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Maternal stress and high-fat diet effect on maternal behavior, milk composition, and pup ingestive behavior

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Abstract

Chronic variable prenatal stress or maternal high-fat diet results in offspring that are significantly heavier by the end of the first postnatal week with increased adiposity by weaning. It is unclear, however, what role maternal care and diet play in the ontogenesis of this phenotype and what contributions come from differences already established in the rat pups. In the present studies, we examined maternal behavior and milk composition as well as offspring ingestive behavior. Our aim was to better understand the development of the obese phenotype in offspring from dams subjected to prenatal stress and/or fed a high-fat (HF) diet during gestation and lactation. We found that dams maintained on a HF diet through gestation and lactation spent significantly more time nursing their pups during the first postnatal week. In addition, offspring of prenatal stress dams consumed more milk at postnatal day (PND) 3 and offspring of HF dams consume more milk on PND 7 in an independent ingestion test. Milk from HF dams showed a significant increase in fat content from PND 10-21. Together these results suggest that gestational dietary or stress manipulations can alter the rat offspring's developmental environment, evidence of which is apparent by PND 3. Alterations in maternal care, milk composition, and pup consumption during the early postnatal period may contribute to long-term changes in body weight and adiposity induced by maternal prenatal stress or high-fat diet.

Keywords

prenatal stress; high-fat diet; obesity; maternal behavior; independent ingestion

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1. Introduction

Obesity has become a major public health concern, particularly as rates continue to increase in children [1, 2]. Childhood obesity often continues into adulthood and can increase risk for type 2 diabetes, cardiovascular disease, and cancer translating into greater health care expenditures and reduced life expectancy [3]. Human and animal studies have shown that adverse conditions *in utero* can lead to obesity and adult chronic disease. Intrauterine growth retardation resulting from maternal dietary restriction has been studied extensively and it is now clear that these conditions predispose an individual towards hypertension, hyperglycemia, hyperinsulinemia and obesity [4-6]. As originally described by Hales and Barker in humans, prenatal growth or dietary restriction could predispose the fetus' metabolism towards fat storage in adulthood; an adaptive mechanism which leaves the offspring ill-suited for Western dietary conditions [5]. Interestingly, maternal obesity can lead to similar adverse metabolic consequences in adulthood [7, 8] and the prevalence of maternal obesity has increased markedly in recent years [9, 10]. Aspects of maternal diet independent of the influence of maternal obesity may also be critical to offspring development. Indeed, offspring of lean rats maintained on high-fat (HF) diet only through gestation and lactation develop an obese phenotype similar to offspring of rats which have been maintained on HF diet for a significant period of time prior to pregnancy [11, 12].

Indeed, animal models have been critical in improving our understanding of possible developmental origins of adult diseases. Rats are born relatively immature without visual or auditory faculties and depend greatly on their mothers, particularly during the first 2 postnatal weeks. Maternal diet has been shown to affect offspring metabolic programming but it is unclear what role nursing behavior might play [12, 13]. Certainly, large differences in the time spent nursing or in the nutritional composition of the dam's milk between groups could contribute to the early pup body weight and metabolic differences.

Maternal care during lactation has also been shown to affect offspring stress response in adulthood [14]. An individual's response to stress is crucial to maintaining homeostasis. In the short-term, changes in activity of the stress-reactive hypothalamic-pituitary-adrenal (HPA) axis can modulate energy balance. Growing evidence also suggests that long-term changes in HPA axis function can have a marked effect on body composition as well [15, 16]. In fact, adrenal corticosteroids have been shown to be closely linked to visceral adiposity to the extent that adrenalectomy can ameliorate genetic or diet-induced obesity in rodents [17]. It is now clear that HPA axis activity can also be programmed by early life experiences and animal models have been used extensively to investigate this phenomenon [14, 18-20].

