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Effects of Synchronous and Asynchronous Embryo Transfer on Postnatal Development, Adult Health, and Behavior in Mice[1]

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ABSTRACT

Asynchronous embryo transfer (ET) is a common assisted reproduction technique used in several species, but its biological effects on postnatal and early development remain unknown. The aim of this study was to determine whether asynchronous ET produces long-term effects in mice. Postnatal development, animal weight, systolic blood pressure (SBP), relative organ weight (liver, spleen, kidneys, heart, lungs, brain, and testicles), and behavior (assessed in open-field and elevated plus maze tests) were assessed in CD1 mice produced by different ET procedures: 1) the transfer of Day 3.5 (D3.5) blastocysts to the uterus (BL-UT); 2) the transfer of D3.5 blastocysts to the oviduct (BL-OV); or 3) the transfer of D0.5 zygotes to the oviduct (Z-OV). In vivo conceived animals served as controls (CT). The transfer of blastocysts to the uterus or zygotes to the oviduct was defined as synchronous, and transfer of blastocysts to the oviduct was defined as asynchronous. Both synchronous and asynchronous ET resulted in increased weight at birth that normalized thereafter with the exception of asynchronous ET females. In this group, female BL-OV, a clear lower body weight was recorded along postnatal life when compared with controls ($P < 0.05$). No effects on animal weight were produced during postnatal development in the synchronous ET groups (BL-UT, Z-OV, and CT). Both synchronous and asynchronous ET had impacts on adult (Wk 30) organ weight. SBP was modified in animals derived from blastocyst but not zygote ET. Effects on behavior (anxiety in the plus maze) were only detected in the BL-UT group ($P < 0.05$). Our findings indicate that zygotes are less sensitive than blastocysts to ET and that both synchronous and asynchronous blastocyst ET may have long-term consequences on health, with possible impacts on weight, arterial pressure, relative organ weight, and behavior.

asynchrony, behavior, blastocyst, early development, embryo-maternal communication, embryo transfer, oviduct, synchrony, uterus, zygote

INTRODUCTION

Embryo transfer (ET) is the final step needed to produce offspring following in vitro fertilization, intracytoplasmic sperm injection, cryopreservation, nuclear transfer, and/or a transgenic procedure [1]. The process of ET has been used extensively in many different species, including humans [2], cows [3], horses [4, 5], pigs [6], and mice, and it is regarded as safe without adverse effects on normal gene expression [7]. In the traditional ET technique, embryos are transferred to the uterus of a recipient female and the process is timed such that there is maximum synchrony between the embryo and recipient to ensure a successful pregnancy. In mice, blastocysts can also be transferred to the oviduct 1 to 3 days before the uterus is receptive. In this procedure, embryos suffer temporary arrest of embryo development until the uterus is ready for implantation. Studies have shown that transferred embryos of different ages will undergo implantation in the uterus at the same time [8] or at least within a several hour period [9]. In addition, the prolonged preimplantation stage experienced by asynchronously transferred embryos gives rise to a more advanced stage of embryonic development at the time of implantation, and this, in turn, leads to a developmental advantage and influences fetal weight [10]. In contrast, others have reported that embryonic development is synchronized in the maternal uterine environment [11]. However, because the transfer of murine blastocysts to the oviduct or uterus gives rise to similar pregnancy outcomes, in most mouse experiments that have examined the long-term effects of ET, transfer to the oviduct or uterus has not been independently considered.

More recently, it has been shown that by manipulating preimplantation development effects are produced on the phenotype of adult animals [12, 13] and that the combination of superovulation, placement in culture media during development to the blastocyst stage, and ET can affect behavior as well as cardiovascular and metabolic measures in rodents [14–16]; and in some case, adults can even transmit these alterations from one generation to the next [17]. When blastocysts are placed in an asynchronous oviduct, they are exposed to abnormal oxygen pressure and carbohydrate and amino acid concentrations for their stage of development and thus have to adapt their physiology and metabolism to this environment. In addition, ET of blastocysts to the oviduct (Day 0.5) causes partially proliferative quiescent state in the embryo, and during the 2.5 days the blastocysts remain in the oviduct, one cell division takes place before the start of quiescent state and the embryo is reactivated only when it passes to the uterus [18]. In vitro studies have revealed that such adaptation to the environment is not devoid of stress for the embryo [7, 12].

