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Original Article

Advanced maternal age induces fetal growth restriction through decreased placental inflammatory cytokine expression and immune cell accumulation in mice

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Abstract. Advanced maternal age is a risk factor for female infertility, and placental dysfunction is considered one of the causes of pregnancy complications. We investigated the effects of advanced maternal aging on pregnancy outcomes and placental senescence. Female pregnant mice were separated into three groups: young (3 months old), middle (8–9 months old), and aged (11–13 months old). Although the body weights of young and middle dams gradually increased during pregnancy, the body weight of aged dams only increased slightly. The placental weight and resorption rate were significantly higher, and live fetal weights were reduced in a maternal age-dependent manner. Although mRNA expression of senescence regulatory factors (p16 and p21) increased in the spleen of aged dams, mRNA expression of p16 did not change and that of p21 was reduced in the placenta of aged dams. Using a cytokine array of proteins extracted from placental tissues, the expression of various types of senescence-associated secretory phenotype (SASP) factors was decreased in aged dams compared with young and middle dams. The aged maternal placenta showed reduced immune cell accumulation compared with the young placenta. Our present results suggest that models using pregnant mice older than 8 months are more suitable for verifying older human pregnancies. These findings suggest that general cellular senescence programs may not be included in the placenta and that placental functions, including SASP production and immune cell accumulation, gradually decrease in a maternal age-dependent manner, resulting in a higher rate of pregnancy complications.
Key words: Aging, Cellular senescence, Inflammaging, Immune cells, Senescence-associated secretory phenotype (SASP)

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Advanced maternal age (> 35 years) is a risk factor for pregnancy complications. Pregnancy at an advanced maternal age is increasing, especially in developed countries [1, 2]. Advanced maternal age is associated with adverse pregnancy outcomes, such as placental dysfunction, fetal growth restriction, perinatal death, stillbirth, and preeclampsia [3–7]. Indeed, the stillbirth rate doubles by the late 30s and increases by 3–4-fold by the mid-40s [8].

Advanced maternal age is associated with reduced fertility, with oocytes exhibiting an age-related decline in quality [9, 10]. This effect is attributed to the exponential increase in chromosome segregation errors in the oocyte with age. Conversely, most complications of later pregnancy occur in the absence of any chromosomal abnormality in the newborn, such as fetal growth restriction, low birth weight, preeclampsia, and stillbirth [2]. These often share underlying pathogenesis rooted in the failure of accurate placentation [11]. Interestingly, Woods *et al*. [2] demonstrated that the development of abnormal embryos in aged female mice is associated with severe placentation defects, which result from major deficits in the decidualization response of the uterine stroma. Importantly, they clarified the impact of maternal age on uterine adaptability to pregnancy as a major contributor to the decline in reproductive success in older dams by experiments in which embryos from older dams were transferred into the uteri of younger dams, demonstrating that a young uterine environment can restore normal placental and embryonic development [2]. Therefore, advanced maternal aging is suggested to affect the uterine environment, such as decidualization and placentation, and is a major problem in pregnancy complications.

Generally, the function of various organs deteriorates with age. Aging is well known as an age-dependent low-grade and chronic inflammatory state that occurs due to the accumulation of immune cells and the production of proinflammatory cytokines [12]. Cellular senescence is recognized as a senescence-associated secretory phenotype (SASP) [13–16]. Various SASPs, including interleukin (IL)-1α, IL-1β, IL-6, and tumor necrosis factor-α (TNFα), clearly exhibit an age-dependent increase in expression [17], which reflect the emerging concept of "inflammaging (inflammation + aging)" [15].

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For example, Uri-Belapolsky *et al*. [18] reported that the deletion of the *Il1a* gene resulted in a higher rate of pregnancy in aged mice than in wild-type aged mice. Conversely, excessive IL-1β can induce cellular senescence and cause pregnancy complications, including increased susceptibility to infectious diseases and inflammation [12, 19–23]. In addition, we previously reported that age-related inflammation occurs in the uterus [24] and oviducts [25], but not in the corpus luteum in the ovary [26], indicating that inflammaging is an organ-dependent phenomenon. However, it is not well understood whether inflammaging occurs in the placenta of the aging mother.

In the present study, we hypothesized that as the mother ages, the mechanism of SASP production and immune function may be dysregulated within the placenta. Therefore, we investigated the effects of advanced maternal age on placental cytokine production associated with inflammaging and senescence in a pregnant mouse model.

Materials and Methods

Animal model

All experiments were approved by the Ethics Committee on Animal Rights Protection and were conducted in accordance with the Tokyo University of Agriculture Guide for Laboratory Animals. ICR wild-type mice were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). Female mice were separated into three groups: young (3 months old, n = 7), middle (8–9 months old, n = 6), and aged (11–13 months old, n = 5). Female mice showing proestrus by smear check were selected and mated with young male mice (3–4 months old), and the presence of a vaginal plug was confirmed the next day, which was designated as embryonic day (E) 0.5. Female mice with no observed plugs were excluded from the experiment. The body weight of pregnant mice was measured at E0.5–16.5. Blood pressure was measured in conscious mice using the tail-cuff method (BP-98A; Softron Ltd., Tokyo, Japan), as described previously [27]. At E16.5, the dams were anesthetized with isoflurane and killed by cardiac exsanguination. The number of viable and resorbed fetuses was counted, and the weights of the placenta, fetus, liver, peritoneal fat, and spleen were recorded. When no clear resorbed fetus was observed, the number of implantation sites (black trace in the endometrium) was counted. The fetal resorption rate was calculated using the following equation: fetal resorption rate = (the number of resorbed fetuses)/(the number of viable fetuses + resorbed fetuses with implantation sites).

Real-time RT-PCR

Total RNA was prepared using ISOGEN II (Nippon Gene Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions, and cDNA was synthesized using a commercial kit (ReverTra Ace; Toyobo Co., Ltd., Osaka, Japan). Real-time RT-PCR was performed using the CFX Connect™ Real Time PCR (Bio-Rad, Hercules, CA, USA) to detect the mRNA expression of *p16*, *p21*, *Il1a*, *Il1β*, *Tnfa*, and *Gapdh*. The primers used are listed in Supplementary Table 1. RT-qPCR was performed in duplicate with a final reaction volume of 20 µl containing 10 µl of SYBR Green (Thunderbird SYBR qPCR Mix, TOYOBO Co., Ltd.), 7.8 µl of distilled water, 0.1 µl of each primer (forward and reverse at 100 µM), and 2 µl of cDNA template. The amplification program consisted of a 5-min denaturation step at 95°C followed by 40 cycles of amplification (95°C for 10 sec, 60°C for 10 sec, and 72°C for 20 sec). The expression levels of each target gene were normalized to the corresponding GAPDH threshold cycle (CT) values using the ΔΔ CT comparative method [28].

