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Institute of Genetics and Animal Biotechnology
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Effects of asynchrony between embryo development and uterine environment on embryo survival and development in rabbits*

María-José Argente[1], Marga Antón[1], Rafik Belabbas[2], Raquel Muelas[1], Ivan Agea[1], María de la Luz García[1]**

[1] Centro de Investigación e Innovación Agroalimentaria y Agroambiental, Universidad Miguel Hernández de Elche, Ctra de Beniel km 3.2, 03312 Orihuela, Spain

[2] University Blida 1, Biotechnology Laboratory of Animal Reproduction, Institute of Veterinary Sciences, Blida, Algeria

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The aim of this study was to estimate the effects of asynchrony between embryo development and uterine environment on embryo survival and subsequent foetal development in rabbits. Embryos at 48 and 72 hours post-coitum (p.c.) were transferred into 22 females at 48 h of pseudopregnancy and into 20 females at 72 h of pseudopregnancy. Similar embryonic survival was obtained in synchronous recipients with embryos transferred at 48 h p.c. (0.40) and asynchronous recipients with embryos transferred at 72 h p.c. (0.47). However, embryonic survival was 26% higher in synchronous recipients with embryos transferred at 72 h p.c. compared to asynchronous recipients with embryos transferred at 48 h p.c. (0.56 vs. 0.30). These findings suggest that embryos have the ability to wait for the favourable uterine environment for implantation and that a less advanced embryo development into a more advanced uterine environment increases implantation failures. Similar foetal survival rates were found in recipients at 72 h p.c. regardless of the embryo development. Nevertheless, foetal survival was 23% higher in synchronous recipients with embryos transferred at 48 h p.c. than in asynchronous recipients with embryos transferred at 72 h p.c. (0.91 vs. 0.68). Most foetal losses in asynchronous recipients with embryos transferred at 72 h p.c. occurred close to implantation, in

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**Corresponding author: mj.argente@umh.es

comparison to synchronous recipients with embryos transferred at 48 h p.c. (38.1 vs. 12.4%). Blood supply seems to be key in implantation losses and foetal development of asynchronous recipient females with embryos transferred at 72 h p.c. The percentage of dead foetuses with placentas receiving fewer than three blood vessels was higher than those with placentas receiving more than three blood vessels in embryos transferred at 72 h p.c. into asynchronous recipient females than in embryos transferred at 48 h p.c. in synchronous recipient females (69% of dead foetus with <3 vessels vs. 17% with >3 vessels in embryos transferred at 72 h p.c. in asynchronous recipients in comparison to 14% with <3 vessels vs 12% with >3 vessels in embryos transferred at 48 h p.c. into synchronous recipients). In conclusion, a less advanced embryonic development than that of the pseudopregnant uterine horn increases pre- and peri-implantation losses, while a more advanced embryonic development than that of the pseudopregnant uterine horn increases foetal losses as a consequence of lower blood supply in each implanted site.

KEYWORDS: asynchrony / available uterine space per foetus / embryonic survival / transferred embryos / rabbit

Litter size is an important and complex physiological trait in prolific species, affected by several sequential components such as the number of corpora lutea, fertilisation rate and embryo survival to term of gestation [Argente 2016]. In rabbits ovulation normally occurs 10-12 hours after the onset of hormonal or mechanical stimulus [Varian *et al.* 1967]. Overlap of cell stage among different embryos is commonly observed, e.g. rabbit embryos are found in the 16-cell and morula stage at 48 h post mating, reaching the compact morula and blastocyst stage at 72 h post mating [Meng *et al.* 2014]. Blastocysts enter the uterus at day 3, move rapidly from day 3 to 5, move slowly and align equidistantly around day 6, and finally implant around day 7 [Böving 1956]. During the first 3 days following ovulation the rabbit embryo acquires a glycoprotein layer known as the mucin coat, which is accumulated during oviductal transport [Fischeret *et al.* 1991, Joung *et al.* 2004]. At a late preimplantation stage complex transformations occur in the mucin coat, incorporating additional material at days 5 and 7 during the passage to the uterus that allows the blastocyst to escape from its zona pellucida and attach to the uterine epithelium [Denker 2000].

Approximately 30% to 40% of ova shed do not result in foetuses at term [reviewed by Blasco *et al.* 1993]. Failures in fertilisation and embryonic losses due to carrying chromosomal abnormalities are low, below 5% [Mocé and Santacreu 2010] and 10% [Fujimoto *et al.* 1974], respectively. There are two critical moments for prenatal survival. The first occurs between days 8 and 12 of gestation with around 14% of total prenatal losses, when endometrial attachment, decidual reaction and the first steps of foetal and haemochorial placental development take place [Adams 1959, Santacreu *et al.* 2005]. The second is between days 17 and 24 with 20-30% of total prenatal losses, corresponding to the period of uterine enlargement, when the haemochorial placenta has finished its development and nutrition of the foetus begins to be controlled by the placenta [Argente *et al.* 2008, Mocé *et al.* 2004]. Available uterine space is key to placenta development and vascular blood supply reaching the placenta [Argente *et al.* 2003, Argente *et al.* 2008, Vicente *et al.* 2013a].