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We speculate that asynchronous murine ET produces alterations in the embryo that may have long-term consequences on the development and health of the animal. In addition, some of the damage caused in the imprinting observed during early embryonic development has been attributed to the transfer process per se [7]. This means that not only ET asynchrony but that the ET process itself could have detrimental effects on the fate of the embryo. The present study was designed to assess the effects of synchronous and asynchronous ET on postnatal development, adult health, and behavior of mice.

MATERIALS AND METHODS

Animals

All the mice used in this experiment were kept in an animal house under controlled conditions of temperature (23°C ± 1°C) and photoperiod (14L:10D) and given free access to water and food. All the experimental procedures using mice were approved by our Institutional Review Board (INIA), permit number CEEA2012/021, and performed according to the Guide for Care and Use of Laboratory Animals endorsed by the Society for the Study of Reproduction and European legislation.

In Vivo Zygote and Blastocyst Production

For embryo production, CD1 females (Harlan Iberica SL) 6- to 8-wk old were mated with fertile CD1 males. If a vaginal plug was detected the next morning, this time point was designated as 0.5 days postcoitum (dpc). Zygotes were recovered at 0.5 dpc by opening the ampulla and briefly incubating its contents with hyaluronidase (300 μg/ml) followed by several washes in M2 medium [19]. Blastocysts were collected on 3.5 dpc by uterine flushing. All the embryos were kept in K^+-modified simplex optimized medium at least 30 min before ET.

Embryo Transfer

Nine-wk-old CD1 female mice were used as surrogate mothers for ET after mating with vasectomized CD1 males. ET was performed under inhalation anesthesia with isoflurane (Isoba Vet Schering-Plough). Groups of 10 embryos, from a pool of different females, were transferred per surrogate mother. Zygotes were transferred at 0.5 dpc into the infundibulum as synchronous transfer between the embryo and maternal environment (Z-OV). Blastocyst stage embryos were randomly divided in two groups: blastocysts transferred to the oviduct to produce asynchrony between the embryo and maternal environment (BL-OV) or blastocysts transferred through the utero-tubal junction into the uterus as synchronous transfer between the embryo and maternal environment (BL-UT). Animals born after natural mating and free delivery formed the control group (CT) (Fig. 1).

Postnatal Growth and Organ Allometry

All the pregnant dams were allowed to deliver spontaneously. The day of delivery was designated as Day 1 of the neonates (error in birth estimates was ± 6 h). On delivery, litter size was recorded for each dam, and each pup was checked for gross abnormalities. Only litters of 7 to 10 animals were used in the experiments. Pups were nursed by their natural dams until weaning. Offspring in the four groups—Z-OV: 30 and 33, BL-OV: 28 and 32, BL-UT: 30 and 22, CT: 12 and 11, females and males, respectively—were weighed weekly from birth until 22 wk of life. At the age of 30 wk, animals were culled by cervical dislocation, and the brain, liver, spleen, kidneys, heart, lungs, and testicles were harvested, freed from fat or any other added tissues, dried with paper, and weighed to ensure the lack of undesirable variations.

Systolic Blood Pressure

Systolic blood pressure (SBP) and heart rates were recorded at 15 and 21 wk of age in conscious mice with an automated multichannel system using the tail-cuff method and a photoelectric sensor (Niprem 546; Cibertec SA) connected to a PowerLab 400 system from AD Instruments. Data were analyzed using the Chart4 package from AD Instruments [16].