Cytokine array

Lysates from placental tissues were prepared using RIPA buffer (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Tissues were washed with cold PBS, incubated with RIPA buffer, and then homogenized on ice. Tissue lysates were subsequently transferred into 1.5 ml tubes and centrifuged at 12000 × g for 20 min at 4°C. Supernatants were transferred to fresh tubes and stored at –80°C before analysis. The extracted protein concentration was measured using a BCA assay (Thermo Fisher Scientific, Waltham, MA, USA). The placental proteins of each individual (n = 5–7 dam/group) were evenly mixed and set to a volume of 500 µg protein and used for the cytokine array.

The Mouse Cytokine Antibody Array was purchased (Membrane, 62 Targets, ab133995, Abcam, Cambridge, UK). The cytokine antibody array was performed according to the manufacturer's protocol. Briefly, the membranes pre-coated with capture antibodies were incubated with the pooled placental protein solution. After washing with a wash buffer and exposure to a detection antibody, the membranes were incubated with streptavidin-HRP and Chemi Reagent Mix. The immunoblot images were captured and visualized using the ImageQuant LAS 4000 (GE Healthcare, UK Ltd., Buckinghamshire, UK), and the intensity of each spot in the captured images was analyzed using the Molecular Dynamics ImageQuant 5.0 software (GE Healthcare).

Determination of expression of cytokines, progesterone, and senescence-associated β-galactosidase

Levels of IL-1β and TNFα in the placenta were determined using a murine ELISA kit (DY401 and DY410, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Plasma progesterone (P4) concentration was measured using a direct enzyme immunoassay (EIA) [29]. Absorbance at 450 nm was measured using a microplate spectrophotometer (DS Pharma Biomedical, Osaka, Japan). Levels of senescence-associated β-galactosidase (SA-β-gal) in the placenta were determined using a cellular senescence assay kit (CBA-213, Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions. Fluorescence at 350 nm (excitation) / 465 nm (emission) was measured using a microplate reader (Spark 10M, Tecan Group Ltd., Mannedorf, Switzerland).

Flow cytometry analysis

To analyze the populations of inflammatory cells within the placental tissue and spleen of pregnant mice, the mice were sacrificed under general anesthesia. Placental and splenic cells were isolated from mice, as described previously [30, 31]. The cells were washed, treated with erythrocyte lysis buffer, and resuspended in PBS supplemented with 3% fetal bovine serum for flow cytometry analysis. Cells were labeled with the following antibodies purchased from eBioscience (San Diego, CA, USA): phycoerythrin (PE)-conjugated anti-Ly6G (Cat 12-9668-82), PE-conjugated anti-CD11b (Cat 12-0112-82), FITC-conjugated anti-F4/80 (Cat 11-4801-82), and allophycocyanin-conjugated anti-CD45 (Cat 17-0451-82). The cells were examined by flow cytometry using a NovoCyte flow cytometer (ACEA Biosciences Inc., San Diego, CA, USA).

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Differences between treatment groups were identified using nonparametric analysis of variance, followed by the Mann-Whitney U-test or Kruskal-Wallis test using StatView software. Statistical significance was set at $P < 0.05$.

Results

Advanced maternal age affects body weight during pregnancy

Young (3 months old, n = 7), middle (8–9 months old, n = 6), and aged (11–13 months old, n = 5) ICR mice were mated with young male mice (3–4 months old), and the impact of advanced maternal age on maternal body weight, blood pressure, and conceptus development were investigated at E16.5. During E0.5-4.5, the body weight of middle and aged female mice was significantly higher than that of young female mice (Fig. 1A). Although the body weight of young and middle dams increased gradually during pregnancy, the body weight of aged dams only slightly increased (Fig. 1A). We calculated the body weight gain rate based on E0.5. The increased ratio of body weight in young dams at E9.5-16.5 was significantly higher than in middle and aged dams, i.e., the increased ratio in middle dams at E13.5-16.5 was significantly higher than that in aged dams (Fig. 1B). Mean blood pressure was stable and did not differ between young and aged dams during pregnancy (Fig. 1C).

Advanced maternal age reduces fetal weight but increases placental weight and resorption rate

In aged dams, the resorption rate was significantly higher than that in the young and middle dams (Fig. 2A). The number of implantations of dams was gradually decreased (but not significantly due to the small number of dams examined) in an age-dependent manner (13.9 ± 1.4 in the young dams, 12.7 ± 2.4 in the middle dams, and 9.7 ± 1.9 in the aged dams). The number of live fetuses and live fetal weight decreased in an age-dependent manner (Fig. 2B and C). In contrast, placental weight was increased in an age-dependent manner (Fig. 2D, 97 placenta / 7 young dams, 70 placenta / 6 middle dams, and 15 placenta / 5 aged dams). A higher incidence of resorption assumed a decrease in ovarian function. In general, plasma P4 concentrations are elevated in the later phase of pregnancy in mice [32]. However, plasma P4 concentration did not differ among the three groups at E16.5 (Fig. 2E). In the maternal organ weight, peritoneal fat and spleen weight were higher in middle or aged dams than in young dams, but there were no differences in the liver weight of dams among the three groups (Figs. 2F–H).

Expression of senescence-regulatory molecules is not increased in the placenta in a maternal age-dependent manner

Cellular and tissue senescence is a state of irreversible cell cycle arrest resulting from high levels of the cyclin kinase inhibitors $p16^{INK4/6/CDKN2}$ and/or $p21^{CDKN1}$ [12, 33]. Therefore, we examined the mRNA expression levels of p16 and p21 in the placenta and spleen as representative examples of the immune system. In the spleen, mRNA expression of p16 and p21 was increased in a maternal age-dependent manner (Figs. 3A and B), suggesting that tissue senescence occurs due to aging. Similar changes in mRNA expression were also observed in the liver tissues (data not shown). However, the mRNA expression of p16 in the placenta did not change among the three groups, and p21 mRNA expression decreased significantly in a maternal age-dependent manner (Figs. 3C and D). Next, we examined the expression levels of SA-β-gal as a senescence marker in the placenta. As a result, the expression levels of SA-β-gal did not change in the placenta between the young, middle, and aged groups (Fig. 3E). These findings suggest that age-dependent changes in senescence-regulatory molecules in the placenta may differ from those in other organs.