An abnormal early embryo development and asynchrony between the development of embryos and the maternal environment would likely explain a large share of preimplantation losses and peri-implantation failures [Laborda *et al.* 2012, Techakumphu *et al.* 1987]. Mucin coat thickness is directly related to successful rabbit embryo implantation [Murakami and Imai 1996]. Likewise, uterine glands secrete critical factors during early pregnancy that stimulate uterine receptivity to embryo implantation and stromal cell decidualisation, such as tissue inhibitor of metalloproteinases 1 (TIMP1) [Hwang *et al.* 2000], oviductal glycoprotein 1 (OVGP1) [Buhi 2002], secretoglobin family 1A member 1 (also known as uteroglobin) [Riffo *et al.* 2007], insulin-like growth factor 1 (IGF1) [Lin *et al.* 2003] and avb3 integrin [Illera *et al.* 2003].

Successful implantation requires synchronisation between the acquisition of implantation competency by the blastocyst and a receptive state in the uterine endometrium. The crosstalk between the blastocyst and the uterus can only occur during a brief period, namely the implantation window [Lim and Dey 2009]. Embryo transfer in synchronous and asynchronous recipients can help us establish the time frame of the implantation window and the limiting points in a successful implantation.

The rabbit is an excellent animal model to study the effects of asynchrony between embryo development state and maternal environmental on pre- and post-implantation losses and success of gestation, as pregnancy can be precisely timed in transferred embryos and recipient females [Harkness *et al.* 2010]. Moreover, the rabbit uterus is constituted by two separate functional uterine horns, allowing the transfer of two sets of embryos into the same recipient female without inter-horn migration [Blasco *et al.* 1994].

The aim of this study was to estimate the effects of asynchrony between embryo development and maternal environment on embryo survival and subsequent foetal development in rabbits.

Material and methods

Material

In this study a total of 44, 77 and 42 multiparous females were used as the control, donors and recipients, respectively. Females came from a divergent selection experiment for litter size variability [Argente *et al.* 2017]. The base population stemmed from a synthetic line (V) selected for 12 generations by litter size at weaning [Estany *et al.* 1989], and then for 10 generations by uterine capacity [Argente *et al.* 1997]. Females were housed at the Miguel Hernández University of Elche farm in individual wire cages. Animals were kept under a controlled 16-h light: 8-h dark photoperiod and were fed a commercial diet.

All experimental procedures involving animals were approved by the Miguel Hernández University of Elche Research Ethics Committee (Reference number DTA-MJA-002-08), in accordance with the International Guiding Principles for

Biomedical Research Involving Animals, as promulgated by the Society for the Study of Reproduction and EU Directive 2010/63/EU.

Embryo recovery

Donor females came from the first generation of a selection experiment on litter size variability. Natural matings were carried out with males from the same generation as the donor females. A total of 38 and 39 females were slaughtered by intravenous (i.v.) injection of sodium thiopental at 50 mg/kg body weight (® Tiobarbital, B. Braun Medical S. A., Barcelona, Spain) at 48 and 72 hours post-coitum (h p.c.), respectively. The entire reproductive tract was removed after slaughter. The oviducts and the first third of the uterine horns were excised and flushed once with 5 mL of Dulbecco´s Phosphate Buffered Saline (®DPBS, Sigma, Alcobendas, Madrid, Spain) supplemented with Cl_2Ca (0.132 g/L), 0.2% of bovine serum albumin (®BSA, Sigma, Alcobendas, Madrid, Spain) and antibiotics (penicillin G sodium 300.000 IU, penicillin G procaine 700.000 IU and dihydrostreptomycin sulphate 1250 mg; ®Penivet 1, Divasa Farmavic, Barcelona, Spain) at room temperature. Embryos were counted and classified using a binocular stereoscopic microscope (Leica MZ75-200x) as normal or abnormal according to morphological criteria; embryos were classified as normal when they presented homogenous cellular mass and intact zona pellucida [Hafez 1993]. Embryo classification was always carried out by the same operator. After recovery, morphologically normal embryos at 48 and 72 h p.c. were kept at room temperature until transfer.

Embryo transfer

Recipient females came from the first generation of a selection experiment on litter size variability. All transfers were carried out after the fourth parity when the females were non-lactating. In rabbits, vulva colour allows us to determine whether a female is receptive [McNitt and Moody 1989]; females that presented vulva colour associated with the receptive status were induced to ovulate by an intramuscular (i.m.) injection of 1μg buserelin acetate (®Hoechst Marion Roussel, S.A., Madrid, Spain) 48 or 72 h before transfer. Rabbits were anaesthetised to perform the transfers with an i.m. injection of Xylazine (®Rompun 2%, Bayer AG, Leverkunsen, Germany) at a rate of 0.2 mL/kg body weight. After 5 min this injection was followed by an i.v. dose of 2-3 mL of Ketamine HCL and chlorbutol (®Imalgéne 500, Merial-Lyon, France) into the marginal ear vein. Embryo transfers were performed by means of an abdominal laparotomy. Only normal embryos were transferred. One of the oviducts of females at 48 h of pseudopregnancy received from five to seven embryos at 48 h p.c. (synchrony 48 h), while another oviduct received from five to seven embryos at 72 h p.c. (asynchrony -24 h). The same procedure was performed in females at 72 h of pseudopregnancy: one oviduct received from five to seven embryos at 48 h p.c. (asynchrony +24 h) and another oviduct received from five to seven embryos at 72 h p.c. (synchrony 72 h, Fig. 1). The control group was composed of females mated

