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Maternal diet modulates the risk for neural tube defects in a mouse model of diabetic pregnancy

Claudia Kappen¹, Claudia Kruger¹, Jacalyn MacGowan^{1,2}, and J. Michael Salbaum²

¹Department of Developmental Biology, Pennington Biomedical Research Center, Louisiana State University System, 6400 Perkins Road, Baton Rouge, LA 70808

²Laboratory of Regulation of Gene Expression, Pennington Biomedical Research Center, Louisiana State University System, 6400 Perkins Road, Baton Rouge, LA 70808

Abstract

Pregnancies complicated by maternal diabetes have long been known to carry a higher risk for congenital malformations, such as neural tube defects. Using the FVB inbred mouse strain and the Streptozotocin-induced diabetes model, we tested whether the incidence of neural tube defects in diabetic pregnancies can be modulated by maternal diet. In a comparison of two commercial mouse diets, which are considered nutritionally replete, we found that maternal consumption of the unfavorable diet was associated with a more than three-fold higher rate of neural tube defects. Our results demonstrate that maternal diet can act as a modifier of the risk for abnormal development in high-risk pregnancies, and provide support for the possibility that neural tube defects in human diabetic pregnancies might be preventable by optimized maternal nutrition.

Keywords

rodent diet; modifier; fat content; protein content; folic acid; NTD; nutrition; diabetes

INTRODUCTION

Pregnancy places high demands on physiology and metabolism of the expecting mother, particularly since the maternal metabolism is the only source of nutrients to sustain embryonic growth and development. Therefore, optimal maternal nutrition is of paramount importance for a successful pregnancy outcome.

Pregnancies complicated by maternal diabetes have an elevated risk for birth defects, collectively referred to as diabetic embryopathy [1–7]. This is a spectrum of birth defects that include heart defects, neural tube defects, and caudal growth defects; all these serious morphogenetic defects show increased frequency in diabetic pregnancies [5–7]. The mechanisms by which these defects arise in the presence of high maternal blood glucose are

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Corresponding author: Claudia Kappen, Dr. rer. nat., Pennington Biomedical Research Center, 6400 Perkins Road, Baton Rouge, LA 70808, Tel: 225-763-2781, Claudia.Kappen@pbrc.edu.

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Conflict of Interest Statement:

The authors declare that there are no conflicts of interest.

being unraveled, and are thought to involve crucial changes of gene expression in the embryo [8–10] as well as the yolk sac [11], with alterations in the Wnt signaling pathway [12], protein Kinase C signaling [13,14], Pax3-dependent [15] and p53-dependent processes [16], and the hypoxia and oxidative stress response systems [17–20].

Supplementation of maternal diet with vitamins C and E [20–23], lipoic acid [24], arachidonic acid [25,26], or myo-inositol [27] has been shown effective in reducing the incidence of developmental defects in rodent diabetic pregnancies [20–28]. Supplementation of maternal diet with the B vitamin folic acid also decreases neural tube defects in rodent embryos exposed to maternal diabetes [29–31], but the underlying mechanisms for the beneficial effects are unclear. In human pregnancies, periconceptual folate supplementation can significantly reduce the risk for birth defects [32,33], although abnormalities in folate levels could not be detected in pregnant women with diabetes [34]. Thus, the higher rate of defects in diabetic pregnancies despite folate supplementation and food fortification warrants consideration of additional factors that modulate the occurrence of developmental defects.

It is interesting to note that the embryos of some mouse strains are more resistant to neural tube defects than in other strains [35–42]. This has been interpreted to imply that the pathogenic mechanisms can be modulated by a so-called modifier [43], [44]. Such modifiers may exist on the side of the embryo, for example, such as represented by the genetic background [45,46]. Consistent with this proposition, genetic loci have been identified that confer increased susceptibility in the neural tube defect prone mouse strain, *SELH/Bc* [41,47]. However, functional modifiers may also be present on the maternal side. Conceivably, this could be conditions that affect the reproductive system or maternal metabolism directly, or factors that act downstream to modulate the consequences of teratogenic insults. Examples are maternal undernutrition, which prevents neural tube defects in the curly tail mutant, a classical model of spina bifida [48] or hyperthermia, which elevates neural tube defect risk [40,49–51]. For the *SELH/Bc* strain of mice, it was recently reported that the frequency of exencephaly is modulated by factors in the maternal diet besides the known beneficial micronutrients [52]. Whether any of these modifiers act on neural tube defects in diabetic pregnancies is presently unknown.

