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Effect of a High Estrogen Level in Early Pregnancy on the Development and Behavior of Marmoset Offspring

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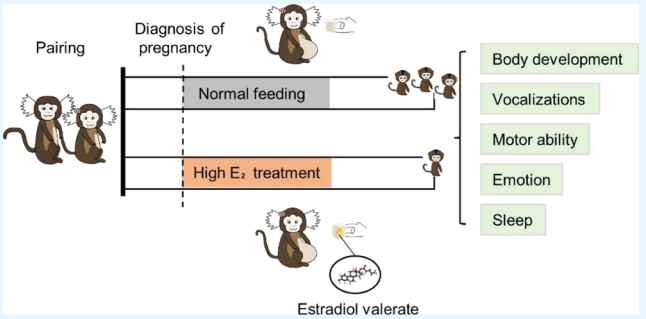
ABSTRACT: The use of assisted reproductive technology (ART) has risen steadily worldwide over the past 3 decades and helps many infertile families. However, ART treatments lead to an abnormal internal environment in the uterus, which may increase the risks of health problems for the offspring. Higher maternal estradiol (E_2) is a notable feature in women who use ART treatments, and this has been suggested as a key factor for the risk of diseases in the offspring. In the current study, we have established a marmoset model with a high E_2 level in early pregnancy to examine its potential risk to the development and behavior of the offspring. In comparison with the normal group, babies of the high E_2 group exhibited lower average survival rates and birth weights. However, those who survived in the high E_2 group demonstrated normal vocal production with rich call repertoires, normal speed during locomotion, and normal behaviors in the home cage. In contrast to the normal group, surviving babies of the high E_2 group spent more time sleeping during development without signs of sleep disorders. In summary, our study revealed that high estrogen in early pregnancy may cause low survival rates and birth weights of the offspring, though the surviving infants did not show obvious behavioral deficiencies during development. The current study is a valuable and highly important non-human primate study for evaluating the safety of ART treatments. However, it is worth noting that some results did not reach the significant level, which may be due to the small sample size caused by animal shortage stemming from the COVID-19 epidemic.



INTRODUCTION

Human and rodent studies have shown that abnormal maternal estrogen levels in the uterus may increase the risk of health problems for both mothers and infants,[1−11] such as low birth weights,[4] premature delivery,[4] thyroid dysfunction,[3,5,7,9] autism spectral disorders,[1,2] and dysfunction of cardiovascular,[11] endocrine,[7,10] and other systems.[8] Assisted reproductive technology (ART) has attracted wide attention since the first in vitro fertilization (IVF) baby was born.[12] Since then, ART treatments have become widespread and have led to more than 5 million births worldwide, which brings great benefits to the infertile population.[12,13] However, concerns about the safety of ART treatments have been raised in recent years. High estradiol (E_2) in the uterus is a notable feature caused by ART treatments such as controlled ovarian hyperstimulation (COH), IVF, and embryo transfer and may lead to health problems for both mothers and infants.[7,14] Take gonadotropin treatments for example, which were widely used to stimulate the ovary and promote the development of multiple follicles.[12−15] Because of the stimulation, maternal E_2 levels can be 10−20 times higher than the physiological levels of a normal pregnancy, and the elevated E_2 levels last throughout early pregnancy.[4,14,15] The ovarian hyperstimulation syndrome is a typical example and shows the negative influence of COH on pregnant women. As embryonic development needs to be strictly controlled over the time, place, and surrounding environment, any slight changes in the environment of the uterus may lead to abnormal development of the fetuses and infants after birth.[16] Human cohort studies showed that high maternal E_2 exposure may affect the growth and development of the offspring.[4,6−8,11] However, there is not enough evidence to deduce to what extent high E_2 exposure in early pregnancy affects the growth and development of the offspring. More animal studies remain required. Non-human primate models with high homology and similar brain structure and functions to humans are especially needed.

Of particular interest to researchers is whether and how high maternal E_2 exposure in the uterus affects the growth and development of the offspring. However, most studies have

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been carried out on animal models such as mice,[4,6−8,10,11] cattle,[17,18] and sheep,[19,20] which greatly differ from humans in the genetic background, life span, hormone secretion, metabolism, and brain structure and function.[21,22] Thus, studies on nonhuman primates are indispensable. The common marmoset is a small body-size New World monkey, with a high reproduction rate and a short development period.[23] In addition, the marmoset is a highly vocal animal with human speech-like turn-taking vocal communications within conspecifics.[24,25] The unique vocal communications and highly social behaviors of marmosets have rendered it a good non-human primate model for the study of cognitive behavior and human mental diseases.[24,26] In terms of reproductive physiology, marmosets' copulation occurs throughout the whole ovarian cycle and pregnancy.[23] Following a 5 month gestation period (~145 days), they often give birth to twins or even triplets and can fertilize again while still nursing the previous set of infants.[23] Thus, the marmoset is a good non-human primate model to study the safety of ART treatments.[27]

To examine how high E_2 levels in early pregnancy influence the development and the behaviors of offspring, we established a high E_2 marmoset model at early pregnancy by orally feeding the female with estradiol valerate after the animals were diagnosed with pregnancy. We found that high E_2 at early pregnancy decreased average survival rates and birth weights of offspring; however, surviving baby marmosets exhibited normal vocalizations, motor ability, and behavior without obvious mental problems during the developmental period. More animals in the high E_2 group are needed to confirm the results in the future.