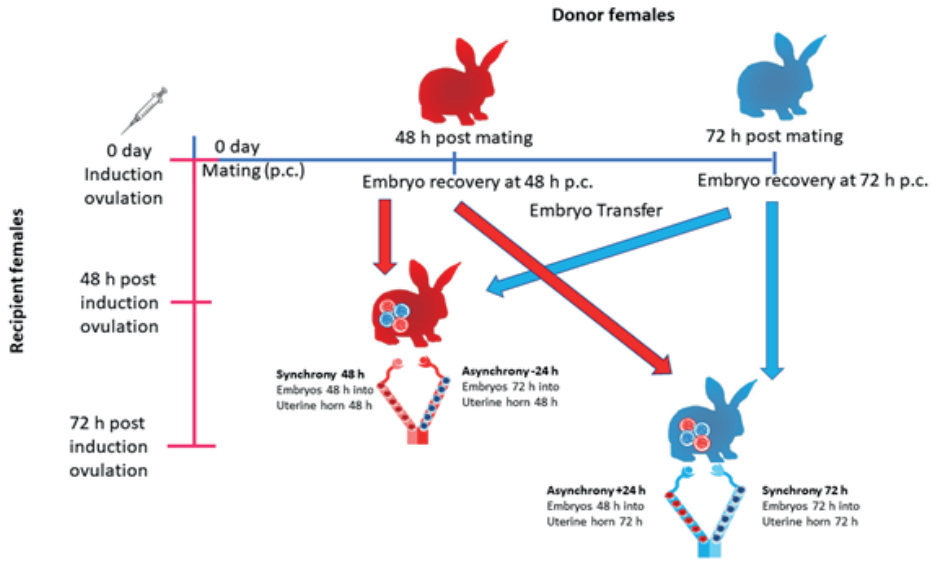

Fig. 1. Protocol for embryo transfer at 48 h and 72 h p.c. into females at 48 h and 72 h of pseudopregnancy.

to fertile males. Thus, 114 embryos at 48 h p.c. and 134 embryos at 72 h p.c. were transferred into a total of 22 females at 48 h of pseudopregnancy, while 129 embryos at 48 h p.c. and 118 embryos at 72 h p.c. were transferred into a total of 20 females at 72 h of pseudopregnancy.

Data collection

Recipient and control females were slaughtered at day 21 of gestation. The ovaries and the uterine tract were collected. The number of corpora lutea in each ovary was counted. An external examination of the uterine horn was performed to locate the implantation site of each foetus. The number of blood vessels reaching each implantation site was used to estimate the vascular supply to each foetus, as in Argente *et al.* [2008]. Foetuses were classified as receiving fewer than three blood vessels, three blood vessels, and more than three blood vessels. Afterwards, the mesometrium was trimmed from the right and left uterine horns. Each uterine horn was opened lengthwise, and the position and status of each foetus were recorded by starting on the ovarian end. There were three uterine positions: oviduct (the first foetus nearest the ovarian end), middle (foetuses in the middle of the uterine horn) and cervix (the last foetus in the uterine horn from the ovarian end). The foetuses were classified according to their status as live (S1), dead foetus, atrophic foetal with maternal placenta (S2), and atrophic maternal placenta or decidual reactions (S3). Implantation sites are identifiable during early gestation and they remain identifiable on day 21 of gestation or later if the embryo dies shortly after implantation site formation. Each

foetus and its foetal placenta were separated from the maternal placenta and then weighed. The distance from the crown to the tail base in each foetus and the length of its foetal placenta were measured using a slide calliper. A .jpg image of the foetal placenta was taken. The length of each maternal placenta and the distances between adjacent maternal placentas and the oviduct and the cervical end were measured on the uterine horn using a slide calliper. Uterine horn space occupied per foetus on the oviduct or cervical end was calculated as the distance from the tip of the uterine horn to the maternal placenta plus the length of its maternal placenta and one half of the distance to the adjacent maternal placenta. Space for other foetuses was considered to be the length of their maternal placentas plus half of the total distance to their two adjacent maternal placentas. This measurement was considered to be the uterine space available per foetus (Fig. 2). Maternal placenta was extracted from the uterine horn and then weighed. A .jpg image of the maternal placenta was taken. AutoCAD 12.0 software was used to measure the area of the foetal and maternal placentas after scaling the .jpg image.

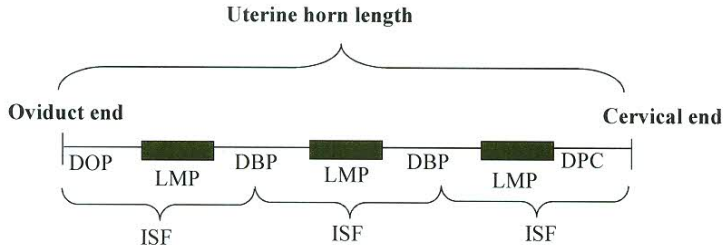

Fig. 2. Individual available space per foetus (ISF) measured on uterine horn. DOP - distance between the oviduct end and the first maternal placenta. LAMP - length of maternal placenta. DB - distance between two adjacent maternal placentas. DPC - distance between the last maternal placenta and the cervical end.

Traits

The following traits were measured: the number of corpora lutea (CL) in each ovary, number of implanted embryos (IE) estimated as the number of implantation sites (S1+S2+S3), number of live foetuses at 21 days of gestation (LF), embryonic survival (ES) estimated as IE/the number of transferred embryos in recipient females or the number of CL in control females, foetal survival at day 21 of gestation (FS) estimated as LF/IE, prenatal survival at day 21 of gestation (PS_{21d}) estimated as LF/ the number of transferred embryos in recipient does females or the number of CL in control females, distance between the oviduct and the first maternal placenta (DOP), and distance between the last maternal placenta and the cervix (DPC).