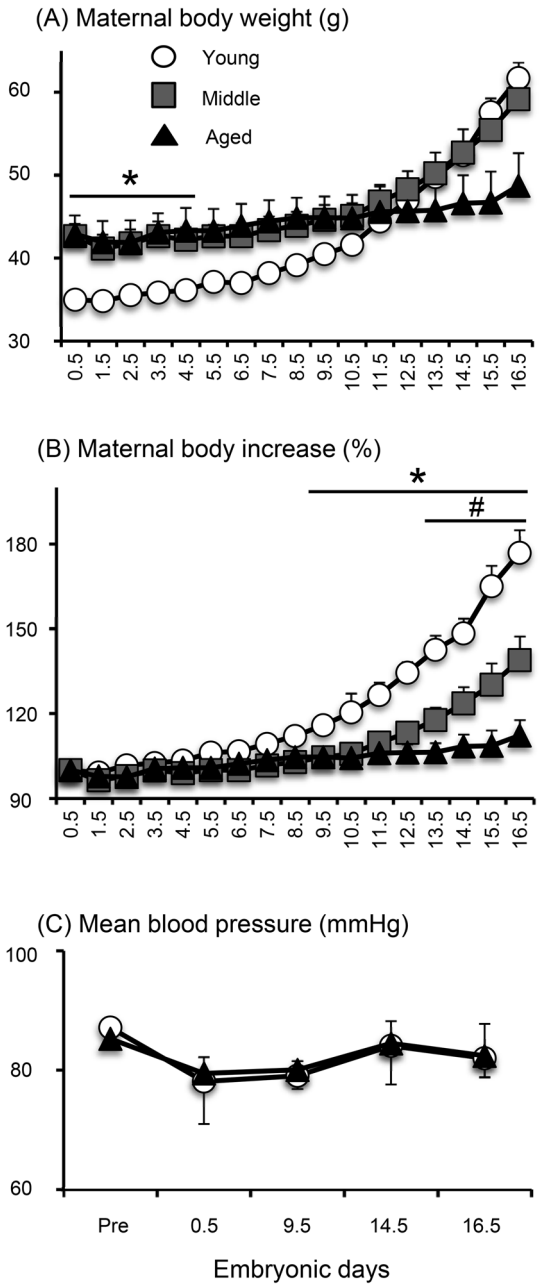

Fig. 1. Effects of aging on maternal body weight and blood pressure during pregnancy. Young (3 months old), middle (8–9 months old), and aged (11–13 months old) ICR mice were used. Maternal body weight (A), increase in maternal body weight (B), and blood pressure (C) were investigated. Data are expressed as the mean ± standard error of the mean (SEM). Significant differences are indicated as *P < 0.05: young *vs*. middle-aged and aged groups, and # P < 0.05: middle *vs*. aged groups.

Production of inflammatory cytokines in the placenta is reduced in a maternal age-dependent manner

We examined various inflammatory cytokines, including major SASPs, using a cytokine array as a screening test. As shown in Supplementary Fig. 1, we measured the area of each dot that corresponds to a cytokine and measured their expression relative to the positive control. For example, the contents of IGFBP-6 and IGFBP-3 proteins were much higher than other proteins, and the levels of these proteins were higher in the young maternal placenta than in the middle

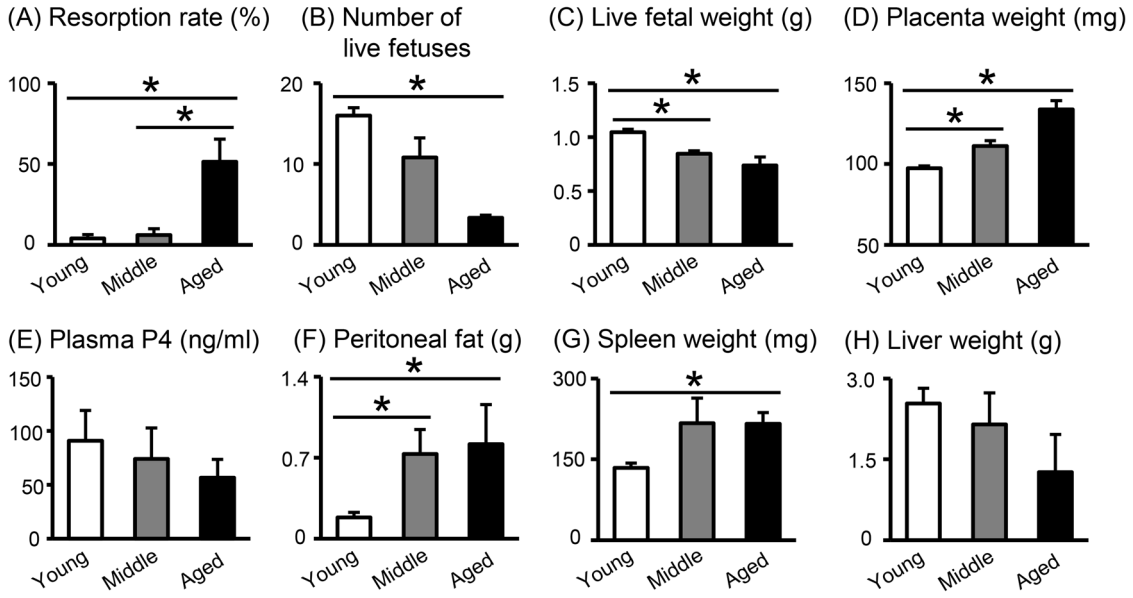

Fig. 2. Effects of maternal aging on pregnant conditions. Young, middle, and aged pregnant mice were used, and the resorption rate, number of live fetuses, and placental, fetal, liver, peritoneal fat, and spleen weights were investigated at E16.5. Significant differences were detected using nonparametric analysis of variance; * $P < 0.05$.