Mice were allowed to acclimatize for 2 h in a room at 28°–30°C before the experiment was carried out. In each mouse, SBP was measured three times when the heart rate indicated the absence of stress.

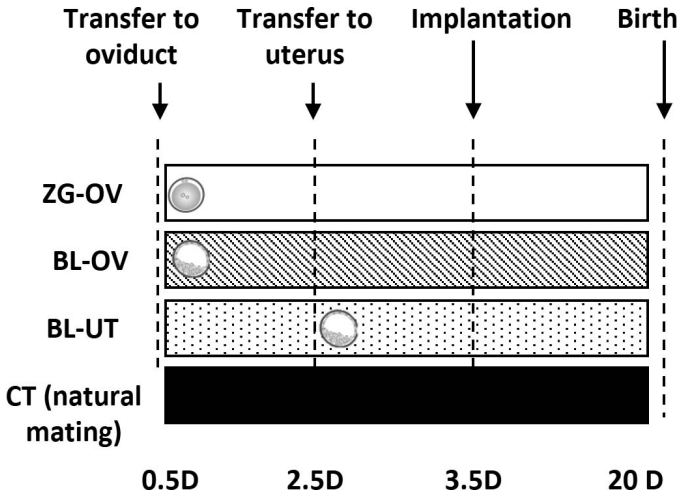

FIG. 1. Experimental groups. Z-OV = Day 0.5 zygotes transferred to the oviduct (n = 63); BL-OV = Day 3.5 blastocysts transferred to the oviduct (n = 60); BL-UT = Day 3.5 blastocysts transferred to the uterus (n = 52); and CT = in vivo conceived animals used as controls (n = 15).

Postnatal Behavior

Two behavioral tests, open field and elevated plus maze, were performed in the offspring at 24 wk of age as previously described [16] by trained observers blind to the experimental groups. The equipment used for the tests was carefully cleaned with a diluted acetic acid solution between animals to avoid olfactory cues.

Data Analysis

Data were analyzed using the software package SigmaStat 3.5 (Jandel Scientific). Differences in body weight, relative and total organ weight, birth weights, and SBP data were compared by one-way repeated-measures ANOVA followed by multiple pairwise comparisons using the Tukey method ($P < 0.05$ was considered significant). When it was necessary normalize the variance, a logarithmic transformation on the data prior to ANOVA was performed. The differences in the rate of life birth were compared between groups by chi-square analysis ($P < 0.05$ was considered significantly). The significance of treatment (ET) effects on behavior was assessed by one-way ANOVA with Tukey post hoc test using the software GraphPad Prism version 5.04 (GraphPad Software Inc.).

RESULTS

Birth Rates

Blastocyst transfer to the oviduct (BL-OV) at 0.5 dpc or to the uterus (BL-UT) at 3.5 dpc produced no differences in litter size and led to good rates of live offspring (43.64% and 42.22%, respectively), indicating good embryo quality and a consistent ET technique. However, the rate of live offspring was greater in response to zygote (ZG-OV) compared to blastocyst (BL-OV) transfer to the oviduct at 0.5 dpc (52.5% vs. 43.64%, respectively; $P < 0.01$) suggesting that the zygote stage is a better time for oviductal transfer than the blastocyst stage.

Postnatal Development

Body weight data for male and female mice produced in vivo or after ET are provided in Figure 2 and Supplemental Table S1 (supplemental data is available online at www.biolreprod.org). We found significant differences in birth weights between control and transfer groups. Although animals derived from synchronous oviductal zygote transfer (ZG-OV) were heavier at birth (males 2.01 ± 0.39 g; females 1.89 ±

