Original Article

Advanced maternal age impairs spatial learning capacity in young adult mouse offspring

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Abstract: Effects of maternal aging on the offspring cognitive function remain controversial in population-based investigations, and information available in animal studies is very limited. We investigated the impact of a delayed first natural pregnancy on pregnancy outcomes in the mouse model. Spatial learning capacity in young adult mouse offspring was observed by step-down passive avoidance task and Morris water maze (MWM). Maternal serum α-klotho was measured by ELISA. Morphological characteristics of fetoplacental unit and offspring brain were identified by H&E and immunohistochemistry. Klotho, VDR and other related genes expression were quantified by real-time-RT-PCR and western blot. We found delayed pregnancy reduced fertility in female mice by three-fold (Young vs. Old: 5.0% vs. 20.7%), and increased adverse pregnant outcomes by eight-fold (Young vs. Old: 3.0% vs. 27.5%). Mice born to old mothers exhibited shorter retention trial latency in passive avoidance task and longer latency to find the platform in MWM, suggesting worse performance on the tests that measure learning and memory. Serum α-klotho level was lower in old female mice before pregnancy, whereas became comparable after pregnancy. Vitamin D receptor (VDR) expression, both in mRNA and protein, markedly decreased during the early stage of fetoplacental unit and offspring brain were identified by H&E and immunohistochemistry. Klotho, VDR and other related genes expression were quantified by real-time-RT-PCR and western blot. We found delayed pregnancy reduced fertility in female mice by three-fold (Young vs. Old: 5.0% vs. 20.7%), and increased adverse pregnant outcomes by eight-fold (Young vs. Old: 3.0% vs. 27.5%). Mice born to old mothers exhibited shorter retention trial latency in passive avoidance task and longer latency to find the platform in MWM, suggesting worse performance on the tests that measure learning and memory. Serum α-klotho level was lower in old female mice before pregnancy, whereas became comparable after pregnancy. Vitamin D receptor (VDR) expression, both in mRNA and protein, markedly decreased during the early stage of fetoplacental unit in old mice, especially in trophoblast giant cells when compared with that of young mice. Importantly, consistent with fetoplacental unit, VDR expression also declined in hippocampus from offspring born to old mice. These results suggest that young adult offspring from aged mothers exhibited worse cognitive function and the reduced VDR expression during fetoplacental development might play an important role.

Keywords: Advanced maternal age, learning and memory, vitamin D receptor, α-klotho, trophoblast giant cells

Introduction

The trend toward delayed first childbirth is increasing across industrial countries. In the United States, the birth rate for women aged 35-39 and 40-44 years was 19.8 and 3.9 births per 1000 women in 1980, but 47.2 and 10.3 in 2011 [1]. Similar trends also have been observed in European Union [2]. The developmental origins of health and disease (DOHaD) suggest that early life exposures may play an essential role in determining the risk of developing various diseases in adulthood [3]. Substantial complications for the fetus have been reported with increasing maternal age, including congenital malformations, preterm delivery, low birth weight, stillbirth or death after delivery, in turn leading to perinatal morbidity and mortality [4].

Apart from physical outcomes, the impact of maternal aging on offspring psychologic and cognitive development, especially after early infancy is not well studied. Furthermore, in contrast to the literature examining the incidence of psychotic and mood disorders [5-7], the research investigating maternal aging and cognitive outcomes in offspring are quite limited, and the associations varied in population-based observations [5, 8-10]. Saha S et al found that offspring from older mothers displayed better performance on tests of neurocognitive function [5]. Fergusson et al [9] showed that older motherhood exerted positive influence on cognitive scores measured in offspring. However, they also found that maternal socioeconomic status at childbirth and child-rearing environment accounted for most of the observed association. On the other hand, Malaspina et al [10] found older maternal age was associated with lower IQ scores in offspring. And population-based Swedish study indicated that offspring from women aged 30 and older had declined cognitive ability after
controlling birth order and birth year [8]. Advanced maternal age has been closely associated with higher socioeconomic status, educational attainment level and total income, all of which have been identified as independent predictors of better academic performance in offspring [11]. Therefore, confirmation of the causative role of maternal aging on offspring cognitive function in human population presents a scientific challenge.

