Effect of Advanced Maternal Age on Pregnancy Outcomes and Vascular Function in the Rat

Alison S. Care, Stephane L. Bourque, Jude S. Morton, Emma P. Hjartarson, Sandra T. Davidge

Abstract—Advanced maternal age is becoming increasingly common in Western societies and is associated with increased maternal and fetal morbidity and mortality. We hypothesized that aging results in impaired vascular function in pregnancy because of increased vascular oxidative stress and resultant scavenging of nitric oxide in both uterine and systemic arteries, causing reduced uteroplacental perfusion and poor pregnancy outcomes. Using aged rats (9.5 months), we investigated the effect of a delayed first natural pregnancy on pregnancy outcomes and uterine and mesenteric artery function on gestational day 20. Delayed pregnancy in the rat reduced fertility by 46%, reduced litter size by 36%, caused fetal growth restriction, increased placental weight, and increased maternal systolic blood pressure (by 16 mm Hg). Uterine arteries from aged dams displayed reduced constriction to phenylephrine (young: 14.3±0.94 mN/mm versus aged: 11.4±0.5 mN/mm, P=0.02) and potassium chloride (124 mmol/L; young: 21.8±1.27 mN/mm versus aged: 14.2±1.7 mN/mm; P=0.01). Methacholine-induced vasodilation was similar in uterine arteries from young and aged dams. However, mesenteric arteries from aged dams had a greater nitric oxide and a reduced endothelial-derived hyperpolarization contribution to methacholine-mediated vasodilation compared with young dams. Both uterine and mesenteric arteries from aged dams had greater active myogenic responses, with area under the curve increased by 228% and 151%, in aged uterine and mesenteric arteries, respectively. These results demonstrate that vascular function is altered at an advanced maternal age and provides further insights into the risks of poor pregnancy outcomes observed in women who delay pregnancy. (Hypertension. 2015;65:00-00. DOI: 10.1161/HYPERTENSIONAHA.115.05167.) ● Online Data Supplement

Key Words: advanced maternal age ■ fetal growth restriction ■ mesenteric arteries ■ myogenic response ■ uterine artery

The age at which women deliver their first child has increased steadily in the past few decades, particularly in Western societies. Births occurring among women aged 35 years and older are increasing and constitute 14% and 18% of total live births in the United States and Canada, respectively.1,2 First births in this age group account for 8% and 11% of all first births in each respective country.1,2 Despite many women being aware of the risks of delaying childbirth, their decision—often borne of personal, economical, and educational reasons—has important clinical ramifications. Advanced maternal age is associated with increased pregnancy complications (e.g., gestational diabetes mellitus,4 preeclampsia,5 placenta previa,4,6 and Caesarean delivery),4 in turn leading to maternal and perinatal morbidity and mortality. Women aged ≥35 years are at increased risk of delivering a preterm infant (<37 weeks)4,6 and are 26.5% more likely to give birth to an infant that is small for gestational age.7

Increased susceptibility to pregnancy-related complications in women of advanced maternal age may stem, in part, from inadequate maternal cardiovascular adaptations during pregnancy. Dramatic maternal hemodynamic changes that occur during gestation include an increased blood volume, enhanced cardiac output, and reduced total peripheral resistance.8 These maternal cardiovascular adaptations enable a progressive increase in blood supply to the placenta (via the uterine artery)9 to support the increasing demands of the growing fetus throughout gestation. The mechanisms of reduced peripheral vascular resistance during normal pregnancy are not completely understood but include increased bioavailability of endothelial-derived vasodilators, such as nitric oxide (NO) and endothelial-derived hyperpolarization.10–13

It is likely that these systemic vasoactive factors work locally to enable pregnancy adaptations at the utero-placental interface, which is essential for healthy pregnancy outcomes. However, in the aged maternal cardiovascular system, normal pregnancy-induced vascular adaptations may be impaired. Aging is associated with cardiovascular impairments, including loss of cardiovascular compliance and altered endothelium-dependent function.14 Moreover, reduced NO bioavailability,
due in part to scavenging by enhanced reactive oxygen species generation, is believed to be an important contributor to age-related endothelial dysfunction. Because of these age-related vascular impairments, the increased cardiovascular demands of pregnancy may exceed the capacity of the aging maternal cardiovascular system, leading to poor pregnancy outcomes. Here, we tested the hypotheses that advanced maternal age results in impaired vascular function and reduced uteroplacental perfusion, which underlie adverse pregnancy outcomes.

Methods

Methods are available in the online-only Data Supplement.

Animals and Treatments

The experimental protocols described herein were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee in accordance with the Canadian Council on Animal Care guidelines. Female Sprague Dawley rats were purchased from Charles River (St. Constant, QC) at 12 weeks of age and allocated to the young or aged group. Young rats were provided ad libitum access to food and water throughout the experiments. Rats in the aged group were maintained on a controlled-feeding regimen until pregnancy, which consisted of 6 pellets of chow per day per rat, based on National Research Council recommendations. Young rats were 3 to 4 months at the time of conception, whereas aged rats were 9.5 to 10 months at the time of conception; the latter corresponds to ≈35 years of age in humans (ie, defined for humans as advanced maternal age) when considering such milestones as weaning, sexual maturity, skeletal maturity, and reproductive senescence. After pregnancy was confirmed by the presence of sperm in vaginal smears, all dams were fed ad libitum.

