Impact of Embryo Transfer on the phenotype of the offspring in pigs

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Research

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Abstract

Background

The increased use of assisted reproductive technologies led to a higher risk of specific alterations in the offspring, as already described in human and other mammalian species. Nonetheless, it is yet to be totally understood how and which specific techniques are inducing these changes. The use of animal models may represent an advantage to find out answers to these questions due to the minimization of confounding factors. The pig is not only a great human reproductive model, but it is also of interest in the livestock industry to develop further technologies that may enhance meat production. This study was designed to decipher the impact of one of these technologies, namely embryo transfer, on mother´s reproductive performance and on the phenotype of the offspring in pigs.

Results

Embryo transfer of *in vivo* derived embryos (ET) showed similar results in terms of pregnancy length, litter size and total litter weight per sow than sows artificially inseminated (AI). Bodyweight at birth was higher for ET-females than AI-females, but this difference was no longer observed at day 15 of postnatal life. Some of the major findings after haematological analyses were that concentration of red blood cells, haemoglobin and haematocrit in female-ET piglets at day 3 showed higher values than AI-females. Additionally, higher concentration of white blood cells was found at day 3 in both ET-derived piglets (males and females) in comparison to AI-piglets. Biochemical analysis showed a higher level of albumin at day 3 for ET-derived male piglets than AI but ET-females showed lower levels of bilirubin than AI in the same day. Despite the statistical differences between groups, all values were within the normal ranges, so that we cannot give them any clinical significance.

Conclusions

Piglets derived from *in vivo* embryo transfer seem to be phenotypically similar, which provides evidence that the embryo transfer procedure is a safe technique, without relevant health implications to the offspring. Additionally, these findings may be of interest to the pig industry regarding the reference values provided for haematological and biochemical parameters in this species.

Background

Compelling evidence about the effects of assisted reproductive technologies (ART) on the short- and long-term health of the offspring (reviewed by [1, 2]) has led to an increase of research in this field, not only in humans but also in other mammalian species. Nowadays, many studies expose the impact that stressful conditions, derived from gametes and embryo manipulation on the first week of preimplantation development, may have on the epigenetic reprogramming that occurs during this period [3]. From ovarian stimulation treatment to embryo transfer (ET), each step in the ART represents a possible alteration in the epigenome with consequences in both transcriptome and physiology of the offspring (reviewed by [4]). Moreover, the phenotype may also be compromised, as it is known that the use of these techniques may affect the birth weight and developmental growth, involving also an increased risk of cardiovascular diseases, diabetes and obesity in adulthood when compared to naturally conceived offspring [5].

While in human ART-derived offspring it is difficult to assess which technology is responsible for the harmful effect(s) (i.e. artificial insemination (AI), *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), ET, etc.) or even distinguish between the effect of the global process versus the parental inherited alterations, animal models offer a simpler way to decipher the isolated impact of one specific ART. Pig, in particular, represents an excellent model to study such impact due to its similar anatomical and physiological characteristics with humans (reviewed by [6]). This creates the opportunity to use the domestic pig as model of ART, since highly selected healthy male and female breeders are available in commercial farms and AI centres, thus enabling a more restricted/controlled genetic variability under similar handling and feeding conditions.

Aside from being an excellent model to study human reproduction, the use of ART in pigs may also be useful at a more commercial level to increase meat production – but apart from AI, these systems are still far from being widely established in the pig industry [7]. The development and extended use of some specific technologies such as ET would not only provide greater genetic selection, but would also decrease the risk of diseases transmission since it avoids the entry of new animals in breeding centres and farms, decrease transportation costs and minimize the effect on animal welfare during transportation (reviewed by [8]). However, ET technique shows some limitations in porcine species since it requires the collection of a large number of viable embryos (ideally cryopreserved) to be transferred to the recipient mothers and, until recent years, it also required the surgical transfer of those embryos [9].

Today, the development of new devices has made it possible to do nonsurgical ET despite the complex anatomy of the sow's genital tract. Embryos can be deposited as deep as possible in one uterine horn of a non-sedated recipient, minimizing the risk of disease transmission and uterine infections [8]. Nevertheless, to date, *in vivo* embryo collection by uterine flushing via transrectal palpation is not yet a reality at the industry level due to the length and tortuousness of the gilts' and sows' uterine horns, something that does not occur in species like cattle and horses (reviewed by [10]).

Despite many studies having been focused on obtaining live piglets through the use of ET technique [11–13], little is known about its possible consequences on the health of the offspring. Thus, the study of the phenotype of piglets derived from ET would provide a valuable information that could be of interest also in the human species. Physiological parameters, including haematological and biochemical, are important indicators of the general state of health. In newborn piglets, very scarce studies have published reference intervals for consistent comparisons [14, 15]. Recently, a very complete report from Ventrella *et al*. [16] has supplied a database to be used as reference, despite showing the high variability occurring in clinical-pathological variables between 5 and 30-day-old piglets.

The present study was conducted to determine, using the pig model, the impact of one of the most commonly used ART, the ET, on weight, average daily weight gain (ADWG), and on the haematological and biochemical profile of the offspring, minimizing the confounders from parental origin or from another ART. The results obtained could help to evaluate the real impact, if any, of the ET technique in the short and long-term health of ART-derived children or other mammalian species.

