UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

FACULTAD DE QUÍMICA

OBESIDAD MATERNA DE LA RATA Y PREDISPOSICIÓN DE LA PROGENIE A ALTERACIONES METABÓLICAS: VENTAJAS DE LA INTERVENCIÓN NUTRICIONAL

T E S I S

QUE PARA OBTENER EL TÍTULO DE

QUÍMICO FARMACÉUTICO BIÓLOGO

PRESENTA

CARLOS ALBERTO IBÁÑEZ CHÁVEZ

MÉXICO, D.F. 2011

UNIVERSIDAD NACIONAL AUTÓNOMA
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FACULTAD DE ESTUDIOS SUPERIORES
ZARAGOZA

Intervención Nutricional en la Obesidad Materna de la Rata: Beneficios en la Conducta y Aprendizaje de la Progenie

TESIS

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QUÍMICA FARMACÉUTICA BIÓLOGA

PRESENTA

RAMÍREZ RODRÍGUEZ ADRIANA

MÉXICO, D.F. 2013

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La obesidad materna de la rata tiene un impacto negativo sobre el aprendizaje asociativo y la motivación en las crías hembra

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LAURA NALLELI GARRIDO CASTILLO

MÉXICO, D.F. OCTUBRE 2013

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OBESIDAD E INTERVENCION NUTRICIONAL ANTES Y DURANTE LA GESTACION Y SUS EFECTOS EN EL METABOLISMO MATERNO

Ibáñez C*. Martínez PM, Rodriguez-González GL, Zambrano E.
Departamento de Biología de la Reproducción, INCMNSZ.

INTRODUCCIÓN. La fisiología y metabolismo fetal y neonatal pueden ser alterados por cambios durante periodos críticos del desarrollo, como la gestación y la lactancia, lo cual puede generar un fenotipo asociado al desarrollo de enfermedades en la vida adulta como la obesidad. Es por esto que el estudio de las condiciones metabólicas en la madre podría ayudar a conocer el origen de las alteraciones en el metabolismo de la descendencia.

OBJETIVO. Evaluar algunas hormonas relacionadas con el metabolismo de lípidos y carbohidratos en ratas gestantes alimentadas con una dieta alta en grasa e intervenidas nutricionalmente previamente y durante la preñez.

MÉTODOS. Se usaron ratas hembras Wistar que fueron alimentadas ad libitum desde los 21 días de edad (d), ya sea con dieta control (5% grasa y 4kcal/g) o alta en grasa (20% de grasa y 5 Kcal/g) y apareadas a los 120 d con un macho de la misma cepa, determinándose el día 0 de gestación al observar espermatozoides en *frotis* vaginal. Se formaron 4 grupos experimentales: control (C) alimentadas con dieta control hasta el día 19 de gestación (dg), graso (G) con dieta alta en grasa hasta el día 19dg, grupo de recuperación previa a la gestación (RPG) con dieta alta en grasa hasta los 90d y control hasta el día 19 dg, grupo de recuperación durante la gestación (RG) con dieta alta en grasa hasta el día 0 dg y control hasta el día 19 dg. Se tomaron muestras sanguíneas con ayuno previo de 6h al día 19 dg para determinar en suero las concentraciones de insulina y leptina por Radio Inmuno-Análisis. Se realizó el análisis estadístico por ANOVA de una vía y prueba de Tukey.

RESULTADOS. El peso corporal entre los grupos, tanto al apareamiento como al 19 dg no mostró diferencia significativa sin embargo al día 19dg los grupos G, RPG y RG presentaron mayores concentraciones de insulina respecto al C, para el caso de la leptina esta fue más elevada para G asi como para RG el cuál es diferente a C y RPG.

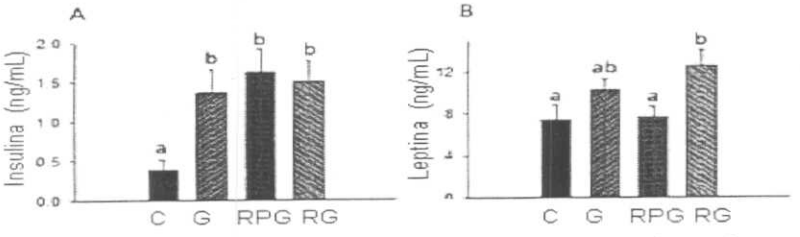

Figura 1. A) Concentraciones de insulina a los 19dg y B) concentraciones de leptina a los 19dg. Media ± EE. Datos que no comparten la misma letra son estadísticamente diferentes, $p<0.05$

CONCLUSIONES. Una dieta alta en grasa previa a la gestación genera una elevación en la concentración de insulina que se observa hasta etapas finales de la preñez, aún en los grupos con intervención nutricional, no así para la leptina cuyas concentraciones elevadas se revierten por la intervención previa a la gestación. De esta manera la dieta materna alta en grasa podría repercutir en el desarrollo de la cría pudiéndose corregir este efecto al menos parcialmente por la intervención nutricional previa a la gestación.