Results

Pregnancy Outcomes and Fetal Biometrics

Aged dams had a higher body weight and a greater percentage of body fat at the time of conception compared with young dams; however, the body weight gained during pregnancy when corrected for the number of pups was similar between the 2 groups (Table S1 in the online-only Data Supplement).

Aged dams had a reduced capacity to carry a viable pregnancy (young: 89.5±7.2% versus aged: 48.2±9.8% carrying viable pregnancies on GD20; P=0.003), which was defined as bearing a litter containing at least 1 live pup. Litter sizes of viable pregnancies were reduced by 36% (Figure 1A; P<0.001), and post-mortality analysis revealed a higher number of fetal resorptions in the aged group (Figure 1B; P=0.009). In viable pregnancies, surviving fetuses from aged dams were growth-restricted (Figure 1C; P=0.02); these pups had reduced abdominal births (young: 4.0±0.0 cm versus aged: 3.8±0.1 cm, P=0.02), but no changes in crown-rump length (young: 3.9±0.1 cm versus aged: 3.8±0.1 cm, P=0.4) or crown-rump length/abdominal girth ratio (Figure 1D; P=0.08) were observed. Fetal organ weights (brain, heart, liver, and kidney) normalized to body weight were not different between offspring from young and aged dams on gestational day 20 (Table S2). Pups from aged dams fall in the 13th percentile of fetal body weights when compared with pups from young dams. Despite lower fetal body weights, placentae from aged pregnancies were 15% heavier (Figure 1E; P=0.04) corresponding to a reduced fetal/placental weight ratio (Figure 1F; P=0.001)—an often used surrogate measure for placental efficiency. Labyrinth area of placental sections from aged dams was significantly greater than that of young dams (Figure S1).

Fetal livers were studied for pimonidazole staining (after treating dams 12 h prior) to assess changes in fetal oxygen delivery; no evidence of hypoxia was observed in either age group (mean fluorescence intensity for young: 0.006±0.0009 versus aged: 0.003±0.002 arbitrary units; P=0.22; Figure S2).

Maternal and Fetal Hemodynamics in Pregnancy

Systolic blood pressure was significantly higher in late gestation in aged dams compared with young dams (young: 114.7±4.4 mm Hg, n=14 versus aged: 130.7±5.9 mm Hg, n=9;...
Using ultrasound biomicroscopy, aged dams were shown to have a trend toward reduced peak systolic velocity in uterine arteries ($P=0.038$), despite no difference before conception or in midgestation (data not shown).

Uterine Artery Function

To gain further insight into the mechanisms causing impaired pregnancy outcomes in aged dams, vascular function was investigated in uterine arteries that undergo extensive remodeling to facilitate the nutrient demands of the fetus. In aged dams, maximal constriction of the uterine artery to the $\alpha$-adrenoceptor agonist phenylephrine was reduced by 21% (Figure 2A and 2B), albeit sensitivity to phenylephrine was not altered ($pEC_{50}$: young: $7.5\pm0.1$ versus aged: $7.3\pm0.1$; $P=0.12$). Maximal constriction to high KCl buffer was reduced by 35% in aged compared with young dams at gestational day 20 ($P<0.05$; Figure 2C).

Interestingly, although constrictor mechanisms seemed to be reduced in response to phenylephrine or KCl, uterine arteries from aged dams had an enhanced active myogenic response compared with uterine arteries from young dams ($P=0.0006$; Figure 3). There were no differences in the circumferential stress–strain relationship (a measure of mechanical changes in the vascular wall) in uterine arteries between young and aged dams ($k$ values; young: $11.3\pm1.2$ versus aged: $9.4\pm1.3$; $P=0.33$; Figure S3A).

Uterine artery responses to methacholine were not different between young and aged dams (Figure 4A). The addition of L-NAME reduced maximal vasodilation to methacholine to a similar extent in arteries from young and aged dams (Figure 4A and 4B). Consistent with this finding, endothelial NO synthase (eNOS) expression in uterine arteries was unaltered (Figure S4A and S4B). In addition, NOS inhibition did not alter phenylephrine constriction (Figure 4B) and uterine artery responses to the NO donor, sodium nitroprusside, were also unchanged between young and aged dams (Figure S5A). These data suggest that in uterine arteries, basal agonist-induced and smooth muscle responsiveness to NO were all unaltered by delayed pregnancy. Further experiments excluded an involvement of inducible NOS, neuronal NOS, and NO scavenging in uterine artery endothelial function (data not shown). Endothelial-derived hyperpolarization-induced vasodilation was unaltered by aging, and prostaglandins were not shown to be involved in vasodilation of young or aged uterine arteries (data not shown).

Maternal Mesenteric Artery Function

In mesenteric arteries (which contribute to total peripheral resistance), neither phenylephrine- nor high KCl-induced maximal constriction was altered in aged dams compared with their younger counterparts (Figure S6A and S6C). Similar to the uterine artery, however, mesenteric arteries from aged dams had an increased active myogenic response compared with young dams ($P=0.004$; Figure 5). There were no changes in the circumferential stress–strain relationship ($k$ values; young: $15.3\pm2.1$ versus aged: $11.5\pm1.7$; $P=0.29$; Figure S3B).