Methods

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich Chemical Company (Madrid, Spain).

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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.

Animals

Crossbred sows (Landrace x Large White) from the same genetic line were used as donors (n = 25, 1–11 parities, 1–5 years old) and as recipients (n = 11, 2–3 parities). All animals were housed and fed under the same conditions and water was provided *ad libitum*.

Estrous synchronization of donor and recipient sows was carried out naturally after weaning. Sows that showed signs of estrous 4–5 days after weaning were used as donors or recipients. Estrous detection was carried out by exposing a mature 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 boars of the same breed and proven fertility. Estrous synchrony of the recipients was between 0 and + 24 h regarding the donors. A total of 32 and 11 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.

In vivo embryo collection

Considering day 0 as the onset of estrous, donors that were on day 6, 7 or 8 of the cycle were sedated by administration of ketamine (10 mg/kg IM, Imalgene® 1000, Boehringer Ingelheim Animal Health, Merial, France), medetomidine hydrochloride (0.02 mg/kg IM, Domitor®, Orion Pharma, Finland) and midazolam (0.2 mg/kg IM, Dormicum®, Roche, Switzerland). Then, sows were euthanized with an overdose of sodium pentobarbital (0.5 mL/kg IV, Eutanax®, Fatro Iberica, Spain) 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 modified TL-Hepes-PVA medium, as described by Funahashi et al. [17], sterile and tempered at 38.5ºC. Embryo's developmental stage and quality were evaluated under a stereomicroscope and morulae, unhatched and/or hatched blastocyst were used for further ET. Embryos were washed in the same medium and remained in a culture dish at 38.5ºC until transferred. In order to get higher number of embryos, each ET was performed using embryos from three donors to a single recipient. The period of time between excising the uterus, flushing, selection of embryos and the non-surgical embryo transfer was between 60 to 90 minutes.

Non-surgical embryo transfer

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. Intrauterine insertion method has been previously described [18]. Briefly, *in vivo* derived embryos were loaded in a 1 ml syringe using the following sequence of aspiration: 0.1 ml TL-Hepes-PVA medium, 0.1 ml air, 0.1 ml TL-Hepes-PVA medium with embryos, 0.1 ml air, and finally 0.1 ml TL-Hepes-PVA medium. Then, the syringe was attached to the catheter and the content introduced. An additional 0.3 ml TL-Hepes-PVA medium was used to wash the catheter. Finally, the catheter was removed and re-washed with the same medium on a culture dish verifying under the stereomicroscope that majority of embryos had been transferred.

The number of embryos per transfer and sow varied between 39 and 99 (Table 1). After the transfer, a dose of amoxicillin (29 mg/kg IM, Clamoxyl LA®; Pfizer, Madrid, Spain) was administrated to each recipient.

Pregnancy diagnosis, farrowing data and collection of piglets' samples

Pregnancy was diagnosed by ultrasound 21–26 days after the onset of estrous. Piglets derived from sows that were artificially inseminated with the same boars 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. Gestation length, farrowing rate, survival rate, and litter size were registered for each sow.

Immediately after birth, piglets from ET and AI group were identified and weighed using a digital hanging scale and the body weight (BW) was registered. After taking the measurements, the piglets were placed immediately with their mother.

On days 3 and 15 of life, all piglets were weighed and average daily weight gain (ADWG) was calculated using the formula:

ADWG = (End Weight - Previous Weight) / (End Date - Previous Date)

Blood samples at days 3 and 15 of life were collected by direct venipuncture of the jugular vein with a 23G x 25 mm needle and lithium heparin tubes (BD Vacutainer®, BD Spain). Blood tubes were transported to the laboratory and haematological analysis was performed using a haematology analyser (Siemens ADVIA® 120, USA). The parameters analysed were concentration of red blood cells (RBC, $_{X}10^{6}$ cells/μL), haemoglobin (HB, g/dl), haematocrit (HCT, %), mean corpuscular volume (MCV, fL), mean

corpuscular haemoglobin (MCH, pg), mean corpuscular haemoglobin concentration (MCHC, g/dl), cell haemoglobin concentration mean (CHCM, g/dl), red blood cell distribution width (RDW, %), haemoglobin concentration distribution width (HDW, g/dl), concentration of 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 (%), mean corpuscular volume of reticulocytes (MCVr, fL) and content of haemoglobin in 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 ($_X10^3$ cells/µL).

Then, blood was centrifuged at 1008 g for 10 min at room temperature and biochemical analysis was performed using a chemistry analyser for plasma (Olympus AU400, Japan). The biochemical 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), lipase (UI/L), total protein (TP, g/dl), albumin, (ALB, g/dl), globulin (GLOB, g/dl), triglycerides (TRIGL, mg/dl) and total bilirubin (TBIL, mg/dl).

Statistical Analysis

Data presented in the manuscript are analysed first by group (AI vs ET), without considering the sex of the piglets, and second by group and sex. All the data were submitted to D´Agostino-Pearson normality test to assess normality. Unpaired t-test was used when data were normally distributed, and non-parametric Mann-Whitney U-test was used in case of non-normal data distribution. 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. Software used was GraphPad Software, version 7 (La Jolla, California, USA).

