory in saline solution containing 100mg/ml kanamycin sulfate. They were subsequently washed at the same temperature, once in 0.04% (w/v) cetrimide solution and twice in saline solution. Cumulus cell-oocyte complexes (COCs) were collected from antral follicles between 3 and 6 mm diameter and washed twice with Dulbecco's PBS (DPBS) supplemented with 1 mg/ml polyvinyl alcohol (PVA) and twice more in maturation medium previously equilibrated for a minimum of 3h at 38.5ºC under 5% CO_2 in air. Maturation medium was NCSU-37 (Petters & Wells, 1993) supplemented with 0.57 mM cysteine, 1 mM dibutyryl cAMP, 5 mg/ml insulin, 50 µM β-mercaptoethanol, 10 IU/ml equine chorionic gonadotropin (eCG; Foligon; Intervet International BV, Boxmeer, Holland), 10 IU/ml human chorionic gonadotropin (hCG; Veterin Corion; Divasa Farmavic, Barcelona, Spain), and 10% (v/v) porcine follicular fluid (NaturARTs® PFF, EmbryoCloud, Murcia, Spain) as additives.

COCs with complete and dense cumulus oophorus were selected, and groups of 50-55 COCs were cultured in 500 µl maturation medium for 22h at 38,5ºC under 5% CO_2 in air. After culture, oocytes were washed twice in fresh maturation medium without dibutyryl cAMP, cAMP, eCG and hCG and cultured for an additional 20-22h (Funahashi et al., 1997).

5.2. In vitro fertilization.

After 44h of maturation, cumulus cells were partially removed mechanically by pipetting and mature oocytes were washed twice in TALP medium previously equilibrated at 38,5ºC under 5% CO_2. TALP medium consisted of 114.06 mM NaCl, 3.2 mM KCl, 8 mM Ca-lactate.5H_2O, 0.5 mM

$MgCl_2.6H_2O$, 0.35 mM NaH_2PO_4, 25.07 mM $NaHCO_3$, 1.85 mM Na-lactate, 0.11 mM Na- pyruvate, 5 mM glucose, 2 mM caffeine, 1 mg/ml PVA and 0.17 mM kanamycin sulfate.

Mature oocytes were divided in two groups according to the IVF medium supplementation: Bovine serum albumin (C-IVP) and Reproductive fluids (RF-IVP). C-IVP was supplemented with 3mg/ml BSA and RF-IVP with 3mg/ml BSA and 1% (v/v) POF from the late follicular (LF) phase of the estrous cycle (NaturARTs® POF-LF, EmbryoCloud, Murcia, Spain) as additives.

Oocytes were put in groups of 50 into each well of a 4-well multidish containing 250 µl TALP medium.

Ejaculated spermatozoa from a fertility-tested boar (1 year old) were transported to the laboratory and diluted at 1:5 in Beltsville thawing solution, BTS (Pursel and Johnson, 1975) remaining stored at 16ºC for 24h until IVF. At 24 hours, the sperm solution was centrifuged (300g, 10min) and the supernatant was collected.

For sperm selection, NaturARTs® PIG sperm swim up medium (EmbryoCloud, Murcia, Spain) was used, previously supplemented with 3mg/ml bovine serum albumin or 10% (v/v) POF-LF (NaturARTs®, EmbryoCloud, Murcia, Spain) for the subsequent IVF of C-IVP and RF-IVP groups, respectively. Then, 1 ml of semen was lay below 1 ml of NaturARTs® PIG sperm swim up medium (http://embryocloud.com) at the bottom of a conical tube. After 20 min of incubation at 37˚C (with the tube at a 45˚ angle), 0.50 ml from the top of the tube were aspirated and diluted in TALP medium. Finally, 250 µl of this solution was added to the wells containing the oocytes, giving a final concentration of 10000 spermatozoa/ml for each corresponding oocyte group. Spermatozoa and oocytes were incubated at 38,5ºC under 5% CO2 for 18-20h.

Then, putative zygotes were transferred to embryo culture medium and a small sample of each group was taken to assess the fertilization rates by fixing and staining the putative zygotes as previously described (P. Coy, Grullon, et al., 2008).

5.3. Embryo culture.

The embryo culture medium was NCSU-23 (Petters & Wells, 1993) supplemented with sodium lactate (5 mM), pyruvate (0.5 mM), non-essential

amino acids and 0.4% BSA for the first 48 post insemination (hpi) (NCSU-23A) or glucose (5.5 mM), essential, non-essential amino acids and 0.4% BSA from 48h to 120-144 hpi (NCSU-23B).

18-20 hpi, putative zygotes were washed once in NCSU23-A and transferred to a culture dishes containing the same medium with or without 1% (v/v) POF from the early luteal (EL) phase (NaturARTs® POF-EL, EmbryoCloud, Murcia, Spain) in the RF-IVP and C-IVP respectively. Each well of the culture dish containing the embryos was covered with parafin oil (Nidoil, Nidacon, Sweden). Putatives zygotes were incubated at 38,5ºC under 5% CO2 and 7% O_2.

After 48 hpi, the cleavage was assessed under the stereomicroscope and the 2-4 cell stage embryos were washed and transferred to NCSU23-B supplemented or not with 1% (v/v) of porcine uterine fluid (PUF) from mid luteal phase of the estrous cycle (NaturARTs® PUF-ML, EmbryoCloud, Murcia, Spain) according to the corresponding group. Each well was also covered with Nidoil and embryos remained here until day 5 or 6 (120-144 hpi) of culture maintaining the same incubation conditions.

6. Surgical embryo transfer (Experiment 2).

6.1. Embryos.

On days 5 or 6 after IVF, embryo morphology was evaluated under stereomicroscope before being transferred to recipients. Then, a commercial medium (BO-Transfer, IVF Bioscience, Denmark) was used to wash and transport the embryos from the main building of the Veterinary Faculty (Department of Physiology, 2nd floor) to the Veterinary farm at the University of Murcia (Spain) on a portable incubator at 38,5ºC.

Emtrac Delphin catheter, (Gynetics®) was selected to transfer the embryos. For it, a 1ml syringe embryo tested (Non-toxic syringe, COOK® Medical, Ireland) was attached to the catheter allowing, by aspiration, embryo loading into it. The procedure used was the same described in Experiment 1 but using BO-Transfer medium (IVF Bioscience, Denmark) in this case.

6.2. Paralumbar laparo-endoscopy single-site.

Considering day 0 as the onset of estrous, on days 4-5, recipients were anesthetized, prior to 24h fast. The protocol was the same described in Experiment 1, however, in this case, anesthetic maintenance and analgesia were necessary through the administration of isoflurane (ISOFLO, Esteve 2-3% O_2) and buprenorphine (0,01mg/kg, Bupredine®, im) respectively.

To perform the surgical approach, the incision area was shaved, washed and disinfected previously with chlorhexidine (Desinclor 1%, Imark, Madrid, Spain).

The technique used to perform the embryo transfer was a paralumbar laparo-endoscopy single-site (LESS) assisted approach with a monoport device (GelPOINT® Advanced Access Platform, Applied Medical, Rancho Santa Margarita, California, USA) formed by a cap of gel and two flexible rings connected by a plastic membrane. Sows were placed in lateral recumbence and a mini-lumbotomy of 5 cm, approximately, was performed. To stabilize the incision in the abdominal wall, one of the flexible rings was placed inside, remaining the second adhered to the abdominal wall externally. Monoport cap was placed on it and two trocars were used to insert the endoscope and a non-traumatic laparoscopy forceps through them into abdominal cavity. Then, abdominal cavity was fill with CO_2 pneumoperitoneum of 8–10 mmHg to facilitate the visualization of the reproductive organs and their handle. The ovaries were located and checked. Later, one of the uterine horns was grasped with the forceps and taken to the port opening. Next, pneumoperitoneum and monoport cap were removed to allow a gentle manipulation and the end of the uterine horn was punctured with dissecting forceps toothed allowing, through a small opening, the introduction of the Emtrac Delphin catheter, (Gynetics®) and deposition of embryos into the lumen of uterine horn. Then, the catheter was removed and re-washed with the same medium on a culture dish to verify no embryos remained inside. The number of embryos per transfer and sow varied between 29 and 93. Finally, the surgical incision was sutured in three planes and a dose of amoxicillin (20mg/kg body weight) was injected intramuscularly (Clamoxyl LA®; Pfizer, Madrid, Spain).

7. Pregnancy diagnosis and farrowing.

Pregnancy was diagnosed by ultrasonography 21-26 days after the onset of estrous, performing, in addition, a monitoring throughout it. Piglets derived from sows that were artificially inseminated in the same farm under the same conditions were used as control (AI group).

All sows were housed in gestation crates located in a parturition unit. However, for Experiment 2, this place was located in the research building of the Veterinary farm at the University of Murcia (Spain), and few days before giving birth, sows were monitored continuously by a camcorder to observe possible signs of parturition as vulvar secretion.

On the farrowing day, umbilical cord (UC) samples were taken of each piglet for gene expression analysis. Then, the UC was disinfected with chlorhexidine (Lainco Clorhexidine 2%, Barcelona, Spain).

To identify placentas individually, a double ligature was made to each umbilical cord as the piglets were expelled, sewing a numbered tag to the end of the cord that would be retracted into the vagina. This method was previously described (Matthew E Wilson et al., 1998). Next, an umbilical cord sample was taken of each piglet and then it was disinfected with chlorhexidine (Lainco Clorhexidine 2%, Barcelona, Spain).

Piglets were weighed using a digital hanging scale and, immediately after, they were placed with their mother. In addition, two additional growth parameters named crown rump length (CRL) and anogenital distance (AGD) were check with a tape measure. CRL refers to the distance between the frontal region of the skull and the rump, at the birth of the tail, while the anogenital distance comprises the space between the center of the anus and the onset of the scrotum.

As the placentas were expelled completely, they were weighed also using a digital hanging scale. Then, they were placed on paper to trace their contour and subsequently measure their surface area

According to the experiment, gestation rate, farrowing rate, survival rate, litter size, body weight, CRL and AGD were analysed.

8. RNA isolation and quantitative Real-time PCR (Experiment 2).

Total RNA was extracted from two different tissues (placenta and umbilical cord) of the two males and females with the highest and lowest weight from each litter, using Trizol® (Invitrogen).

Briefly, at each time point the collected tissues were washed with PBS, and immediately immersed and stored in liquid nitrogen until further use. Afterword, the tissues were immersed in Trizol reagent, proteins were removed with chloroform extraction, and the RNA pellets were washed once with isopropyl alcohol (Sigma, USA), followed by a washing with 70% ethanol solution prepared with RNAse, DNAse-free water (Gibco, Invitrogen). The total RNA pellets were reconstituted in RNAse free water (Gibco, Invitrogen). A microspectrophotometer (NanoDrop 2000c, Thermo Scientific, USA) was used to quantify the extracted RNA (ng/µL).

The single-strand cDNA synthesis by RT-PCR was performed using SuperScript™ III Reverse Transcriptase kit (Invitrogen) according to manufacturer's protocol. Briefly, a reaction mixture consisting of 1X iScript Reaction Mix, 1 µL iScript Reverse Transcriptase, RNA template (1ug total RNA) and nuclease-free water was prepared, for the final volume of 20 µL. The reaction was performed under the following conditions: 25ºC for 5min, 42ºC for 30 min., and 85ºC for 5min.

The expression of selected genes was measured by quantitative real-time PCR (q-PCR) on a CFX96 Touch System (Bio-Rad). SYBR® Green PCR Master Mix (Applied Biosystems™) was used to perform Real-time PCR, according to the manufacturer´s indications.

Reactions were prepared for the final reaction volume of 25 mL, using a dilution of 1/20 of cDNA products, 1 µM specific primer (primer sets listed in Table 2), 1X iQ SYBR Green Supermix and nuclease-free water. The primers for each selected gene were designed using Primer3 web application (http://primer3.ut.ee/).

The cycling conditions followed were an initial denaturation at 95ºC for 10 min followed by 40 cycles of denaturation at 95ºC for 10 s, annealing at 60ºC

for 10 s with an extension at 72ºC for 12s, and finally a Melting curve consisting of 95ºC for 20s and 65ºC for 20s. The gene expression study for each gene per sample was performed for triplicate, and reactions were performed on the C1000 Touch Thermal Cycler (Bio-Rad). The fold change in gene expression of selected target genes relative to the housekeeping gene β-actin (*ActB*) was evaluated by the Livak (2 -ΔΔCT) method (Livak & Schmittgen, 2001).

Different genes with different functions such as imprinted, angiogenesis, transcription, and glucose transport were selected as shown in Table 2 (Livak & Schmittgen, 2001).

***Table 2**. Primers sequences for quantitative real-time PCR*

Gene	Functional role	Description	Primer sequence (5´-3´)	Product size (bp)	GenBank ID
IGF2	Imprinted gene	Insulin like growth factor 2	Forward: ACACCCTCCAGTTTGTCTGC Reverse: AAGCAGCACTCTTCCACGAT	100	NC_010444.4
IGF2R	Imprinted gene	Insulin like growth factor 2 receptor	Forward: GCCGGCGAATACACCTATTA Reverse: CATCTTCAACACCCCGTTCT	180	NM_001244473
H19	Imprinted gene	Imprinted maternally expressed	Forward: GGCCGGAGAATGGGAAAGAAG G Reverse: CGCAGTGCTGCGTGGGAACG	148	NM_100624888
PEG3	Imprinted gene	Paternally expressed 3	Forward: GTCGCAGAAGAGTCACACCA Reverse: AGCTGCGAAGAACAGACCTC	128	NC_010448.4
GRB10	Imprinted gene	Growth factor receptor bound protein 10	Forward: CTTCCCCGAACAGATGGTTA Reverse: CCCTTCGTGGAGCAGTAGAG	192	NC_010451.4

MEST	Imprinted gene	Mesoderm specific transcript	Forward: AAGGGACTGCGCATCTTCTA Reverse: AGGGTCAGACCTTCCCAGAT	125	NC_010460.4
SLC7A1	Imprinted gene	Solute carrier family 7 member 1	Forward: CATCAAAAACTGGCAGCTCA Reverse: TGGTAGCGATGCAGTCAAAG	194	NM_001012613
GRIN2C	Imprinted gene	Glutamate ionotropic receptor NMDA type subunit 2C	Forward: TGTCTGGCCTCAGTGACAAG Reverse: GTGCATGTCACGGTAGTTGC	122	NC_010454.4
LUM	Angiogenesis	Lumican precursor	Forward: TCTGCTGGAGCTGGATCTCT Reverse: CGCAAATGTTTGATCTTGGA	165	NC_010447.5
VIM	Angiogenesis	Vimentin	Forward: ATGCTTCTTTGGCACGTCTT Reverse: GATTTGGACGTGCTGTTCCT	134	NC_010452.4
PEG10	Imprinted gene	Paternally expressed 10	Forward: AGCGATCCCACTACCTGATG Reverse: CGTTCCAATCCAGATCCTGT	138	NC_010451.4

JUN	Transcription	Transcription factor AP-1	Forward: CCCAAGATCCTGAAGCAGAG Reverse: GATGTGCCCGTTACTGGACT	174	NC_010448.4
Slc2A1	Glucose transport	Solute carrier family 2 member 1	Forward: GCAGGAGATGAAGGAGGAGAGC Reverse: ACGAACAGCGACACGACAGT	258	NC_010448.4

9. Placental parameters (Experiment 2).

Three different parameters were analysed: placental weight (g), placental area (cm^2) and placental efficiency (g/g). For the first, each placenta, previously identified with its corresponding piglet, was weighed using a digital hanging scale. The placental surface area was analysed by ImageJ 1.52a software (National Institute of Health, USA), subsequently multiplying the area by two. Finally, the placental efficiency, which is defined as the ratio of birth weight to placental weight and reflects grams of fetus produced per gram of placenta, was calculated,

10. Growth parameters (Experiment 3).

All piglets were weighed the same way described earlier, and Average Daily Weight Gain (ADWG) was calculated by the following formula:

$$ADWG = \frac{Weight\ at\ day\ X - Weight\ at\ the\ previous\ day\ assessed}{Days\ from\ first\ to\ second\ weight\ measure}$$

The Body weight (BW) and ADWG (g) were measured on days 3 and 15 of life for Experiment 1.

For Experiment 3, BW, ADWG, CRL (cm) and AGD (cm) were checked at different days of life: 0, 3, 9, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165 and 180. The weight was obtained with two different types of scales for different ages. The animals were weighed until day 45 using a digital hanging scale, tying a rope to the animal back end behind the hock. From day 84 onwards, they were weighed on a mobile scale.

11. Blood collection (Experiment 3).

Blood samples were collected by direct venipuncture of the jugular vein with a Vacutainer® system using a 20G x 25mm needle and BD

Vacutainer® lithium heparin tubes. Blood tubes were transported to the laboratory and haematological analysis was performed using a haematology analyzer (Siemens ADVIA® 120, USA). The parameters analysed were concentration of erythrocytes (RBC, $_X10^6$ cells/µL), hemoglobin (HB, g/dl), hematocrit (HCT, %), mean corpuscular volume (MCV, fL), mean corpuscular hemoglobin (MCH, pg), mean corpuscular hemoglobin concentration (MCHC, g/dl), cell hemoglobin concentration mean (CHCM, g/dl), hemoglobin concentration distribution width (HDW, g/dl), white blood cells (WBC, $_X10^3$ cells/µL), neutrophils ($_X10^3$ cells/µL), lymphocytes ($_X10^3$ cells/µL), monocytes ($_X10^3$ cells/µL), eosinophils ($_X10^3$ cells/µL), basophils ($_X10^3$ cells/µL), reticulocytes indices: percentage of reticulocytes (%), average size of reticulocytes (MCVr, fL) and average hemoglobin content of reticulocytes (CHr, pg), platelets ($_X10^3$ cells/µL) and platelets indices: platelecrit (PCT, %), mean platelet volume (MPV, fL), platelet distribution width (PDW, %), platelet component distribution width (PCDW, g/dl), mean platelet mass (MPM, pg), platelet mass distribution width (PMDW, pg) and large platelets (Large PLT, $_X10^3$ cells/µL).

In Experiment 1, in addition, different biochemical parameters were also analysed. For that, blood was centrifuged at 1008G for 10 min at room temperature and then, biochemical analysis was performed using a chemistry analyzer (Olympus AU400, Japan). The parameters analysed were: creatinine (CREA, mg/dl), urea (mg/dl), amylase (UI/L), creatine kinase (CK, UI/L), cholesterol (mg/dl), alkaline phosphatase (ALP, UI/L), gamma-glutamyl transferase (GGT, UI/L), glucose (mg/dl), aspartate aminotransferase (AST, UI/L), alanine aminotransferase (ALT, UI/L), lipases (UI/L), total proteins (TP, g/dl), albumin, (ALB, g/dl), globulins (GLOB, g/dl), triglycerides (TRIGL, mg/dl) and total bilirubin (TBIL, mg/dl).

The study performed during Experiment 3 lasts from birth (day 0) to an age of 180 days, and due to the casualties suffered and the abnormal individuals eliminated, only 74 live piglets (42 females and 32 males) remained at the end of the study.

During this experiment, the animals lived in different units and had *ad libitum* access to food and water. At birth, they were in the parturition unit, where

all the sows near the birth were located. In this unit, after birth, the piglets remained until day 28, the date on which weaning occurred. After this, they changed location to the transition unit. On day 84, the last relocation of the piglets was carried out and they were transferred to the fodder unit, where they remained until the end of the study.

12. Oral glucose tolerance test (Experiment3).

The oral glucose tolerance test (OGTT) was performed at day 45 of life in four litters of relative animals per group. To do this, the two males and two females of highest and lowest weight per litter were selected for the study (N=14, N=15, and N=16 for RF-IVP, C-IVP, and AI, respectively).

After an 18 h overnight fast, water was withdrawn and, 1 h later, all selected piglets ingested 1.75 g/kg BW of glucose solution. Then, blood samples were collected from the auricular lateral vein before and 5, 10, 15, 20, 30, 45, 60, 90, 120 and 150 min after glucose intake. Blood glucose concentration (BGC) was immediately measured by test strips with a glucometer (GlucoMenLX Plus+).

13. Statistical Analysis.

All growth parameters, including AGD in males, were analysed using the R studio software version 3.4.4 (R core Team 2018). A robust mixed analysis of variance (ANOVA) was used to find differences for BW and ADWG data while for CRL and AGD a mixed ANOVA was used, since the Mauchly's test for sphericity was significant (p<0.05) for both cases, but the corrections Greenhouse-Geisser (GGe) and Huyhn Feldt (HFe) were not valid for the first two.

BW and ADWG data were analysed by sex and group following a robust mixed ANOVA, since the Mauchly's test for sphericity was significant (p<0.05) for both cases, but the GGe and HFe corrections were invalid.

The differences for CRL were also investigated through a robust mixed ANOVA in the case of males, while in the case of females it was used a mixed ANOVA with GGe correction.

To search for differences in AGD a mixed ANOVA was used, with GGe correction in the case of males.

All these parameters were studied each sampling day. It was performed by means of a one-way ANOVA when there was normality and homoscedasticity and, by heterocestic ANOVA when there was normality but not homoscedasticity.

In addition, to know if there was a correlation between the litter size and the BW of the individuals at days 0, 30, 75, 120 and 180, a two-way ANOVA was performed for these days in the case of males. In females, a two-way ANOVA was performed for days 0, 120 and 180, and a Welch-James test for days 30 and 75, since there was normality but no homoscedasticity. In all the parameters significant differences were considered those that obtained probability levels of $p<0.05$.

All blood parameter data analyses were performed using GraphPad Software, version 7, La Jolla California USA. D´Agostino & Pearson normality test was performed to asses normality of the data. Unpaired t-test was used when data were normally distributed, and Mann-Whitney U-test was used in case of non-normal data distribution for Experiment 1. Instead, for Experiment 2, One-way ANOVA was used when data were normally distributed and Kruskal-Wallis test when data did not follow a normal distribution. Dunn´s multiple comparison test was used to assess differences between groups.

For the analysis of gene expression results, GraphPad Software, version 7 was also used. D´Agostino & Pearson normality test, and Shapiro-Wilk test were performed to asses normality of the data. One-way ANOVA was used when data were normally distributed and Kruskal-Wallis test for a non-normal distribution of data. Dunn´s multiple comparison test was used to assess differences between groups.

The results are presented as mean ± SD (standard deviation). Values of $p<0.05$ were considered significant. To study the OGTT results, the software used was IBM SPSS Statistics (v22.0). One-way ANOVA was performed to analyze the differences between data. Tukey post-hoc tests were applied when ANOVA showed significant differences ($p<0.05$). Then, Pearson correlation coefficient was used to detect litter influence. The results are presented as mean ± SEM (standard error of the mean).

14. Experimental design

Experiment 1.

In vivo embryos were collected from artificially inseminated sows and transferred by non-surgical approach to recipients. Gestation length, farrowing rate, survival rate, litter size and phenotypical traits of the offspring at short term (birth weight, average daily weight gain, haematological and biochemical parameters) were analysed.

Experiment 2.

Embryos were produced by *in vitro* fertilization, cultured in media with different protein sources and surgically transferred to recipients at the blastocyst stage. Outcomes of the embryo development *in vitro*, pregnancy rates and parturition issues were analysed. Furthermore, placental parameters (placental weight, surface area and placental efficiency) as well as placental and umbilical cord expression of selected genes were evaluated.

Experiment 3.

The phenotype of the offspring derived from experiment 2 was analysed until month 6 of age, through growth and haematological profile, and glucose tolerance.

Results

Experiment 1.

1.1. Pregnancies and farrowing rate.

Three of the 13 ET-recipients that were transferred with around 40 or more embryos became pregnant and ten returned to oestrus at 17-25 days (Table 3). One of the three pregnant sows presented a synchrony of 0h regarding the donor, and the other two of +24h.

Gestation period was within expected normal range and piglets born after *in vivo* embryo transfer did not present any anatomical abnormality. The total piglets born were 39 (Table 5).

1.2. Piglets.

Piglets born from a total of three artificially inseminated sows were used as control as shown in Table 4. In the total litter size, whose range was from 6 to 19 and from 10 to 17 in the ET and AI groups, respectively. There were no significant differences between groups (p=0.9999).

Table 5 shows the data at birth from piglets derived from ET and those derived from AI. The total number of piglets born by ET was 39 while 41 were born by AI. None of them presented morpho-anomalies, therefore being anatomically normal and weighing as expected.

Birth weight was analysed separately by sex in piglets. Only females showed significant differences with those in ET group weighing more than in the AI group. However, no differences were observed between males.

Analyzing the total Kg of piglets obtained at birth per sow in both groups, those recipients delivering ET piglets presented a higher value compared with those artificially inseminated, although no significant differences were observed (Table 5), (p>0,05).

Despite the differences observed between females from ET and AI group at birth weight (Table 5), body weight was within the normal range and no

significant differences were observed on days 3 and 15 of life when piglets were separated by sex or group. AGD was slightly higher in the AI group in both sexes, but not significantly different from ET (Figure 1).

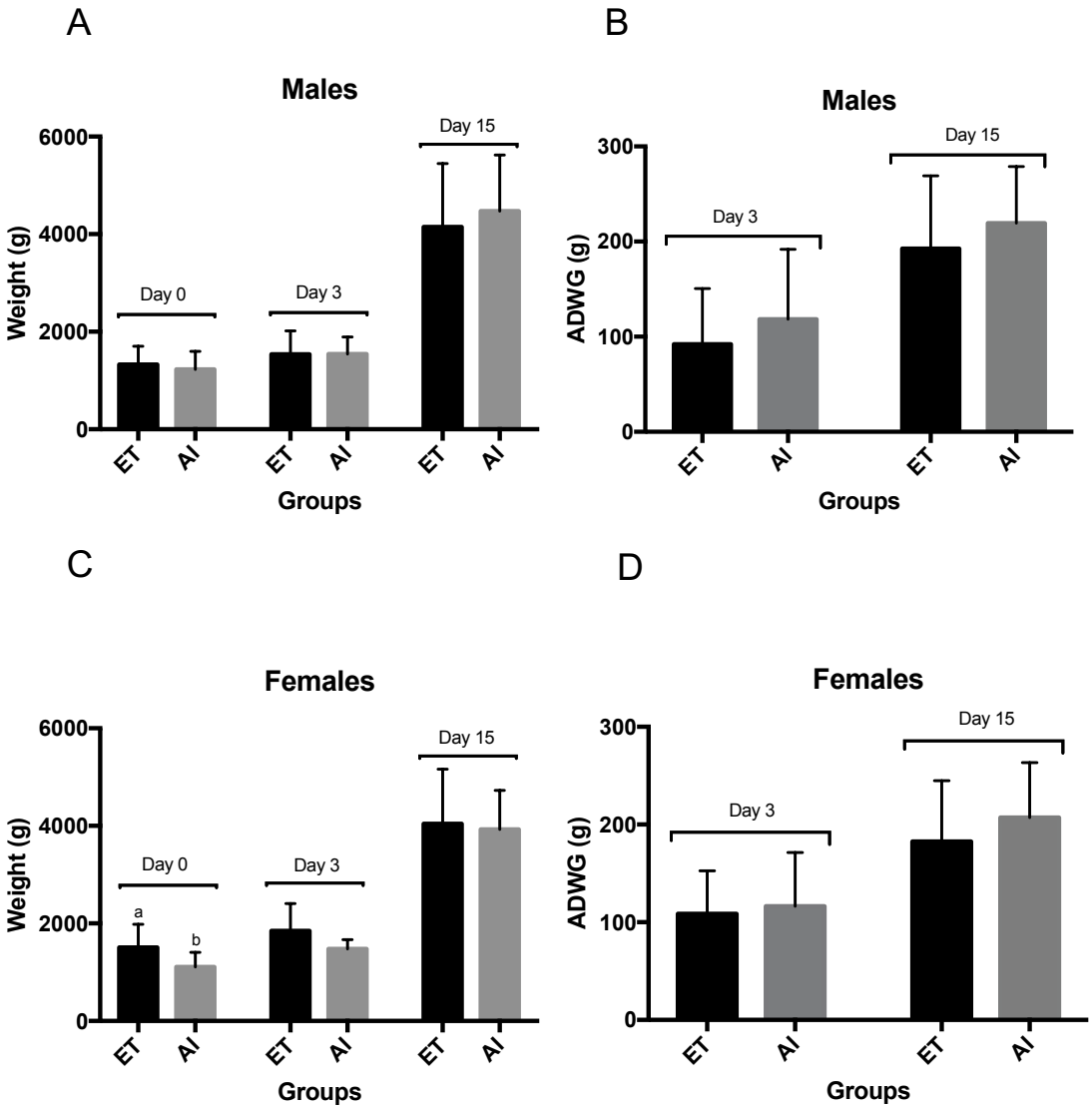


Figure 1. *Differences in piglets growth at day 0, day 3 and day 15. Weight and average daily gain weight (ADWG) were analysed separately by sex. Significant differences between mean values are presented with different letters (a, b), p<0.05. Males did not present differences in weight at birth or on days 3 and 15 of life when compared by groups (Fig. 1A). Similarly, no differences were found when ADG was measured on days 3 and 15 between groups (Fig. 1B). However, females showed significant differences at birth weight (Fig. 1C), being higher in ET group compared to AI, but no differences were observed on days 3 and 15. In the same way, when ADG was compared between females (Fig. 1D), no differences were observed.*

A total of four piglets from the ET group and one from the AI group died during the study.

***Table 3**. Pregnancy and farrowing results after non-surgical embryo transfer*

Donor ID	**Age (years)**	**No. of parities (n)**	**No. of embryos collected (n)**	**Embryonic stage**	**Age of embryos (days)**	**No. of embryos transferred per recipient (n)**	**Synchrony of recipient (hours)**	**Pregnancy (+/-)**	**Gestation period (days)**	**Total piglets born (n)**
1	4	9	14	M						
2	3	7	19	B	6-7	40	+24	+	108	6
3	1	1	10	B						
4	4	9	36	HB						
5	4	9	37	HB	7-8	99	+24	+	103	20
6	4	10	35	HB						
7	4	9	16	HB						
8	4	8	23	HB	8-9	60	+24	-	-	-
9	4	10	35	HB						
10	3	7	32	HB						
11	2	3	21	HB	6-7	46	0	-	-	-
12	4	9	27	HB						
13	4	10	39	B	6-7	39	0	-	-	-
14	4	9	35	HB	7-8	50	0	+	105	13
15	4	9	21	HB						
16	4	10	31	HB	7-8	50	0	-	-	-
17	4	8	15	HB						

18	3	6	18	HB	7-8	47	0	-		-
19	4	9	14	HB						
20	4	7	39	HB	7-8	51	0	-	-	-
21	4	8	26	HB						
22	3	7	18	HB	7-8	42	+24	-	-	-
23	5	10	23	HB						
24	5	11	27	HB	7-8	47	+24	-	-	-
25	4	10	25	HB						

M=Morulae, B=Blastocyst, HB=Hatched Blastocyst.

Table 4. *Litter size of live-born piglets (mean □ SD)*

Group	**No. total of live-born piglets (litters)**	**Litter size**	**Range**
ET	37 (3)	12.33±6.51	6-19
AI	41 (3)	13.67±3.51	10-17

Table 5*. Data at birth of piglets derived from transferred embryos (ET group) vs artificial insemination (AI)*

	ET	**AI**
Total No. of piglets born (live-born piglets)	39 (37)	42 (41)
Sex		
Males (live-born males)	14	27 (26)
Females (live-born females)	25 (23)	15
Birth Weight (mean in *g ± SD)*		
Total Males born (live-born males)	1326.0±374.7 (1326.0±374,7)	1248.0±374.7 (1229.0±368.7)
Total Females born (live-born females)	1450.0±496.1[a] (1510.0±471.1[a])	1109.0± 299.2[b] (1109.0±299.2[b])
Total Kg of piglets per sow (mean in *Kg ± SD)*	18.27±6.09	16.78±7.79

[a-b] Values in the same row with different superscripts are significantly different (p<0,05)

1.3. Haematological and biochemical parameters

All the blood haematological and biochemical parameters were first analysed on the total population including males and females. Values are shown in Supplementary Tables S1-S4. Males and females were also separately studied, and those differences found in both sexes are shown below graphically, but in more detail in Supplementary Tables S5-S12.

1.3.1. Red Blood Cells

Red blood cell count (RBC)

No significant differences were found in males for RBC (Figure 2 A). Instead, as we can see in Figure 2 B, significantly higher differences were found in females from ET group compared with those derived from AI. Although the total RBD count was still higher in the ET group on day 15, no significant differences were found.

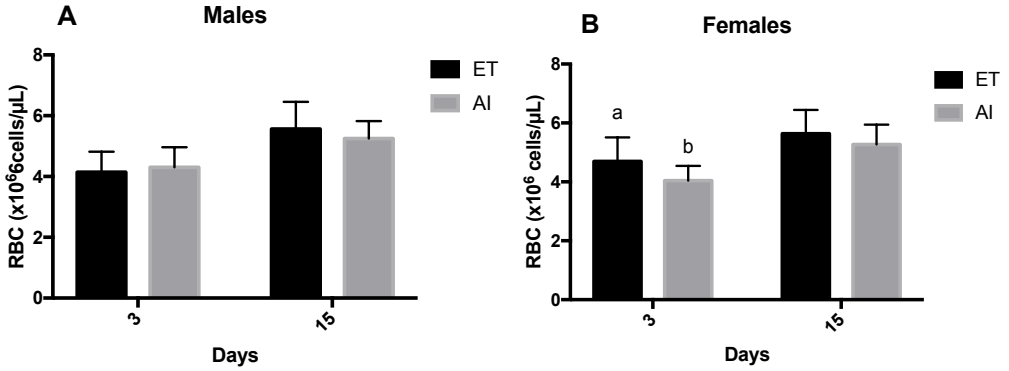


Figure 2. *Bar plot of RBC (x106cells/ µl) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly higher differences are showed at day 3 between females from the ET and AI groups (B). No differences are found in males (A). Different letters (a, b) indicate significant differences (p<0.05).*

Hemoglobin (Hb)

Although the Hb values were slightly higher in males from the ET group compared to those in the AI group, no significant differences were seen on both days (Figure 3 A). However, when females were analysed, significantly higher differences were found for ET group compared to AI. These differences were not maintained at day 15 (Figure 3 B).

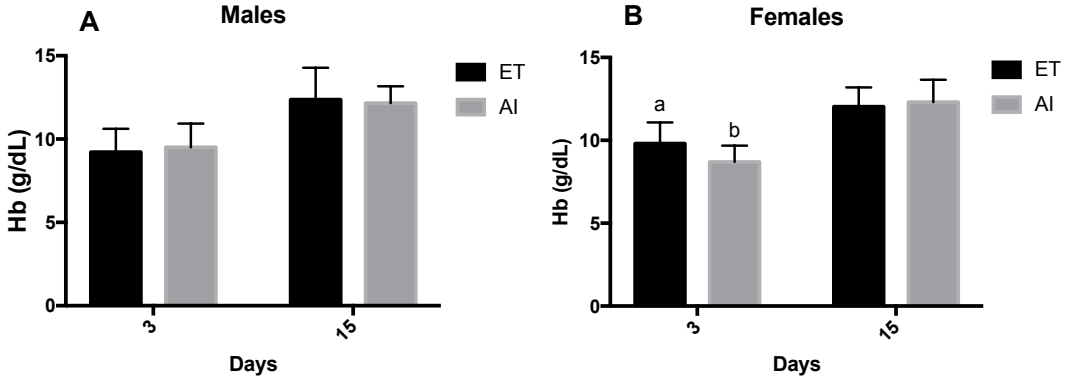


Figure 3. *Bar plot of Hb (g/dL) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly higher differences are showed at day 3 in females from the ET group compared with AI (B). No differences are found in males (A). Different letters (a, b) indicate significant differences (p<0.05)*

Hematocrit (HTC)

Similar to the findings on RBC and Hb, when HTC was analysed, significantly higher differences were observed for females from ET at day 3 compared to those derived from AI (Figure 4 B).

Although the HTC values were slightly higher in males from the ET group regarding AI, no significant differences were seen at both days (Figure 4 A).