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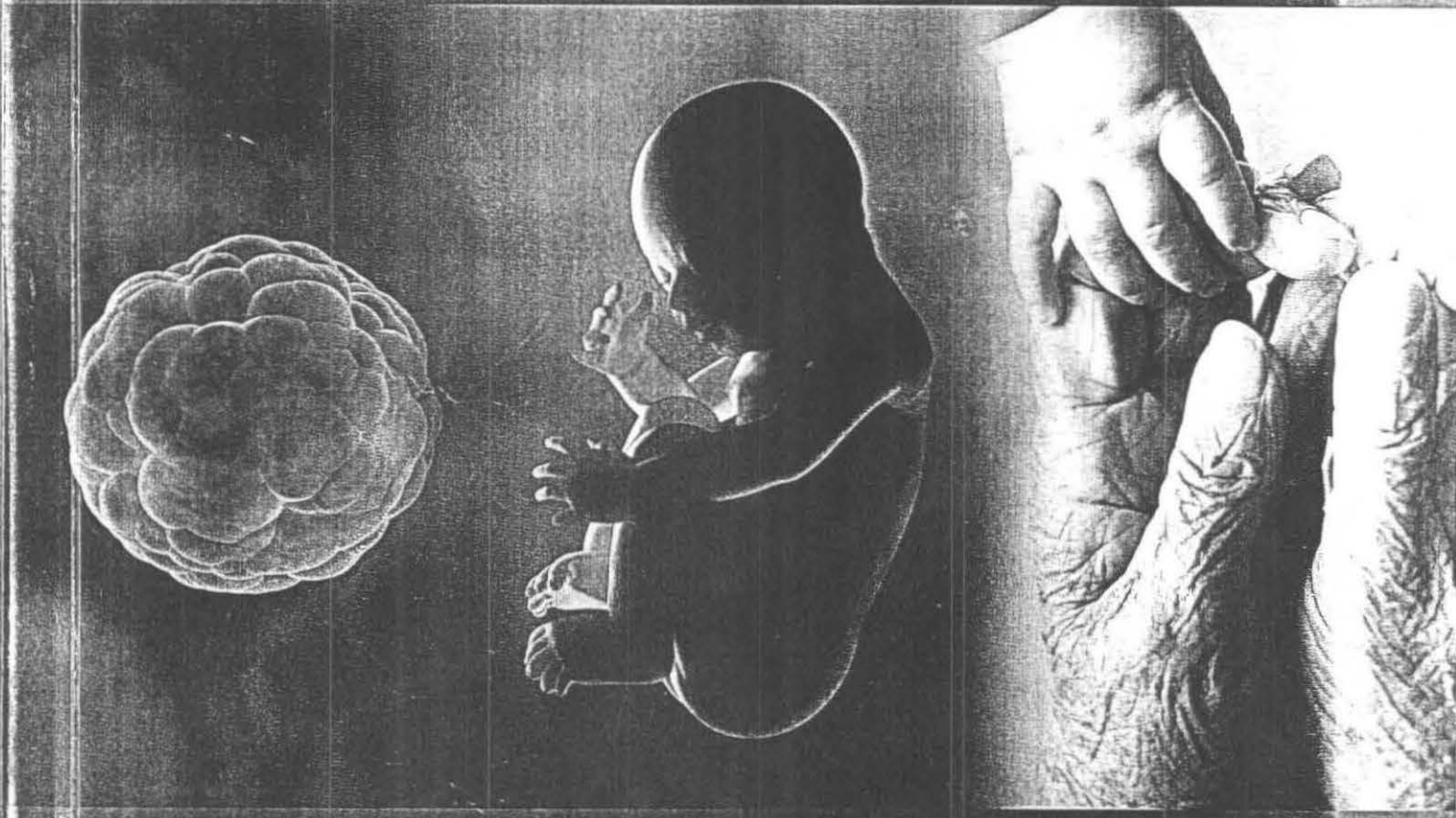

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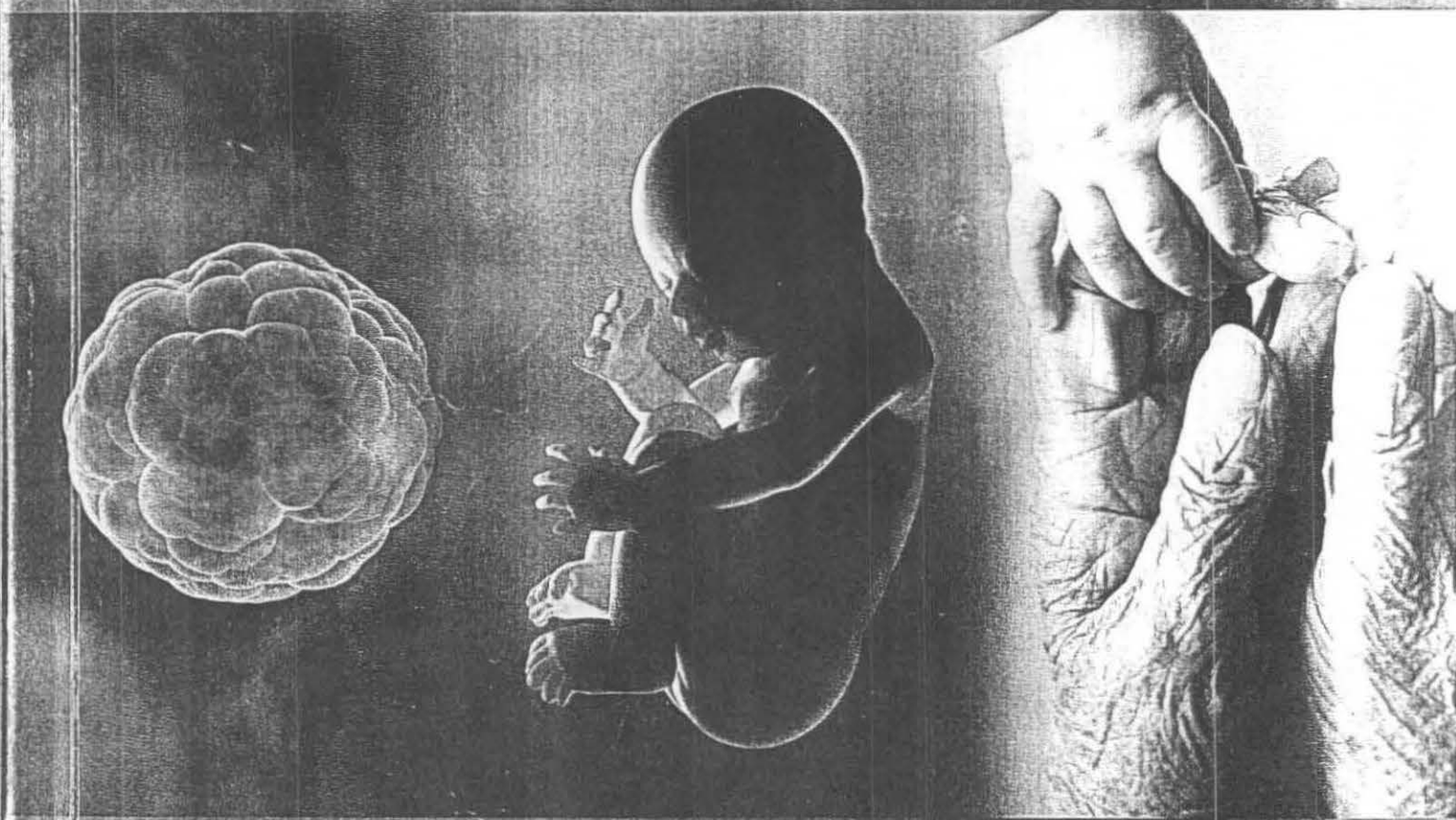

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Objective: Preconceptional nutritional status of women, calculated using body mass index (P-BMI), and gestational weight gain (GWG), are important determinants of birth weight[1]. Their joint effects have not been assessed in Chilean data using recently developed criteria for classifying P-BMI and GWG.