Results

Pregnancies and farrowing rate

Three out of 11 ET-recipients became pregnant and 8 returned to oestrus at 17–25 days (Additional file 1). One of the three pregnant sows presented a synchrony of 0 h regarding the donor, and the other two of + 24 h. The number of embryos transferred was 40 to 99 from morulae to hatched blastocyst stage, resulting in a total of 39 piglets being born (Additional file 1). Gestation period was within expected normal range and piglets born after *in vivo* ET did not present any visible anatomical abnormality.

Piglets

There were no significant differences between ET and AI groups ($p = 0.9999$) in the total piglets born nor mean litter size, whose range was from 6 to 21 and from 11 to 18 in ET and AI groups, respectively (Table 2).

Table 1. Data related to non-surgical embryo transfer procedures (donors, embryos, synchrony, pregnancy, gestation and piglets).

Embryo transfer stage[1] procedure	Donors (n) (+/-)[2] collected (n)	Total embryos (n)	Embryos transferred (days)	Embryonic embryos donor (hours)	Age of recipient vs. (days)	Synchrony of length born (n)	Pregnancy piglets	Gestation	Total
1	3	43	40	M & B	6-7	+24	+	108	6
2	3	108	99	HB	7-8	+24	+	103	21
3	3	74	60	HB	8-9	+24	-	-	-
4	3	80	46	HB	6-7	0	-	-	-
5	1	39	39	B	6-7	0	-	-	-
6	2	56	50	HB	7-8	0	+	105	13
7	2	46	46	HB	7-8	0	-	-	-
8	2	32	32	HB	7-8	0	-	-	-
9	2	65	51	HB	7-8	0	-	-	-
10	2	41	41	HB	7-8	+24	-	-	-
11	2	52	47	HB	7-8	+24	-	-	-

- M=Morulae, B=Blastocyst, HB=Hatched Blastocyst
- (+) positive pregnancy detection at 21-26 days after onset of estrous; (-) negative pregnancy detection at 21-26 days after onset of estrous

Table 2
Piglets born, litter size mean and range, after in vivo embryo transfer or artificial insemination[1]

Group	Live-born piglets/Born piglets	Litter size[2]	Litter size range[2]
ET	38/40	13.33±7.50	6–21
AI	42/44	14.67±3.51	11–18
[1]No statistical differences were found between groups			
[2] Litter size and range considered live-born and stillborn piglets			

Table 3 discriminates the data concerning sex, birth weight and Kg per sow from piglets derived from ET and AI at birth. Thirteen males and 22 females from ET group, and 25 males and 14 females from AI group were born, and none of them presented any visible external morpho-anomalies, therefore being anatomically normal and weighing as expected. Birth weight in ET females was significantly higher than females from AI group. No differences were observed between males.

Table 3
Piglets' sex per group, birth weight and total Kg of piglets per sow[1]

Group	Piglet's sex	n	Birth weight (g)	Kg of piglets per sow
ET	Males	13	1368.00±354.10	17.19±5.21
	Females	22	1535.00±465.20[a]	
AI	Males	25	1285.00±360.80	16.03±7.59
	Females	14	1142.00±282.40[b]	
[a–b] Values in the same column with different superscripts are significantly different ($p < 0.05$)				
[1]Stillbirths and piglets that did not survive the first 48 h were excluded from this analysis (n = 5 for for embryo transfer – ET – and artificial insemination - AI).				

Analysing the total Kg of piglets obtained at birth per sow in both groups, no significant differences were observed.

A total of 5 animals from the ET group and 5 from the AI group were excluded from this study, due to being stillbirths or dying of natural causes (i.e. low weight or crushed by the sow).

Figure 1 shows the data regarding weight and ADWG of the piglets, separated by sex. Males did not present differences in weight at birth or on days 3 and 15 of life when compared by groups. Similarly, no differences were found when ADWG was measured on days 3 and 15 between groups. However, females showed significant differences at birth weight, being heavier in ET group compared to AI, but no differences were observed on the following days. In the same way, when ADWG was compared between females, no differences were observed.

Haematological and Biochemical parameters

Data presented in the manuscript are analysed by group and sex of the piglets. An additional analysis, independent of the sex, is presented as tables in Additional File 2 (Tables S1 to S4). For more detail on mean values and to address the full haematological and biochemical analysis, data analysed by sex are also presented in the form of tables on Additional File 2 (Tables S5 to S12). Representative graphs of the principal results are shown below.

Red Blood Cells

Red blood cell count (RBC)

No significant differences were found in male piglets for RBC (Fig. 2, RBC males). On the contrary, significantly higher values were found in females from ET group compared with those derived from AI on day 3, but the difference was lost on day 15 (Fig. 2, RBC females).

Haemoglobin (Hb)

No significant differences were observed between males of ET and AI in both days (Fig. 2, Hb males). Female piglets from ET group had higher values when compared to AI, but only on day 3 (Fig. 2, Hb females).

Haematocrit (HTC)

No differences were found in male piglets born by ET or AI in both days 3 and 15 (Fig. 2, HTC males). Female ET piglets showed significant higher values at day 3 compared to those derived from AI (Fig. 2, HCT females).

