Effects of uteroplacental insufficiency and reducing litter size on maternal mammary function and postnatal offspring growth

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O’Dowd R, Kent JC, Moseley JM, Wlodek ME. Effects of uteroplacental insufficiency and reducing litter size on maternal mammary function and postnatal offspring growth. Am J Physiol Regul Integr Comp Physiol 294: R539–R548, 2008. First published December 12, 2007; doi:10.1152/ajpregu.00628.2007.—Human intrauterine growth restriction is often associated with uteroplacental insufficiency and a decline in nutrient and oxygen supply to the fetus. This study investigated the effects of uteroplacental insufficiency and intrauterine growth restriction (Restricted) or reducing litter size for normally grown pups (Reduced Litter) on maternal mammary development and function, milk composition, offspring milk intake, and their resultant effects on postnatal growth. Uteroplacental insufficiency was surgically induced by bilateral uterine vessel ligation on day 18 of gestation in the Wistar Kyoto rat. At birth, a group of sham control rats had their litter size reduced to five (Reduced Litter) to match that of the Restricted group. Cohorts of rats were terminally anesthetized on day 20 of gestation or day 6 of lactation, and a third group was studied throughout lactation. Restricted pups had a lower birth weight (by 16%) and litter size (by 36%) compared with controls, as well as reduced mammary parathyroid hormone-related protein content and milk ionic calcium concentrations associated with reduced total pup calcium. Restricted dams with lower circulating progesterone experienced premature lactogenesis, producing less milk per pup with altered composition compared with controls, as well as reduced mammary parathyroid hormone-related protein content and milk ionic calcium concentrations associated with reduced total pup calcium. Restricted dams with lower circulating progesterone experienced premature lactogenesis, producing less milk per pup with altered composition compared with controls, as well as further slowing growth during lactation. Reducing litter size of pups born of normal birth weight (Reduced Litter) was associated with decreased pup growth, highlighting the importance of appropriate controls. The present study demonstrates that uteroplacental insufficiency impairs mammary function, compromises milk quality and quantity, and reduces calcium transport into milk, further restraining postnatal growth.

An adequate development of the lactating mammary gland, effective mammary nutrient transfer, and delivery of calcium-rich milk to the suckling offspring are essential for normal postnatal growth and development. The structural and functional development of the mammary gland during pregnancy and lactation requires coordinated actions of several hormones and growth factors, as well as local epithelial-mesenchymal interactions (32). Mammary secretory cells develop during pregnancy, illustrating that the secretory capacity is determined before birth and can influence milk production during lactation (32). Milk production near term is triggered by a decline in circulating maternal progesterone concentrations (25, 32). Appropriate coordination of these events ensures timely initiation of lactation and production of adequate quality and quantity of milk to ensure normal growth of the offspring after birth. In rodents, alterations in litter size and the suckling stimulus directly affect the quality and quantity of milk produced during lactation (4, 43).

Parathyroid hormone-related protein (PTHrP) is a factor present in the mammary gland, breast milk, as well as maternal and offspring circulation during lactation in various species. PTHrP acts to stimulate mammary branching morphogenesis, increase calcium transport from blood to milk, regulate mammary blood flow and myoepithelial cell tone, and control maternal and postnatal calcium homeostasis (23, 29, 39). Targeted overexpression and knockout studies have revealed the critical importance of PTHrP for perinatal survival, as well as for mammary development and lactation (22, 24, 60, 62). Overexpression of PTHrP has been shown to disrupt branching morphogenesis (61). In light of these important roles of PTHrP, we hypothesized that impaired mammary development and function may involve altered PTHrP action and may contribute to poor postnatal offspring growth in an experimental model of growth restriction.

In Western society, uteroplacental insufficiency is associated with reduced oxygen and nutrient delivery across the placenta and is the most common feature in human pregnancies that are complicated by intrauterine growth restriction. Postnatally, maternal nutrient restriction and altered mammary function commonly limit nutrient delivery to the suckling offspring for whom mother’s milk is the only source of nutrition. We have used the well-characterized paradigm of prenatal growth restriction in rats, caused by bilateral uterine artery and vein ligation, that mimics uteroplacental insufficiency in humans (56, 58). The effects of uteroplacental insufficiency in the rat

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on postnatal growth, blood pressure, and insulin sensitivity are variable (21, 27, 45, 56). This may be due to variations in technical approach and experimental design, including whether one or both uterine vessels are ligated (21), using reduced litter size groups as controls (21, 27, 45), and cross-fostering onto unoperated control mothers (45). We suggest that these approaches can all have consequences for lactation and postnatal offspring growth (56), possibly through the influence on lactation that may further confound outcomes. The studies proposed here overcome these limitations by using appropriate controls with normal mammary function (e.g., control sham surgery with intact litter size), as well as control for the lower litter size that our laboratory has reported following uteroplacental insufficiency (56, 58).