Total methacholine-induced vasodilation was not different in mesenteric arteries from young or aged dams (Figure 6A). L-NAME reduced the sensitivity to methacholine to a similar extent in both young and aged dams (Figure 6C); however, maximal relaxation was reduced only in aged dams (Figure 6B), suggesting a greater NO modulation in the aged mesenteric arteries. Because responses to the NO donor, sodium nitroprusside, were not different between aged and young dams (Figure S5B), smooth muscle signal transduction did not contribute to altered NO-mediated vasodilation. Conversely, the addition of L-NAME caused greater maximal phenylephrine-induced constriction in only young dams (Figure S6B), without affecting sensitivity ($pEC_{50}$: young vehicle: $6.6\pm0.09$ versus young L-NAME: $6.8\pm0.1$; aged vehicle: $6.5\pm0.04$ versus aged L-NAME: $6.8\pm0.1$; $P=0.046$; 2-way ANOVA group effect of L-NAME treatment). Although these data would suggest less basal NO production in aged dams, eNOS protein expression was not altered in mesenteric arteries (Figure S4C and S4D). Furthermore, the addition of a peroxynitrite scavenger/superoxide dismutase mimetic did
Hypertension
June 2015

not alter methacholine-induced vasodilation, excluding scavenging of NO as a contributing factor. In our study, vascular production of NO could be attributed largely to eNOS because inhibitors of inducible NOS and neuronal NOS had no effect on mesenteric artery constrictor or vasodilator capacity (data not shown).

In young dams, endothelial-derived hyperpolarization inhibition via apamin and TRAM-34 reduced maximal methacholine-induced vasodilation compared with vehicle (young vehicle: 97.2±1.3% versus young apamin/TRAM-34: 86.2±2.6%; P=0.02), whereas no effect was observed on maximal vasodilation in aged dams (aged vehicle: 90.8±3.7% versus aged apamin/TRAM-34: 82.3±7.4%; P=0.32). Neither young nor aged dams demonstrated an involvement of prostacyclin pathways (data not shown).

Discussion

In this study, we tested the hypothesis that aging constitutes a cardiovascular stressor that impairs cardiovascular adaptations to pregnancy resulting in adverse pregnancy outcomes. We demonstrated that dams of advanced age had (1) reduced capacity to sustain a pregnancy, (2) adverse pregnancy outcomes in surviving fetuses, (3) elevated blood pressure in late gestation, and (4) altered vascular function in both uterine and mesenteric resistance arteries. These data suggest that inadequate maternal cardiovascular function may contribute to but not fully account for the age-related decline in pregnancy success.

Advanced Maternal Age and Pregnancy Outcomes

Studies in women have shown that advanced maternal age is associated with reduced reproductive success, as well as intrauterine growth restriction, increased pregnancy loss, and increased placental weight.4–7,18,19 In our rat model of advanced maternal age, we observed similar adverse pregnancy outcomes, including reduced fertility, a higher number of fetal resorptions, and fetal growth restriction with increased placental weight in surviving offspring. Given that our animal model recapitulates the salient features of pregnancy at advanced maternal age in humans, it provides a suitable model to study age-related changes in vascular function in pregnancy. We chose to study first pregnancy because women having their first child at advanced maternal age account for 1 in every 9 births in Canada and 1 in every 12 in the United States.1,2 Moreover, primiparity is associated with a greater risk of complications in this age group.20 Finally, studying first pregnancy avoids the confounding factors of potential lasting maternal cardiovascular adaptations from previous pregnancies.21

Despite implementing a control-feeding regime from 3 months of age, aged dams were larger (because skeletal growth is still occurring in the young dams) with a higher body fat percentage. However, the quantity of food provided was based on National Research Council recommendations,16 and further reductions could affect nutrient status at conception and alter pregnancy outcomes.22,23

The reduced litter sizes in dams of advanced maternal age could not be fully explained by the increased resorption rate and, therefore, may be attributed to implantation failure or peri-implantation loss; consistent with this idea, 52% of aged dams had no evidence of pregnancy at term. Previous studies have shown that advanced maternal age is associated with an impaired decidual reaction and reduced uterine prostaglandin synthesis, as well as changes in the microstructure of the uterine luminal epithelium, particularly in the microvillous architecture. This may affect the ability of the blastocyst to attach and of trophoblast cells to invade into the underlying decidua.24 In addition, impaired oocyte developmental potential and a suboptimal intrauterine environment contribute to reduced embryo developmental competence, all of which have been described in advanced maternal age.24,25

![Figure 3. Uterine artery overall active myogenic response was assessed in late gestation (GD20) from young (3–4 months) and aged (9.5–10 months) dams. Data presented as mean±SEM. n=4 to 5 dams per group.](http://hyper.ahajournals.org/)