Methods: Prospectively collected anonymous data from a Santiago maternity ward surveillance system was used. Single, term, non-smoking and uncomplicated pregnancies from 11,266 women delivering their newborns between 2000 and 2004 were included in the analysis. The new criteria to classify women's nutritional status based on P-BMI[1], was applied: underweight (<18.5), normal (18.5–24.9), overweight (25–29.9) and obese (≥30). A recently used criteria for GWG classification was also applied, as: low (< 10 kg), medium (10–15 kg), high (16–19 kg), very high (≥20 kg)[2]. Relative risks (RR CI 95%) for birth weight <3000 g, as a proxy for IUGR, and ≥4000 g, as a proxy for FM, were calculated for each category of the combined P-BMI and GWG classifications; non-risky subjects were defined as those born from normal P-BMI women having medium GWG. Appropriate institutional ethics committee clearance was obtained.

Results: A significant reduction in the RR of IUGR was observed with high and very high GWG in underweight women: RR (CI 95%) = 0.42 (0.2–0.89) and 0.34 (0.15–0.79), respectively. In normal P-BMI women the RR of IUGR was inversely related to GWG: RR (CI 95%) = 1.41 (1.19–1.66), 0.80 (0.67–0.96) and 0.48 (0.36–0.62) for low, high and very high GWG, respectively. Also in normal women the RR of FM was directly related to GWG: RR (CI 95%) = 0.38 (0.26–0.55), 1.40 (1.16–1.70) and 2.35 (1.95–2.82) for low, high and very high GWG, respectively. On the other hand, high and very high GWG was related to an increased risk of FM in overweight women: RR (CI 95%) = 1.44 (1.16–1.80) and 2.11 (1.70–2.61), respectively. These effects were similar in obese women. Interestingly, a low GWG in both overweight and obese women was associated to a reduction of FM risk: RR (CI 95%) = 0.76 (0.61–0.96) and 0.56 (0.44–0.73), respectively. However, restriction of GWG in these over nourished women was associated to an increased RR of IUGR: RR (CI 95%) 1.29 (1.00–1.68) and 1.66 (1.01–2.74), for overweight and obese women, respectively.

Conclusions: Heavier women may benefit from avoiding high and very high GWG, which is associated with a relatively low increase in the risk of IUGR; the later results may be improved using proportionate to maternal height GWG[3]. High GWG in underweight women does not appear to have deleterious consequences for their infants and low GWG was clearly associated with IUGR, as recently reported[2].

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3. F. Mardones, P. Rosso. *Matern Child Nutr*, 1:77–90, 2005.

P-7A-314

Intervention that decreases pre-pregnancy obesity recuperates effects of maternal obesity and high fat diet on adipose tissue and glucose tolerance of rat male offspring

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[1]*Department of Biology of Reproduction, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, México, D.F;* [2]*Center for Pregnancy and Newborn Research, University of Texas Health Sciences Center San Antonio, TX, USA*

Objective: Maternal pre-pregnancy BMI is a major determinant of adverse rat male offspring (OFF) metabolic outcomes resulting from maternal obesity (MO)[1]. Although rodent models of developmental programming of OFF, adipose tissue (AT) and glucose tolerance (GT) have been extensively studied no studies exist on effectiveness of interventions to reduce food intake prior to pregnancy on OFF outcomes. We induced MO prior to rat pregnancy with high fat diet (F) and determined if MO reversal before mating recuperates effects on OFF AT and GT.

Methods: After weaning female Wistar rats randomly received either control (C) rodent diet 5001 (Teklad – 5%F) or high fat (F −25%F added to C). One month before breeding 50% of F females were recuperated (R) on C diet for the rest of the study including pregnancy (P) and lactation (L) while remaining F were fed F during P and L. At postnatal day (PND) 120 all three groups were bred and remained on their pre-pregnancy diet in P and L. Litters were adjusted to10 pups/dam. Body and subcutaneous fat weight, serum leptin (RIA) and triglycerides (TG) were averaged in two random male OFF per litter at weaning (21 PND). OFF visceral fat cell size and gonadal F mass were measured at 150 PND. An iv GTT was performed at 120 PND. Data M ± SEM, analysis ANOVA, n = 5 mothers/group.

Results: Maternal breeding weight was higher in F vs C with R intermediate (C: 213 ± 9^{a}, F: 262 ± 13^{b} and R: 232 ± 7^{ab} g, $p < 0.01$). F maternal serum leptin at weaning was higher than C and R (C: 0.8 ± 0.1^{a}, F: 3.8 ± 0.1^{b}, R: 1.2 ± 0.1^{a} $ng.ml^{-1}$, $p < 0.001$). Pup weights were similar at birth and weaning (Fig. 1A) when F pups had more fat (Fig. 1B), serum leptin (Fig. 1C) and TG (Fig. 1D). 150 PND visceral fat cell size was greatest in F, least in C with R intermediate (Fig. 1E) as was gonadal fat mass (data not shown). 120 PND serum GTT glucose was recuperated in R (Fig. 1F) (insulin in progress).