We hypothesized that uteroplacental insufficiency and a reduction in litter size (to match that following uteroplacental insufficiency) impair milk production, which further restrains growth after birth. Growth and milk intake were analyzed separately for males and females, as there are known sex differences in the programming of growth and disease risk for babies born small. We suggest that lower maternal progesterone concentrations during pregnancy may contribute to altered lactation and lower milk supply to offspring. The alterations in milk composition, and hence nutrient supply to the pup after birth, are proposed to be due, in part, to the impaired mammary function, as well as altered activity of the PTHrP and calcium axes in the mother, mammary gland, and pups.

MATERIALS AND METHODS

Experimental protocol. Wistar Kyoto (WKY) rats (9–13 wk of age) were obtained from the Australian Resources Centre (Murdoch, WA, Australia) and provided with standard food pellets and tap water ad libitum. Rats were housed in a 12:12-h light-dark cycle (lighting from 0530 to 1730) at 19–22°C. Rats were mated after using a vaginal progesterone impedance reader (model MK-10B, Muromachi Kikai, Osaka, Tokyo, Japan), which measures the electrical impedance of the epithelial cell layer of the vaginal mucosa. This was routinely performed in the afternoon, and a reading of >4 kΩ indicated that females were in proestrus and presumably in estrus overnight. The presence of sperm in the vaginal smear the following morning indicated successful mating and was taken as day 1 of gestation. This study was approved by The University of Melbourne’s Animal Experimentation Ethics Sub-Committee.

On day 18 of gestation, pregnant rats were randomly allocated into three study groups: pregnant (Control, n = 12; Restricted, n = 14), postnatal Control (n = 16; Restricted, n = 11; Reduced Litter, n = 12), and lactation (Control, n = 8; Restricted, n = 9; Reduced Litter, n = 8) groups. The pregnant and postnatal experimental studies investigated mothers and pups at postmortem on day 20 of gestation and in a separate group on day 6 of lactation, respectively. The third experimental lactation group studied mothers and pups sequentially from 3 days after birth to 3 wk of age. Dams in the Restricted treatment groups underwent uteroplacental insufficiency surgery with a bilateral uterine artery and vein ligation on day 18 of gestation (term = 22 days) (56, 58). Sham surgery produced Controls (56, 58).

Our laboratory has shown that uteroplacental insufficiency reduces litter size to approximately five (56). To control for growth differences induced by altered litter size following uteroplacental insufficiency, a separate group of pregnant females that underwent sham control surgery had their litter size randomly reduced to five. This experimental group is referred to as Reduced Litter (7). Before aseptic surgery, rats were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg body wt; Parnell Laboratories, Alexandra, NSW, Australia) and ilium xylazil-20 (10 mg/kg body wt; Troy Laboratories, Smithfield, NSW, Australia). The sham control surgery that produced the Control and Reduced Litter groups was performed in the same manner, except the uterine vessels were not ligated, thereby controlling for the influence of variables associated with surgical and anesthetic procedures that may affect growth and development. The duration of surgery was ~10 min, and time under anesthesia ~40 min.

Pregnant study measurements. On the morning of day 20 of gestation, pregnant females and their fetuses (Control and Restricted groups) were terminally anesthetized with an intraperitoneal injection of ketamine and illium-xylazil (as above). Maternal blood was obtained by cardiac puncture. Mammary tissue was dissected, weighed, and snap frozen in liquid nitrogen and stored at ~80°C. Fetal weight data have been previously published, together with fetal and placental measurements (58).

Postnatal study measurements. All pups remained with their own mothers from birth to day 6 of lactation. On the morning of day 6, pups (Control, Restricted, and Reduced Litter groups) were removed from their mother and immediately euthanized by decapitation. Mothers were terminally anesthetized 4–6 h after removal of the pups to allow milk to accumulate. Approximately 200 µl of milk were then collected following gentle massage of the left mammary gland and nipples (59), without the need for hormonal stimulation. Maternal blood was obtained by cardiac puncture, whereas blood from pups was obtained using heparinized capillary tubes. For each litter, ~1–2 ml of pup blood were collected, pooled, and placed into two heparinized tubes for either PTHrP or calcium and electrolyte analysis. Aprotinin (5.2 trypsin inhibitor units/mg solid, Sigma Chemical St. Louis, MO) was added to the blood tubes used for PTHrP analysis. Pup body weight, crown rump length, head width, and hindlimb length (using calipers with 0.01 mm accuracy) were measured. Ponderal index was calculated as body weight (in g) × 100/crown rump length (in cm²) (58). One pup per litter was frozen for whole body calcium analysis and stored at −20°C. Pup heart, liver, spleen, and kidney weights were recorded for the remaining postnatal pups. Maternal uterine, ovarian, mammary, and heart weights were measured. The mammary glands were dissected and weighed, and the right mammary gland was snap-frozen in liquid nitrogen and stored at ~80°C. Milk was stored at ~20°C until analyzed.