Similar to human beings, offspring from the older mother in rodent species exhibited similar effect in behavioral features that closely resemble those of humans. Herein, our study used a mouse model to investigate the effects of maternal aging on cognitive function in young adult offspring. Furthermore, we also measured genes expression in fetoplacental development and offspring brain tissue to explore the potential mechanism underlying the relationship between early life exposure and cognitive impairment in offspring.

Materials and methods

Animals and sample collection

All experimental protocols involving the use of animals were approved by Wuhan University Research Ethics Board in accordance with the regulation of Guide for the care and use of laboratory animals published by the US National Institutes of Health. All animals were acclimatized to the experimental environment for one week prior to behavioral characterization.

Virgin C57BL/6J Young (9-12 weeks) and Old (32-35 weeks) female mice, mated with young C57BL/6J males (aged 10-13 weeks), were all purchased from the Hubei Provincial Center for Disease Control and Prevention, China. All mice were housed in a specific pathogen-free area at 25°C±2°C, humidity of 60%±2% and 12 h light: dark cycle. Animals had free access to standard laboratory chow diet and water. Female and male mice (ratio 2:1) were placed into the same cage at 19:00 and separated the next morning. Successful mating was confirmed by the presence of a vaginal plug and the day was recorded as E0.5 (Embryonic day). On E7.5 and E18.5, pregnant mice (both Young females and Old females) were killed by cervical dislocation and placentas were immediately dissected, part of samples was frozen in liquid nitrogen and stored at -80°C until analyzed, part of samples was fixed in 4% paraformaldehyde for histological analyses.

Offspring analysis

Offspring from both Young and Old mice were weaned three weeks after birth and separated based on sex. In total, there were 31 litters from Young females and 29 from Old females. Offspring from Young females labeled as Off-Y, from Old females labeled as Off-O. At postnatal week 6, behavior tests for spatial learning and memory were conducted. Two days after the tests, half of the offspring were killed by cervical dislocation, and brain tissues were immediately frozen in liquid nitrogen for further analysis, and half of the offspring were perfused with phosphate-buffered saline and then 4% paraformaldehyde. After perfusion, mice were sacrificed, and brains were harvested for histological study and immunohistochemistry analysis.

Morphological and histological analyses

Macroscopic abnormalities of placentas at E7.5 and E18.5 were evaluated for both Young and Old females. The absence of embryonic tissue in the implantation chamber was classified as early resorption site, whereas the presence of an embryo without a beating heart was classified as late resorption. For histological analysis, implantation sites at the same gestational age were collected and fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. The embedded sites were cut in 4 μm sections and stained with H&E for visualization.

For immune-histochemical analysis, the placental sections were waxed and rehydrated in xylol and a graded alcohol series containing decreasing concentrations of ethanol, followed by heat-induced antigen retrieval in citrate buffer. The sections were then treated with 3% hydrogen peroxide to quench endogenous peroxidase activity, and incubated with 5% goat serum. After incubation with the primary antibodies and HRP-linked secondary antibody (Servicebio, GB23303), the color reaction was developed using 3,3’-diaminobenzidine (Servicebio, G1211). The sections were mounted, air-dried, dehydrated, cover-slipped, and observed under a TS100 light microscope (Nikon Instruments, Tokyo, Japan). The density of phosphorylated KL and VDR staining was mea-
Serum klotho by ELISA

Blood samples were taken from the inner canthus vein from mice before pregnancy, during the early and late stage of pregnancy under the condition of ether anesthesia. Serum was harvested after blood clotting and centrifuged at 6000 rpm for 10 minutes, and stored at -80°C until analyzed. Serum samples were required 50-fold dilution, and α-klotho levels were measured using a mouse ELISA Kit (SEH757Mu; Cloud-Clone Corp. Wuhan USCN Business Co. Ltd.).

RNA isolation and qRT-PCR

Total mRNA was isolated from 50 mg frozen placenta using TRIzol reagent (15596; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription to synthesize cDNA was accomplished using PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Japan). Real-time PCR was performed using SYBR Premix Ex Taq II (Tli RNaseH Plus (Code No.RR820A/B, Takara, Japan) and StepOnePlus Real-Time PCR System (Life Technologies)). mRNA expression of target genes was calculated using a comparative Ct (ΔCt) method and normalized by β-actin. The sets of primers used for RT-PCR are provided in the Supplementary Table 1.