Conclusions: Dietary intervention that decreases maternal weight from 123% of C to 109% of C before P continuing

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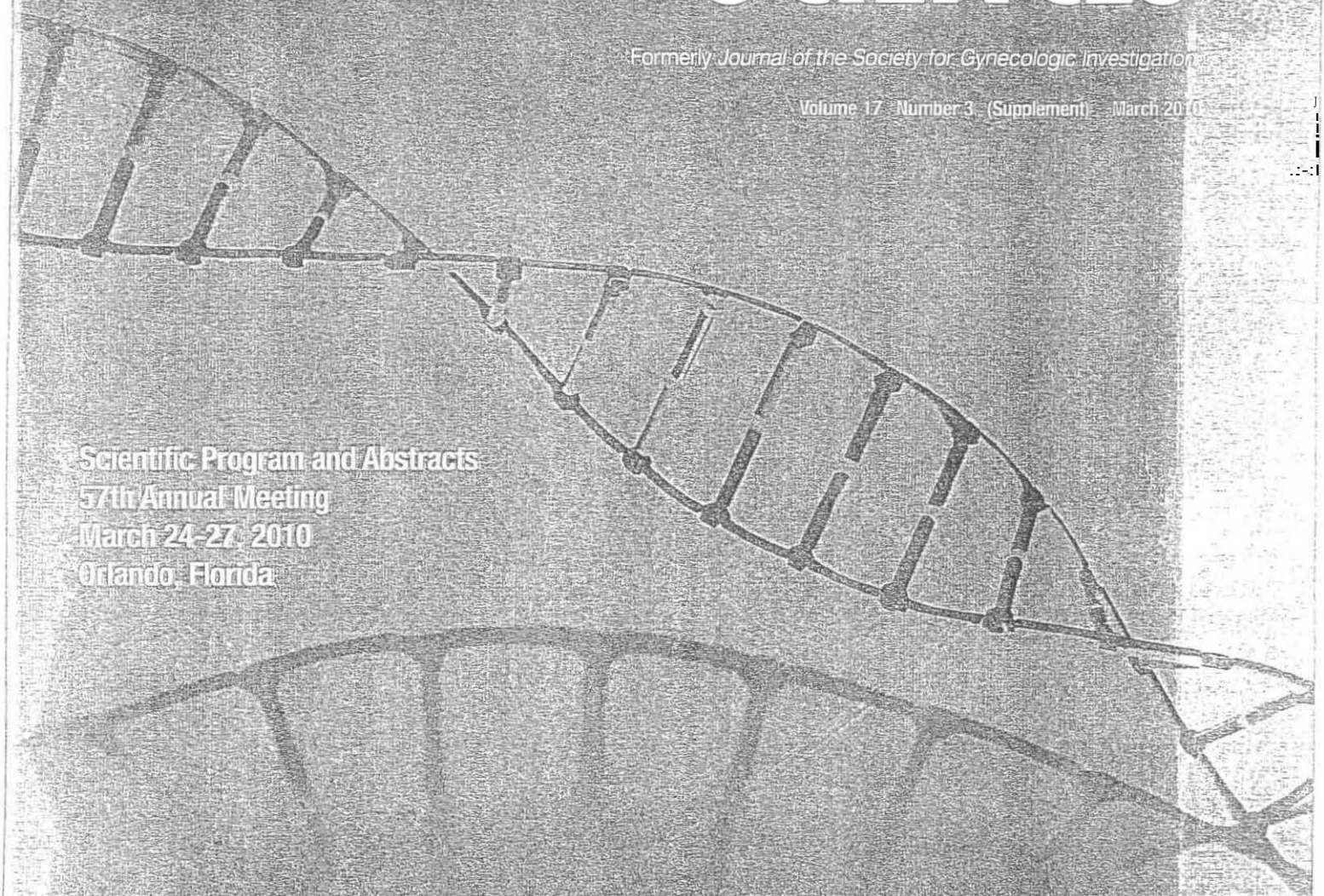

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Dietary Interventions To Reverse Effects of Maternal (M) Obesity (MO) and High Energy Obesogenic Diets (HEOD) Have Very Different Outcomes According to the Timing of the Intervention. Peter W Nathanielsz,[1] Paola Martinez,[2] Carlos Ibanez,[2] Guadalupe L Rodriguez,[2] Elena Zambrano.[2] *[1]Dept. OB/GYN, UTHSCSA, San Antonio, TX, USA; [2]Reproductive Biology, INNSZ, Mexico, DF, Mexico.*

Introduction. MO and HEOD prior to and during gestation have adverse effects on both M and fetus (F). There is currently much clinical interest in the development of effective dietary interventions to reverse these adverse outcomes. We produced MO by feeding a HEOD. We hypothesized that prepregnancy recuperation (PPR) on a normal diet for one month before breeding results in different M and F outcomes to recuperation during pregnancy (PR).

Methods. After weaning (CTR, n=5) female Wistar rats were fed control rodent diet 5001 (5% fat, 4.0 Kcal/g) and 15 received HEOD (25% fat, 4.9 Kcal/g) One month before breeding 5 HEOD rats were returned to CTR diet (PPR). Another 5 HEOD rats were returned to the CTR diet at conception (PR). 5 MO rats remained on HEOD through pregnancy. Rats were euthanized at 19 d gestation (dG), M liver weight serum leptin and insulin (RIA), placental and F weight were measured. Data M ± SEM. Analysis ANOVA.

Results. There were no differences in M body weight at conception or 19 dG (Fig 1A). At 19dG MO liver weight was lower vs all other groups (data not shown). Maternal serum insulin was higher and not different between MO, PPR and PR groups compared with CTR (Fig. 1B). Maternal leptin was recuperated in PPR but not PR (Fig. 1C). There were no differences between groups in placental and F weight, but total placental and F weight per mother was also recuperated in PPR (CTR: 23.4 ± 1.4a, MO: 27.3 ± 1.9bc, PPR: 23.9 ± 3.3ab, PR: 30.5 ± 1.5c, g, $p<0.05$).

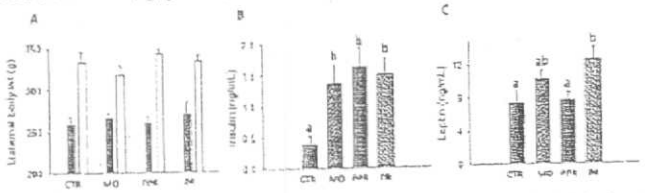

Fig1. A) Maternal weight at conception (black bars) and 19dG (white bars); B) plasma insulin at 19d and C) plasma leptin at 19d. CTR, MO, PPR and RC defined in text. M ± SEM; n=5. Different letters, $p < 0.05$.