Lactation study measurements. Separate groups of rats were used for the lactation study, as described above, and all pups in the litter were studied from birth to postnatal day 21. Milk intake of individual pups was measured on days 3, 6, 10, 14, and 17 of lactation (59). Pup weights were monitored during a 1-h maternal separation period followed by a 3-h refeeding period. All pups in the litter were removed, so the impact on maternal behavior was common to all groups. Weight gain of pups over the refeeding period provides an indirect measure of mother-to-pup milk transfer. Milk intake was also calculated as a percentage of body weight. As an index of milk consumption during lactation, area under the milk intake curve was calculated between days 3 and 17 of lactation for pups that had data available on all days (3, 6, 10, 14, and 17). Body weight of individual pups was measured on days 3, 6, 10, 14, 17, and 21 of lactation.

Tissue, plasma, and milk analyses. Protein was extracted from mammary and uterine tissue using previously established techniques, and the extract was analyzed for PTHrP concentrations by radiimunoassay (57–59). Plasma, milk, and tissue concentrations of PTHrP were quantified by a sensitive N-H₂-terminal radiimmunoassay that does not recognize parathyroid hormone (18, 57–59). Total calcium concentrations were determined using a Beckman Synchron CX-5 Clinical System with colorimetric spectrometry (Beckman Coulter, Fullerton, CA) and ionic calcium concentrations using ion-selective electrodes correcting for pH (Ciba-Corning model 644, Medfield, MA) from milk. Total and ionic calcium was also measured in pup and maternal plasma. Total calcium concentration in the pup body was determined after ashing using the Beckman CX-5 analyzer (59).
Maternal plasma progesterone concentrations were measured using a DPC Coat-A-Count radioimmunoassay kit (Biomediq, Doncaster, Victoria, Australia) with a detection limit of 0.06 nmol/l. The concentration of fat in milk was measured as esterified fatty acids using an adaptation of the method of Stern and Shapiro (48). The concentration of total protein in skim milk was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) adapted by Atwood and Hartmann (2). The method of Kuhn and Lowenstein (26), as modified by Arthur et al. (1), was used to measure the concentration of lactose in skim milk.

Quantitative real-time PCR. Real-time PCR was used to quantitate milk protein gene expression on day 20 of pregnancy as markers of lactogenesis. Total RNA was extracted from frozen mammary tissue using the Qiagen RNeasy kit (Qiagen, Clifton Hill, Victoria, Australia). Reverse transcription and quantitative real-time PCR was performed as previously described for α-lactalbumin and β-casein. Quantitative real-time PCR was performed on the Rotorgene 3000 (Corbett Research, Mortlake, NSW, Australia). Taqman primers and probes were used following optimization. The ribosomal RNA, 18S (forward primer: 5′-CGGCTACCACATCCAAGGAA-3′; reverse primer: 5′-GCTGGAATTACCCGGCT-3′; probe: VIC 5′-TGCTG-GCACCGACTTTCCTC-3′) (Applied Biosystems, Scoresby, Victoria, Australia) was used as an endogenous control and multiplexed with the milk protein genes, β-casein (Rn00567460_m1, Applied Biosystems) and α-lactalbumin (Rn00561447_m1, Applied Biosystems). For the relative quantitation of gene expression, a multiplex comparative threshold cycle method (56, 58) was employed.

Statistical analysis. Data for individual pups were analyzed by two-way (with age and group as factors) analysis of variance (SPSS-X 14.0, SPSS), as appropriate. Where a significant interaction between factors was detected, data were then analyzed by one-way analysis of variance. Differences across the groups were determined by a post hoc Student-Newman-Keuls test. Differences between ages (pregnant vs. postnatal) were determined using Student’s t-test. Pearson’s correlation was used to determine the association between particular variables. Data are presented as means ± SE, and P < 0.05 was taken as being statistically significant.