Mean corpuscular volume (MCV)

No significant differences were found between male piglets born through ET or AI (Fig. 2, MCV males). Female ET piglets showed lower values on day 15 when compared to AI (Fig. 2, MCV females).

Mean corpuscular haemoglobin (MCH)

No significant differences were found in MCH from males at any time but females from ET group showed lower levels when compared to AI group, on day 15 (Additional File 2, Tables S5 and S9).

Haemoglobin concentration distribution width (HDW)

ET male piglets showed higher mean value than AI piglets on day 15 (Additional File 2, Table S5), but female ET piglets showed lower values at day 3 when compared to AI (Additional File 2, Table S9).

Reticulocytes

No differences were found in male ET vs. AI piglets (Fig. 3. Reticulocytes males), but female ET piglets showed significant lower values in comparison to AI female piglets on day 15 (Fig. 3, reticulocytes females).

Mean corpuscular volume of reticulocytes (MCVr)

Significant differences were found between both groups on day 15, with ET male piglets showing lower values than their AI counterparts (Fig. 3, MCVr males) similarly to females (Fig. 3, MCVr females). However, only females showed the same pattern on day 3, with ET females showing lower values than AI females.

Haemoglobin content of reticulocytes (CHr)

Both male and female piglets from ET group showed significant lower values than AI counterparts on day 15 (Additional File 2, Tables S1 & S4 and S5 & S9). Significant differences were found between both groups, on day 15, with ET male piglets showing lower values than their AI counterparts.

White Blood Cells

White blood cells count (WBC)

The total number of WBC was significantly higher in the ET group for both males and females on day 3 of life (Fig. 4, WBC males and females); while at day 15, these differences were only significant between females.

Neutrophils *w*ere significantly higher in males from ET group vs. AI at day 3, with no further difference (Additional File 2, Tables S6 and S10).

Lymphocytes were also significantly higher for female ET piglets, in both days 3 and 15 (Additional File 2, Tables S6 and S10).

Platelets

Platelets (PLT)

No significant differences in 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 (Additional File 2, Tables S7 and S11).

Platelecrit (PCT)

As shown in Fig. 4 (PCT, males and females), ET-males and females showed significant lower values compared to AI on days 3 and 15 of life, respectively.

Mean platelet volume (MPV)

Male ET piglets had higher values at day 15 than AI piglets (Fig. 4, MPV males). Females did not show any difference between groups (Fig. 4, MPV females).

Mean platelet mass (MPM) *s*howed only significant differences for males derived from the ET group at day 3 regarding AI (Additional File 2, Table S7).

Platelet mass distribution width (PMDW)

The only difference found was regarding day 15, where ET male piglets had higher values than AI piglets (Additional File 2, Table S7).

Large PLT

No significant differences were found in males (Suppl. Table S7) for large PLT but females ET-derived showed lower values in comparison with females AI-derived on day 15 (Additional File 2, Table S11).

Biochemical analysis

Urea

Males did not present significant differences between groups (Fig. 5, Urea males). Conversely, females from the ET group showed values below those derived from AI, being significant different on day 15 of life (Fig. 5, Urea females).

Alkaline phosphatase (ALP)

Both males and females derived by ET showed values above AI-piglets on day 3 (Fig. 5, ALP males and females), however, only males from the ET group showed significant differences regarding the AI group. On the other hand, on day 15 of life, no significant differences were found for piglets of any sex.

Albumin (ALB)

The ALB concentrations were significantly lower on day 3 in males from the ET group compared to those from the AI group (Fig. 5, ALB males).

Total bilirubin (TBIL)

Both males and females showed a lower concentration of TBIL on day 3, with only females from the ET group showing significant differences compared to those from the AI group (Fig. 5, TBIL males and females). However, on day 15 only trend was observed ($p = 0.0551$) between females.

Gamma-glutamyl transferase (GGT)

Piglets (males and females) born by ET presented values above those born by AI on days 3 and 15 of life, with only males from ET showing significant differences compared to those born by AI on day 15 (Additional File 2, Tables S8 and S12).

Aspartate aminotransferase (AST)

Higher values were detected in ET-derived male piglets' vs. AI on day 15 (Additional File 2, Table S8).

Lipases

On day 15 of life, piglets from both groups considerably decreased their concentration of lipases compared to day 3, however, only significantly differences were found in males from ET regarding those from AI (Additional File 2, Table S8).

Globulins (GLOB)

Although no significant differences were found, when the concentration of globulins was analysed, a trend was observed between males from ET having higher values compared to AI on day 15 ($p = 0.0538$).

Discussion

The research and development of 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 of genetic material without risk of disease transmission and reducing transportation costs (reviewed by [19]); second, for the human species, there is compelling evidence that the pig is an useful model to decipher the long term impact of each aspect of the ART without confounding factors such as those related to the fertility of the parents, 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 [20].

In this context, this study focuses on one of the ART, the embryo transfer, using the porcine model to shed light on its consequences on the pregnancy and farrowing rates of the mothers and on the phenotype of the offspring from birth to day 15 of life.

The present study 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 would be 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, were 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 [5, 21, 22], but most of them have been in mice, due to its easy handling, short gestation length, and other beneficial characteristics [23]. 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 performed in the current study.