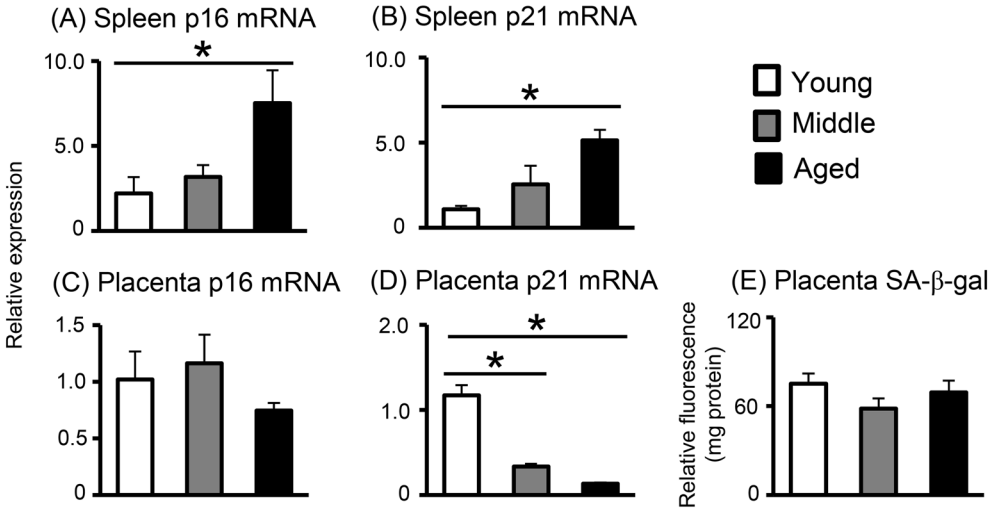

Fig. 3. Effects of maternal aging on p16 and p21 mRNA expression and SA-β-gal expression in the placenta and spleen. The placenta and spleen were collected from young, middle, and aged pregnant mice. The mRNA expression levels of *p16* and *p21* and SA-β-gal levels were determined. Significant differences were detected using nonparametric analysis of variance; * $P < 0.05$.

and aged maternal placenta (Supplementary Fig. 1). In contrast, the expression of CTACK (CCL27) and CXCL16 was higher in the aged maternal placenta than in the young and middle maternal placenta (Suppplementary Fig. 1). Furthermore, representative inflammatory cytokines and SASPs were extracted from a total of 62 factors of protein expression (Fig. 4A), and their relative expression levels in the young maternal group were calculated (Fig. 4B). We selected 18 inflammation-related cytokines, and the protein levels of 16 factors, excluding 2 factors (CTACK and CXCL16), were decreased in a maternal age-dependent manner (Fig. 4A). The levels of these 16 factors in the placenta derived from middle and aged dams were reduced by approximately 50% when compared with those in the placenta derived from young dams (Fig. 4B).

SASP production is reduced in a maternal age-dependent manner in the placenta, but not in the spleen

We further selected major SASPs, including IL-1α, IL-1β, and TNFα, from the above cytokine array results (Fig. 5). Interestingly, mRNA expression of these SASPs was significantly decreased in the middle or aged maternal placenta compared with that in the young maternal placenta (Figs. 5A, C, and E). In contrast, in the spleen, mRNA expression of IL-1α and IL-1β was increased in the aged group than in the young group, and TNFα mRNA expression did not change among the three groups (Figs. 5B, D, and F). Therefore, we determined the protein concentrations of IL-1β and TNFα in the placenta (Figs. 5G and H). Confirming the results of cytokine array and mRNA expression, the protein concentration of IL-1β

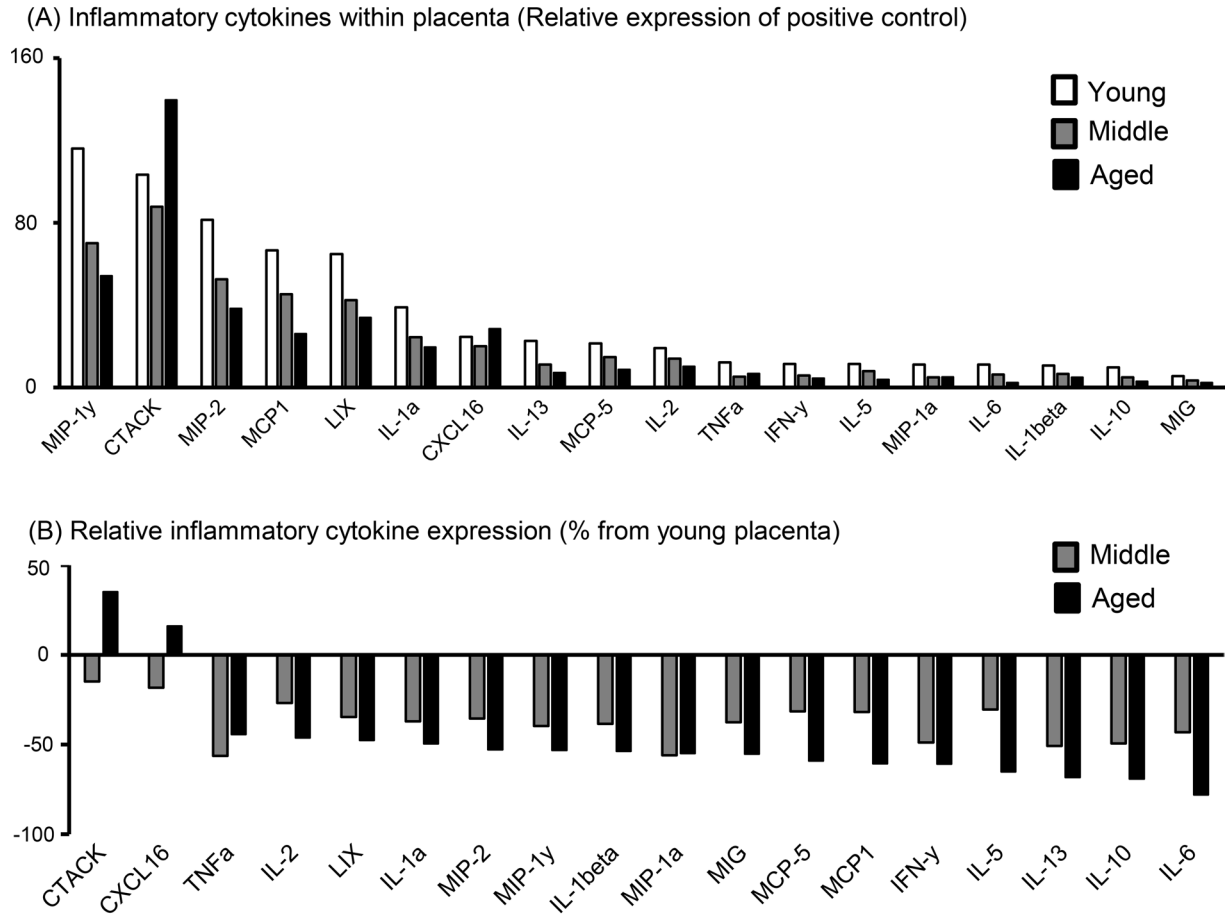

Fig. 4. Effects of maternal aging on inflammatory cytokine expression in the placenta. The placenta was collected from young, middle, and aged pregnant mice. Cytokine arrays were conducted using placental tissue proteins. (A) Expression of inflammatory cytokines was calculated (A) and relative inflammatory cytokine expression in the young placenta was calculated (B).