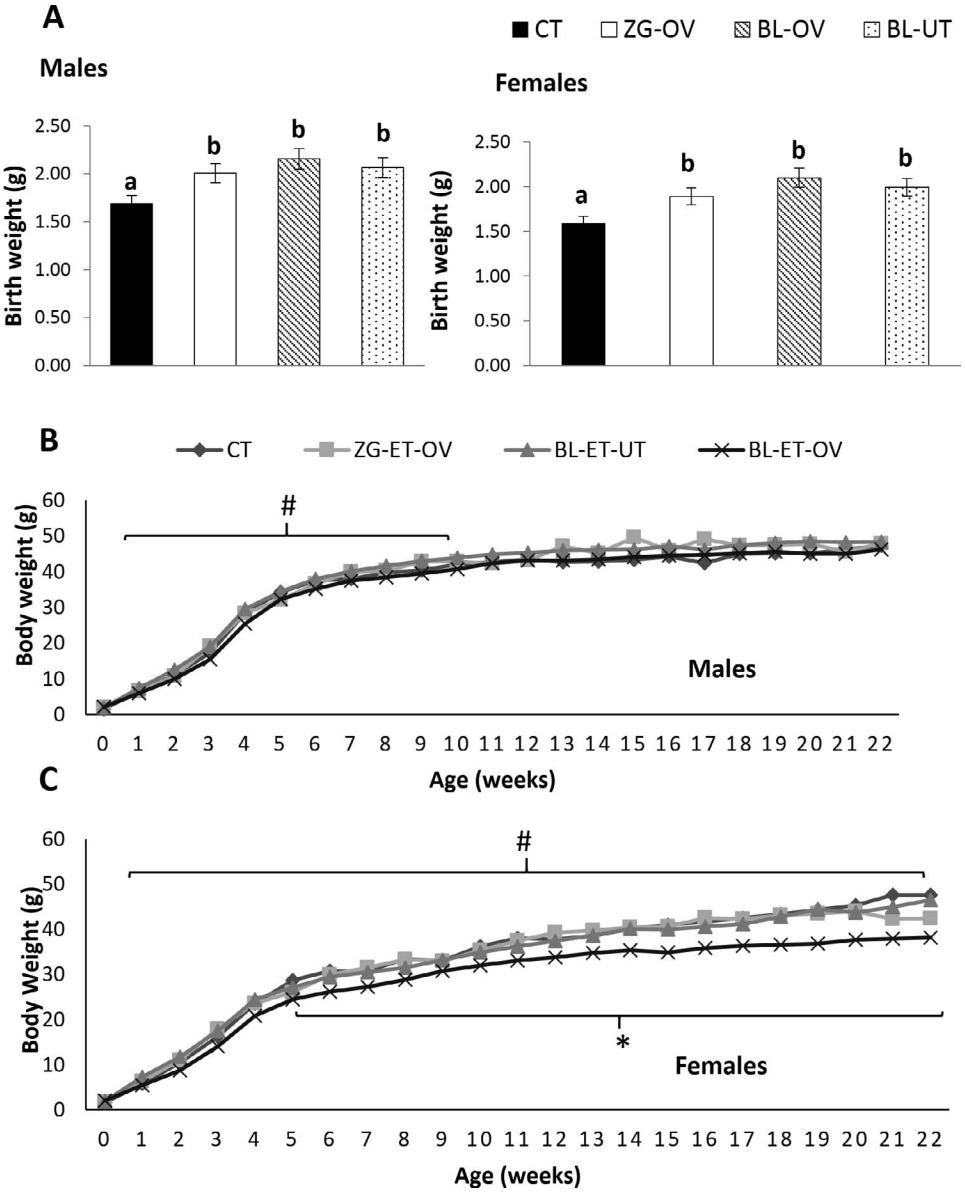

FIG. 2. Average animal birth weights (**A**) and postnatal body weight in males (**B**) and females (**C**) produced by synchronous or asynchronous ET. Z-OV = Day 0.5 zygotes transferred to the oviduct; BL-OV = Day 3.5 blastocysts transferred to the oviduct; BL-UT = Day 3.5 blastocysts transferred to the uterus; and CT = in vivo conceived animals. (*) BL-OV versus CT ($P < 0.05$). (#) BL-UT versus BL-OV ($P < 0.05$). Different superscripts ($^{a,b,*,\#}$) indicate significant differences ($P < 0.05$).