Retrospective studies of human mothers exposed to physical or psychological stress during pregnancy have shown offspring to have altered stress responses and increased rates of obesity and cardiovascular disease in adulthood [19, 21]. Changes in the development of rodent offspring's stress response following prenatal stress appear to result from exposure to high levels of corticosterone *in utero* [22]. Normally, the fetus is protected from excess circulating glucocorticoids by the placental enzyme 11-beta-hydroxysteroid dehydrogenase (11- β HSD) which catalyzes the metabolism of corticosterone to inactive 11-keto steroids [23, 24]. Administration of excess or synthetic glucocorticoids, such as dexamethasone, to the pregnant rat can overcome this barrier and expose the fetus to high glucocorticoid levels. This can have deleterious developmental consequences and can induce hyperglycemia, hyperinsulinemia, and hypertension in the adult offspring [25]. Stress to the mother, particularly during the last week of gestation in the rat, can produce similar results. In the present studies we used a chronic variable stress paradigm from gestation day 14-21 which

precludes habituation and thus induces secretion of high levels of glucocorticoids throughout this time period [26].

We previously reported that prenatal stress or high-fat diet lead to significant increases in body weight as early as postnatal day (PND) 7 and produce offspring that have impaired glucose tolerance and increased adiposity by weaning on PND 21 [12]. In this study we sought to further investigate the underpinnings of this phenotype and the potential contributions from the dam and the pups themselves during early postnatal life through examination of maternal behavior and milk composition as well as pup independent ingestion during the pre-weaning period.

2. Materials and Methods

Timed-pregnant female Sprague-Dawley rats (Charles River) arrived on gestation day (GD) 2 and were housed individually in standard plastic tub cages with *ad libitum* food and water access. All rats were housed in the same environmentally controlled room with a 12h light/dark cycle (0600-1800h light). All animal procedures were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

Upon arrival, pregnant rats were assigned to either standard chow ("CHOW"; Harlan Teklad 2018, 17% kcal from fat; $n = 24$) or high-fat diet ("HF"; Research Diets, D12492, 60% kcal from fat, $n = 26$) groups. In each group, half of the rats were subdivided into stress (STRESS) and control (CON) groups. Body weight and food intake were monitored weekly. The day that a litter was found before the end of the light cycle was designated postnatal day (PND) 0. On the morning of PND 1, pups were counted, sexed and weighed. Litters were normalized to 8-10 pups each (with equal numbers male and female pups where possible) and only litters of at least 8 pups were included in the following studies. Three cohorts of animals were used in these studies. Each cohort was treated in the same manner and animals within each cohort were divided evenly among the four treatment groups.

2.1. Variable Stress

The variable stress paradigm used in this study is summarized in Table 1 and has been previously described in detail [12]. Unlike some prenatal stress methods such as repeated restraint stress, rats do not habituate to these stressors due to their variety and the variable timing of their presentation [27, 28]. Additionally, stressors applied during the last week of gestation in the rat (GD 14-21) have been shown to produce the most robust effects in the offspring [18].

2.2. Maternal Behavior

We analyzed maternal behavior during the first two postnatal weeks using a procedure similar to that used by Schroeder *et al.* 2006 [29]. To avoid disturbing the litters, video recordings were made of three-hour periods during the middle of the light (1200-1500hrs) and dark periods (000-0300hrs) during week 1 (PND3-6) and during week 2 (PND10-13). Over the three-hour observation period, nursing postures, feeding/drinking, dam activity, and grooming behaviors were observed for 2 sec every 7.5 minutes and recorded. Data for each behavior are presented as a percentage of the total number of behavioral observations. Eight litters per group were observed during the first week and 4 litters per group during the second week.

2.3. Independent Ingestion

Testing took place on postnatal days 3, 7, and 10 using a protocol similar to Blumberg *et al.* 2006 [30]. Briefly, on the morning of testing, one male pup was marked and separated from

each litter and together they were placed in a warm, humidified incubator. After 5 hours, urination and defecation were manually stimulated with a soft cotton gauze pad and the pups were weighed to the nearest 0.01 g. Two sheets of filter paper were placed on the floor of small glass containers and soaked with warm, sweetened (10% sucrose) half-and-half (Land-O-Lakes, 13.3% fat w/v) in a humidified incubator maintained at 37 °C. Each pup was placed in an individual container for the 30-min test. All pups began to lick the warm sweetened milk within one minute of the start of the test. Throughout testing warm milk was added as needed. After the 30-min test, pups were carefully towel-dried and re-weighed. The difference between the starting and ending body weight was recorded as milk consumption. Following the testing session the pups were killed by decapitation.