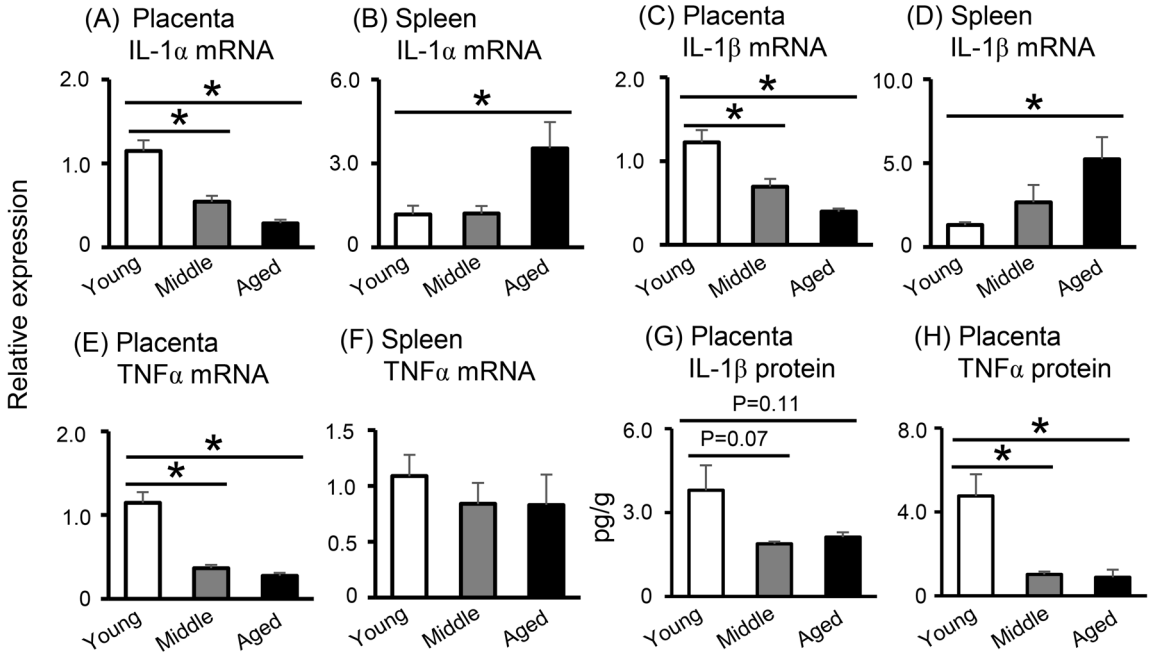

Fig. 5. Effects of maternal aging on senescence-associated secretory phenotype (SASP) expression in the placenta and spleen. The placenta and spleen were collected from young, middle, and aged pregnant mice. The mRNA expression of *IL-1α*, *IL-1β*, and *TNFα* was determined (A–F). Using ELISA, placental protein levels of *IL-1β* and *TNFα* were determined (G and H), and significant differences were detected using nonparametric analysis of variance; * $P < 0.05$.

slightly decreased, but the protein concentration of TNFα significantly decreased in middle or aged maternal placenta compared with that in the young maternal placenta.

Advanced maternal age changes immune cell accumulation and population in the placenta

Generally, senescent cells secrete SASP factors and are associated with immune cell accumulation to remove senescent cell own within the tissues. The spleen ratio of $CD45^+$ immune cells did not differ between young and aged dams (Fig. 6A). In addition, there was no change in immune cell populations, such as $Ly6G^+$ neutrophils, $CD11b^+$ neutrophils or macrophages, and $F4/80^+$ macrophages within the spleen between young and aged dams (Figs. 6B–D). These data indicate that maternal aging does not affect immune cell accumulation and population within the spleen.

In contrast, in the placenta, the ratio of $CD45^+$ immune cells was significantly reduced in aged dams than in young dams (Fig. 6E). In addition, within the $CD45^+$ immune cell population, the ratio of $Ly6G^+$ and $CD11b^+$ cells was significantly higher, but the number of $F4/80^+$ cells was significantly lower in the placenta derived from aged dams than that in the placenta derived from young dams (Figs. 6F–H). These results indicate that the number of placental immune cells is reduced, and the immune cell populations are changed in a maternal age-dependent manner.

Discussion

In the present study, ICR mice, which are prolific and have a low rate of miscarriage and embryo resorption during pregnancy, were used as a model. Recently, Furuya *et al*. [34, 35] established an advanced maternal age model using 6-month-old ICR mice and showed that these old ICR pregnant mice manifested declining fertility, fetal growth restriction, a higher rate of intrauterine fetal death, and increased blood pressure at late gestation. However, the placental weight of 6-month-old ICR pregnant mice did not differ from that of 2-month-old young mice, whereas placental weight increases in aged pregnant women (in the late 30s and 40s) [1, 36]. Flurkey *et al*. [37] provided comparable mouse ages for humans and compared the maturational rates of both humans and mice; 3–6-month-old mice as a model of 20–30-year-old humans and 10–14-month-old mice as a model of 38–47-year-old humans. Therefore, we set up further older pregnant ICR mice as models of humans: middle (8–9 months old) as a model of the late 30s and aged (11–13 months old) as a model of 40s of pregnant women. As a result of this setting, similar to the result of aging pregnant women, these middle or aged pregnant mice showed increased placental weight, low fetal weight, and increased intrauterine fetal mortality (resorption rate). Similar to the present study, pregnant C57BL/6 mice aged 8 months or older showed increased placental weight, fetal growth restriction, and placental dysfunction [1]. These findings suggest that models using pregnant mice older than 8 months are more suitable for verifying older human pregnancies.