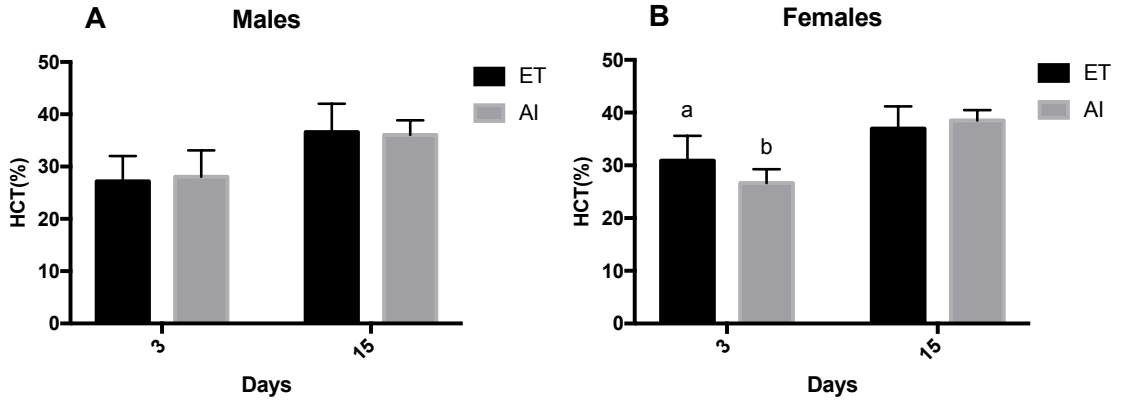


Figure 4. *Bar plot of HTC (%) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly higher differences are showed at day 3 in females from the ET group compared with the AI group (B). No differences are found in males (A). Different letters (a, b) indicate significant differences (p<0.05).*

Mean corpuscular volume (MCV)

Although males presented slightly higher values in the ET group compared to AI both days, no significant differences were found (Figure 5 A). However, when females were analysed, lower values were observed in those derived from the ET group both days, showing significant differences on day 15.

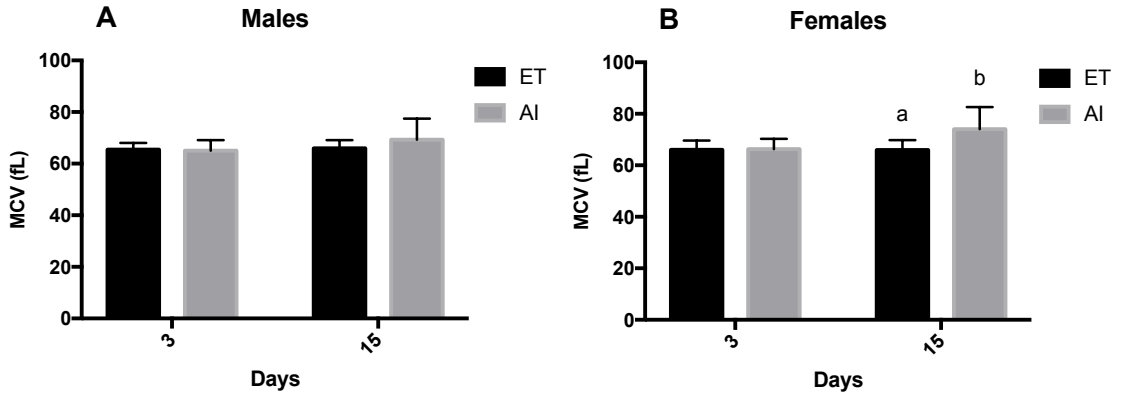


Figure 5. *Bar plot of MCV (fL) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly lower differences are showed at day 15 in females from ET group regarding AI (B). No differences are found in males (A). Different letters (a, b) indicate significant differences (p<0.05).*

Mean corpuscular hemoglobin (MCH)

Similar to MCV, on day 15 of life, females from ET groups showed significantly lower levels of MCH compared to females derived from AI (Figure 6 B). No significant differences were found in males none of days (Figure 6 A)

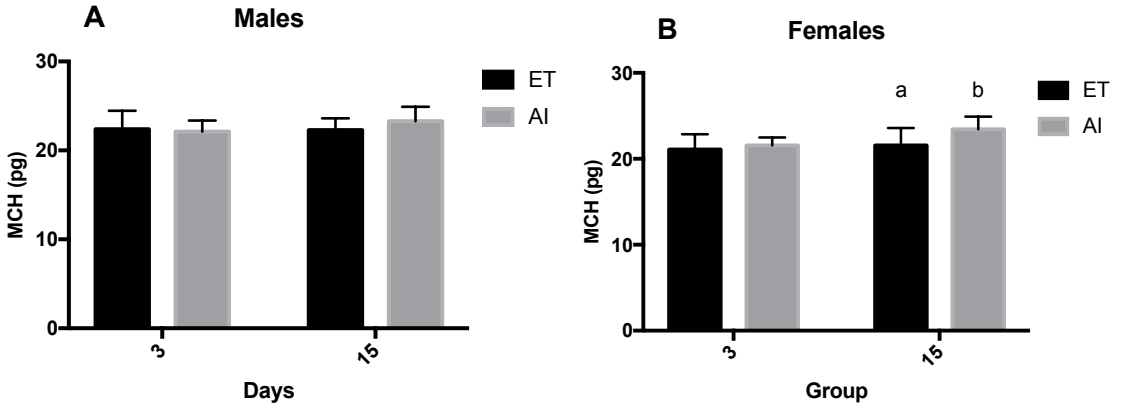


Figure 6. *Bar plot of MCH (pg) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly lower differences are showed at day 15 in females from the ET group regarding AI (B). No differences are found in males (B). Different letters (a, b) indicate significant differences (p<0.05).*

Hemoglobin concentration distribution width (HDW)

As we can see in Figure 7, when HDW was analysed, both males and females from the ET group showed slightly lower values at day 3 compared to AI, however, only significant differences were found in females this day (Figure 7 B).

Contrary, on day 15 of life, both males and females from the ET group showed slightly higher values regarding AI, although significant differences were only observed in males (Figure 7 A).

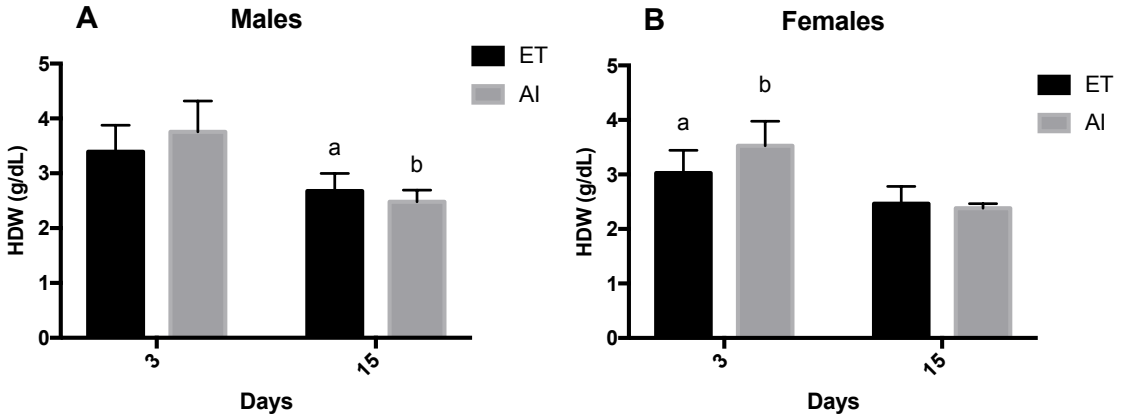


Figure 7. *Bar plot of HDW (g/dL) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly higher differences are showed at day 15 in males from the ET group compared with the AI group(A). On day 3 of life, females from the ET group showed significantly lower differences regarding AI (B). Different letters (a, b) indicate significant differences (p<0.05).*

Reticulocytes

Figure 8 shows the percentages of reticulocytes in males and females. Despite the fact that on day 3 females derived from the ET group showed a slightly lower percentage than those derived from AI, it was not until day 15 of life that significant differences were observed between both groups (Figure 8 B).
In contrast, males from the ET group showed slightly higher values on day 3, and conversely, lower values on day 15 compared to males from the AI group, although no significant differences were observed (Figure 8 A).

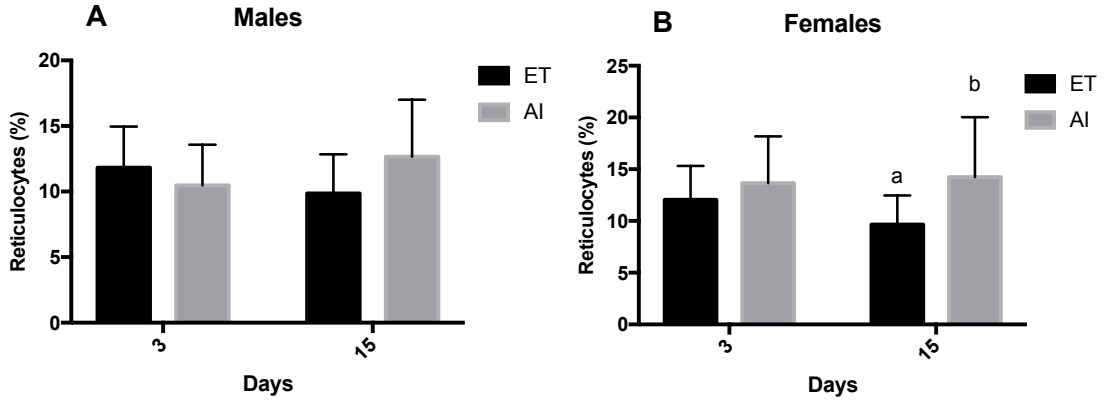


Figure 8. *Bar plot of Reticulocytes (%) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly lower differences are showed at day 15 in females from the ET group compared with AI (B). No differences are found in males (A). Different letters (a, b) indicate significant differences (p<0.05).*

Average size of reticulocytes (MCVr) and Hemoglobin content of reticulocytes (CHr)

When MCVr and CHr reticulocyte parameters were analysed, the same differences were found both days for males and females. As we can see in Figure 9 A, and Figure 10 A, males from the ET group showed significantly lower values compared to those derived from AI. However, females from the ET group showed significantly lower values both days compared to the AI group (Figure 9 B, and Figure 10 B).

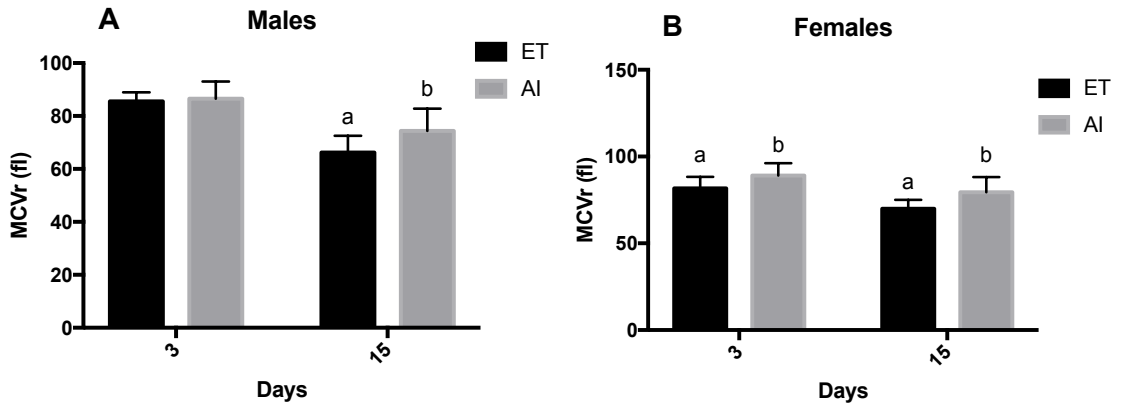


***Figure 9**. Bar plot of MCVr (%) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly lower differences are showed between females from the ET and AI groups at day 3 (B). At day 15, both males and females show the same differences (A, B). Different letters (a, b) indicate significant differences (p<0.05).*

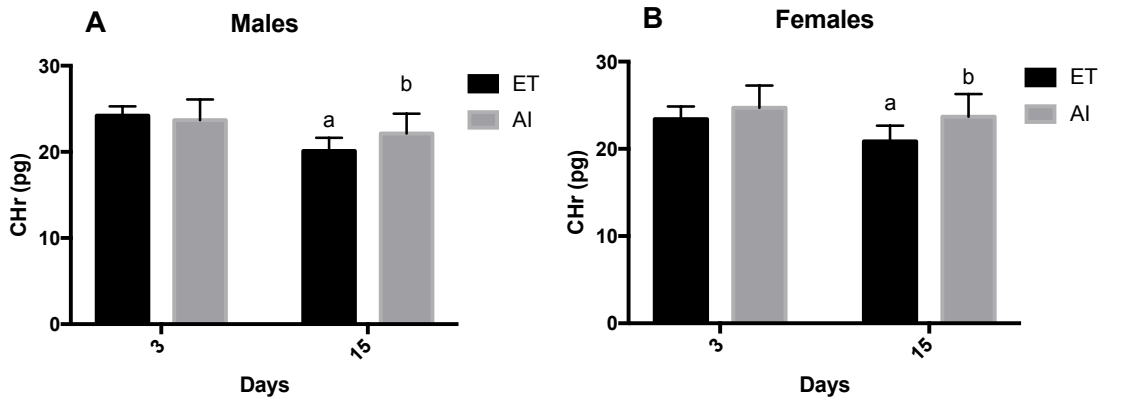


***Figure 10.** Bar plot of CHr (pg) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly lower differences are showed in both males and females on day 15 of life for the ET group compared with the AI group (A, B). Different letters (a, b) indicate significant differences (p<0.05).*

The numerical values of Red blood cells parameters in males and females can be found in Supplementary Tables S5 and S9 respectively.

1.3.2. White Blood Cells (WBC)

The total number of WBC was significantly higher in the ET group both males and females on day 3 of life (Figure 11 A, B); Instead, at day 15, these differences were maintained only in females (Figure 11 B).

On the other hand, significantly higher differences were found at day 3 in neutrophils (p=0,0075) and lymphocytes (p=0,0243) for males and females respectively; Females maintained the differences found in the lymphocyte concentration also at day 15 (p=0,0038).

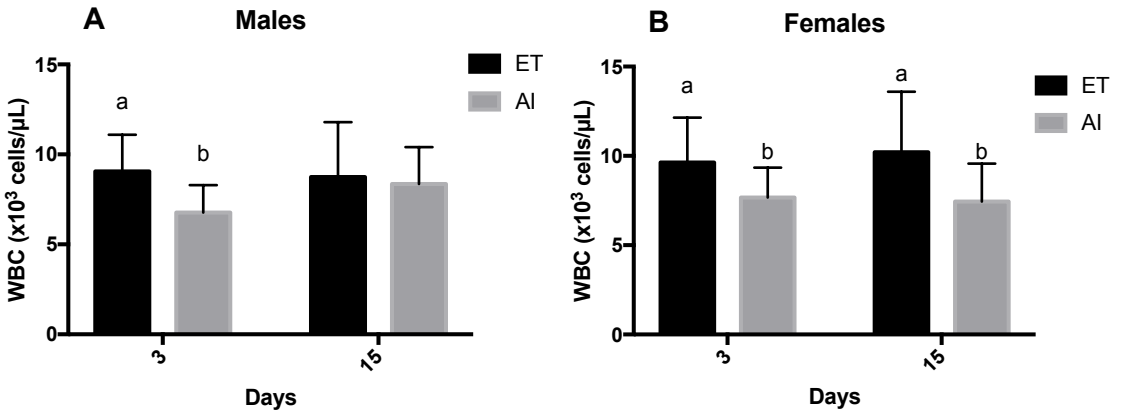


Figure 11. *Bar plot of WBC (x103cells/ µl) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly higher differences are showed in both males and females at day 3 between the ET and AI groups (A, B). On day 15 of life, significantly higher differences are found in females derived from ET compared with AI (B). Different letters (a, b) indicate significant differences (p<0.05).*

The numerical values of white blood cells parameters in males and females can be found in Supplementary Tables S6 and S10, respectively.

1.3.3. Platelets (PLT)

No significant differences in the PLT concentration were found between the two groups, although a trend was observed at day 3 in females (*p*=0,0549), having those derived from the ET group a slightly lower value than those derived from the AI group.

Platelecrit (PCT)

As we can see in Figure 12 (A, B), both males and females from the ET group showed lower values than AI when PCT was analysed. However, only males, and females from the ET group showed significantly lower differences compared to AI on days 3 and 15 of life, respectively.

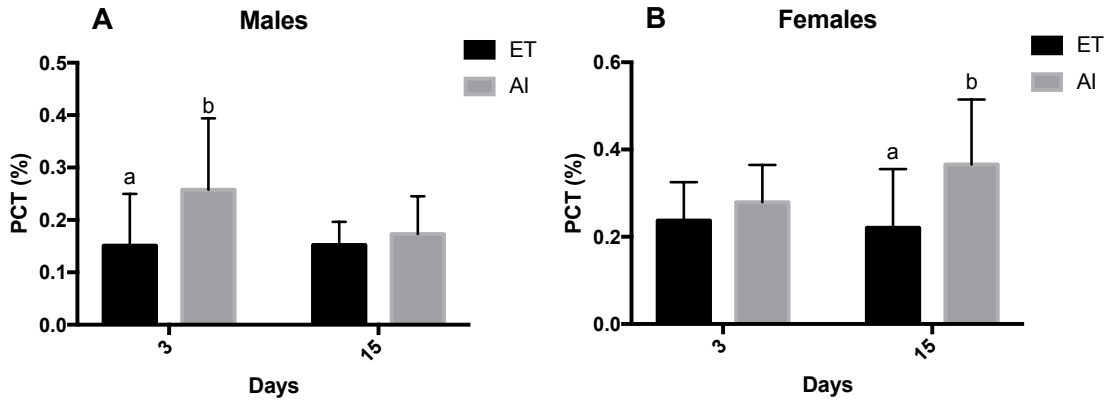


Figure 12. *Bar plot of PCT (%) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly lower differences are showed in males from ET group at day 3 regarding AI (A). On day 15 of life, significantly lower differences are found in females from the ET group compared with females from AI (B). Different letters (a, b) indicate significant differences (p<0.05).*

Mean platelet volume (MPV) and Platelet mass distribution width (PMDW)

Similarly, when MPM was analysed, only significant lower differences were found for males derived from the ET group at day 3 regarding AI. On the other hand, on day 15, this group showed slightly higher values, although no significant differences were observed (Figure 13 A). Similarly, no significant differences were found in females (Figure 13 B).

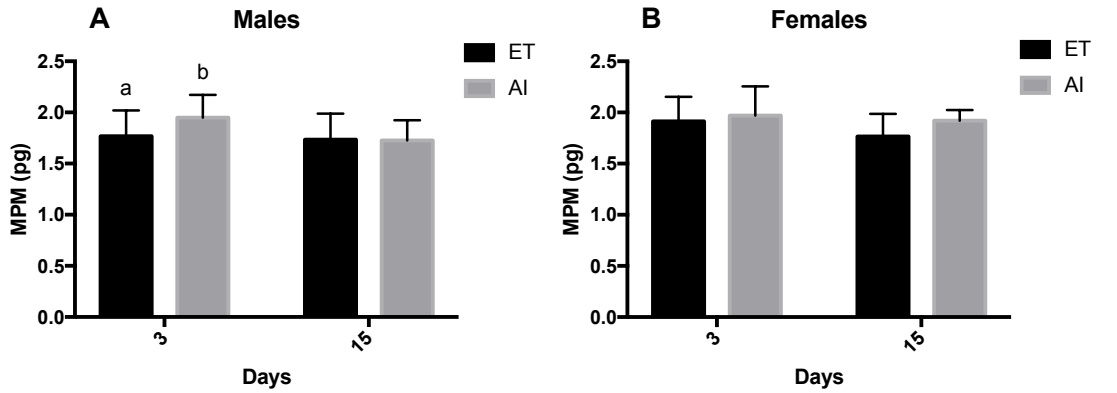


Figure 13*. Bar plot of MPM (pg) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly lower differences are showed in males derived from ET group at day 3 compared with males from the AI group (A). No differences are found for females (B). Different letters (a, b) indicate significant differences (p<0.05).*

When MPV and PMDW platelet parameters were analysed, the same differences were found, since those males derived from the ET group showed significantly higher values compared to those males derived from AI. (Figure 14 A and Figure 15 A).

On the other hand, although females from the ET group presented a similar pattern to males from the same group with slightly higher values, no significant differences were found on days 3 or 15 (Figure 14 B and Figure 15 B).

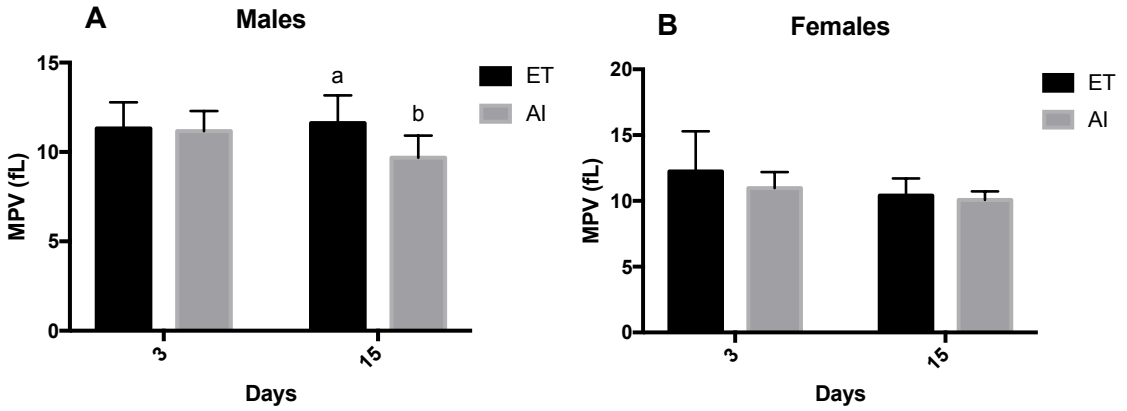


Figure 14. *Bar plot of MPV (fL) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly higher differences are showed between males derived from ET and AI groups at day 3 (A). No differences are found in females. Different letters (a, b) indicate significant differences (p<0.05).*

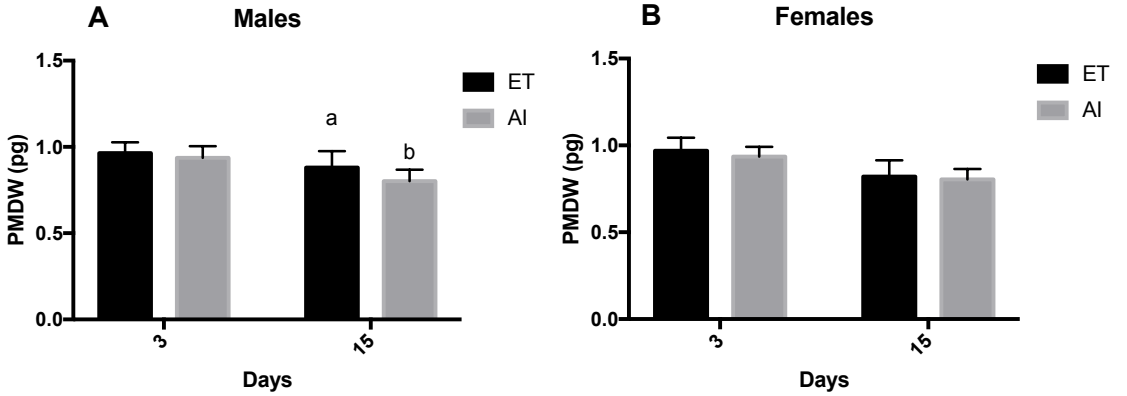


Figure 15. *Bar plot of PMDW (pg) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly higher differences are showed in males derived from ET group compared with males from the AI group at day 3 (A). No differences are found in females (B). Different letters (a, b) indicate significant differences (p<0.05).*

Large PLT

Finally, and following the same kinetic both males and females on days 3 and 15 of life, only significantly lower differences in Large PLT were observed at day 15 between females from the ET and AI groups (Figure 16 B). No significant differences were found in males (Figure 16 A).

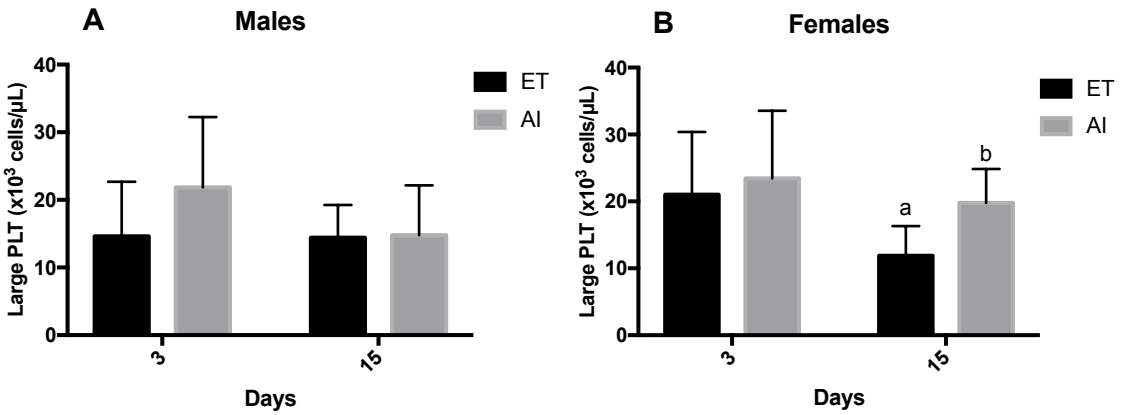


Figure 16. *Bar plot of Large PLT (x103cells/ µl) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly lower differences are showed between females from the ET and AI groups at day 15 (B). No differences are found in males (A). Different letters (a, b) indicate significant differences ($p<0.05$).*

The numerical values of Platelets parameters in males and females can be found in Supplementary Tables S7 and S11, respectively.

1.3.4. Biochemical analysis

Urea

As we can see in Figure 17, males from the ET group showed slightly higher values compared to males from the AI group, although significant differences were found (Figure 17 A). Conversely, females from the ET group showed values below those derived from AI, showing only significant differences on day 15 of life (Figure 17 B)

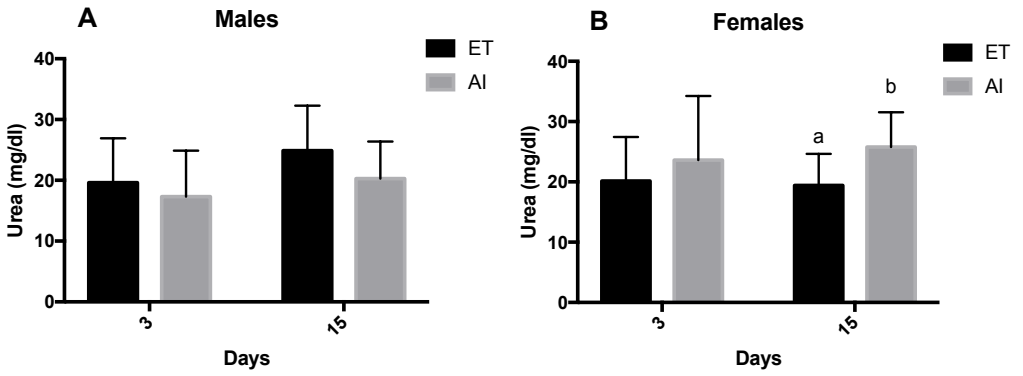


Figure 17. *Bar plot of Urea (mg/dl) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly lower differences are showed between females from the ET and AI groups at day 15 (B). No differences are found in males (A). Different letters (a, b) indicate significant differences (p<0.05).*

Alkaline phosphatase (ALP)

Both males and females derived by ET showed values above those piglets born by AI on day 3 (Figure 18 A, B), however, only males from the ET group showed significant differences regarding the AI group at day 3 (Figure 18 A). On the other hand, on day 15 of life, piglets of both sexes born by ET, showed lower values than those born by AI, although no significant differences were found (Figure 18 A, B).

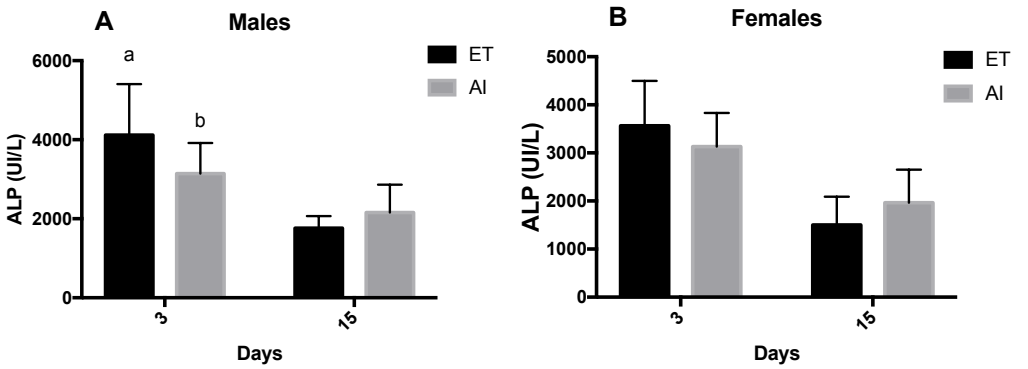


Figure 18. *Bar plot of ALP (UI/L) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly higher differences are showed between males from the ET and AI groups at day 3 (A). No differences are found in females (B). Different letters (a, b) indicate significant differences (p<0.05).*

Gamma-glutamyl transferase (GGT)

Figure 19 shows how piglets (males and females) born by ET showed values above those born by AI on days 3 and 15 of life, however, only males from ET showed significant differences compared to those born by AI (Figure 19 A). No differences were found in females (Figure 19 B)

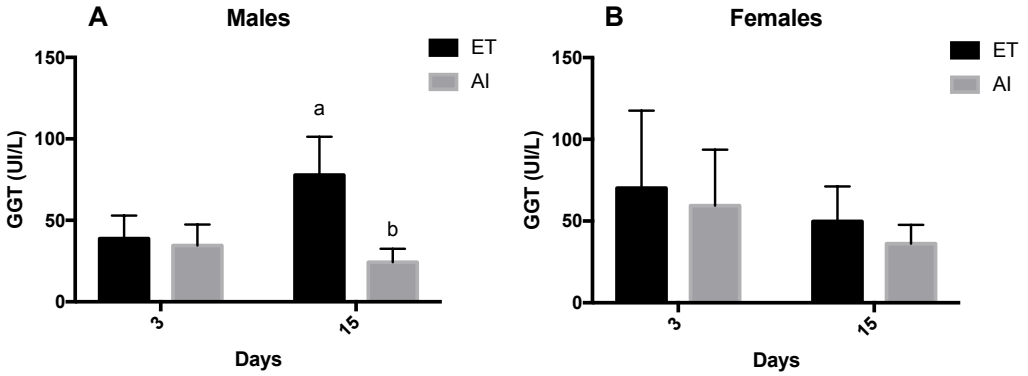


Figure 19. *Bar plot of GGT (UI/L) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly higher differences are showed between males from the ET and AI groups at day 15 (A). No differences are found in females (B). Different letters (a, b) indicate significant differences (p<0.05).*

Aspartate aminotransferase (AST)

As we can see in Figure 20, the same differences found for GGT were also shown when AST was analysed, being those males from ET significantly higher compared to those derived by AI (Figure 20 A). No differences were found in females (Figure 20 B).

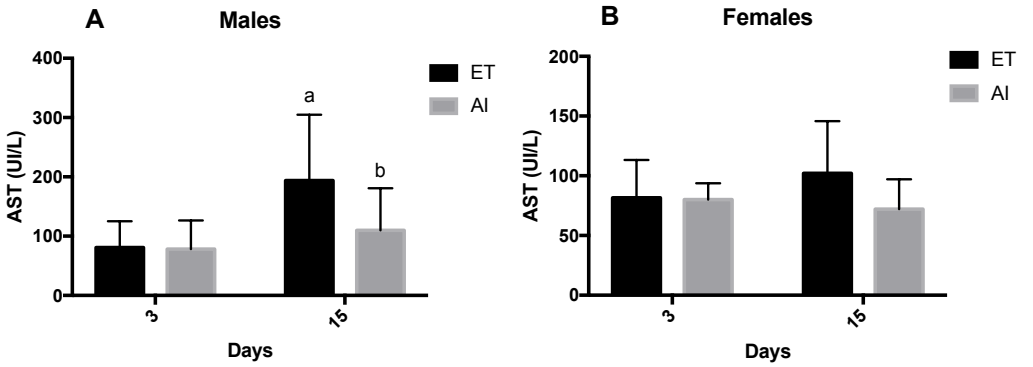


***Figure 20**. Bar plot of AST (UI/L) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly higher differences are showed between males from the ET and AI groups at day 15 (A). No differences are found in females (B). Different letters (a, b) indicate significant differences (p<0.05).*

Lipases

Both males and females did not show significant differences at day 3 when lipases were analysed (Figure 21), On day 15 of life, piglets from both groups considerably decreased their concentration of lipases compared to day 3, however, only significantly lower differences were found in males from ET regarding those from AI (Figure 21 A). No differences were observed in females (Figure 21 B)

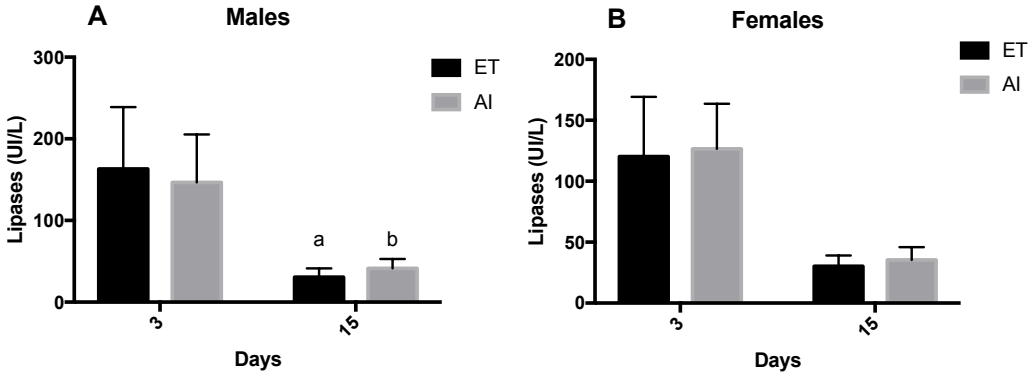


***Figure 21**. Bar plot of Lipase (UI/L) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly lower differences are showed between males from the ET and AI groups at day 15 (A). No differences are found in females (B). Different letters (a, b) indicate significant differences (p<0.05).*

Albumin (ALB)

The ALB concentrations were significantly lower on day 3 in males from the ET group compared to those from the AI group (Figure 22 B). However, although on day 15 this concentration increased in both males and females from the two groups, no significant differences were observed (Figure 22 A, B)

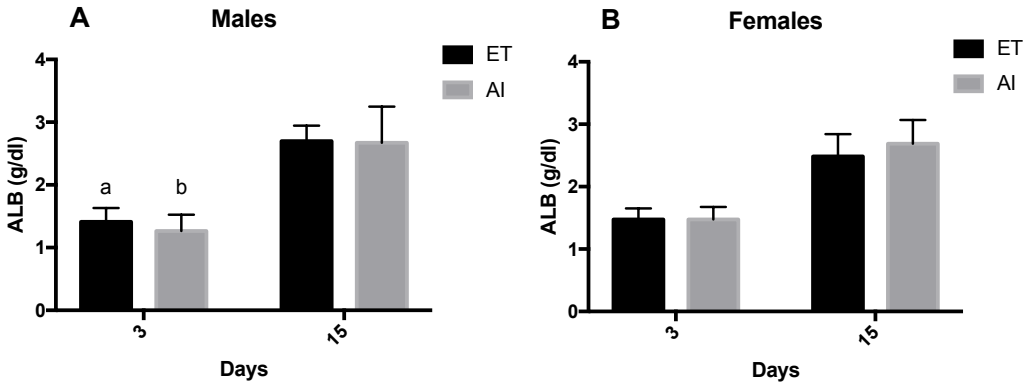


Figure 22. *Bar plot of ALB (UI/L) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly higher differences are showed between males from the ET and AI groups at day 3 (A). No differences are found in females (B). Different letters (a, b) indicate significant differences (p<0.05).*

Total bilirubin (TBIL)

Figure 23 (A, B) shows how both males and females showed a lower concentration of TBIL on day 3, only females from the ET group showing significant differences compared to those from the AI group. However, on day 15 a trend was observed (*p*=0,0551), although no significant differences were observed (Figure 23 B). On the other hand, despite the fact that males from ET increased their TBIL concentration compared to males from AI, no significant differences were observed either (Figure 23 A).