Variables measured in foetuses included the individual weight of the foetus (IWF), its foetal (IWFP) and maternal placenta (IWMP), crown-rump length (CRL), length of the foetal (LFP) and maternal placenta (LMP), area of foetal (AFP) and maternal placenta (AMP), available uterine space per foetus (ISF), and efficiency of foetal placenta (EP) estimated as IWF/IWFP.

Statistical analyses

Differences between control, synchronous and asynchronous recipient females in terms of prenatal traits

Only data from gestating females at day 21 were included in the analyses. CL, IE, LF, ES, FS, PS_{21d}, DOP and DPC were analysed with the following linear model:

$$y_{ijkl} = \mu + T_i + L_j + S_k + m_{ijkl} + e_{ijkl}$$

μ – the overall mean;

T_i – the fixed treatment effect (with five levels: synchrony of recipient oviduct with embryos transferred at 48 h p.c., asynchrony of +24h between recipient oviduct with embryos transferred at 48 h p.c., asynchrony of -24 h between recipient oviduct with embryos transferred at 72 h p.c., synchrony of recipient oviduct with embryos transferred at 72 h p.c., and control females mated to fertile males);

L_j – the fixed uterine side effect (with two levels: right or left uterine horn);

S_k – the fixed season effect (with four levels: spring, summer, autumn and winter);

m_{jkl} – the random effect of female;

e_{ijkl} – the random residual term.

Asynchrony between transferred embryos and uterine horn recipient, blood supply and uterine position in terms of embryonic survival and development

The distribution of percentages of live and dead foetuses in relation to asynchrony embryos and recipient uterine horn, the number of blood vessels reaching each implantation site and locations of the foetuses were analysed using the χ^2 test on the basis of the contingency tables.

Differences between control, synchronous and asynchronous recipient females in terms of foetal traits

IWF, CRL, IWFP, LFP, AFP, IWMP, LMP, AMP, ISF and EP were analysed using the following mixed model:

$$y_{ijklmno} = \mu + T_i + L_j + S_k + P_l + V_m + (TV)_{im} + m_{ijklmn} + e_{ijklmno}$$

where:

μ – the overall mean;

T_i – the fixed treatment effect;

L_j – the fixed uterine side effect;

S_k – the fixed season effect;

P_l – the fixed effect of the position of the foetus in the uterine horn (with three levels: oviduct, middle or cervix);

V_m – the fixed effect of the number of blood vessels reaching the foetal implantation site (with three levels: fewer than three blood vessels, three blood vessels, and more than three blood vessels);

$(TV)_{im}$ – the effect of the interaction of treatment by number of blood vessels;

m_{ijklmn} – the random effect of female;

$e_{ijklmno}$ – the random residual term.

Bayesian methodology was used for all analyses. Bounded uniform priors were used for all effects with the exception of the female effect, which was considered normally distributed with mean 0 and variance I, where I is an identity matrix and is the variance of the female effect. Female and residual effects were independent. Residuals were a priori normally distributed with mean 0 and variance I. (is the residual variance). The priors for the variances were also bounded uniform. Features of the marginal posterior distributions for all unknowns were estimated using Gibbs sampling. A chain of 60,000 samples was used, with a burn-in period of 10,000. Only one out of every 10 samples was saved for inferences. Convergence was tested using the Z criterion of Geweke and Monte Carlo sampling errors were computed using time-series procedures.

These computations were performed with the use of the Rabbit package program [Blasco, Mekkawy, González-Peña] developed by the Institute for Animal Science and Technology (Valencia, Spain).

Results and discussion

Effect of embryo manipulation and asynchrony between embryo and uterine environment on prenatal survival

No differences were found for the number of CL among the control, synchronous and asynchronous females (see Fig. 3). However, control females had a higher number of implanted embryos per uterine horn (4.76 embryos) than synchronous females at 48 h p.c. (2.58 embryos) and 72 h p.c. (3.10 embryos), as a consequence of a greater embryonic survival (0.72 vs. 0.40 and 0.56, respectively). Foetal survival was high and similar among control (0.87) and synchronous recipient females at 48 h (0.91) and 72 h p.c. (0.83). These results show that embryo manipulation mainly affects embryo survival, increasing preimplantation losses. However, once attachment of the blastocyst to the endometrium takes place, the transferred embryo has a similar chance of survival as a non-manipulated embryo.