Using the well-established STZ diabetic mouse model, we investigated whether composition of maternal diet modifies birth defect incidence associated with maternal diabetes. We here report that a mouse diet that is commonly and successfully used to support breeding colonies, is thought to be nutritionally replete, and has no ill effects on development under normal conditions, has seriously detrimental consequences under conditions of maternal diabetes. Thus, diet appears to play the role of a modifier. Our findings imply a cooperative effect of diet and maternal diabetes on development of the embryo, and also suggest that besides proper control of blood glucose, careful attention to diet may be beneficial to improve outcomes of diabetic pregnancies.

MATERIALS AND METHODS

Animals and Diets

All experiments used FVB inbred mice purchased from Charles River Laboratories Inc. (Wilmington, MA). Mice were housed in a 12-hour light-dark cycle, with access to food and water *ad libitum*. The animals were fed Purina 5001 (referred to as "chow diet") until they were placed into the experiment, when one group was fed Purina 5015 (referred to as "breeder diet"). Diet was kept constant for the duration of the experiment. All females were fed the respective diet for at least four weeks before conception. According to the manufacturer (Lab Diet, Purina Mills Inc., Gray Summit, MO), 'chow' diet provides 28.5%

of calories from protein, 13.5% of calories from fat, and 58% of calories from carbohydrates, and 'breeder diet' provides 19.8% of calories from protein, 25.3% of calories from fat, and 54.8% of calories from carbohydrates. Detailed information on composition of the diets is available from the manufacturer (http://www.labdiet.com/rodent_diet.html).

Streptozotocin treatment

Diabetes was induced in 7–9 week old female FVB mice by two intraperitoneal injections of 100 mg/kg body weight Streptozotocin in 50 mM sodium citrate buffer at pH 4.5 (STZ; Sigma, St. Louis, MO) within a one-week interval [9,12]. Mice that exhibited blood glucose levels exceeding 250 mg/dL were used for mating to normal FVB males no earlier than 7 days after the last treatment.

Glucose and weight measurements

Glucose levels in tail vein blood were measured in the morning by a glucometer (Bayer Contour). Weight was recorded in the morning; no corrections were made for weight of embryos or litter size in a given dam, dam weight was recorded prior to dissection.

Matings and Isolation of Embryos

Matings between FVB control or diabetic females and FVB inbred males were set up with 2 females and one male per cage in the afternoon. Males were removed from the cage the next morning; when a copulation plug was observed, this was considered gestational day 0.5. Dams were sacrificed at the designated time points by CO₂ asphyxiation. Concepti were isolated from the uterus by microdissection under a Leica MZ6 stereomicroscope, and after removal of extraembryonic membranes, embryos were assessed for developmental stage and evaluated for the presence of morphogenetic defects. Neural tube defects were scored on the day of isolation for all embryos isolated at gestational day 10.5 (E10.5) or later. Photography was performed on a Z16 Leica Macroscope with sagittal view of each embryo in the presence of a metric ruler.

Size determination of embryos

Width and length of embryos was determined from sagittal view photographs using the Leica Application Software, with scales adjusted to the ruler marks on the photographs. Length was measured as distance from crown to rump, width was measured as the longest dorso-ventral distance as perpendicular to length as possible. For embryos isolated at E8.5, we counted the number of somites as a proxy for size.

Statistical evaluation

Results were evaluated for statistical significance using Fisher's exact test for categorical data (incidence of neural tube defects) and one-way ANOVA for scaled data (embryo length, dam weights and glucose levels). P-values smaller than 0.05 were considered statistically significant.