0.34 g) when compared with controls (males 1.68 ± 0.06 g; females 1.59 ± 0.16 g), weights were balanced during postnatal development (Fig. 2, B and C). Synchronous ET of blastocysts to the uterus (BL-UT) produced heavier offspring than controls on the delivery day (males: 2.06 ± 0.35 g vs. 1.68 ± 0.06 g; females: 1.99 ± 0.21 g vs. 1.59 ± 0.16 g) (Fig. 2A), but the differences from Wk 1 to 22 were suppressed for both sexes compared to controls (Fig. 2, B and C). Asynchronous oviductal ET (BL-OV) led to higher birth weights compared with controls in both males and females (males: 2.15 ± 0.21 g vs. 1.68 ± 0.06 g; females: 2.10 ± 0.34 g vs. 1.59 ± 0.16 g). Moreover, during development, asynchronous oviductal ET females (BL-OV) showed lower weights from Wk 5 to 22 than controls and from Wk 1 to 22 than synchronous uterine ET females (BL-UT) (Fig. 2C), and males also showed lower weights from Wk 1 to 10 compared with synchronous BL-UT males (Fig. 2B).

Organ Allometry

Postmortem examination of selected organs—liver, spleen, kidneys, heart, lungs, brain, and testicles—at 30 wk revealed relative organ weight, defined as absolute organ weight (mg)/body weight (g), differences among groups despite no morphological abnormalities. In the synchronous Z-OV group, which showed the smallest organ size differences, the heart was significantly heavier in males (0.728 ± 0.134) compared with control males (0.577 ± 0.06) while relative organ weights in females were similar to those recorded in controls (Table 1).

Greater organ weight alterations were recorded in the synchronous BL-UT group. In males from the BL-UT group, relative weights of liver, spleen, and heart were greater than those in controls (Table 1), while females had a larger spleen and smaller lungs than controls (Table 1). Finally, the offspring resulting from BL-OV (asynchronous) transfer showed higher

TABLE 1. Relative organ weights recorded in mice produced by synchronous or asynchronous embryo transfer.*

Group†	Liver	Spleen	Left kidney	Right kidney	Heart	Lungs	Brain	Testicles
Males								
BL-UT	5.30 ± 0.67[b]	0.36 ± 0.10[b]	0.84 ± 0.11	0.89 ± 0.14	0.73 ± 0.12[b]	0.65 ± 0.11	1.06 ± 0.12	0.55 ± 0.09
BL-OV	4.98 ± 0.52[ab]	0.36 ± 0.08[b]	0.82 ± 0.09	0.84 ± 0.08	0.71 ± 0.13[b]	0.68 ± 0.06	1.16 ± 0.11	0.62 ± 0.07
Z-OV	4.84 ± 0.68[ab]	0.25 ± 0.05[a]	0.80 ± 0.09	0.83 ± 0.12	0.73 ± 0.13[b]	0.72 ± 0.08	1.08 ± 0.12	0.59 ± 0.11
CT	4.52 ± 0.48[a]	0.26 ± 0.05[a]	0.76 ± 0.06	0.79 ± 0.05	0.58 ± 0.06[a]	0.62 ± 0.06	1.09 ± 0.12	0.54 ± 0.06
Females								
BL-UT	4.84 ± 0.62[ab]	0.40 ± 0.10[b]	0.57 ± 0.11	0.57 ± 0.08	0.52 ± 0.09	0.59 ± 0.09[b]	1.20 ± 0.26	
BL-OV	5.11 ± 0.58[b]	0.43 ± 0.10[b]	0.60 ± 0.09	0.59 ± 0.07	0.55 ± 0.14	0.73 ± 0.18[a]	1.37 ± 0.23	
Z-OV	4.47 ± 0.52[a]	0.27 ± 0.05[a]	0.57 ± 0.07	0.60 ± 0.05	0.62 ± 0.18	0.72 ± 0.17[a]	1.13 ± 0.23	
CT	4.78 ± 0.40[ab]	0.33 ± 0.06[a]	0.56 ± 0.08	0.57 ± 0.25	0.56 ± 0.03	0.71 ± 0.15[a]	1.19 ± 0.17	