RESULTS

Body and organ weights. Pup weight on day 20 of gestation was reduced by 16% in the Restricted group compared with Control (P < 0.0001; Fig. 1) (58). On day 20 of gestation, litter size for the Restricted group was reduced by 25% compared with Control (P < 0.002; Fig. 2). Gestational age at delivery was 22 days for all rats. At birth, the number of live pups born was further reduced in the Restricted group to 36% of that of Control, with a further 10% reduction between birth and day 6 of lactation (P < 0.001; Fig. 2). Litter size at birth for the Control and Reduced Litter groups was not significantly different. The reduction in the number of offspring in the litter between day 20 of gestation and day 6 of lactation was significant for the Restricted group (P < 0.0001; Fig. 2). Sham control surgery was associated with a 21% decrease in litter size between day 20 of gestation and birth (P < 0.05; Fig. 2), with no further reduction at day 6 of lactation.

At day 6 of lactation, Restricted pup weight was further reduced to 36% of that of Control and Reduced Litter groups (P < 0.05; Fig. 1). Crown rump length followed the same trend (P < 0.0001; Fig. 1). Despite the decrease in Restricted pup body weight and crown rump length, head width, hindlimb length, and pup organ weights (kidney, spleen, heart, and liver, as a percentage of body weight) were not different between the groups (Table 1). Ponderal index for the Restricted Litter, but not Restricted pups, was lower than for Controls (P < 0.05; Table 1). On day 6 of lactation, maternal age, body weight, heart, kidney, uterus, and ovary weights (expressed as a percentage of body weight) were not different between the groups (data not shown).

Maternal and fetal plasma, milk composition, and mammary PTHrp content. On day 20 of gestation, maternal plasma progesterone was reduced by 46% in pregnant Restricted animals compared with Controls (P < 0.001; Fig. 3A). Maternal mammary gland α-lactalbumin mRNA expression (P < 0.05; Fig. 3B) in Restricted animals was increased by 113% compared with Control animals. There were no significant differences in maternal mammary gland β-casein mRNA expression between groups (Fig. 3C). At day 6 of lactation, there were no significant differences in maternal plasma PTHrp, progesterone, ionic calcium and total calcium concentrations, and pup plasma PTHrp and total calcium concentrations between the three groups (Table 2). Milk concentrations of PTHrp, total calcium, total protein, and the percentage of fat in milk were not different between Control, Reduced Litter, and Restricted groups (Table 2).

Maternal mammary weight (as a percentage of maternal body weight) (P < 0.008; Fig. 4A) and milk lactose [as an index of lactogenesis II (1)] (P < 0.01; Fig. 4B) were significantly lower on day 6 of lactation in Restricted animals compared with Controls, with Reduced Litter values being intermediate. Mammary weight (Fig. 4A) was correlated with

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litter size (Fig. 2) on day 6 of lactation ($r^2 = 0.642; P < 0.02$). In the Restricted groups, milk sodium concentrations were higher and potassium concentrations were decreased in the Restricted group compared with Controls ($P < 0.01$; Fig. 4C). Mammary PTHrP tissue content increased 2.5-fold between day 20 of gestation (data not shown) and day 6 of lactation (Fig. 5A) in the Control group ($P < 0.001$), but this was not observed in the Restricted group. Both Reduced Litter and Restricted mammary PTHrP content were lower than Control ($P < 0.01$; Fig. 5A), and both group values were not different from Control on day 20 of gestation (data not shown). Maternal mammary PTHrP content was correlated with milk lactose concentrations ($r^2 = 0.453; P < 0.002$) on day 6 of lactation. There were no differences in uterine PTHrP tissue content across the groups (Table 2). On day 6 of lactation, milk and pup ionic calcium concentrations tended to be lower in the Restricted group compared with Reduced Litter ($P < 0.05$; Fig. 5B). Pup ionic calcium concentrations were decreased in the Reduced Litter group compared with Control ($P < 0.02$; Fig. 5C). Pup body calcium was lower in both the Reduced Litter and Restricted pups compared with Controls ($P < 0.01$; Fig. 5D).