RESULTS

Establishment of the High E_2 Marmoset Model at Early Pregnancy. Marmoset monkeys that were 2−6 years old with stable fertility were used in this study. To examine the effect of material high E_2 levels on the next generation, we divided the animals into two groups: a high E_2 group and a normal control group. The high E_2 group was established by orally feeding the females with estradiol valerate (800 μg/kg, once per day) for 45 days after they were diagnosed with pregnancy (Figure 1A). The treatment for the normal group is the same as that of the high E_2 group except that the normal group was not fed estradiol valerate during early pregnancy. Blood samples (0.5 mL) were collected 2 weeks after estradiol valerate treatment, and two additional samples were collected once per week to confirm E_2 levels if necessary. We found that the E_2 group showed significantly higher serum E_2 levels than the normal group (Figure 1B, p = 0.0005, rank-sum test). The result proved that we established the high E_2 marmoset model successfully.

Lower Survival Rates and Birth Weights in Offspring of High E_2 Female Marmosets. This study is difficult to carry out because the high E_2 female marmosets are more likely to have a spontaneous miscarriage. Eventually, only three high E_2 female marmosets delivered babies, as shown in Table 1. Female marmosets in the normal group had an average litter size of 1.9, consistent with previous reports on the average litter size in common marmosets (1.86−2.69).[28] Although the average numbers of babies produced by the high E_2 and normal females were similar (high E_2 group, 2.00 ± 1.00, n = 3 and normal group, 2.21 ± 0.89, n = 14), the average number of surviving babies produced by the high E_2 females was much lower than that of normal females (high E_2 group, 1.00 ± 0, n = 3 and normal group, 1.86 ± 0.86, n = 14). Thus, the average survival rate of offspring in the high E_2 group was much lower than that in the normal group (Table 1; high E_2 group, 61%, n = 3 and normal group, 86.9%, n = 14). However, due to the small sample size of the high E_2 group, no significant difference was found between the two groups in both the number of offspring (P = 0.1500, rank-sum test) and the survival rate of offspring (P = 0.1103, rank-sum test). We carefully traced a pair of marmosets through nine pregnancies with eight being normal controls and the penultimate pregnancy as a high E_2 treatment (Animal ID:1707F1, Table 2). In the first few

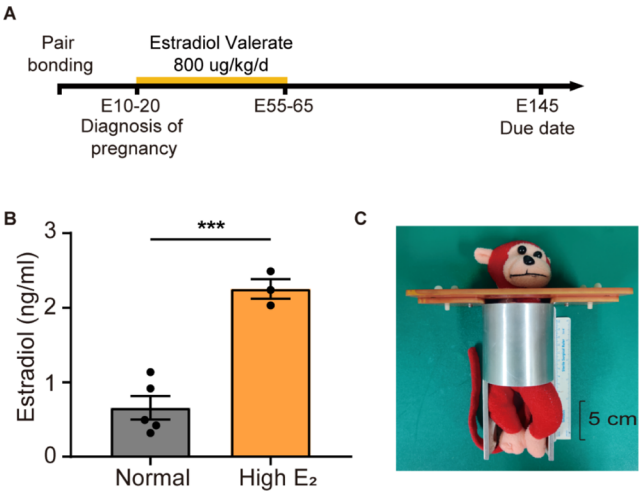

Figure 1. Establishment of the marmoset model with a high E_2 level at early pregnancy. (A) Experiment design for the establishment of the high E_2 marmoset model at early pregnancy. Female marmosets were administered with 800 μg/kg of estradiol valerate per day for 45 days starting from 10 to 20 days after pregnancy (E10−20). If the high E_2 treatment was started at embryonic day 10 (E10), it was finished at embryonic day 55 (E55). The gestation period of the common marmoset is generally 145 days. E145, embryonic day 145. (B) Average serum E_2 levels during early pregnancy in the high E_2 group (high E_2, n = 3 animals) and the normal group (normal, n = 5 animals). Results are expressed as means ± SE. (P = 0.0005, rank-sum test). ***P < 0.001. (C) Head restraint device for blood collection from awake adult marmosets.