The senescence program is coordinately regulated by the p53/p21 and p16 pathways that promote and sustain growth arrest in senescent cells [38]. Although short-term effects of cellular senescence are needed for tumor suppression, tissue injury restriction, and embryonic development, the long-term accumulation of senescent cells is associated with detrimental complications such as age-related pathologies. Cellular senescence pathways are also induced in the placenta and are essential for triggering syncytiotrophoblast fusion during pregnancy [38, 39]. In addition, the activation of cellular senescence pathways in the human placenta are increased in a pregnancy-phase-dependent manner, including an increase in p16-, p21-, and SA-β-gal-positive senescent cells [39, 40]. However, little information has been reported on the maternal age-related cellular senescence pathway in the placenta. We showed that p16 and p21 mRNA expression and SA-β-gal levels did not increase, and the expression of SASP-related factors was clearly decreased in the placenta of middle and aged maternal groups. Likewise, placental p53 mRNA expression did not differ between young and aged mice

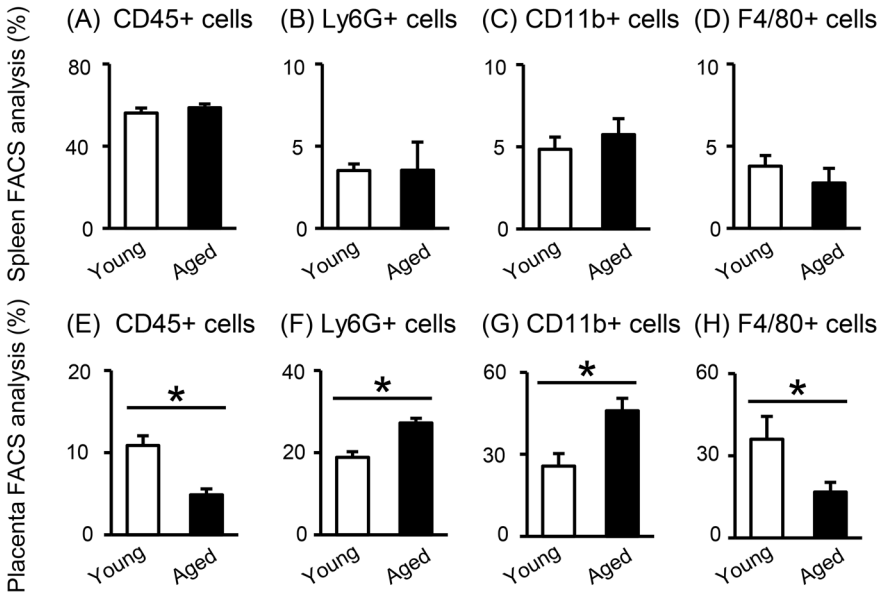

Fig. 6. Effects of maternal aging on immune cell populations in the placenta and spleen. The placenta and spleen were collected from young, middle, and aged pregnant mice. Percentages of immune cells ($CD45^+$ cells), neutrophils ($CD45^+/Ly6G^+$ cells or $CD45^+/CD11b^+$ cells), macrophages ($CD45^+/F4/80$ cells) in the placenta and spleen were analyzed by flow cytometry (A–H). Significant differences were detected using nonparametric analysis of variance; * $P < 0.05$.

[41]. The generally observed phenomenon of increased SA-β-gal and SASP expression in tissues associated with aging may not occur in the placenta. Notably, Gal *et al*. [38] reported that senescence pathways are downregulated in the human placenta during intrauterine growth restriction, and the absence of cellular senescence molecules such as p21 leads to functional and morphological abnormalities in the murine placenta. Thus, the cellular senescence program may be essential for maintaining the structure and function of the placenta.

In general, senescent cells secrete various SASPs, and activation of SASPs can promote interactions with the immune system and reinforce the growth arrest of the adjacent senescent cells [12]. In contrast, as the placenta did not undergo age-related cellular senescence, the production of SASP-related factors from the placenta clearly decreased in a maternal age-dependent manner, suggesting that cellular senescence is not associated with maternal age-dependent changes in placental function. Moreover, the total number of accumulated immune cells was also reduced in the placenta derived from aged dams. From these results, we suggest two possibilities: 1) low levels of SASP-related factors in aged placental cells result in the decrease in the number of accumulated immune cells because SASPs include various chemokines and 2) due to the decrease in immune cell migration via maternal aging, immune cells cannot accumulate in the aged placenta, resulting in a decrease in SASP production. In the present study, we have not clarified which possibility occurs at what timing, but we suggest that the combined occurrence of these possibilities may be associated with pregnancy complications.

Specific immune cells regulate the recognition of the embryo, implantation, and success of pregnancy. Recently, Woods *et al*. [2] demonstrated that uterine immune cell composition, especially at the maternal-fetal interface, is affected by maternal age because maternal aging leads to an overall reduction in the number of leukocytes, such as macrophages and dendritic cells, in mice. Importantly, they reported that by transferring embryos from aged females to young foster mothers, the frequent developmental abnormalities of aged mothers were rescued by the environment of a young foster mother [2]. They demonstrated that maternal age affects the hormonal responsiveness of uterine stromal cells by interfering with the progesterone response [2]. These results indicate that most developmental problems in aged females to be of maternal origin but not the “old” oocytes, and an unfavorable maternal uterine environment may lead to placental dysfunction and pregnancy complications.

In this study, it is unclear why the placental weight was higher in aged dams despite fetal growth restriction because poor placentation is generally associated with fetal growth restriction in humans. In this regard, the placental function of nutrient transport is significantly higher in placentas from mothers aged > 40 years than in younger ones, and the placental weight of aged mothers is higher than that of younger mothers [1]. In the morphology of mouse placentas, an imbalance of spongio-trophoblasts and the labyrinth zone is observed in aged dams compared with younger dams, and the higher expression of placental development-associated factors, such as insulin-like growth factor-1 and bone morphogenetic protein-2, is observed in the placenta from aged dams [2]. These findings suggest that the nutritional supply-related system in the placenta may change in aged dams and that increased placental growth is an adaptation to promote fetal growth and survival.