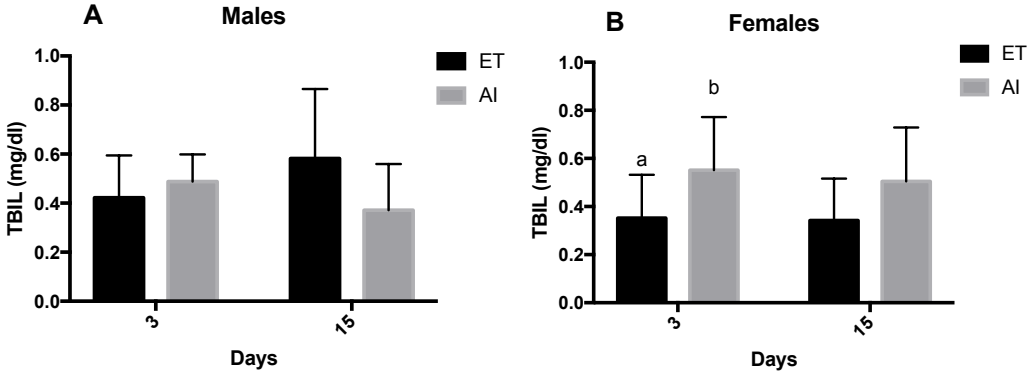


Figure 23. *Bar plot of TBIL (mg/dl) in males (A) and females (B) from the two groups of piglets analysed: ET and AI, on days 3 and 15 (mean±SD). Significantly lower differences are showed between females from the ET and AI groups on days 3 and 15 (B). No differences are found in males (A). Different letters (a, b) indicate significant differences (p<0.05).*

Although no significant differences were found, when the concentration of globulins was analysed, a trend was observed between males from ET compared to those from AI on day 15 (ET: 2,59±0,72; AI: 2,18±0,38, *p*=0,0538).

The numerical values of Biochemical parameters in males and females can be found in Supplementary Tables S8 and S12, respectively.

Experiment 2.

2.1. In vitro production of embryos.

Table 6 shows the cleavage rate and blastocyst yield obtained after the *in vitro* embryo production by using or not reproductive fluids as additives for the culture media. As we can see, no significant differences were found between both groups (p>0.05).

Table 6. *Cleavage rate, blastocyst yield and total mean of embryos transferred after IVP with or without reproductive fluids as an additional source of protein to the culture media. Data are expressed as mean±SD.*

Group	Blastocyst (N)	Cleavage (%)	Blastocyst/Cleavage (%)	Blastocyst/Oocyte (%)
RF-IVP	724	69.41± 6.97	21.0±3.93	14.66±3.76
C-IVP	660	66.92±18.36	22.55±8.2	13.99±2.9

2.2. Pregnancies and farrowing rates.

Five of thirteen recipients (38,5%) and four of eleven recipients (36,4%) became pregnant in the RF-IVP and C-IVP groups respectively. The rest of the sows returned to estrous at 19-22 days after being transferred.

The farrowing rate was 80% in the RF-IVP group, since the abortion of one of the sows occurred on the 28th day after transfer. On the other hand, all the pregnancies in the C-IVP group came to term, with the farrowing rate being 100%. No significant differences were found between groups although induction of parturition was necessary in 1 animal from the AI group as well as in 2 and 4 animals from the C-IVP and RF-IVP groups, respectively.

As can be seen in Table 7, two of the pregnant recipients from RF-IVP group and one from C-IVP group presented a synchrony of -24h regarding the embryos; conversely, 3 of the pregnant recipients in the first group (RF-IVP) and 3 of those in the second group (C-IVP) were at -48h regarding the embryos.

All the resulting positive pregnancies corresponded to the transfer of blastocyst and/or hatched blastocysts embryo stages.

Gestation period ranged between 111-121 days in IVP groups and 116-119 days in AI group. Nevertheless, when gestation period between RF-IVP, C-IVP and AI groups was compared separately, no significant differences were found, as well as when both IVP groups and AI were analysed, although a tendency to shorter gestational lengths was observed in RF-IVP vs. AI group (p=0.0539).

Table 7. *Pregnancy and farrowing results after transfer of in vitro produced embryos*

Recipient	Group	No. of parities (n)	Estrous cycle day (days)	Age of embryos (days)	Synchrony recipients-embryos (h)	No. of embryos transferred (n)	Embryonic stage	Pregnancy (+/-)	Gestation period (days)	Total piglets born (n)
1	C-IVP	0	4	6	-48	93	B	-		
2	C-IVP	1	5	6	-24	61	B	-		
3	C-IVP	4	5	6	-24	31	B-ExB	-		
7	C-IVP I	1	4	6	-48	40	B	+	115	10
5	C-IVP	5	4	6	-48	33	B	-		
6	C-IVP	0	5	6	-24	38	ExB	-		
13	C-IVP	3	4	5	-24	34	M-B	-		
15	C-IVP	9	5	6	-24	47	B-ExB	+	116	6
17	C-IVP	5	5	6	-24	47	B	-		
19	C-IVP	5	4	6	-48	42	B	+	121	5
22	C-IVP	6	4	6	-48	47	ExB	+	115	7

3	RF-IVP	2	5	6	-24	45	M-ExB	-		
4	RF-IVP	5	4	6	-48	40	B	-		
5	RF-IVP	5	5	6	-24	32	ExB	+	113	5
8	RF-IVP	6	4	6	-48	52	B	+	111	3
10	RF-IVP	0	5	6	-24	42	B- ExB	-		
12	RF-IVP	5	5	6	-24	41	B- ExB	-		
14	RF-IVP	6	4	5	-24	31	B	-		
16	RF-IVP	6	4	6	-48	26	B-ExB	-		
18	RF-IVP	4	5	6	-24	45	B	-		
20	RF-IVP	1	4	6	-48	29	B	-		
21	RF-IVP	7	4	6	-48	42	ExB	+	115	3
23	RF-IVP	7	5	6	-24	45	ExB	+	Abortion (day 28)	
24	RF-IVP	5	4	6	-48	59	ExB-HB	+	114	7

M=Morulae, B=Blastocyst, ExB=Expanded Blastocyst, HB=Hatched Blastocyst

The total number of piglets born by RF-IVP and C-IVP was 19 (11 males, 8 females) and 30 (11 males, 19 females) respectively (Table 8). Piglets born from a total of four artificial insemination sows were used as control (AI group), resulting in 59 animals (25 males, 34 females). In brackets, the total number of live born piglets in each group is shown. None of them presented morpho-anomalies and all weighed as expected.

One out of 19 animals from RF-IVP group, as well as 2 out of 30 from C-IVP and 4 out of 59 from AI were born dead. On the other hand, when the litter size was analysed, significant differences were observed between RF-IVP vs AI and between C-IVP vs AI as shown in Table 8.

Table 8. *Total piglets derived from IVP (RF-IVP, C-IVP) vs Artificial Insemination (AI) and litter size analysis (mean±SD)*

	RF-IVP	**C-IVP**	**AI**
Total No. of piglets born (live-born piglets)	19 (18)	30 (28)	59 (55)
Males (live-born males)	11(10)	11 (11)	25 (24)
Females (live-born females)	8 (8)	19 (17)	34 (31)
Litter size			
Total litter size *(males+females)*	4.5±1.91[a]	7.0± 2.6[a]	14.0±5.42[b]
Males	2.5±0.58	2.75±1.71	6.0±3.27
Females	2.0±1.41	4.25±3.30	7.75±3.50

The number of live born piglets appears in brackets.

[a-c] Values in the same row with different superscripts are significantly different (p<0,05)

2.3. Placental parameters

As can be observed in Table 9, when different placental parameters were analysed, significantly higher surface areas were found in the C-IVP group regarding the other two. However, only the RF-IVP showed a significantly lower placental efficiency when compared with the C-IVP and AI groups.

Table 9. *Analysis of placental parameters (mean±SD)*

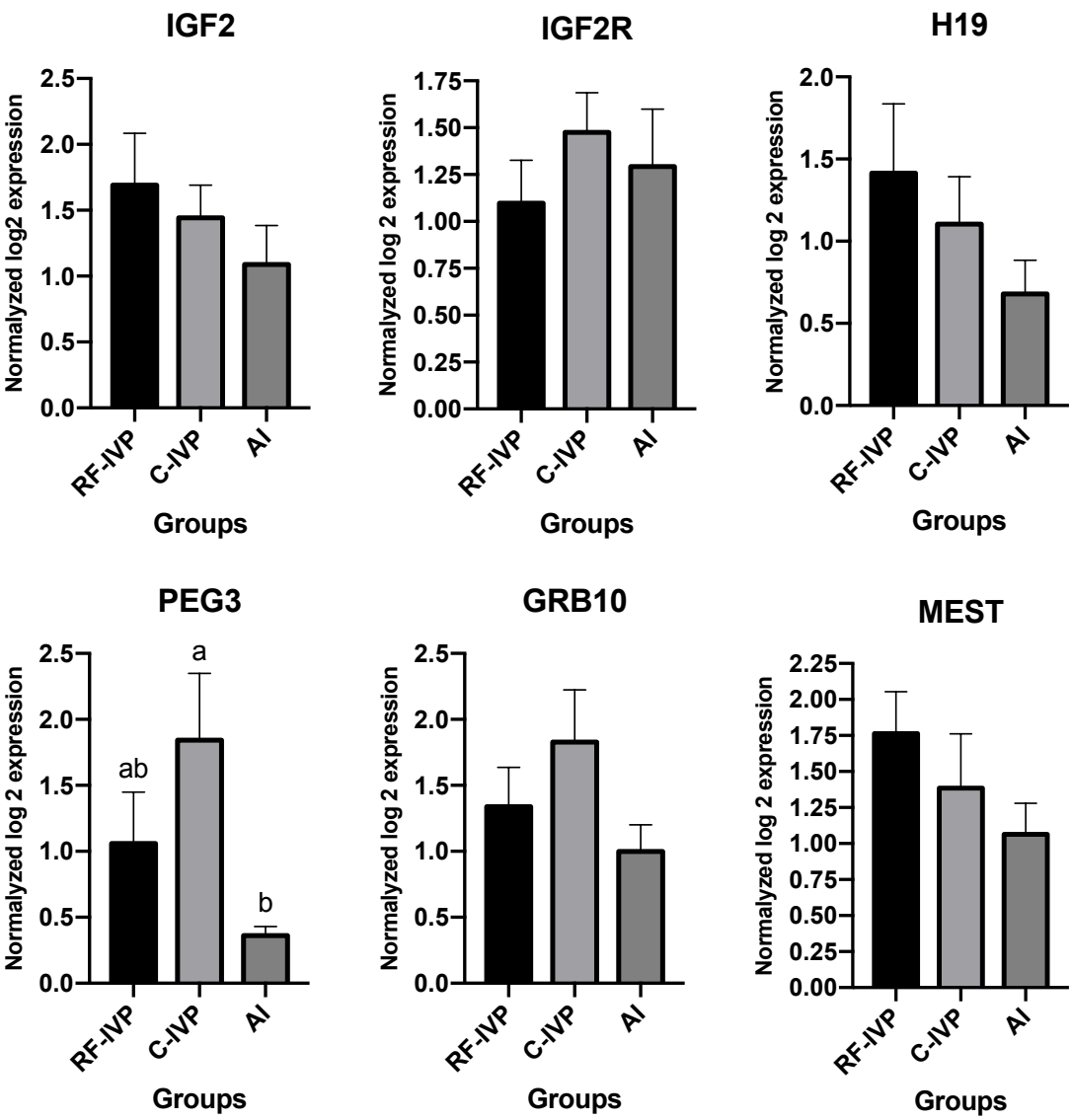
	RF-IVP	**C-IVP**	**AI**
Placental weight (g)	196.3±70.89	182.3±46.93	151.9±48.33
Placental surface area (cm^2)	1700.0±473.0[a]	2150.0±815.6[b]	1625.0±353.8[a]
Placental efficiency (g/g)	7.42±1.67[b]	9.0±2.62[a]	9.7±2.63[a]

[a-b] Values in the same row with different superscripts are significantly different (p<0,05)

2.4. Gene expression levels in placenta and umbilical cord.

When gene expression levels were assessed in placenta, *PEG3* showed a significantly increased level in C-IVP compared with AI (p <0,05) (Figure 24). Similarly, *LUM* also had a significantly increased expression level in C-IVP compared with AI (p<0,05) (Figure 24). However, no differences were found in the transcription levels of the rest of genes analysed (p >0,05) (Figure 24).

On the other hand, when umbilical cord expression levels for the different genes were analysed, no differences were found (p> 0,05) (Figure 25).


IGF2
Normalyzed log2 expression
2.5
2.0
1.5
1.0
0.5
0.0
RF-IVP
C-IVP
AI
Groups
IGF2R
Normalyzed log 2 expression
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
RF-IVP
C-IVP
AI
Groups
H19
Normalyzed log 2 expression
2.0
1.5
1.0
0.5
0.0
RF-IVP
C-IVP
AI
Groups
PEG3
Normalyzed log 2 expression
2.5
2.0
1.5
1.0
0.5
0.0
ab
a
b
RF-IVP
C-IVP
AI
Groups
GRB10
Normalyzed log 2 expression
2.5
2.0
1.5
1.0
0.5
0.0
RF-IVP
C-IVP
AI
Groups
MEST
Normalyzed log 2 expression
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
RF-IVP
C-IVP
AI
Groups

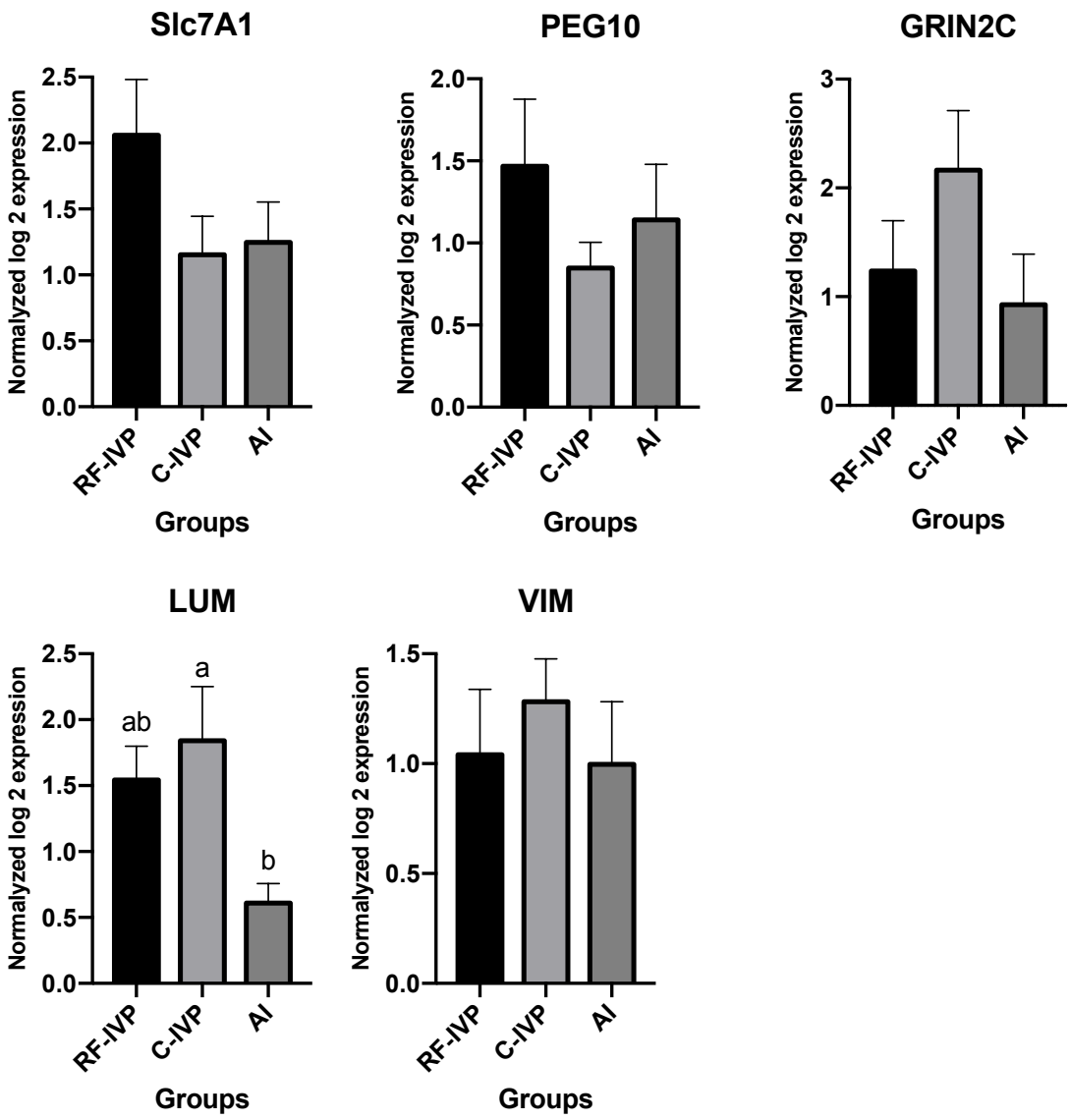


Figure 24. *Expression of selected genes from placental tissue derived from the three groups of piglets analysed: RF-IVP, C-IVP and AI. The graphs show significant upregulation in PEG3 (D) and LUM (J) genes for C-IVP group compared with AI group. All data are presented as mean ± SD. Different letters (a, b, c) indicate significant differences (p<0.05).*

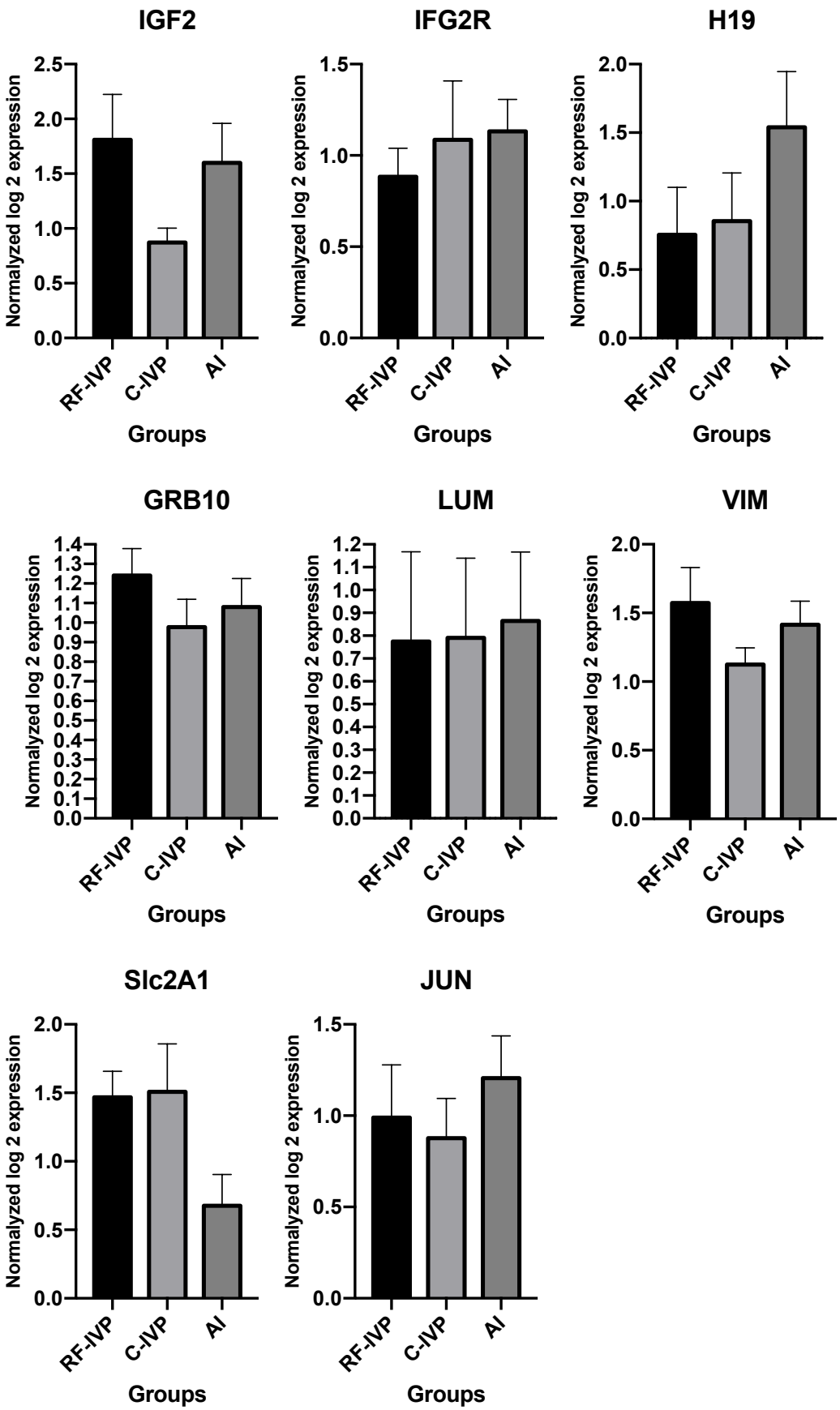


Figure 25. *Expression of selected genes from umbilical cord tissue derived from the three groups of piglets analysed: RF-IVP, C-IVP and AI. No significant differences are found for each gene in the different groups. All data are presented as mean ± SD.*

Experiment 3.

6.1. Morphometric parameters of piglets at birth.

According to the results obtained, at day 0 we found significant differences (p<0.05) between piglets born from AI group and those derived from IVP (RF-IVP, C-IVP) (Table 10). However, when piglets were separated by sex/group, both males and females from C-IVP were the heaviest, also showing significant differences regarding AI group, while there were no differences between males from RF-IVP group and the other two. Instead, females from RF-IVP showed significant differences regarding AI group but not with C-IVP.

The weight of all piglets was within the normal range.

Table 10. *Birth weight (g) of piglets derived from IVP (RF-IVP, C-IVP) and Artificial Insemination (AI) (mean ± SD)*

	RF-IVP	C-IVP	AI
Total piglets (males + females)	1381.0±425.4^{a}	1524.0±340.1^{a}	1170.0±281.7^{b}
Males	1407.0± 460.9^{ab}	1627.0±267.5^{a}	1191.0±293.7^{b}
Females	1345.0±399.2^{a}	1461.0±370.6^{a}	1153.0±275.6^{b}

$^{a-c}$ Values in the same row with different superscripts are significantly different (p<0,05)

When CRL was analysed (Table 11), we found significant differences between AI and C-IVP groups (p<0.05), while none was found with group RF-IVP.

Similarly, separating the data by sex, CRL exhibited significant differences between AI and C-IVF groups, without significant differences between RF-IVF and the other two groups.

Table 11. *Crown Rump Length at birth (cm ± SD)*

	RF-IVP	C-IVP	AI
Total piglets	31.13± 3.10^{ab}	32.22±2.58^{a}	29.66±2.28^{b}
Total Males born	31.64± 3.23 ^{ab}	32.23± 2.58 ^{a}	29.58± 2.17^{b}
Total Females born	30.44± 2.98 ^{ab}	32.22±2.66 ^{a}	29.73±2.4^{b}

$^{a-c}$ Values in the same row with different superscripts are significantly different (p<0,05)

By last, no differences were found between AI and IVP groups when AGD of males was compared, (Table 12).

Table 12*. Anogenital distance at birth (cm ± SD)*

	RF-IVP	C-IVP	AI
Total Males	1.518±0.5492	1.955±0.6105	2.00±0.5083

$^{a-c}$ Values in the same row with different superscripts are significantly different (p<0.05)

6.2. Growth parameters from day 3 to day 180 of life.

The results of growth parameters at the different days and, separating males and females, are available in Supplementary Tables S13-S16 and S17-S19 respectively.

Males

Weight

The differences found at birth weight were also maintained on days 3 and 9 of life, although the RF-IVP group did not show significant differences with the AI group (p=0.756) or with the C-IVP group (p=0.464).

On day 15 of life, the C-IVP group had an average weight of 6.299,09±1.443,98 g, practically double the average weight of the AI group (3.291,18±791,52 g) (p<0.05). In addition, the C-IVP group also presented significant differences with respect to the RF-IVP group (p<0.05).

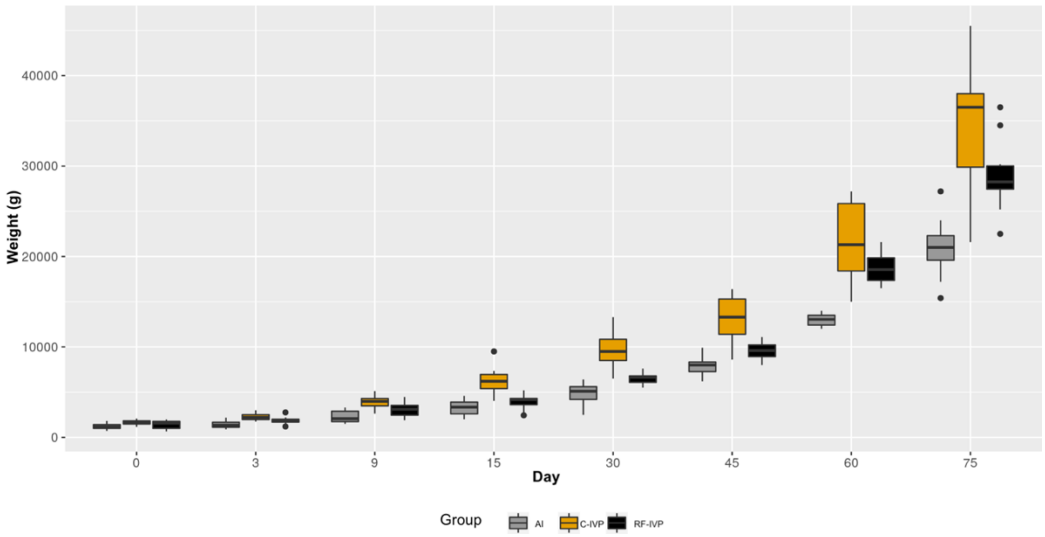


Figure 26. *Box plot of male weights per group from day 0 to day 75. Significant differences from day 0 to day 9 are showed between C-IVP and AI groups. from day 15 to day 45, C-IVP shows significantly higher differences compared to AI and RF-IVP groups. On days 60 and 75, C-IVP remains the heaviest group, although significant differences are found between all groups ($p<0.05$). All data are presented as the mean ± SD.*

These differences were maintained in the measurements at days 15, 30 and 45. As for day 60 of life, the three experimental groups presented statistical differences among them ($p<0.05$).

On day 75, the situation was similar to that on day 60, with the three groups presenting significant differences among them ($p<0.05$).

Figure 26 shows the weights of the males until day 75, where the heaviest group at birth (C-IVP) remained the heaviest until day 75, while group RF-IVP always presented intermediate values between the AI group and the C-IVP group. In addition, it can be observed that at day 60 the weight of group RF-IVP is more similar to the weight of the C-IVP group according to the results of the analysis, appearing for the first time on this day differences between the group AI and RF-IVP. However, although RF-IVP has a weight closer to that of C-IVP, both on day 60 and 75, it showed significant differences with the C-IVP and AI groups.

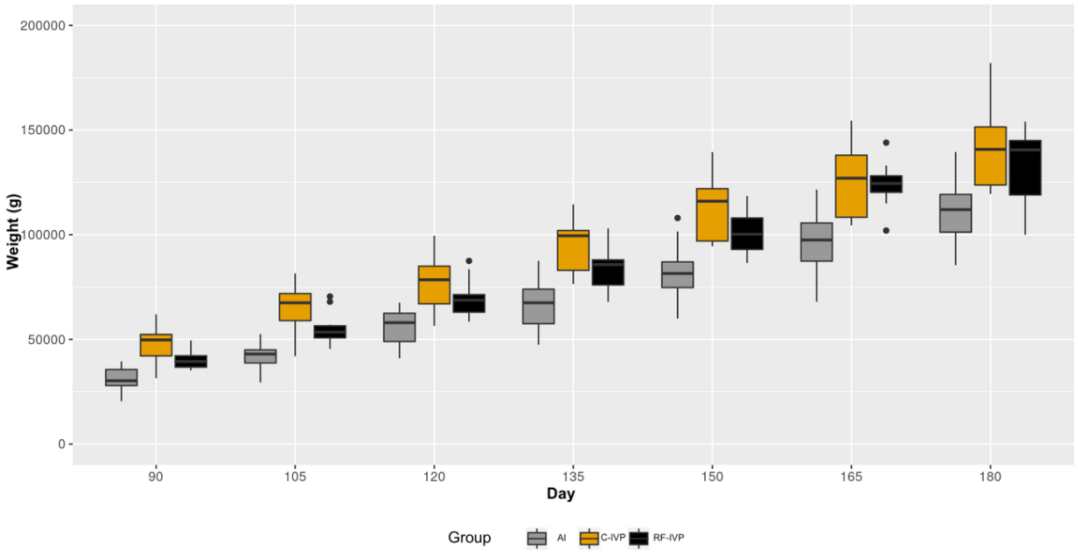


Figure 27. *Box plot of male weights per group from day 90 to day 180. Significantly lower differences are shown in the AI group compared to C-IVP and RF-IVP groups from day 90 to day 180 ($p>0.05$). No significant differences are found between C-IVP and RF-IVP groups. All data are presented as the mean ± SD.*

On day 90 significant differences were found between the AI group and the C-IVP group, and also with RF-IVP group, while no significant differences appeared between the two *in vitro* groups (p=0.131). This situation, where significant differences appeared between the AI group and the two experimental groups, remained until the end of the experiment (day 180).

Figure 27 shows the weight from day 90 to 180, appearing a similar scenario to the one at day 75, where the C-IVP group remained the heaviest group, the AI group continued to be the one with the lowest weight and the RF-IVP group showed intermediate values. On day 90 of life, there were no statistical differences between RF-IVP and C-IVP, but between these two groups and AI significant differences were found. In addition, no correlation was found between litter size and weight on any of the days 0, 30, 75, 120 and 180.

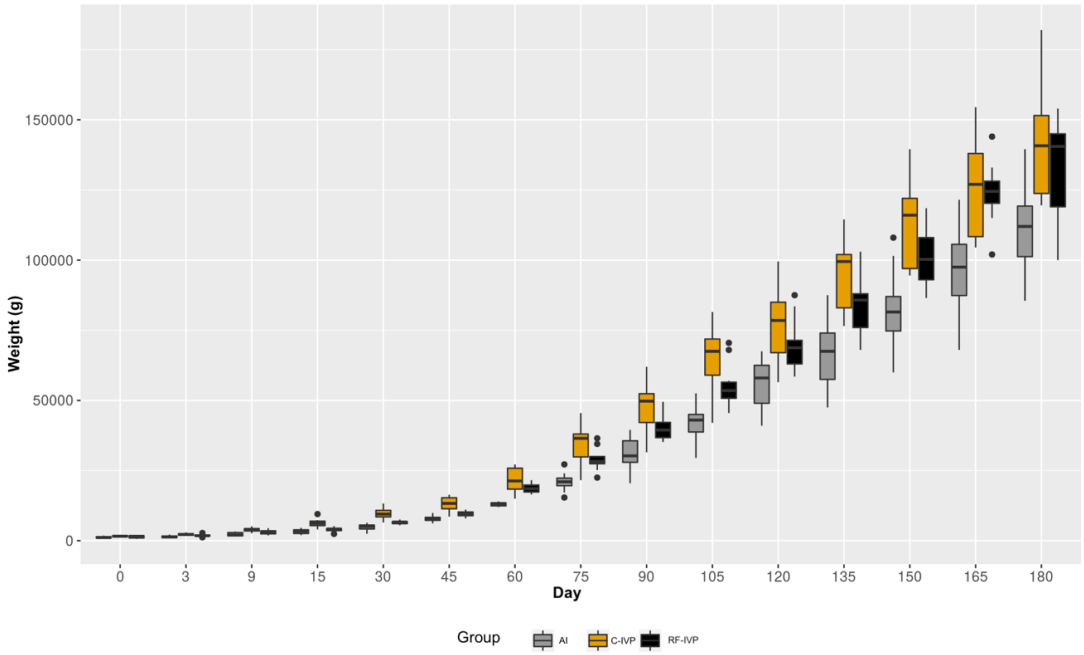


Figure 28. *Box plot of male weights per group from day 0 to day 180. Comparison of global piglet growth. The C-IVP group remained the heaviest throughout the study, followed by the RF-IVP group, which remained with intermediate values, with the AI group being the least weight. All data are presented as the mean ± SD.*

If we look at the global growth curve (Figure 28), we can see that there is a tendency of the weights to be equalized, a trend that is more marked in the case of RF-IVP group. All male weight data are shown in Supplementary Table S13.

Average daily weight gain (ADWG).

According to the robust mixed ANOVA considering all the data, there are no significant differences between the different groups regarding the ADWG. However, when analyzing them day by day, differences appeared between some groups.

Since it was not meaningful to consider day 0 for this measurement, we started to describe it on day 3, where we found significant differences ($p<0.05$; Table S14) between the AI and C-IVP groups; the latter showing differences also with the RF-IVP group, but no differences were found between the AI and RF-IVP groups ($p= 0.209$).

These differences remained until day 15. At day 30, we found no significant differences between RF-IVP and C-IVP groups, while they continue to exist between AI and C-IVP ($p<0.05$), having the control group the highest value.

While no significant differences were found on day 45, they reappeared on day 60 between the AI control group and the RF-IVP experimental groups and C-IVP.

From day 75 to 180, no significant differences were found between any group, except at days 105, 120 and 135, where differences appeared between the AI and C-IVP groups ($p<0.05$). The average daily weight gain per group and day can be seen in Supplementary Table S14.

Crown Rump Length (CRL)

At day 3 of life there were differences between C-IVP and AI groups ($p<0.05$), while RF-IVP group had a length between both groups. At day 9 of life, in addition to the difference between AI and C-IVP, significant differences were observed between AI and RF-IVP groups ($p<0.05$).

At day 15 of life, RF-IVP and C-IVP groups presented significant differences. Figure 29 shows the differences described in the C-IVP group with respect to the other two. This pattern was maintained until day 45, where differences were found in all the groups among themselves ($p<0.05$).

At day 60 of life, only the RF-IVP and C-IVP groups showed differences with the AI group ($p<0.05$). At day 75 of life, all groups presented differences between them ($p<0.05$), highlighting the largest difference in size between C-IVP and AI. Then, at day 90 and 105, we only found significant differences between the AI and C-IVP groups ($p<0.05$).

Finally, from day 120 to day 180, there were only differences between AI and the other two experimental groups. Figure 29 shows the tendency of the lengths for C-IVP and RF-IVP groups to reach similar values from day 120 onwards, even reaching RF-IVP higher values than C-IVP group at day 180, while the AI group maintained much shorter lengths. The numerical data of the CRL measurements can be found in Supplementary Table S15.