![Figure 4. Uterine artery endothelium-dependent (methacholine [MCh]) function was assessed in late gestation (GD20) from young (3–4 months) and aged (9.5–10 months) dams in the presence or absence of the nitric oxide synthase inhibitor, L-NAME (100 μmol/L). A, MCh concentration–response curve. B, MCh E_max dilation. Data are presented as mean±SEM. n=4 to 5 dams per group. Veh indicates vehicle control.](http://hyper.ahajournals.org/)
Interestingly and despite reduced litter sizes, pups from aged dams were nevertheless growth-restricted, suggesting inadequate nutrient or oxygen delivery to the fetus. A reduced litter size would be expected to ensure a better blood supply to the fewer surviving pups, thereby limiting growth restriction. The observation that placentae from aged dams were larger than their young counterparts suggests an attempt to compensate and increase nutrient and oxygen supply to the fetus, and the enlarged labyrinth zone supports this notion. The finding that fetuses from aged dams were not hypoxic may be indicative that this compensation was, at least in part, successful. However, the marked growth restriction despite no evidence of hypoxia indicates that alternate mechanisms were involved. It is possible that blood flow (and hence nutrient delivery) to the placenta was reduced, albeit oxygen delivery was sufficient to prevent placental or fetal hypoxia. We reasoned that the observed fetal growth restriction may be because of a failure of the aged maternal hemodynamic system to adequately adapt during pregnancy, even to supply a smaller number of fetuses. However, this is unlikely to be the only mechanism involved, and future studies investigating placental nutrient exchange would shed light on other mechanisms contributing the fetal and placental changes occurring with advanced age. Notwithstanding the cause, the finding that fetuses from aged dams were not hypoxic may be indicative that this compensation was, at least in part, successful. However, the marked growth restriction despite no evidence of hypoxia indicates that alternate mechanisms were involved. It is possible that blood flow (and hence nutrient delivery) to the placenta was reduced, albeit oxygen delivery was sufficient to prevent placental or fetal hypoxia. We reasoned that the observed fetal growth restriction may be because of a failure of the aged maternal hemodynamic system to adequately adapt during pregnancy, even to supply a smaller number of fetuses. However, this is unlikely to be the only mechanism involved, and future studies investigating placental nutrient exchange would shed light on other mechanisms contributing the fetal and placental changes occurring with advanced age. Notwithstanding the cause, the finding that fetuses from aged dams exhibited intrauterine growth restriction has important implications for the long-term health of the offspring, and puts these offspring at increased risk for chronic diseases and reduced lifespan.

**Advanced Maternal Age and Altered Uterine Artery Function During Pregnancy**

We observed age-related changes in both maternal hemodynamic and vascular function. The functional changes in the uterine artery are particularly notable given its role in supplying blood to the conceptus. Although there was a minimal myogenic response in the uterine arteries of young dams, this response was increased in aged dams. This enhanced constriction in response to intraluminal pressure could be detrimental to pregnancy, resulting in reduced blood flow to the placenta. This finding is consistent with enhanced myogenic reactivity in the reduced uteroplacental perfusion rat model of placental insufficiency. Aged uterine arteries also had reduced maximum constrictor capacity—a well-described phenomenon in aging blood vessels.

Despite these changes in vasoconstrictor capacity of uterine arteries, vasodilator capacity was largely unchanged between young and aged dams during pregnancy. NO is a major contributor to uterine artery vasodilation, and treatment with the NOS inhibitor L-NAME reduced maximal vasodilation to methacholine to the same extent in young and aged dams. Furthermore, no changes in eNOS protein expression were evident between young and aged uterine arteries. These observations are perhaps not surprising, given that the dams were relatively young at 10 to 11 months of age by late gestation and healthy without comorbidities. Similarly, we also observed no differential effects of the PGHS-inhibitor meclofenamate on uterine artery relaxation, which is consistent with previous studies showing a lack of involvement of prostaglandins in the vascular dysfunction observed in pregnancy complications in rats.

**Advanced Maternal Age and Altered Systemic Artery Function During Pregnancy**

We observed enhanced myogenic responses in mesenteric arteries of aged dams compared with young dams, resulting in a greater vasoconstrictor phenotype with increased pressure. The more
constrictive systemic vasculature may underlie the higher systolic blood pressure observed in aged dams, particularly when challenged by the hemodynamic demands of pregnancy. Interestingly, unlike in the uterine arteries, L-NAME pretreatment potentiated phenylephrine-induced vasoconstriction in mesenteric arteries of young dams that was absent in aged dams. In contrast to other studies, we did not observe a change in the sensitivity to phenylephrine in the presence of L-NAME.30 However, L-NAME potentiation of the maximum response to phenylephrine is indicative of greater basal NO modulation of constriction in the young dams. This may be contributing to differences observed in the myogenic response in young versus aged dams.

The data herein showed no overall increase in endothelium-dependent vasodilation in mesenteric arteries in pregnancy between young and aged dams. Interestingly, pretreatment of vessels with L-NAME revealed a greater NO contribution to agonist-induced vasodilation in the aged mesenteric vasculature than in the young. This was likely mediated by eNOS because selective neuronal NOS or inducible NOS inhibitors failed to produce a similar effect. The enhanced NO contribution in aged mesenteric arteries could not be attributed to changes in vascular smooth muscle sensitivity to NO because vascular responses to the NO-donor sodium nitroprusside revealed no differences. Furthermore, we could not attribute these differences to excess vascular oxidative stress diminishing bioavailability of NO because treatment with the cell-permeable superoxide dismutase mimetic/peroxynitrite scavenger, MnTBAP, also revealed no differences. It may be that the increased constriction in the wake of L-NAME treatment unmasks the enhanced constrictor phenotype in these vessels, as described above. An alternative explanation is that non-NO pathways compensate in young vessels, whereas NO-dependent pathways predominate in aged dams. Consistent with this hypothesis, we observed a role for endothelial-derived hyperpolarization in mesenteric artery vasodilation in young but not in aged dams.