Table 1. Pup weights and dimensions

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Restricted</th>
<th>Reduced Litter</th>
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<tbody>
<tr>
<td>Head width, mm</td>
<td>15.3±0.8</td>
<td>14.4±1.0</td>
<td>15.8±0.8</td>
</tr>
<tr>
<td>Distal hindlimb length, mm</td>
<td>16.6±0.9</td>
<td>14.9±1.1</td>
<td>17.6±1.1</td>
</tr>
<tr>
<td>Kidney weight, g/%body wt</td>
<td>1.25±0.02</td>
<td>1.31±0.04</td>
<td>1.26±0.04</td>
</tr>
<tr>
<td>Spleen weight, g/%body wt</td>
<td>0.43±0.02</td>
<td>0.48±0.02</td>
<td>0.47±0.02</td>
</tr>
<tr>
<td>Heart weight, g/%body wt</td>
<td>0.61±0.02</td>
<td>0.59±0.03</td>
<td>0.63±0.01</td>
</tr>
<tr>
<td>Liver weight, g/%body wt</td>
<td>2.63±0.09</td>
<td>2.65±0.15</td>
<td>2.67±0.08</td>
</tr>
<tr>
<td>Ponderal index, g × 100/cm³</td>
<td>8.24±0.26†</td>
<td>7.68±0.24++</td>
<td>7.38±0.20*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Pup head width, distal hindlimb length, and kidney, spleen, heart, and liver weights (as a % of body weight), as well as ponderal index for Control, Restricted, and Reduced Litter (control litter size reduced at birth) groups on day 6 of lactation are shown. *†Different symbols indicate significant differences, such that data with an “+” are different from data with a “-”; the same as data with a “++*/†”.

Milk intake. There were significant differences in pup milk intake between day 3 and 17 lactation and differences between groups at given ages ($P < 0.05$; Fig. 6). Female pup milk intake was significantly lower in the Restricted and Reduced Litter groups compared with Control on day 6 of lactation and lower in the Restricted group compared with Control and Reduced Litter groups on day 10 of lactation ($P < 0.01$; Fig. 6A). Male pup milk intake was significantly lower in the Restricted group compared with Control and Reduced Litter groups on day 6 and lower in Restricted and Reduced Litter groups compared with Controls on day 14 ($P < 0.05$; Fig. 6B). Similar trends were present when milk intake was corrected for pup body weight for the females on both days 6 and 10 and for the males on day 6 ($P < 0.05$, data not shown).
Table 2. Maternal and fetal plasma, maternal uterine PTHrP content, and milk composition

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Restricted</th>
<th>Reduced Litter</th>
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<tr>
<td>Maternal plasma</td>
<td></td>
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<tr>
<td>PTHrP, pmol/l</td>
<td>4.9±0.6</td>
<td>6.3±0.6</td>
<td>5.8±0.6</td>
</tr>
<tr>
<td>Ionic calcium, mmol/l</td>
<td>0.82±0.05</td>
<td>0.81±0.07</td>
<td>0.68±0.04</td>
</tr>
<tr>
<td>Total calcium, mmol/l</td>
<td>2.5±0.04</td>
<td>2.6±0.10</td>
<td>2.4±0.06</td>
</tr>
<tr>
<td>Maternal uterus</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PTHrP, fmol PTHrP/mg protein</td>
<td>4.1±0.7</td>
<td>2.1±0.5</td>
<td>2.0±0.5</td>
</tr>
<tr>
<td>Pup plasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTHrP, pmol/l</td>
<td>25.4±1.9</td>
<td>27.1±2.2</td>
<td>26.2±0.8</td>
</tr>
<tr>
<td>Total calcium, mmol/l</td>
<td>1.71±0.13</td>
<td>1.60±0.11</td>
<td>1.46±0.16</td>
</tr>
<tr>
<td>Milk</td>
<td></td>
<td></td>
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<tr>
<td>PTHrP, pmol/l</td>
<td>1,922±144</td>
<td>1,922±148</td>
<td>1,607±258</td>
</tr>
<tr>
<td>Total calcium, mmol/l</td>
<td>58.1±2.9</td>
<td>54.3±3.6</td>
<td>62.8±2.7</td>
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<tr>
<td>Total protein, g/l</td>
<td>84±7.8</td>
<td>74±1.6</td>
<td>74±5.2</td>
</tr>
<tr>
<td>Fat, %</td>
<td>16.7±2.5</td>
<td>16.7±0.9</td>
<td>18.5±1.9</td>
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</table>

Values are means ± SE. Maternal plasma parathyroid hormone-related protein (PTHrP) and ionic and total calcium, maternal uterus PTHrP content, pup plasma PTHrP and total calcium, milk PTHrP, total calcium, total protein, and percentage fat for Control, Restricted and Reduced Litter (control litter size reduced at birth) groups on day 6 of lactation are shown. There were no significant differences for any parameter between the groups.