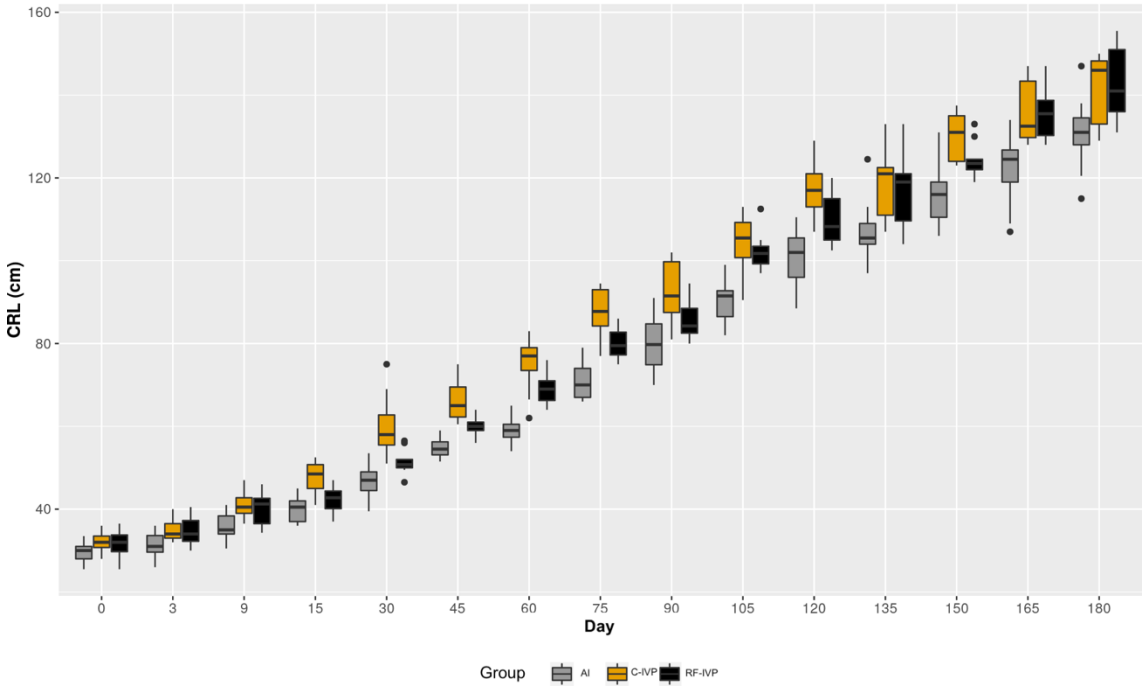


***Figure 29**. Box plot of male Crown-Rump-Length (CRL) per group from day 0 to day 180. Significantly lower differences are shown for AI groups compared to both experimental groups on days 9, 60 as well as from day 120 to day180 of life. On day 3 and from day 90 to 105, significantly higher differences are found between C-IVP and AI groups. Differences between both experimental groups are shown on day 30. All groups present significant differences on days 45 and 75. All data are presented as the mean ± SD.*

Anogenital distance (AGD)

The first differences in AGD appeared on day 9 between AI and C-IVP groups (p<0.05), differences that remained apparent until day 30 where, in addition to the above, differences appeared between AI and RFIVP groups (p<0.05). The existing difference between the AI group and both experimental groups can be seen in Figure 30. At day 45, we found differences only between AI and C-IVP groups (p<0.05). If we observe the representation of the AGD (Figure 30), we can see that, on this day, the AGD of RF-IVP and AI groups are very similar, having a value of 3.93±0.57 cm and 3.65±0.47 cm, respectively. Meanwhile, on day 60, significant differences appeared between C-IVP and the other two (p<0.05), presenting this group the highest AGD values on this day (5.95±1.77 cm).

At day 75 no significant differences appeared between any group, while on day 90 we found differences between AI and C-IVP groups (p<0.05). At day 105, in addition, differences between AI and both IVP groups, (p<0.05) were observed.

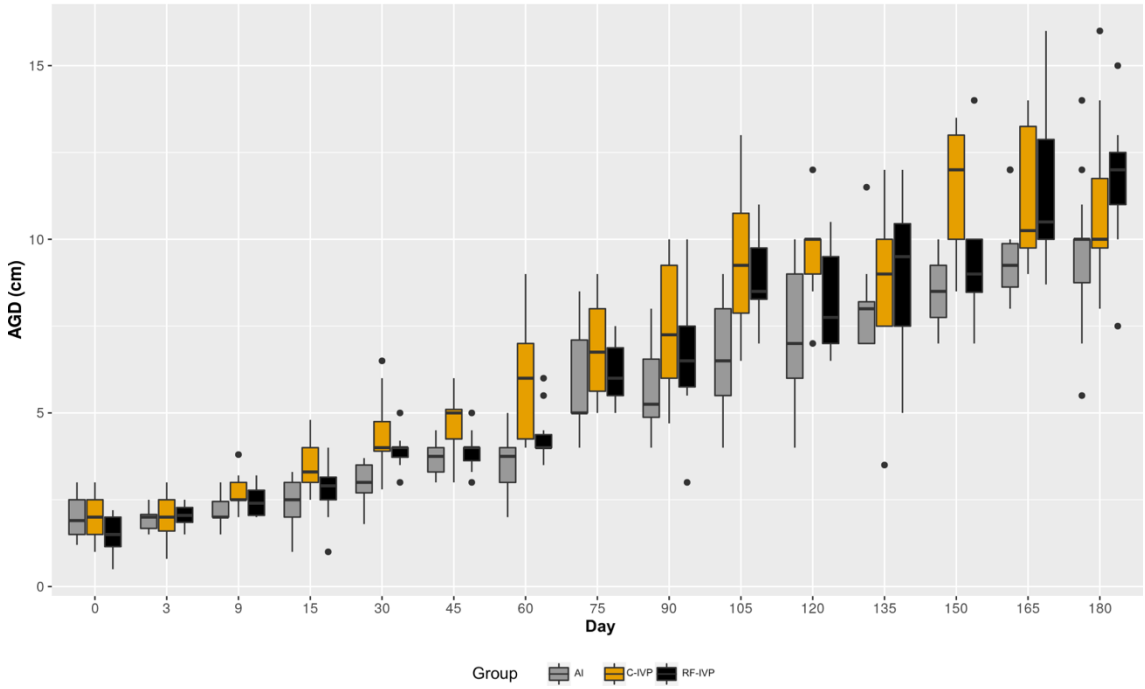


Figure 30. *Box plot of male Anogenital Distance (AGD) per group from day 0 to day 180. Significantly lower differences are shown between AI group and both experimental groups (C-IVP and RF-IVP) on days 30, 105 and 165. The AI group maintains these differences regarding C-IVP on days 9, 15, 45, 90, 120 and 150. In addition, C.IVP shows significantly higher differences on day 60 compared to AI and RF-IVP groups. No differences are found on days 3, 75, 135 and 180 ($p<0.05$). All groups present significant differences on days 45 and 75. All data are presented as the mean ± SD*

At day 120, we only found significant differences between AI and C-IVP groups, while on day 135 there were no significant differences between any group. At day 150 we also found significant differences between AI and C-IVP ($p<0.05$), while RF-IVP group did not show significant differences with any other group ($p>0.05$).

At day 165, in addition to the differences between AI and C-IVP groups, there were also differences between AI and RF-IVP groups ($p<0.05$). However, at the end of the experiment, at day 180, we found no significant differences between any groups. The numerical values of the AGD measurements can be found in Supplementary Table S16.

Females

Weight

The differences found at day 0 in females were also maintained until day 9. On day 15 of life, the animals in AI and C-IVP groups showed significant differences, as well as C-IVP and RF-IVP groups (p<0.05).

In the growth curve up to day 75 (Figure 31) we can see that the differences between the groups seemed to be accentuated on day 30, with significant differences appearing between all groups (p<0.05). Mean weights of 4,615.22±988.18 g, 8,991.25±1,952.04 g and 6,754.29±977.73 g, respectively were found for groups AI, C-IVP and RF-IVP; these differences continued being observed until day 45.

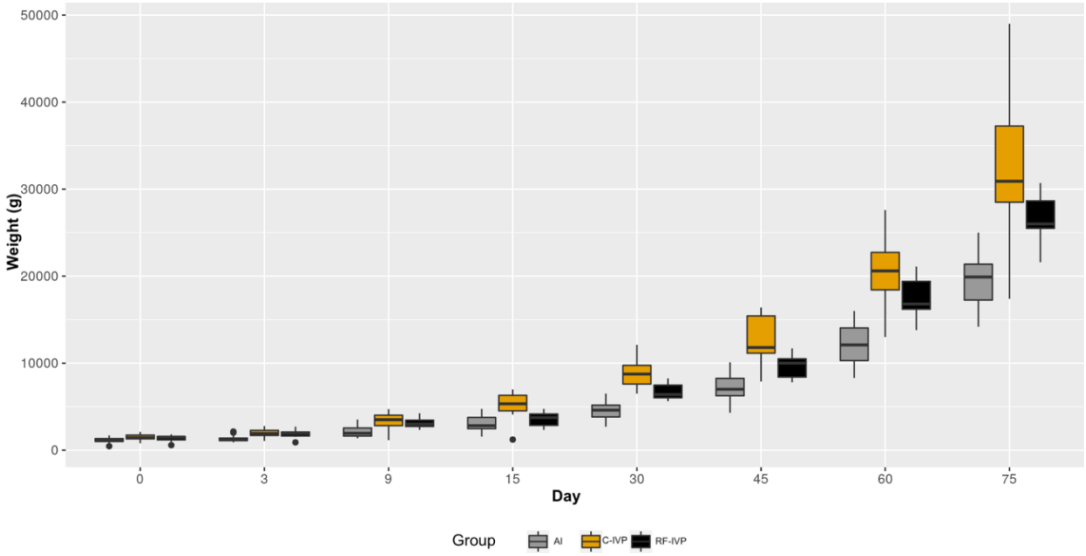


Figure 31. *Box plot of female weights per group from day 0 to day 75. Significantly lower differences are shown from birth to day 9 of life, and on days 60 and 75 for AI group compared with both experimental groups (C-IVP and RF-IVP). On day 15, only significantly higher differences are observed in C-IVP compared to AI and RF-IVP. Significant differences are showed for all groups on days 30 and 45. All data are presented as the mean ± SD.*

At day 60, no differences were found between groups RF-IVP and C-IVP (p=0.073), with differences existing only between AI and both experimental groups, which lasted until day 120.

At day 135 (Figure 32) a greater difference could be observed in the weights of all groups with 61,075.00±10,367.30 g, 89,031.25±12,546.54 g 76,000.00±7,670.29 g for AI, C-IVP and RF-IVP groups, respectively.

Finally, from day 150 to 180, only significant differences appeared between AI and the two experimental groups (p<0.05), ending the study with

weights of 101,263.16±10,227.10 g, 124,937.50±17,224.86 g and 125,057.14±10,697.64 g for AI, C-IVP and RF-IVP groups, respectively.

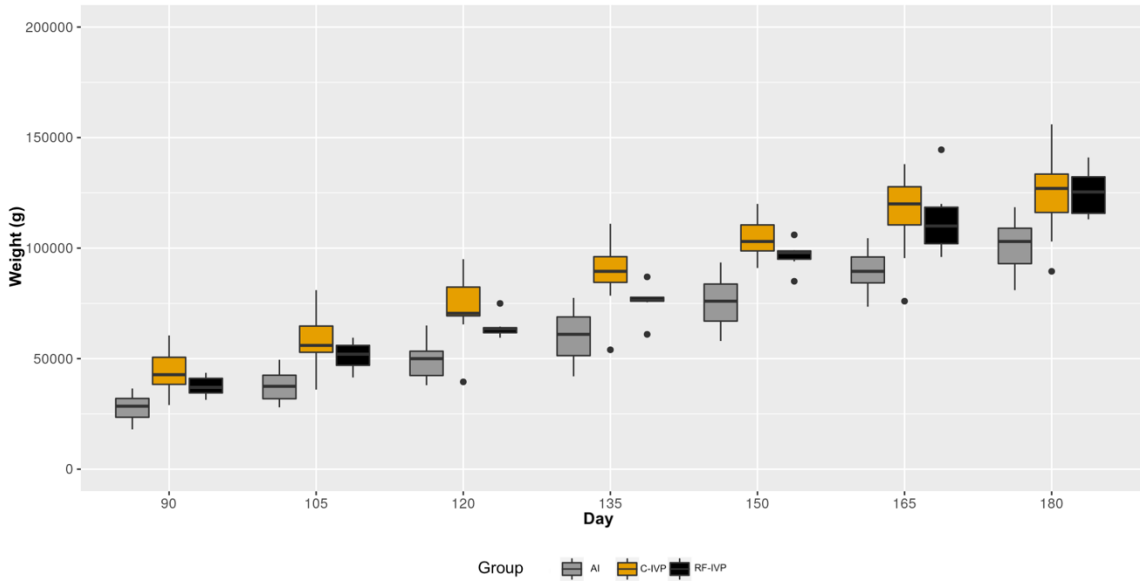


Figure 32. *Box plot of female weights per group from day 90 to day 180. Significantly lower differences are shown for AI group compared to C-IVP and RF-IVP from day 90 to day 120. Significant differences are found between all groups from day 135 to day 180, remaining the heaviest the C-IVP group, and the least weight the AI group. All data are presented as the mean ± SD.*

Figure 33 shows the complete growth curve for females, from day 0 to day 180. In that plot, a situation similar to that of males can be observed, where the heaviest group at birth remains in that place for almost all the experiment, with the exception of day 180, where the average weight of this group (124,937.50±17,224.86 g) is overtaken by the weight of group RF-IVP (125,057.14±10,697.64 g). Meanwhile, the weight of the AI group always remained below the weight of the other groups.

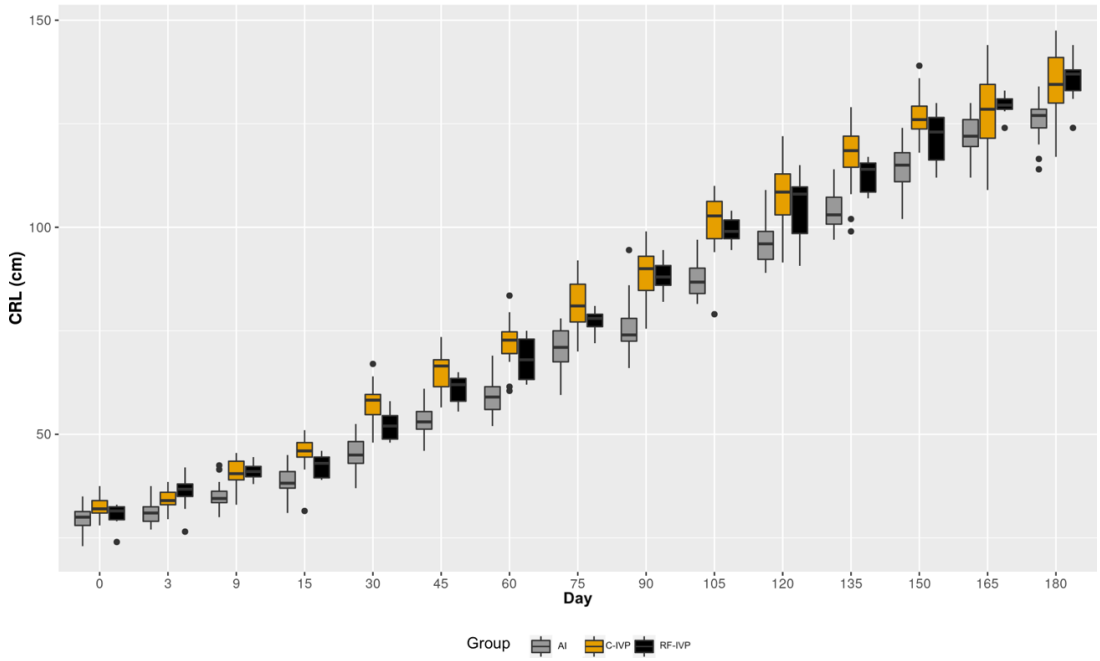


***Figure 33**. Box plot of female weights per group from day 0 to day 180. Comparison of global piglet growth. Except on day 3, where RF-IVP group was the heaviest, the C-IVP group remained the heaviest throughout the study, followed by RF-IVP group, which remained with intermediate values, with the AI group being the least weight. All data are presented as the mean ± SD*

As in the case of males, there seems to be a tendency for the weights to reach similar values, although in this case the AI group is much more distant from the other two groups. Furthermore, no correlation was found between litter size and weight at days 0, 75, 120 and 180, while it was found on day 30.

The mean weights of each group per day can be found in Supplementary Table S17.

Average daily weight gain (ADWG)

According to the robust mixed ANOVA considering all the data, there were no significant differences (p>0.05) between the different groups regarding ADWG. However, when analyzing the data day by day, differences were found between some groups.

As it has been described for males, since it was meaningless to consider day 0 for this measurement, we started to describe it on day 3, where we found significant differences (p<0.05), between AI group, and the two experimental group. On day 9, there were still significant differences between AI and C-IVP groups (p<0.05), but not between AI and RF-IVP groups (p=0.09), while new

differences appeared between RF and C-IVP (p<0.05). These differences between AI and RF-IVP group respect the C-IVP group were observed again on day 15. On day 30, significant differences were found between AI and both experimental groups (p<0.05). At day 45, only significant differences were found between AI group and C-IVP group (p<0.05). At day 60, significant differences were found between AI and the two experimental groups (p<0.05). At day 75 and 90, no statistical differences were found between AI and RF-IVP groups (p>0.05), with the significant differences only appearing between AI and C-IVP (p<0.05).

No significant differences were found between any group in the interval from day 105 to day 180 (p>0.05).

The mean ADWG of each group per day is shown in Supplementary Table S18.

CRL

After studying the results obtained day by day, we found significant differences between all groups (p<0.05) at day 3 of life. On this day, group RF-IVP outnumbers C-IVP group.

On the 9 day of piglets' life, significant differences were found between AI and the two experimental groups (p<0.05), still being RF-IVP group as the largest group, followed by C-IVP.

At day 15 of life, significant differences were again found between all groups (p<0.05). At this time, C-IVP had a longer length than RF-IVP group. In the following measurement, on day 30, there were still significant differences between all groups (p<0.05).

In Figure 34 it can be seen how that, from day 45 to day 180, the CRL of RF-IVP remained at a value very similar to C-IVP group, without significant differences between them (p>0.05). In this interval, the CRL of AI group always remained below the values of the other two, while in most of the time RF-IVP remained in intermediate values between AI and group C-IVP. Towards the end of the experiment, in the last two measurements, RF-IVP presented a greater length than C-IVP. The numerical values of the CRL measurements can be found in Supplementary Table S19.

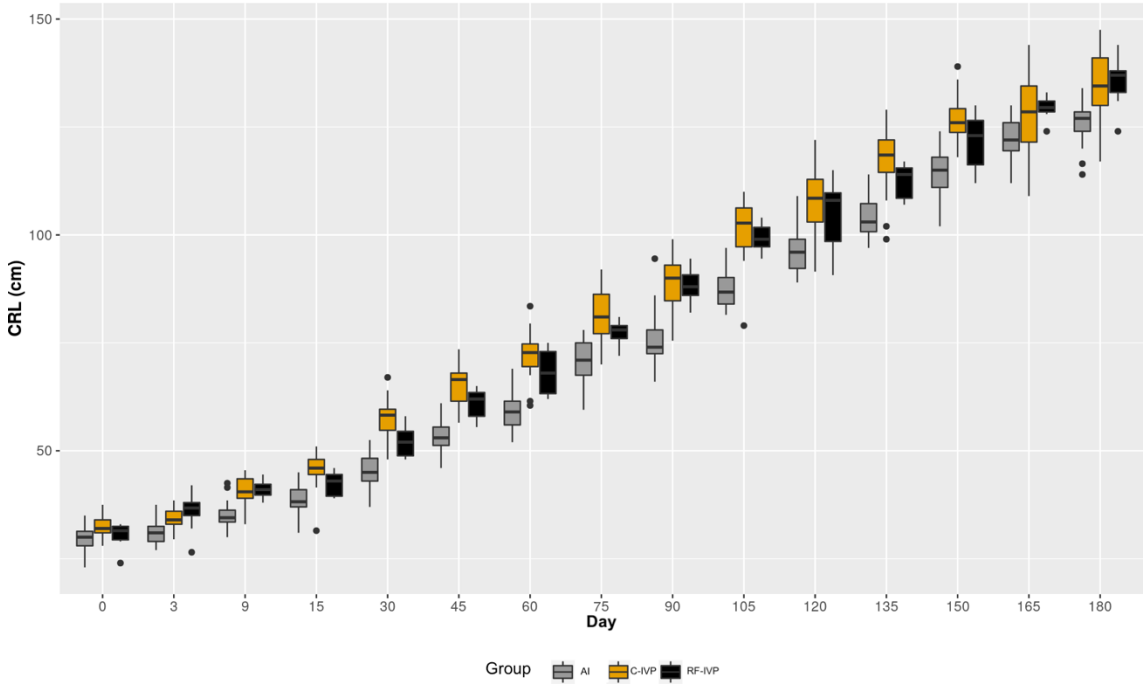


Figure 34. *Box plot of female CRL per group from day 0 to day 180. Significant differences are found in all groups on days 3, 15 and 30 of life. On day 9 and from day 45 to 180, piglets from AI groups shows a significantly lower values compared to those derived from C-IVP and RF-IVP. All data are presented as the mean ± SD.*

6.3. Haematological parameters

All the blood haematological parameters were first analyses on the total population including males and females. Values are showed in Supplementary Table S20-S22. When significant differences were found between some of the different parameters, males and females were separately studied. Those differences maintained for a certain period of time are described below.

3.3.1. Red blood cells parameters

Red blood cells count (RBC)

RBC in males showed significant differences on days 28, 60 and 90 of life (Figure 35, A) between C-IVP and the other two groups except on day 60 where no differences were observed with RF-IVP. Moreover, C-IVP represented the highest value these days.

Females instead, showed significantly higher differences between RF-IVP and AI at day 9 as we can see in Figure 35, B. In addition, as described in males, on day 28, significantly higher differences were also found between C-IVP and the other two groups. Subsequently, only on day 120, significant differences were observed between the AI group and both experimental groups.

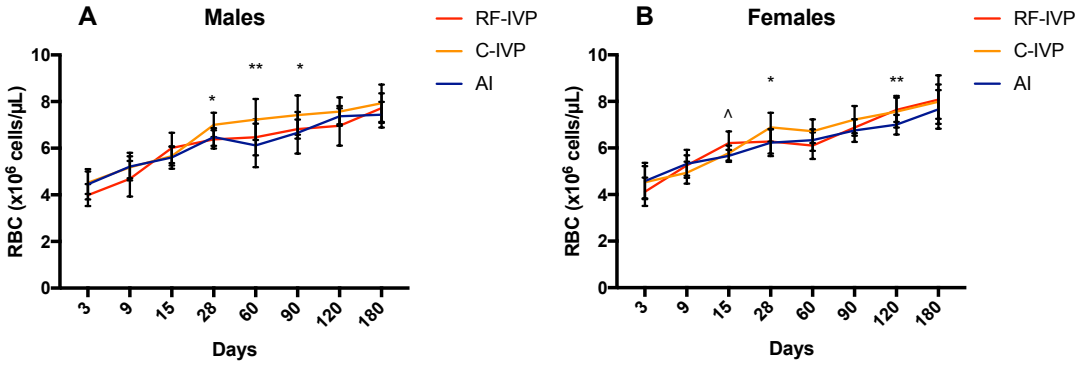


Figure 35. *Kinetics of RBC (x106cells/ µl) in males* ***(A)*** *and females* ***(B)*** *from the three groups of piglets analysed: RF-IVP, C-IVP and AI from day 0 to day 180 (mean±SD). Different symbols indicate significant differences (p<0.05). (*) C-IVP* vs RF-IVP/AI; (**) AI vs RF-IVP/C-IVP; (^) AI vs RF-IVP.

Hemoglobine (Hb)

As we can see in the Figure 36 A, the Hb concentration in males was significantly higher in the C-IVP group on days 3 and 9 compared with RF-IVP. However, the most interesting findings correspond to a first peak at day 15 for the RF-IVP group, which showed significantly higher differences compared with C-IVP and AI. But in addition, on day 28, a second peak was observed, showing in this case the C-IVP group a significantly higher value compared to the other groups. This pattern was also observed when females were analysed (Figure 36 B), although no significant differences were observed. On the other hand, on day 90, males from C-IVP showed a significantly higher value, but in this case when it was compared with AI.

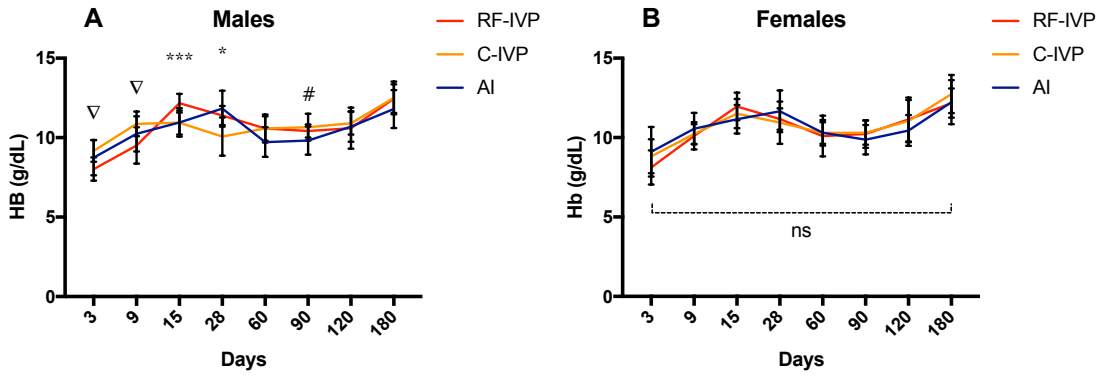


Figure 36. *Kinetics of Hb (g/dL) in males **(A)** and females **(B)** from the three groups of piglets analysed: RF-IVP, C-IVP and AI from day 0 to day 180 (mean±SD). Different symbols indicate significant differences (p<0.05). (*) C-IVP vs RF-IVP/AI; (***) RF-IVP vs C-IVP/AI; (∇) RF-IVP vs C-IVP.*

Hematocrit (HCT)

Figure 37 A shows significantly higher differences between RF-IVP and AI at day 15, being also the highest peak found throughout the entire study in this group. Although a peak was also observed on day 15 in females from the RF-IVP group, no significant differences were observed throughout the study (Figure 37 B).

On the other hand, at day 28, males from AI showed significantly higher differences regarding C-IVP, being the second highest peak throughout the study. Instead, on day 90, the C-IVP value was significantly lower compared with AI (Figure 37 A).

Similarly, when females were studied, those from RF-IVP group showed a peak, although no differences were found throughout the study (Figure 37 B).

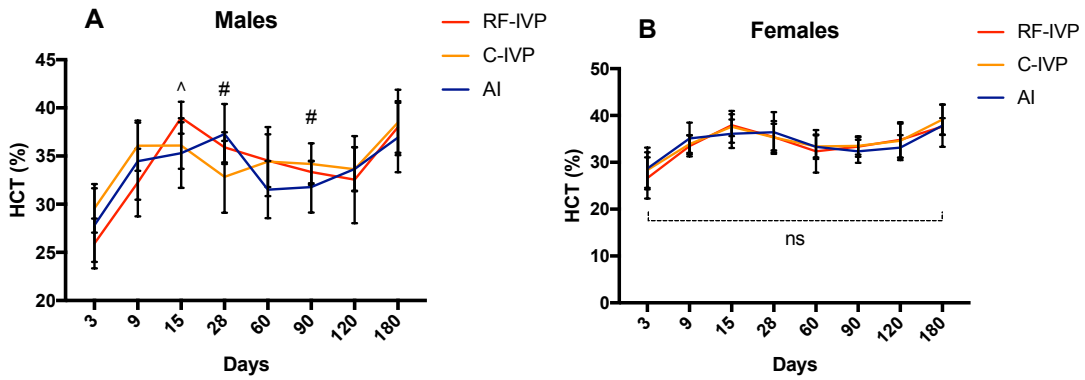


Figure 37. *Kinetics of HCT (%) in males **(A)** and females **(B** from the three groups of piglets analysed: RF-IVP, C-IVP and AI from day 0 to day 180 (mean±SD). No significant differences are found in females (ns). Different symbols indicate significant differences (p<0.05). (^) AI vs RF-IVP; (#) C-IVP vs AI.*

Mean corpuscular volume (MCV)

MCV concentration in males was significantly lower for the AI group on day 3 regarding the other two experimental groups (Figure 38 A). However, on days 28 and 60 of life, C-IVP was significantly lower compared with the other two groups. At day 90, these differences were maintained at day 90 but only between the C-IVP and AI groups.

On the other hand, as we can see in Figure 38 B, females from C-IVP showed a peak on day 9, being significantly higher compared with RF-IVP. Similar to males, at day 28, females from the C-IVP group showed significantly lower differences, but only regarding AI. However, at day 60, the same differences found in males between C-IVP and the other two groups were also observed when females were analysed.

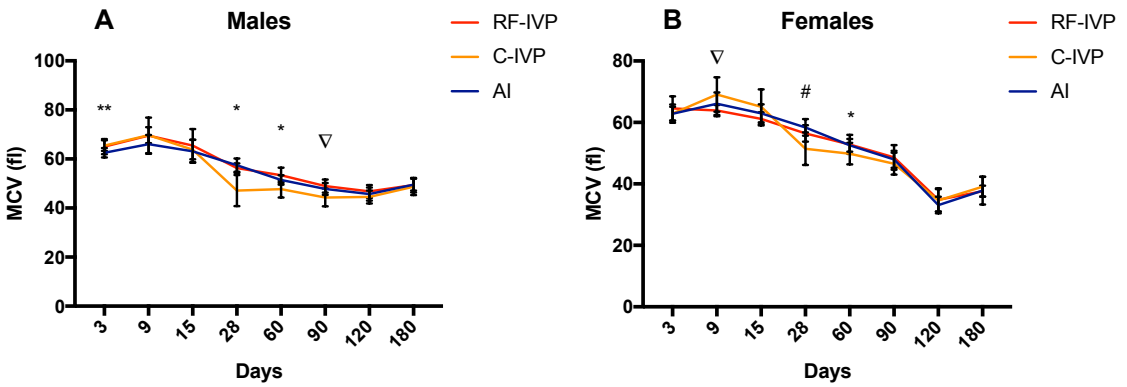


Figure 38. *Kinetics of MCV (fl) in males* ***(A)*** *and females* ***(B)*** *from the three groups of piglets analysed: RF-IVP, C-IVP and AI from day 0 to day 180 (mean±SD). Different symbols indicate significant differences (p<0.05). (*) C-IVP vs RF-IVP/AI; (**) AI vs RF-IVP/C-IVP; (∇) RF-IVP vs C-IVP; (#) C-IVP vs AI.*

Mean corpuscular hemoglobin (MCH)

As we can see in Figure 39 A, males from C-IVP showed significantly higher differences regarding AI, but it was not until the 28th day of life when this group decreased significantly compared to the other two. This pattern was also observed in females, although only differences were found between the C-IVP and AI groups (Figure 39 B).

However, curiously, both males and females maintained these differences until day 60, being the control group significantly lower compared to AI and RF-IVP.

Only males from RF-IVP showed significantly higher differences at day 90 compared with the C-IVP and AI groups (Figure 39 A).

On the other hand, there was an increase in females from day 120 to 180 although no significant differences were detected between groups (Figure 39 B).

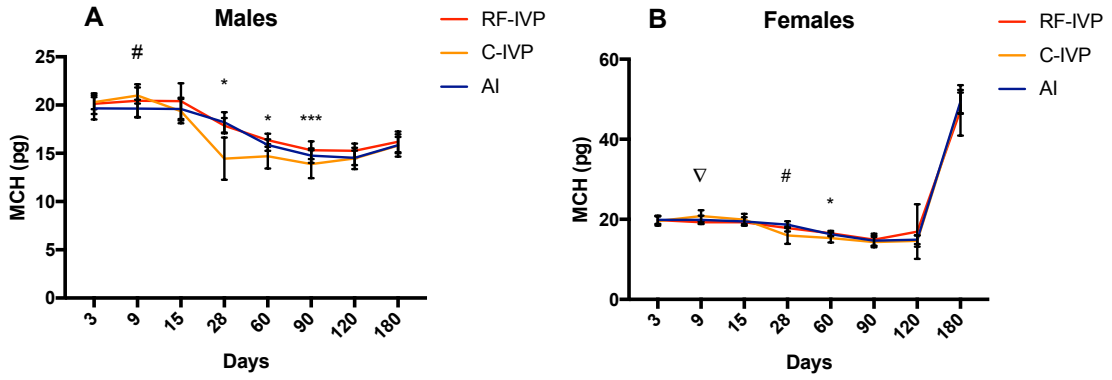


Figure 39. *Kinetics of MHV (pg) in males **(A)** and females **(B)** from the three groups of piglets analysed: RF-IVP, C-IVP and AI from day 0 to day 180 (mean±SD). Different symbols indicate significant differences (p<0.05). (*) C-IVP vs RF-IVP/AI; (***) RF-IVP vs C-IVP/AI; (∇) RF-IVP vs C-IVP; (#) C-IVP vs AI.*

Red blood cell distribution width (RDW)

When RDW was analysed, different fluctuations were observed in males (Figure 40 A); first, at day 3, the RF-IVP group showed significantly higher differences compared with AI. Similarly, when females were analysed, these differences were observed in all groups, being RF-IVP the highest value (Figure 40 B).

Males did not show differences at day 9, however, females from C-IVP were significantly higher compared with RF-IVP and AI groups.

Later, at day 15, males from C-IVP was significantly higher compared with the other two groups, remaining the RF-IVP value in the middle. This day no differences were observed for females.

However, on day 28, significantly lower differences were observed between AI and both experimental groups. In addition, this day the C-IVP also reached the highest peak of the entire study. This peak was also observed in the females (Figure 40 B), where in addition to being observed between the C-IVP and AI groups, they remained until day 90.

Males from C-IVP instead showed significantly higher differences on day 60 compared with RF-IVP and AI (Figure 40 A). In addition, at day 90, significantly

lower differences were found for RF-IVP group compared with the other two. At day 120 this difference was maintained but only regarding the C-IVP group.

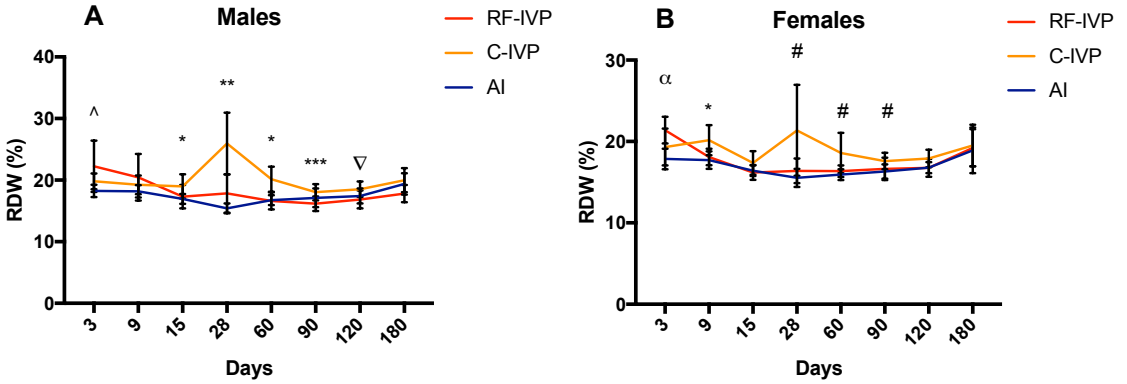


Figure 40. *Kinetics of RDW (%) in males* ***(A)*** *and females* ***(B)*** *from the three groups of piglets analysed: RF-IVP, C-IVP and AI from day 0 to day 180 (mean±SD). Different symbols indicate significant differences (p<0.05). (*) C-IVP vs RF-IVP/AI; (**) AI vs RF-IVP/C-IVP; (***) RF-IVP vs C-IVP/AI; (∇) RF-IVP vs C-IVP ; (#) C-IVP vs AI; (^) AI vs RF-IVP; (α) AI vs RF-IVP vs C-IVP.*

Hemoglobin concentration distribution width (HDW)

As we can see in Figure 41 A, significantly higher differences were found in males from RF-IVP group regarding AI at day 9, but then, on day 15, it was males from C-IVP group that showed significantly higher differences compared with the other two, remaining the RF-IVP value in the middle. Subsequently, on day 28, a peak was observed for the C-IVP group, although differences were observed between all groups.

Similarly, females showed the same pattern (Figure 41 B), although only differences were found between C-IVP and AI groups, the RF-IVP group remaining between both groups.