It is important to note that the percentage of animals that became pregnant after embryo transfer in our study (3 out of 11, 27.27%) 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 + 24 h of asynchrony in estrous regarding the donors and, as Angel et al. [18] demonstrated, the ideal time frame of asynchrony must be between 0 and − 48 h. Despite this issue, 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 heavier body weight in absolute terms than AI piglets 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*. [11], 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 the weight at birth higher in ET than in AI, as others reported [11], but such differences disappeared on days 3 and 15. This fact, however, should be kept in mind for future studies on the long-term phenotypes of ET animals 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 large offspring syndrome [24, 25] as well as to the Beckwith–Wiedemann syndrome in humans [26], although it has not been described in pigs until now. Another reason not to obviate this finding is that the differences were observed only in females, which are the animals kept in the farm as future mothers for next generations. The likely impact of the increased birth weight of a mother on her further offspring is unknown and deserves future research. Still, 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 [18, 27].

As for ADWG, 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 [28].

Although there are few references to haematological and biochemical parameters in the porcine species [29], there are various studies in cloned pigs where different blood parameters have been evaluated [30–32]. In addition, other studies in piglets of two age groups with and without iron supplementation [16] have recently provided some reference intervals that could help us interpret the results obtained. The different parameters analysed to assess the general health status of the piglets showed some differences but also high variability. Few authors attribute this finding to the immune system development during the growth of piglets [15, 33]. 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. [34], 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, lower values 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 lower in males derived from ET. Godyn et al. [35] reported that these parameters might be indicators of the existence of iron deficiency; however, none of the values were outside the normal range nor was the Hb concentration altered. On the other hand, Bhattarai et al. [36] 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.

WBC 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 [37] and these values were within the normal range, we cannot, again, give any clinical significance to these differences.

Pliszczak-Król et al. [38] 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 being found in the PLT concentration, significantly lower values 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 values 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 [39], the clinical significance, reference values and usefulness of most of the platelet indices are still under investigation [40] 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, including 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 day. According to some authors, an increase in ALP levels is associated with increased production of osteoblasts due to the growth of piglets [14, 41]. In contrast, a decrease in the concentration of this enzyme is related to a decrease in phosphorus levels in diet [15]. On the other hand, Stone [42] reported that high ALB levels are associated with the physiological maturation of the liver but it should also be noted that, the ratio of albumin:globulin decreases from day 3 to 15, as expected after colostrum intake during the first days of life [43].

In addition, despite an increase in AST concentration being associated with increased physical activity or the existence of muscle damage [44], all values were within the normal range. According to Yu *et al.*, [45], 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. Besides, Dubreuil and Lapierre [46] 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 were found in CK, our results showed a high variability in this parameter, also being influenced by the age and sex of the animals [47, 48]. 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 [29].

Conclusions

Even though some differences were noted between ET-piglets and their AI counterparts, the embryo transfer technique, in absence of other ART, does not seem to affect the growth, haematological or biochemical parameters of the offspring at birth, day 3 or day 15 of age, in terms of general health and expected development. This study provides evidence that the embryo transfer is a safe procedure and even more interestingly, that the development of this technique in pigs can be a useful tool for the industry sector.

Abbreviations

ART
Assisted reproductive technologies
ET
Embryo transfer
AI
Artificial insemination
IVF
In vitro fertilization
ICSI
Intracytoplasmic sperm injection
ADWG
Average daily weight gain
IM
Intramuscular administration

IV
Intravenous administration
PVA
Polyvinyl alcohol
BW
Body weight
RBC
Red blood cells
HB
haemoglobin
HCT
Haematocrit
MCV
Mean corpuscular volume
MCH
Mean corpuscular haemoglobin
MCHC
Mean corpuscular haemoglobin concentration
CHCM
Cell haemoglobin concentration mean
RDW
Red blood cell distribution width
HDW
Haemoglobin distribution width
WBC
White blood cells
MCVr
Mean corpuscular volume of reticulocytes
CHr
Content of haemoglobin in reticulocytes
PCT
Platelecrit
MPV
Mean platelet volume
PDW
Platelet distribution width
PCDW
Platelet component distribution width
MPM
Mean platelet mass
PMDW
Platelet mass distribution width
CREA
Creatinine
CK
Creatine kinase
ALP
Alkaline phosphatase
GGT

Gamma-glutamyl transferase
AST
Aspartate aminotransferase
ALT
Alanine aminotransferase
TP
Total protein
ALB
Albumin
GLOB
Globulin
TRIGL
Triglycerides
TBIL
Total bilirubin

Declarations

Ethics approval

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

Consent for publication

All the authors read and agree to the content of this paper and its publication.

Availability of data and material

Supplementary Tables (S1 to S12).

Competing interests

The authors declare no competing interest.

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Author’s contributions

PC designed the experiment; all authors conducted the research; EPO, JG and SC analysed the data; EPO, SC and PC wrote the manuscript; all authors reviewed the manuscript and approved the final version.