Western blotting

Proteins were extracted from collected samples with RIPA buffer containing protease and phosphatase inhibitors. 30 µg of proteins were separated by 10% SDS-PAGE gel and then transferred onto a PVDF membrane (Thermo Scientific, Bleiswijk, The Netherlands). Membranes were blocked with 5% non-fat milk in TBST buffer for 2 h and then probed with the following primary antibodies diluted in 5% non-fat milk in TBST at 4°C overnight: anti-Klotho (Abcam ab181373, 1:2000), anti-VDR (Proteintech, 14526-1-AP, 1:1000), anti-GAPDH (Proteintech, 10494-1-AP, 1:2000). Membranes were then rinsed and incubated with an anti-rabbit HRP-linked antibody (Servicebio, GB23303) diluted 1:3000 in TBS-Tween. Immunoreactive bands were visualized using the chemiluminescent substrate (Roche Diagnostics, Mannheim, Germany) and quantified on an Image J software (National Institutes of Health).

Spatial learning and memory

Passive avoidance task: Modified from Kim [12], a step-through passive avoidance task was carried out in identical illuminated and non-illuminated compartments (20×20×20 cm) with an electrifiable grid floor of 2-mm stainless steel rods. These compartments were separated by an entrance shutter (5×5 cm). For the acquisition trial, mice were initially placed in the illuminated compartment, and the shutter was opened after 10 s of acclimatization. After the mice entered the dark compartment, an electrical foot shock (0.8 mA) was delivered through the stainless steel rods.

For the retention trial, the mice were again placed in the illuminated compartment 24 h after the acquisition trial. In both trials, we defined latency as the time between the door being opened and the mouse entering the non-illuminated compartment and recorded until 300 s as the maximum latency time.

Morris water maze: Testing was based on an established protocol [13-15]. The water maze was a black pool with a diameter of 90 cm and a height of 90 cm. The pool was filled with water to a depth of 60 cm at temperature of 22°C±1°C controlled by aquarium heaters. The temperature of the room was controlled at 24°C. The maze was divided into four equal quadrants, with release points in each quadrant designated as southwest, northwest, northeast, and southeast. A black platform with a diameter of 9 cm was located 1 cm beneath the water's surface. And this platform was kept in the northwest quadrant. A variety of distal extramaze cues were positioned around the laboratory (e.g., wall posters, racks of equipment, lights).

During the training trials, the mice were allowed to find a hidden escape platform in the water pool in 60 s and were allowed to stay on the platform for 15 s. The mice failed to locate the platform in 60 s will be guided to the platform by the experimenter. The latency to find the hidden platform and the escape path length (distance traveled to the hidden platform) were recorded. Each mouse performed four trials daily for four consecutive days. On the fifth day, probe trials were conducted to assess spatial memory performance, during which the platform was removed, and mice were able to swim freely for 60 s. Time to find the platform (s), length of swim path (cm), time spent within a
Advanced maternal age lead to worse reproductive outcome

The first natural pregnancy outcomes in young (9-12 weeks) and aged female (32-35 weeks) mice paired with males (10-13 weeks) were observed. As shown in Table 1, delayed pregnancy markedly reduced female fertility (Young vs. Old: 5.0% vs. 20.7%, P=0.029), increased adverse outcomes which including spontaneous abortion, hard labor and stillbirth (Young vs. Old: 3.0% vs. 27.5%, P=0.005). Aged mother exhibited a reduced capacity to carry a viable pregnancy, and postmortem analysis showed that they had a higher number of fetal resorptions (Supplementary Figure 1, Young vs. Old: 13.0% vs. 52.2%, P=0.000, Table 1; Supplementary Figure 1). Furthermore, dams from the old mother group had lower body weight gain during pregnancy when compared with young mother counterparts (Yong vs. Old: 17.98±0.67 vs. 14.89±1.80, P=0.0548).

Delayed motherhood had no significant effect on litter size. Body weight of pups on weaning day was lower in the old mother group but did not reach the significant level (Table 1). Nonetheless, pups from the old mother group displayed a higher number of dying by natural causes during postnatal days 1-5 (Table 1).