It is evident that pregnancy in the rat at 9.5 to 10 months (=35 years of age in humans) is sufficient to induce notable changes in vascular function. Although the age investigated in our study does not seem to be sufficient to reduce endothelium-dependent relaxation,31,32 it is likely that pathophysiological changes are already underway. This was supported by the observance of slight alterations in uterine blood flow velocity in vivo and further by the development of growth restriction in the offspring, suggesting that the vascular pathological alterations may be at a subthreshold level. In this regard, the imposition of additional stressors (eg, obesity and hypertension) may cause these underlying deficits to manifest. The imposition of such stressors is relevant, given that women of advanced maternal age are more prone to developing comorbidities. Overall, our results demonstrate that vascular function is altered at an advanced maternal age and provides further insights into the risks of poor pregnancy outcomes observed in women who delay pregnancy.

Perspectives
Women delaying pregnancies until later in life is an increasing phenomenon in Western societies. We show that rats aged 9.5 to 10 months share many pregnancy complications with women that delay childbirth and therefore provides a model to study the mechanisms underpinning the adverse pregnancy outcomes and altered cardiovascular adaptations. Although these dams were fertile, they exhibited alterations in uterine and mesenteric artery function that may contribute to the poor pregnancy outcomes observed. This study shows a clear effect of advanced maternal age on fetal growth and development. Because developmental stressors are known to affect long-term health of the offspring, the clinical implications of delaying pregnancy may be greater than previously thought. However, for many women, the decision to delay pregnancy until advanced maternal age is not borne of convenience. Therefore, further research using suitable animal models is needed to better understand the cause of the increased maternal and fetal morbidity associated with advanced maternal age to enable the development of therapeutic strategies to improve outcomes for these mothers, as well as the survival and long-term health of their children.

Acknowledgments
We gratefully acknowledge Anita Quon and Dr Brennan for their technical assistance and expertise and Amy J. Barr for running the EchoMRI.

Sources of Funding
This work was funded by grants from the Canadian Institutes of Health Research (CIHR) and the Women and Children’s Health Research Institute (WCHRI) through the generous contributions of the Stollery Children’s Hospital Foundation and the Royal Alexandra Hospital Foundation. A.S. Care was supported by fellowships from the Heart and Stroke Foundation of Canada and Alberta Innovates-Health Solutions (AIHS), S.L. Bourque was funded by fellowships from CIHR and AIHS, E.P. Hjartarson was supported by a summer studentship from WCHRI, S.T. Davidge is a Canada Research Chair in Maternal and Perinatal Cardiovascular Health.

Disclosures
None.

References
Hypertension
Novelty and Significance

What Is New?
- This study describes a rat model of advanced maternal age that recapitulates many of the salient features seen in women.
- Advanced maternal age in the rat is associated with pregnancy complications and fetal growth restriction.
- Vascular adaptations to pregnancy are impaired in rats of an advanced maternal age.

What Is Relevant?
- Delaying pregnancy until later in life is an increasing phenomenon in Western society. Understanding the causes of the pregnancy complications associated with advanced maternal age will enable the development of therapeutic strategies to improve outcomes for women and the long-term health of their children.

Summary
We demonstrate that pregnancy outcomes are perturbed in a rat model of advanced maternal age, which may be due, in part, to altered maternal vascular function.

References
Effect of Advanced Maternal Age on Pregnancy Outcomes and Vascular Function in the Rat
Alison S. Care, Stephane L. Bourque, Jude S. Morton, Emma P. Hjartarson and Sandra T. Davidge

Hypertension, published online April 27, 2015;

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2015/04/26/HYPERTENSIONAHA.115.05167

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2015/04/27/HYPERTENSIONAHA.115.05167.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/
Online Supplementary information for:

EFFECT OF ADVANCED MATERNAL AGE ON PREGNANCY OUTCOMES AND VASCULAR FUNCTION IN THE RAT

Alison S. Care,1,3,4 Stephane L. Bourque,2,3,4 Jude S. Morton,1,3,4 Emma P. Hjartarson,1,3 Sandra T. Davidge1,3,4

To whom correspondence should be addressed:

Dr. Sandra Davidge,

Department of Obstetrics and Gynecology,

University of Alberta.

Edmonton, Alberta, Canada, T6G 2S2

Tel: 780-492-1864; Fax: 780-492-1308

Email: sandra.davidge@ualberta.ca
Methods

Animals and treatments

The experimental protocols described herein were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee in accordance with the Canadian Council on Animal Care guidelines. Female Sprague Dawley rats were purchased from Charles River (St. Constant, QC) at 12 weeks of age, and housed in the Animal Care Facility at the University of Alberta, which maintained a 10:14h light dark cycle, in an ambient temperature of 22±1°C. All rats were given at least 1 week to acclimatize to their surroundings prior to mating.

Rats were allocated to a young or aged group. Young rats were provided ad libitum access to food and water throughout the experiments. Rats in the aged group were maintained on a controlled-feeding regimen until pregnancy, which consisted of 6 pellets of chow per day, based on NRC recommendations; this was done to reduce excessive body fat accumulation with ad libitum-feeding. Rats in the aged pregnancy group were aged between 9.5 to 10 months at the time of conception, corresponding to approximately 35 years of age in humans (i.e. defined for humans as advanced maternal age) when considering such milestones as weaning, sexual maturity, skeletal maturity and reproductive senescence. Young rats (3-4 months of age) and aged rats (9.5-10 months of age) were mated with 3-5 month old males. Pregnancy was confirmed by the presence of sperm in vaginal smears; this was considered gestational day (GD) 0. After confirmation of pregnancy, all dams were fed ad libitum.