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Figures

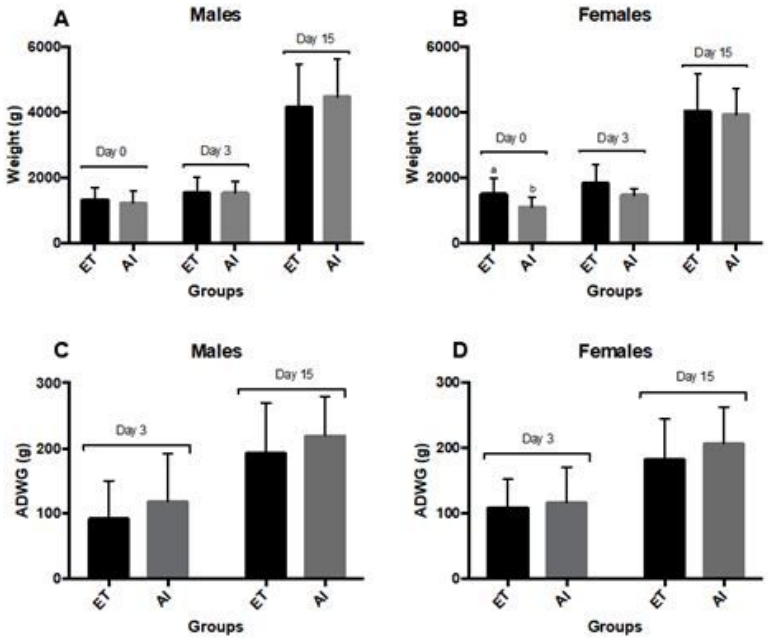

Figure 1

Weight and average daily weight gain (ADWG) of piglets born by ET or AI. Data are mean±SD, separated by group (ET or AI) and sex (males or females). Significant differences ($p<0.05$) are represented with different letters (a, b).

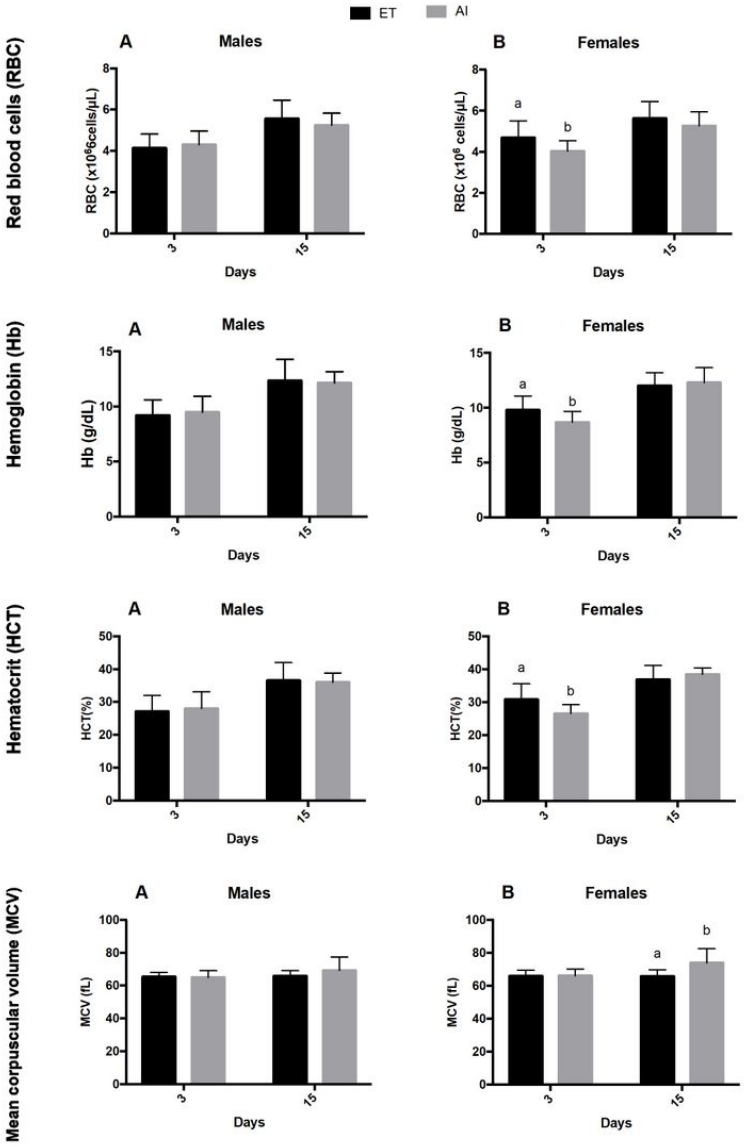

Figure 2

Haematological analysis of red blood cell parameters of piglets born by ET or AI. Red blood cells count, hemoglobin, hematocrit and mean corpuscular volume in embryo transfer (ET, black bar) or artificial insemination (AI, grey bar) groups. Values are represented by means±SD and separated by age (days) and sex. Significant differences ($p<0.05$) are represented with different letters (a, b).