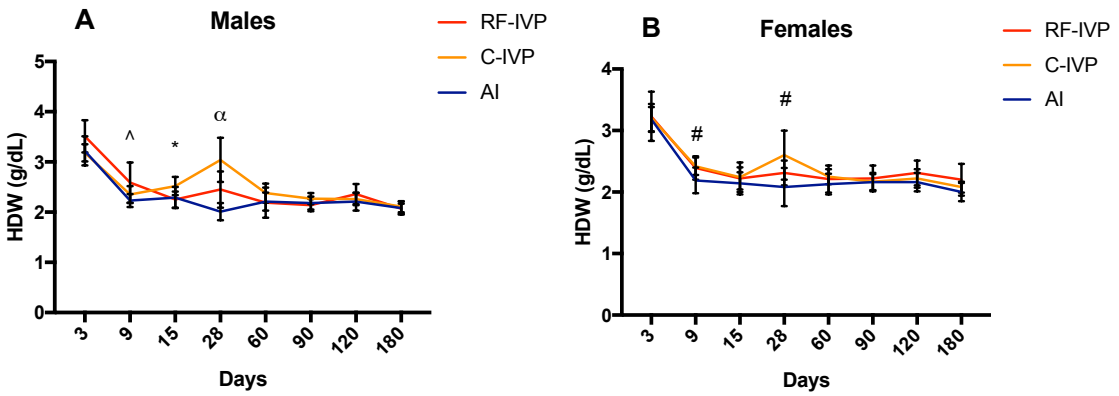


***Figure 41**. Kinetics of HDW (g/dL) in males **(A)** and females **(B)** from the three groups of piglets analysed: RF-IVP, C-IVP and AI from day 0 to day 180 (mean±SD). Different symbols indicate significant differences (p<0.05). (*) C-IVP vs RF-IVP/AI; (#) C-IVP vs AI; (^) AI vs RF-IVP; (α) AI vs RF-IVP vs C-IVP.*

Reticulocyte parameters

When the reticulocyte percentage was analysed, males from the AI group showed significantly lower differences at day 3 compared with both experimental groups (Figure 42 A). These differences can also be observed the same day for MCVr and CHr (Figure 43 A and Figure 44 A, respectively). However, at day 9, C-IVP reaches the highest peak in the reticulocyte percentage, showing significant differences with RF-IVP and C-IVP (Figure 42 A). Then, at day 15, the percentage of reticulocytes decreased sharply for RF-IVP group, showing significant differences with the other two. Later, on day 120 of life, significantly lower differences were found between C-IVP and RF-IVP.

Similarly, when females were analysed (Figure 42 B), significant differences were found between all groups, showing RF-IVP the highest value. In addition, these differences were also seen in MCVr at day 3.

As observed in males, on day 9 of life, females from C-IVP group reaches the highest peak in the reticulocyte percentage, showing significant differences with RF-IVP and C-IVP, also maintaining this difference until day 15 of life.

On the other hand, both males and females showed significantly lower differences at day 28 in MCVr between C-IVP regarding RF-IVP and AI (Figure 43 A, B).

When CHr concentration was evaluated (Figure 44 A), significant differences between C-IVP and the other two groups were maintained in males from day 9 to 60, showing C-IVP, the lowest value. On the other hand, females from C-IVP showed significantly higher differences regarding RF-IVP and AI

groups (Figure 44 A), although it was not until day 28 and 120 when significant differences were observed between C-IVP and AI group as well as C-IVP and RF-IVP groups respectively.

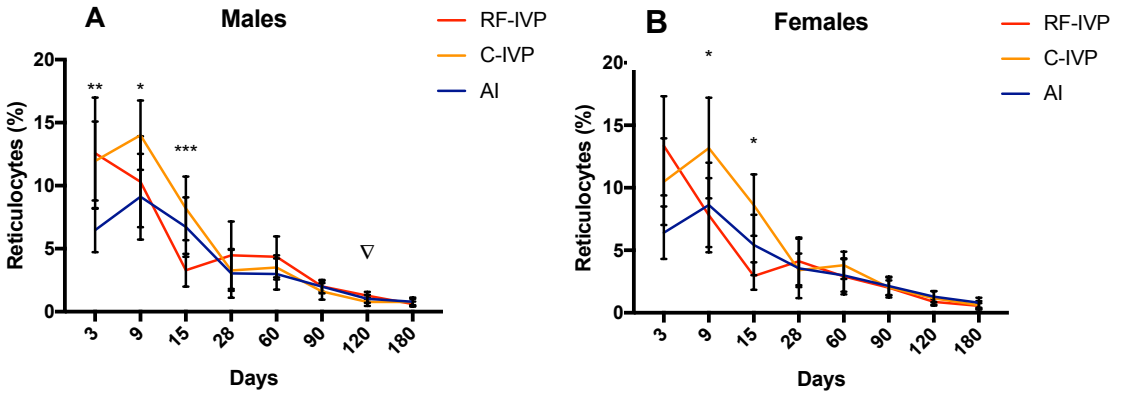


Figure 42. *Kinetics of Reticulocytes (%) in males* ***(A)*** *and females* ***(B)*** *from the three groups of piglets analysed: RF-IVP, C-IVP and AI from day 0 to day 180 (mean±SD). Different symbols indicate significant differences (p<0.05). (*) C-IVP vs RF-IVP/AI; (**) AI vs RF-IVP/C-IVP; (***) RF-IVP vs C-IVP/AI; (∇) RF-IVP vs C-IVP; (α) AI vs RF-IVP vs C-IVP.*

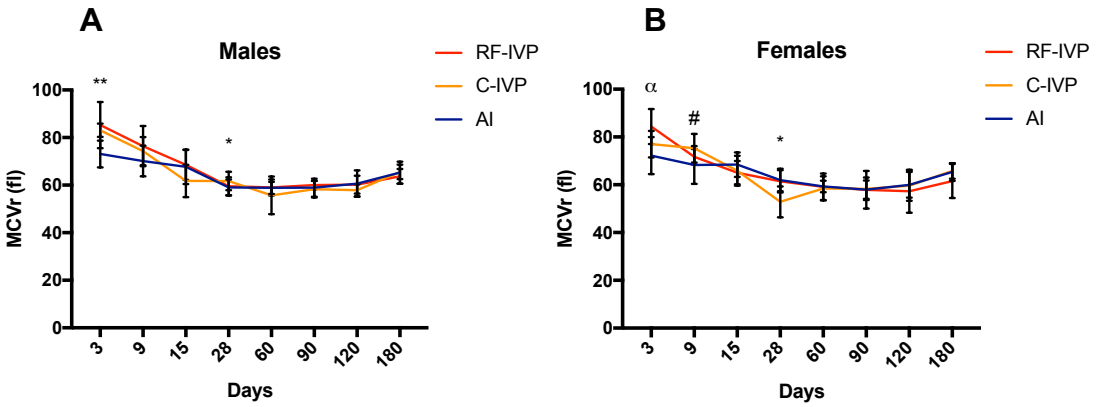


Figure 43*. Kinetics of MCVr (fl) in males* ***(A)*** *and females* ***(B)*** *from the three groups of piglets analysed: RF-IVP, C-IVP and AI from day 0 to day 180 (mean±SD). Different symbols indicate significant differences (p<0.05). (*) C-IVP vs RF-IVP/AI; (**) AI vs RF-IVP/C-IVP; (#) C-IVP vs AI; (α) AI vs RF-IVP vs C-IVP.*

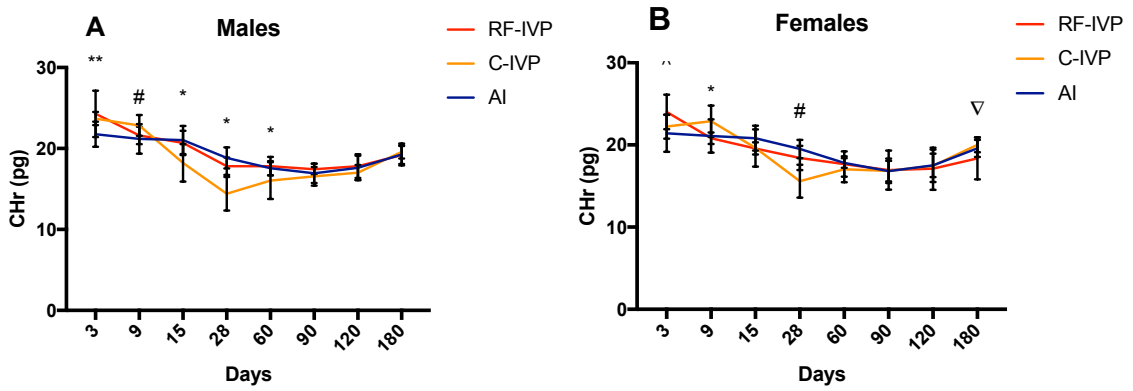


Figure 44. *Kinetics of CHr (pg) in males* ***(A)*** *and females* ***(B)*** *from the three groups of piglets analysed: RF-IVP, C-IVP and AI from day 0 to day 180 (mean±SD). Different symbols indicate significant differences (p<0.05). (*) C-IVP vs RF-IVP/AI; (**) AI vs RF-IVP/C-IVP; (∇) RF-IVP vs C-IVP (#) C-IVP vs AI; (^) AI vs RF-IVP.*

The numerical values of Red blood cells parameters in males and females can be found in Supplementary Tables S23 and S26, respectively.

3.3.2. White blood cells parameters

WBC

When WBC was analysed, significant lower values were found in males from AI group regarding RF-IVP at day 3, however, at day 9, significant lower differences were found regarding the C-IVP group (Figure 45 A). No differences were observed when females were studied (Figure 45 B).

It was on day 9 of life, in addition, when significant lower values were found for monocytes, eosinophils and basophils between males from AI and C-IVP groups, remaining the RF-IVP value in the middle. In addition, significant lower values were also found on day 28 between C-IVP and the other two groups when neutrophils and basophils were analysed. The same day, only differences were found between C-IVP and AI groups in the lymphocytes concentration (p=0,0127), being C-IVP the highest value (Supplementary Table S21).

On the other hand, very isolated differences were found when leucocytes were analysed in females; on day 3, significant higher differences were found in lymphocytes for RF-IVP group compared with AI (p=0,0057). Then, at day 60, females from C-IVP showed significantly higher differences in basophils compared with AI (p=0,0384), however, at day 90, these differences were

observed again between RF-IVP and AI when eosinophils were analysed (p=0,0324), (Supplementary Table S21).

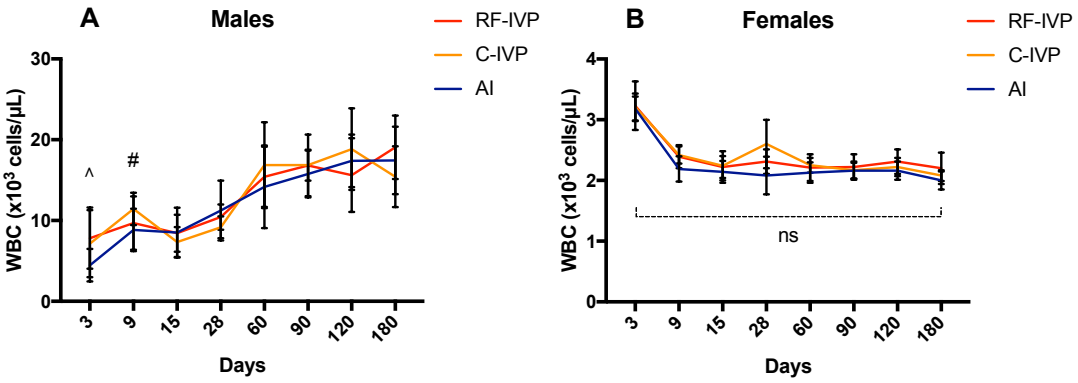


Figure 45. *Kinetics of WBC (X10^3 cells/ µl) in males (**A**) and females (**B**) from the three groups of piglets analysed: RF-IVP, C-IVP and AI from day 0 to day 180 (mean±SD). No significant differences are found in females (ns). Different symbols indicate significant differences (p<0.05). (#) C-IVP vs AI; (^) AI vs RF-IVP.*

The numerical values of WBC parameters in males and females can be found in Supplementary Tables S24 and S27, respectively.

3.3.3. Platelets

As we can see in Figure 46 A, when PLT was analysed, significant lower values were found at day 3 between males from AI and RF-IVP groups. At day 9, instead, a first peak was observed in all groups, although no significant differences were observed. At day 15, significantly higher differences were found between C-IVP and AI groups. Then, on day 28 of life, a second peak was observed, showing C-IVP significant higher differences, but in this case, compared with the other two groups.

Similar to males, females followed the same pattern described previously; A first peak at day 9 where no differences were found and, a second peak where C-IVP showed significantly higher values compared with RF-IVP and AI (Figure 46 B).

A similar pattern was found in PCT both in males and females. As we can see in Figure 48 (A, B) two different peaks can be seen on days 9 and 28, although only females showed significantly higher differences in C-IVP regarding RF-IVP and AI groups. Males only showed significantly higher differences

between C-IVP and AI at day 15. On the other hand, at day 9, females showed significantly lower differences in AI group compared with RF-IVP.

On the other hand, in males, significant differences for AI group were found at day 90 in MPV and PMDW parameters compared with RF-IVP and C-IVP (Figure 47 A and Figure 50 A, respectively). However, differences in MPM were observed between C-IVP and the other two groups, being C-IVP the lowest value (Figure 49 A).

When MPV and PMDW were analysed in females (Figure 47 B and Figure 50 B, respectively), significantly higher differences for AI compared with RF-IVP and C-IVP were maintained from day 60 to 120. Similarly, for MPM, as we can see in Figure 49 B, these differences were also observed, although, at day 120, only differences were found between AI and RF-IVP groups.

Finally, when large PLT was analysed, both males and females showed significantly higher differences for C-IVP when it was compared with AI and both AI and RF-IVP groups in males and females respectively (Figure 51 A, B).

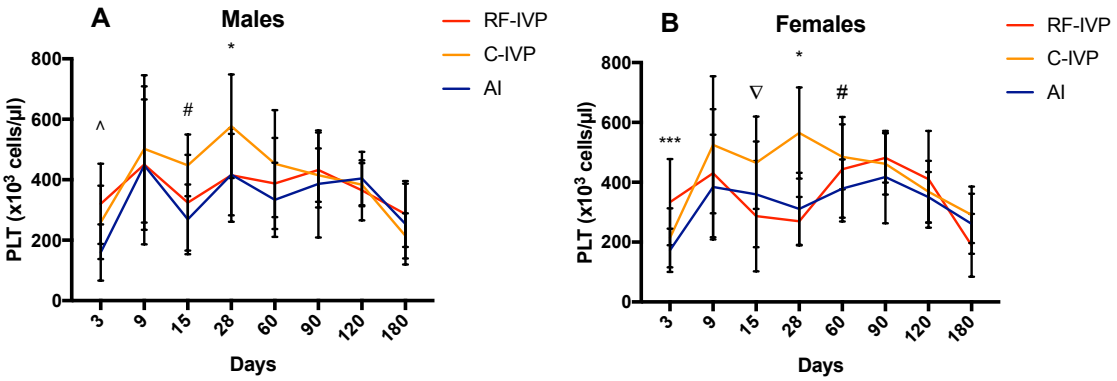


Figure 46. *Kinetics of PLT (X10^3 cells/ µl) in males **(A)** and females **(B)** from the three groups of piglets analysed: RF-IVP, C-IVP and AI from day 0 to day 180 (mean±SD). Different symbols indicate significant differences (p<0.05). (*) C-IVP vs RF-IVP/AI; (***) RF-IVP vs C-IVP/AI; (∇) RF-IVP vs C-IVP; (#) C-IVP vs AI; (^) AI vs RF-IVP.*

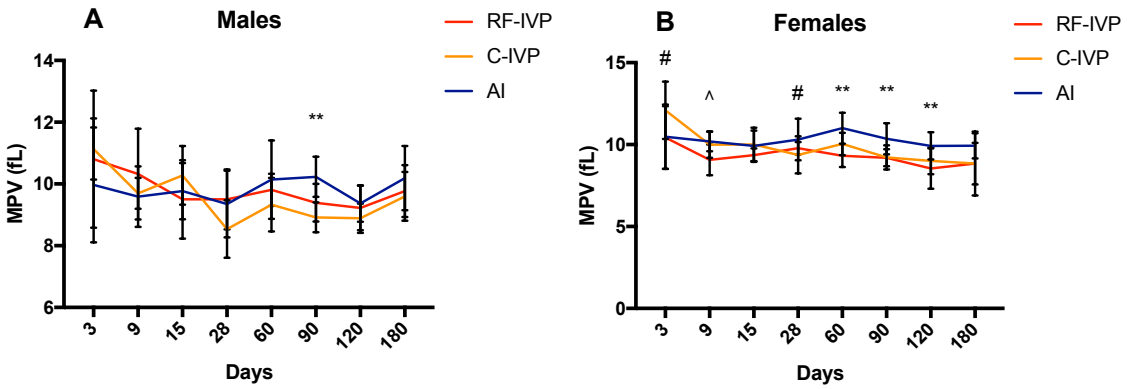


Figure 47. *Kinetics of MPV (fl) in males **(A)** and females **(B)** from the three groups of piglets analysed: RF-IVP, C-IVP and AI from day 0 to day 180 (mean±SD). Different symbols indicate significant differences (p<0.05). (**) AI vs RF-IVP/C-IVP; (#) C-IVP vs AI; (^) AI vs RF-IVP.*

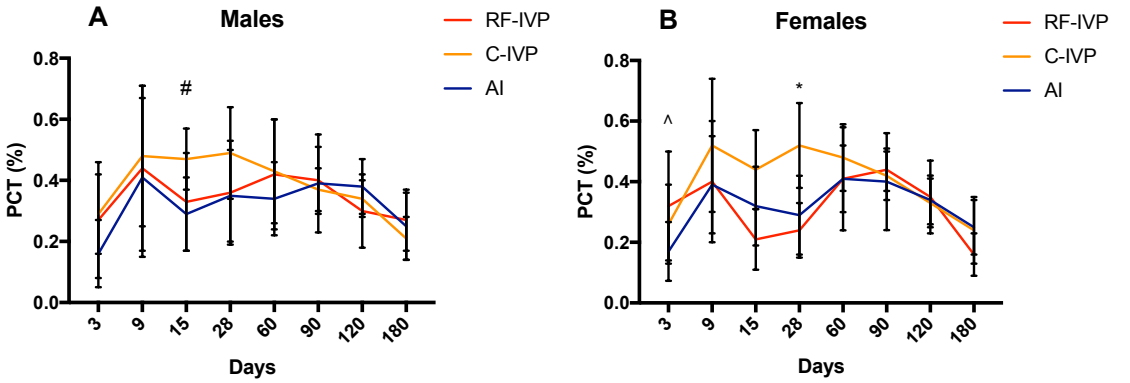


Figure 48. *Kinetics of PCT (%l) in males **(A)** and females **(B)** from the three groups of piglets analysed: RF-IVP, C-IVP and AI from day 0 to day 180 (mean±SD). Different symbols indicate significant differences (p<0.05). (*) C-IVP vs RF-IVP/AI; (#) C-IVP vs AI; (^) AI vs RF-IVP.*

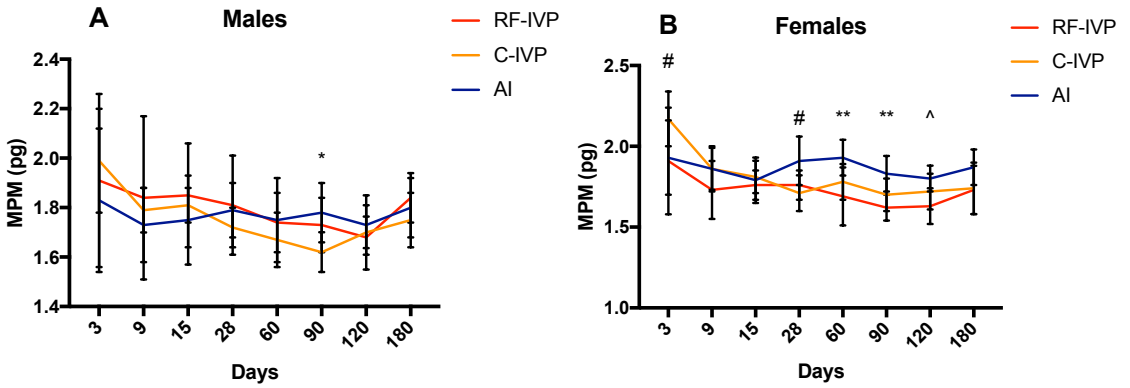


Figure 49. *Kinetics of MPM (pg) in males **(A)** and females **(B)** from the three groups of piglets analysed: RF-IVP, C-IVP and AI from day 0 to day 180 (mean±SD). Different symbols indicate significant differences (p<0.05). (*) C-IVP vs RF-IVP/AI; (**) AI vs RF-IVP/C-IVP; (#) C-IVP vs AI; (^) AI vs RF-IVP.*

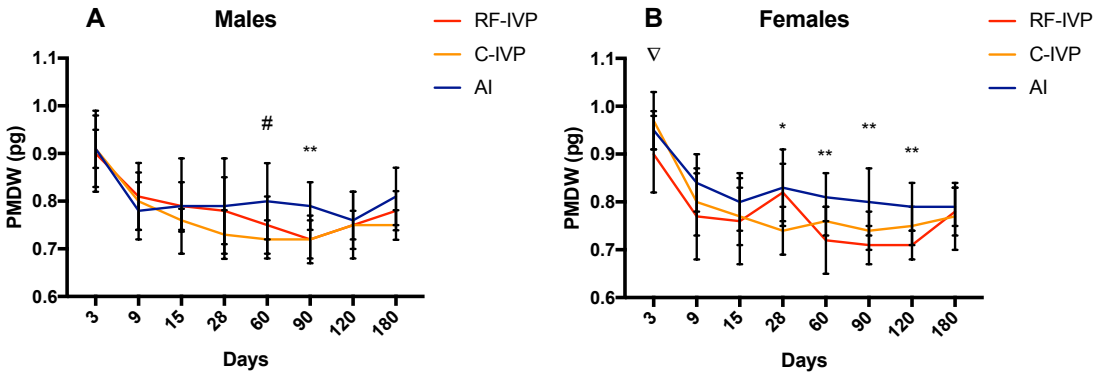


Figure 50. *Kinetics of PMDW (pg) in males **(A)** and females **(B)** from the three groups of piglets analysed: RF-IVP, C-IVP and AI from day 0 to day 180 (mean±SD). Different symbols indicate significant differences (p<0.05). (*) C-IVP vs RF-IVP/AI; (**) AI vs RF-IVP/C-IVP; (∇) RF-IVP vs C-IVP; (#) C-IVP vs AI.*

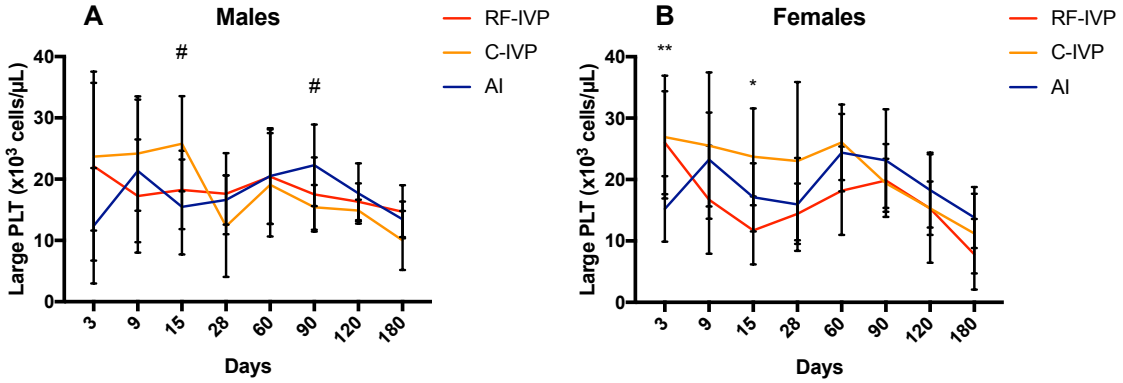


Figure 51. *Kinetics of Large PLT (X103 cells/ µl) in males (A) and females (B) from the three groups of piglets analysed: RF-IVP, C-IVP and AI from day 0 to day 180 (mean±SD). Different symbols indicate significant differences (p<0.05). (*) C-IVP vs RF-IVP/AI; (**) AI vs RF-IVP/C-IVP; (#) C-IVP vs AI.*

The numerical values of Platelet parameters in males and females can be found in Supplementary Tables S25 and S28, respectively.

6.4. Oral glucose tolerance test

A positive correlation between BGC value and weight was found. Moreover, a monophasic BGC curve was observed in the three groups (Figure 52), since BGC steadily increased, reaching a maximum at 45 min after glucose intake, subsequently decreasing to basal values (range 74,13-78,67mg/dl). In addition, differences between the AI and RF-IVP groups were observed at 15, 20 and 30 min, with RF-IVP showing greater values.

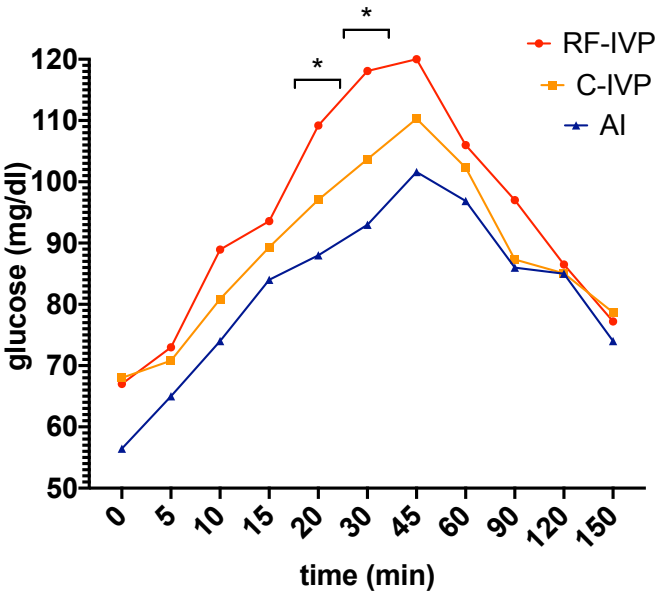


Figure 52. *Oral glucose tolerance test. Blood glucose levels at different times from the three groups of piglets: RF-IVP, C-IVP and AI. Differences between AI and RF-IVP mean values are represented with an asterisk (*), (p <0,05) at 20 and 30 min.*

When the analyses were repeated splitting the animals by sex, both males and females showed a monophasic curve (Figure. 53 A, B) with similar basal levels, and the glucose peak at 45 min in all groups (Males: range 98.43-115.0mg/dl; Females: range 100.88-128.20mg/dl).

In males, differences between AI and RF-IVP groups were present at 20 min and between RF-IVP vs. AI and C-IVP groups at 30 min. In contrast, females showed differences between AI and RF-IVP groups before glucose intake and at 20 min.

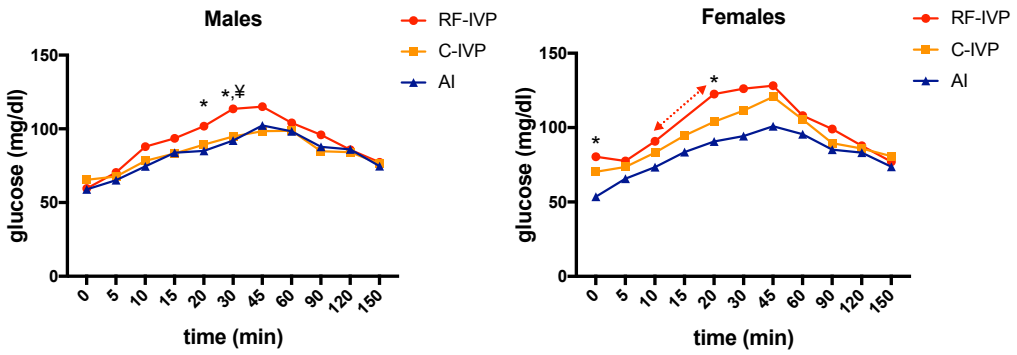


Figure 53*. Oral glucose tolerance test. Blood glucose levels at different times per group and sex.* ***Males*** *(Figure 53A). Differences between AI and RF-IVP mean values are represented with an asterisk (*), (p <0.05) at 20 and 30 min. Differences between RF-IVP and C-IVP are showed at 30 min (¥).* ***Females*** *(Figure 53B). Differences (p<0.05) between AI and RF-IVP at 0 and 20 min are represented with an*

asterisk (*). The dashed lines with arrowheads between t10 and t20 represent insufficient data for the RF-IVP group at time 15.

Despite these findings, all the basal and peak values were always within the physiological range.

Discussion

The research in, and the development of, the different ART protocols in the porcine species, with the final objective of improving their efficiency in a holistic sense, represents an area of increasing interest for two main reasons: first, for the porcine species, the potential existence of an embryo market, allows farmers the exchange genetic material without risk of disease transmission and reduced transportation costs (Fowler *et al.,* 2018); second, for the human species, there is compelling evidence that the pig as an useful model to decipher the long term impact of each aspect of the ART procedure without confounding factors such as those related to the fertility of the parents (Cánovas *et al.,* eLife 2017), a fact that has been recently reinforced with the discovery of the similarities between the pig and the human regarding the DNA methylation reprogramming events during the first week of development (Ivanova *et al.,* 2020).

In this context, this thesis focuses on two of the main aspects of ART procedures using the porcine model - embryo transfer and the *in vitro* culture of embryos - to shed light on their immediate efficiency and their biological consequences.

To better understand, interpret and summarize the main findings of this study, the results of the three experiments will be discussed separately, although many inter-relations and connections will be commented upon, where appropriate, throughout the manuscript.

1. The embryo transfer does not affect the growth, haematological or biochemical parameters of the offspring at birth, day 3 or day 15 of age.

The first experiment was designed to assess the isolated effect of the removal of the embryos from the uterus of their mother and their rapid transfer into the uterus of a recipient. Non-surgical transfer was used to avoid confounders, so that the surgical stress in the recipient sow was abolished. Doing so, we expected that any difference between the experimental and control (artificially inseminated sibling sows) groups in terms of gestational length,

farrowing rate, survival rate, litter size or phenotypical traits of the offspring at short term, was due to the effect produced in the embryos by the mere act of the transfer.

Our results represent, to the best of our knowledge, the first approach to describe the impact derived from the use of non-surgical ET in pigs, not only at birth but also at days 3 and 15 of age. Indeed, there have been previous studies which refer to the effect on birth weight, growth parameters, metabolism, etc. in ART offspring (Berntsen et al., 2019; Castillo et al., 2019; Feuer & Rinaudo, 2017), but most of them have been in mice, due to its easy handling, short gestation length, etc. (Gutierrez et al., 2015). In addition, the few studies performed in pigs have evaluated some productive parameters in IVP-derived piglets such as farrowing rate, litter size or birth weight, but none of them have described the haematological and biochemical profile of the piglets before weaning, as was done in the current study.

Because it has been shown that each technology applied during embryonic development might have some impact on the neurodevelopmental and physical health of the offspring throughout life (Mintjens et al., 2019), we aimed for our experimental design to help us see if differences found by studying the haematological and biochemical profiles, as well as the weight and ADWG of the offspring during the first days of age, could have long-term implications.

First of all, it is important to note that the percentage of animals that became pregnant after embryo transfer (3 out of 13) in our study was below the normal expected average, but the explanation for this can be found in the synchronization protocol used, since the recipients were selected among those animals between 0 and +48h of asynchrony in estrous regarding the donors and, as Angel et al. demonstrated (and we confirm in our experiment 3), the ideal time frame of asynchrony must be between 0 and -48h (Angel *et al.,* 2014). Despite this problem, the rest of the reproductive parameters (gestation length, litter size, live-born piglets, sex proportion or morphology) were all within the normal ranges and not different among ET and AI groups.

Regarding growth parameters, our results showed that, although ET piglets had a slightly higher body weight in absolute terms than AI at birth as well as days 3 and 15 of age, no differences were found between groups ($p>0.05$). This is in disagreement with (Ducro-Steverink et al., 2004), who detected

differences in non-surgical ET, showing higher birth weights in comparison with AI piglets. However, these differences could be attributed to the reduced size of non-surgical ET litters in that study, contrary to our results where that difference was not found.

In our attempt to investigate if the birth weight was affected by sex, we found that only females showed differences when the data were analysed separately, being with the weight at birth higher in ET than in AI, as (Ducro-Steverink et al., 2004) found, but such differences disappeared at days 3 and 15. In the same way, males did no show differences at birth or on days 3 and 15. This fact, however, should be kept in mind and considered again when data from Experiment 3 are analysed because, as it has been shown in different studies, increased birth weight is one of the most common findings in ART-derived calves and it has been related to the LOS (Z. Chen et al., 2013; Y. Li et al., 2019) as well as to the BWS in humans (Weksberg et al., 2010), although it has not been described in pigs until now. Thus, the study of pig females born by IVP to find out if they are indeed of greater birth and postnatal weights than their AI counterparts and their long-term monitoring in case, they are heavier, would be of particular interest.

Nevertheless, weight at birth in all cases was within the normal range and no anatomical abnormalities were detected in any piglet, which is in agreement with other studies (Angel, Gil, Cuello, Sanchez-osorio, et al., 2014; Yoshioka et al., 2012a).

As for ADWG, again no differences were found between ET and AI groups, something expected considering the absence of differences in body weight. This is consistent with a study in mice, where males and females from IVF had a similar growth phenotype compared to the control group (Donjacour et al., 2014).

Although there are few references to haematological and biochemical parameters in the porcine species (Perri et al., 2017), there are various studies in cloned pigs where different blood parameters have been evaluated (Mir *et al.*, 2005; Greg *et al.,*2014; Gu *et al.*, 2019). In addition, (Ventrella et al., 2017) have recently provided some reference intervals that could help us interpret the results obtained.

Different parameters were analysed to assess the general health, status, presenting, in general terms, the haematological and biochemical profiles with

some differences and high variability between some of them. A few authors attribute this finding to the immune system development during the growth of piglets (Faustini et al., 2003; Perri, 2015).

On day 3 postnatally, females derived from the ET group showed significantly greater values in the concentration of RBC, Hb and HTC compared to the AI group. Conversely, on the same day a decrease in the percentage of reticulocytes, MCVr and CHr was observed, although only significantly lower differences were seen in MCVr for the ET group. This is in agreement with Ekert Kabalin *et al.* (2008), who previously described that an increase of erythrocytes, due to increased erythropoiesis, is accompanied by a decrease in the reticulocyte count in newborn piglets.

On the other hand, at day 15, significantly smaller differences were found in the percentage of reticulocytes, MCVr and CHr for females from the ET group compared to those derived by AI, the latter two parameters also being significantly less in males derived by ET. Godyń *et al.* (2016) reported that these parameters are indicators of the existence of iron deficiency; however, none of the values were outside the normal range nor was the Hb concentration was altered. On the other hand, Bhattarai and Nielsen (2015) reported that due to the differences in weight, most reticulocyte indices vary depending on the size of the piglets, and thus our data may not be considered as indicative of any anomaly.

WBCB was significantly greater at day 3 in males and females from ET due to an increase in the levels of neutrophils and lymphocytes, respectively, being elevated in females also on day 15. Since this population of cells is expanding during piglet growth (Cooper et al., 2014) and these values were within the normal range, we cannot, again, give any clinical significance to these differences.

Pliszczak-Król et al. (2016) described that PLT variability in piglets may be due to the rapid growth of these animals and the maturation of their hematopoietic system. Despite no differences found in the PLT concentration, significantly lower differences were found on day 3 in PCT and MPM for males from ET compared to those derived by AI. On the other hand, significantly higher differences were seen in males from the ET group on day 15. However, although MPV is an index with some clinical relevance and an increased MPV indicates increased platelet diameter, which can be used as a marker of production rate and platelet activation

(Budak *et al.,* 2016), the clinical significance, reference values and usefulness of most of the platelet indices are still under investigation (Kim et al., 2014) and, in our study, we cannot affirm that any of them could be interpreted as potential markers of any kind of phenotypical difference between our two groups of piglets.

