Effect of Normal Pregnancy Followed by Lactation on Long-Term Maternal Health in a Mouse Model

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Abstract

Although it has been widely accepted that pregnancies with complications are associated with increased maternal cardiovascular risk later in life, there is no consensus if noncomplicated pregnancy followed by lactation plays a protective role or is a risk factor. The objective of this study was to investigate the effects of normal pregnancy and lactation on long-term maternal health in a mouse model. CD-1 mice were allocated to breeding (primigravid [PG]) and nonbreeding (nulligravid [NG]) groups. The PG group proceeded through normal pregnancy and delivery. Using a telemetry system, blood pressure (BP) was analyzed in the PG group at 6 months postpartum and in age-matched NG mice. Serum analytes, gene expressions, and protein levels were determined using appropriate analysis methods. Primigravid mice had significantly lower systolic and diastolic BP and fasting glucose levels. Circulating oxytocin (OXT) levels were significantly higher in PG mice. *Oxt* gene expression was significantly higher in the heart and aorta and lower in visceral adipose tissue (VAT) from PG mice. The oxytocin receptor (*Oxtr*) gene expression was significantly higher in the heart, aorta, and VAT from PG animals. The level of *Oxtr* DNA hypermethylation and the expression of *mmu-miR-29a* were significantly lower in the hearts of PG mice. In PG VAT, glucose transporter-4 expression was significantly higher. Our study demonstrates that a history of normal pregnancy followed by lactation was associated with lower maternal cardiovascular risk factors later in life in female mouse.

Keywords

pregnancy, maternal, cardiovascular

Introduction

Metabolic syndrome (MetS)—a cluster of cardiovascular risk factors, including hyperglycemia, obesity, hypertension, and dyslipidemia—affects 1 in 3 women in the United States.¹ The prevalence of MetS increases with age, reaching 54.4% for women ≥ 60 years.¹ Additionally, women with MetS have a 2.6-fold higher risk of cardiovascular disease (CVD) compared to women without MetS.² This association is stronger among women than men, for whom MetS confers a 2-fold risk of CVD, suggesting that gender-specific factors may moderate the underlying pathophysiology of CVD. The growing prevalence of MetS and its contribution to the leading cause of death in women thus warrant concentrating research into female-specific exposures.

We propose that pregnancy could be one of these gender-specific factors. Literature is inconclusive whether pregnancy plays a protective role against maternal CVD and MetS later in life or whether it increases the risk of maternal chronic diseases.^{3,4} Parous women have been shown to have higher CVD prevalence and a higher risk of carotid artery plaque when compared with nulligravid (NG) women.^{3,5} High parity was also associated with lower high-density lipoprotein

cholesterol (“the good cholesterol”) levels and higher glucose/insulin ratios in older women.³ Contrarily, other studies have found that NG women have a significantly increased risk of developing hypertension and cerebrovascular disease.⁴ In addition, a significantly higher risk of CVD mortality in NG than women with 4 or more pregnancies was reported.⁶ Comparison of short-term outcomes between primiparous and nulliparous women revealed decreased arterial pressure and reduced arterial stiffness following pregnancy.⁷ However,

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Figure 1. Experimental design of the study.

studies in humans are complicated by the multiple physiological and socioeconomic determinants.

Given conflicting evidence from epidemiologic studies, animal models are essential for investigating this association. There are few animal studies reporting long-term comparisons between animals with and without previous pregnancies.^{8,9} None of these studies reported parameters associated with cardiometabolic risk, such as blood pressure (BP), glucose, and triglyceride levels. A study from our laboratory several years ago demonstrated that mice that lactated had better postpartum maternal cardiovascular outcomes than mice that did not lactate.¹⁰

The objective of the current study was to establish a mouse model to examine the effect of parity followed by lactation on cardiometabolic health in later life using age-matched virgin control mice. During pregnancy and lactation, animals are exposed to high levels of oxytocin (OXT). Oxytocin is known to have multiple salutary effects on cardiovascular risk factors, such as lowering BP and reducing obesity.¹¹⁻¹⁴ Thus, we also investigated OXT and OXT receptor (*Oxtr*) levels and *Oxtr* epigenetic modifications in peripheral tissues as possible mechanisms underlying differences between primigravid (PG) and NG animals.

Materials and Methods

Animals

The study protocol was approved by the Institutional Animal Care and Use Committee at The University of Texas Medical Branch at Galveston (protocol # 0411077). The animals were housed separately in temperature- and humidity-controlled quarters with constant 12:12-hour light–dark cycles and were provided with food and water ad libitum. Female and male CD-1 mice were obtained from Charles Rivers Laboratories (Wilmington, Massachusetts) and bred in our facility. The animals were killed using CO₂ inhalation in accordance with the Animal Care and Use Committee and the American Veterinary Medical Association guidelines.