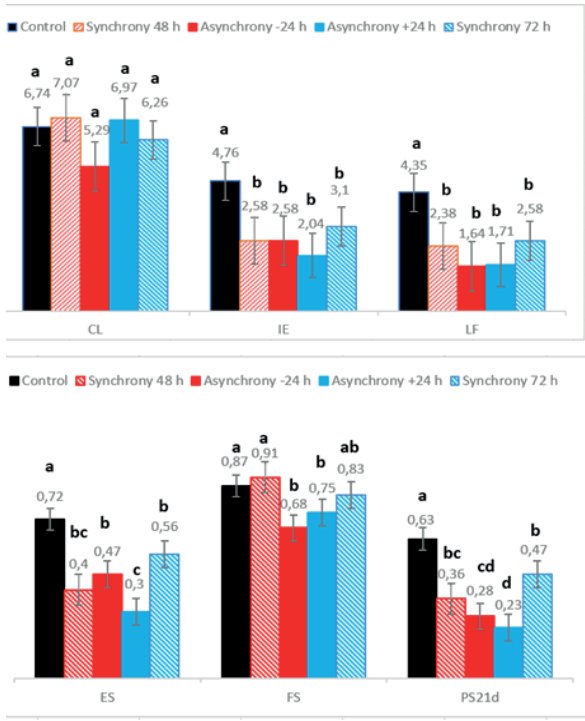

Fig. 3. Number of corpora lutea (CL), number of implanted embryos (IE), number of live foetuses at day 21 of gestation (LF), embryonic survival (ES), foetal survival (FS) and prenatal survival at day 21 of gestation (PS_{21d}) for control, synchrony and asynchrony groups. Bars represent the standard errors of the means. Different letters indicate significant differences.

Asynchronous females showed a lower embryonic survival than control females and embryonic survival was lower in asynchronous females with embryos at 48 h p.c. than those with embryos at 72 h p.c. (0.30 vs. 0.47, Fig. 3). Mucin coat thickness is essential in embryo development, as it is related to time spent by the embryo in the oviduct. Lower embryonic survival rates in asynchronous recipient females with embryos of 48 h p.c. would be consistent with a thinner mucin coat. Concerning foetal survival asynchronous females with embryos at 48 h p.c. and 72 h p.c. displayed similar values, but lower than in control females (0.68 and 0.75 vs. 0.87, Fig. 3).

Similar embryonic survival was obtained in recipient females at 48 h p.c., independently of embryo development (0.40 in synchrony at 48 h vs. 0.47 in asynchrony at -24 h, Fig. 3), with embryonic survival by 26% higher in asynchrony at 72 h than in asynchrony at +24 h (0.30 vs 0.56, Fig. 3). These findings suggest that embryos have the ability to wait for the uterine environment to become favourable for implantation and the development of a less advanced embryo in a more advanced uterine environment increases implantation failures.

Similar foetal survival was shown in recipient females at 72 h p.c., regardless of embryo development (0.83 in synchrony at 72 h vs 0.75 in asynchrony at +24 h, Fig. 3), with foetal survival 23% higher in synchronous recipients with embryos at 48 h p.c. compared with that in asynchronous recipients with embryos at 72 h p.c. (0.91 in synchrony at 48 h vs. 0.68 in asynchrony at -24 h, Fig. 3). Most foetal losses

Table 1. Percentage of live foetuses (S1), dead foetuses, atrophic foetal and maternal placenta (S2) and atrophic maternal placenta or decidual reaction (S3) in control and recipient females at 48 and 72 h p.c. with embryos transferred at 48 and 72 h p.c.

Item	Control ($N = 44$)	Synchrony at 48 h ($N = 22$)	Asynchrony at -24 h ($N = 22$)	Asynchrony at +24 h ($N = 20$)	Synchrony at 72 h ($N = 20$)
S1	87.2 (n=183)	87.6 (n=42)	61.9 (n=39)	79.5 (n=35)	72.4 (n=42)
S2	10.9 (n=23)	6.2 (n=3)	12.7 (n=8)	11.4 (n=5)	12.1 (n=7)
S3	1.9 (n=4)	6.2 (n=3)	25.4 (n=16)	9.1 (n=4)	15.5 (n=9)
Significance	a	ac	b	c	bc

N – number of females; n – number of observations.
a, b, c – different letters indicate statistical differences between groups at P <0.05.

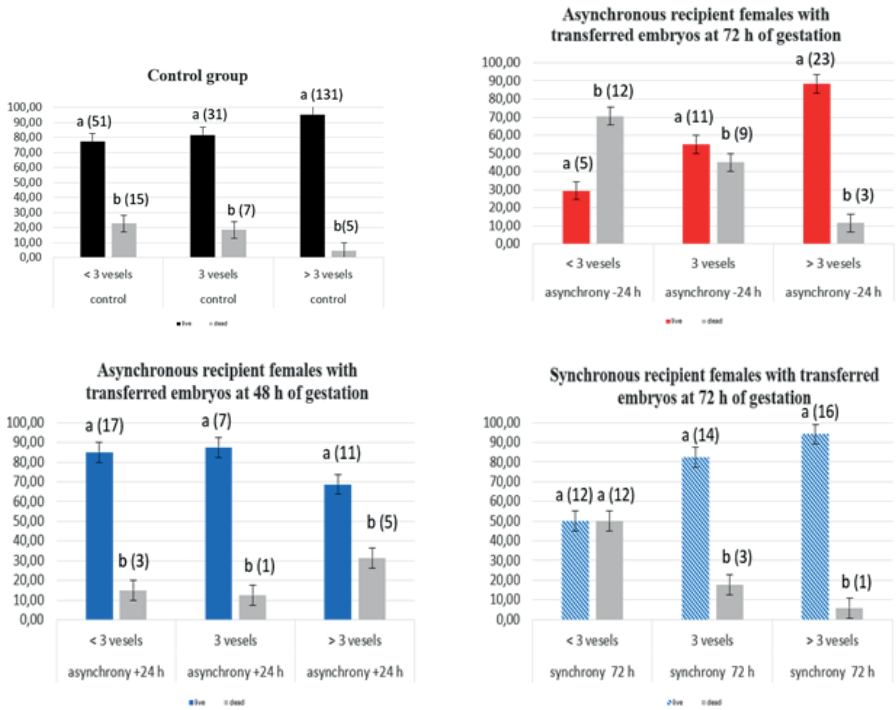

Fig. 4. Percentage of live and dead foetuses per number of blood vessels (fewer than 3, 3, more than 3 blood vessels) reaching each implantation site for control, synchrony and asynchrony groups. Bars represent standard errors of means. Different letters indicate significant differences in control and recipient females at 48 and 72 h of pseudopregnancy with embryos transferred at 48 and 72 h p.c. Number of data in parentheses.