2.4. Milk Composition

To avoid complicating behavioral analysis with maternal separation, litters were sacrificed on the day milk was collected from the dam. At each time point (PND 3, 7, 10, 16, and 21), dams were removed from their home cages and were deeply anesthetized with a 4:3 mixture of ketamine:xylazine (0.1 ml/100 g body weight, i.p.) and injected i.p. with 4 I.U. oxytocin (Sigma). Fifteen minutes later, milk was collected into glass capillary tubes (Drummond Scientific, Broomall, PA) by gently massaging the mammary glands. Samples were stored in microcentrifuge tubes and frozen at -80°C for later analysis. Following milk collection, dams were killed by decapitation.

Total lipid content was analyzed for milk collected at PND 3, 7, 10, 16, and 21. In addition, sugar, protein and water content were assessed in PND 21 milk samples. Lipid content was determined through sequential ether-extractions in a Rose-Gottlieb procedure modified for micro amounts of fat [31]. Water content was determined gravimetrically as the inverse of dry mass and sugar content was compared to lactose monohydrate standards in a phenol-sulfuric acid method [32]. Nitrogen was measured by an elemental analyzer (Model 2400, Perkin Elmer, Norwalk, CT); protein was estimated by multiplying N content by 6.38 [31]. Gross energy content (GE) of PND 21 milk samples was calculated assuming values of 3.95 kcal/g for sugar, 5.86 kcal/g for protein and 9.11 kcal/g for fat [31].

Similar to previous studies reporting milk leptin and insulin content [13], we used commercially available radioimmunoassay kits according to the manufacturer's protocol (Millipore Billerica, MA).

2.5. Statistical Analysis

Data are presented as mean $\pm$ SEM and were analyzed using Statistica 7.0 (Systat, Tulsa, OK). Milk composition was analyzed by Student's *t* test. Body weight, maternal behavior, and independent ingestion data were analyzed by ANOVA. Additional post-hoc comparisons between groups used Newman-Keuls procedures.

3. Results

3.1. Animal Subjects

There was no significant difference between groups in dams' gestational weight gain or caloric intake. Following parturition, we similarly found no differences in litter size, male-female ratio, or birth weight (Figure 1). At PND 3, pups from HF or STRESS litters were significantly heavier than controls (HF vs CHOW, $F(1, 11)=9.3249$, 11.0 ± 0.2 g vs 10.0 ± 0.2 g $P < 0.05$; STRESS vs CON, $F(1, 11)=7.6949$, 11.0 ± 0.2 vs 10.1 ± 0.2). At PND 7 and 16, an overall effect of HF diet on body weight was apparent (PND 7: $F(1, 28)=17.070$, 20.7 ± 0.4 g vs 18.5 ± 0.4 g $P < 0.05$; PND 16: $F(1, 10)=50.532$, 50.2 ± 0.9 g vs 40.7 ± 0.9 g; $P < 0.05$). Three litters were excluded from analyses because less than 8 pups were born.

3.2. Maternal Behavior

The level of care individual rat mothers provide to their litters is naturally variable and yet crucial to the pups' development. In this study we observed the behavior of dams both during light and dark cycles on postnatal day 3-6 and 10-13 (Figure 2). No significant differences were found between groups in any behavior during the light cycle periods or during postnatal week 2.