Table 1. Records of Baby Birth Rates in Normal and High E_2 Female Marmosets

animal ID	groups	baby number	surviving babies	survival rate (%)
1806F1	control	1	1	100
1806F1	control	1	1	100
1806F1	high E_2	1	1	100
1806F2	control	3	3	100
1806F2	control	1	1	100
1707F1	control	1	1	100
1707F1	control	3	1	33.3
1707F1	control	2	1	50
1707F1	control	3	2	66.7
1707F1	control	3	3	100
1707F1	control	2	2	100
1707F1	control	3	3	100
1707F1	high E_2	3	1	33.3
1707F1	control	2	2	100
1707F2	control	3	3	100
1707F2	control	3	2	66.7
H	high E_2	2	1	50

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Table 2. Records of Babies Produced by 1707F1

animal ID	birth serial number	age of the mother at birth (years/months)	group	number of babies	number of survivals	survival rate (%)
1707F1	1	2/8	control	1	1	100
1707F1	2	3/1	control	3	1	33.3
1707F1	3	3/6	control	2	1	50
1707F1	4	3/1	control	3	2	66.7
1707F1	5	4/4	control	3	3	100
1707F1	6	4/9	control	2	2	100
1707F1	7	5/2	control	3	3	100
1707F1	8	5/10	high E_2	3	1	33.3
1707F1	9	6/11	control	2	2	100

pregnancies (first−fourth), only one baby survived, which is low but normal for a new mother. The same female produced two−three babies stably in the following pregnancies (fifth−seventh) until treated by high E_2 during early pregnancy (8th) with a 33.3% baby survival rate, which was in sharp contrast to the last normal pregnancy with a 100% baby survival rate. 1707F1 produced babies with a lower survival rate after it was administered with estradiol valerate orally at early pregnancy.

Next, we measured the basic physical parameters of the newborn babies and those during development and compared them between the normal and high E_2 groups (Figure 2). In comparison with the normal group, babies of the high E_2 group showed decreased birth weights (Figure 2A, $P = 0.011$, rank-sum test, and Table 3). However, no significant differences were found in the body lengths (Figure 2B, $P = 0.536$, rank-sum test), head circumstance (Figure 2C, $P = 0.393$, rank-sum test), and crown−rump length (Figure 2D, $P = 0.500$, rank-sum test) between offspring of normal and high E_2 groups. Our results were consistent with what was found in material high E_2 rodent models,[10] indicating that high E_2 exposure in the uterus may lead to the lower body weight of the fetus. Moreover, we measured the body weight of offspring produced by females of the normal group and high E_2 group, respectively, during their body development. Interestingly, no significant difference was found when they were 1 month old (Figure 2E, $P = 0.8593$, rank-sum test) and 3 months old (Figure 2F, $P = 0.8571$, rank-sum test). These results indicated that the lower birth weight in babies of the high E_2 group diminished during postnatal body development.

Babies of the High E_2 Group Showed Normal Vocal Production with a Rich Call Repertoire. The common marmoset is a highly vocal non-human primate, communicating with each other mainly through vocalizations.[24,25] Normal baby marmosets can produce different types of calls, including four major types (phee, trill, trill-phee, and twitter), three infant-specific calls (cry, compound-cry, and sub-harmonic), and other calls such as tsik and ekk. We wondered whether maternal high E_2 exposure altered the vocal production of marmoset babies. To answer this question, we recorded the vocalizations of newborn baby marmosets in the high E_2 group and normal group, respectively. We found newborn babies of the high E_2 group exhibited normal vocal production with a rich call repertoire, which is similar to those of the normal group (Figure 3A,B). The offspring of the high E_2 group can produce vocalizations with distinct acoustic features, which can be used to distinguish the animal identity during social interactions (Figure 3B). In comparison with that of the normal group, the number of call types recorded from offspring of the high E_2 group was similar (Figure 3C). These results