in asynchronous recipients with embryos at 72 h p.c. occurred close to implantation, compared to synchronous recipients with embryos at 48 h p.c. (38.1% in asynchrony at -24 h vs. 12.4% in synchrony at 48 h, Tab. 1). The blood supply appears to be key in the post-implantation survival of foetuses for transferred embryos with more advanced development. In this regard, we observed that the percentage of dead foetuses with placentas receiving fewer than three blood vessels was higher than those with placentas receiving more than three blood vessels in embryos transferred at 72 h p.c. into asynchronous recipients at 48 h p.c. compared to embryos transferred at 48 h p.c. into synchronous recipients (69% dead foetuses with <3 blood vessels and 17% dead foetuses with >3 blood vessels in asynchronous females vs. 14% dead foetuses with <3 blood vessels and 12% dead foetuses with >3 blood vessels in synchronous females).

Differences between live foetuses of control, synchronous and asynchronous recipient females

Embryo development and receptivity of the uterus can determine the speed of embryo migration and therefore the distance between oviduct and first implantation site (DOP), distance between last implantation site and cervical end (DPC) and available uterine space per foetus (ISF) could be variable. We found a smaller DOP in recipient females at 72 h p.c. than in recipient females at 48 h p.c. and similar to control females (Fig. 5). DPC seems to be higher in recipient females at 72 h p.c. than in recipient females at 48 h p.c. (Fig. 5). A smaller DOP and a larger DPC would be related to earlier receptivity to embryo implantation in recipient females at 72 h p.c. than in recipient females at 48 h p.c., in agreement with a lesser distance covered by the first and the last embryo at implantation in these females. However, ISF was not affected by speed of embryo migration and uterine receptivity of the female, as no differences were found between foetuses of control, synchronous and asynchronous females (Fig. 5).

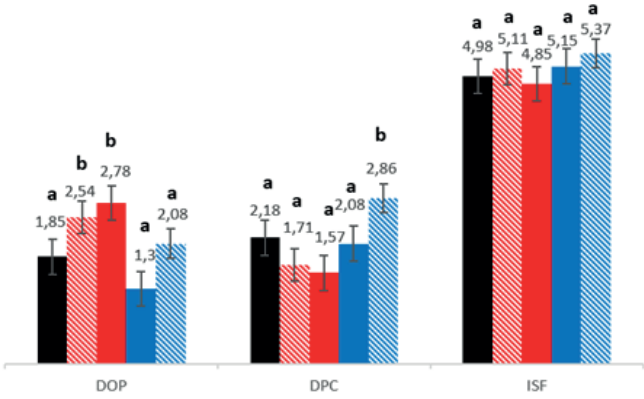

Fig. 5. Distance between the oviduct and the first maternal placenta (DOP, cm), distance between the last maternal placenta and the cervix (DPC, cm), and available uterine space per foetus (ISF, cm.) for the control, synchrony and asynchrony groups. Bars represent standard errors of means. Different letters indicate significant differences between groups.

Individual weight of the live foetus, foetal crown-rump length and efficiency of foetal placenta were greater in control females (5.67 g, 4.37 cm and 2.12, respectively, see Fig. 6) than in synchronous and asynchronous females. Pseudopregnant females at 48 h p.c. showed heavier and longer foetuses (5.05 g and 5.20 g 4.18 cm and 4.19 cm, respectively, for synchronous vs. asynchronous females). The respective data for pseudopregnant females at 72 h p.c. were 3.65 g and 3.72 cm for asynchronous and 4.48 g and 4.07 cm for synchronous females. In general, asynchrony +24 h showed lower weight, length and area of foetal and maternal placenta than the other groups (Fig. 6).