Blood Pressure Assessments

Blood pressure was assessed by tail-cuff plethysmography (Coda System, Kent Scientific, Torrington, CT) before pregnancy, in mid gestation (GD13-14) and late gestation (GD19). On each experimental day, twenty consecutive measurements were taken and averaged for each rat. Rats were trained in restraint tubes for 10 minutes on the day prior to each measurement.

Echo MRI

Quantitative nuclear magnetic resonance technology (EchoMRI, Echo Medical Systems) was used to assess body composition as described previously.

Ultrasound Biomicroscopy

Ultrasound biomicroscopy was performed to assess uterine artery as well as fetal umbilical artery and vein hemodynamic parameters, as previously described. Briefly, rats were anaesthetized with isoflurane (5% induction, 2.5% maintenance, in air). Hair on the abdomen was removed and pre-warmed gel was used as an ultrasound coupling medium. Rats were imaged transcutaneously using an ultrasound biomicroscope (model Vevo 2100, VisualSonics, Toronto, ON, Canada) with a 16-21 MHz MicroScan array transducer probe. A 0.2 to 0.5 mm pulsed Doppler gate was used, and the angle between the Doppler beam and the vessel was <45°. Doppler waveforms were obtained from uterine arteries near the lateral-inferior margin of the utero-cervical junction close to the iliac artery on each side, as well as from the umbilical artery and vein from three fetuses per dam. Peak systolic velocity and end diastolic velocity averages were obtained from a minimum of three consecutive cardiac cycles.
Pregnancy Outcomes and Fetal and Placental Biometrics

On GD20, rats were anesthetized with isoflurane (dosed to effect by inhalation) and euthanized by exsanguination via a cardiac puncture. Fetuses were quickly removed and placed on ice. Fetal biometric parameters, including crown-rump length and abdominal circumference, as well as body weights and placental weights were measured. Fetuses were then decapitated, and organs were collected, weighed and frozen in liquid nitrogen. Fetal hypoxia was assessed in a subset of pregnancies by treating dams on GD19 with Pimonidazole HCl (60 mg/kg, PO; Hypoxyprobe Inc. Burlington MA, USA); dams were euthanized 12 h later, and tissues were fixed in formalin, sectioned, and stained for immunofluorescence as previously described. Histological preparations of the samples were performed by the Alberta Diabetes Institute Histology Core. Slides were stained with mouse anti-pimonidazole IgG (Hypoxyprobe, Burlington, MA, USA; 1:50), followed by goat anti-mouse IgG Alexa Fluor 488 (Invitrogen, Burlington, ON, USA; 1:250) as the secondary antibody. Coverslips were mounted with fluorescent mounting medium containing DAPI (Vector, Burlington). Images were taken (Olympus IX81) and the mean fluorescence intensity relative to a secondary-only control was measured using the ImageJ image analysis system (NIH).

Transverse placental sections were stained with hematoxylin and eosin by the Alberta Diabetes Institute Histology Core. Area of the labyrinth zone was quantified using ImageJ.

Ex vivo assessment of vascular function

Wire myography

Vascular function was assessed in uterine and mesenteric resistance arteries according to established procedures. The uterus and mesentery was rapidly excised and placed in iced HEPES-buffered physiological saline solution [PSS; in mmol/L: 142 NaCl, 4.7 KCl, 1.17 MgSO4, 4.7 CaCl2, 1.18 K2PO4, 10 HEPES, 5.5 glucose, pH 7.4]. The main uterine artery and second-order mesenteric arteries were isolated and mounted in an isometric myograph system (DMT, Copenhagen, Denmark), using 40 μm tungsten wire. Arteries were normalized through a series of stepwise increases in diameter. To assess viability, vessels were twice treated with phenylephrine (PE; 10 μmol/L; Sigma) and once with methacholine (MCh; 3 μmol/L; Sigma) to assess functionality of the vascular smooth muscle and endothelium. Following a washout period, arteries were treated for a minimum of 30 minutes with either vehicle; N^G-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L; Sigma), a pan inhibitor of NO synthase (NOS); 1400W (10 μmol/L; Sigma), an inhibitor of inducible NOS; L-NPA (2 μmol/L; Sigma), an inhibitor of nNOS; meclofenamate (10 μmol/L; Sigma), a nonselective inhibitor of prostaglandin H synthase (PGHS); a combination of the potassium channel blockers apamin [100 nmol/l, small-conductance, Ca^{2+}-activated potassium channel (SK_{Ca}) blocker] and 1-[2-chlorophenyl] diphenylmethyl]-1H-pyrazole (TRAM-34) [10 μmol/L, intermediate-conductance, Ca^{2+}-activated potassium channel (IK_{Ca}) blocker], to inhibit EDH-mediated vasodilation; or MnTBAP (10 μmol/L; Calbiochem), a cell-permeable superoxide dismutase mimetic and peroxynitrite scavenger. Cumulative concentration response curves to PE were then generated (0.01-100 μmol/L; Sigma). For MCh or sodium nitroprusside (SNP) cumulative concentration responses (0.003 - 3 μmol/L and 0.0001 - 3 μmol/L, respectively; Sigma), vessels were pre-constricted to 80% of maximal constriction with PE and given a minimum of 5 min before administration of vasodilators. At the end of each experiment, 5 ml of 124 mmol/L potassium chloride solution (high KCl buffer; in mmol/L: 10 HEPES, 24 NaCl, 124 KCl, 2.4 MgSO4, 4.9
CaCl₂, 1.18 KH₂PO₄, 5.5 glucose; pH 7.4) was added to each vessel segment and the maximal constriction determined.