Study Design

Four- to 5-week-old female CD-1 mice were fed regular chow for 12 weeks (Figure 1). Females were then randomly allocated to breeding and nonbreeding groups: PG and NG, respectively. The PG mice were allowed to deliver. The pups were weaned from their mothers at the usual age of 3 weeks postdelivery. Food and drink intake, animal care, and other activities were not regulated during the study. We assessed outcomes in the 2 groups at 6 months postdelivery when animals were about 12 months old. In order to establish an age-related analog to the human model, we used the following metric. Mice live on average for 2 years when in captivity. Therefore, at 12 months of age, mice could be considered mature adults. If the human life span is about 80 years and mice live about 2 years, then 12 months in mice would be the equivalent to about 40 human years. A postdelivery period of 6 months in mice would be equivalent to about 20 years.

Blood Pressure In Vivo

Mice were anesthetized with a mixture of ketamine (Ketalar; Parke-Davis, Morris Plains, New Jersey) and xylazine (Gemini; Rugby, Rockville Center, New York), and telemetric BP transducers (PA-C10 model; Data Sciences, St Paul, Minnesota) were implanted as described previously.¹⁵ Telemetric transducers were implanted in the first part of the day. Mice were kept warm and closely monitored. Data were recorded for the next 7 days for 30 seconds at 10-minute intervals using the Dataquest ART data acquisition system (DSI, St Paul, Minnesota). During the first 3 days, mice were allowed to fully recover from the surgery. The recordings of the last 4 days were analyzed. Nine postpartum and 6 virgin mice had telemetry devices implanted; however, 3 and 2 animals, respectively, were excluded from final analysis due to technical problems, such as death during recording, obstructed catheter, and so on. The mice were then killed, and blood was collected via heart puncture. Half of the blood was collected in microtubes containing K2EDTA and was spun down at 4°C for 20 minutes at 10 000 rpm. Plasma was removed and stored at

–80°C until analysis for OXT levels, as recommended by the manufacturer. The other half was allowed to clot at room temperature, spun to obtain serum, and stored at –80°C until analysis for triglyceride levels. The right carotid artery was carefully dissected for vascular reactivity experiments. Abdominal visceral adipose tissue (VAT) from mesenteric, perigonadal, and perirenal fat depots was collected, pooled together for each mouse, weighed, and stored at –80°C for further analyses. Visceral adipose tissue weight was used to calculate adiposity—the percentage of visceral fat per total body weight. Abdominal aorta and heart were also collected and stored at –80°C for further experiments.

Vascular Reactivity Studies

The optimal diameter, the passive tension applied to the carotid arteries, the protocol, and concentrations used in these studies were determined in our previous experiments.^{15–17} In short, 2 to 3 rings per mouse obtained from the right carotid artery were mounted in a wire myograph (model 410A; J.P. Trading I/S, Aarhus, Denmark) using 25- μ m tungsten wires. The length of each ring segment (3 mm) was measured using an intraocular ruler. The force was continuously recorded by an isometric force transducer and analyzed using the PowerLab System and Chart 5 data acquisition and playback software (AD Instruments, Castle Hill, Australia). After stabilization of tone, the vessels were contracted twice with 60 mM potassium chloride (KCl) for 10 minutes to obtain a nonspecific contractile response and to enhance reproducibility. To evaluate endothelial function, the response to a single concentration of acetylcholine (ACh; 10^{-6} mmol/L) in vessels precontracted with phenylephrine (PE; 10^{-6} or 3×10^{-6} mmol/L) was determined. After 1 hour of equilibration, relaxant responses to the endothelium-dependent vasorelaxant ACh (10^{-10} – 10^{-5} mmol/L), the endothelium-independent vasorelaxant sodium nitroprusside (SNP; 10^{-10} – 10^{-5} mmol/L), and the β -adrenoreceptor agonist isoproterenol (ISO; 10^{-10} – 10^{-5} mmol/L) were obtained after precontraction of the vessels with PE (10^{-7} – 10^{-6} mmol/L). Vessels were washed out and given 1 hour rest, and then contractile responses to the α 1-adrenergic agonist PE (10^{-10} – 10^{-5} mmol/L), thromboxane A₂ mimetic U46619 (Tbx; 10^{-10} – 10^{-5} mmol/L), and 5-hydroxytryptamine or serotonin (5-HT, 10^{-10} – 10^{-5} mmol/L) were also assessed. Relaxant and contractile agents were added in half-log concentration at 10-minute intervals.

Serological Analysis

Glucose blood levels were measured using OneTouch Ultra, a blood glucose monitoring system (LifeScan, Milpitas, California), after mice had fasted overnight (16–18 hours). Commercially available kits were used according to the manufacturer's instructions to determine serum levels of triglycerides (cat # ETGA-200; BioAssay Systems, Hayward, California) and plasma concentration of OXT (cat # RMNPMAG-83K-06, minimum detectable concentration 7 pg/mL, intraassay

coefficient of variation (CV) 4%, interassay CV 9%; EMD Millipore Corporation, Billerica, Massachusetts).

