Reproductive Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Reproductive Biology

journal homepage: www.elsevier.com/locate/repbio

Original article

The decline of pregnancy rate and abnormal uterine responsiveness of steroid hormones in aging mice

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ARTICLE INFO

Keywords:
Aging
Uterus
Pregnancy
Mice
Hormonal responsiveness

ABSTRACT

Reproductive capacity in animals and women declines with increasing age. Although ovarian aging is considered as a main cause for the decline of pregnancy rate, whether uterine aging occurs remains unclear. Even if blastocysts are transferred from young donors to older pseudopregnant recipients, the rate of implantation is still low, suggesting the occurrence of uterine aging. In this study, we compared the pregnancy rate and the uterine responsiveness of steroid hormones in ovariectomized mice at age between 2- and 12-month-old. Compared to 2-month-old mice, there is a significant decrease of both pregnancy rate and the number of implantation sites in 12-month-old mice. In ovariectomized mice, the uterine responsiveness of steroid hormones is also significantly different between 2- and 12-month-old mice. On day 4, Muc1 and PR level in 12-month-old mice is significantly higher than that in 2-month-old mice, while Hand2 level is significantly lower in 12-month-old mice. Our data suggest that the abnormal responsiveness of steroid hormones may contribute to the decline of pregnancy rate in 12-month-old mice.

1. Introduction

Aging is characterized by a progressive decline in multiple physiological functions [1]. Reproductive potential in women declines with age [2]. For women in their early 20s, less than 10% of natural pregnancies result in spontaneous abortion, but spontaneous cumulative pregnancy rates start to decline more than 50% for women age 40 and older [3,4]. Spontaneous pregnancy rate declines to zero soon after 45 years of age [5]. Furthermore, primates undergo ovarian failure and loss of sex steroids at the end of reproductive life [6]. Advanced age is also related with poorer pregnancy rate following in vitro fertilization (IVF) and embryo transfer and gamete intrafallopian transfer [7].

A key aspect of reproductive aging is the decrease in oocyte quality and quantity **because** the poor pregnancy rate in old women can be rescued through egg donation from young women in IVF [8–10]. Ovarian aging is accompanied by a remarkable decline in the ovarian follicle pool and oocyte reserve, and an increase in low-quality oocytes [11–13]. Nevertheless, implantation rate decreases and miscarriage rate increases with the increase of maternal age [14]. In 12-month-old mice, there is a significant reduction in the number of implantation sites during early pregnancy [15]. When blastocysts are transferred from young donors to older recipients, the rate of implantation failure is also high [16,17]. Furthermore, older mice show an impairment of artificially induced decidual response [18,19]. This impaired decidual response may be in part related to reduced progesterone secretion in older mice [15]. The accumulated evidences suggest the occurrence of uterine aging. Uterine aging should be responsible for a decline in fecundity in most mammals. The luteal-phase progesterone secretion declines in older women and in aged golden hamsters [20,21]. For the comparison of young (4 months) and aged (10 months) ovariectomized mice, the decidual response to a standardized stimulus is reduced in the older animals even if there is an equivalent uterine growth in response to estrogen and progesterone [19]. In rodents, there is a decrease in the expression of endometrial estrogen and progesterone receptor in older ones [21,22].

In this study, we hypothesized that there is a significant reduction of pregnancy rate and steroid hormonal responsiveness during uterine aging. Therefore, we examined reproductive characteristics between 2- and 12-month-old mice. We showed that the abnormal uterine hormone responsiveness in aging mice may disturb implantation-related pathways and lead to impaired embryo implantation.

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http://dx.doi.org/10.1016/j.repbio.2017.09.001
Received 13 April 2017; Received in revised form 14 August 2017; Accepted 1 September 2017

2. Materials and methods

2.1. Animal treatments

Adult CD1 mice used in this study were purchased from Hunan SJA Laboratory Animal Co. and housed in a SPF facility with a controlled environment. All animal procedures were approved by the Institutional Animal Care and Use Committee of South China Agricultural University (Guangzhou, China). All of the experiments were carried out in accordance with the approved guidelines by South China Agricultural University. In this study, 2- and 12-month-old female mice were used for a comparison. Mice were sacrificed by cervical dislocation.

2.2. Analysis of implantation sites

Adult female mice (2 and 12 months of age) were mated naturally with fertile CD1 males (3–5-months old) to induce pregnancy (one male for one female), respectively. Day 1 of pregnancy (D1) is the day when vaginal plug was detected. The implantation sites on day 5 (D5) were visualized as blue bands by intravenous injection of Chicago Blue dye solution (Sigma, MO, USA) (2 months of age, N = 4 and 12 months of age, N = 6). Uteri were collected on day 8 (D8) to record the numbers of implantation sites (2 months of age, N = 3 and 12 months of age, N = 4). Each experiment was divided into two groups (2 and 12 months of age). The pregnancy rates on days 5 and 8 were the percentage of mice with implantation sites over total mice on days 5 and 8 of pregnancy, respectively.