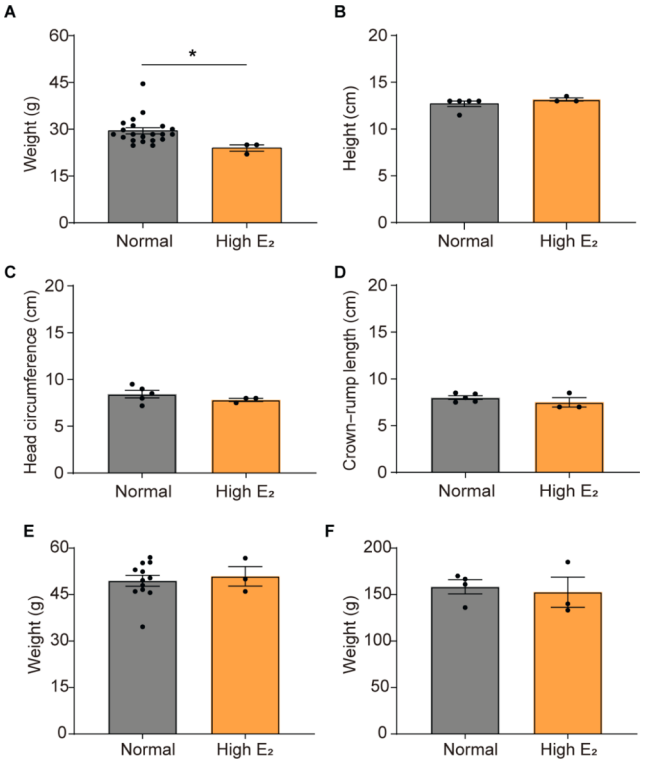

Figure 2. Physical measurements of baby marmosets produced by normal and high E_2 females. (A) Birth weight of marmoset triplets produced by female marmosets in normal and high E_2 groups (babies of normal females, $n = 21$ and babies of high E_2 females, $n = 3$), respectively. (B) Body length of newborn marmosets produced by normal and high E_2 females (babies of normal females, $n = 5$ and babies of high E_2 females, $n = 3$). (C) Head circumference of newborn marmosets produced by normal and high E_2 females (babies of normal females, $n = 5$ and babies of high E_2 females, $n = 3$). (D) Crown−rump length of newborn marmosets produced by normal and high E_2 females (babies of normal females, $n = 5$ and babies of high E_2 females, $n = 3$). The data in B−D were collected from the same animals. (E) Body weight of marmoset babies (1 month old) produced by female marmosets in normal and high E_2 groups (babies of normal females, $n = 12$ and babies of high E_2 females, $n = 3$). (F) Body weight of marmoset babies (3 months old) produced by female marmosets in normal and high E_2 groups (babies of normal females, $n = 4$ and babies of high E_2 females, $n = 3$). Results are expressed as means ± SE. *$P < 0.05$.

suggest that high E_2 exposure in early pregnancy may not affect the vocalizations of marmoset infants.

Offspring of the High E_2 Female Exhibited Normal Mental and Motor Behaviors. Previous human and animal studies have shown that abnormal maternal E_2 levels may lead

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Table 3. Birth Weight of Triplets Produced by 1707F1[a]

	weight (g)			
group	baby 1	baby 2	baby 3	average weight (g)
control	27.6			
control	27.4	26.8		27.1
control	28.4	26.4	35.4	30.1
control	30	28.4	24.8	27.7
high E_2	25 (√)	22 (x)	25 (x)	24

[a] √ indicates that this baby survived and x indicates that this baby did not survive.

to mental disorders in offspring, such as ASD[1,2] and anxiety accompanied by movement disorders.[8] ASD has core symptoms including deficits in social interactions and speech communications as well as restricted and repetitive behaviors. To examine whether material high E_2 levels may lead to mental diseases, we performed video recordings in the animal cage for the offspring of the normal and high E_2 female marmosets when they grew up to 8 months old. First, restricted and repetitive behaviors were not found in both groups (Figure 4A), suggesting that offspring of the high E_2 female marmosets did not have autism-like stereotyped behaviors. Second, offspring of the high E_2 female marmosets exhibited normal locomotion with a similar moving speed to that of offspring produced by the normal female marmosets (Figure 4B, P = 0.623, rank-sum test). Furthermore, to identify the anxiety level of the offspring in the high E_2 group, we calculated the time that the animal stayed on the top half of the home cage, which to some extent reflects the animal's anxiety level. No significant difference was found between the two groups of animals (Figure 4C, P > 0.999, rank-sum test). Thus, our results indicated that maternal high E_2 levels did not alter the motor ability of offspring during the development period and did not increase the risk of ASD and anxiety levels.

Offspring of the High E_2 Female Marmosets Slept Longer and Displayed More Naps. Sleep is an indispensable part of life for humans and animals. People cannot function properly without sleep.[29] Sleep is important for brain and body development,[30] memory formation and consolidation,[31] and the erasure of undesired memories.[32] Many studies showed that learning abilities, memory, and perceptual skills are improved after sleep.[33−35] We wondered whether marmoset offspring of normal and high E_2 females have any difference in sleep. To assess the sleep of marmoset offspring in two groups, we measured the sleep time, sleep quality, and daily activity patterns by having the animal subjects (9−12 months old) wear a Mini ActiWatch (CamNtech) on their neck for 7 days (Figure S1). Interestingly, we found that the offspring of the high E_2 group slept longer time than those of the normal group at the same age (Figure 5B, P = 0.048, rank-sum test). Moreover, the high E_2 group showed a tendency for long naps in the daytime, and the difference was marginally significant (Figure 5E, P = 0.095, rank-sum test; and Figure 5A). Regarding sleep quality at night, there was no significant difference between the two groups, with offspring in the high E_2 group showing similar sleep bouts (Figure 5C, P = 0.548, rank-sum test), sleep efficiency (Figure 5D, P = 0.714, rank-sum test), sleep stability (Figure 5F, P > 0.999, rank-sum test), and sleep fragmentation index (Figure 5G, P = 0.262, rank-sum