RESULTS

In this study, we investigated the effects of different animal diets on embryonic development in a well-established mouse model of diabetic pregnancy. The baseline for this comparison was Laboratory Rodent Diet #5001 (see Materials and Methods). This is a rodent maintenance diet typically referred to as 'chow', and provides 28.5% of calories from protein, 13.5% of calories from fat, and 58% of calories from carbohydrates. The comparison diet was Laboratory Rodent Diet #5015. This diet is recommended for breeding colonies (hence 'breeder diet'), and provides 19.8% of calories from protein, 25.3% of

calories from fat, and 54.8% of calories from carbohydrates. Both diets have similar carbohydrate content, yet differ in their protein and fat content, with chow being comparatively higher in protein and lower in fat. With mice having ad libitum access, both diets are considered to be nutritionally replete. Female mice were placed on the respective diet at least 4 weeks before diabetes induction. Diabetes was induced using the Streptozotocin model [9]; diabetic as well as control female mice were mated, and neural tube defects in embryos from both control and diabetic pregnancies were recorded and compared for both dietary modalities. The results of this comparison are summarized in Table 1.

We find that normal pregnancies were not affected by either diet with respect to neural tube defect frequency, which was found to be 0.8% for chow, and 0.4% for the breeder diet; this difference was not statistically significant. For diabetic pregnancies we observed, as expected in comparison to normal pregnancies, a statistically significant increase of neural tube defects: embryos from diabetic dams on chow diet exhibited a neural tube defect incidence of 5.6%. In contrast, we observed a high incidence of neural tube defects in diabetic pregnancies on breeder diet: embryos from such dams displayed neural tube defects at a frequency of 21.6%. This difference in the frequency of neural tube defects on the two diets is statistically significant, and strongly suggests that, in a diabetic pregnancy, maternal diet may act as a modifier of the risk for birth defects, such as neural tube defects.

We conducted morphometric measurements on embryos from normal as well as diabetic pregnancies maintained on the respective diets. Comparison of crown-rump length and width of embryos at gestational day 10.5 revealed a high correlation both in embryos from normal (Figure 1A) as well as from diabetic pregnancies (Figure 1B). These results indicate that crown-rump length is a suitable proxy for overall embryo size regardless of metabolic status. When dams were maintained on the chow diet, embryos from the diabetic dams exhibited statistically significant differences in size to normal embryos only at late stages of gestation (E15.5 and E18.5), whereas no significant differences were observed at earlier stages (Figure 1C). In contrast, when dams were on breeder diet, embryos from diabetic pregnancies were smaller at all stages of gestation, and this was statistically significant at all stages except for E8.5 (Figure 1D). At E8.5, there appears to be a trend towards lower somite counts, but this was not statistically significant, likely owing to the combination of variation and sample number. In normal pregnancies (Figure 1E), significant differences in embryo size were found for embryos from dams fed breeder diet only at E9.5 and E18.5, and these differences were less than 10% of the average embryo length. In diabetic pregnancies, significant differences were apparent at all time points except E10.5, with a consistent trends towards smaller size when the dam was on breeder diet. Thus, maternal diet had minor effects on embryo growth in normal pregnancies, but breeder diet was associated with reduced embryo size in diabetic pregnancies.

A comparison of litter sizes between normal and diabetic pregnancies on the respective diets revealed only minor effects (Table 2). Litter sizes were different at E8.5 between controls on chow and controls on the breeder diet, as well as between controls and diabetics on breeder diet, but it should be noted that the number of informative pregnancies was small. Further differences were observed at E18.5, with control litters being larger than diabetic litters on chow diet. Diabetic litters had a ~2-fold higher rate of resorptions than controls on chow (7% versus 3.7%, $P=0.031$), with a tendency to greater loss at later time points. When dams were on breeder diet, the controls had a rate of 2.1% resorptions, and the diabetic dams 14.2% ($P=1.6\times 10^{-8}$). This is significantly higher than in diabetic pregnancies on chow diet, and again, affected the E15.5 and E18.5 time points most. Although some embryos are lost in late in diabetic pregnancy, and this is more pronounced in dams fed the breeder diet, our data indicate that fertility of the females is not adversely affected by diabetes, diet, or both,

at early postimplantation time points. Similarly, resorptions are less frequent at earlier stages and therefore cannot account for the increased rate of neural tube defects in diabetic pregnancies, or the diet effect. Our data imply that differential litter size or rate of resorptions are unlikely to be critical parameters for neural tube defect incidence in diabetic pregnancies; however, they do not exclude adverse effects of diabetes and diet at later stages of pregnancy. Taken together, our data show that maternal diet modulates size and growth of the embryo in diabetic pregnancies, and the incidence of neural tube defects.