The biochemical analysis showed higher differences on day 3 in ET-derived males compared to males from AI for different parameters, included ALP, GGT, AST and ALB. Interestingly while on day 15 the concentrations of GGT, AST and ALB remained higher in the ET group compared to the AI, a decrease was observed for ALP. However, no statistically significant differences were found on this this day. According to some authors, an increase in ALP levels is associated with increased production of osteoblasts due to the growth of piglets (Casas-Díaz et al., 2015; Kabalin et al., 2012). In contrast, a decrease in the concentration of this enzyme is related to a decrease in phosphorus levels in diet (Perri, 2015).

On the other hand, Stone (Stone, 1984) reported that high ALB levels are associated with the physiological maturation of the liver.

In addition, despite an increase in AST concentration being associated with increased physical activity or the existence of muscle damage (Verheyen et al., 2007), all values were within the normal range. On the other hand, according to Yu *et al.,* (Yu et al., 2019), a high concentration in newborn calves is an indication that these enzymes are absorbed from colostrum, at least in the case of GGT and AST. In addition, Dubreuil and Lapierre (Dubreuil & Lapierre, 1997) reported an increase in CK and AST of growing pigs up to week 8 of age, both parameters being related to muscle growth. Despite no differences found in CK, our results showed a high variability this parameter, also being influenced by the age and sex of the animals (Heffron *et al.,* 1976; Grindem, 2011).

On day 15, females from the ET group showed significantly lower values for urea compared with those from the AI group, which has been associated with a decrease in protein intake (Perri et al., 2017).

Due to the observation that all parameters were within the normal range, a clinical significance cannot be established.

2. Protein source of the culture media does not alter the yield of the in vitro embryo production system, nor the pregnancy and parturition

outcomes, but influences the placental efficiency and some molecular traits in placenta and umbilical cord.

Results from our Experiment 2 showed that, when the reproductive fluids were introduced in the culture media at the different steps of the *in vitro* production system, the percentage of cleavage was similar to that obtained in the absence of fluids. Similarly, the percentage of blastocysts, assessed from the total oocytes fertilized or from the cleaved zygotes, was not different between groups. As the use of reproductive fluids is not yet a common practice in the field, the only work previously published to compare with is that from our own group (Cánovas *et al*., eLife 2017), showing a 5% higher cleavage rate in the control group than in the group using reproductive fluids, although both values were below the values in the present study (lower than 50% in Cánovas *et al.* for both groups vs. higher than 65% in the present study for both groups). As in our case, the percentage of blastocysts in the Cánovas *et al*. study was not different regarding the presence or absence of reproductive fluids. These results confirm what was previously proposed about the lack of increase in the final number of embryos obtained when reproductive fluids are used, but cannot, at this point, confirm yet the higher quality of the embryos described by Canóvas *et al.* because no other parameters where analysed in the present study. Instead, we transferred most of the embryos to investigate their ability to implant and develop to term.

First, we will call attention regarding the pregnancy rate after transfers, with a greater percentage (>35% in both, C-IVP and RF-IVP groups) of positive pregnancies compared to those in our experiment 1. As mentioned, the recommended range of asynchrony between donor and recipients must be between 0 and -48 h (Angel *et al.,* 2014; Hazeleger *et al.,* 2000), and this was the rule we followed in this experiment, so that we assume this was the reason for the higher pregnancy and parturition rates obtained compared with Experiment 1. Despite this, the percentage of non-pregnant animals after embryo transfer in our study was still higher than 64% in both groups and, as has been recently proposed, this could be associated with a dysregulation of pro- and anti-inflammatory cytokine levels in recipient sows that, in turn, induce embryonic mortality (Cristina A. Martinez et al., 2020). However, many other factors related

to either the quality of the embryos or the recipients' status and age could have been affecting the rate of unsuccessful transfers (Martínez *et al*., 2019). On the one hand, and as for our own ET procedure, the reported levels of embryonic mortality by using non-surgical procedures are approximately of 70% (Ducro-Steverink *et al.,* 2004; Martínez *et al.,* 2014), whereas using surgical laparoscopic procedures Wieczorek *et al.,* (Wieczorek et al., 2015) reported 50% of successful pregnancies after transferring *in vivo* produced embryos. This is another crucial factor to be considered because all the above referenced rates derive from embryos produced *in vivo* and ours were produced *in vitro*. In fact, for most of the researchers, the transfer of embryos produced *in vivo* is the only ET procedure with "possible short-term use in pig production" (Martinez *et al.*, 2019) although, from our results, this statement should probably be reconsidered.

Actually, in our experiment we observed high farrowing rates, with only one miscarriage in the RF-IVP group at day 24 post-transfer (which is considered the time frame for implantation). This high farrowing rate and low miscarriage rate can be considered good indexes of the quality of the embryos transferred, although more studies with higher sample sizes are needed to confirm this statement.

Gestation length depends on the litter size, but it is well known that some other factors such as farm, parity, number of inseminations or genetic line can affect it (Ketchem et al., 2017). While short gestation lengths are associated with higher number of stillborns, extended gestation lengths are not desired by the farmers and the advantages of inducing and attending farrowings compared to letting the sows farrow on their own are a matter of current debate (Ketchem et al., 2017). In our experience, inductions were necessary in 1, 2 and 4 animals from the AI, C-IVP and RF-IVP groups, respectively, mainly because of delays in deliveries between the first and following piglets. However, our presence during parturition, in order to take the individualized umbilical cord and placental samples, could have acted as an additional stressor factor contributing to the delays and, consequently, we cannot affirm at this point if such problems were related to the embryo transfer procedure, the embryo source or our own presence. The litter size, in our case, was not a factor seeming to affect the gestation length because the sow with the longer gestation (121 days) delivered only 5 piglets whereas the sow with higher litter size (10 piglets) delivered at day

115. Similarly, the fact that embryos were *in vitro* produced was not a factor affecting gestation length because the AI animals showed similar periods of pregnancy to the IVP groups. All in all, the small sample size in our experiment, the final goal of which was not to compare the reproductive indexes between groups but the phenotypes of the offspring, makes it impossible to reach valid conclusions in these aspects.

Umbilical and placental abnormalities are relatively common in clones but information about the impact of porcine *in vitro* derived embryos on these defects is still limited. In Experiment 2, the relationship between birth weight, placental development and umbilical cord were also analysed in the two IVP groups (C-IVP, RF-IVP) vs. the AI group. The fetal growth of the piglets is highly dependent on its placenta, and fetal weight is found to be proportional to placental weight in several studies (Leenhouwers et al., 2002; Rampersad, R., M. Cervar-Zivkovic, 2011; Van Rens et al., 2005). Reduced placental weight, on the other hand, has been reported in somatic cell nuclear transfer-derived piglets vs. those produced by AI (Zheng et al., 2017). However, while this parameter has been studied in pigs in production settings (Wilson *et al.*, 1998; Rootwelt *et al.*, 2013), in other species such as mice, changes in placental weight have been related to exposure to stressors during *in vitro* production (Bloise *et al.*, 2015b), although no data about the impact of *in vitro* production on placental weight is available in pig. Our results show no differences in the placental weight between the experimental groups (C-IVP and RF-IVP) and the AI group.

Nonetheless, placental area is considered a good marker for postpartum piglets viability, and it is highly associated with birth weight (Rootwelt et al., 2012). Lower placental area was reported in piglets dead at weaning vs piglets alive at weaning (Rootwelt et al., 2013). In our study, the C-IVP group displayed larger placental area, and heavier piglets, than AI group, while the litter size was lower in C-IVP than AI group. This is in accordance with the negative association reported between placental weight and live litter size (Rootwelt et al., 2013). By contrast, in the RF-IVP group, the birth weight and placental weight were similar to those of the C-IVP group, but placental area was lower than in C-IVP, even with equivalent litter size.

Although placental area has been suggested as a good marker for piglet viability, placental efficiency (PE; the ratio of fetal weight:placental weight, with

greater fetal weight per unit of placental weight considered more “efficient”) (M E Wilson & Ford, 2001) could provide complementary information about placental function, with high PE values associated with greater nutrient or respiratory gas transport capacity. PE was signficantly decreased in the RF-IVP vs C-IVP and AI. Nonetheless, this parameter shows natural variation in pigs and between breeds. Even within litters, PE can vary significantly, with similar piglet birthweights but very different placental weight (up to 25%) (Wilson *et al.*, 1998; Krombeen *et al.*, 2019). Moreover, the use of PE as a selection tool to increase litter size is debatable, because an increase in litter size could result in reduced birth weight and higher mortalities. It is controversial regarding animal welfare, even in the hypothetical situation that global production outcome would remain beneficial.

As for the molecular analyses, it is known that placental nutrient transport capacity can be associated with gene expression variation of transporter genes, with special interest in amino acid and glucose transporters. For example, in mice, *Slc2a1 (GLUT1 g*lucose transporter) and *Slc38a2 (SNAT2* amino acid transporter) were upregulated in the lightest placentas, confirming that placentas with high PE adapt and increase nutrient transport efficiency. Contrary, *SLC7A1 (CAT1), a* cationic amino acid transporter, was found negatively related to PE (Krombeen et al., 2019). Placentas in the RF-IVP group, with the lowest PE, showed twice the SCL7A1 expression than C-IVP or AI, but these differences were not statistically significant, perhaps due to the low number of samples. In the umbilical cord, SCL2A1 expression did not show differences, but there was a tendency (p=0,0502), with RF-IVP and C-IVF showing expression values greater than AI.

Placentas from ART derived animals exhibit higher probability of perturbations in genomic imprinting, and expression of imprinted genes is also altered, as reported by different laboratories (mouse, (B. Li et al., 2016); pig (Zheng *et al.,* 2017); bovine (Z. Chen et al., 2013); human (Turan et al., 2010)). However, under our experimental conditions, there were no differences in the expression of imprinted genes in placenta and umbilical cord, except for *PEG3* in placenta. Expression of this imprinted gene (paternally expressed) was upregulated in C-IVP embryos vs AI, whilst the RF-IVP group showed

intermediate values. *PEG3* expression level is sexually biased with two-fold higher levels in males than females (Mohammad F., Hana K., 2014).

This could not explain our results since, in C-IVP and AI groups the number of males obtained was very similar (40% vs 44%; Table 8). Thus, increased *PEG3* expression in the C-IVP group could be a consequence of the culture media used in this group, and the supplementation with reproductive fluids to IVF and EC media could mitigate this effect.

3. *In vitro* production of embryos affects birth weight and growth, with reproductive fluids palliating this effect.

In the porcine species, the composition and physicochemical characteristics of the culture medium have been shown to directly affect the development of blastocysts and the methylation and expression pattern of their genes (Cánovas et al., 2017). Our results show that, in addition, the culture medium used may have long-term consequences on pig growth.

In this study, males in the AI group showed a mean birth weight similar to that previously reported for this breed (Whittemore, 1996) but lower than that of the experimental groups RF-IVP and C-IVP, with these differences in body weight maintained throughout the study period, the C-IVP group always the heaviest. In the case of females, we found a similar scenario, with the exception that on day 180, weight of group RF-IVP was higher than weight of group C-IVP. In both cases, throughout the study, group RF-IVP showed intermediate weight values, compared to C-IVP and AI groups. In the study of García-Vázquez et al. (2010), who transferred embryos obtained by intracytoplasmic injection of genetically modified sperm into the oviductal lumen, the weight at birth of the piglets was similar to that of our control group but lower than that of our experimental groups. This may be due to the different type of technique used, since, in our case, the embryos were produced *in vitro* and transferred at the blastocyst stage to the uterine lumen, increasing the time in which they were exposed to a stressful environment outside the maternal body. However, our data are consistent with those reported in calves, where animals obtained from embryos produced *in vitro* had a higher birth weight than those conceived through

artificial insemination (Jacobsen et al., 2000; van Wagtendonk-de Leeuw et al., 2000).

Furthermore, in this study we observed different effects of fluid addition depending on sex. In males, differences appeared between C-IVP and the other two groups at the initial time points (days 0-90). By contrast, during the second half of the experiment (from day 90 onwards), differences only appeared between the AI and the two experimental groups. In females the differences between the AI group and the experimental groups occurred at all the time points analysed. These differences agree with the report of Feuer et al. (2014) in mice, where, although exposure of the embryos to a stressful environment caused lower body weights, the effect differed depending on sex. In our study the animals obtained through conventional IVP (C-IVP) had a greater body weight than the AI group, with the RF-IVP group having an intermediate weight between the other two groups. This seems to indicate that the addition of reproductive fluids to IVF and EC media could mitigate the effects of embryonic stress in the case of males, providing the offspring with a phenotype similar to those born through AI, although this mitigation disappeared in the second half of the study (from day 90), where the body weights between groups RF-IVP and C-IVP tend to equalize. However, in the case of females, although group RF-IVP showed intermediate values between group AI and C-IVP, there were differences from birth between group AI and RF-IVP, and there were only differences between group RF-IVP and C-IVP at few specific days, indicating that for this gender, the benefits of adding reproductive fluids are less pronounced.

Regarding the ADWG, in general we did not find differences between the groups, whereas they were found when the data were analysed within each day. At almost every time point, the ADWG was higher in pigs in the C-IVP group for both sexes, followed by group RF-IVP, with group AI being the one that usually gained the least weight.

The quantitative values of ADWG in our study can be compared during the first 75 days of age with those reported in the literature for this breed, since Andersson et al. (1994) reported an average gain of 323 g / day from birth to 30 kg at 90 days of age. This is in accordance to our results in both males and females from the RF-IVP group, since if we perform a mean value for the first 90 days, a mean daily gain of 366.7±196.0 g / day and 341.4±263.1 g/ day,

respectively would be obtained, being instead greater for the C-IVP group (Males: 450.8±270.8; Females: 408.5±241.5) and lesser for the AI group (Males: 261.4±196.0; Females: 240.4±174.6). In addition, our data do coincide with those reported by Whittemore (1996) since, in all cases, the ADWG exceeded 750 g / day from day 120 onwards, when the animals had mostly exceeded 70 kg of weight.

As for the CRL, at most time points for both sexes the C-IVP group had the longest CRL, followed by group RF-IVP and finally AI being the shortest. Human studies have found that during the first two years of age, the type of culture medium used in ART can affect the height of children (Kleijkers et al., 2014). These results coincide with those observed in piglets from C-IVP and RF-IVP groups, where animals were significantly longer than those born through AI. Furthermore, observing both the weight and length data, we found that piglets born through ART show faster growth, coinciding with the report by Ceelen *et al.,* (Ceelen *et al.;*2009). However, although piglets in group RF-IVP also grow faster than those of group AI, they maintain intermediate CRL measurements between the other two groups. However, at the end of the experiment, in both sexes, group RF-IVP outperformed CRL in groups C-IVP and AI. This seems to support the idea that the benefits of adding fluids affect only the first months.

In the anogenital distance results, we observed that the C-IVP group was the one with the highest AGD, followed by group RF-IVP and finally the AI group. Various studies have linked AGD with seminal quality in humans, being directly proportional (Eisenberg *et al.,* 2011; Mendiola *et al.,* 2011). This indicates that the seminal quality of boars might not be affected by the use of IVF, although in order to make this statement it would be necessary to carry out a study relating the seminal quality and the anogenital distance in this species. Furthermore, it has been suggested that testicular volume could be a more reliable indicator of fertility than AGD (Eisenberg and Lipshultz, 2015), so it would be interesting to add this measure in future studies. We have not found in the literature AGD data in pigs that allow us to compare our results with those of other authors.

In summary, the present study offers the first comparative data on the growth of animals produced by means of three assisted reproduction techniques

(artificial insemination, transfer of embryos produced *in vitro* in culture media supplemented with reproductive fluids, and transfer of embryos produced in vitro in media culture supplemented exclusively with BSA), demonstrating that there are differences between the groups for both males and females. Overall, there is a tendency to have a larger size at birth and faster growth in animals from *in vitro* fertilization and embryo culture, although this trend is diminished by the addition of reproductive fluids to the culture media.

4. Piglets from embryos produced in vitro with reproductive fluids show haematological parameters more similar to those born by artificial insemination than piglets from embryos produced in vitro without reproductive fluids.

As described for Experiment 1, there are very few haematological parameters in the literature for growing piglets on which make firm comparisons using a reference interval. However, in a study by Casas-Díaz *et al.,* (Casas-Díaz et al., 2015), reference intervals for wild boar are established for three age ranges: piglets (0-6 month), juveniles (6-12 months), and adults (>12 months). In that study, the majority of the data in piglets was similar to those described by Ventrella et al., (Ventrella et al., 2017) for piglets between 5 and 30 days old.

To compare the haematological parameters obtained with the reference intervals established by Casas-Díaz et al. (Casas-Díaz et al., 2015), we based our comparisons on the body weight of the animals since, according to their study, juvenile wild boars, for example, presented a weight of around 30 kg, which is the approximate weight of our piglets between days 75 and 90 postnatally.

However, although there are not enough data in the bibliography that provide us with information on parameters in growing and adult domestic pigs, more recently, Gu *et al.,*(Gu et al., 2019) provided some parameters for cloned pigs of up to 72 weeks of age, and their results for growing pigs were similar to those obtained in our study.

Analyzing the erythrocyte indices, despite some exceptions, similar patterns were observed throughout the study in both males and females, with the C-IVP group being particularly striking compared with the other two groups. Interestingly, both AI and RF-IVP had a peak in Hb on days 15 and 28 of age,

respectively, and both days also coincided with a drop in the percentage of reticulocytes. By contrast, the C-IVP group presented a first rise in Hb and HTC on day 9 of age, reaching the lowest levels of reticulocytes on day 28 of age. In addition, as previously described for Experiment 1, and according to Ekert *et al.* (2008), an increase in RBC was accompanied by a decrease in reticulocyte parameters. However, a drop in the concentrations of HB, HTC, MCV and MCH was also observed on day 28 for the C-IVP group, which was associated with an increase in RDW and HDW. It should be noted that this finding coincides with the weaning of the piglets, although previously, in all groups 7-8 days before weaning, a solid feed was introduced to the diet in addition to the sow's milk. According to Ventrella *et al.,* (Ventrella et al., 2017), despite the fact that all the parameters were within the established range, this change in diet, along with a maturation of the gastrointestinal system, could explain the differences observed, showing that the AI and RF-IVP groups had a similar pattern of changes.

It is known that Hb is an indicator of iron status (Miller., 1977); therefore, a decrease in blood Hb concentration could be related to an iron deficiency. However, taking into account the age of the piglets, the immaturity of iron metabolism could be another of the possible causes (Ventrella et al., 2017). Furthermore, this phenomenon was accompanied by a decrease in MCV and MCHC, with both parameters being significantly lower in the C-IVP group compared to the AI group.

Similarly, a reduction in RBC, Hb, HTC, MCHC and MCV, as well has an increase in RDW, was also reported by Yeom *et al.* (2012) in miniature young piglets, being compatible with iron deficiency anemia.

However, taking into account that all the parameters were within the established range, according to Ventrella et al. (2017) this finding in the first month of age seems to be a paraphysiological phenomenon.

White blood cell count was also within the normal range, with the variations found for the lymphocytes a possible consequence derived from stress due to manipulation during the blood collection (Dubreuil & Lapierre, 1997; Grindem, 2011).

When platelets were analysed, both PLT and PCT presented greater values in the C-IVP group compared to RF-IVP and AI. However, the only significant differences were found on day 28. An increase in the number of

platelets is related to rapid growth (Stockham SL, 2008). Since those piglets in the C-IVP group showed a higher body weight and ADWG than the RF-IVP and AI groups, this could explain the increase in the platelet count. On the other hand, this finding has also been related to an iron deficiency (Evstatiev et al., 2014), which could also be explained due to the decrease in the Hb concentration. As described in Experiment 1, most of the platelet indices are still under investigation (Kim et al., 2014) and thus a clinical significance cannot be attributed to the differences found.

In addition, all of the parameters were within the normal range, so a definitive clinical diagnosis cannot be established.

5. Glucose tolerance in growing piglets is affected by the embryonic origin and sex.

Changes in glucose metabolism are subtle, yet significant, in ART-derived offspring in several species like the mouse or human (M. Chen et al., 2014; Donjacour et al., 2014; Vrooman & Bartolomei, 2017). Both IVF and EC take place during a critical stage of embryonic development and entail stressful conditions which could modulate embryonic programming leading to impaired glucose tolerance in adult life. Suboptimal *in vitro* culture conditions have revealed a significant impact in glucose metabolism in mice, whereas optimized culture conditions restored normal glucose levels (Donjacour *et al.*, 2014). In our study, oral glucose tolerance was evaluated in piglets from *in vitro*- (C-IVP and RF-IVP) and *in vivo*-(AI)-derived embryos at day 45 of age, as part of the phenotypical characterization of the offspring and allowing us to evaluate the impact of *in vitro* culture using two different media.

Even oral ingestion of glucose required previous training of the piglets, and was used as it triggers a more physiological and greater insulin release because gastric inhibitory polypeptide and glucagon-like peptide-1 (GLP-1) are also released (Manell et al., 2016)

In vivo and *in vitro* groups displayed a similar tendency after glucose intake, even though some differences were observed between the AI and RF-IVP groups during the ascendant phase. Nonetheless, the area under the curve (AUC), which is an index of whole glucose excursion and provides more

information about glucose tolerance than glucose levels at a single time point (Sakaguchi et al., 2016) did not show differences between the groups after 90 min. Two hours is the analysis period in human for glucose intolerance in OGTT, but this period is extended up to 180 or 240 min in pigs (Manell et al., 2016; Pluschke et al., 2016). Our study stopped blood sample collection at 150 min because glycemic values were similar in all groups at this time. In addition, all the basal and peak values were in the physiological range, but we are not able to discount differences among groups which could emerge later into adulthood.

Conclusions

In summary, the research developed in this thesis showed that:

→ The growth as well as the haematological and biochemical profiles of piglets is not affected by ET during the first 15 days of age. Nonetheless, females showed higher weight at birth in ET than in AI, however, those differences disappeared at day 15 of age.

→ Although reproductive yield is not affected by the protein source of the culture media, there is an influence on placental efficiency and some molecular traits in this transitory organ. Amongst the imprinted genes analysed in placenta and umbilical cord, *PEG3* was upregulated in placental tissues from C-IVP embryos vs AI, whilst the RF-IVP group showed intermediate values. It could be a consequence of the culture media used in this group, and the supplementation with RF to IVF and EC media could mitigate this effect.

→ There is an effect derived from the *in vitro* production of embryos on growth and some haematological parameters, which is mitigated by the addition of RF to the culture media. In addition, effects of fluid addition were dependent on sex. The addition of RF to IVF and EC media could partially mitigate the effects of embryonic stress in the case of males, providing the offspring with a phenotype similar to those born through AI, while the benefits of adding RF are less pronounced in female embryos.

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Supplementary files

Table S13. *Comparison of Red Blood Cells parameters at days 3 and 15 in piglets born by ET and AI*

	Day 3		Day 15	
Parameter	ET group (Mean ± SD)	AI group (Mean ± SD)	ET group (Mean ± SD)	AI group (Mean ± SD)
RBC (x10^6 cells/µL)	4.47±0.80	4.22±0.62	5.61±0.82	5.25±0.59
Hb (g/dL)	9.57±1.33	9.24±1.33	12.15±1.45	12.2±1.11
HCT (%)	29.39±5.04	27.58±4.40	36.85±4.58	36.84±2.76
MCV (fL)	65.78±3.18	65.47±4.02	**65.98±3.54[a]**	**70.79±8.43[b]**
MCH (pg)	21.61±2.0	21.92±1.17	21.82±1.82[a]	23.33±1.56[b]
MCHC (g/dL)	32.86±2.67	33.58±2.08	33.06±1.95	33.17±2.51
HDW (g/dL)	**3.17±0.47[a]**	**3.68±0.53[b]**	2.54±0.33	2.45±0.19
CHCM (g/dL)	**30.65±1.07[a]**	**31.49±1.97[b]**	32.04±1.50	32.18±1.83
RDW (%)	20.58±1.83	19.72±1.66	18.66±2.10	18.67±1.80
Reticulocytes (%)	11.97±3.156	11.54±3.88	**9.48±3.07[a]**	**13.64±5.50[b]**
MCVr (fL)	**83.25±5.76[a]**	**87.38±6.72[b]**	**68.58±5.75[a]**	**75.98±8.64[b]**
CHr (pg)	23.72±1.35	24.03±2.45	**20.58±1.73[a]**	**22.61±2.47[b]**

[a-b] Values in the same row with different superscripts are significantly different (p<0.05)

Table S2. *Comparison of White Blood Cells parameters at days 3 and 15 in piglets born by ET and AI (mean ± SD)*

	Day 3		Day 15	
Parameter	ET group (Mean ± SD)	AI group (Mean ± SD)	ET group (Mean ± SD)	AI group (Mean ± SD)
WBC (x10^3 cells/µL)	**9.64±2.66^a**	**7.07±1.61^b**	9.10±2.50	8.07±2.08
Neutrophils (x10^3 cells/µL)	**4.70±1.80^a**	**3.41±1.25^b**	2.73±1.25	2.63±1.16
Lymphocytes (x10^3 cells/µl)	**4.34±1.68^a**	**3.18±1.31^b**	**5.86±1.73^a**	**4.77±1.27^b**
Monocytes (x10^3 cells/µl)	0.27±0.16	0.24±0.10	0.32±0.21	0.33±0.37
Eosinophils (x10^3 cells/µl)	0.08±0.04	0.07±0.04	0.09±0.05	0.09±0.07
Basophils (x10^3 cells/µl)	0.08±0.06	0.06±0.04	0.13±0.09	0.08±0.07

$^{a-b}$ Values in the same row with different superscripts are significantly different (p<0.05)

Table S3. *Comparison of Platelets parameters at days 3 and 15 in piglets born by ET and AI (mean ± SD)*

	Day 3		Day 15	
Parameter	ET group (Mean ± SD)	AI group (Mean ± SD)	ET group (Mean ± SD)	AI group (Mean ± SD)
PCT (%)	0.20±0.11	0.26±0.12	0.20±0.16	0.27±0.18
PLT (x10^3 cells/µl)	**173.0±93.38[a]**	**230.8±116.9[b]**	202.9±175.7	273.8±194.3
MPV (fL)	11.87±2.55	11.44±3.21	**10.84±1.5[a]**	**9.8±1.1[b]**
MPC (g/dL)	22.56±1.70	23.51±2.05	22.12±2.39	22.82±1.8
PCDW (g/dL)	**6.47±0.86[a]**	**5.97±1.0[b]**	6.70±0.81	6.38±0.75
MPM (pg)	1.85±0.25	1.95±0.24	1.75±0.23	1.78±0.2
PMDW (pg)	0.97±0.07	0.94±0.06	0.84±0.10	0.80±0.06
Large_PLT (x10^3 cells/µl)	19.2±12.23	22.94±12.55	14.04±6.30	15.86±7.47

[a-b] Values in the same row with different superscripts are significantly different ($p<0.05$)

Table S4. *Comparison of Biochemical parameters at days 3 and 15 in piglets born by ET and AI (mean ± SD)*

	Day 3		Day 15	
Parameter	ET group (Mean ± SD)	AI group (Mean ± SD)	ET group (Mean ± SD)	AI group (Mean ± SD)
Creatinine (mg/dl)	0.68±0.13	0.695±0.22	**0.92±0.13[a]**	**0.81±0.14[b]**
Urea (mg/dl)	21.01±9.31	21.21±15.89	22.38±8.41	22.05±6.45
Amylase (UI/L)	1505±483.1	1299±333.4	2051±676.1	1761±575.9
CK (UI/L)	661.9±415	602±423	929.6±637.2	788.1±552.5
Cholesterol (mg/dl)	138.9±27.26	123.7±32.92	155.3±43.59	139±41.36
ALP (UI/L)	**3687±985.6[a]**	**3138±744[b]**	**1478±582.5[a]**	**2168±793.6[b]**
GGT (UI/L)	**65.96±46.35[a]**	**41.91±23.75[b]**	**85.54±59.23[a]**	**52.5±46.43[b]**
Glucose (mg/dl)	86.84±24.52	92.51±27.82	117.9±21.68	119.5±23.75
AST (UI/L)	81.27±36.23	78.78±40.85	**157.4±107.3[a]**	**97.7±62.31[b]**
ALT (UI/L)	50.29±11.25	51.93±17.37	48.69±24.27	39.54±14.99
Lipases (UI/L)	150.8±82.97	141±53.55	**29.62±10.09[a]**	**37.37±8.914[b]**
TP (g/dl)	5.664±1.139	5.354±1.04	5.35±0.52	5.07±0.64
ALB (g/dl)	**1.45±0.19[a]**	**1.32±0.26[b]**	2.56±0.33	2.70±0.52
GLOB (g/dl)	4.21±1.05	4.03±0.90	**2.79±0.66[a]**	**2.39±0.51[b]**
TRIGL (mg/dl)	**145.0±88.41[a]**	**184.1±72.09[b]**	102.5±33.46	108.6±41.01
TBIL (mg/dl)	0.37±0.18[a]	**0.508±0.1527[b]**	0.37±0.25	0.41±0.27

[a-b] Values in the same row with different superscripts are significantly different ($p<0.05$)

Table S5. *Comparison of Red blood cells parameters at days 3 and 15 in male piglets born by ET and AI (mean ± SD)*

	Day 3		Day 15	
Parameter	ET group (Mean ± SD)	AI group (Mean ± SD)	ET group (Mean ± SD)	AI group (Mean ± SD)
RBC (x10^6 cells/µL)	4.14±0.7	4.30±0.66	5.60±0.9	5.25±0.6
Hb (g/dL)	9.22±1.4	9.50±1.42	12.37±1.91	12.16±1.01
HCT (%)	27.17±4.84	28.05±5.03	36.61±5.42	36.07±2.8
MCV (fL)	65.43±2.6	65.08±4.04	65.99±3.15	69.33±8.14
MCH (pg)	22.38±2.07	22.1±1.26	22.29±1.31	23.28±1.63
MCHC (g/dL)	34.23±3.19	34.05±2.32	33.77±1.47	33.76±2.2
HDW (g/dL)	3.40±0.5	3.8±0.6	**2.70±0.32[a]**	**2.48±0.21[b]**
CHCM (g/dL)	31.08±1.02	31.73±2.01	32.48±1.11	32.54±1.76
Reticulocytes (%)	11.83±3.13	10.47±3.11	9.90±2.98	12.65±4.36
MCVr (fL)	85.53±3.39	86.51±6.50	**66.21±6.34[a]**	**74.41±8.4[b]**
CHr (pg)	24.2±1.06	23.69±2.34	**20.08±1.54[a]**	**22.13±2.31[b]**

[a-b] Values in the same row with different superscripts are significantly different (p<0.05)

Table S6. *Comparison of White blood cells parameters at days 3 and 15 in male piglets born by ET and AI (mean ± SD)*

	Day 3		Day 15	
Parameter	ET group (Mean ± SD)	AI group (Mean ± SD)	ET group (Mean ± SD)	AI group (Mean ± SD)
WBC (x10^3 cells/µL)	**9.05±2.04^a**	**6.77±1.52^b**	8.74±3.05	8.35±2.05
Neutrophils (x10^3 cells/µL)	**4.10±0.97^a**	**3.17±0.90^b**	2.90±1.60	2.66±1.10
Lymphocytes (x10^3 cells/µl)	3.86±1.60	3.13±1.23	5.46±2.13	5.0±1.30
Monocytes (x10^3 cells/µl)	0.20±0.07	0.26±0.10	0.28±0.17	0.25±0.14
Eosinophils (x10^3 cells/µl)	0.08±0.04	0.08±0.05	0.10±0.04	0.08±0.04
Basophils (x10^3 cells/µl)	0.03±0.02	0.05±0.02	0.10±0.05	0.08±0.04

$^{a-b}$ Values in the same row with different superscripts are significantly different (p<0.05)

Table S7. *Comparison of Platelets parameters at days 3 and 15 in male piglets born by ET and AI (mean ± SD)*

	Day 3		Day 15	
Parameter	ET group (Mean ± SD)	AI group (Mean ± SD)	ET group (Mean ± SD)	AI group (Mean ± SD)
PCT (%)	**0.15±0.10[a]**	**0.26±0.14[b]**	0.15±0.04	0.17±0.0
PLT (x10^3 cells/µl)	154.80±107.70	218.20±130.0	125.80±50.24	154.40±90.18
MPV (fL)	11.62±1.55	9.70±1.23	**22.49±1.40[a]**	**23.61±1.80[b]**
MPC (g/dL)	22.49±1.40	23.61±1.8	21.93±2.13	22.47±2.02
PCDW (g/dL)	6.70±0.94	6.07±1.10	6.98±0.84	6.55±0.83
MPM (pg)	**1.80±0.25[a]**	**1.95±0.22[b]**	1.73±0.25	1.72±0.20
PMDW (pg)	1.0±0.06	0.94±0.10	**1.0±0.10[a]**	**0.80±0.07[b]**
Large_PLT (x10^3 cells/µl)	14.6±8.10	21.85±10.41	14.4±5.0	14.79±7.40

[a-b] Values in the same row with different superscripts are significantly different ($p<0.05$)

Table S8. *Comparison of Red blood cells parameters at days 3 and 15 in male piglets born by ET and AI*

	Day 3		Day 15	
Parameter	ET group (Mean ± SD)	AI group (Mean ± SD)	ET group (Mean ± SD)	AI group (Mean ± SD)
Creatinine (mg/dl)	0.70±0.16	0.71±0.26	0.96±0.14	0.82±0.15
Urea (mg/dl)	19.63±7.29	17.33±7.60	24.89±7.40	20.28±6.12
Amylase (UI/L)	1461.0±501.0	1305.0±346.70	2156.0±795.40	1806.0±572.90
CK (UI/L)	572.80±323.0	580.1±392.8	827.9±714	896±591.8
Cholesterol (mg/dl)	132.60±28.49	119.70±33.25	151.30±41.81	133.10±44.34
ALP (UI/L)	**4118.0±1287.0[a]**	**3140.0±778.80[b]**	1761.0±306.0	2155.0±708.50
GGT (UI/L)	38.87.0±14.05	34.51±12.89	**77.85±23.47[a]**	**24.36±8.23[b]**
Glucose (mg/dl)	94.15±30.12	96.63±28.92	115.70±21.45	119.40±21.40
AST (UI/L)	80.85±44.37	78.23±48.33	**193.90±111.0[a]**	**109.80±71.18[b]**
ALT (UI/L)	48.91±7.45	48.58±13.71	48.30±20.06	40.34±12.28
Lipases (UI/L)	163.10±75.72	146.70±58.71	**30.60±10.88[a]**	**41.28±11.56[b]**
TP (g/dl)	5.08±1.20	5.0±0.98	5.30±0.53	4.85±0.60
ALB (g/dl)	**1.41±0.22[a]**	**1.30±0.26[b]**	2.70±0.25	2.67±0.60
GLOB (g/dl)	3.67±1.14	3.74±0.82	2.59±0.72	2.18±0.38
TRIGL (mg/dl)	176.50±94.86	189.20±73.70	111.20±42.84	104.40±44.04
TBIL (mg/dl)	0.42±0.17	0.50±0.11	0.60±0.28	0.37±0.19

[a-b] Values in the same row with different superscripts are significantly different ($p<0.05$)

(mean ± SD)

Table S9. *Comparison of Red blood cells parameters at days 3 and 15 in female piglets born by ET and AI (mean ± SD)*

	Day 3		Day 15	
Parameter	ET group (Mean ± SD)	AI group (Mean ± SD)	ET group (Mean ± SD)	AI group (Mean ± SD)
RBC (x10^6 cells/µL)	**4.70±0.81^a**	**4.04±0.50^b**	5.63±0.81	5.30±0.67
Hb (g/dL)	**9.81±1.27^a**	**8.70±0.98^b**	12.03±1.2	12.30±1.36
HCT (%)	**30.88±4.71^a**	**26.65±2.64^b**	36.99±4.21	38.54±1.92
MCV (fL)	66.01±3.60	66.25±4.05	**65.98±3.82^a**	**74.04±8.62^b**
MCH (pg)	21.09±1.78	21.56±0.92	**21.56±2.04^a**	**23.44±1.46^b**
MCHC (g/dL)	31.94±1.84	32.63±1.02	32.66±2.11	31.87±2.84
HDW (g/dL)	**3.03±0.42^a**	**3.52±0.45^b**	2.46±0.32	2.40±0.08
CHCM (g/dL)	30.36±1.03	31.01±1.90	31.80±1.61	31.37±1.81
Reticulocytes (%)	12.06±3.26	13.66±4.53	**9.66±2.80^a**	**14.23±5.80^b**
MCVr (fL)	**81.73±6.56^a**	**89.12±7.12^b**	**69.90±5.11^a**	**79.48±8.70^b**
CHr (pg)	23.41±1.50	24.72±2.54	**20.85±1.81^a**	**23.69±2.60^b**