In both male and female pups, milk intake increased in Control and Reduced Litter groups between days 3 and 6 of lactation, but this age-related increase was significantly delayed to day 10 in the Restricted group (P < 0.05; Fig. 6). Female milk intake for all groups plateaued by days 14–17, whereas male milk intake for all groups decreased between days 14 and 17 (P < 0.05), suggesting delayed weaning in females. The area under the milk intake curve between days 3 and 17 of lactation was significantly lower in male and female Restricted pups (male 1.10 ± 0.09 g/day, female 1.20 ± 0.05 g/day) compared with both Control (male 1.40 ± 0.10 g/day, female 1.60 ± 0.10 g/day) and Reduced Litter (male 1.30 ± 0.10 g/day, female 1.40 ± 0.09 g/day) groups (P < 0.05). There was a significant delay in the day of maximal milk intake in female Restricted (14.2 ± 0.7 days) and Control (13.7 ± 0.6 days) pups compared with Reduced Litter (11.7 ± 0.9 days) (P < 0.05). On this day of maximal intake, Restricted female pups consumed significantly less milk (0.51 ± 0.04 g) than did Control (0.70 ± 0.05 g) or Reduced Litter (0.63 ± 0.05 g) pups (P < 0.05). A similar, but nonsignificant, trend was observed in male pups.

Postnatal growth measurements. In the lactation group, separate experimental cohorts were studied from birth to day 21, and mean litter size in the Reduced Litter group at birth (4.8 ± 0.3 from 9 mothers, n = 47 pups) and Restricted (4.6 ± 0.4 from 8 mothers, n = 36 pups) groups was significantly lower than that in Controls (10.1 ± 0.8 from 8 mothers, n = 81 pups) (P < 0.05). The mean number of pups in the Reduced Litter group was less than five as a consequence of natural death or cannibalism by the mother in the early postnatal period. The proportion of male (45.6 ± 3.7%) and female (53.3 ± 3.9%) pups in the litters was not significantly different overall, or within any of the groups, suggesting consistent sex ratios. All pups remained with their own mothers from birth until day 21, and litter size was not altered in the Control and Restricted groups during lactation. In the first week of postnatal life (days 3 and 6), Restricted male and female pups were significantly growth restricted (by 28% compared with Control) compared with Reduced Litter (by 10% compared with Control), which were smaller than Controls (P < 0.05; Fig. 7). Male and female Restricted pup weights (as for milk intake) did not increase from day 3 to day 6, while the Reduced Litter and Control pups increased their weight significantly during this period (P < 0.05; Fig. 7). Restricted female pup weights had caught up and were not different from Reduced Litter by days 10 and 14 of lactation (P < 0.05; Fig. 7A). By day 21, Restricted and Reduced Litter female pup weights were not different from Controls (P < 0.05; Fig. 7A). In contrast, Restricted male pup weights remained significantly lower than those of Reduced Litter, which was less than Control offspring throughout lactation to day 21 (Fig. 7B).

**DISCUSSION**

**Litter size.** The number of suckling offspring influences lactational performance, and, therefore, litter size was determined in all experimental groups. In the Restricted group, a reduction in litter size was evident by day 20 of gestation, 2 days after uteroplacental insufficiency surgery, with a further reduction in litter size being evident by day 21. Male and female Restricted offspring were significantly growth restricted (by 28% compared to Controls) compared with Reduced Litter (by 10% compared with Control), which were smaller than Controls (P < 0.05; Fig. 7). Male and female Restricted pup weights (as for milk intake) did not increase from day 3 to day 6, while the Reduced Litter and Control pups increased their weight significantly during this period (P < 0.05; Fig. 7). Restricted female pup weights had caught up and were not different from Reduced Litter by days 10 and 14 of lactation (P < 0.05; Fig. 7A). By day 21, Restricted and Reduced Litter female pup weights were not different from Controls (P < 0.05; Fig. 7A). In contrast, Restricted male pup weights remained significantly lower than those of Reduced Litter, which was less than Control offspring throughout lactation to day 21 (Fig. 7B).
reduction by day 6 of lactation. We suggest that this litter size reduction was a result of the in utero decrease in nutrient and oxygen supply across the placenta and early lactation losses consistent with other studies (20, 41, 58). The Reduced Litter animals, which had undergone sham control surgery and had their litter size reduced to five at birth, served as a litter size control for the Restricted group during lactation. It is known that changes in litter size or suckling pressure can alter milk production (3, 4), consistent with our data. Large reductions of litter size to ~25% of normal are often used as an experimental model of postnatal overnutrition and obesity (15, 40, 53) as a result of increased pup milk intake (4). Many of these studies include male pups only and often alter litter size a few days after birth when lactation is becoming established for a given litter number. Our reduction in litter size is much less severe, includes equal sex ratios, with males and females investigated separately, and is performed 1 day after birth so that lactation can adjust to the demands of the offspring. Our data highlight the need for adequate control groups in which growth and the influences on mammary function are investigated.