2.3. Treatment of steroid hormones

Female mice were ovariectomized and rested for 2 weeks. Ovariectomized mice (29–31 g, body weight) were treated with an single subcutaneous injection of progesterone (P4, 1 mg/mouse, Sigma) or estradiol-17β (E2, 100 ng/mouse, Sigma), respectively as previously described [23]. The control group of mice was injected with 0.1 mL of sesame oil. E2 (100 ng) or P4 (1 mg) was dissolved and diluted in 100 μL sesame oil. These mice were sacrificed by cervical dislocation 24 h after treatments with steroid hormones for further analysis.

2.4. Analysis of telomerase expression

Aging was detected by telomerase. Several observations have established the role of telomeres in cellular aging. The accumulation of short telomeres with age in humans may contribute to age-dependent processes [24,25]. In this study, the uterine expression of telomerase was examined by real time PCR to analyze the difference after these mice were treated with estrogen or progesterone between 2- and 12-month-old mice.

2.5. RNA extraction and real-time PCR

Uteri was collected, snap frozen in liquid nitrogen and kept immediately at −70 °C. Total RNAs were extracted using TRI Reagent (Sigma). Extracted total RNAs were digested with RQ1 deoxyribonuclease I (Promega, Fitchburg, WI) for 30 min at 37 °C. Then 5 μg of digested total RNAs were reverse-transcribed into cDNA with PrimeScript reverse transcriptase reagent kit (TaKaRa, Dalian, China). Real-time PCR was performed using a SYBR Premix Ex Taq kit (TaKaRa) as described previously [26]. Rpl7, a house-keeping gene, was used for normalization. Data from real-time PCR were analyzed by the $2^{-\Delta\Delta Ct}$ method. Primer pairs specific for each gene were designed using Primer preimer 5.0. Primers used for real-time PCR were provided in Table 1.

2.6. Immunohistochemistry

Paraffin-embedded uterine sections (5 μm thick) were deparaffinized in xylene, rehydrated through a graded series of ethanol, and washed in distilled water. Antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0) by microwaving for 15 min and then cooling to room temperature. Endogenous horseradish peroxidase (HRP) activity was blocked with 3% H_2O_2 for 15 min. After non-specific binding was blocked with 10% horse serum at 37 °C for 1 h, sections were incubated with rabbit anti-Muc-1 (1:400; Thermo), rabbit anti-PR (1:1000, Thermo), or goat anti-Hand2 (1:200, Santa) diluted in PBS at 4 °C overnight, respectively. Followed by washing and incubating with biotin-labeled rabbit anti-goat IgG antibodies or goat anti-rabbit IgG antibodies (Zhongshan Golden Bridge, Beijing, China) for 30 min, respectively, sections were incubated with streptavidin-HRP complex (Zhongshan Golden Bridge) for 30 min. The positive signals were visualized using DAB Horseradish Peroxidase Color Development Kit according to the manufacturer's protocol (Zhongshan Golden Bridge) as a reddish-brown color. The sections were counterstained with hematoxylin.

2.7. Statistical analysis

At least three replicates were conducted for each treatment. The significance of difference between two groups was assessed by Student's *t* test. Data are presented as the mean ± standard deviation. P value < 0.05 was considered statistically significant.

3. Results

3.1. Fertility in natural aging mice

Pregnant mice were sacrificed on days 5 and 8 of pregnancy to examine the pregnancy rate. On day 5, the pregnancy rate was 100% in 2-month-old mice and 33.3% in 12-month-old mice. The number of implantation sites on day 5 was 14.5 in 2-month-old mice and 7.5 in 12-month-old mice (Fig. 1A and B). On day 8, pregnancy rate was 100% in 2-month-old mice and 50% in 12-month-old mice. The number of implantation sites on day 8 of pregnancy was 14 in 2-month-old mice and 2.5 in 12-month-old mice (Fig. 1C and D).

3.2. Expression of implantation-related proteins on day 4 of pregnancy

Muc-1(Mucin 1), an anti-attachment molecule, is down-regulated in the receptive stage [27]. On day 4 of pregnancy, Muc1 level in 12-month-old mice was strongly expressed in the glandular epithelium compared with that in 2-month-old mice (Fig. 2A and B). At the time of receptive phase, PR expression in the luminal epithelium was rapidly decreased [28,29]. Compared to 2-month-old mice, PR level in the luminal epithelium were slightly increased in 12-month-old mice (Fig. 2A and B). Hand2 (Heart and neural crest derivatives expressed transcript 2) protein was strongly expressed in the subluminal stromal cells on days 3 and 4 of pregnancy. Uterine deficiency of Hand2 resulted in impaired implantation [30]. Uterine Hand2 expression in 12-month-old mice was significantly reduced compared to 2-month-old mice (Fig. 2A and B).

3.3. Effects of steroid hormones on the expression of estrogen-responsive genes

In order to investigate whether aging has effects on the uterine responsiveness to estrogen or progesterone, ovariectomized mice of both 2 and 12-month-old mice were treated with estrogen or progesterone for 24 h. The mRNA levels of estrogen-responsive genes [Lactoferrin (Ltf) and complement C3 (C3)] were analyzed by real-time PCR. When ovariectomized mice were treated with sesame oil, there was no detectable difference for the expression levels of both Ltf and C3 between 2- and 12-month-old2-month-old mice (Fig. 3A and B). No difference was also detected between 2 groups for both Ltf an C3 after