Conclusions. Dietary intervention one month prior to but not at conception decreased adverse effects of MO and HEOD on total weight of products of conception and M leptin but not insulin. Grant by SMNE (Mexico), NIH-HD21350 USA).

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Efficacy of Yoga in Reducing Maternal Anxiety in Pregnancy. JJ Newham,[1] JD Aplin,[1] A Wittkowski,[2] M Westwood.[1] *[1]Maternal & Fetal Health Research Group, Manchester University; [2]Psychology, Manchester University.*

Background Maternal anxiety during pregnancy has been associated with an increased risk of preterm birth, low birth weight and delayed infant development (Glover, 2006). Furthermore, mothers suffering from antenatal anxiety are more likely to experience delivery complications (Da Costa, 2000) and develop postnatal depression (Austin, 2007). Consequently, the National Institute of Clinical Evidence have emphasised the need for non-pharmacological interventions to help reduce antenatal anxiety. Yoga may be a suitable intervention as it incorporates relaxation techniques with physical exercise that can be customised for pregnant women and is known to reduce anxiety in non-pregnant women (Javnbakh, 2009).

Aim Analysis of women attending antenatal yoga classes to assess the potential effectiveness of yoga as an intervention to reduce antenatal anxiety and fear of delivery

Method Participants (n=26; maternal age (mean±SD)=33±4y; gestational age=23±4 weeks) completed a questionnaire measuring anxiety (State Trait Anxiety Inventory (STAI)) both before and after a baseline and endpoint (10 weeks later) yoga session. Prior to each session, participants also completed the Wijma Delivery Expectancy Questionnaire (W-DEQ), which measures fear of delivery, and the Edinburgh Postnatal Depression Scale (EPDS), which screens for antenatal depression.

Results At both the baseline and end-point sessions, there was a significant decrease in STAI scores between pre- and post-session ($p>0.01$). Pre-session anxiety scores were significantly lower at endpoint compared to baseline ($p=0.01$). There was a decrease in fear of delivery between baseline and endpoint which, although only a significant trend, showed a large effect size ($p=0.095$; eta squared=0.15). W-DEQ scores significantly correlated with STAI scores ($r=.538$; $p=0.01$) but not EPDS scores ($r=2.83$; $p=0.24$). The lower pre-anxiety and fear of delivery scores at endpoint is contradictory to the elevated anxiety and fear levels usually expected as gestation advances (Lee, 2007).

Conclusion The significant correlation between anxiety and fear of delivery suggests that measuring anxiety, rather than solely screening for depression, may be more reflective of the mother's fears about childbirth. Furthermore, attending yoga may, by actively lowering anxiety levels, reduce such fears. Further research is required to determine which aspects of prenatal yoga may be most effective in preparing a woman for labour.

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The Maternal MDR-1 C3435T Polymorphism and Lipid Profile in Association with the Risk of Congenital Heart Disease. Sylvia A Obermann-Borst,[1] Emma A van Walsem,[1] Aaron Isaacs,[2] Ron HN van Schaik,[3] Cornelia M van Duijn,[2] Eric AP Steegers,[1] Regine PM Steegers-Theunissen.[1,2,4,5] *[1]Obstetrics and Gynecology, Erasmus MC, Rotterdam, Netherlands; [2]Epidemiology/ Genetic Epidemiology Unit, Erasmus MC, Rotterdam, Netherlands; [3]Clinical Chemistry, Erasmus MC, Rotterdam, Netherlands; [4]Pediatrics, Erasmus MC, Rotterdam, Netherlands; [5]Clinical Genetics, Erasmus MC, University Medical Center, Rotterdam, Netherlands.*

Objective: To investigate whether the maternal MDR-1 C3435T polymorphism affects maternal lipid levels thereby influencing the risk of a child with perimembranous ventricular septal defect (pVSD).

Methods: In a cross-sectional case-control study we evaluated 52 mothers of a child with pVSD and 255 with nonmalformed offspring. Maternal MDR-1 CC, CT,and TT genotypes and lipid levels (cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides, apolipoprotein A1 (ApoA1) and apolipoprotein B (ApoB)) were determined at 17 months after the index-pregnancy. Median lipid levels for both cases and controls were calculated and stratified per genotype. Univariate and multivariate logistic regression analyses were performed and odds ratios with 95% confidence intervals were calculated.

Results: Maternal carriership of the MDR-1 TT genotype resulted in a significantly higher risk of pVSD offspring (OR 2.75, 95% CI [1.13-6.69]). Lipid levels did not differ significantly between case and control mothers. In case mothers, the ApoA1 levels were significantly higher in MDR-1 TT carriers than in case mothers with the MDR-1 CT/TT reference genotypes (156.3 mg/dL vs. 130.9 mg/dL P=0.015). The risk for pVSD offspring in MDR-1 TT carriers reduced after adjusting for ApoA1 levels (OR 2.04, 95% CI [1.24-3.37]).

Conclusion: Maternal MDR-1 3435TT carriership is associated with an increased risk of a child with pVSD and higher serum levels of ApoA1. Lowering of the maternal ApoA1 level in combination with MDR-1 TT carriership seems to reduce the risk of pVSD which warrants further investigation.

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Weight Retention in Teen Mothers: Do Nine Months Stay with You Forever? Loral Patchen,[1] Erica K Berggren,[2] Erin M Conroy,[3] Dennis Amini,[1,3] Jason G Umans,[1,3] Menachem Miodovnik.[1] *[1]Obstetrics and Gynecology, Washington Hospital Center, Washington, DC, USA; [2]Obstetrics and Gynecology, University of North Carolina, Chapel Hill, NC, USA; [3]Obstetrics and Gynecology, Georgetown University, Washington, DC, USA.*

OBJECTIVE: To determine whether pregnant teens return to their pre-pregnancy weight within 18 months after delivery.