In addition, alterations in litter size can cause stress to the mother during lactation, although late pregnant and lactating rats have been found to be hyporesponsive to stress caused by external stimuli (9, 51), unless the external stimuli involve her offspring (12). Stress during pregnancy is known to alter...
Litter pups. The asymmetric growth restriction, with a pre-birth body weight reduction for the Restricted pups increased to 36% uteroplacental insufficiency studies (20, 37, 52, 56, 58). The species and is consistent with many, but not all, experimental degree of growth restriction observed in most human pregnancy reflects the associated with slowed postnatal growth. The combination of impaired mammary development, premature lactogenesis, and delivery of smaller and fewer offspring could lead to changes in milk composition, as well as reduced milk production for Restricted offspring. Milk production was measured in this study using the weigh-suckle-weigh method, which has previously been validated against a milking machine and oxytocin administration in sheep (8). Milk production was influenced by both the number of pups a rat dam is suckling and the size of the pups suckling, which supports previous studies on suckling frequency and pressure (11, 19) and further implicates milk removal as a major contributor to milk production. The male and female Reduced Litter pups born of normal birth weight consumed less milk on days 7 and 14, respectively, which may have contributed to their slowed growth. The Restricted female pups had a lower milk intake on days 6 and 10 of lactation, an overall reduction in milk intake during lactation as calculated by area under the milk intake curve (over days 3–17 of lactation), as well as a delay in the timing and quantity of maximal intake, which were associated with slowed postnatal growth. Male Restricted pups had a lower milk intake on day 14 of lactation, which was later in lactation than observed in females. The lower overall milk intake (area under the milk intake curve) of the male Restricted group was associated with a greater degree of growth restriction. We cannot exclude the possibility that these male offspring experience accelerated growth later in life. The lower milk intake for Restricted offspring could also be caused by a reduced suckling stimulus, which may delay peak milk production. Poor milk intake in pups born small thus amplifies any preexisting deficits in offspring growth later in life. The lower milk intake of the male Restricted group was associated with a lower overall milk intake (area under the milk intake curve) of the male Restricted group was associated with a lower overall milk intake (area under the milk intake curve) of the male Restricted group was associated with a lower overall milk intake (area under the milk intake curve) of the male Restricted group was associated with a lower overall milk intake (area under the milk intake curve) of the male Restricted group was associated with a lower overall milk intake (area under the milk intake curve) of the male Restricted group was associated with a lower overall milk intake (area under the milk intake curve) of the male Restricted group was associated with a lower overall milk intake (area under the milk intake curve) of the male Restricted group was associated with a lower overall milk intake (area under the milk intake curve) of the male Restricted group was associated with a lower overall milk intake (area under the milk intake curve). Mammary function. Our evidence in Restricted mothers, which have undergone uteroplacental insufficiency surgery, suggests that mammary function is impaired, which can further compromise postnatal growth of offspring. This result led us to explore the mechanisms by which milk production and composition can influence offspring growth during lactation. Maternal milk samples were collected via manual palpation and milking, not by exogenous oxytocin administration to stimulate milk ejection. As the endocrine status of the Restricted rat dams was altered during pregnancy, it is important not to artificially stimulate the mammary gland, as this may impact on milk production and composition. Lactogenesis can be separated into two stages. It begins in midpregnancy with the synthesis of milk components (lactogenesis I) and is followed by the onset of copious milk secretion associated with the
decrease in progesterone and an increase in prolactin and glucocorticoids around parturition (lactogenesis II) (25, 35). Progesterone withdrawal has been identified as the initial trigger of lactogenesis II in rodents (10, 25, 35, 42, 46, 54). This is supported by the finding that lactogenesis II is impaired as a result of delayed progesterone withdrawal when placental fragments are retained within the uterus of humans and dairy cattle (30). Our Restricted rat dams had a 46% decrease in plasma progesterone on day 20 of gestation, and it was likely to be a large enough decrease to serve as a trigger for premature lactogenesis II before delivery of pups at term. We suggest that, in the absence of suckling by pups, theRestricted rats may have induced the mammary gland to undergo early involution before delivering their offspring, which resulted in the lower mammary weight compared with Controls. The milk protein α-lactalbumin is required for the synthesis of lactose (17, 36). In the Restricted rat dams, the increase in maternal mammary gland α-lactalbumin gene expression on day 20 of gestation, compared with Controls, is consistent with the premature initiation of lactogenesis II. In contrast, maternal mammary gland β-casein gene expression did not change when α-lactalbumin was elevated. This result is consistent with a previous mouse study that showed that mammary β-casein gene expression is unaffected by progesterone withdrawal (49). Further analysis is required to understand the mechanisms that led to this early lactogenesis phenotype and to establish whether the mammary gland exposed to uteroplacental insufficiency did undergo involution. In Restricted dams, it is hypothesized that lactogenesis II was reinitiated after birth by the suckling pups. The lower milk lactose concentrations on day 6 of lactation, indicative of low milk production, supports the reinitiation of lactogenesis II (1). The rate of lactose synthesis determines the volume of milk secreted, as this process involves water being osmotically drawn into the Golgi vesicles of the mammary secretory cells (31). Reduced Litter dams, with a lower litter size than Control, had mammary weight and milk lactose concentrations intermediate between Control and Restricted groups, indicating that mammary gland function may be influenced by litter size. Once lactation is established, the rate of milk production relates to the demands of the offspring, primarily by a feedback mechanism related to the amount of milk remaining in the mammary alveoli at termination of suckling (55). This demand for milk is likely to be regulated not only by the size, but also the number of suckling pups (55). Despite the reduction in milk lactose in the Restricted group, there was no change in the percentage of milk fat and total protein, although other milk components were not measured due to the limited volume of milk available.