We further evaluated whether maternal parameters were affected by the different diets. On chow diet, weight did not differ between controls and diabetic females for any early and mid-gestation stages of the pregnancy; statistically significant differences were only observed at late stages (at E15.5 and E18.5, Figure 2A), where diabetic dams exhibited lower weights, and slower weight gain, compared to normal dams. A similar pattern was found in dams on the breeder diet (Figure 2B), where diabetic dams had decreased weights at late stages of the pregnancy. In addition, there were fluctuations of female weight at earlier stages (E8.5 and E9.5), but no consistent changes in one particular direction were observed. Notably, the weights at the beginning of the pregnancy were not different between normal dams fed either chow or breeder diet (Figure 2E). At E8.5 and E9.5, statistically significant differences were found, but sample numbers are low; by E18.5, dams on breeder diet weighed less than dams on chow. Taken together, these data indicate that breeder diet does not induce obesity at any of the time points investigated. For diabetic dams (Figure 2F), there were no statistically significant differences in weight at any stage of the experiments. We therefore conclude that the different maternal diets did not modulate the way in which diabetes affects weight or weight gain in pregnant dams.

However, it appears that the breeder diet had an aggravating effect on blood glucose levels in diabetic females (Figure 2C and D). At mating, diabetic dams on chow diet had an average glucose level of 306.23 ± 81.81 mg/dL ($n=56$). Diabetic dams on breeder diet had glucose levels of 349.65 ± 97.79 mg/dL ($n=48$). The difference between the two conditions was significant ($P=0.015$), and may explain the lower weight of diabetic dams at mating. Glucose levels at various stages of pregnancy (measured at the time of sacrifice for embryo analysis) were found to be consistently higher in diabetic dams on breeder diet when compared to diabetic dams on the chow diet. In contrast, blood glucose levels of normal non-diabetic dams on either diet were highly similar, indicating that diet alone did not produce hyperglycemia in pregnant females. Taken together, our results strongly indicate that maternal diet during pregnancy constitutes a modifying condition that interacts with maternal diabetes by affecting blood glucose levels, and that maternal diet modulates the frequency of neural tube defects in diabetic pregnancies.

DISCUSSION

Maternal diabetes is a well-recognized teratogen that elevates the risk for birth defects in humans, particularly of the heart and the neural tube. Neural tube defects also occur in rodent diabetes models, including the well-established STZ model. Intriguingly, we found an approximately 3-fold higher incidence of neural tube defects in diabetic pregnancies when the dams were maintained on Purina 5015 (breeder diet) compared to Purina 5001 (chow diet). These pregnancies occurred in the same facility and over the same period of time, excluding seasonality as a potential source of the variation. Furthermore, our results do not provide support for the possibility that litter size is correlated with neural tube defect risk in diabetic pregnancies. We also did not detect any unusual clustering of neural tube defects, as the incidence of neural tube defects per litter in diabetic dams on either diet followed the Poisson distribution (Figure 3, Panels A and B). Thus, the higher rate of defects with breeder diet is very likely due to the influence of this particular diet as compared to chow.

Embryo growth, however, was affected by both maternal diabetes and maternal diet. In diabetic dams that were maintained on chow diet, embryos were smaller on E15.5 and E18.5, and newborns were also smaller than progeny from control pregnancies (unpublished observations). Yet, there were no significant differences in embryo size at earlier stages of gestation. Somite counts at E8.5, prior to neural tube closure, were also very similar in embryos derived from control and diabetic pregnancies on chow diet, excluding differential growth as the cause for neural tube defects in these diabetic pregnancies.

In contrast, when diabetic dams were on breeder diet, embryos were smaller than controls at all developmental stages. In normal pregnancies, embryos from breeder diet-fed dams were slightly larger at E9.5. This is consistent with the report for SELH/Bc embryos, which had higher somite counts at E9.4 when the dam was fed breeder diet [53]. The authors propose that this accelerated development could be responsible for the higher incidence of neural tube defects with breeder diet in this strain. However, this is unlikely to be the case with maternal diabetes, since we observe a reduction of embryo size in diabetic dams on breeder diet at E9.5. This is foreshadowed by lower somite numbers at E8.5, indicative of developmental delay rather than accelerated growth. Furthermore, the reduction by maternal diabetes brings the size average back to that of embryos from chow-fed normal dams, which do not incur a high rate of neural tube defects. Thus, in diabetic pregnancies, the effect of breeder diet on embryo growth is different from the situation in SELH/Bc, despite similarly high neural tube defect rates. It is therefore likely that the mechanisms underlying the neural tube closure defects are different between the genetic and the diabetes model.