**Pressure myography**

Pressure myography was used to study the myogenic and mechanical properties of arteries by measuring vessel diameter and wall thickness with increasing intraluminal pressure. Uterine and mesenteric arteries were collected from young and aged dams on GD20 as described above. Arteries were mounted on two glass cannulas in a two-bath pressure myograph (Living Systems, Burlington, VT). Intravascular pressure was monitored and adjusted using a pressure servo control PS/200 and perfusion pressure monitor.

Vessels were given a 40-min equilibration period during which they were exposed to a stepwise increase in pressure from 60 to 80 mmHg, as described previously.¹¹,¹² Vessel viability was tested by the addition of PE (10 μmol/L) followed by MCh (3 μmol/L). Drugs were washed out and vessels rested for 10 min before vascular active characteristics were assessed using pressures from 4 to 160 mmHg, with 2 min intervals between steps. Passive characteristics were assessed by complete dilation of the arteries, achieved using papaverine (0.1 μmol/l; Sigma) and EGTA-Ca²⁺-free PSS (in mmol/L: 142 NaCl, 4.7 KCl, 1.17 MgSO₄, 1.18 KH₂PO₄, 10 HEPES, 2 EGTA). Following a 20 min equilibration, pressure steps were repeated as described above. Active and passive curves were plotted as the change in diameter from an unpressurised state. Analysis of circumferential wall stress and wall strain was performed as described previously.¹¹,¹² An exponential curve was fitted to the stress-strain data from each animal, from which the rate constant (k) was derived.¹¹,¹²

**Western Blot**

Protein homogenates of uterine and mesenteric arteries from young and aged rats were used to determine eNOS expression using Western blots. Total protein was determined using the BCA protein assay (Pierce, Rockford, IL, USA). Approximately 25-50 μg (45 μL/well) of total protein was loaded onto a 7.5% SDS-polyacrylamide gel. The separated proteins were then transferred onto nitrocellulose membranes (0.2 μm: Biorad, Mississauga, ON, Canada). Expression was determined using a primary antibody for eNOS (BD Transduction Laboratories; mouse monoclonal antibody, 1:250). Alpha-tubulin was loaded as a control (Abcam; Rabbit α-tubulin antibody; 1:2500). IRDye 800CW donkey anti-mouse IgG and IRDye 680RD donkey anti-rabbit IgG (LI-COR Bioscience, Lincoln, NE, USA; 1:1000) were used as secondary antibodies. Protein bands were imaged and analyzed using the Li-Cor Odyssey version 3.0 imager and software system v3.0 (Mandel Scientific Company). Expression data were presented as ratios of α-tubulin expression.

**Statistical Analyses**

Data are presented as mean±SEM and were plotted and analyzed using GraphPad Prism software version 6.0. Data for fetal biometrics were compared by Student’s t-test. For comparison of vascular function data, concentration-response curves were fitted to the Hill equation from which a sigmoid plot was generated by non-linear least squares regression analysis. The mean effective concentration that produced 50% of the maximal response (EC₅₀) was then determined. Comparison of EMAX and EC₅₀ were made using a Student’s t-test or two-way ANOVA with Bonferroni’s post-hoc test for multiple comparisons, as required. The
Grubb’s test was used to exclude outlying data points. To assess differences in overall active myogenic responses, the area under the curve (AUC) was calculated and analysed by Student’s t-test. A $P<0.05$ was considered statistically significant and $n$ values reflect the numbers of litters represented.
References
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Young</th>
<th>Aged</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dam Fat wt./ Body Wt (%)*</td>
<td>8.0±0.7 (6)</td>
<td>15.4±1.6 (6)</td>
<td>0.002</td>
</tr>
<tr>
<td>Dam Body Wt (GD0)(g)</td>
<td>309±7 (13)</td>
<td>389±8 (13)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Dam Body Wt (GD20)(g)</td>
<td>434±8 (14)</td>
<td>483±14 (12)</td>
<td>0.003</td>
</tr>
<tr>
<td>Pregnancy Body Wt Gain (g)</td>
<td>122±8 (11)</td>
<td>93±11 (10)</td>
<td>0.047</td>
</tr>
<tr>
<td>Pregnancy Body Wt Gain/per pup (g)</td>
<td>9.9±1.6 (11)</td>
<td>9.6±1.3 (10)</td>
<td>0.89</td>
</tr>
</tbody>
</table>