RNA Isolation and Reverse Transcriptase Reaction

Tissue samples from each mouse (VAT, heart, and aorta) were analyzed individually. These samples were homogenized utilizing the Bullet Blender from Next Advance (Averill Park, New York). Total RNA was isolated using TRIzol and Zymo RNA isolation kits (Ambion, Austin, Texas and Zymo Research Corporation, Irvine, California), according to the manufacturers' instructions. Quantification of RNA was performed by measuring the absorbance of RNA sample solutions at 260 nm. For the reverse transcription reaction, the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California) was used according to the manufacturer's instructions (25°C for 10 minutes → 37°C for 120 minutes → 85°C for 5 minutes → 4°C for infinity).

Quantitative Real-Time Polymerase Chain Reaction

Messenger RNA (mRNA) expression of *Oxt* and *Oxtr* in VAT, heart, and aorta and glucose transporter-4 (*Glut4*) and lipoprotein lipase (*Lpl*) in VAT were quantified using TaqMan primer-probe gene expression assays for the specific genes listed above (Mm00726655_s1, Mm01182684_m1, Mm01245502_m1, and Mm00434764_m1, respectively; Life Technologies, Grand Island, New York) according to the manufacturer's instructions for quantitative real-time polymerase chain reaction (PCR) performed in 7500 Fast Real-Time PCR System (Applied Biosystems). Gene expression was calculated as the mRNA of the targeted gene relative to β -actin mRNA levels (primer-probe assay Mm00607939_s1; Life Technologies) in each specific sample (relative unit). Each reaction was carried out in triplicate. Relative quantification was determined using 7500 software, version 2.06 (Applied Biosystems).

Oxytocin Receptor Methylation Experiments

For DNA methylation experiments, heart and VAT were purified using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, California). Insufficient tissue was available to test for DNA methylation in the aorta. Genomic DNA was amplified by real-time PCR with the 7500 Fast Real-Time PCR System (Applied Biosystems). EpiTect Methyl II PCR Primer Assay for mouse *Oxtr* (CpG Island 109491, located on chromosome 6: 112439018–112439877, cat # EPMM109491-1A; Qiagen) was used to assay methylation according to the manufacturer's instructions. Genomic DNA was digested overnight with methyl-sensitive, methyl-dependent, or both enzyme mixes (EpiTect Methyl II DNA Restriction Kit, cat # 335452, Qiagen). A mock-digested sample was analyzed in parallel. Enzymes were inactivated, and the digested samples were used to seed quantitative PCR (qPCR) reactions using SYBR Green MasterMix (RT² SYBR Green/ROX qPCR MasterMix, cat # 330520; Qiagen) and an *Oxtr* profiler primer. Changes in

critical threshold values from the qPCR reaction were analyzed using a methyl profiler data analysis template and reported as percentages of methylation of samples run in triplicate.

Quantitative Real-Time PCR *mmu-miR-29a* Analysis

MicroRNAs (miRNAs) are short (approximately 20–23 nucleotides in length) endogenous noncoding RNA molecules that inhibit translation. MicroRNA for the *Oxtr* 3′ untranslated region was identified using the Web-based bioinformatics software—TargetScanHuman 6.2 (release 6.2, June 2012, www.targetscan.org, accessed July 2013). This software suggested 3 conserved miRNAs for *Oxtr*—*mmu-miR-29a*, *mmu-miR-29b*, and *mmu-miR-29c*. In the heart, *mmu-miR-29a* had been shown to be involved in cardiomyocyte proliferation and the cell cycle gene expression. It is the only biomarker for both hypertrophy and fibrosis in patients with hypertrophic cardiomyopathy.^{18,19} Thus, we chose to examine the gene expression of *mmu-miR-29a*.

The same extracted total mRNA from the heart tissue, noted above, was used. Transcription master mix was prepared using 10× miScript Nucleic Mix, RNase-free water, and 5× miScript HiSpec Buffer (all at room temperature) with 5× miScript HiSpec Buffer, 10× miScript Nucleic Mix, miScript Reverse Transcriptase Mix, Template RNA, and variable RNase-free water on ice. The mix was incubated for 60 minutes at 37°C, followed by 5-minute incubation at 95°C to inactivate miScript Reverse Transcriptase Mix. The mRNA analysis was done using SYBR Green detection with miScript Primer Assays and miScript SYBR Green PCR Kit according to the manufacturer's instructions (Qiagen). A quantitative real-time PCR procedure was performed using 2× QuantiTect SYBR Green PCR Master Mix, 10× miScript, Universal Primer, 10× Mm_miR-29a_1 miScript Primer Assay (cat # MS00001372; Qiagen), template complementary DNA (cDNA), and RNase-free water at room temperature. The assay reaction mix contained everything except the template cDNA. The template cDNA was dispensed into the individual 96-well plate, and the reaction mix was then added to the wells. After carefully and tightly sealing it with film, the plate was centrifuged to remove bubbles. The real-time cycler was programmed for initial activation step (15 minutes at 95°C) and 3-step cycling (denaturation 15 seconds at 94°C, annealing 30 seconds at 55°C, and extension 30 seconds at 70°C) for 40 cycles. snRNA RNU6B was used as an internal control to obtain relative quantitative data.