Decreased TGCs in junctional zones of placenta from mice with advanced maternal age

Histological examination of a fetoplacental unit at E7.5 showed that the area of parietal trophoblast giant cells (P-TGCs) zone and the number of P-TGCs between the two groups did not

### Table 1. Pregnancy outcomes of Young and Old female mice

<table>
<thead>
<tr>
<th>Maternal outcomes</th>
<th>Young</th>
<th>Old</th>
<th>t/X²/F</th>
<th>P</th>
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<tr>
<td>Adverse pregnancy outcomes (n/N, %)²</td>
<td>1/33, 3.0</td>
<td>11/40, 27.5</td>
<td>7.882</td>
<td>0.0050</td>
</tr>
<tr>
<td>Infertility (n/N, %)²</td>
<td>2/40, 5.0</td>
<td>12/58, 20.7</td>
<td>4.759</td>
<td>0.0290</td>
</tr>
<tr>
<td>Fetal absorption (n/N, %)²</td>
<td>7/54, 13.0</td>
<td>35/67, 52.2</td>
<td>20.353</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gestational Weight Gain (g)²</td>
<td>1.44±0.15</td>
<td>1.89±0.29</td>
<td>2.120</td>
<td>0.1583</td>
</tr>
<tr>
<td>Pregnancy at E7.5</td>
<td>6.26±0.26</td>
<td>5.89±0.86</td>
<td>0.309</td>
<td>0.5836</td>
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<tr>
<td>Pregnancy at E12.5</td>
<td>17.98±0.67</td>
<td>14.89±1.80</td>
<td>4.076</td>
<td>0.0548</td>
</tr>
</tbody>
</table>

### Statistical analysis

Continuous data were presented as means ± standard deviation (SD), categorical data were expressed as frequencies and percentages (n, %). Differences between two groups were analyzed using the Chi-square analysis or Student’s t-test for categorical or continuous variables. ANOVA and post hoc test were applied for multiple groups comparison. Repeated measures ANOVA was conducted for indicates with multiple time points. Statistical significance in this study was set at *P<0.05. All analyses were performed with IBM SPSS Statistics 20.0 software.

### Results

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Delayed pregnancy and offspring cognitive function

At E18.5, the placenta showed typical architecture with three compartments: maternal decidua (MD), junctional zone (JZ) and labyrinth zone (LZ). However, the number of TGCs in Junctional area of placental sections from old mother was significantly lower than that of young mother (Figure 1B). The TGCs as the first cell type to terminally differentiate during embryogenesis are located at the uterine trophoblastic border. They are indispensable for implantation and decidualization because of the ability to invade and promote a production of progesterone [16]. Therefore, reduced number of TGCs in old motherhood might affect the formation of a fetal-maternal interface which leads to dysfunction of fetoplacental development.

Offspring from aged mice exhibits impaired spatial learning capacity

To evaluate the effect of advanced maternal age on cognitive alterations in the offspring, a passive avoidance task and Morris water maze test were performed. Repeated measures two-way ANOVA revealed no significant gender effects, therefore data from male and female mice were combined.

Advanced maternal age had a negative impact on spatial learning capacity of mice offspring. After experiencing fewer electric shocks, time to enter the dark compartment was significantly increased in the offspring from the young mother group than that from the old mother, suggesting that the memory capacity of mice offspring in the young mother group was superior to that of their counterparts (Figure 2A, 2B).

The Morris water maze test was performed the next day after the step-through passive avoid-
Figure 2. Mice offspring born to the young mother perform better in Learning and Memory tests. A: Mice born to the young mother had longer passive escape latency in Step-through Passive avoidance test. B: Offspring from the young and old mother did not exhibit difference in passive escape errors. C: Offspring from young mothers had
longer escape latency in Morris Water Maze. D: Offspring from the young group had a higher number of platform crosses. E: Offspring from both groups spent comparable time in target quadrant. F: Offspring born to young mother travelled longer distance in target quadrant. G: Representative swim paths in Morris Water Maze suggesting mice from old mother tended to use a random pattern. *, P<0.05 between the two groups; a, b, c, P<0.05 intragroup difference among offspring born to young mother; I, II, III: P<0.05 intragroup difference among offspring born to the old mother.