During the dark cycle observations on PND 3-6, designated "Postnatal Week 1", HF dams spent more total time nursing ($F(1, 44)=6.3120$, $38.5 \pm 3.4\%$ vs $25.8 \pm 3.9\%$; $P < 0.05$) and more time in the preferable arched-back nursing posture ($F(1, 44)=9.4948$, $26.4 \pm 2.3\%$ vs $15.3 \pm 2.7\%$; $P < 0.05$) than CHOW dams. Moreover, HF dams spent less time resting ($F(1, 44)=5.1501$, 24.3 ± 3.3 vs 35.7 ± 3.9 , $P < 0.05$) which indicates more time spent caring for their litters. Multiple recordings were made for each litter and the behavioral observation results were averaged. Data are expressed as a percent of total observations $\pm$ SEM.

3.3. Independent Ingestion

For the first two postnatal weeks, the rat pup's diet consists almost exclusively of its mother's milk [33]. This time-period coincides with the development of hypothalamic circuits which will later play a large role in the offspring's control of food intake and regulation of body weight [34, 35]. We used an independent ingestion test to measure pups' milk intake independent of the dam. We measured milk intake over the course of a 30-minute testing session in individuals from litters on PND 3, 7, and 10.

Following a 5-hour maternal separation, pups were individually given access to warm, sweetened milk and intake was measured by change in body weight (Figure 4). Post hoc analysis revealed that both the CHOW-STRESS and HF-STRESS pups consumed more milk per gram of body weight at PND 3 than CHOW-CON pups (STRESS vs CON: $F(1, 29)=3.8325$, 3.35 ± 0.31 vs 2.55 ± 0.26 , $P < 0.05$). At PND 7, there was an overall effect of maternal HF diet with these pups consuming more than the pups from CHOW litters (HF vs CHOW: $F(1, 28)=5.4616$, 2.48 ± 0.24 vs 1.66 ± 0.26 , $P < 0.05$). By PND 10, there were no longer any significant differences among the groups.

3.4. Maternal Milk Analysis

We analyzed fat, sugar, and water content of PND 21 milk from dams maintained on chow or HF diet from gestation day 2 onward. In addition, fat content was measured on PND 3, 7, 10, and 16 (Table 2). No differences were found between milk from STRESS and CON dams so samples were grouped by maternal diet condition only.

We found milk from HF dams to have a significantly higher fat content (%w/v) than that of CHOW dams from PND 10 to PND 21 ($F(1, 44)=8.3725$, 14.18 ± 0.82 vs 11.30 ± 0.56 , $n = 23$, $P < 0.05$). Furthermore, HF dam milk at PND 21 had higher protein content (10.82 ± 0.38 vs 9.43 ± 0.35 , $n = 21$, $P < 0.05$), lower water content than milk from CHOW dams (66.84 ± 1.22 vs 72.44 ± 0.75 , $n = 21$, $P < 0.05$), and greater gross energy content (2.13 ± 0.11 kcal/g vs 1.83 ± 0.07 kcal/g, $n = 21$, $P < 0.05$). There was no difference between groups in sugar content (CHOW: 2.34 ± 0.20 vs HF: 2.47 ± 0.22 , $n = 23$).

In addition, we measured hormone levels in milk samples at PND 21 (Figure 3). No differences were found in milk leptin content. There was an overall significant increase in milk insulin content in HF dams and post hoc analysis showed HF-STRESS dams to have greater insulin concentration compared to CHOW-CON ($F(1,36)=4.6135$, $P < 0.05$).

4. Discussion

Growing evidence suggests that a predisposition towards obesity and adult chronic diseases can result from a suboptimal early life environment. Maternal exposure to stress or poor diet during fetal development or in the early postnatal period can have long-term health consequences for the offspring. Animal models have been used extensively to better our understanding of the potential developmental origins of adult chronic disease. We previously found that gestational stress or high-fat diet can increase body weight and adiposity as well as impair glucose tolerance in neonatal rats [12]. In addition, rats exposed to either prenatal stress or maternal high-fat diet gain more weight when weaned to high-fat diet than controls perhaps indicating a deficit in their ability to compensate for the higher energy density of the high-fat chow. These early disturbances appear to be critical in shaping the offspring's metabolism yet the mechanisms involved are unclear.