METHODS: We analyzed data from a prospective cohort of 183 pregnant adolescents, aged 12 to 18, who enrolled in the Teen Alliance for Prepared Parenting (TAPP), a program for pregnant and parenting teens. Participants were classified as underweight (BMI <18.5 kg/m^2), normal (BMI 18.5 – 24.9 kg/m^2), overweight (BMI 25 – 29.9 kg/m^2), and obese (BMI ≥ 30 kg/m^2). Participants were enrolled during pregnancy and followed through 18 months after delivery. Statistical analysis included one-way ANOVA and t-test, with $p<0.05$ considered significant.

al to 0.48 ± 0.10ng/ml and 0.41 ± 0.08ng/ml at 6 and 12 months respectively after RYGB surgery. Evaluation of additional samples will be completed and presented at the meeting.

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Dietary Interventions To Reverse Effects of Maternal (M) Obesity (MO) and High Energy Obesogenic Diets (HEOD) Have Very Different Outcomes According to the Timing of the Intervention. Peter W Nathanielsz,[1] Paola Martinez,[2] Carlos Ibanez,[3] Guadalupe L Rodriguez,[2] Elena Zambrano.[2] [1]*Dept. OB/GYN, UTHSCSA, San Antonio, TX, USA;* [2]*Reproductive Biology, INNSZ, Mexico, DF, Mexico.*

Introduction. MO and HEOD prior to and during gestation have adverse effects on both M and fetus (F). There is currently much clinical interest in the development of effective dietary interventions to reverse these adverse outcomes. We produced MO by feeding a HEOD. We hypothesized that prepregnancy recuperation (PPR) on a normal diet for one month before breeding results in different M and F outcomes to recuperation during pregnancy (PR).

Methods. After weaning (CTR, n=5) female Wistar rats were fed control rodent diet 5001 (5% fat, 4.0 Kcal/g) and 15 received HEOD (25% fat, 4.9 Kcal/g) One month before breeding 5 HEOD rats were returned to CTR diet (PPR). Another 5 HEOD rats were returned to the CTR diet at conception (PR). 5 MO rats remained on HEOD through pregnancy. Rats were euthanized at 19 d gestation (dG), M liver weight serum leptin and insulin (RIA), placental and F weight were measured. Data M ± SEM. Analysis ANOVA.

Results. There were no differences in M body weight at conception or 19 dG (Fig 1A). At 19dG MO liver weight was lower vs all other groups (data not shown). Maternal serum insulin was higher and not different between MO, PPR and PR groups compared with CTR (Fig. 1B). Maternal leptin was recuperated in PPR but not PR (Fig. 1C). There were no differences between groups in placental and F weight, but total placental and F weight per mother was also recuperated in PPR (CTR: 23.4 ± 1.4a, MO: 27.3 ± 1.9bc, PPR: 23.9 ± 3.3ab, PR: 30.5 ± 1.5c, g, $p<0.05$).

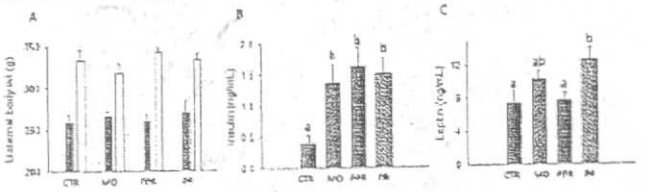

Fig1. A) Maternal weight at conception (black bars) and 19dG (white bars); B) plasma insulin at 19d and C) plasma leptin at 19d. CTR, MO, PPR and RC defined in text. M ± SEM; n=5. Different letters, $p < 0.05$.

Conclusions. Dietary intervention one month prior to but not at conception decreased adverse effects of MO and HEOD on total weight of products of conception and M leptin but not insulin. Grant by SMNE (Mexico), NIH-HD21350 USA).

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Efficacy of Yoga in Reducing Maternal Anxiety in Pregnancy. JJ Newham,[1] JD Aplin,[1] A Wittkowski,[2] M Westwood.[1] [1]*Maternal & Fetal Health Research Group, Manchester University;* [2]*Psychology, Manchester University.*

Background Maternal anxiety during pregnancy has been associated with an increased risk of preterm birth, low birth weight and delayed infant development (Glover, 2006). Furthermore, mothers suffering from antenatal anxiety are more likely to experience delivery complications (Da Costa, 2000) and develop postnatal depression (Austin, 2007). Consequently, the National Institute of Clinical Evidence have emphasised the need for non-pharmacological interventions to help reduce antenatal anxiety. Yoga may be a suitable intervention as it incorporates relaxation techniques with physical exercise that can be customised for pregnant women and is known to reduce anxiety in non-pregnant women (Javnbakh, 2009).

Aim Analysis of women attending antenatal yoga classes to assess the potential effectiveness of yoga as an intervention to reduce antenatal anxiety and fear of delivery

Method Participants (n=26; maternal age (mean±SD)=33±4y; gestational age=23±4 weeks) completed a questionnaire measuring anxiety (State Trait Anxiety Inventory (STAI)) both before and after a baseline and endpoint (10 weeks later) yoga session. Prior to each session, participants also completed the Wijma Delivery Expectancy Questionnaire (W-DEQ), which measures fear of delivery, and the Edinburgh Postnatal Depression Scale (EPDS), which screens for antenatal depression.

Results At both the baseline and end-point sessions, there was a significant decrease in STAI scores between pre- and post-session ($p>0.01$). Pre-session anxiety scores were significantly lower at endpoint compared to baseline ($p=0.01$). There was a decrease in fear of delivery between baseline and endpoint which, although only a significant trend, showed a large effect size ($p=0.095$; eta squared=0.15). W-DEQ scores significantly correlated with STAI scores ($r=.538$; $p=0.01$) but not EPDS scores ($r=2.83$; $p=0.24$). The lower pre-anxiety and fear of delivery scores at endpoint is contradictory to the elevated anxiety and fear levels usually expected as gestation advances (Lee, 2007).

Conclusion The significant correlation between anxiety and fear of delivery suggests that measuring anxiety, rather than solely screening for depression, may be more reflective of the mother's fears about childbirth. Furthermore, attending yoga may, by actively lowering anxiety levels, reduce such fears. Further research is required to determine which aspects of prenatal yoga may be most effective in preparing a woman for labour.