* Relative organ weights reflect absolute organ weight (mg)/body weight (g) data expressed as mean ± SD.
† BL-UT = Day 3.5 blastocysts transferred to the uterus (males n = 13, females n = 16); BL-OV = Day 3.5 blastocysts transferred to the oviduct (males n = 24, females n = 19); Z-OV = Day 0.5 zygotes transferred to the oviduct (males n = 15, females n = 11); CT = in vivo conceived animals (males n = 9, females n = 14).
[a,b] Different superscript letters in the same column indicate significant differences by ANOVA ($P < 0.05$).

relative weights of heart and spleen in males and liver and spleen in females than their respective controls (Table 1).

Systolic Blood Pressure (SBP)

Systolic blood pressure was determined in mmHg at Wk 15 and 21 (Fig. 3). At both time points, this variable was similar in control and ZG-OV males (105.4 ± 27 vs. 113.1 ± 25, and 114.3 ± 14 vs. 113.7 ± 19, respectively) as well as control and ZG-OV females (108.51 ± 16 vs. 101.1 ± 10 and 109.7 ± 14 vs. 101.2 ± 27, respectively). In contrast, blastocyst transfer, whether synchronous or asynchronous, led to reduced SBP in male offspring compared with control and ZG-OV males. In 15-wk-old female offspring, SBP difference was lower in the BL-OV group, but not BL-UT, compared with ZG-OV, indicating a greater effect in asynchronous transfer (Fig. 3). These results indicate that blastocyst transfer to a synchronous or asynchronous maternal environment produced hypotension in both sexes compared with zygote transfer or natural mating.

Postnatal Behavior

No significant differences were detected in the results of the open field test among groups (Supplemental Fig. S1), indicating no effects of the ET procedure on the mobility or locomotor activity of the mice. In contrast, elevated plus maze test scores indicated behavioral differences in the synchronous BL-UT group. In both males and females, we observed fewer open arm entries and less time spent in the open arm (Fig. 4). The observations reflect increased anxiety in these animals.

DISCUSSION

The results of our study indicate that ET gives rise to a higher birth weight compared to newborns derived from natural mating. In mice, it has been described that ET causes the misexpression of several imprinted genes in extraembryonic tissues (i.e., yolk sac and placenta) [7], possibly related to a higher weight of newborns. A newborn's phenotype is a critical measure of damage incurred during embryonic and fetal development [20]. The effects of factors related to the maternal environment on birth weight during pregnancy have been widely described, including nutrition [21–23], stress [24, 25], and assisted reproductive technologies [26–28]. In contrast, the effects of transfer protocols on the adult phenotype have not been addressed. An elevated birth weight has been also reported in an in vitro cow production model. This phenom-