Parallel to reduced embryonic growth towards the end of diabetic pregnancies, we also find that, irrespective of diet, weight gain during pregnancy is reduced in diabetic dams. Based upon our results, we consider it unlikely that differential maternal weight gain could be responsible for the dramatically higher neural tube defect incidence with breeder diet in diabetic dams. In early stages of normal pregnancies, weight gain appears to be delayed in the dams on breeder diet, but given the small sample number, this aspect needs follow-up. From our finding that weight gain overall was independent of diet condition, we conclude that the combined effects of maternal diet and diabetes on neural tube closure are likely independent of maternal weight, and instead mediated by specific metabolic or molecular mechanisms.

Interestingly, there appears to be a trend towards higher glucose levels in the diabetic dams when they are maintained on breeder diet as compared to chow, possibly from the time of mating. Statistical evaluation of our results is hampered by the fact that glucose measurements often exceeded upper limits of the scale on the glucometer, indicating severe hyperglycemia. The number of dams exceeding the limit was higher at all time points when dams were fed breeder diet than when on chow. Glucose levels resembling those in diabetic dams on breeder diet at E8.5 and E9.5 were reached in diabetic dams on chow diet only by day E10.5. Thus, if diabetic dams on chow developed exceedingly high glucose levels, they did so later in the pregnancy than dams on breeder diet. However, the propensity to exceed limits was not predictable from the glucose level prior to pregnancy, since there was no correlation. When we conducted a retrospective analysis of individual diabetic pregnancies with and without neural tube defects (Figure 3, Panels C and D), we found that increases in blood glucose levels between start and end of pregnancy were similar. While Breeder diet was associated with slightly higher prepregnancy glucose levels in diabetic dams that had NTD-affected embryos, any differences to diabetic pregnancies with normal embryos were not statistically significant. Overall, it is important to note that glucose levels in all diabetic dams exceeded 250 mg/dL from the start of pregnancy, and that the diabetes was uncontrolled, and as reflected by the glucose levels at sacrifice, severe.

We currently do not know whether the breeder diet influences the action of STZ in induction of diabetes, or whether it aggravates the hyperglycemia only after the dam becomes diabetic. We favor the latter interpretation, since glucose levels were not elevated in control dams fed the breeder diet. This is in contrast to what was reported for the comparison of these same two diets in the neural tube defect prone SELH/Bc mouse strain [53]. The discrepancy between the SELH/Bc strain and the FVB inbred mice we used may suggest that one or more of the genetic modifiers for neural tube defects in SELH/Bc could be a factor regulating maternal glucose levels in response to diet, and that this is unaffected by diet in FVB. More detailed measurements of metabolic parameters, including metabolites such as free fatty acids, or ketones, in controls as well as in diabetic females would be required to determine to which extent the two diets affect maternal metabolism.

The major differences between diets are in macronutrient composition, particularly fat and protein content. This is accompanied by a higher energy density of the breeder diet (4.68 kcal/gm) compared to chow (4.07 kcal/gm). In addition, chow diet has a higher content of folic acid (7.1 ppm) than breeder diet (2.9 ppm), but both diets are considered replete for rodent dietary requirements [53]. Overall, the diets therefore differ in composition in micro- and macronutrients, making it difficult to identify specific reasons for the dramatically different incidence of neural tube defects in diabetic pregnancies. Custom formulated diets made from defined ingredients [54] will be needed to modify each potential parameter, such as protein content and composition, fat content and composition, and concentrations of vitamins and other micronutrients.