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The Maternal MDR-1 C3435T Polymorphism and Lipid Profile in Association with the Risk of Congenital Heart Disease. Sylvia A Obermann-Borst,[1] Emma A van Walsem,[1] Aaron Isaacs,[2] Ron HN van Schaik,[3] Cornelia M van Duijn,[2] Eric AP Steegers,[1] Regine PM Steegers-Theunissen.[1,2,4,5] [1]*Obstetrics and Gynecology, Erasmus MC, Rotterdam, Netherlands;* [2]*Epidemiology/ Genetic Epidemiology Unit, Erasmus MC, Rotterdam, Netherlands;* [3]*Clinical Chemistry, Erasmus MC, Rotterdam, Netherlands;* [4]*Pediatrics, Erasmus MC, Rotterdam, Netherlands;* [5]*Clinical Genetics, Erasmus MC, University Medical Center, Rotterdam, Netherlands.*

Objective: To investigate whether the maternal MDR-1 C3435T polymorphism affects maternal lipid levels thereby influencing the risk of a child with perimembranous ventricular septal defect (pVSD).

Methods: In a cross-sectional case-control study we evaluated 52 mothers of a child with pVSD and 255 with nonmalformed offspring. Maternal MDR-1 CC, CT,and TT genotypes and lipid levels (cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides, apolipoprotein A1 (ApoA1) and apolipoprotein B (ApoB)) were determined at 17 months after the index-pregnancy. Median lipid levels for both cases and controls were calculated and stratified per genotype. Univariate and multivariate logistic regression analyses were performed and odds ratios with 95% confidence intervals were calculated.

Results: Maternal carriership of the MDR-1 TT genotype resulted in a significantly higher risk of pVSD offspring (OR 2.75, 95% CI [1.13-6.69]). Lipid levels did not differ significantly between case and control mothers. In case mothers, the ApoA1 levels were significantly higher in MDR-1 TT carriers than in case mothers with the MDR-1 CT/TT reference genotypes (156.3 mg/dL vs. 130.9 mg/dL P=0.015). The risk for pVSD offspring in MDR-1 TT carriers reduced after adjusting for ApoA1 levels (OR 2.04, 95% CI [1.24-3.37]).

Conclusion: Maternal MDR-1 3435TT carriership is associated with an increased risk of a child with pVSD and higher serum levels of ApoA1. Lowering of the maternal ApoA1 level in combination with MDR-1 TT carriership seems to reduce the risk of pVSD which warrants further investigation.

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Weight Retention in Teen Mothers: Do Nine Months Stay with You Forever? Loral Patchen,[1] Erica K Berggren,[2] Erin M Conroy,[2] Dennis Amini,[1,3] Jason G Umans,[1,3] Menachem Miodovnik.[1] [1]*Obstetrics and Gynecology, Washington Hospital Center, Washington, DC, USA;* [2]*Obstetrics and Gynecology, University of North Carolina, Chapel Hill, NC, USA;* [3]*Obstetrics and Gynecology, Georgetown University, Washington, DC, USA.*

OBJECTIVE: To determine whether pregnant teens return to their pre-pregnancy weight within 18 months after delivery.

METHODS: We analyzed data from a prospective cohort of 183 pregnant adolescents, aged 12 to 18, who enrolled in the Teen Alliance for Prepared Parenting (TAPP), a program for pregnant and parenting teens. Participants were classified as underweight (BMI <18.5 kg/m^2), normal (BMI 18.5 – 24.9 kg/m^2), overweight (BMI 25 – 29.9 kg/m^2), and obese (BMI ≥ 30 kg/m^2). Participants were enrolled during pregnancy and followed through 18 months after delivery. Statistical analysis included one-way ANOVA and t-test, with $p<0.05$ considered significant.

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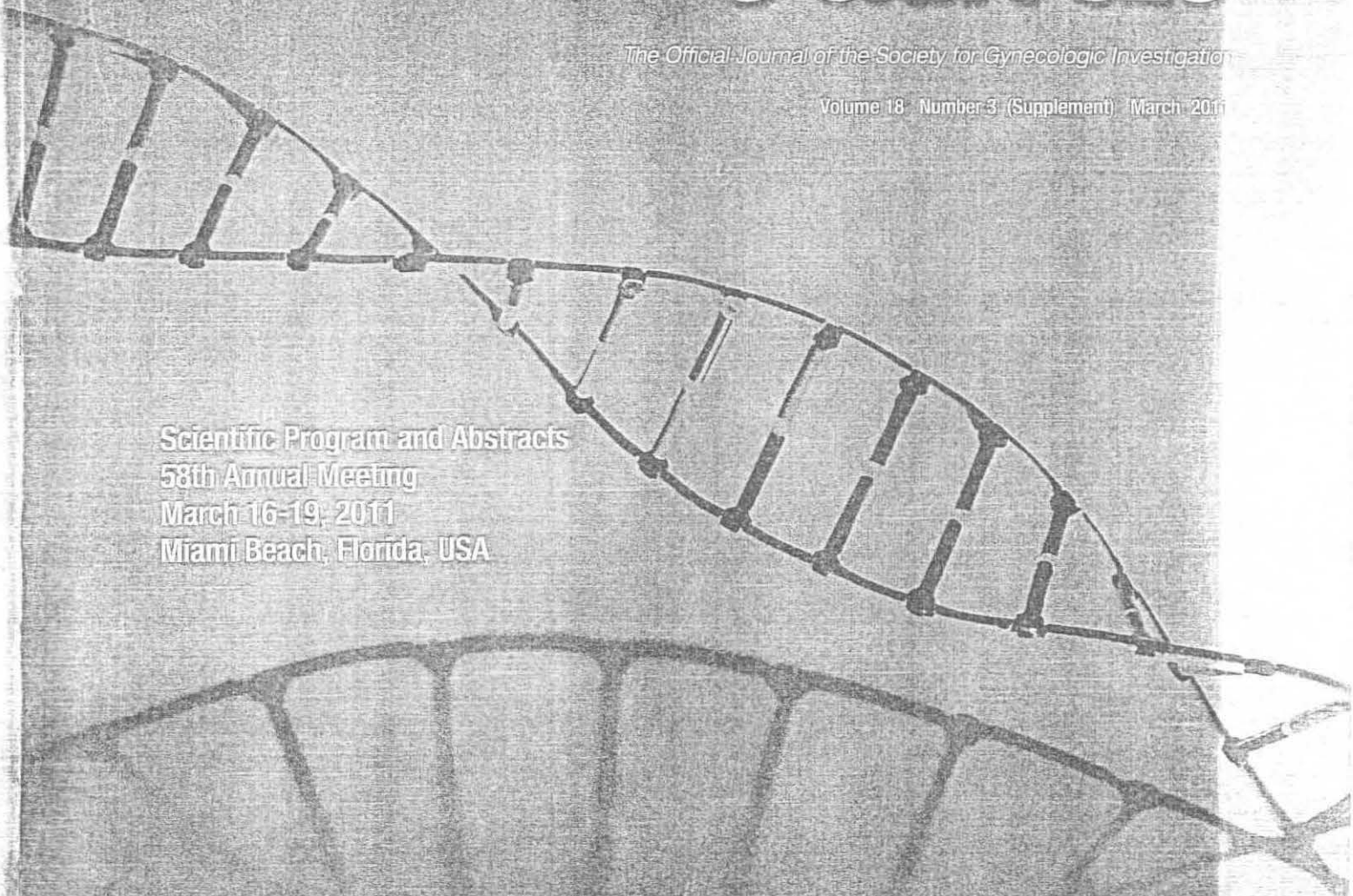