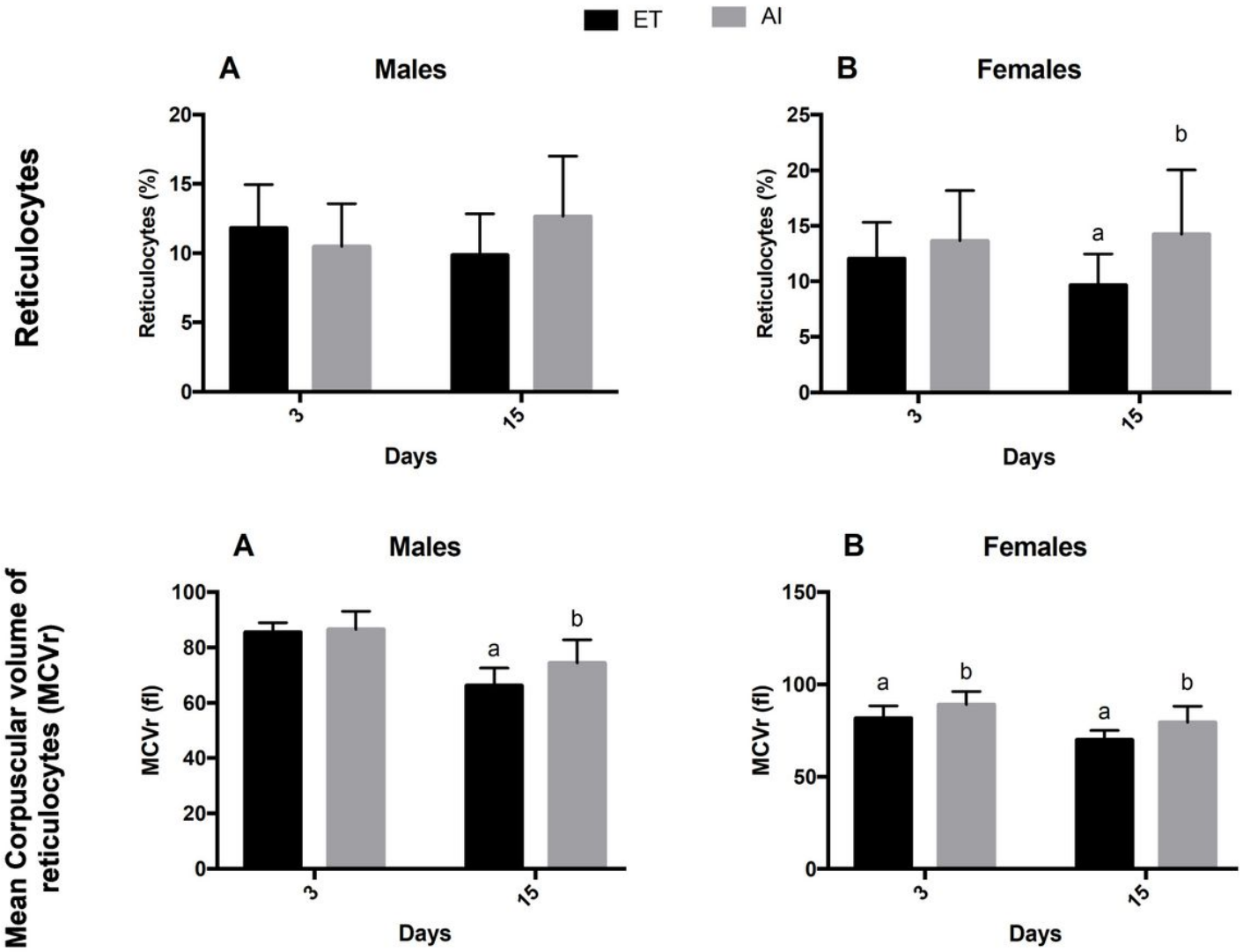

Figure 3

Haematological analysis of reticulocytes parameters of piglets born by ET or AI. Reticulocytes percentage and mean corpuscular volume of reticulocytes of piglets born by in vivo embryo transfer (ET, black bar) or artificial insemination (AI, grey bar). Values are represented by means±SD and separated by age (days) and sex. Significant differences ($p<0.05$) are represented with different letters (a, b).