In these studies we examined the neonatal environment in an attempt to gain a better understanding of possible contributing factors to the observed phenotype. We found that dams maintained on HF diet throughout gestation and lactation spent more time nursing their pups than CHOW fed dams during the first week following parturition. In fact, even in the absence of the dam, we found that within the first postnatal week pups exposed to prenatal stress or maternal high-fat diet will consume more milk in tests of independent ingestion than controls. Analysis of the nutritional composition of milk from HF diet and CHOW-fed dams revealed an increase in total fat content from PND 10-21, and higher gross energy content and protein at PND 21 in milk samples from dams on the HF diet. Together, these differences in maternal behavior, pup ingestive behavior, and early nutritional environment could contribute to lifelong metabolic changes.

The mechanisms underlying metabolic programming are unclear, but it is likely that changes in eating behavior early in life are important for later development. Even accounting for body weight differences, pups exposed to prenatal stress or maternal HF diet increased the amount of milk they will consume in the presence of an unlimited supply. This could have a bearing on how satiety mechanisms are established later in life. Unlike adults, neonatal rats do not respond to the nutritional composition of milk until approximately PND 9 and gastric fill is likely the chief inhibitor of ingestion until this age [36-38]. We have previously reported that offspring of STRESS or HF dams have impaired glucose tolerance and gain more weight when challenged with a HF diet post-weaning. These results suggest that gestational stress or maternal HF diet or a combination of the two may be changing the offspring's ingestive behavior as well.

Diet and maternal adiposity have been shown to affect the nutritional composition of the dam's milk [13, 39-41]. Certainly large differences between groups in the nutritional composition of the milk provided by the dam could contribute to offspring body weight differences. While there were no differences in milk fat content at PND 3 or 7, from PND 10-PND 21 the milk from HF dams had a significantly higher fat content than that of CHOW dams. It is unlikely, however, that this difference in milk fat content is solely responsible for the pups' increased body weight. Our previous data has shown the pups' body weights from HF litters to diverge as early as PND 7 [12]. Del Prado's 1997 study similarly found offspring body weight increases to precede increases in maternal milk fat content. This group also showed that the dams maintained on a high-energy diet produced more milk at PND 14 [40] which could be the case with our animals as well. Based on the results of our independent ingestion tests it is possible that given a greater supply of milk the pups from HF or STRESS litters might consume more. Additionally, Schroeder and colleagues have shown that in a genetic model of early-onset obesity (the OLETF rat that lacks a functional CCK1 receptor), differences in suckling efficiency can also be a

contributing factor [42]. Furthermore, a similar study by Doerflinger and Swithers showed that pups from dams exposed to a high-fat diet throughout gestation and lactation initiated independent ingestion of solid food 1 day earlier than those litters given standard chow [43]. Epidemiological data indicate that infants who start consuming solid foods earlier are at greater risk to develop obesity and other associated metabolic disorders although the underlying mechanisms remain unclear [44, 45]. Together these results suggest that there are many factors related to milk composition and ingestion, all of which might contribute to body weight differences in neonatal rats.

Finally, in analysis of milk hormone content at PND 21 we found increased insulin concentration in HF-STRESS dam's milk although no differences were found in leptin concentration. Leptin has been shown to be an important trophic factor mediating the development of hypothalamic circuitry [46]. Another group studying obese and lean rat mothers with a genetic predisposition to obesity (DIO) also found no difference in leptin concentrations between the milk of obese or lean rats [13]. This group did find insulin levels to be elevated in the milk of both lean and obese DIO rats at PND 7. Here, we report a similar finding in dams maintained on HF diet at PND 21. Previous studies have shown exposure to higher than normal insulin levels can affect the development of hypothalamic circuitry [47] and that insulin and other circulating factors have been shown to pass through milk and can affect the neonate [48]. Further studies examining insulin and leptin levels in maternal milk at earlier time points could shed light on this and other potential contributing factors to the pup body weight and food intake differences we have observed.