Interestingly, assisted reproductive technologies (ARTs), such as in vitro fertilization (IVF) and embryo culture, induced placental overgrowth, reduced fetal weight, and reduced placental DNA methylation in a mouse model. In contrast, compared to IVF and embryo culture, the technique of embryo transfer had no bigger problem for these changes. These data indicate that embryo culture is the major factor contributing to most placental abnormalities [42]. Since the placental and fetal phenotypes induced by ARTs are very similar to those of the aging mouse model in the present study, placental hyperplasia associated with aging is also considered a type of dysfunction. However, placental tissue structure was not analyzed in the present study. In addition, Woods *et al*. [2] demonstrated that although embryos from aged dams were allowed to develop in young dams using the embryo transfer technique (without embryo culture), placental weight was not described. Therefore, it is necessary to investigate this issue in the future.

In summary, in this study, advanced maternal aging affected the intrauterine environment, resulting in increased placental weight, fetal growth restriction, decreased number of live fetuses, and increased resorption in ICR mice (8 months or older). In the placenta, general cellular senescence programs may not be active, and both SASP production and immune cell accumulation gradually decrease in a maternal age-dependent manner. These changes in the placenta may be associated with a higher rate of pregnancy complications.

Conflict of interests: The authors declare no conflict of interest.

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References

1. **Lean SC, Heazell AEP, Dilworth MR, Mills TA, Jones RL.** Placental dysfunction underlies increased risk of fetal growth restriction and stillbirth in advanced maternal age women. *Sci Rep* 2017; **7**: 9677. [Medline] [CrossRef]
2. **Woods L, Perez-Garcia V, Kieckbusch J, Wang X, DeMayo F, Colucci F, Hemberger M.** Decidualisation and placentation defects are a major cause of age-related reproductive decline. *Nat Commun* 2017; **8**: 352. [Medline] [CrossRef]
3. **Abel EL, Kruger M, Burd L.** Effects of maternal and paternal age on Caucasian and Native American preterm births and birth weights. *Am J Perinatol* 2002; **19**: 49–54. [Medline] [CrossRef]
4. **Jolly M, Sebire N, Harris J, Robinson S, Regan L.** The risks associated with pregnancy in women aged 35 years or older. *Hum Reprod* 2000; **15**: 2433–2437. [Medline] [CrossRef]
5. **Delbaere I, Verstraelen H, Goetgeluk S, Martens G, De Backer G, Temmerman M.** Pregnancy outcome in primiparae of advanced maternal age. *Eur J Obstet Gynecol Reprod Biol* 2007; **135**: 41–46. [Medline] [CrossRef]
6. **Salihu HM, Wilson RE, Alio AP, Kirby RS.** Advanced maternal age and risk of antepartum and intrapartum stillbirth. *J Obstet Gynaecol Res* 2008; **34**: 843–850. [Medline] [CrossRef]
7. **Lamminpää R, Vehviläinen-Julkunen K, Gissler M, Heinonen S.** Preeclampsia complicated by advanced maternal age: a registry-based study on primiparous women in Finland 1997-2008. *BMC Pregnancy Childbirth* 2012; **12**: 47. [Medline] [CrossRef]
8. **Hansen JP.** Older maternal age and pregnancy outcome: a review of the literature. *Obstet Gynecol Surv* 1986; **41**: 726–742. [Medline] [CrossRef]
9. **Malhi PS, Adams GP, Mapletoft RJ, Singh J.** Oocyte developmental competence in a bovine model of reproductive aging. *Reproduction* 2007; **134**: 233–239. [Medline] [CrossRef]
10. **Nelson SM, Telfer EE, Anderson RA.** The ageing ovary and uterus: new biological insights. *Hum Reprod Update* 2013; **19**: 67–83. [Medline] [CrossRef]
11. **Norwitz ER.** Defective implantation and placentation: laying the blueprint for pregnancy complications. *Reprod Biomed Online* 2006; **13**: 591–599. [Medline] [CrossRef]
12. **Watanabe S, Kawamoto S, Ohtani N, Hara E.** Impact of senescence-associated secretory phenotype and its potential as a therapeutic target for senescence-associated diseases. *Cancer Sci* 2017; **108**: 563–569. [Medline] [CrossRef]
13. **Lim MA, Lee J, Park JS, Jhun JY, Moon YM, Cho ML, Kim HY.** Increased Th17 differentiation in aged mice is significantly associated with high IL-1β level and low IL-2 expression. *Exp Gerontol* 2014; **49**: 55–62. [Medline] [CrossRef]
14. **Schmitt V, Rink L, Uciechowski P.** The Th17/Treg balance is disturbed during aging. *Exp Gerontol* 2013; **48**: 1379–1386. [Medline] [CrossRef]
15. **Goto M.** Inflammaging (inflammation + aging): A driving force for human aging based