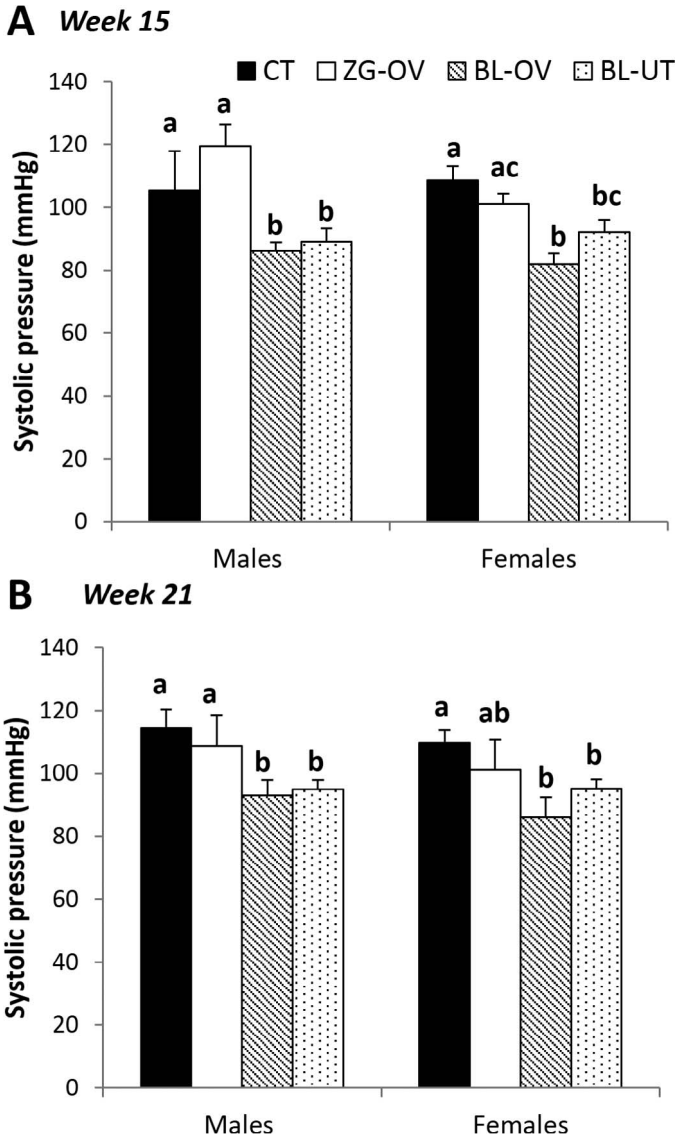

FIG. 3. Effects of synchronous or asynchronous ET on systolic blood pressure in male and female mice. Z-OV = Day 0.5 zygotes transferred to the oviduct; BL-OV = Day 3.5 blastocysts transferred to the oviduct; BL-UT = Day 3.5 blastocysts transferred to the uterus; and CT = in vivo conceived animals. (**A**) Wk 15 and (**B**) Wk 21. Different superscripts ([a,b,c]) indicate significant differences ($P < 0.05$).

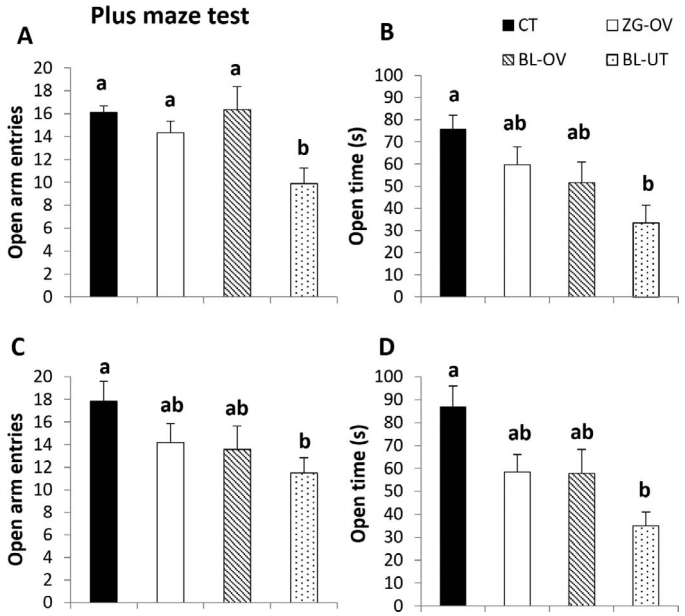

FIG. 4. Effects of synchronous or asynchronous ET on behavior. Plus maze test results for males (**A** and **B**) and females (**C** and **D**) derived from Day 3.5 blastocysts transferred to the uterus (BL-UT); 3.5 Day blastocysts transferred to the oviduct (BL-OV); 0.5 Day zygotes transferred to the oviduct (Z-OV); or natural mating (CT). Different superscripts (^{a,b}) indicate significant differences ($P < 0.05$).

enon, known as large offspring syndrome, observed in in vitro culture and subsequent transfer of blastocysts [29] was related to suboptimal embryo culture. However, as given embryos were not cultured in our experiments, we can attribute this effect to the ET process per se.