Importantly, this study has some limitations which should be addressed. First, while ours and other previous studies have shown long-term effects of PS, few developmental differences are reported here. The early-life HF diet exposure results in a much more robust phenotype which is expressed almost immediately. Our previous studies suggest that exposure to PS imparts a susceptibility to later challenges; weaning PS animals to a HF diet results in more weight-gain than control animals that did not have the prenatal stress experience [12]. Furthermore, future studies may be helpful in understanding maternal-pup interactions and in further analyzing milk composition at earlier time-points. While videotaping ensured we did not disturb the litters, it does not allow for the observation of more detailed behaviors such as the initiation of nursing or potential sex differences. Growing evidence for gender-specific effects of prenatal stress [49, 50] and early-life diet [51-53] necessitate further investigation.

5. Conclusions

Early environmental factors are critical in myriad aspects of an individual's development and a growing body of evidence suggests food intake and metabolism could be influenced in this manner. Prenatal stress or maternal high-fat diet during gestation and lactation can produce similar phenotypes in adult offspring. These phenotypes appear to be the result of early changes in the dam and her pups and future studies are needed to further elucidate these mechanisms. These data provide a better understanding for how these manipulations can indirectly change the offspring's early environment and potentially lead to adult chronic disease.

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Figure 1.

Body weight. Pups from HF or STRESS litters were significantly heavier than CHOW-CON pups on PND 3 and an overall effect of prenatal HF diet on PND 7 and 16 (* $P < 0.05$ vs CHOW).

Figure 2a & b.

Maternal behavior during postnatal week 1. a) We found no differences in maternal behavior between groups during the light cycle. b) During the dark cycle HF dams spent significantly more time in arched-back nursing (ABN) and spent more time nursing overall than CHOW dams. No significant differences were found in PNW 2 (data not shown). * $P < 0.05$ vs CHOW group. (ABN, arched-back nursing; L/G, licking and grooming pups; Passive Nursing, nursing in any posture except ABN; Total Nursing, sum of ABN and Passive Nursing.)

Figure 3. Milk Hormones

No differences were found in milk leptin concentrations between groups. Milk from HF-STRESS dams contained higher concentration of insulin than that of CHOW-CON at PND 21 (* $P < 0.05$ vs CHOW-CON).

Figure 4. Independent Ingestion

At PND 3, offspring of STRESS dams consumed significantly more of the sweetened milk per gram body weight whereas at PND 7, offspring of HF dams ingested more than offspring of CHOW dams. By PND 10, no effect is apparent. (* $P < 0.05$ vs CHOW-CON; # $P < 0.05$ vs CHOW).

Table 1

Variable stress paradigm (adapted from Tamashiro et al. 2009)

Gestation Day	Morning	Noon	Afternoon	Overnight
14	1-hr restraint	15-min swim	1-hr restraint	
15	6-hr @ 4°C			
16	15-min swim	1-hr restraint	15-min swim	
17		15-min swim		lights on overnight
18				social stress overnight
19	1-hr restraint	15-min swim	1-hr restraint	
20	6-hr @ 4°C			
21	15-min swim	1-hr restraint	15-min swim	

Table 2

Milk composition. CHOW and HF groups contain samples from STRESS and CON animals since no difference in fat content was found between these groups. On average, milk from HF dams had significantly more fat content than milk from CHOW dams from PND 10-21. Milk from HF dams had significantly less water content on PND 21 (* $P < 0.05$ vs CHOW).

Total % Fat	Chow	HF
PND 3	18.06 ± 0.95	17.53 ± 0.94
PND 7	9.20 ± 0.52	9.42 ± 0.88
PND 10	9.01 ± 1.96	12.74 ± 0.17
PND 16	10.73 ± 0.66	13.65 ± 1.36
PND 21	12.54 ± 0.67	14.89 ± 1.29
P10-21 mean	11.30 ± 0.56	14.18 ± 0.82*

PND 21	Chow	HF
% Water	72.44 ± 0.75	66.84 ± 1.22*
% Sugar	2.34 ± 0.20	2.47 ± 0.22
% Protein	9.43 ± 0.35	10.82 ± 0.38*
Gross Energy (kcal/g)	1.78 ± 0.07	2.13 ± 0.11*