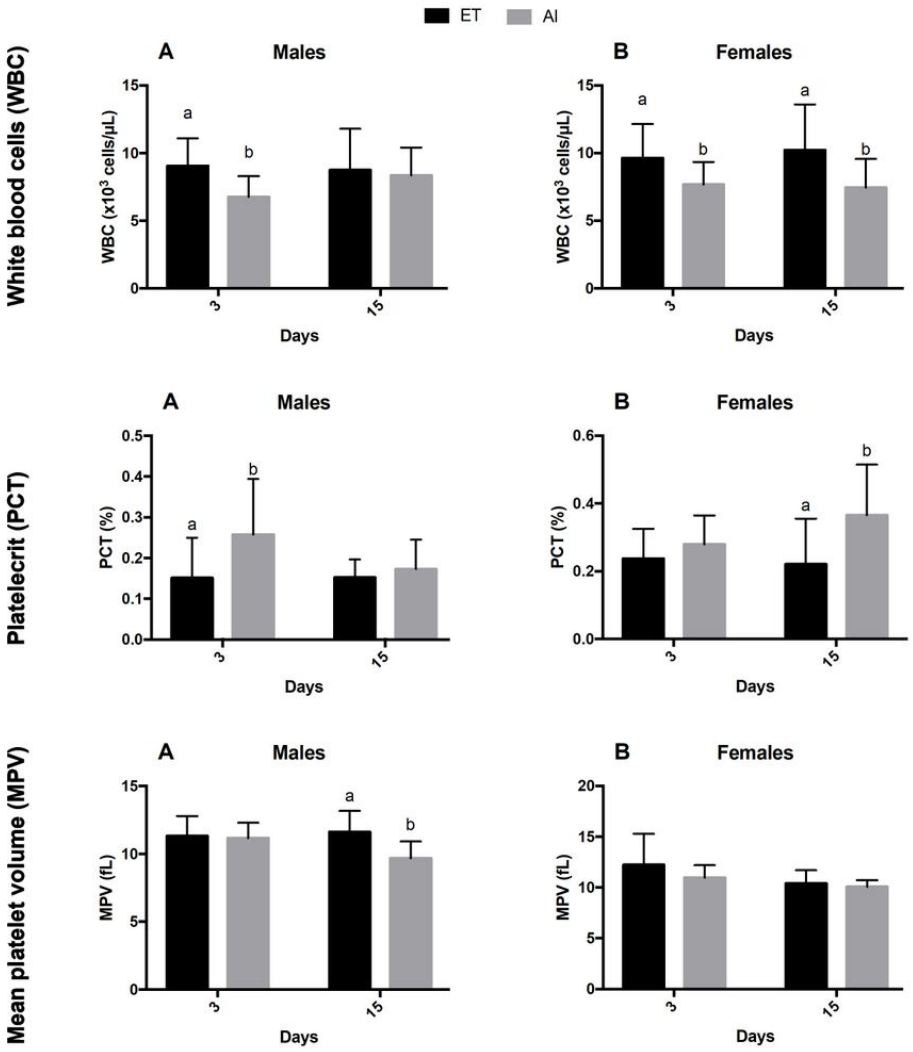

Figure 4

Haematological analysis of white blood cell count and platelets parameters of piglets born by ET or AI. Platelecrit and mean platelet volume of piglets born by in vivo embryo transfer (ET, black bar) or artificial insemination (AI, grey bar). Values are represented by means±SD and separated by age (days) and sex. Significant differences ($p<0.05$) are represented with different letters (a, b).

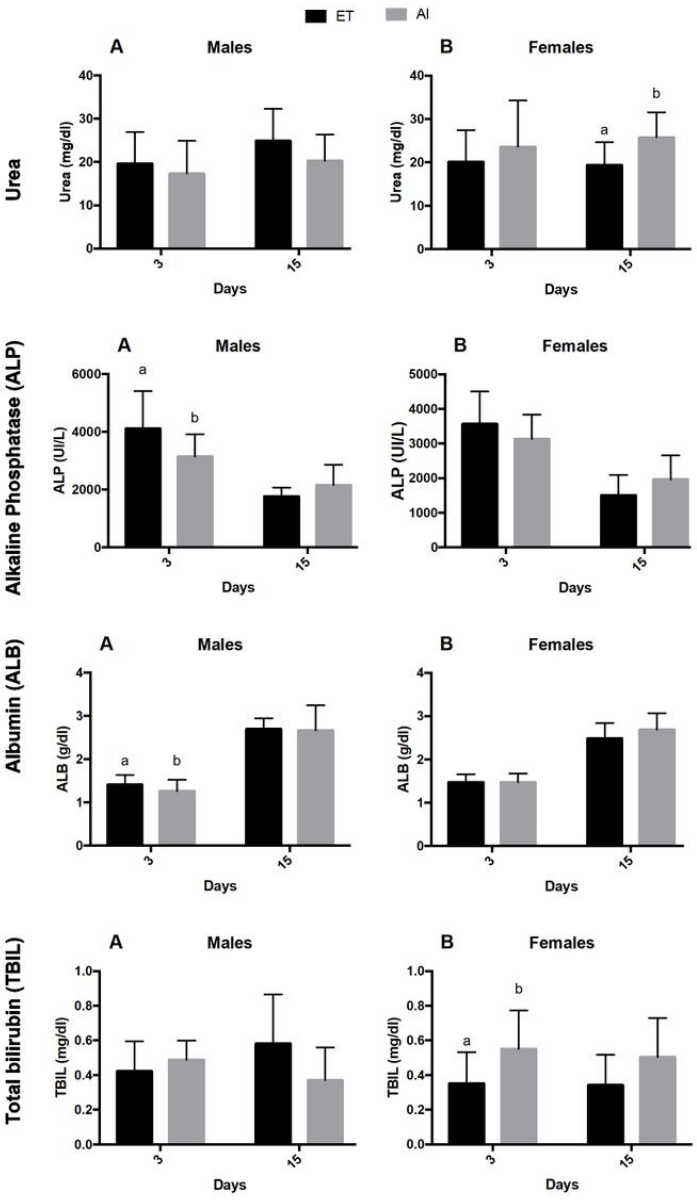

Figure 5

Biochemical analysis of piglets born by ET or AI. Concentrations of urea, alkaline phosphatase, albumin and total bilirubin in piglets born by in vivo embryo transfer (ET, black bar) or artificial insemination (AI, grey bar). Values are represented by means±SD and separated by age (days) and sex. Significant differences ($p<0.05$) are represented with different letters (a, b).

Supplementary Files

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