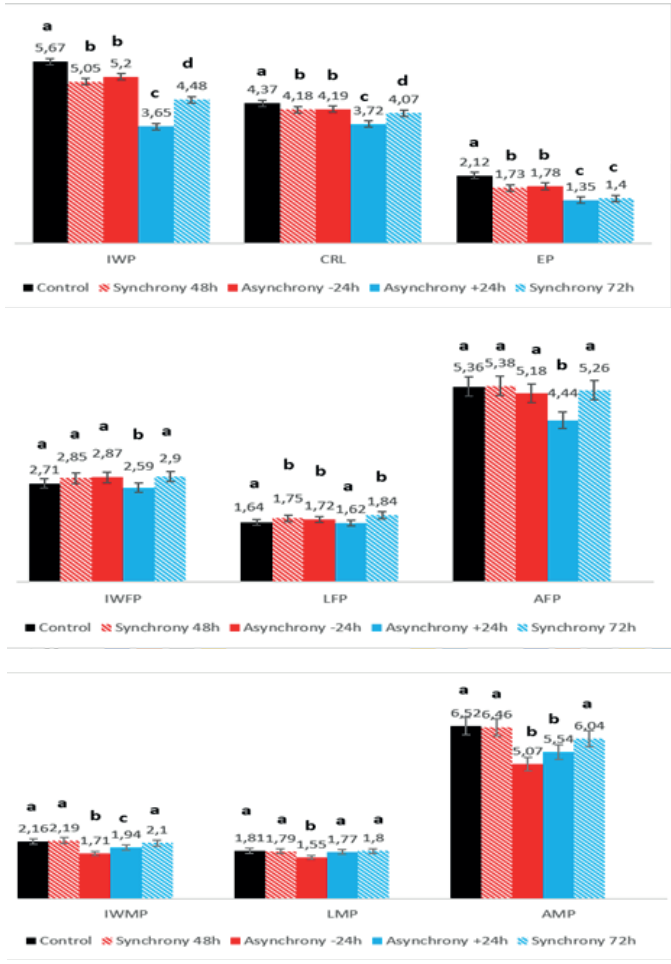

Fig. 6. Individual weight of the foetus (IWF, g), crown-rump length (CRL, cm), foetal placenta efficiency (EP), individual weight (IWMP, g), length (LMP, cm) and individual area of maternal placenta (AMP, cm^2), individual weight (IWFP, g), length (LFP, cm) and area of foetal placenta (AFP, cm^2) for the control, synchrony and asynchrony groups. Bars represent standard errors of means. Different letters indicate significant differences between groups.

Pseudopregnant females at 48 h p.c. had a higher percentage of live foetuses with placentas receiving more than three blood vessels when compared to pseudopregnant females at 72 h p.c. Moreover, foetal placentas that received more than 3 blood vessels were heavier in pseudopregnant females at 48 h p.c. than in pseudopregnant females at 72 h p.c., regardless of the number of blood vessels. The same pattern was also reported for weight of live foetuses (Fig. 7). A greater weight of foetal placentas which received more than 3 blood vessels was related to a greater weight in their foetuses. Better irrigation and a greater development of foetal placenta in live foetuses of females at 48 h p.c. than at 72 h p.c. would be directly related to the greater weight and length of live foetuses.

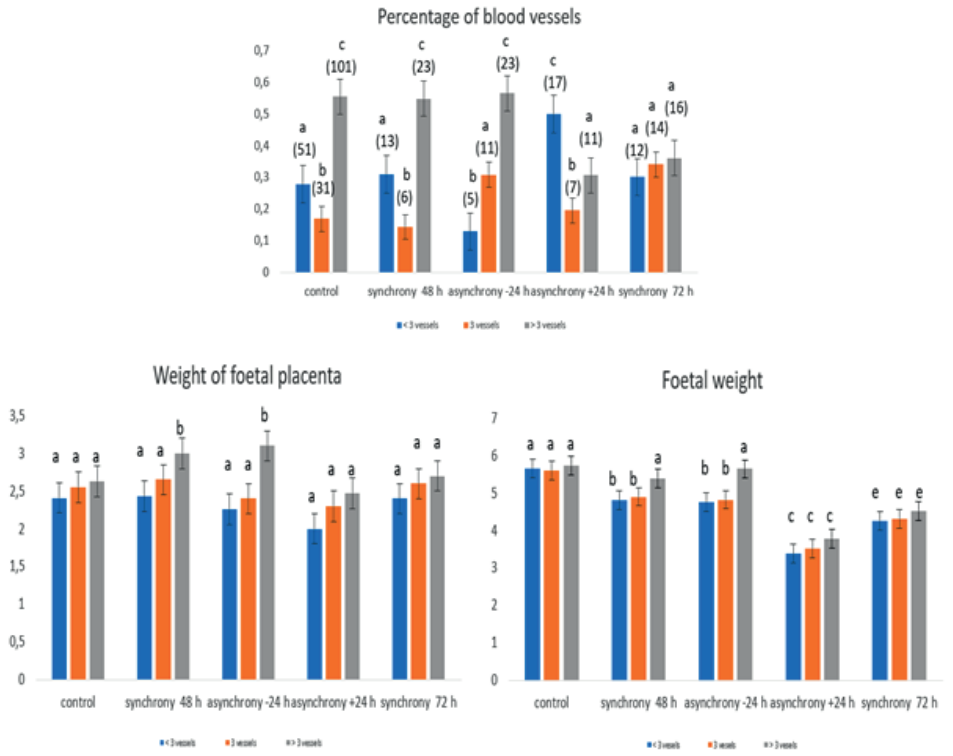

Fig. 7. Distribution of irrigation (< 3 blood vessels, 3 blood vessels or > 3 blood vessels) in live foetuses, weight of live foetuses (g) and their foetal placenta (g) in the control, synchrony and asynchrony groups. Bars represent standard errors of means. Different letters indicate significant differences between groups and numbers of blood vessels.