$^{a-b}$ Values in the same row with different superscripts are significantly different (p<0.05)

Table S10. *Comparison of White blood cells parameters at days 3 and 15 in female piglets born by ET and AI (mean ± SD)*

	Day 3		Day 15	
Parameter	ET group (Mean ± SD)	AI group (Mean ± SD)	ET group (Mean ± SD)	AI group (Mean ± SD)
WBC (x10^3 cells/µL)	**9.63±2.51^a**	**7.70±1.70^b**	**10.21±3.40^a**	**7.44±2.12^b**
Neutrophils (x10^3 cells/µL)	5.06±2.10	4.23±1.40	2.65±1.06	2.54±1.41
Lymphocytes (x10^3 cells/µl)	**4.36±1.35^a**	**3.30±1.51^b**	**6.10±1.50^a**	**4.25±1.11^b**
Monocytes (x10^3 cells/µl)	0.27±0.14	0.25±0.10	0.27±0.13	0.28±0.12
Eosinophils (x10^3 cells/µl)	0.10±0.03	0.06±0.03	0.08±0.04	0.10±0.04
Basophils (x10^3 cells/µl)	0.07±0.03	0.06±0.03	0.10±0.042	0.06±0.02

$^{a-b}$ Values in the same row with different superscripts are significantly different (p<0.05)

Table S11. *Comparison of Platelets parameters at days 3 and 15 in female piglets born by ET and AI (mean ± SD)*

	Day 3		Day 15	
Parameter	ET group (Mean ± SD)	AI group (Mean ± SD)	ET group (Mean ± SD)	AI group (Mean ± SD)
PCT (%)	0.24±0.09	0.28±0.08	**0.22±0.13[a]**	**0.36±0.15[b]**
PLT (x10^3 cells/µl)	191.90±80.77	256.0±84.99	351.50±199.60	388.90±138.10
MPV (fL)	12.24±3.05	10.97±1.21	10.41±1.30	10.07±0.65
MPC (g/dL)	22.61±1.92	23.30±2.56	22.23±2.60	23.60±0.65
PCDW (g/dL)	6.33±0.80	5.77±0.90	6.50±0.76	6.0±0.3
MPM (pg)	1.91±0.24	2.0±0.30	1.80±0.22	1.92±0.10
PMDW (pg)	1.0±0.07	0.93±0.06	0.82±0.09	0.80±0.06
Large_PLT (x10^3 cells/µl)	21.0±9.40	23.45±10.1	**11.88±4.42[a]**	**19.78±5.10[b]**

[a-b] Values in the same row with different superscripts are significantly different ($p<0.05$)

Table S12. *Comparison of Biochemical parameters at days 3 and 15 in female piglets born by ET and AI (mean ± SD)*

	Day 3		Day 15	
Parameter	ET group (Mean ± SD)	AI group (Mean ± SD)	ET group (Mean ± SD)	AI group (Mean ± SD)
Creatinine (mg/dl)	0.70±0.11	0.70±0.07	0.90±0.13	0.80±0.12
Urea (mg/dl)	20.15±7.30	23.61±10.64	**19.42±5.22[a]**	**25.78±5.80[b]**
Amylase (UI/L)	1533.0±482.10	1286.0±319.60	2070.0±564.90	1780.0±534.30
CK (UI/L)	642.40±385.90	654.20±394.10	980.40±622.30	596.30±440.90
Cholesterol (mg/dl)	142.70±26.43	133.30±32.13	157.80±45.83	142.6.0±43.19
ALP (UI/L)	3564.0±934.0	3132.0±699.20	1500.0±591.30	1963.0±688.70
GGT (UI/L)	70.24±47.36	59.49±34.15	49.78±21.42	36.20±11.45
Glucose (mg/dl)	82.30±19.77	82.23±23.38	119.10±22.36	119.60±29.54
AST (UI/L)	81.53±31.68	80.09±13.72	102.10±43.58	72.12±24.89
ALT (UI/L)	51.02±12.91	52.63±12.97	44.92±21.38	32.07±5.21
Lipases (UI/L)	120.20±49.05	126.6±37.06	30.23±8.90	35.43±10.55
TP (g/dl)	6.024±0.96	6.23±0.56	5.385±0.53	5.53±0.50
ALB (g/dl)	1.47±0.18	1.47±0.20	2.48±0.36	2.70±0.38
GLOB (g/dl)	4.55±0.86	4.76±0.58	2.90±0.62	2.80±0.47
TRIGL (mg/dl)	126.80±62.94	172.10±71.39	97.43±26.70	117.50±34.35
TBIL (mg/dl)	**0.35±0.18[a]**	**0.55±0.22[b]**	0.34±0.17	0.50±0.22

[a-b] Values in the same row with different superscripts are significantly different ($p<0.05$)

Table S13. *Average weight of males per group and day (g ± SD)*

Day	RF-IVP	C-IVP	AI
3	1,846.00±437.98[ab]	2,256.36±377.34[b]	1,410.00±380.22[a]
9	3,022.00±801.75[a]	3,890.91±674.11[b]	2,276.11±609.97[a]
15	3,895.00±833.72[a]	6,299.09±1,443.98[b]	3,291.18±791.52[a]
30	6,557.00±658.82[c]	9,710.91±2,246.85[b]	4,870.59±983.39[a]
45	9,580.00±947.28[a]	13,072.73±2,723.27[b]	7,818.75±946.02[a]
60	18,710.60±1,633.15[c]	21,809.09±4,384.62[b]	12,993.75±714.11[a]
75	28,990.00±4,091.31[c]	34,560.00±6.721.64[b]	20,976.92±3,003.10[a]
90	40,270.00±4,461.20[b]	47,800.00±9,171.82[b]	30,837.50±5,635.23[a]
105	55,450.00±7,963.28[b]	64,950.00±11,712.88[b]	41,366.67±6,528.69[a]
120	69,550.00±9,458.83[b]	77,000.00±13,007.21[b]	55,038.46±9,319.47[a]
135	84,050.00±10,912.40[b]	95,111.11±13,651.41[b]	65,000.00±12,338.96[a]
150	101,150.00±11,148.12[b]	113,777.78±17,614.11[b]	80,733.33±13,652.40[a]
165	123,937.50±12,289.48[b]	125,812.50±18,564.44[b]	96,250.00±13,768.34[a]
180	132,444.44±17,369.95[b]	142,125.00±21,692.25[b]	110,466.67±14,404.20[a]

[a-c] Values in the same row with different superscripts are significantly different ($p<0.05$)

Table S14. *Average daily weight gain of males per group and day (g ± SD)*

Day	RF-IVP	C-IVP	AI
3	112.87±65.62[a]	209.70±47.92[b]	75.83±42.42[a]
9	196.67±78.96[a]	287.27±62.97[b]	127.41±60.37[a]
15	148.88±112.90[a]	382.94±138.43[b]	139.50±53.80[a]
30	201.14±47.59[ab]	253.97±134.38[b]	135.66±41.12[a]
45	184.10±29.35	193.84±100.81	166.07±44.15
60	575.25±79.14[b]	614.68±182.79[b]	340.70±46.02[a]
75	733.97±188.62	802.90±239.06	538.35±185.96
90	780.33±138.02	860.76±213.81	567.54±186.77
105	944.96±258.03[ab]	1,077.05±238.44[b]	712.93±380.40[a]
120	963.50±288.19	792.22±285.41	883.13±342.10
135	1,001.19±312.23[ab]	1,348.77±287.27[b]	746.79±443.69[a]
150	1,123.81±313.37	1,244.44±357.85	1,043.14±269.00
165	1,195.61±735.41	827.08±724.95	944.39±471.61
180	1,161.04±506.85	1,122.09±373.93	985.71±254.77

[a-c] Values in the same row with different superscripts are significantly different ($p<0.05$)

Table S15. *Average CRL in males per group and day (cm ± SD)*

Day	RF-IVP	C-IVP	AI
0	31.64±3.23	32.23±2.58	29.58±2.17
3	34.60±3.43^{ab}	34.95±2.55^{b}	31.12±2.83^{a}
9	40.13±4.12^{b}	41.00±2.83^{b}	35.54±3.06^{a}
15	42.20 ±3.28^{a}	47.64±3.87^{b}	40.03±3.07^{a}
30	51.40±3.01^{a}	60.14±7.06^{b}	46.32±3.97^{a}
45	59.70±2.41^{c}	66.27±5.04^{b}	54.66±2.28^{a}
60	69.40±4.22^{b}	75.14±6.18^{b}	59.41±3.24^{a}
75	80.15 ±3.86^{c}	87.35±6.30^{b}	70.55±3.97^{a}
90	86.00±5.09^{ab}	92.90±7.52^{b}	79.91±6.80^{a}
105	102.00±4.55^{b}	103.85±7.73^{b}	90.47±4.54^{a}
120	109.80±6.30^{b}	116.83±6.85^{b}	100.15±6.84^{a}
135	117.60±9.68^{b}	119.22±8.66^{b}	106.42±7.23^{a}
150	124.40±4.14^{b}	129.78±5.91^{b}	116.00±7.59^{a}
165	135.80±6.70^{b}	135.81±7.75^{b}	122.04±8.00^{a}
180	143.50±8.54^{b}	141.50±8.93^{b}	130.63±7.90^{a}

$^{a\text{-}c}$ Values in the same row with different superscripts are significantly different ($p<0.05$)

Table S16. *AGD of males per group and day (cm ± SD)*

Day	RF-IVP	C-IVP	AI
0	1.52±0.55	1.95±0.61	2.00±0.51
3	2.04±0.36	2.00±0.71	1.95±0.34
9	2.45±0.42^{ab}	2.75±0.48^{b}	2.18±0.48^{a}
15	2.75±0.83^{ab}	3.48±0.71^{b}	2.44±0.69^{a}
30	3.92±0.51^{b}	4.39±1.08^{b}	2.99±0.58^{a}
45	3.93±0.57^{ab}	4.75±0.83^{b}	3.65±0.47^{a}
60	4.30±0.82^{a}	5.95±1.77^{b}	3.56±0.83^{a}
75	6.10±0.88	6.85±1.47	5.87±1.53
90	6.65±1.84^{ab}	7.42±2.04^{b}	5.67±1.19^{a}
105	8.97±1.31^{b}	9.35±2.01^{b}	6.73±1.62^{a}
120	8.12±1.50^{ab}	9.50±1.37^{b}	7.38±2.06^{a}
135	9.16±2.27	8.83±2.49	8.05±1.25
150	9.43±1.88^{ab}	11.44±1.99^{b}	8.53±1.01^{a}
165	11.53±2.46^{b}	11.19±2.14^{b}	9.43±1.28^{a}
180	11.61±2.07	11.00±2.67	9.47±2.07

$^{a\text{-}c}$ Values in the same row with different superscripts are significantly different ($p<0.05$)

Table S17. *Average weight of females per group and day (g ± SD)*

Día	RF-IVP	C-IVP	AI
0	1,345.00±399.25[b]	1,461.11±370.59[b]	1,153.44±275.61[a]
3	1,872.50±532.27[b]	1,991.76±518.46[b]	1,298.26±318.57[a]
9	3,134.29±667.90[b]	3,416.47±914.80[b]	2,114.78±617.60[a]
15	3,548.57±890.76[ab]	5,292.94±1,421.07[b]	3,040.87±844.22[a]
30	6,754.29±977.73[c]	8,991.25±1,952.04[b]	4,615.22±988.18[a]
45	9,614.29±1,479.22[c]	12,431.25±2,623.92[b]	7,247.83±1,393.45[a]
60	17,557.14±2,560.51[b]	20,637.50±3,610.70[b]	12,265.22±2,254.81[a]
75	26,657.14±2,986.59[b]	32,237.50±7,230.66[b]	19,459.09±2,985.47[a]
90	37,600.00±4,572.38[b]	44,000.00±8,047.77[b]	27,442.86±5,789.61[a]
105	51,285.71±6,607.18[b]	57,968.75±11,121.25[b]	37,750.500±6,510.11[a]
120	64,214.29±5,081.48[b]	73,531.25±12,292.91[b]	49,583.33±8,262.69[a]
135	76,000.00±7,670.29[c]	89,031.25±12,546.54[b]	61,075.00±10,367.30[a]
150	96,642.86±6,342.41[b]	105,300.00±8,837.42[b]	75,394.74±1,.254.51[a]
165	113,071.43±16,621.84[b]	116,733.33±16,937.77[b]	88,947.37±9,655.01[a]
180	125,057.14±10,697.64[b]	124,937.50±17,224.86[b]	101.263.16±10,227.10[a]

[a-c] Values in the same row with different superscripts are significantly different ($p<0.05$)

Table S18. *Average daily weight gain of females per group and day (g ± SD)*

Day	RF-IVP	C-IVP	AI
3	139.25±67.41[b]	189.41±76.63[b]	52.90±47.66[a]
9	178.37±87.08[a]	241.76±73.62[b]	130.75±56.26[a]
15	66.36±93.93[a]	306.74±99.11[b]	132.30±49.47[a]
30	243.25±76.77[b]	261.88±88.75[b]	137.72±49.29[a]
45	172.97±42.93a[b]	205.02±75.24[b]	156.47±47.95[a]
60	515.42±135.91[b]	558.04±120.62[b]	323.54±91.84[a]
75	658.16±96.40a[b]	762.60±249.00[b]	479.37±96.29[a]
90	757.07±155.80[ab]	742.33±255.50[b]	510.43±305.96[a]
105	843.56±182.13	933.09±277.56	765.91±258.05
120	850.06±318.98	970.15±311.40	767.07±202.80
135	801.02±480.97	1085.43±331.92	729.40±203.57
150	1,353.22±574.15	928.89±231.58	874.72±322.29
165	900.03±1207.66	904.76±603.79	906.39±375.76
180	585.40±736.49	608.28±455.16	821.05±269.94

[a-c] Values in the same row with different superscripts are significantly different ($p<0.05$)

Table S19. *Average CRL of females per group and day (cm ± SD)*

Day	RF-IVP	C-IVP	AI
0	30.44±2.98^{ab}	32.22±2.66^{b}	29.73±2.40^{a}
3	36.00±4.92^{c}	34.21±2.61^{b}	31.11±2.65^{a}
9	41.07±2.15^{b}	40.38±3.81^{b}	34.92±3.05^{a}
15	42.28±2.91^{c}	45.44± 4.40^{b}	38.75±3.44^{a}
30	52.10±4.03^{c}	57.41±5.02^{b}	45.37±4.07^{a}
45	60.79±3.78^{b}	65.22±4.66^{b}	53.59±3.66^{a}
60	68.21±5.51^{b}	72.19±6.02^{b}	59.30±4.76^{a}
75	77.29±3.04^{b}	81.28±6.89^{b}	70.75±4.92^{a}
90	88.29±4.34^{b}	89.22±6.42^{b}	75.90±6.35^{a}
105	99.36±3.34^{b}	101.16±7.85^{b}	87.67±4.93^{a}
120	104.31±8.57^{ab}	108.31±8.53^{b}	96.64±5.71^{a}
135	112.29±4.15^{b}	116.91±8.27^{b}	104.58±5.08^{a}
150	121.50±6.76^{b}	127.13±5.60^{b}	114.26±5.50^{a}
165	129.36±2.93^{b}	127.47±9.50^{b}	122.32±4.90^{a}
180	135.29±6.32^{b}	134.78±8.62^{b}	125.68±4.96^{a}

$^{a-c}$ Values in the same row with different superscripts are significantly different ($p<0.05$)

Table S20. *Comparison of Red Blood Cells parameters in piglets per group and day (mean ± SD)*

	Day 3			Day 9			Day 15		
Parameters	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
RBC (x10^6 cells/µL)	**4.05±0.53^{a}**	**4.53±0.62^{ab}**	**4.53±0.72^{b}**	4.92±0.69	5.03±0.48	5.28±0.59	**6.10±0.58^{a}**	**5.73±0.35^{ab}**	**5.70±0.45^{b}**
Hb (g/dL)	**8.07±0.88^{a}**	**8.95±0.95^{b}**	**8.95±1.37^{b}**	9.76±1.06	10.48±0.75	10.42±1.04	**12.08±0.71^{a}**	**11.29±0.89^{b}**	**11.07±0.89^{b}**
HCT (%)	26.25±3.41	28.8±3.4	28.31±4.16	32.76±3.04	34.76±2.42	34.81±3.63	**38.52±1.98^{a}**	**37.0±3.09^{ab}**	**35.77±3.27^{b}**
MCV (fL)	**64.81±3.4^{a}**	**63.82±2.94^{ab}**	**62.71±2.09^{b}**	**67.19±6.32^{ab}**	**69.33±4.75^{a}**	**66.09±3.63^{b}**	63.58±5.55	64.61±5.02	63.04±3.73
MCH (pg)	19.99±1.05	19.85±1.06	19.78±1.06	**19.96±1.43^{a}**	**20.89±1.23^{b}**	**19.77±1.0^{a}**	19.91±1.51	19.71±1.37	19.52±1.09
MCHC (g/dL)	30.87±1.49	31.11±0.77	31.55±1.53	29.76±0.92	30.13±0.61	29.93±0.76	**31.36±0.55^{a}**	**30.52±0.65^{b}**	**30.98±0.92^{a}**
HDW (g/dL)	**3.40±0.37^{a}**	**3.20±0.20^{ab}**	**3.2±0.24^{b}**	**2.51±0.34^{a}**	**2.40±0.16^{a}**	**2.21±0.18^{b}**	**2.24±0.17^{ab}**	**2.37±0.28^{a}**	**2.22±0.24^{b}**
CHCM (g/dL)	31.6±1.35	31.48±0.91	31.76±0.78	**30.79±1.1^{ab}**	**31.02±0.62^{a}**	**30.35±1.05^{b}**	31.92±0.60	31.4±0.75	31.77±1.30
RDW (%)	**21.87±3.25^{a}**	**19.5±1.95^{b}**	**18.04±1.16^{c}**	**18.89±2.07^{ab}**	**19.80±1.76^{a}**	**17.92±1.06^{b}**	**16.83±1.62^{a}**	**18.0±1.83^{b}**	**16.73±0.92^{a}**
Reticulocytes (%)	**12.95±4.09^{a}**	**10.93±3.35^{a}**	**6.44±1.93^{b}**	**9.14±3.46^{a}**	**13.52±3.53^{b}**	**8.84±3.35^{a}**	**3.12±1.17^{a}**	**8.44±2.45^{b}**	**5.97±2.45^{c}**
MCVr (fL)	**84.87±8.52^{a}**	**79.19±5.53^{b}**	**72.6±6.81^{c}**	**74.46±7.05$_{ab}$**	**74.90±5.86^{a}**	**69.10±7.29^{b}**	66.99±5.78	64.26±6.64	68.12±6.01
CHr (pg)	**24.16±2.48^{a}**	**22.76±1.44^{ab}**	**21.58±1.95^{b}**	**21.23±0.98^{a}**	**22.87±1.66^{b}**	**21.14±1.92^{a}**	**20.2±1.33^{ab}**	**19.08±2.34^{a}**	**20.91±1.60^{b}**

$^{a-c}$ Values in the same row with different superscripts are significantly different (p <0.05)

	Day 28			Day 60			Day 90		
Parameters	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
RBC (x10^6 cells/µL)	**6.34±0.44^{a}**	**6.94±0.57^{b}**	**6.34±0.51^{a}**	**6.33±0.49^{a}**	**6.93±0.66^{b}**	**6.25±0.51^{a}**	**6.85±0.40^{a}**	**7.43±0.61^{b}**	**6.71±0.53^{a}**
Hb (g/dL)	**11.3±0.63^{ab}**	**10.59±1.33^{a}**	**11.73±1.22^{b}**	10.40±0.98	10.41±0.77	10.06±0.90	10.34±0.62	10.27±1.14	9.86±0.90
HCT (%)	**35.69±2.11 ab**	**34.3±3.73^{a}**	**36.82±3.80^{b}**	33.71±3.55	33.81±2.90	32.58±2.86	33.35±1.6	33.2±3.40	32.11±2.52
MCV (fL)	**56.34±2.19^{a}**	**49.69±6.04^{b}**	**57.97±2.71^{a}**	**53.18±3.0^{a}**	**48.97±3.56^{b}**	**52.09±2.10^{a}**	**48.82±3.17^{a}**	**45.72±3.70^{b}**	**47.91±2.60^{ab}**
MCH (pg)	**17.85±0.80^{a}**	**15.36±2.25^{b}**	**18.47±0.97^{a}**	**16.43±0.63^{a}**	**15.08±1.20^{b}**	**16.1±0.56^{a}**	**15.16±1.17^{a}**	**14.15±1.34^{b}**	**14.71±1.12^{ab}**
MCHC (g/dL)	**31.69±0.80^{a}**	**30.84±0.95^{b}**	**31.86±0.80^{a}**	30.92±0.91	30.8±0.72	30.93±0.88	31.02±0.78	30.94±0.85	30.73±1.81
HDW (g/dL)	**2.39±0.31^{a}**	**2.78±0.46^{a}**	**2.06±0.26^{b}**	**2.20±0.27^{ab}**	**2.30±0.17^{a}**	**2.16±0.18^{b}**	2.17±0.16	2.21±0.14	2.17±0.13
CHCM (g/dL)	**32.94±0.96^{a}**	**31.67±0.99^{b}**	**32.99±1.15^{a}**	31.85±1.07	31.71±0.82	32.02±1.03	32.28±0.95	32.27±0.81	32.2±0.86
RDW (%)	**17.25±2.62^{a}**	**23.02±5.73^{a}**	**15.48±1.0^{b}**	**16.53±1.15^{a}**	**19.23±2.39^{b}**	**16.26±0.82^{a}**	**16.36±1.25^{a}**	**17.76±1.13^{b}**	**16.65±1.24^{a}**
Reticulocytes (%)	3.92±1.63	3.36±1.44	3.08±1.95	3.51±1.37	3.70±1.03	3.0±1.26	2.01±0.49	1.87±0.76	2.08±0.67
MCVr (fL)	**60.32±4.13^{a}**	**51.37±6.75^{b}**	**61.87±4.31^{a}**	59.07±4.13	57.35±6.37	59.1±2.45	59.35±5.21	58.32±4.20	58.7±4.26
CHr (pg)	**18.06±1.25^{a}**	**15.1±2.08^{b}**	**19.2±1.23^{c}**	**17.76±1.25^{a}**	**16.63±1.92^{b}**	**17.71±0.72^{a}**	17.36±1.66	16.73±1.40	16.95±1.33

$^{a-c}$ Values in the same row with different superscripts are significantly different (p <0.05)

	Day 120			Day 180		
Parameters	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
RBC (x10^6 cells/µL)	6.96±0.85	7.57±0.61	7.37±0.34	7.72±0.63	7.93±0.80	7.44±0.55
Hb (g/dL)	10.6±1.29	10.91±0.72	10.7±0.96	12.46±0.90	12.5±1.03	11.8±1.19
HCT (%)	32.56±4.52	33.64±2.29	33.66±2.25	38.02±2.67	38.49±3.40	36.93±3.60
MCV (fL)	46.76±2.65	44.53±2.62	45.68±2.68	49.34±2.99	48.7±3.40	49.53±2.39
MCH (pg)	15.26±0.74	14.47±1.11	14.54±14.54	16.19±1.08	15.81±1.15	15.85±0.84
MCHC (g/dL)	32.64±0.928	32.46±0.75	31.79±0.84	**32.79±0.54^{a}**	**32.47±0.53^{ab}**	**31.97±0.32^{b}**
HDW (g/dL)	2.36±0.20	2.26±0.12	2.21±0.18	2.07±0.10	2.11±0.11	2.08±0.13
CHCM (g/dL)	33.73±0.59	33.53±0.77	33.13±0.90	33.46±0.55	33.33±0.60	32.91±0.57
RDW (%)	**16.85±1.45^{a}**	**18.53±1.24^{b}**	**17.43±1.21^{ab}**	17.82±1.41	19.99±1.95	19.38±1.74
Reticulocytes (%)	**1.28±0.29^{a}**	**0.78±0.32^{b}**	**1.02±0.32^{ab}**	0.60±0.20	0.77±0.25	0.79±0.33
MCVr (fL)	60.28±3.76	57.84±2.51	60.66±5.56	63.68±3.17	65.57±3.07	65.25±4.65
CHr (pg)	17.82±1.47	17.02±0.94	17.61±1.50	19.19±1.11	19.59±0.82	19.26±1.34

[a-c] Values in the same row with different superscripts are significantly different (p <0.05)

Table S21. *Comparison of White Blood Cells parameters in piglets per group and day (mean ± SD)*

	Day 3			**Day 9**			**Day 15**		
Parameter	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
WBC (x10^3 cells/μL)	7.0±2.92	6.69±2.25	5.20±2.27	9.10±2.81	10.01±2.36	9.10±2.70	8.72±2.32	7.80±2.0	8.56±2.82
Neutrophils (x10^3 cells/μL)	3.74±1.93	4.17±2.04	3.11±1.97	3.64±1.77	4.78±1.49	4.33±2.05	3.24±1.08	2.75±0.96	2.82±1.26
Lymphocytes (x10^3 cells/μl)	**2.70±0.93^{a}**	**2.35±1.07^{ab}**	**1.71±0.55^{b}**	4.35±1.34	4.56±1.05	4.03±1.15	5.04±1.76	4.33±1.36	4.78±1.82
Monocytes (x10^3 cells/μl)	**0.19±0.1^{ab}**	**0.26±0.14^{a}**	**0.16±0.08^{b}**	**0.30±0.17^{ab}**	**0.40±0.15^{a}**	**0.25±0.15^{b}**	0.22±0.15	0.26±0.13	0.32±0.22
Eosinophils (x10^3 cells/μl)	0.07±0.04	0.08±0.04	0.06±0.03	0.11±0.04	0.13±0.04	0.13±0.05	0.15±0.06	0.12±0.05	0.14±0.07
Basophils (x10^3 cells/μl)	0.05±0.04	0.04±0.04	0.04±0.03	0.06±0.03	0.08±0.03	0.06±0.03	0.06±0.02	0.04±0.02	0.06±0.02

[a-c] Values in the same row with different superscripts are significantly different (p <0.05)

	Day 28			Day 60			Day 90		
Parameter	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
WBC (x10^3 cells/µL)	9.98±2.04	9.77±1.89	11.14±3.27	15.40±3.70	15.89±5.49	14.03±4.08	16.09±3.42	16.0±4.26	15.86±3.34
Neutrophils (x10^3 cells/µL)	**3.56±1.12^{ab}**	**2.58±1.1^{a}**	**4.49±1.95^{b}**	4.33±1.68	3.31±1.15	4.11±1.81	5.82±2.25	5.10±1.56	6.25±2.84
Lymphocytes (x10^3 cells/µl)	**5.60±1.42^{ab}**	**6.40±1.60^{a}**	**5.23±2.22^{b}**	9.60±2.20	9.52±2.70	8.26±2.40	8.55±2.04	8.49±1.87	8.33±2.42
Monocytes (x10^3 cells/µl)	0.34±0.14	0.34±0.13	0.36±0.16	0.91±0.38	0.97±0.50	0.86±0.44	1.01±0.25	0.85±0.26	0.88±0.21
Eosinophils (x10^3 cells/µl)	0.18±0.07	0.16±0.07	0.16±0.06	0.18±0.07	0.19±0.083	0.16±0.05	**0.46±0.10^{b}**	**0.28±0.09^{a}**	**0.24±0.10^{a}**
Basophils (x10^3 cells/µl)	0.06±0.029	0.07±0.04	0.07±0.03	0.09±0.04	0.11±0.04	0.09±0,04	0.09±0.04	0.05±0.01	0.1±0.06

[a-c] Values in the same row with different superscripts are significantly different ($p < 0.05$)

	Day 120			Day 180		
Parameter	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
WBC (x10^3 cells/µL)	15.62±4.56	18.84±5.05	17.38±3.25	19.06±3.92	15.42±3.77	17.44±4.18
Neutrophils (x10^3 cells/µL)	4.05±1.65	5.87±1.81	5.47±0.88	6.20±2.21	3.36±1.83	4.73±2.45
Lymphocytes (x10^3 cells/µl)	9.88±2.85	9.66±2.23	10.26±2.53	10.47±2.30	10.31±3.06	10.71±2.12
Monocytes (x10^3 cells/µl)	0.92±0.40	0.84±0.21	0.95±0.38	0.80±0.29	0.78±0.35	0.67±0.24
Eosinophils (x10^3 cells/µl)	0.51±0.29	0.49±0.19	0.39±0.16	**1.05±0.28^{a}**	**0.38±0.23^{b}**	**0.80±0.38^{ab}**
Basophils (x10^3 cells/µl)	0.08±0.05	0.08±0.04	0.05±0.02	0.12±0.03	0.13±0.09	0.13±0.08

[a-c] Values in the same row with different superscripts are significantly different (p <0.05)

Table S22. *Comparison of Platelet parameters in piglets per group and day (mean ± SD)*

	Day 3			**Day 9**			**Day 15**		
Parameter	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
PCT (%)	**0.29±0.18[a]**	**0.27±0.13[a]**	**0.20±0.10[b]**	0.42±0.24	0.51±0.23	0.40±0.21	**0.27±0.14[a]**	**0.46±0.12[b]**	**0.30±0.12[a]**
PLT (x10^3 cells/µl)	**326.9±133.2[a]**	**243.5±119.8[a]**	**159.8±84.77[b]**	468.3±184.2	497.2±206.4	398.8±161.3	**275.7±137.8[a]**	**440.9±125.9[b]**	**310.8±143.3[a]**
MPV (fL)	**10.63±2.04[ab]**	**11.74±1.60[a]**	**10.25±1.91[b]**	9.81±1.40	9.87±0.69	9.93±0.84	9.44±1.0	10.11±1.0	9.86±0.92
MPC (g/dL)	22.26±1.70	23.4±1.27	22.55±2.17	21.88±1.83	22.18±1.10	22.08±1.69	23.05±2.36	22.04±0.68	21.98±1.66
PCDW (g/dL)	6.40±0.65	6.28±0.59	6.46±0.78	6.36±0.67	6.61±0.31	6.35±0.59	6.54±0.67	6.65±0.37	6.55±0.45
MPM (pg)	**1.91±0.33[ab]**	**2.10±0.20[a]**	**1.90±0.26[b]**	1.80±0.28	1.83±0.12	1.80±0.16	1.81±0.20	1.81±0.09	1.80±0.16
PMDW (pg)	0.90±0.08	0.95±0.06	0.93±0.06	0.81±0.07	0.80±0.08	0.80±0.06	0.78±0.10	0.80±0.05	0.79±0.07
Large_PLT (x10^3 cells/µl)	**23.93±12.37[a]**	**24.72±11.56[a]**	**12.82±8.214[b]**	18.14±7.8	23.96±9.50	22.45±9.46	**15.2±6.71[a]**	**24.42±7.752[b]**	**16.42±6.51[a]**

[a-c] Values in the same row with different superscripts are significantly different (p <0.05)

	Day 28			Day 60			Day 90		
Parameter	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
PCT (%)	**0.31±0.15^{a}**	**0.51±0.14^{b}**	**0.32±0.14^{a}**	0.42±0.17	0.46±0.14	0.38±0.12	0.40±0.11	0.37±0.07	0.39±0.16
PLT (x10^{3} cells/µl)	**357.1±146.3^{a}**	**569.7±157.2^{b}**	**357.9±135.8^{a}**	**410.0±157.0^{ab}**	**472.1±138.3^{a}**	**360.4±109.4^{b}**	432.0±124.0	415.3±88.58	386.1±177.1
MPV (fL)	**9.62±0.78^{ab}**	**9.03±1.11^{a}**	**9.88±1.27^{b}**	**9.62±0.62^{a}**	**9.752±0.83^{a}**	**10.65±1.14^{b}**	**9.39±0.61^{a}**	**8.92±0.48^{a}**	**10.23±0.65^{b}**
MPC (g/dL)	22.23±2.12	21.54±1.14	22.24±1.51	21.21±1.22	21.15±0.82	21.31±1.17	21.72±1.51	21.44±1.40	21.49±1.14
PCDW (g/dL)	**6.21±0.46^{ab}**	**6.46±0.31^{a}**	**6.20±0.41^{b}**	**6.03±0.25^{a}**	**6.29±0.23^{b}**	**6.16±0.39^{ab}**	6.20±0.33	6.35±0.23	6.24±0.29
MPM (pg)	**1.79±0.16^{ab}**	**1.72±0.10^{a}**	**1.86±0.15^{b}**	**1.72±0.15^{a}**	**1.73±0.12^{a}**	**1.86±0.17^{b}**	**1.73±0.11^{a}**	**1.62±0.08^{b}**	**1.78±0.12^{a}**
PMDW (pg)	**0.79±0.07^{a}**	**0.73±0.05^{b}**	**0.81±0.09^{a}**	**0.74±0.06^{a}**	**0.74±0.04^{a}**	**0.81±0.06^{b}**	**0.72±0.05^{a}**	**0.72±0.05^{a}**	**0.79±0.05^{b}**
Large_PLT (x10^{3} cells/µl)	16.59±8.54	18.04±9.83	15.42±7.01	19.56±7.36	24.08±6.58	22.70±7.16	**17.50±6.06^{ab}**	**15.40±3.66^{a}**	**22.27±6.64^{b}**

$^{a-c}$ Values in the same row with different superscripts are significantly different (p <0.05)

	Day 120			Day 180		
Parameter	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
PCT (%)	0.301±0.12	0.34±0.06	0.38±0.09	0.27±0.10	0.21±0.07	0.25±0.11
PLT (x10^3 cells/µl)	364.8±99.23	383.7±71.49	404±88.22	286.6±109.2	214.4±74.88	253.4±133.5
MPV (fL)	9.22±0.72	8.89±0.47	9.37±0.59	9.77±0.84	9.60±0.79	10.19±1.04
MPC (g/dL)	21.66±1.24	22.69±1.24	21.74±1.27	**23.09±1.16[b]**	**21.61±1.14[a]**	**21.74±1.05[a]**
PCDW (g/dL)	6.30±0.15	6.25±0.20	6.21±0.31	6.21±0.37	6.36±0.22	6.17±0.35
MPM (pg)	1.68±0.13	1.70±0.064	1.73±0.12	1.84±0.10	1.75±0.11	1.80±0.12
PMDW (pg)	0.75±0.07	0.75±0.03	0.76±0.060	0.78±0.041	0.75±0.03	0.03±0.06
Large_PLT (x10^3 cells/µl)	16.33±3.0	14.89±1.76	17.67±4.92	14.67±4.3	10.0±4.83	13.46±2.90

[a-c] Values in the same row with different superscripts are significantly different (p <0.05)

Table S23. *Comparison of Red Blood Cells parameters in male piglets per group and day (mean ± SD)*