Data Analysis

Systolic, diastolic, and mean BP; heart rate; and pulse pressure data for each animal were collected and averaged over 24-hour periods. Results were plotted as mean values over a 24-hour period and expressed as means ± standard error of the mean (SEM) using GraphPad Prism 6 software version 6.07 for Windows (GraphPad Software, La Jolla, California). For statistical analysis of telemetry data, 2-way repeated-measures analysis

of variance (ANOVA) with Bonferroni and Sidak multiple comparison tests were applied.

In the vascular reactivity studies, responses to KCl in the 2 to 3 rings from 1 animal were averaged and analyzed using Student *t* test. To obtain the concentration–response curves (CRC) to Ach, SNP, ISO, PE, Tbx, and 5-HT, results were expressed as mean ± SEM for each concentration; *n* represented the number of mice used in each experiment. The second response to KCl was used as a reference to calculate the percentage of contraction achieved by the contractile agents studied, while PE precontraction was used to obtain the percentage of relaxation induced by the vasorelaxant agents. The 2-way ANOVA and Sidak's multiple comparison test were used for analysis of CRCs. The maximal effect (*E*_{max}, absolute and as percentage of KCl contraction), logarithm of the concentration producing 50% of the max effect, and the area under the concentration–response curves (AUC) were also calculated and analyzed using Prism 6.07 and SigmaPlot 12 (Systat 2011, San Jose, California). Data are reported as mean ± SEM or median with interquartile range when specified. The Shapiro-Wilk test was used to assess for normality. If passed and with equal variance, Student *t* test was used; otherwise, the Mann-Whitney rank sum test was employed.

Serological data were obtained for each mouse and compared between the 2 groups. Relative expression for each gene in each animal was calculated using the following formula: Fold(RQ) = 2^{−ddCt}. Methylation results were reported as percentages of unmethylated and hypermethylated promoter regions. For statistical analysis, Student *t* test and Mann-Whitney test were applied as appropriate (GraphPad Prism 6.07). A probability value (*P* value) of ≤.05 was considered statistically significant.

Results

Weights

The PG mice were weighed upon receipt from the supplier and then again on day 1 of gestation for PG animals and on age-matching day for NG mice (Figure 1). There were no differences in total body weight on either of those time points (on arrival PG 19.4 ± 0.8 g vs NG 19.3 ± 0.6 g, *P* = .9; on day 1 PG 33.8 ± 2.1 g vs NG 32.3 ± 1.0 g, *P* = .5). There were no significant statistical differences in total body weight, adiposity (percentage of excised visceral fat, including mesenteric and perirenal fat, from total weight), heart weight, and heart weight/total body weight ratio between the PG at 6 months postdelivery and age-matched NG mice (*P* = .09, *P* = .22, *P* = .12, and *P* = .60, respectively; Table 1).

Blood Pressure

Systolic, diastolic, and mean arterial BPs were significantly lower in PG mice as compared to NG animals (*P* = .002, *P* = .01, *P* = .007, respectively; Figure 2A–C). Heart rate and

Table 1. Characteristics of Primigravid at 6 Months Postdelivery and Age-Matched Nulligravid Mice.^a

Parameters	NG Group	PG Group	P Value
Total body weight (g)	37.5 ± 2.1 (n = 9)	41.9 ± 1.3 (n = 11)	.09
Adiposity (% of excised visceral fat from total body weight)	5.2 ± 1.4 (n = 9)	7.3 ± 1.0 (n = 11)	.22
Heart weight (g)	0.15 ± 0.005 (n = 9)	0.17 ± 0.007 (n = 11)	.12
Heart weight/total body weight	0.004 ± 0.0002 (n = 9)	0.004 ± 0.0001 (n = 11)	.60
Fasting glucose (mg/dL)	109.4 ± 4.3 ^b (n = 9)	85.6 ± 8.8 (n = 7)	.02
Triglycerides (mg/mL)	226.1 ± 36.7 (n = 8)	199.0 ± 26.1 (n = 10)	.54

Abbreviations: NG, nulligravid; PG, primigravid; SEM, standard error of the mean.

^aValues are expressed as mean ± SEM.

^bStatistically significance differences ($P \leq .05$) in bold.

Figure 2. (A) Systolic, (B) diastolic, and (C) mean blood pressures; (D) heart rate; and (E) pulse pressure in PG (n = 6) and age-matched NG (n = 4) mice. Values are given as mean ± SEM. *Statistically significant differences ($P < .05$) between the PG and NG groups. NG indicates nulligravid; PG primigravid; SEM, standard error of the mean.