Figure 3. Profiles of Klotho and related genes expression during fetal-placental development. A, B: Placenta from old female expressed higher level of Klotho mRNA and protein. C: Young female had higher level of serum α-Klotho before pregnancy but comparable with old female after pregnancy. D: FGF23 mRNA reduced in placental tissues from the old female mice. E, F: VDR mRNA and protein expression decreased in fetoplacental units from old mother. G: ADAM17 mRNA increased in old female placenta. H: VEGF mRNA increased in old female placenta. E7.5: Embryonic day 7.5. E18.5: Embryonic day 18.5. *, P<0.05 between the two groups; a, compared to young mother before pregnancy. BP: Before Pregnancy.
ance task. During the training period of 4 consecutive days, all mice showed a modest but progressive reduction of the average escape latencies (Figure 2C) identified by repeated-measures one-way ANOVA. Offspring born to the old mice took a longer time to find the hidden platform (Figure 2C), had a lower total number of platform crosses (Figure 2D), and significantly shorter distance travelled in the target quadrant (Figure 2F), indicating impaired acquisition of spatial information as compared with their counterparts. The offspring of both groups did not differ in their swim speed or in a proportion of time spending for target quadrant searching (Figure 2E). Examples of representative swim paths during probe trials show that offspring born to the old mother searched for the hidden platform in a less precise manner (Figure 2G).

Maternal serum α-Klotho did not alter during early and late stage of pregnancy

Klotho, a protein with anti-aging properties, first reported in mice by Kuro-o group. Knockout mice for α-klotho are infertile [17], a population-based study showed that higher serum α-klotho level during preimplantation closely associated with higher maturation of oocytes and clinical fertilization rate after adjusting women’s age, ethnicity and BMI [18]. Therefore, we speculate that Klotho might play a role in reproductive outcome for aged mice. Our findings showed that young female mice had a higher level of serum α-klotho than that of their counterparts; however, serum α-klotho concentration became comparable between the two groups during the early and later stage of pregnancy (Figure 3C). Higher Klotho mRNA and protein expression were observed in placental tissue at E18.5 from old female when compared with that of young counterparts (Figure 3A, 3B). Enhanced A Disintegrin and Metalloproteinase 17 (ADAM17, Figure 3G) expression, which responsible for the shedding of α-klotho to the extracellular fluid [19], might explain the increased level of α-klotho in the old mice during pregnancy. FGF23, a critical component of FGFR-αKlotho complex in regulating vitamin D and phosphate metabolism, markedly reduced in placenta from the old mothers (Figure 3D).

To further identify Klotho’s distribution in fetoplacental development, we performed immuno-histochemical analysis found Klotho in a placent at the late stage of pregnancy, but not in a fetoplacental unit at E7.5 (Figure 4A). Although the overall intensity in the two groups was comparable, significantly stronger staining was observed in MD and LZ areas of placentas from the young mother (Figure 4B).

VDR expression were decreased in fetoplacental unit

It is well investigated that 1,25-dihydroxyvitamin D₃ (vitamin D), also have anti-aging properties [20]. Vitamin D functions are mediated through the nuclear vitamin D receptor (VDR) binding directly to its responsive elements. During fetal-placenta development, significant reduced VDR mRNA, but not protein expression, were observed in Old mice (Figure 3E, 3F). Immuno-histological analysis showed that the fetoplacental unit at E7.5 exhibited a significant difference in VDR expression in the two groups, with higher expression in fetal-placental units from Young dams (Figure 5A). In contrast, VDR expression in the placenta at E18.5 did not exhibit the difference between the two groups (Figure 5B).

Reduced expression of VDR in offspring hippocampus

VDR expression was markedly reduced in fetoplacental unit from the aged mothers during the early stage of pregnancy. Given that the critical association between vitamin D deficiency and cognitive decline [21], we speculated that impaired learning and memory capacity in offspring born to old mother might attribute to the downregulation of brain VDR expression. As to our expected, VDR mRNA and protein expression of the whole brain (cortex and hippocampus) from mice offspring born to the old mother.
Delayed pregnancy and offspring cognitive function

A

E7.5

Embryos VDR

TGCs VDR

B

E18.5

Placenta VDR

Young

Old

Young

Old
Delayed pregnancy and offspring cognitive function

Figure 5. Vitamin D receptor (VDR) protein markedly increased in fetoplacental units in young female mice. A: Embryo at Day 7.5 (×30) had higher VDR protein expression. Upper panel: Strong positive VDR observed in embryo-placenta unit; Middle panel: Higher magnification of embryo (×900); Lower panel: Quantification of VDR intensity in embryo and TGCs. B: VDR expression in Placenta at Day 18.5 is comparable in both groups. Upper panel: Panorama view (30×); Middle panel: Higher magnification of placenta (×900); Lower panel: Quantification of VDR intensity in placenta. *: P<0.05 compared to Young mother group.