*EchoMRI measurements obtained in non-pregnant animals. Data presented as mean±SEM. The number of young (3-4 months) and aged (9.5-10 months) dams are shown in parentheses.
Table S2: Organ weights for GD20 fetuses from young and aged dams.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Young</th>
<th>Aged</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute Brain Wt (mg)</td>
<td>189.9±2.8</td>
<td>178.6±2.5</td>
<td>0.021</td>
</tr>
<tr>
<td>Brain Wt/Body Wt (mg/g)</td>
<td>48.7±1.0</td>
<td>51.2±1.9</td>
<td>0.32</td>
</tr>
<tr>
<td>Absolute Heart Wt (mg)</td>
<td>25.6±0.8</td>
<td>22.5±0.8</td>
<td>0.027</td>
</tr>
<tr>
<td>Heart Wt/Body Wt (mg/g)</td>
<td>6.6±0.3</td>
<td>6.4±0.3</td>
<td>0.80</td>
</tr>
<tr>
<td>Absolute Liver Wt (mg)</td>
<td>343.6±5.4</td>
<td>317.6±9.0</td>
<td>0.055</td>
</tr>
<tr>
<td>Liver Wt/Body Wt (mg/g)</td>
<td>88.0±1.7</td>
<td>90.6±2.9</td>
<td>0.48</td>
</tr>
<tr>
<td>Absolute Kidney Wt (mg)</td>
<td>30.9±0.9</td>
<td>29.2±1.6</td>
<td>0.42</td>
</tr>
<tr>
<td>Kidney Wt/Body Wt (mg/g)</td>
<td>7.9±0.1</td>
<td>8.3±0.3</td>
<td>0.32</td>
</tr>
</tbody>
</table>

Data presented as mean±SEM. n=4-5 dams/group. 4 pups/litter (when there were ≥4 pups available).
**Table S3:** *In vivo* assessment of uterine and umbilical artery blood flow velocity in young and aged dams on GD20.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Young</th>
<th>Aged</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uterine artery PSV (mm/s)</td>
<td>831.2±70.04 (6)</td>
<td>629.8±60.3 (11)</td>
<td>0.06</td>
</tr>
<tr>
<td>Uterine artery EDV (mm/s)</td>
<td>267.4±16.9 (5)</td>
<td>242.6±36.7 (11)</td>
<td>0.66</td>
</tr>
<tr>
<td>Uterine artery RI</td>
<td>0.63±0.03 (6)</td>
<td>0.62±0.03 (11)</td>
<td>0.87</td>
</tr>
<tr>
<td>Uterine artery PI</td>
<td>1.10±0.05 (5)</td>
<td>1.03±0.06 (9)</td>
<td>0.47</td>
</tr>
<tr>
<td>Umbilical artery PSV (mm/s)</td>
<td>171.6±18.9 (8)</td>
<td>167.3±15.9 (11)</td>
<td>0.86</td>
</tr>
<tr>
<td>Umbilical artery EDV (mm/s)</td>
<td>6.2±0.7 (7)</td>
<td>10.4±2.0 (10)</td>
<td>0.11</td>
</tr>
<tr>
<td>Umbilical artery RI</td>
<td>0.96±0.005 (7)</td>
<td>0.94±0.007 (10)</td>
<td>0.06</td>
</tr>
<tr>
<td>Umbilical artery PI</td>
<td>1.8±0.07 (7)</td>
<td>1.77±0.05 (9)</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Data presented as mean±SEM. The number of young (3-4 months) and aged (9.5-10 months) dams are shown in parentheses. UT; uterine artery. UM; umbilical artery. PSV; peak systolic velocity. EDV; end diastolic velocity. RI; resistance index. PI; pulsatility index.
Figure S1
Hematoxylin and eosin staining of GD20 placenta from young (3-4 months; A) and aged (9.5-10 months; B). Labyrinth and junctional zone (Jz) areas are labelled and the labyrinth area was measured (C). Data presented as mean±SEM.
Figure S2
Representative images of fetal liver on GD20 from young (3-4 months; A) and aged dams (9.5-10 months; B) following immunohistochemistry to detect pimonidazole. Green; anti-pimonidazole staining. Blue; DAPI staining of nuclei.
Figure S3
The circumferential stress-strain relationship in (A) uterine arteries and (B) mesenteric arteries, from young (3-4 months) and aged dams (9.5-10 months) at GD20. Data presented as mean±SEM.
Figure S4
Absolute eNOS expression was determined in late gestation (GD20) in uterine (A-B) and mesenteric arteries (C-D) from young (3-4 months) and aged (9.5-10 months) dams. Representative blots showing eNOS and α-tubulin expression in (A) uterine arteries, and (B) mesenteric arteries. eNOS expression normalized to α-tubulin in (C) uterine arteries and (D) mesenteric arteries. Data presented as mean±SEM. n=5 dams/group for both uterine and mesenteric arteries.
Figure S5
Sodium nitroprusside (SNP) concentration-response curves were conducted to assess endothelium-independent function in the (A) uterine artery and (B) mesenteric artery, in late gestation (GD20) from young (3-4 months) and aged (9.5-10 months) dams. Data are presented as mean±SEM. n=4-5 dams/group.
Figure S6
Mesenteric artery vasoconstriction to phenylephrine (PE) or high potassium buffer (KCl, 124 mmol/L) was assessed in late gestation (GD20) from young (3-4 months) and aged (9.5-10 months) dams in the presence or absence of the nitric oxide synthase inhibitor, L-NAME (100 µmol/L). (A) PE concentration-response curve. (B) PE $E_{\text{MAX}}$ constriction. (C) KCl $E_{\text{MAX}}$ constriction. Data are presented as mean±SEM. $n=5-6$ dams/group. Veh; vehicle control. Int; interaction. ** refers to multiple comparisons post-hoc, where $P<0.01$. 