Figure 3. The effect of KCl on carotid artery rings from NG ($n = 9$) and age-matched PG ($n = 13$) mice. The box extends from 25th percentile to the 75th percentile, with a line at the median (50th percentile). Whiskers show the highest and lowest values. KCl indicates potassium chloride; NG, nulligravid; PG primigravid.

pulse pressure did not differ between the groups ($P = .15$ and $P = .27$, respectively; Figure 2D and E).

Vascular Reactivity

Carotid artery responses to nonspecific contractile agent, KCl, were significantly lower in PG mice as compared to NG animals ($P = .001$; Figure 3). The vasorelaxant and vasoconstrictive CRCs to Ach, SNP, ISO, PE, Tbx, and 5-HT were not different between the PG and NG mice (Figure 4). Data for Emax, AUC, and logarithm of concentration producing 50% of the maximal effect (logIC50) are summarized in Tables 2 and 3. Only logIC50 in response to Tbx was significantly lower in PG groups when compared to the NG group ($P = .02$; Table 3), indicating higher sensitivity to Tbx in carotid arteries obtained from mice that underwent pregnancy and lactation earlier in life.

Because the KCl-induced reference contractions were significantly lower in PG mice, we also analyzed the absolute responses to contractile agents. Concentration–response curves, Emax, AUC, and logIC50 to PE and ISO remained the same (data not shown). Concentration–response curve, Emax, and AUC to Tbx did not change (data not shown), while logIC50 to Tbx remained significantly lower in PG mice ($-5.5 [-6.1 \text{ to } -5.1]$ vs $-6.4 [-6.8 \text{ to } -5.7]$, $P = .014$).

Blood Analytes

Significantly lower fasting glucose levels were found in the PG group as compared to the NG mice ($P = .02$; Table 1). Triglyceride levels were not statistically significantly different between the groups ($P = .54$; Table 1).

Plasma OXT Levels

We determined significantly higher levels of OXT in PG mice than in NG mice ($P = .03$; Figure 5).

Expression of Oxt and Oxtr Gene

The Oxt mRNA expression was significantly higher in the aorta and heart of PG mice compared with NG mice ($P = .05$ and $P = .03$, respectively; Table 4). In VAT, Oxt mRNA expression was significantly lower in PG mice ($P = .04$; Table 4). The Oxtr expression was greater in aorta, heart, and VAT in PG animals ($P = .05$, $P = .04$, and $P = .008$, respectively; Table 4).

Oxytocin Receptor Methylation

Given the higher *Oxtr* mRNA expression found in PG mice, we investigated whether epigenetic modifications differ in PG compared to NG mice. When compared to NG animals, PG DNA unmethylation level was significantly higher in the heart ($91.9\% \pm 7.8\%$ vs $100.0\% \pm 0.0002\%$, respectively; $P = .02$), but not in VAT ($99.8\% \pm 0.04\%$ vs $99.9\% \pm 0.02\%$, respectively; $P = .6$). Hypermethylation was significantly lower in the hearts from PG mice ($0.15\% \pm 0.06\%$ vs $0.0007\% \pm 0.0002\%$, respectively; $P = .03$), with no difference in VAT ($0.15\% \pm 0.04\%$ vs $0.1\% \pm 0.03\%$, respectively; $P = .6$). These results suggest that differences in *Oxtr* methylation levels could explain the higher mRNA expression of *Oxtr* in PG mice.

mmu-miR-29a Levels

We determined that the expression of mmu-miR-29a was significantly lower in the hearts of PG mice than in NG animals, indicating another plausible mechanism for OXTR silencing in NG mice ($P = .04$; Figure 6).

Expression of Glut4 and Lpl Gene

The *Glut4* gene expression in VAT was significantly elevated in PG mice as compared to NG mice ($P = .03$; Figure 7). There was no difference in *Lpl* expression between PG and NG mice ($P = .7$; Figure 8).

Discussion

In this study, we explored the role of pregnancy with lactation in the long-term health of females. Mice that underwent normal pregnancy followed by lactation demonstrated significantly better CVD outcomes and had lower risk factors for MetS (BP, vascular reactivity, and fasting glucose) than age-matched animals without a history of parity and lactation. We also found higher levels of OXT, *Oxt*, and *Oxtr* in animals with exposure to pregnancy and lactation, suggesting that OXT, among many other factors, may mediate the observed differences in phenotypes between NG and PG mice. Moreover, we found that epigenetic changes involving *Oxtr* could play a role as well. Genes downstream from OXT (*Glut4*) were also affected. Overall, our results favor the hypothesis that normal pregnancy with lactation lowers the risk of maternal CVD and MetS in later life.