All the ET groups produced higher birth weight compared to newborns derived from natural mating. However, weight gains observed until 22 wk of postnatal development were only affected in our asynchronous ET group (BL-OV). Noyes and Dickmann [30] were the first to propose that the stage of embryonic development at the time of transfer could affect fetal weight. These authors reported that asynchronous transfer of rat blastocysts (Day 4 embryo, Day 3 uterus) produced significantly heavier fetuses than their synchronously transferred counterparts (Day 3 embryo, Day 3 uterus). Similarly, Aitken et al. [10] recorded greater fetal weights on Gestation Day 16 when early cleavage embryos were synchronously transferred on Days 2 or 3. These authors suggested that the implantation and development of Day 3 blastocysts was unhindered in Day 2 uteri and that the higher fetal weights observed on Day 16 of pregnancy merely reflected a more advanced stage of pregnancy. However, no significant differences in gestation length were observed between groups.

The differences observed here between the synchronous and asynchronous ET of blastocysts could be related to a partially proliferative quiescent state induced by the transfer of blastocysts to the oviduct. The asynchronous transfer of blastocysts to a 0.5 dpc oviduct in foster mothers induces a state of metabolic and proliferative quiescence in the embryo lasting 2–3 days. This occurs because blastocyst-stage embryos are ready for implantation in the uterus, but it is not until a rise in estrogen levels provokes the opening of the utero-tubal junction and prepares the uterus for implantation that the quiescent blastocyst is reactivated. This phenomenon is similar to the obligate embryo diapause [31, 32]. In prior work [18], we examined the effect of quiescence during the 2.5 days blastocysts remained in the oviduct, where they underwent one cell division before entering quiescence followed by passage to the uterus. In the uterus, the quiescent embryos had double the number of cells (96.4, n = 14) than in vivo produced Day 3.5 blastocysts (48.8, n = 15) suggesting an incomplete state of quiescence.

In our study, both the synchronous and asynchronous transfer of embryos led to greater birth weights compared with control newborns (Fig. 2A). However, differences between the two groups were observed in postnatal development. Thus, body weight gains during development in BL-UT were similar to those recorded in control animals yet weight gains were reduced in the asynchronous ET group (BL-OV). These differences in adaptation could indicate adult phenotypic differences.

Synchronous ET did not affect weights during development, but when these animals were culled at 24 wk of age, we observed higher heart/body weight ratios when compared with controls in all males. In offspring derived from synchronous blastocyst transfer to the uterus, relative spleen and liver weights were also modified in males and relative spleen and lung weights were modified in females. Abnormal large organs has been also reported in cattle with large offspring syndrome produced by suboptimal in vitro culture or by nuclear transfer [29] and in mice produce by suboptimal in vitro culture [12]. Similarly, systolic blood pressure (hypotension) and behavior patterns (more anxiety) differed more from those observed in controls in animals produced by blastocyst transfer than those produced by zygote transfer. The hypotension observed could be related to the increased organ/body weight ratios detected.

Although we do not know whether the phenotypes observed in our model will be reproducible in other species, our findings indicate that in the mouse, any slight manipulation arising from asynchronous ET or from ET per se may have adverse consequences for the development and health of the adult animal. In our study, such impacts took the form of different weight gains during development, and in adults, factors such as SBP or relative organ weights were affected. Our findings indicate that blastocyst stage embryos are more sensitive than zygotes to ET and highlight the importance of considering the ET procedure when using a mouse model to analyze offspring phenotypes.

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