A

Call types \ Animal ID	M	L	O	S	T	U	AH	AK
Phee	√	√	√	√	√	√	√	√
Trill-phee			√	√	√	√		√
Twitter	√	√		√	√	√	√	√
Trill	√	√	√	√	√	√	√	√
Tsik	√	√	√	√	√	√	√	√
Ekk		√						
Cry		√		√			√	
Compond-cry	√	√	√		√	√		
Sub-harmonic	√	√	√	√		√	√	√
Total	6	8	6	7	6	7	6	6

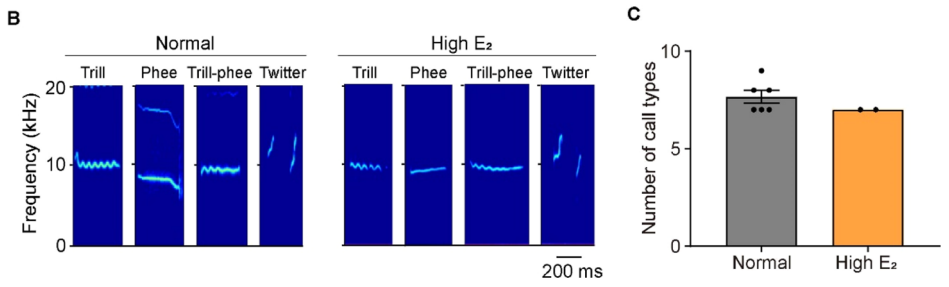

Figure 3. Vocal production of the offspring produced by normal and high E_2 female marmosets. (A) Different types of marmoset calls recorded from newborn marmosets at postnatal day one (P1). M, L, O, S, T, U, AH, and AK are animal IDs. Phee, trill-phee, twitter, trill, tsik, ekk, cry, compound-cry, and sub-harmonics are different types of marmoset calls. √ indicates that this type of call was recorded from the animal listed. Blank indicates that the animal did not produce this type of call during vocal recordings. (B) Spectrograms of marmoset calls (trill, phee, trill-phee, and twitter) recorded from newborn offspring of normal and high E_2 females. Left panel, calls produced by the offspring of normal female (normal); right panel, calls produced by the offspring of high E_2 female (high E_2). (C) Average number of call types emitted by offspring of normal and high E_2 females (offspring of normal females, n = 6 and offspring of high E_2 females, n = 2).

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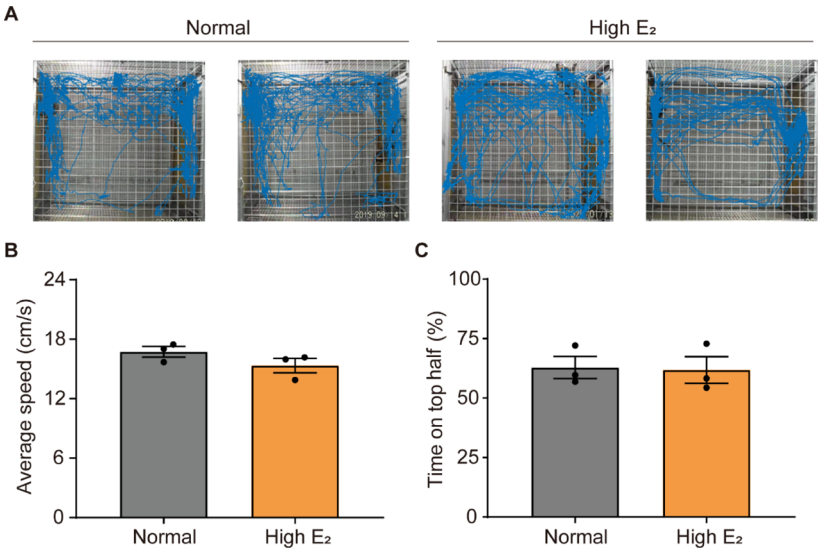

Figure 4. Free movement in the home cage recorded from the offspring of normal and high E_2 female marmosets. (A) Locomotion trajectories (blue traces) of marmoset offspring (8 months old) produced by normal and high E_2 females. The videos were captured using a high-definition video camera, which was positioned in front of the animal cage to record the animal's daily activities in the cage without external disturbance. For each animal, the recording was performed for 10 min per day for 5 times. (B) Average speed of marmoset offspring (8 months old) produced by normal and high E_2 females (offspring of normal females, $n = 3$ and offspring of high E_2 females, $n = 3$). For each animal, the locomotion speed was calculated by the distance traveled per second and averaged from 5 day recordings. (C) Time staying on the top half of the animal cage in marmoset offspring (8 months old) produced by normal and high E_2 females (offspring of normal females, $n = 3$ and offspring of high E_2 females, $n = 3$). For each animal, the time staying on the top half of the cage was averaged from 5 day recordings. The staying time on the top was usually used as one behavioral index of marmosets for stress. (B,C) were calculated from the same video recordings. Results are expressed as means ± SE.

test). These results indicated that offspring of the high E_2 group slept longer while not showing signs of sleep disorder.