Figure 4. Concentration–response curves to relaxation agents (A) Ach (NG, $n = 9$; PG, $n = 13$), (B) SNP (NG, $n = 9$; PG, $n = 12$), and (C) ISO (NG, $n = 9$; PG, $n = 12$; $P = .04$) and to contractile agents (D) PE (NG, $n = 9$; PG, $n = 13$), (E) Tbx (U46619, NG, $n = 9$; PG, $n = 13$), and (F) 5-HT (NG, $n = 9$; PG, $n = 10$). Symbols represent mean $\pm$ SEM. Ach indicates acetylcholine; 5-HT, 5-hydroxytryptamine or serotonin; ISO, isoproterenol; NG, nulligravid; PE, phenylephrine; PG primigravid; SEM, standard error of the mean; SNP, sodium-nitroprusside; Tbx, thromboxane A₂ mimetic U46619.

During pregnancy, substantial physiological maternal cardiovascular adaptations take place—blood volume, cardiac output, and maternal heart rate increase, while arterial BP and systemic vascular resistance decrease.²⁰ Most of these changes almost fully reverse. However, it was found that mean arterial pressure and arterial stiffness significantly decreased in postpartum women compared to prepregnancy values, while there was no change in women who did not become pregnant during the study.⁷ The observation was relatively short—on average 14 months. Thus, whether difference remains later in life needs to be investigated in humans.

Our study demonstrates that in a mouse model, the significantly favorable effect of pregnancy on maternal BP and vascular function is sustained into midlife, which would be 20 years postpartum in women. Although carotid artery efficacy and sensitivity (except to Tbx) did not differ between the NG and PG mice, contraction to KCl was significantly lower in mice with previous pregnancy. We propose that this response could be a proxy for decreased carotid artery resistance. Further investigations into pulse wave velocity (the gold standard for measuring arterial stiffness) using Doppler ultrasound would clarify this hypothesis.^{21,22}

Table 2. Maximal Effect, logIC50, and AUC of Ach, SNP, and ISO in the Carotid Arteries of PM at 6 Months Postdelivery and Age-Matched NG Mice.^a

	Ach	SNP	ISO
Emax			
NG	101.8 (98.0-105.9)	101.8 (100.9-103.5)	92.8 ± 2.3
PG	98.6 (95.5-104.4)	99.7 (96.7-103.9)	91.8 ± 4.8
logIC50			
NG	-8.3 ± 0.1	-7.9 ± 0.1	-7.5 ± 0.2
PG	-8.5 ± 0.2	-8.1 ± 0.1	-7.5 ± 0.1
AUC			
NG	344.8 ± 16.1	312.8 ± 16.4	247.7 ± 17.6
PG	318.4 ± 16.0	332.4 ± 22.4	265.5 ± 25.3

Abbreviations: Ach, acetylcholine; AUC, area under the concentration–response curve; Emax, absolute maximal effect; ISO, isoproterenol; logIC50, logarithm of concentration producing 50% of the maximal effect; NG, nulligravid; PG, primigravid; SEM, standard error of the mean; SNP, sodium-nitroprusside.

^aData are expressed as mean ± SEM or median (interquartile range, ie, 25 and 75 percentiles).

Physiological BP and vascular function changes during pregnancy are mediated by estrogen, relaxin, atrial and brain natriuretic peptides, nitric oxide (NO), prostaglandins, growth hormone, and other molecules.²⁰ Since OXT does impact both pregnancy and lactation, we propose that differences in OXT could possibly, in part, explain the lower BP following pregnancy and lactation in mice. The most well-known actions of peripheral OXT are stimulation of uterine smooth muscle contraction during labor and milk ejection during lactation. Oxytocin also exerts cardiovascular and antiobesity effects through central and peripheral OXTR.^{23,24} OXTR has been identified in the brain, kidney, heart, vascular endothelium, pancreas, adipocytes, and other tissues.²⁴ Animal studies show that chronic OXT administration lowers BP, and these changes persist long after discontinuation of hormone administration.^{14,25}

Moreover, central oxytocinergic fibers inhibit sympathetic and promote parasympathetic stimulation of the heart, leading to a lower heart rate and less forceful contractions.²⁶⁻²⁹ The cardiovascular activities of OXT also include vasodilatation, negative cardiac inotropy and chronotropy, and reduction in myocardial infarct size and ventricular arrhythmias.^{30,31} A dose-dependent decrease in body weight gain, increased adipose tissue lipolysis and fatty acid β -oxidation, and reduced glucose intolerance and insulin resistance have been reported after chronic central OXT administration to diet-induced obese rats.¹² These and other reports clearly support a role for OXT in the prevention of CVD and MetS.