	Day 3			Day 9			Day 15		
Parameters	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
RBC (x10^6 cells/µL)	3.99±0.47	4.52±0.48	4.45±0.65	4.69±0.76	5.18±0.47	5.21±0.59	6.01±0.65	5.66±0.40	5.60±0.48
Hb (g/dL)	**8.02±0.73^{a}**	**9.17±0.69^{b}**	**8.74±1.11^{ab}**	**9.51±1.14^{a}**	**10.87±0.78^{b}**	**10.24±1.11^{ab}**	**12.17±0.59^{ab}**	**10.95±0.76^{a}**	**10.96±0.89^{a}**
HCT (%)	25.93±2.58	29.57±2.52	27.83±3.82	32.25±3.52	36.07±2.60	34.46±4.00	**38.98±1.65^{a}**	**36.09±2.41^{ab}**	**35.31±3.62^{b}**
MCV (fL)	**65.03±3.11^{a}**	**65.47±2.14^{a}**	**62.53±1.89^{b}**	69.51±7.37	69.72±3.26	66.08±3.68	65.46±6.73	63.83±3.94	63.15±4.70
MCH (pg)	20.14±1.07	20.31±0.73	19.66±1.16	**20.45±1.70^{ab}**	**20.99±0.83^{a}**	**19.63±0.92^{b}**	20.41±1.86	19.37±1.26	19.59±1.18
MCHC (g/dL)	30.99±1.45	31.06±0.48	31.42±1.40	29.49±0.98	30.1±0.44	29.74±0.89	31.23±0.49	30.36±0.52	31.07±1.09
HDW (g/dL)	3.51±0.32	3.18±0.17	3.22±0.29	**2.59±0.40^{a}**	**2.35±0.17^{ab}**	**2.23±0.13^{b}**	**2.25±0.16^{a}**	**2.52±0.18^{b}**	**2.29±0.21^{a}**
CHCM (g/dL)	31.75±1.13	31.53±1.10	31.78±0.85	30.49±0.98	30.99±0.67	30.28±1.24	31.83±0.48	31.61±0.52	31.93±1.39
RDW (%)	**22.25±4.18^{a}**	**19.81±1.28^{ab}**	**18.25±1.00^{b}**	20.46±3.78	19.24±1.51	18.18±1.00	**17.32±1.91^{a}**	**18.98±1.98^{b}**	**16.94±0.80^{a}**
Reticulocytes (%)	**12.58±4.41^{a}**	**11.96±3.13^{a}**	**6.48±1.75^{ab}**	**10.31±3.61^{a}**	**14.01±2.75^{b}**	**9.12±3.40^{a}**	**3.29±1.30^{b}**	**8.20±2.53^{a}**	**6.72±2.36^{a}**
MCVr (fL)	**85.26±9.73^{a}**	**83.08±2.82^{a}**	**73.09±5.63^{ab}**	76.41±8.43	74.3±5.90	70.15±6.46	68.5±6.26	61.76±6.77	67.71±7.22
CHr (pg)	**24.28±2.86^{a}**	**23.7±0.810^{a}**	**21.76±1.55^{ab}**	**21.61±1.07^{ab}**	**22.84±1.29^{a}**	**21.19±1.82^{b}**	**20.69±1.52^{a}**	**18.22±2.31^{b}**	**21.04±1.74^{a}**

$^{a-c}$ Values in the same row with different superscripts are significantly different (p <0.05)

	Day 28			Day 60			Day 90		
Parameters	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
RBC (x10^6 cells/µL)	**6.38±0.39^{a}**	**7.00±0.52^{b}**	**6.48±0.38^{a}**	**6.47±0.39^{ab}**	**7.23±0.74^{a}**	**6.12±0.55^{b}**	**6.82±0.45^{a}**	**7.42±1.29^{b}**	**6.66±0.59^{a}**
Hb (g/dL)	**11.39±0.60^{a}**	**10.07±1.20^{b}**	**11.83±1.13^{a}**	10.58±0.77	10.58±0.88	9.72±0.93	**10.42±0.41^{ab}**	**10.66±0.84^{a}**	**9.82±0.89^{b}**
HCT (%)	**35.9±1.56^{ab}**	**32.85±3.73^{a}**	**37.29±3.12^{b}**	34.53±2.74	34.42±3.58	31.52±2.96	**33.34±1.17^{ab}**	**34.17±2.16^{a}**	**31.79±2.66^{b}**
MCV (fL)	**56.29±1.94^{a}**	**47.11±6.32^{b}**	**57.47±2.72^{a}**	**53.38±3.06^{a}**	**47.69±3.39^{b}**	**51.44±1.94^{a}**	**48.96±2.66^{a}**	**44.33±3.64^{b}**	**47.79±2.38^{ab}**
MCH (pg)	**17.86±0.79^{a}**	**14.45±2.19^{b}**	**18.21±1.06^{a}**	**16.36±0.68^{a}**	**14.69±1.26^{b}**	**15.86±0.587^{a}**	**15.32±0.92^{a}**	**13.88±1.45^{b}**	**14.75±0.75^{ab}**
MCHC (g/dL)	**31.73±0.87^{a}**	**30.64±0.61^{b}**	**31.71±0.93^{a}**	30.69±0.82	30.82±1.03	30.86±0.69	31.27±0.59	31.28±0.83	30.91±1.15
HDW (g/dL)	**2.45±0.36^{a}**	**3.04±0.44^{b}**	**2.01±0.17^{c}**	2.19±0.30	2.38±0.19	2.21±0.18	2.14±0.12	2.27±0.11	2.18±0.13
CHCM (g/dL)	**32.88±1.08^{a}**	**31.4±0.73^{b}**	**33.02±1.19^{a}**	31.67±1.01	31.49±1.02	32.01±0.85	32.51±0.79	32.47±0.84	32.29±0.89
RDW (%)	**17.84±3.11^{a}**	**25.94±5.01^{a}**	**15.41±0.81^{b}**	**16.63±1.37^{a}**	**20.15±2.04^{b}**	**16.74±0.80^{a}**	**16.17±1.18^{a}**	**18.04±1.31^{b}**	**17.13±1.53^{ab}**
Reticulocytes (%)	4.48±2.67	3.28±1.63	3.04±1.93	4.35±1.63	3.51±0.96	2.99±1.23	2.01±0.44	1.61±0.66	1.99±0.54
MCVr (fL)	**59.53±3.72^{a}**	**49.23±6.88^{b}**	**61.73±3.89^{a}**	59.02±3.39	55.71±7.93	58.84±2.56	60.01±1.78	58.32±3.49	58.93±3.78
CHr (pg)	**17.81±1.07^{a}**	**14.41±2.08^{b}**	**18.83±1.30^{a}**	**17.82±1.14^{a}**	**16.03±2.27^{b}**	**17.56±0.88^{a}**	17.44±0.69	16.57±1.15	16.93±1.19

$^{a-c}$ Values in the same row with different superscripts are significantly different (p <0.05)

	Day 120			Day 180		
Parameters	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
RBC (x10^6 cells/µL)	6.96±0.85	7.57±0.61	7.37±0.34	7.72±0.63	7.93±0.80	7.44±0.55
Hb (g/dL)	10.6±1.29	10.91±0.72	10.7±0.96	12.46±0.90	12.5±1.03	11.8±1.19
HCT (%)	32.56±4.52	33.64±2.29	33.66±2.25	38.02±2.67	38.49±3.40	36.93±3.60
MCV (fL)	46.76±2.65	44.53±2.62	45.68±2.68	49.34±2.99	48.7±3.40	49.53±2.39
MCH (pg)	15.26±0.74	14.47±1.11	14.54±14.54	16.19±1.08	15.81±1.15	15.85±0.84
MCHC (g/dL)	32.64±0.928	32.46±0.75	31.79±0.84	**32.79±0.54^{a}**	**32.47±0.53^{ab}**	**31.97±0.32^{b}**
HDW (g/dL)	2.36±0.20	2.26±0.12	2.21±0.18	2.07±0.10	2.11±0.11	2.08±0.13
CHCM (g/dL)	33.73±0.59	33.53±0.77	33.13±0.90	33.46±0.55	33.33±0.60	32.91±0.57
RDW (%)	**16.85±1.45^{a}**	**18.53±1.24^{b}**	**17.43±1.21^{ab}**	17.82±1.41	19.99±1.95	19.38±1.74
Reticulocytes (%)	**1.28±0.29^{a}**	**0.78±0.32^{b}**	**1.02±0.32^{ab}**	0.60±0.20	0.77±0.25	0.79±0.33
MCVr (fL)	60.28±3.76	57.84±2.51	60.66±5.56	63.68±3.17	65.57±3.07	65.25±4.65
CHr (pg)	17.82±1.47	17.02±0.94	17.61±1.50	19.19±1.11	19.59±0.82	19.26±1.34

[a-c] Values in the same row with different superscripts are significantly different (p <0.05)

Table S24. *Comparison of White Blood Cells parameters in male piglets per group and day (mean ± SD)*

	Day 3			Day 9			Day 15		
Parameter	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
WBC (x10^3 cells/μL)	**7.82±3.79^{a}**	**7.13±4.15^{ab}**	**4.47±2.01^{b}**	**9.69±3.3^{ab}**	**11.45±2.00^{a}**	**8.83±2.63^{b}**	8.43±2.29	7.33±1.85	8.50±3.10
Neutrophils (x10^3 cells/μL)	4.53±2.92	3.55±2.55	2.43±1.76	4.74±2.80	5.53±1.18	4.39±2.67	3.05±1.57	2.88±1.04	3.00±1.74
Lymphocytes (x10^3 cells/μl)	**2.82±1.19^{a}**	**3.07±1.51^{a}**	**1.72±0.63^{b}**	4.36±1.63	4.93±1.33	3.87±1.12	4.80±1.62	3.95±1.25	4.541±1.53
Monocytes (x10^3 cells/μl)	0.20±0.099	0.23±0.10	0.16±0.08	**0.23±0.12^{ab}**	**0.36±0.12^{a}**	**0.25±0.18^{b}**	0.20±0.17	0.20±0.08	0.27±0.17
Eosinophils (x10^3 cells/μl)	0.06±0.039	0.05±0.02	0.05±0.02	**0.12±0.05^{ab}**	**0.24±0.12^{a}**	**0.11±0.03^{b}**	0.13±0.06	0.12±0.06	0.13±0.07
Basophils (x10^3 cells/μl)	0.03±0.03	0.04±0.02	0.02±0.014	**0.06±0.034^{ab}**	**0.10±0.034^{a}**	**0.05±0.02^{b}**	0.05±0.013	0.03±0.02	0.06±0.03

$^{a-c}$ Values in the same row with different superscripts are significantly different (p <0.05)

	Day 28			Day 60			Day 90		
Parameter	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
WBC ($x10^3$ cells/μL)	10.43±1.56	9.16±1.34	11.23±3.70	15.42±3.72	16.85±5.31	14.17±5.11	16.82±3.81	16.85±1.91	15.76±2.91
Neutrophils ($x10^3$ cells/μL)	**4.01±1.14[a]**	**2.09±0.75[b]**	**4.50±1.52[a]**	4.21±1.69	4.23±2.48	4.54±2.76	6.50±2.54	6.24±1.36	5.78±2.58
Lymphocytes ($x10^3$ cells/μl)	**5.54±0.95[ab]**	**6.37±1.40[a]**	**4.68±1.90[b]**	9.85±2.26	9.80±1.91	8.29±2.79	8.55±2.04	8.49±1.87	8.33±2.42
Monocytes ($x10^3$ cells/μl)	0.46±0.24	0.30±0.11	0.34±0.16	0.86±0.37	1.04±0.49	0.83±0.45	1.01±0.25	0.85±0.26	0.88±0.21
Eosinophils ($x10^3$ cells/μl)	0.22±0.08	0.18±0.19	0.18±0.09	0.19±0.06	0.22±0.16	0.15±0.045	**0.46±0.10[b]**	**0.28±0.09[a]**	**0.24±0.10[a]**
Basophils ($x10^3$ cells/μl)	**0.06±0.02[a]**	**0.02±0.01[b]**	**0.05±0.02[a]**	0.09±0.05	0.09±0.05	0.09±0.05	0.09±0.0	0.05±0.01	0.1±0.06

[a-c] Values in the same row with different superscripts are significantly different ($p < 0.05$)

	Day 120			Day 180		
Parameter	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
WBC ($x10^3$ cells/μL)	15.62±4.56	18.84±5.05	17.38±3.25	19.06±3.92	15.42±3.77	17.44±4.18
Neutrophils ($x10^3$ cells/μL)	4.05±1.65	5.87±1.81	5.47±0.88	6.20±2.21	3.36±1.83	4.73±2.45
Lymphocytes ($x10^3$ cells/μl)	9.88±2.85	9.66±2.23	10.26±2.53	10.47±2.30	10.31±3.06	10.71±2.12
Monocytes ($x10^3$ cells/μl)	0.92±0.40	0.84±0.21	0.95±0.38	0.80±0.29	0.78±0.35	0.67±0.24
Eosinophils ($x10^3$ cells/μl)	0.51±0.29	0.49±0.19	0.39±0.16	**1.05±0.28[a]**	**0.38±0.23[b]**	**0.80±0.38[ab]**
Basophils ($x10^3$ cells/μl)	0.08±0.05	0.08±0.04	0.05±0.02	0.12±0.03	0.13±0.09	0.13±0.08

[a-c] Values in the same row with different superscripts are significantly different (p <0.05)

Table S25. *Comparison of Platelet parameters in male piglets per group and day (mean ± SD)*

	Day 3			**Day 9**			**Day 15**		
Parameter	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
PCT (%)	0.27±0.19	0.29±0.13	0.16±0.11	0.44±0.27	0.48±0.23	0.41±0.26	**0.33±0.16^{ab}**	**0.47±0.10^{a}**	**0.29±0.12^{b}**
PLT (x10^3 cells/µl)	**320.4±132.7^{a}**	**259.0±121.4^{ab}**	**159.4±92.84^{b}**	414.1±233.2	502.1±243.6	425.1±271.2	**324.0±158.5^{ab}**	**447.7±102.1**	**268.8±115.3^{b}**
MPV (fL)	10.80±2.22	11.13±0.99	9.97±1.86	10.32±1.47	9.70±0.50	9.59±0.98	9.50±1.27	10.28±0.95	9.77±0.91
MPC (g/dL)	22.21±1.92	22.74±1.44	21.88±2.47	22.11±1.9	22.08±0.87	21.23±1.05	23.31±2.78	22.06±0.42	21.67±1.96
PCDW (g/dL)	6.40±0.62	6.51±0.52	6.29±0.71	6.40±0.72	6.70±0.34	6.28±0.54	6.48±0.78	6.77±0.18	6.51±0.40
MPM (pg)	1.91±0.35	1.99±0.21	1.83±0.29	1.84±0.33	1.79±0.09	1.73±0.15	1.85±0.21	1.81±0.07	1.75±0.1
PMDW (pg)	0.90±0.08	0.91±0.04	0.91±0.08	0.81±0.07	0.80±0.06	0.78±0.06	0.79±0.10	0.76±0.024	0.79±0.05
Large_PLT (x10^3 cells/µl)	22.13±15.41	23.67±12.04	12.39±9.41	17.25±9.22	24.18±9.34	21.35±11.62	**18.25±6.41^{ab}**	**25.78±7.77**	**15.47±7.76^{b}**

$^{a-c}$ Values in the same row with different superscripts are significantly different (p <0.05)

	Day 28			Day 60			Day 90		
Parameter	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
PCT (%)	0.36±0.17	0.49±0.15	0.35±0.15	0.42±0.18	0.43±0.17	0.34±0.12	0.40±0.11	0.37±0.07	0.39±0.16
PLT (x10^3 cells/µl)	**415.3±154.4^{a}**	**577.1±171.4^{b}**	**417.1±134.5^{a}**	387.7±150.5	453.1±177.1	333.8±122.8	432.0±124.0	415.3±88.58	386.1±177.1
MPV (fL)	9.50±0.97	8.54±0.93	9.35±1.08	9.81±0.52	9.33±0.87	10.14±1.27	**9.39±0.61^{a}**	**8.92±0.48^{a}**	**10.23±0.65^{b}**
MPC (g/dL)	22.23±2.55	21.62±0.88	21.89±1.18	21.23±1.29	21.05±0.89	21.28±1.53	21.72±1.51	21.44±1.40	21.49±1.14
PCDW (g/dL)	**6.0±0.44^{a}**	**6.55±0.16^{b}**	**6.22±0.40^{a}**	6.03±0.29	6.21±0.25	6.25±0.37	6.20±0.33	6.35±0.23	6.24±0.29
MPM (pg)	1.81±0.20	1.72±0.08	1.79±0.11	1.74±0.12	1.67±0.11	1.75±0.17	**1.73±0.11^{a}**	**1.62±0.08^{b}**	**1.78±0.12^{a}**
PMDW (pg)	0.78±0.07	0.73±0.051	0.79±0.10	**0.75±0.06^{ab}**	**0.72±0.04^{a}**	**0.80±0.08^{b}**	**0.72±0.05^{a}**	**0.72±0.05^{a}**	**0.79±0.05^{b}**
Large_PLT (x10^3 cells/µl)	17.63±6.63	12.33±8.27	16.6±4.01	20.4±7.70	19.09±8.44	20.50±7.81	**17.50±6.06^{ab}**	**15.40±3.66^{a}**	**22.27±6.64^{b}**

$^{a-c}$ Values in the same row with different superscripts are significantly different (p <0.05)

	Day 120			Day 180		
Parameter	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
PCT (%)	0.301±0.12	0.34±0.06	0.38±0.09	0.27±0.10	0.21±0.07	0.25±0.11
PLT (x10^3 cells/µl)	364.8±99.23	383.7±71.49	404±88.22	286.6±109.2	214.4±74.88	253.4±133.5
MPV (fL)	9.22±0.72	8.89±0.47	9.37±0.59	9.77±0.84	9.60±0.79	10.19±1.04
MPC (g/dL)	21.66±1.24	22.69±1.24	21.74±1.27	**23.09±1.16[b]**	**21.61±1.14[a]**	**21.74±1.05[a]**
PCDW (g/dL)	6.30±0.15	6.25±0.20	6.21±0.31	6.21±0.37	6.36±0.22	6.17±0.35
MPM (pg)	1.68±0.13	1.70±0.064	1.73±0.12	1.84±0.10	1.75±0.11	1.80±0.12
PMDW (pg)	0.75±0.07	0.75±0.03	0.76±0.060	0.78±0.041	0.75±0.031	0.03±0.06
Large_PLT (x10^3 cells/µl)	16.33±3.0	14.89±1.76	17.67±4.92	14.67±4.33	10.0±4.83	13.46±2.90

[a-c] Values in the same row with different superscripts are significantly different (p <0.05)

Table S26. *Comparison of Red Blood Cells parameters in female piglets per group and day (mean ± SD)*

	Day 3			Day 9			Day 15		
Parameters	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
RBC (x10^6 cells/µL)	4.12±0.61	4.53±0.69	4.58±0.78	5.25±0.44	4.94±0.47	5.32±0.60	**6.21±0.50^{a}**	**5.78±0.32^{ab}**	**5.66±0.26^{b}**
Hb (g/dL)	8.12±1.08	8.82±1.07	9.11±1.56	10.11±0.8	10.23±0.62	10.56±0.99	11.96±0.87	11.51±0.92	11.16±0.90
HCT (%)	26.65±4.40	28.36±3.79	28.7±4.46	33.49±2.24	33.91±1.91	35.09±3.37	37.93±2.32	37.59±3.40	36.1±3.04
MCV (fL)	64.54±3.93	62.89±2.97	62.85±2.28	**63.89±1.84^{a}**	**69.08±5.59^{b}**	**66.1±3.66^{ab}**	61.17±2.11	65.12±5.67	62.95±2.94
MCH (pg)	19.8±1.07	19.59±1.14	19.88±0.98	**19.26±0.36^{a}**	**20.82±1.45^{b}**	**19.88±1.06^{ab}**	19.26±0.42	19.93±1.43	19.47±1.04
MCHC (g/dL)	30.71±1.62	31.14±0.91	31.34±0.82	30.14±0.71	30.15±0.71	30.08±0.63	31.51±0.62	30.62±0.72	30.91±0.78
HDW (g/dL)	3.23±0.40	3.21±0.22	3.18±0.20	**2.39±0.19^{ab}**	**2.42±0.14^{a}**	**2.19±0.21^{b}**	2.22±0.18	2.24±0.24	2.14±0.18
CHCM (g/dL)	31.41±1.65	31.45±0.81	31.74±0.74	**31.23±1.37^{a}**	**31.04±0.61^{ab}**	**30.4±0.88^{b}**	32.03±0.75	31.26±0.86	31.65±1.26
RDW (%)	**21.4±1.64^{a}**	**19.32±2.26^{b}**	**17.86±1.26^{c}**	**18.09±1.01^{a}**	**20.17±1.85^{b}**	**17.71±1.07^{a}**	16.2±0.91	17.36±1.44	16.41±0.65
Reticulocytes (%)	**13.36±3.96^{a}**	**10.49±3.46^{b}**	**6.41±2.10^{c}**	**7.813±2.97^{a}**	**13.18±4.026^{b}**	**8.63±3.37^{a}**	**2.94±1.10^{a}**	**8.61±2.46^{b}**	**5.42±2.42^{a}**
MCVr (fL)	**84.38±7.34^{a}**	**77.01±5.52^{b}**	**72.2±7.75^{c}**	**71.69±3.247^{ab}**	**75.28±5.97^{a}**	**68.29±7.91^{b}**	65.06±4.86	65.87±6.22	68.43±5.10
CHr (pg)	**24.01±2.09^{a}**	**22.23±1.47^{ab}**	**21.42±2.25^{b}**	**20.8±0.70^{a}**	**22.89±1.90^{b}**	**21.09±2.03^{a}**	19.57±0.74	19.63±2.26	20.82±1.52

$^{a-c}$ Values in the same row with different superscripts are significantly different (p <0.05)

	Day 28			Day 60			Day 90		
Parameters	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
RBC (x10^6 cells/µL)	**6.28±0.52^{a}**	**6.89±0.62^{b}**	**6.23±0.58^{a}**	6.10±0.57	6.72±0.51	6.34±0.46	6.88±0.35	7.22±0.58	6.75±0.49
Hb (g/dL)	11.17±0.68	10.94±1.33	11.66±1.31	10.1±1.28	10.29±0.68	10.3±0.81	10.23±0.87	10.31±0.76	9.87±0.93
HCT (%)	35.39±2.842	35.3±3.487	36.43±4.303	32.35±4.55	33.4±2.35	33.33±2.59	33.37±2.18	33.51±1.86	32.35±2.45
MCV (fL)	**56.41±2.66^{ab}**	**51.46±5.32^{a}**	**58.38±2.70^{b}**	**52.85±3.10^{ab}**	**49.86±3.51^{a}**	**52.55±2.06^{b}**	48.61±4.0	46.59±3.58	47.99±2.79
MCH (pg)	**17.84±0.86^{ab}**	**15.98±2.13^{a}**	**18.67±0.85^{b}**	**16.53±0.59^{a}**	**15.34±1.10^{b}**	**16.27±0.49^{a}**	14.94±1.50	14.32±1.28	14.68±1.34
MCHC (g/dL)	**31.64±0.72^{ab}**	**30.99±1.13^{a}**	**31.98±0.67^{b}**	31.3±0.99	30.78±0.42	30.98±1.00	30.67±0.93	30.73±0.81	30.6±2.18
HDW (g/dL)	**2.31±0.20^{ab}**	**2.60±0.40^{a}**	**2.08±0.31^{b}**	2.21±0.22	2.25±0.12	2.13±0.17	2.22±0.21	2.17±0.14	2.16±0.13
CHCM (g/dL)	**33.01±0.82^{ab}**	**31.85±1.12^{a}**	**32.96±1.14^{b}**	32.15±1.21	31.86±0.64	32.02±1.16	31.94±1.12	32.15±0.78	32.14±0.86
RDW (%)	**16.4±1.51^{ab}**	**21.37±5.58^{a}**	**15.53±1.11^{b}**	**16.35±0.71^{ab}**	**18.59±2.47^{a}**	**15.93±0.66^{b}**	**16.63±1.38^{ab}**	**17.59±1.01^{a}**	**16.31±0.88^{b}**
Reticulocytes (%)	4.12±1.91	3.40±1.34	3.548±2.37	2.91±1.44	3.80±1.09	2.99±1.30	2.01±0.59	2.03±0.80	2.14±0.75
MCVr (fL)	**61.44±4.69^{a}**	**52.84±6.46^{ab}**	**61.99±4.72^{a}**	59.15±5.53	58.48±5.02	59.28±2.41	57.89±7.87	58.32±4.70	57.98±3.84
CHr (pg)	**18.41±1.47^{ab}**	**15.58±2.00^{a}**	**19.50±1.11^{b}**	17.67±1.53	17.04±1.58	17.80±0.59	16.93±2.38	16.84±1.49	16.80±1.25

$^{a-c}$ Values in the same row with different superscripts are significantly different (p <0.05)

	Day 120			Day 180		
Parameters	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
RBC (x10^6 cells/µL)	**7.64±0.52[a]**	**7.56±0.68[a]**	**7.0±0.42[b]**	8.08±1.04	7.99±0.74	7.66±0.83
Hb (g/dL)	11.14±1.38	11.04±1.33	10.45±0.97	12.17±0.92	12.74±1.20	12.23±1.39
HCT (%)	34.8±3.78	34.62±3.66	33.13±2.67	37.65±1.80	39.13±3.20	37.84±4.53
MCV (fL)	45.69±5.33	45.83±3.68	47.32±2.76	47.23±6.28	49.04±2.64	49.43±2.90
MCH (pg)	16.93±6.82	14.61±1.42	14.91±1.08	47.23±6.28	49.04±2.64	49.43±2.90
MCHC (g/dL)	32.35±1.34	31.86±0.76	31.53±0.91	32.35±1.34	32.54±0.68	32.38±0.88
HDW (g/dL)	2.31±0.20	2.22±0.15	2.16±0.15	2.20±0.26	2.08±0.09	2.0±0.15
CHCM (g/dL)	33.09±1.12	33.15±0.88	32.62±0.76	33.42±1.323	33.44±0.80	33.13±0.90
RDW (%)	16.76±1.09[ab]	17.91±1.08[a]	16.78±0.67[b]	19.22±2.28	19.51±2.56	18.92±2.80
Reticulocytes (%)	0.88±0.22	1.16±0.58	1.29±0.44	0.55±0.23	0.60±0.34	0.8±0.40
MCVr (fL)	57.27±8.98	59.59±6.27	59.99±5.38	61.57±7.10	65.8±3.26	65.34±3.70
CHr (pg)	17.1±2.57	17.47±1.97	17.51±1.43	**18.37±2.55[a]**	**20.01±0.90[b]**	**19.58±1.03[ab]**

[a-c] Values in the same row with different superscripts are significantly different (p <0.05)

Table S27. *Comparison of White Blood Cells parameters in female piglets per group and day (mean ± SD)*

	Day 3			**Day 9**			**Day 15**		
Parameter	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
WBC (x10^3 cells/μL)	6.92±2.93	7.71±2.45	5.82±2.34	8.11±1.39	9.07±2.12	9.31±2.78	9.10±2.48	8.10±2.09	8.60±2.69
Neutrophils (x10^3 cells/μL)	3.6±1.89	4.90±2.13	3.69±1.98	2.99±1.30	4.04±1.69	4.46±2.44	3.06±0.85	2.90±1.34	2.88±1.20
Lymphocytes (x10^3 cells/μl)	**2.84±0.95[a]**	**2.20±1.10[ab]**	**1.70±0.50[b]**	4.33±0.88	4.32±0.76	4.25±1.10	5.35±2.01	4.58±1.41	4.95±2.01
Monocytes (x10^3 cells/μl)	0.31±0.24	0.27±0.15	0.16±0.08	0.33±0.22	0.38±0.17	0.25±0.12	0.24±0.10	0.30±0.14	0.35±0.24
Eosinophils (x10^3 cells/μl)	0.06±0.035	0.09±0.04	0.07±0.03	0.12±0.07	0.12±0.04	0.15±0.07	0.22±0.16	0.12±0.05	0.17±0.10
Basophils (x10^3 cells/μl)	0.04±0.01	0.03±0.02	0.03±0.01	0.06±0.04	0.10±0.08	0.09±0.06	0.08±0.03	0.07±0.05	0.06±0.03

[a-c] Values in the same row with different superscripts are significantly different (p <0.05)

	Day 28			Day 60			Day 90		
Parameter	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
WBC ($x10^3$ cells/µL)	9.33±2.58	10.19±2.13	11.07±2.96	15.37±4.0	14.19±3.70	13.93±3.30	15.04±2.69	16.14±4.59	15.94±3.68
Neutrophils ($x10^3$ cells/µL)	2.92±0.77	2.92±1.20	4.47±2.22	4.54±1.81	3.37±1.20	4.38±1.99	4.84±1.36	4.64±1.24	5.81±2.51
Lymphocytes ($x10^3$ cells/µl)	5.67±2.0	6.41±1.75	5.68±2.41	9.17±2.21	9.33±3.13	8.23±2.14	8.63±1.29	8.89±2.03	8.00±2.19
Monocytes ($x10^3$ cells/µl)	0.31±0.15	0.37±0.13	0.37±0.15	0.98±0.43	0.86±0.46	0.88±0.45	0.89±0.21	0.76±0.27	0.69±0.23
Eosinophils ($x10^3$ cells/µl)	0.16±0.06	0.18±0.07	0.16±0.06	0.16±0.08385	0.22±0.09	0.17±0.06	**0.39±0.12[a]**	**0.28±0.10[a]**	**0.25±0.13[b]**
Basophils ($x10^3$ cells/µl)	0.06±0.04	0.08±0.04	0.08±0.04	**0.08±0.04[ab]**	**0.12±0.03[a]**	**0.09±0.03[b]**	0.11±0.05	0.10±0.03	0.16±0.10

[a-c] Values in the same row with different superscripts are significantly different (p <0.05)

	Day 120			Day 180		
Parameter	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
WBC ($x10^3$ cells/μL)	16.62±4.14	15.64±2.86	15.76±3.24	16.83±4.0	16.23±3.01	15.69±2.46
Neutrophils ($x10^3$ cells/μL)	4.37±2.09	4.22±1.49	4.80±1.56	3.9±0.89	4.77±1.68	3.87±1.43
Lymphocytes ($x10^3$ cells/μl)	10.41±1.83	9.00±2.76	9.27±2.45	10.06±2.13	9.79±2.36	9.45±2.19
Monocytes ($x10^3$ cells/μl)	1.00±0.26	0.98±0.44	0.90±0.26	0.67±0.37	0.76±0.19	0.74±0.30
Eosinophils ($x10^3$ cells/μl)	0.58±0.35	0.51±0.23	0.44±0.22	0.50±0.08	0.49±0.16	0.52±0.22
Basophils ($x10^3$ cells/μl)	0.08±0.03	0.06±0.03	0.06±0.01	0.10±0.02	0.13±0.05	0.10±0.06

[a-c] Values in the same row with different superscripts are significantly different (p <0.05)

Table S28. *Comparison of Platelet parameters in female piglets per group and day (mean ± SD)*

	Day 3			Day 9			Day 15		
Parameter	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
PCT (%)	**0.32±0.18[a]**	**0.26±0.13[ab]**	**0.170.097[b]**	0.40±0.20	0.52±0.22	0.39±0.16	0.21±0.10	0.44±0.13	0.32±0.13
PLT (x10^3 cells/µl)	**333.4±144.0[a]**	**234.3±122.1[ab]**	**166.1±76.9[b]**	430.1±213.9	525.1±228.9	384.1±175.2	**286.7±184.2[a]**	**465.2±154.2[b]**	**359.6±176.7[ab]**
MPV (fL)	**10.43±1.91[ab]**	**12.09±1.75[a]**	**10.48±1.96[b]**	**9.07±0.94[a]**	**9.99±0.79[ab]**	**10.2±0.61[b]**	9.36±0.41	10.01±1.02	9.92±0.94
MPC (g/dL)	22.31±1.46	23.78±1.03	23.09±1.76	21.56±1.76	22.25±1.24	22.75±1.81	22.71±1.86	22.03±0.82	21.87±1.76
PCDW (g/dL)	6.37±0.72	6.15±0.60	6.51±0.68	6.3±0.65	6.55±0.2	6.4±0.64	6.61±0.54	6.56±0.43	6.58±0.48
MPM (pg)	**1.91±0.33[ab]**	**2.17±0.17[a]**	**1.93±0.23[b]**	1.73±0.18	1.86±0.13	1.86±0.14	1.76±0.09	1.81±0.10	1.79±0.14
PMDW (pg)	**0.9±0.08[a]**	**0.97±0.06[b]**	**0.95±0.04[ab]**	0.77±0.09	0.80±0.07	0.84±0.06	0.76±0.09	0.77±0.06	0.80±0.0
Large_PLT (x10^3 cells/µl)	**26.0±8.38[a]**	**26.93±10.0[a]**	**15.22±5.34[b]**	16.71±8.79	25.53±11.92	23.26±7.66	**11.71±5.53[a]**	**23.71±7.88[b]**	**17.1±5.55[a]**

[a-c] Values in the same row with different superscripts are significantly different (p <0.05)

	Day 28			Day 60			Day 90		
Parameter	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
PCT (%)	**0.24±0.09[a]**	**0.52±0.14[b]**	**0.29±0.13[a]**	0.41±0.17	0.48±0.11	0.41±0.11	0.44±0.07	0.42±0.08	0.40±0.16
PLT (x10^3 cells/µl)	**269.8±80.97[a]**	**564.7±152.3[b]**	**311.1±120.3[a]**	**443.5±174.7[ab]**	**485.1±108.7[a]**	**378.9±97.47[b]**	481.4±82.6	461.8±102.6	417.5±154.2
MPV (fL)	**9.78±0.38[ab]**	**9.37±1.13[a]**	**10.31±1.27[b]**	**9.32±0.69[a]**	**10.04±0.68[a]**	**11.0±0.94[b]**	**9.2±0.52[a]**	**9.22±0.74[a]**	**10.36±0.95[b]**
MPC (g/dL)	22.23±1.48	21.48±1.32	22.53±1.71	21.18±1.22	21.22±0.79	21.33±0.88	20.87±0.76	21.77±0.99	21.7±1.07
PCDW (g/dL)	6.5±0.33	6.39±0.37	6.18±0.43	**6.03±0.17[a]**	**6.35±0.20[b]**	**6.09±0.40[ab]**	6.33±0.25	6.39±0.23	6.35±0.31
MPM (pg)	**1.76±0.09[ab]**	**1.71±0.11[a]**	**1.91±0.15[b]**	**1.69±0.18[a]**	**1.78±0.11[a]**	**1.93±0.11[b]**	**1.62±0.08[a]**	**1.70±0.10[a]**	**1.83±0.11[b]**
PMDW (pg)	**0.82±0.06[a]**	**0.74±0.05[b]**	**0.83±0.08[a]**	**0.72±0.07[a]**	**0.76±0.03[a]**	**0.81±0.05[b]**	**0.71±0.04[a]**	**0.74±0.04[a]**	**0.80±0.07[b]**
Large_PLT (x10^3 cells/µl)	14.43±4.93	23.0±12.88	15.95±7.58	18.17±7.195	26.06±6.148	24.38±6.289	19.86±5.93	19.38±4.01	23.1±8.36

[a-c] Values in the same row with different superscripts are significantly different (p <0.05)

	Day 120			Day 180		
Parameter	RF-IVP	C-IVP	AI	RF-IVP	C-IVP	AI
PCT (%)	0.35±0.12	0.33±0.08	0.34±0.08	0.16±0.07	0.24±0.11	0.25±0.09
PLT (x10^3 cells/µl)	410.1±161.8	368.3±103.1	350±84.08	189.3±105.0	291.1±94.42	261.2±100.5
MPV (fL)	**8.54±1.23[a]**	**9.01±0.81[a]**	**9.92±0.83[b]**	8.85±1.95	8.84±1.27	9.93±0.77
MPC (g/dL)	22.67±1.11	22.71±1.2	21.75±1.13	23.22±1.64	22.67±1.49	22.94±1.04
PCDW (g/dL)	6.329±0.30	6.26±0.29	6.09±0.26	6.18±0.53	6.26±0.47	5.97±0.38
MPM (pg)	**1.63±0.11[a]**	**1.72±0.11[ab]**	**1.8±0.08[b]**	1.73±0.15	1.74±0.16	1.87±0.11
PMDW (pg)	**0.71±0.03[a]**	**0.75±0.04[a]**	**0.79±0.05[b]**	0.78±0.05	0.77±0.07	0.79±0.04
Large_PLT (x10^3 cells/µl)	15.29±8.84	15.33±4.34	18.28±6.09	7.83±5.74	11.21±6.48	13.82±4.96

[a-c] Values in the same row with different superscripts are significantly different (p <0.05)