DISCUSSION

ART is traditionally used for infertility treatments to achieve pregnancy. The demand and the range of scenarios for ART application have been steadily increasing due to delays in marriage and the increasing desire to preserve fertility for fertile populations.[36] Human and animal studies have shown that various ART procedures employed may result in high E_2 levels in the uterus of pregnant women.[7,14] It has been reported that an abnormal intrauterine environment influences fetal development because the speed of fetal development is much faster than any other stage of life,[16,19] and it may also be associated with an increased risk of various chronic pathology in the offspring.[19] For example, the studies on human beings showed that an abnormal maternal environment affected not only the physical health including low birth weight and preterm birth[37] but also the mental health of the offspring, for example, autism.[2] Experiments on animal models showed that maternal high E_2 levels increased the risk of infants with low birth weight and endocrine abnormalities.[3,5,7−10] However, these findings remain not consistently demonstrated, and controversy has surrounded the strength of these associations.

In the present study, we have established a maternal high E_2 non-human primate model to examine its potential risks to the development and behavior of the offspring. Our results revealed that high E_2 exposure in early pregnancy may cause low survival rates and birth weights of offspring, though the surviving infants did not show behavioral deficiencies during development. Our results suggest that high E_2 exposure in early pregnancy mainly affected embryo and fetal development in the uterus, which is demonstrated by a higher probability of miscarriage, stillbirth, and lower body weight of newborns. Thus, although ART treatments give great benefits to both infertile and fertile human populations, the manipulations and dosage of medicines applied should be strictly controlled to decrease the risks of health problems for the next generation. Interestingly, we found that surviving babies of the high E_2 group slept longer during development without signs of sleep disorders. As sleep is crucial for brain and body development,[30] sufficient sleep that surviving babies of the high E_2 group spent may be a compensation to make up for the deficiencies of physical and brain development. In addition, the current study found no significant difference between normal and high E_2 groups in the body weight of the offspring at 1 month old and 3 months old (Figure 2E,F), although the birth weight of the offspring produced by high E_2 female marmosets was much lower than that of normal female marmosets (Figure 2A). These results suggest that deficiency of physical development in the fetus period may be complemented during postnatal development. In mammals, epigenetic reprogramming is involved in early embryonic development.[7,38−40] A high E_2 environment in early life may alter epigenetic reprogramming and influence the body development and behavior of offspring. Thus, further studies in non-human primates are needed to explore the underlying in vivo mechanisms.

In summary, our study revealed that high estrogen levels in early pregnancy may cause low survival rates and birth weights of offspring, though the surviving infants did not show behavioral deficiencies during development.

Limitations of the Current Study. There are several limitations in the current study. First, we only tested the E_2 level 2 weeks after estradiol valerate administration and we did not test it throughout the pregnancy due to concern for animal health and welfare. Second, the sample size in the current study was small because we have nowhere to purchase marmoset monkeys due to the COVID-19 epidemic. The small sample

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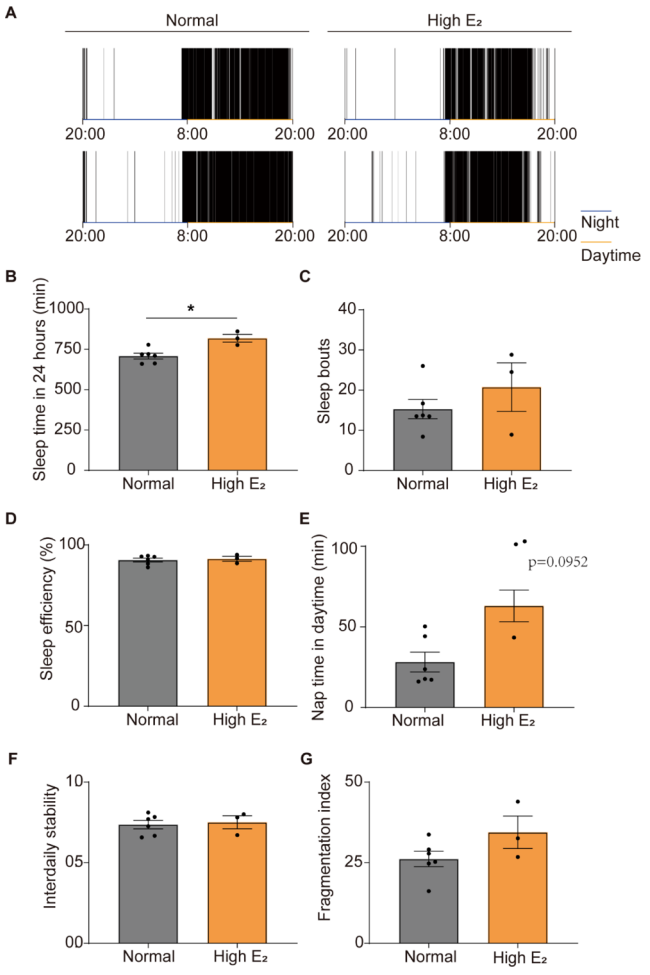