The content of isoflavones in commercial animal diets has been considered a source of variation for reproductive parameters due to the potential action as phytoestrogens. Wang et al. [55] showed that Purina 5001, having a higher phytoestrogen content than other mouse diets, produced higher rates of implantation in CD-1 mice. While Purina 5015 was not investigated in that particular study, the implication for our studies would be that implantation success should be lower with this diet. Yet, we observe a trend towards lower litter size in dams on breeder diet only at E8.5, and in diabetic dams on this diet, we even found larger litter sizes (however, a serious caveat is the small sample size). At all other time points during neural tube closure, we did not observe diet effects on litter size. Significant differences in the rates of resorptions were only apparent at late pregnancy stages, and thus our data do not support the notion of differential implantation success. Similarly, high fat content in mouse diet has been linked to skewed sex ratio, presumably due to selectively higher survival rates for males [56]. If female embryos were disadvantaged in a dam on breeder diet, this could present as an apparently higher rate of neural tube defects, if accompanied by selective loss of normally developing embryos of one sex. Yet, skewed sex ratios were only observed at much higher ratios of fat content [56] than present in either of our two diets. In fact, that study used Purina 5015 as the baseline diet, with a sex ratio of 53.3% males to 46.7% females (in first parity) from dams of the NIH Swiss mouse strain. It is thus unlikely that breeder diet would have altered the sex ratio or embryonic survival in our experiments; again, the low rates of resorptions at early pregnancy stages argue against embryonic loss as a cause for the considerably higher neural tube defect incidence.

There are also variations in content of amino acids, free fatty acids, minerals and vitamins between the two diets, with the most notable being a greater than 10-fold difference in carotene with 2.3 ppm in Purina 5001, and 0.2 ppm in Purina 5015. However, the specific carotene forms are not indicated, and thus, it is unclear which effect this might have on vitamin A metabolism. While excess vitamin A is known to be associated with neural tube defects [57–59], deficiency in vitamin A is linked to patterning defects, most prominently in the hindbrain, rather than neural tube closure defects [60–62]. Vitamin A itself is present at

comparable levels in the diets (15 IU/g in Purina 5001, 19 IU/g in Purina 5015); thus, it is unclear whether availability to the embryo would be affected by the different carotene levels. Reduced mobilization of vitamin A from the liver has been documented in diabetic patients [63], and diabetic rats have low vitamin A status [64]. It is thus conceivable that, in combination with maternal diabetes, differential vitamin A availability could modulate the risk for aberrant development in diabetic pregnancies. Intriguingly, the diets also differ in cholesterol content, with 200 ppm in chow and 28 ppm in breeder diet. This could be associated with reduced transport of cholesterol to the developing embryo, and reduced availability of cholesterol has been linked to embryonic defects, most prominently in Smith-Lemli-Opitz Syndrome [OMIM #270400]. Yet, neural tube closure defects are not typical for this phenotype, and in normal pregnancies, the difference between the diets in cholesterol content does not appear to elevate defect risk. On the other hand, analyses of several mutants for cholesterol synthesis genes [65–67] have shown that embryos which are unable to synthesize cholesterol exhibit neural tube defects, demonstrating that at least some endogenous cholesterol is required for successful neural tube closure. Reduced cholesterol levels are associated with reduced folate retention in some mouse strains [68], but whether this also applies to the FVB strain, or during pregnancy, has not been established. Nevertheless, it is possible that reduced cholesterol availability could be a risk factor for abnormal development under condition of exposure to maternal diabetes.

Few studies report on a comparison of the two diets we used here relative to birth defect incidence. The most relevant is the finding that maternal diet modulates neural tube defect incidence in the SELH/Bc strain [52,53], a mouse strain prone to neural tube defects [41]. In this strain, at least three major genetic loci have been identified that contribute to the liability for exencephaly [69], which was observed in up to 10% of the progeny. This rate was constant over at least 10 years, with dams fed chow diet (Purina 5001), presumably from various production lots. In contrast, when females of this strain were fed Purina 5015 (breeder diet), the incidence of neural tube defects was considerably higher, with ~25% of embryos exhibiting exencephaly. In detailed measurements of embryonic growth in this model, the authors came to the conclusion that in comparison to chow, breeder diet appears to accelerate development overall but delay midbrain closure [53], thus causing temporal asynchrony in the development of major tissues of the embryo, which in turn could be predisposing to neural tube defects. Differences to our model are the genetic liability and a different strain background, yet, the two diets affect neural tube defect incidence in a similar manner as we found for diabetic pregnancies, i.e. breeder diet increases risk for developmental defects over chow diet. Another difference are the higher glucose levels in SELH/Bc dams on breeder diet [53], a finding that was not confirmed for non-diabetic dams in our model; even though we kept our dams at least 4 weeks on either diet prior to pregnancy, we detected elevated glucose levels only in dams that were diabetic. It has to be noted, however, that in the SELH/Bc strain, the dam herself also carries the genetic susceptibility loci, thus metabolism of the SELH/Bc dam may be different from normal pregnant FVB females.