Figure 6. VDR Expression and Localization in mice offspring brain. A: Higher brain VDR mRNA were detected in mice offspring born to the young mother. B: Higher VDR protein level in brain of mice offspring born to the young mother. C: Panorama view of brain section (×30) showed higher intensity of VDR in hippocampal sub-regions of offspring born to Young mother. D, E: Showed quantification of VDR intensity in CA1, CA2, CA3 & DG regions (×400).

Discussion

Women delaying their first pregnancies become an increasing trend, and primiparity at advanced maternal age is an independent risk factor of pregnancy complications. We established mice model of the first natural pregnancy to avoid the confounders of potential adaptations from previous pregnancies. Our study indicated that ageing motherhood not only exerted a negative impact on reproductive capability, also on embryonic, placental and postnatal development. We demonstrated that mice offspring born to old dams performed significantly worse in passive avoidance task and Morris water maze test compared to their counterparts, indicating impaired cognitive function. Reduced VDR expression in E7.5 embryos and in the hippocampus of mice offspring suggested that lower VDR in the early pregnancy might partially contribute to offspring cognitive impairment. Furthermore, reduced number of TGCs in placentas from old dams, which indicating impaired fetoplacental units, might also explain the influ-
ence of maternal aging on postnatal development.

Advanced maternal age is usually accompanied by higher socioeconomic status, educational attainment level, and total income [11], therefore, population-based studies examining maternal age and offspring cognitive function are problematic due to these confounders [22]. By contrast, the laboratory research investigating maternal aging and psychologic and cognitive outcomes in offspring is far from sufficient. In fruit flies, offspring born to aged mother were significantly less social in D. melanogaster [23]. In mice, Sampino S found pups conceived by older mice tended to social isolation compared with pups conceived by young mothers [24]. Lerch S observed that maternal age affected emotionality in the offspring by dark-light box and social recognition tests [25]. Our mice model is able to rule out the social-environmental impact on offspring’s behavior from motherhood. However, one study in mouse model found the mother with advanced age built better quality nest than young mother did while maternal activity was unaffected [25], indicating the necessity to provide detail information of breeding protocols for any animal study.

VDR expresses in placenta, decidua of pregnant mice [26], and regulates trophoblasts proliferation, migration, and differentiation in vitro [27] and in vivo [28], which is vital for a healthy fetal outcome. Vitamin D exerts a variety of biological functions by binding to its nuclear hormone receptor, VDR. Rats born to vitamin D deficient mothers had profound alterations in brain–reduced neurotrophins and disturbed neuronal differentiation [29]. One human study demonstrated a direct link between maternal hypovitaminosis D during pregnancy and offspring language impairment at early childhood [30]. Prenatal hypovitaminosis D disrupted brain development, which in turn, leads to brain dysfunction in adulthood. Other preliminary studies also suggested that gestational hypovitaminosis D could be linked to adult cognitive disorders [31, 32]. On the other hand, Vitamin D prevents cognitive decline and enhances hippocampal synaptic function in aging rats [21]. Significantly decreased VDR in early embryos from the old mother may cause a negative impact on fetoplacental units due to insufficient differentiation of trophoblasts in the critical period.

Although declined VDR expression was identified in offspring hippocampus from old mothers, the comparable level of VDR in placenta from both groups during the late stage of pregnancy indicates that VDR is likely one of the mechanisms involved. Future studies using suitable animal models to examine the “cause-effect” relationship should be performed to identify the contribution of maternal aging to offspring cognitive function.

Conclusion

To the best of our knowledge, we are the first to report that maternal aging had an adverse effect on offspring cognitive function. Given that maternal aging also affect long-term postnatal development, great attention should be paid when considering delayed pregnancy.

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Disclosure of conflict of interest

None.

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References

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Supplementary Table 1. RT-PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
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<tr>
<td>β-actin</td>
<td>GGCTGTATTCCCTCCATCG</td>
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<td>KL</td>
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Supplementary Figure 1. Embryonic development. A: Normal embryonic development in Young female. B: Early embryonic development stagnated in Older female. C: Metaphase fetal development stagnated in Older female.