Figure 5. The offspring of the high E_2 females slept longer and displayed more naps. (A) Examples showing the daily activity and sleep patterns of the offspring produced by the normal (Left) and high E_2 female marmosets (Right) captured using a Mini ActiWatch. The data were continuously collected for 10−14 days from animals at the age of 9−12 months old. The sleep phase is indicated by a black bar and the awake phase is indicated by a white bar. Daytime: 8 AM to 8 PM, orange bar. Nighttime: 8 PM to 8 AM, blue bar. (B) Average sleep time per day for the offspring of normal and high E_2 marmosets (offspring of normal females, n = 6 and offspring of high E_2 females, n = 3). For each animal, the sleep time was defined as the total sleep time in 24 h and was averaged over 10−14 days during recordings. (C) Sleep bouts during the night time (8 PM to 8 AM) for the offspring of normal and high E_2 marmosets (offspring of normal females, n = 6 and offspring of high E_2 females, n = 3). (D) Sleep efficiency of the offspring produced by normal and high E_2 marmosets (offspring of normal females, n = 6 and offspring of high E_2 females, n = 3). Sleep efficiency is defined as the proportion (%) of time that animals spent sleep during the night. (E) Naps during daytime for the offspring of normal and high E_2 marmosets (offspring of normal females, n = 6 and offspring of high E_2 females, n = 3). (F) Interdaily stability showing the variation in the sleep time and sleep phase across the recording days (10−14 days) for the offspring of normal and high E_2 marmosets (offspring of normal females, n = 6 and offspring of high E_2 females, n = 3). (G) Fragmentation index showing the restlessness state of the offspring of normal and high E_2 marmosets (offspring of normal females, n = 6 and offspring of high E_2 females, n = 3). B−G was calculated from the same sleep data set. Results in B−G are expressed as means ± SE. *P < 0.05.

size may be the reason why some results in the current study statistically did not reach a significant level.

PROCEDURES

All experimental procedures were approved by the Zhejiang University Animal Care and Use Committee.

Animals. Five female marmosets (2−6 years old, 350−450 g) and 24 infant marmosets (0−8 months, 20−250 g) of both sexes were used in this study.

In our facility, two pairs of adult marmosets were donated by the Tianjin Medical University in 2017, and five pairs of adult marmosets were bought from Jiangsu Johnsen Bioresource Co., Ltd. (JOHNBIO, Shuyang, Jiangsu, China) in 2018. The marmoset monkeys were housed, maintained, and bred at the Zhejiang University Interdisciplinary Institute of Neuroscience and Technology (ZIINT) Non-Human Primate Center located at the Huajiachi Campus, Hangzhou, Zhejiang Province, China. All animals were held in pairs or family groups in cages (850 × 800 × 800 mm) with sufficient space and fresh air in colony rooms with a 24 h ventilation system. Resting boards, perches, swings, and hammocks were provided in the cages. The colony room was maintained under a 12:12 h day/night cycle, 26−28 °C temperature, and 45−55% relative humidity. Animals had free access to fresh water and were fed daily with 30−40 g of food, including cereal, eggs, sweet potatoes, honey, fruit, vegetables, and mealworms. Additional treats such as marshmallows, biscuits, and yogurt were used as positive reinforcements when transferring animals from their home cage to animal carriers. Veterinarians and experimenters inspected the animals at least once per day and dealt with sick individuals immediately. Breeding pairs were housed in separate rooms with a larger space and minimal human disturbance. Additional food such as mealworms, eggs, yogurt, and dried fruit was given to the animals in breeding cages to guarantee that the females were provided with a complete and sufficient diet. All experimental procedures were approved by the Animal Use and Care Committee of Zhejiang University following the National Institutes of Health guidelines. The protocol number is ZJU20190131.

Establishment of the Marmoset Model with a High E_2 Level at Early Pregnancy. Male and female marmosets at the age of 2−6 years old were paired in the home cage of the marmoset colony. If diagnosed as pregnant by palpation (typically 10−20 days after the start of the embryonic period, E10−20), female marmosets were fed 800 μg/kg of estradiol valerate (Aladdin, E129414-1G) dissolved in corn oil and dripped onto a marshmallow each morning for 45 days (Aladdin C116025-500ML). The dosage, frequency, and timing of administration were in accordance with previous studies.[7,23,41] Estradiol valerate ($C_{23}H_{32}O_3$) is a long-acting estrogen preparation used as a supplemental treatment for inadequate estrogen levels. The hormone treatment can last for 2 weeks after injection and indirectly affects follicular development and ovulation, and thus, it can simulate high estradiol levels under ART treatment. Females in the normal group were treated with marshmallows mixed with the same volume of corn oil. We further calculated and confirmed the date of pregnancy based on the birth date of the babies. Maternal blood was collected from the femoral vein every week to measure the serum E_2 concentration. The newborn marmoset babies were weighed after birth and were raised by their parents.