Oxytocin is one of the few peptides that in physiological situations, such as pregnancy and lactation, induces its own synthesis and central and peripheral release.²³ It is already well known that during pregnancy and lactation, OXT levels increase due to cervical stretching, nipple stimulation, and mother–pup interaction. Animal models have established that *Oxt* expression in the brain increases during pregnancy and postpartum.³² Data indicating a possible cardioprotective role of OXT among early postpartum women have also been reported.³³ Consistent with observations in humans, we found higher OXT plasma levels and *Oxt* and *Oxtr* mRNA expression in the heart and VAT of postpartum mice when compared with NG mice. Our data are the first to demonstrate differential OXT, *Oxt*, and *Oxtr* levels in 2 different phenotypes defined by a history of pregnancy. Thus, we hypothesize that the increase in OXT production is sustained for a long-time postpartum, and therefore, OXT levels are significantly higher in postpartum dams as we demonstrated in this study. This continued OXT production in postpartum is due to modifications in the peripheral expression of OXT and OXTR. These alterations together with factors outside the scope of our study lead to lower maternal risks associated with metabolic disease. One of the interesting results is that in VAT from NG mice, the expression of the *Oxt* gene was significantly higher, meaning increased production; however, this increase did not translate

Table 3. Maximal Effect, LogIC50, and AUC of PE, Tbx, and 5-HT in the Carotid Arteries of Primigravid at 6 Months Postpartum and Age-Matched Nulligravid Mice.^a

	PE	Tbx	5-HT
Emax			
NG	24.7 (14.6 to 60.7)	151.8 ± 22.5	32.3 (16.9 to 84.0)
PG	31.4 (13.4 to 81.2)	166.8 ± 25.9	24.0 (21.8 to 66.9)
logIC50			
NG	-6.5 (-6.6 to -6.1)	-5.6 (-6.2 to -5.1)	-7.3 ± 0.06
PG	-6.1 (-6.4 to -5.6)	-6.4 (-6.8 to -5.7) ^b	-7.4 ± 0.1
AUC			
NG	33.2 (25.7 to 100.7)	115.1 (55.6 to 193.4)	118.7 ± 23.6
PG	52.1 (33.2 to 108.4)	102.9 (50.1 to 407.3)	134.9 ± 21.5

Abbreviations: AUC, area under the concentration–response curve; Emax, absolute maximal effect; 5-HT, 5-hydroxytryptamine or serotonin; logIC50, logarithm of concentration producing 50% of the maximal effect; NG, nulligravid; PE, phenylephrine; PG, primigravid; SEM, standard error of the mean; Tbx, thromboxane A2.

^aData are expressed as mean ± SEM or median (interquartile range, ie, 25 and 75 percentiles).

^bStatistically significant differences between NG and PG groups ($P \leq .05$).

Figure 5. Plasma OXT concentrations in NG ($n = 6$) and age-matched PG ($n = 5$) mice at 10 months. The box extends from 25th percentile to the 75th percentile, with a line at the median (50th percentile). Whiskers show the highest and lowest values. NG indicates nulligravid; OXT, oxytocin; PG primigravid.

Table 4. Relative Oxt and Oxtr mRNA Expression in the Heart, Aorta, and VAT From PG at 6 Months Postdelivery and Age-Matched NG Mice.^a

Tissue	NG Group	PG Group	P Value
Oxt mRNA (relative expression)			
Aorta	0.07 ± 0.02^b ($n = 7$)	0.6 ± 0.3 ($n = 5$)	.03
Heart	0.46 ± 0.1^b ($n = 4$)	2.0 ± 0.7 ($n = 3$)	.05
VAT	1.7 ± 0.3^b ($n = 6$)	0.6 ± 0.3 ($n = 6$)	.04
Oxtr mRNA (relative expression)			
Aorta	0.3 ± 0.1^b ($n = 6$)	1.0 ± 0.3 ($n = 7$)	.04
Heart	0.5 ± 0.1^b ($n = 4$)	0.9 ± 0.2 ($n = 4$)	.05
VAT	0.5 ± 0.1^b ($n = 5$)	1.4 ± 0.2 ($n = 6$)	.008

Abbreviations: VAT, visceral adipose tissue; NG, nulligravid; PG, primigravid; Oxt, oxytocin; Oxtr, oxytocin receptor; SEM, standard error of the mean.

^aData are expressed as mean $\pm$ SEM.

^bStatistically significance differences ($P \leq .05$) in bold.

into higher levels of circulating OXT. Therefore, this finding needs further investigation. In addition, the role of OXT and OXTR in pregnancy alone versus pregnancy with lactation needs to be determined.

Other novel findings in our study are epigenetic differences in virgin versus parous mice. Traditional epigenetic studies focus on the prenatal period; however, growing evidence suggests that epigenetic modifications are not restricted to the developmental period. Throughout the life span, both internal and external environmental factors modify genetic expression. Social behaviors, pollution, smoking, nutrition, and even changes in circadian rhythm have direct effects on adult epigenome.³⁴ Studies in monozygotic twin pairs demonstrate methylation discordance within the pair, which further confirms a role for unique environmental factors.³⁵ However, there are also events in which physiological, behavioral, nutritional, social, and environmental modalities determine future health. We propose that pregnancy is one such event.