on an evolutionarily antagonistic pleiotropy theory? *Biosci Trends* 2008; **2**: 218–230. [Medline]
16. **Jagger A, Shimojima Y, Goronzy JJ, Weyand CM.** Regulatory T cells and the immune aging process: a mini-review. *Gerontology* 2014; **60**: 130–137. [Medline] [CrossRef]
17. **Alvarez-Rodríguez L, López-Hoyos M, Muñoz-Cacho P, Martínez-Taboada VM.** Aging is associated with circulating cytokine dysregulation. *Cell Immunol* 2012; **273**: 124–132. [Medline] [CrossRef]
18. **Uri-Belapolsky S, Shaish A, Eliyahu E, Grossman H, Levi M, Chuderland D, Ninio-Many L, Hasky N, Shashar D, Almog T, Kandel-Kfir M, Harats D, Shalgi R, Kamari Y.** Interleukin-1 deficiency prolongs ovarian lifespan in mice. *Proc Natl Acad Sci USA* 2014; **111**: 12492–12497. [Medline] [CrossRef]
19. **Orjalo AV, Bhaumik D, Gengler BK, Scott GK, Campisi J.** Cell surface-bound IL-1alpha is an upstream regulator of the senescence-associated IL-6/IL-8 cytokine network. *Proc Natl Acad Sci USA* 2009; **106**: 17031–17036. [Medline] [CrossRef]
20. **Acosta JC, Banito A, Wuestefeld T, Georgilis A, Janich P, Morton JP, Athineos D, Kang TW, Lasitschka F, Andrulis M, Pascual G, Morris KJ, Khan S, Jin H, Dharmalingam G, Snijders AP, Carroll T, Capper D, Pritchard C, Inman GJ, Longerich T, Sansom OJ, Benitah SA, Zender L, Gil J.** A complex secretory program orchestrated by the inflammasome controls paracrine senescence. *Nat Cell Biol* 2013; **15**: 978–990. [Medline] [CrossRef]
21. **Dinarello CA.** Biologic basis for interleukin-1 in disease. *Blood* 1996; **87**: 2095–2147. [Medline] [CrossRef]
22. **Shirasuna K, Karasawa T, Takahashi M.** Exogenous nanoparticles and endogenous crystalline molecules as danger signals for the NLRP3 inflammasomes. *J Cell Physiol* 2019; **234**: 5436–5450. [Medline] [CrossRef]
23. **Shirasuna K, Karasawa T, Takahashi M.** Role of the NLRP3 inflammasome in pre-eclampsia. *Front Endocrinol (Lausanne)* 2020; **11**: 80. [Medline] [CrossRef]
24. **Tanikawa N, Ohtsu A, Kawahara-Miki R, Kimura K, Matsuyama S, Iwata H, Kuwayama T, Shirasuna K.** Age-associated mRNA expression changes in bovine endometrial cells in vitro. *Reprod Biol Endocrinol* 2017; **15**: 63. [Medline] [CrossRef]
25. **Tanaka H, Ohtsu A, Shiratsuki S, Kawahara-Miki R, Iwata H, Kuwayama T, Shirasuna K.** Age-dependent changes in inflammation and extracellular matrix in bovine oviduct epithelial cells during the post-ovulatory phase. *Mol Reprod Dev* 2016; **83**: 815–826. [Medline] [CrossRef]
26. **Hori K, Matsuyama S, Nakamura S, Iwata H, Kuwayama T, Miyamoto A, Shirasuna K.** Age-related changes in the bovine corpus luteum function and progesterone secretion. *Reprod Domest Anim* 2019; **54**: 23–30. [Medline] [CrossRef]
27. **Usui F, Shirasuna K, Kimura H, Tatsumi K, Kawashima A, Karasawa T, Yoshimura K, Aoki H, Tsutsui H, Noda T, Sagara J, Taniguchi S, Takahashi M.** Inflammasome activation by mitochondrial oxidative stress in macrophages leads to the development of angiotensin II-induced aortic aneurysm. *Arterioscler Thromb Vasc Biol* 2015; **35**: 127–136. [Medline] [CrossRef]
28. **Livak KJ, Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; **25**: 402–408. [Medline] [CrossRef]
29. **Miyamoto A, Okuda K, Schweigert FJ, Schams D.** Effects of basic fibroblast growth factor, transforming growth factor-beta and nerve growth factor on the secretory function of the bovine corpus luteum in vitro. *J Endocrinol* 1992; **135**: 103–114. [Medline] [CrossRef]
30. **Shirasuna K, Usui F, Karasawa T, Kimura H, Kawashima A, Mizukami H, Ohkuchi A, Nishimura S, Sagara J, Noda T, Ozawa K, Taniguchi S, Takahashi M.** Nanosilica-induced placental inflammation and pregnancy complications: Different roles of the inflammasome components NLRP3 and ASC. *Nanotoxicology* 2015; **9**: 554–567. [Medline] [CrossRef]
31. **Sano M, Shimazaki S, Kaneko Y, Karasawa T, Takahashi M, Ohkuchi A, Takahashi H, Kurosawa A, Torii Y, Iwata H, Kuwayama T, Shirasuna K.** Palmitic acid activates NLRP3 inflammasome and induces placental inflammation during pregnancy in mice. *J Reprod Dev* 2020; **66**: 241–248. [Medline] [CrossRef]
32. **Murr SM, Stabenfeldt GH, Bradford GE, Geschwind II.** Plasma progesterone during pregnancy in the mouse. *Endocrinology* 1974; **94**: 1209–1211. [Medline] [CrossRef]
33. **Redman CW, Sargent IL.** Pre-eclampsia, the placenta and the maternal systemic inflammatory response—a review. *Placenta* 2003; **24**(Suppl A): S21–S27. [Medline] [CrossRef]
34. **Furuya K, Kumasawa K, Nakamura H, Nishimori K, Kimura T.** Novel biomarker profiles in experimental aged maternal mice with hypertensive disorders of pregnancy. *Hypertens Res* 2019; **42**: 29–39. [Medline] [CrossRef]
35. **Furuya K, Kumasawa K, Nakamura H, Kimura T.** Endothelin-1 profiles in advanced maternal age complicated with hypertensive disorders of pregnancy. *Biochem Biophys Res Commun* 2019; **516**: 941–944. [Medline] [CrossRef]
36. **Haavaldsen C, Samuelsen SO, Eskild A.** The association of maternal age with placental weight: a population-based study of 536,954 pregnancies. *BJOG* 2011; **118**: 1470–1476. [Medline] [CrossRef]
37. **Flurkey K, Currer JM.** D.E. H. Mouse models in aging research. The Mouse in Biomedial Research 2nd ed 2007; 3:2000-2009.
38. **Gal H, Lysenko M, Stroganov S, Vadai E, Youssef SA, Tzadikevitch-Geffen K, Rotkopf R, Biron-Shental T, de Bruin A, Neeman M, Krizhanovsky V.** Molecular pathways of senescence regulate placental structure and function. *EMBO J* 2019; **38**: e100849. [Medline] [CrossRef]
39. **Higuchi S, Miyamoto T, Kobara H, Yamada S, Asaka R, Kikuchi N, Kashima H, Ohira S, Shiozawa T.** Trophoblast type-specific expression of senescence markers in the human placenta. *Placenta* 2019; **85**: 56–62. [Medline] [CrossRef]
40. **Bonney EA, Krebs K, Saade G, Kechichian T, Trivedi J, Huaizhi Y, Menon R.** Differential senescence in feto-maternal tissues during mouse pregnancy. *Placenta* 2016; **43**: 26–34. [Medline] [CrossRef]
41. **Napso T, Hung YP, Davidge ST, Care AS, Sferruzzi-Perri AN.** Advanced maternal age compromises fetal growth and induces sex-specific changes in placental phenotype in rats. *Sci Rep* 2019; **9**: 16916. [Medline] [CrossRef]
42. **Vrooman LA, Rhon-Calderon EA, Chao OY, Nguyen DK, Narapareddy L, Dahiya AK, Putt ME, Schultz RM, Bartolomei MS.** Assisted reproductive technologies induce temporally specific placental defects and the preeclampsia risk marker sFLT1 in mouse. *Development* 2020; **147**: 147. [Medline] [CrossRef]