In Vivo Serum Levels of E_2 Hormones. Blood samples (<0.5 mL) were collected under awake conditions from the femoral vein of pregnant female marmosets in the high E_2 and

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control groups, respectively. Blood collection was carried out 2 weeks after the animals were treated with estradiol valerate or corn oil with gaps of at least 1 week between two times of blood collection, and for each animal, blood was collected at most three times during the early pregnancy. We closely monitored the animals' behavior after blood collection and provided additional food. The serum was separated from whole blood via centrifugation. Serum E_2 levels were analyzed by an EIA method (SM-3 automatic EIA analyzer) by the Beijing North Institute of Biological Technology (Beijing, China). The intra- and inter-assay coefficients of variation for the determination of all biochemical variables were less than 10%.

Vocal Recordings of Marmoset Calls. The calls of marmoset babies were recorded using the microphone (Audio-Technica AT2020) in a soundproof chamber that had walls covered completely in acoustic attenuating foam and a carpeted floor. Before vocal recordings, both the offspring and parent were transferred to the recording room and allowed 30 min to acclimate to the new environment. The infant was then separated from its parent and placed on a stuffed toy monkey to reduce the stress of parental separation. We recorded vocalizations for 5−10 min and then returned the infant to its parent. Because marmosets exhibit spontaneous altruistic behaviors, for example, nursing babies other than their own and rescuing infants from dangerous environments.[42] After the infant and parent were returned to their home cage, they were monitored for 30 min to ensure that the infant was carried and nursed. The marmosets were transferred to the soundproof chamber by a carrier, and the room temperature was maintained at 28 °C. When the recording was started, the animals were put in the experimenter's hand. The microphone was positioned 0.5 m in front of the animals. During the recordings, the experimenter touched the animals so that more types of calls could be recorded. Adobe Audition and Raven Pro 1.6 were used to record and analyze the calling. The type of call was identified according to the spectrogram.

Video Recordings in the Home Cage. Video recordings were carried out in the marmoset colony. Before video recording, the animals were transferred to a video recording cage, similar to their home cage except that one wall was transparent. The animals were allowed to acclimate to the new environment for 20 min for 3 days. They could see other marmosets in the same room similar to their home cage. Food and water were not provided in the video recording cage. During the recording session, subjects were transferred to the recording cage and allowed to move freely. No humans were in the room. The video recording started 5 min after the animal entered the video recording cage. The movement of the subjects was recorded using a video camera (ORDRO HDV-Z80) 1.5 m from the recording cage. Video recording lasted 10 min per day for each animal subject.

Daily Activity Patterns and Sleep Recordings. Offspring of the high E_2 group and the normal group participated in this experiment. Daily activity patterns and sleep of the animal subjects were assessed by activating (CamNtech).[43] The recording lasted 12−16 days, and the data were analyzed using ActiWatch Activity and Sleep Analysis 7. A Mini ActiWatch (CamNtech) was worn on the neck of the marmosets like a necklace. Animals were gradually habituated to wearing it over 1 week. 12−16 days of data collection were performed during which normal husbandry and feeding continued. At the end of the trial, the animals were lured into a transfer box, and the Mini ActiWatch was removed. The Mini ActiWatch is a data logger, that batches data into 15 s epochs. The activity counts from the first day and last day of the collection were removed from the analysis as these represented handling and cage manipulation. Average counts per hour were calculated for the 12 h of light (day) and 12 h of dark (night) and statistically compared for the offspring of the high E_2 group and normal control group.

Data Analysis. All values are expressed as the means ± standard error (SE) unless otherwise specified. Data in the results were plotted using Prism GraphPad and Matlab (MathWorks). A rank-sum test (Mann−Whitney U test), which is a non-parametric statistical test, was performed for comparison of data between the high E_2 group and normal control group. P values <0.05 were considered statistically significant for all the analyses and were indicated with asterisks, such as *p values < 0.05, **p values < 0.01, and ***p values < 0.001. The movement of the subjects was tracked using a code implemented in Matlab (MathWorks). Sleep data were analyzed using ActiWatch Activity and Sleep Analysis 7 software (CamNtech).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c03263.

Recordings of sleep and daily activity in marmoset offspring of normal and high E_2 groups (PDF)

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Author Contributions

L.G. and X.L supervised the project. L.Z., X.C., X.W, R.Q., Y.Y., and R.C. performed the experiments. X.C., X.W., R.Q., and G.S analyzed data. L.G. and X.C. wrote the manuscript. All authors reviewed and edited the manuscript and approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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