In analogy to the genetic sensitization by susceptibility loci in the SELH/Bc strain, we may consider the hyperglycemia-exposed embryo as sensitized by maternal diabetes and diet. In the diabetic dams on breeder diet, this affects embryo growth, and further studies will be required to investigate whether this abnormal growth is characterized by temporal asynchronies. Intriguingly, our study revealed a dramatic effect of diet on the outcome of diabetic pregnancies, with different diets varying as much as three-fold in risk of neural tube defects. Our data thus establish maternal diet as an important risk factor for neural tube defects in high-risk pregnancies. Identification of specific adverse or beneficial components in the diets that modulate neural tube defect risk will have implications for the etiology of

neural tube defects in human diabetic pregnancies, and provide insight into how maternal diet possibly can be optimized to provide better prevention against neural tube defects.

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Figure 1. Effect of maternal diet on embryo growth in diabetic pregnancies

All embryos were photographed in the presence of a metric ruler. Measurements of length and width were obtained with the Leica Application Suite using the ruler for scale. A: Correlation of width to crown-rump length of embryos in normal pregnancies at E10.5. B: Correlation of width to length of embryos in diabetic pregnancies at E10.5. The strong correlation of width to length in embryos of control as well as diabetic pregnancies indicates that embryo crown-rump length is an appropriate proxy for overall size of embryos. C: Embryo size over time in control and diabetic pregnancies of dams on chow diet. D: Embryo size over time in control and diabetic pregnancies of dams on breeder diet. It is noteworthy that in dams on chow diet (C), embryos are of similar size early in the pregnancy, but diabetic embryos are significantly smaller towards the end of gestation. In dams consuming breeder diet (D), diabetic embryos are significantly smaller at all stages of gestation. E, F: Plots of the same data as depicted in C and D, regressed to facilitate direct comparison between diets. Except for E8.5 and E18.5, no significant differences were found for the size of embryos from dams on chow or breeder diet. In diabetic pregnancies, embryos from dams on breeder diet were smaller at all time points except for E10.5. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

Figure 2. Effects of maternal diet on dam weight and glucose levels in diabetic pregnancies

All dams were weighed at mating (prepregnancy) and at the time of sacrifice; glucose levels were obtained before mating and at the time of sacrifice, except for control dams. Only those dams were used in diabetic pregnancies that had a glucose level exceeding 250 mg/dL prior to mating. A: Dam weight of control and diabetic dams during pregnancy when fed the chow diet. B: Dam weight of control and diabetic dams during pregnancy when fed the breeder diet. Significant differences between control and diabetic dams were found only at last stages of gestation, applicable to both diets. Note the small number of dams at E8.5. C, D: Plots of the same data as depicted in A and B, regraphed to facilitate direct comparison between diets. No significant differences in dam weight were found between respective groups at each stage on the different diets, except for normal dams at E8.5 and E18.5 (see text). E: Glucose levels in dams fed the chow diet. F: Glucose levels in dams fed the breeder diet. Glucose levels are plotted as individual values; measurements that exceeded the scale of the glucometer (>600) are plotted as points above the red dotted line. G, H: Plots of the same data as depicted in E and F, regraphed to facilitate direct comparison between diets. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$.

Figure 3. Effects of maternal diet on neural tube defect incidence in diabetic pregnancies