Figure 6. Relative mRNA expression of the mmu-miR-29a in the heart from NG ($n = 6$) and age-matched PG ($n = 5$) mice at 10 months. The box extends from 25th percentile to the 75th percentile, with a line at the median (50th percentile). Whiskers show the highest and lowest values. mRNA indicates messenger RNA; NG, nulligravid; PG, primigravid.

Figure 7. Relative mRNA expression of the *Glut4* in the VAT from NG ($n = 4$) and age-matched PG ($n = 5$) mice at 10 months. The box extends from 25th percentile to the 75th percentile, with a line at the median (50th percentile). Whiskers show the highest and lowest values. *Glut4* indicates glucose transporter-4; mRNA, messenger RNA; NG, nulligravid; PG, primigravid; VAT, visceral adipose tissue.

Since OXTR has CpG islands that are susceptible to DNA methylation and we found that *Oxtr* is unmethylated in mice with previous parity, we propose that *Oxtr* silencing via methylation could, in part, explain BP differences between the groups.^{36,37} Moreover, we found that gene expression of mmu-miR-29a, another mechanism of epigenetic silencing specifically targeting translation of OXTR, is lower in the hearts of mice with a history of pregnancy. We speculate that physiological adaptations associated with pregnancy followed by lactation affect the maternal epigenome, improving future health.

Experimental studies in rats have shown that administration of either central or peripheral OXT protects against high-fat diet-induced glucose intolerance and insulin resistance and promotes an increase in *Lpl* gene expression in VAT.¹² *Glut4*

Figure 8. Relative mRNA expression of the *Lpl* in the VAT from NG (n = 5) and age-matched PG (n = 6) mice at 10 months. The box extends from 25th percentile to the 75th percentile, with a line at the median (50th percentile). Whiskers show the highest and lowest values. *Lpl* indicates lipoprotein lipase; mRNA, messenger RNA; NG, nulligravid; PG, primigravid; VAT, visceral adipose tissue

and *Lpl* are the downstream receptors from OXT regulating glucose and fatty acid transport in VAT, respectively.¹² We found a significantly higher gene expression of *Glut4* in PG mice as compared to NG, indicating elevated glucose uptake into VAT—a finding in line with lower fasting glucose levels in PG mice. Furthermore, higher gene expression of *Glut4* in PG mice indirectly indicates lower insulin resistance. The expression of *Lpl*, a triglyceride transporter in VAT also activated by OXT, was not different between the 2 groups of mice, reflecting no difference found in circulating triglyceride levels.

The strengths of our study are: telemetry was used to collect BP measurements in vivo for several consecutive days, animals were age-matched, and epigenetic parameters had not been investigated in virgin versus postpartum mice. Our study had several limitations: the results are limited to just one age group; food intake and weight gain after pregnancies were not controlled; since we concentrated on local tissues, expression of *Oxt* and *Oxtr* was not measured in the brain; and the vascular reactivity in the resistance vasculature, such as mesenteric arteries, as well as lumen diameter or wall thickness in carotid arteries were not evaluated.

In summary, based on the literature and data presented here, we propose the following concept: activation of the OXT-OXTR system during pregnancy and lactation is associated with lower maternal BP and fasting glucose levels in later life. The mechanisms of OXT action are complex, and here we present a simplified version. During pregnancy with lactation, physiologically elevated levels of OXT—either by itself or via OXTR in VAT activate the GLUT4—transcription factor involved in glucose transport through membranes into adipocytes, leading to lower circulating glucose levels, improved glucose tolerance, and insulin sensitivity. In the heart and vasculature, elevated OXT levels could lead to increased endothelial NO synthase and NO release, resulting in better vasodilation and lower BP. In future investigations, the role of OXT's maternal long-term cardiovascular risk needs to be

further defined. Studies in OXT and OXTR knockout mice and the use of OXTR antagonist could provide invaluable information and possibly lead to therapeutic measures to prevent CVD in women.

In conclusion, our data indicate that pregnancy followed by lactation reduces maternal risk of CVD and MetS in later life when compared to virgin animals. We propose that pregnancy's and lactation's effects on cardiometabolic health could, in part, be mediated by OXT levels, epigenetic OXTR status, and downstream gene expression. This report lays the groundwork for further mechanistic explorations of the role of pregnancy and lactation in maternal metabolic health. In addition, mechanisms activated during pregnancy and lactation (such as the release of OXT) may directly translate into gender-specific interventions that may prevent cardiovascular and metabolic disease in women.

Authors' Note

This study was conducted at The University of Texas Medical Branch at Galveston. EBP conceived the idea for this project, obtained animals and samples, analyzed the data, and completed the manuscript. AMS provided critical guidance on the project and aided in the interpretation of findings. DO, TK, and HY were responsible for developing and performing the experiments. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIAID, NICHD, OD, or the National Institutes of Health.

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