Tight junctions are formed in the mammary epithelium during lactogenesis, before copious milk secretion, and become “leaky” following mammary involution (47). Tight junctions are responsible for establishing contact between cells in epithelial tissues and preventing paracellular movement of ions and small molecules between adjacent cells from blood to the apical side of the cell and vice versa (47). The premature initiation of lactation seen in the Restricted group in association with the early withdrawal of progesterone in late pregnancy may have caused the tight junctions to become leaky, which would cause a decrease in milk lactose concentrations. The suggested early mammary involution may have also caused apoptosis and resulted in leaky tight junctions (47). The increase in milk Na/K seen on day 6 of lactation in the Restricted and Reduced Litter rat dams is suggestive of “leaky” tight junctions in the mammary gland epithelium. The increased milk Na/K in the Reduced Litter group could be explained by a decrease in suckling stimulus caused by fewer suckling pups altering milk production (55). Milk collection at multiple ages may clarify the mechanisms associated with the changes in the altered milk composition following uteroplacental insufficiency.

PTHRp and calcium. We suggest that the lower mammary PTHrP content in the Restricted and Reduced Litter groups may have reduced calcium transport from maternal blood into milk, resulting in the lower pup plasma ionic calcium and pup body calcium on day 6 of lactation and, therefore, may be indirectly related to restricted pup growth. Any PTHrP and calcium effects shown were mammary specific, as uterine PTHrP was not altered between experimental groups. In support of our findings, our laboratory has previously shown that the spontaneously hypertensive rat has impaired mammary function and altered milk PTHrP and calcium concentrations that were associated with reduced postnatal growth (59). Many small human babies have low circulating calcium concentrations (50), supporting the suggestion that reduced delivery of calcium to milk and offspring may contribute to growth restriction through mammary PTHrP-mediated mechanisms.

Perspectives and significance. The novel finding of this study was that there is an associated impairment of mammary development during pregnancy and lactation after birth in rat dams with pregnancies compromised by uteroplacental insufficiency, which further slows postnatal growth. We suggest that such adverse effects on lactation may occur in other experimental models of growth restriction in which maternal physiology is altered (e.g., altering maternal nutrition). The findings also demonstrate that reducing litter size at birth for pups born of normal weight can have adverse consequences for offspring growth. Our results have significant implications for the developmental programming field, as many investigators have failed to include appropriate litter size controls. Cross-foster studies are also required to determine whether the slowed postnatal growth of offspring exposed to uteroplacental insufficiency may be due to, not only the compromised pregnancy, but also lactational constraint that we have reported in this study. In addition, such cross-fostering studies may be able to investigate whether the mammary gland defect can be rescued postnatally. This study, which examined the effect of uteroplacental insufficiency on postnatal growth of offspring and the role of lactation, has highlighted many important prenatal and postnatal factors regulating growth, with implications for investigating the developmental origins of later health and disease.

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REFERENCES


41. Rajakumar PA, Jing H, Simmons RA, Devaskar SU. Effect of utero

42. Rotchchild I, Billiar RB, Kline JT, Pepe G. The persistence of postgr