A: Plot of the distribution of neural tube defects per diabetic pregnancy when dams were fed Chow Diet. Open bars: no defective embryos (n=27); filled bars: pregnancies with neural tube defects (n=17). B: Plot of the distribution of neural tube defects in diabetic pregnancies when dams were fed Breeder diet (n=18 for no defect; n=22 for pregnancies with NTD). No significant clustering of neural tube defects was detected. The distributions follow Poisson distributions with a proportion of linear covariance of $r^2=0.975$ for Chow diet and $r^2=0.839$ for Breeder diet. C: Comparison of maternal glucose levels between diabetic dams with normal pregnancies (dashed lines) and dams with neural tube defect affected pregnancies (solid lines), that were fed Chow diet. Each line connects an individual dam's pre-pregnancy measurement (pre) to the measurement at time of sacrifice. D: Comparison of maternal glucose levels between diabetic dams that were fed Breeder diet and had normal or neural tube defect associated pregnancies. The dotted line at 600 mg/dl indicates the upper limit of detection for the glucometer. The 27 dams that were fed Chow diet and had normal pregnancies exhibited an average prepregnancy glucose level of 315 mg/dl (± 73.2), the average for the 17 dams with NTD-affected pregnancy that were fed Chow diet was 328 mg/dl (± 88.9), the 18 dams that had normal pregnancies with Breeder diet had glucose levels of 340 mg/dl (± 77.5), and prepregnancy glucose levels in 22 diabetic dams that had NTD-affected embryos after Breeder diet and were 379 mg/dl (± 97.3). The averages were not statistically significantly different from each other.

Table 1

Effect of maternal diet on neural tube incidence in diabetic pregnancies.

Diet	Modality			Diabetes-exposed				P-value [CD]
		Control						
	NTD	total	%	NTD	total	%		
Chow	3	362	0.8	17	302	5.6	3.5×10^{-4}	
Breeder	1	249	0.4	45	208	21.6	6.4×10^{-16}	
P-value [CB]		0.65			7.7×10^{-8}			

The number of neural tube defects was recorded relative to all embryos recovered in each modality. The incidence of neural tube defects does not differ significantly among embryos from normal dams on either diet. However, neural tube defects were significantly more frequent in embryos from diabetic dams compared to control dams [CD]; furthermore, there were significantly more neural tube defects in embryos isolated from dams on breeder diet as compared to dams on chow diet [CB]. Statistical evaluation was done by two-tailed Fisher's exact test, and retrospective power analysis with alpha at 0.05 indicated that statistical power exceeded 80% in all comparisons except between diets in normal dams.

Table 2

Effect of maternal diet on litter size in control and diabetic pregnancies.

Diet	Modality Gestation day	n dams	Embryos in litter $\pm$ SD	P-value [CB]	P-value [CD]	Modality Gestation day	n dams	Embryos in litter $\pm$ SD	P-value [CB]	P-value [CD]
Chow	Control					Diabetic				
	8.5	4	10.2 $\pm$ 2.3	0.022		8.5	5	9.0 $\pm$ 1.9		
	9.5	6	8.0 $\pm$ 3.0			9.5	9	8.1 $\pm$ 3.2		
	10.5	15	8.5 $\pm$ 2.4			10.5	14	8.5 $\pm$ 1.7		
	12.5	11	8.1 $\pm$ 3.0			12.5	10	8.0 $\pm$ 2.4		
Breeder	15.5	14	8.1 $\pm$ 3.4			15.5	12	6.8 $\pm$ 3.0		
	18.5	10	10.5 $\pm$ 2.3		0.039	18.5	10	7.5 $\pm$ 3.6		0.039
	Control					Diabetic				
	8.5	4	6.5 $\pm$ 2.4	0.022	0.038	8.5	3	12.7 $\pm$ 3.5		0.038
	9.5	5	7.6 $\pm$ 2.6			9.5	4	6.5 $\pm$ 1.9		
	10.5	11	7.5 $\pm$ 4.1			10.5	6	9.7 $\pm$ 1.5		
	12.5	7	7.7 $\pm$ 2.7			12.5	12	7.3 $\pm$ 3.1		
	15.5	8	7.4 $\pm$ 3.3			15.5	11	7.6 $\pm$ 3.9		
	18.5	10	9.1 $\pm$ 2.2			18.5	12	8.6 $\pm$ 2.5		

Litter size per dam was recorded on each day and excludes resorptions, for which no significant differences were found. Embryo numbers are given as the average at a specific gestational age $\pm$ standard deviation (SD) from the mean. P-values are given where $p < 0.05$ for the comparison of litter sizes from dams on control diet to dams on breeder diet [CB] and the comparison of litter sizes between control dams and diabetic dams [CD]. Statistically significant differences were only found at E 18.5 and E8.5 (however, note the small number of dams at this latter time point).