Effect of embryo manipulation and asynchrony between embryo and uterine environmental on prenatal survival

Our results show that an intramuscular dose of 1μg buserelin acetate makes it possible to obtain comparable numbers of CL as in natural stimulation, since the number of corpora lutea was similar between control and pseudopregnant females of 48 h and 72 h. This dose had already been employed in previous rabbit studies [Viudes-de-Castro *et al.* 2007, Mocé *et al.* 2010, Rebollar *et al.* 2012]. The number of corpora lutea was within the range of values reported in other maternal lines such as A, V and Heterogeneous lines [Khalil and Baselga 2002, García *et al.* 2021]. Results obtained after fresh embryo transfer showed a lower implantation rate than in control females and a similar foetal survival in synchronous recipient females at 48 and 72 h p.c. Saenz de Juano *et al.* [2016] found that embryo recovery and transfer alter OCT4, EMP1, C1QTNF1 and TNFAIP6 mRNA expression in late blastocysts, affecting placenta development and successful embryo implantation, but no subsequent foetus survival, which is in agreement with our findings. In our study, prenatal survival of synchronous recipient females is within the range of values reported by other authors with vitrified embryos [Mehaisen *et al.* 2006, Mocé *et al.* 2010, Vicente *et al.* 2013a]. A more advanced embryonic stage, close to the blastocyst, is more susceptible to the negative effects of vitrification. Embryo vitrification alters subsequent placental development, with the embryo failing to develop to term [Vicente *et al.* 2013b]. Our study showed that the embryo at 72 h p.c. is an inadequate state for transfer of fresh embryos into synchronous females.

In relation to pre- and peri-implantation losses, embryo and endometrial development are two independent processes and accordingly a lesser or more advanced development of embryo compared to endometrial development can impair success in implantation and subsequent embryo survival [Geisert and Schmitt 2002]. This would agree with lower embryonic survival rates in asynchronous recipient females compared to the control females in our study.

Within asynchronous receptive females we found that embryonic survival was even lower in those with embryos at 48 h of gestation compared to embryos at 72 h of gestation. Rabbit embryos have a mucin layer that is deposited during passage through the oviduct. Several studies have reported a positive relationship between rabbit embryo mucin coat thickness and successful implantation [Murakami and Imai 1996, Joung *et al.* 2004]. Mucin coat deposition is limited to the oviduct for 3 days following ovulation. Therefore, embryos at 48 h p.c. have a thinner mucin coat than those at 72 h p.c. [García *et al.* 2016], being more sensitive to manipulation during collection and transference.

Once embryos are deposited in the oviduct, they enter the uterus and move unidirectionally while separating and they achieve equal spacing just before implantation [Böving 1956, Tsutsumi and Hafez 1974]. This is in agreement with similar available uterine space per foetus in synchronous, asynchronous and control females. However, speed in progress into the uterus is different between embryos at

48 h and 72 h of gestation, being quicker in those at 48 h of gestation than those at 72 h of gestation, perhaps due to their smaller diameter [Techakumphu *et al.* 1987]. Moreover, contractions in the oviduct are gradually increasing in intensity until 72 h p.c., in order to enable embryos to enter the uterus. Our hypothesis is that the pseudopregnant uterine horn at 72 h p.c. allows a much faster transit of the embryos to the uterus than the pseudopregnant uterine horn at 48 h p.c. and the deposition of the mucin coat in the embryo would be lesser, which would deteriorate implantation rate. In addition, there is evidence indicating that the embryo developmental stage may be more advanced than the uterine environment in pseudopregnant females, but not the opposite, as the favourable uterine environment cannot wait for the embryos to reach the right stage for implantation [Yoshinaga 2018]. Therefore, embryos at 48 h of gestation transferred into pseudopregnant females at 72 h p.c. would have a lower chance of survival and implantation than those at 72 h of gestation transferred into pseudopregnant females at 48 h p.c., due to both a lower thickness of the mucin coat in the embryo and uterine environment being more advanced than the development that would not wait for its implantation.

Irrigation and the supply of nutrients to the foetus have an important role in its development and survival [Argente *et al.* 2003 and 2008], affecting morphometric characteristics of foetal and maternal placenta, decreasing foetal survival [García *et al.* 2021]. In this sense, we found that poor irrigation abruptly increases the percentage of dead foetuses during implantation in more developed embryos (72 h of gestation), as they can be more sensitive to poorer irrigation than those less developed (48 h of gestation). This effect is more relevant in receptive females with a uterine environment less advanced than the embryo development.

Differences among live foetuses of control, synchronous and asynchronous recipient females

Although control females showed a slightly higher degree of uterine overcrowding than recipient females (around 2 foetuses per uterine horn), no differences were found for available uterine space between groups. However, other authors have reported that an increase in the number of foetuses reduces the uterine space available per foetus and foetal development [Breuer and Claussen 1977, Argente *et al.* 2003]. Weight, length and area of maternal placentas were similar in control females to those of the synchronous recipients. Nevertheless, the development of maternal placentas was lesser in asynchronous recipients than in control females. Thus, the asynchrony between transferred embryo and uterine environment appears to adversely affect the development of maternal placenta.

Note that despite the greater crowding in control females, their foetuses were heavier and larger than those of synchronous and asynchronous recipient females as a consequence of higher efficiency of foetal placenta, defined as the ratio of the weight of the foetus to that of its placenta. Previous results have indicated that foetal weight is the most important parameter to ensure foetal survival during gestation [Argente *et al.* 2003, García *et al.* 2021]. Foetuses of pseudopregnant females at 48 h p.c. showed

greater weight and height than those of pseudopregnant females at 72 h p.c., as a consequence of more advanced development of foetal placentas and better irrigation of foetuses.

Conclusions

Asynchrony between the development of the embryo and the pseudopregnant uterine horn increases prenatal losses. Embryonic development less advanced than that of the pseudopregnant uterine horn increases pre- and peri-implantation losses, while a more advanced embryonic development than that of the pseudopregnant uterine horn increases foetal losses as a consequence of lower blood supply in each implanted site.

Conflicts of interest

Authors declare no conflict of interest.

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