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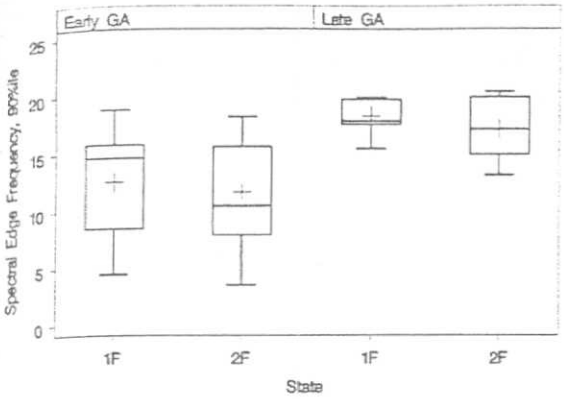


The figure also shows that the mean SEFs changed with GA by similar amounts in both the 1F and 2F states, consistent with the finding of a non-significant GA-by-state interaction (P=0.16). With the interaction removed, the SEF increased from early to late GA (P=0.0014), but decreased slightly from the 1F to 2F state without reaching statistical significance (P=0.087). The mean SEF in early GA for 1F and 2F states were 12.4 and 11.4 Hz respectively. Similarly the mean SEF for late GA for 1F and 2F states was 18.9 and 17.9 Hz respectively.

Conclusion: Our analysis replicates results reported by Bell et al, (1991) where they reported mean SEF of 10 Hz in preterm babies and 24 Hz in term babies. Similar trend in our data in the early and late GA fetuses implies that there is an overall increase in the SEF with increasing GA. The quantification of fetal MEG with SEF is a promising tool to investigate fetal brain maturation.

[1] Bell AH, etal. Biol Neonate. 1991;60(2):69-74.
[2] Nijhuis JG. Ultrasound in obstetrics and gynecology. 1993. p. 447-55.

T-066

Gene Expression in the Hypothalamus of Ovine Fetuses Treated with Estradiol-3-Sulfate in Late Gestation. Maria B Rabaglino, Elaine M Richards, Maureen Keller-Wood, Charles E Wood. *Dept. Physiology and Functional Genomics, University of Florida, Gainesville, FL, USA.*

Introduction: In ruminant fetuses, estrogen plays an essential role stimulating the hypothalamus-pituitary-adrenal axis in the fetal brain. In fetal blood, estrogen is present mostly as sulfoconjugated estrogen that needs to be deconjugated before cellular action. Our objective was to identify the genomics of estradiol sulfate action in the ovine fetal hypothalamus.

Methods: Four sets of chronically-catheterized ovine twin fetuses were studied with one infused with estradiol-3-sulfate intracerebroventricularly (1 mg/day) and the other remained untreated (control fetus). mRNA was extracted from hypothalamus after euthanasia. RNA purification was achieved according the RNA STAT-60 Protocol. Purified RNA samples were analyzed with the Agilent 2100 Bioanalyzer to determine RNA integrity. Microarray was performed according Agilent protocol for 1-color 8x15 microarrays. The 8 samples were hybridized into one slide containing 8 arrays (15744 probes each), then analyzed using an Agilent Scanner. Microarray data analysis was performed using the JMP Genomics 4.1 software. A functional enrichment analysis was done to identify the ontological biological processes significantly associated with the genes up-regulated by estradiol sulfate. The analysis was performed by PAGE test and the p-value was adjusted by Bonferroni method.

Results: A total of 2462 genes were differentially regulated (FDR<0.05%). From them, 808 genes were positively induced by the treatment with estradiol sulfate. The biological processes significantly up-regulated ($p<0.05$) were: adult feeding behavior, sensory perception of taste, hormone-mediated signaling synaptic transmission, polyamine catabolic process and striated muscle contraction. These processes were related with the following genes that showed the higher folder change compared to the control: agouti-related protein (AGRP), neuropeptide y (NPY), transmembrane 9 superfamily member 2 (TM9SF2), oxytocin receptor (OXTR), glutamate receptor 4 precursor (GRIA4), glial high affinity glutamate transporter 2 (SLC1A2), glutamate neutral amino acid transporter 4 (SLC1A4), basic fibroblast growth factor (FGF2), heavy chain skeletal muscle (MYH1), myosin heavy chain 2a (MYH2) and heavy chain skeletal perinatal (MYH3).

Conclusion: We conclude that estradiol sulfate influences both estrogen and non-estrogen-responsive pathways in the late gestation fetal hypothalamus.

T-067

Ontogeny of Global Gene Expression in the Ovine Fetal Hypothalamus in Late Gestation. Maria B Rabaglino,[1] Elaine M Richards,[1] Maureen Keller-Wood,[1] Charles E Wood.[1] *[1]Dept. Physiology and Functional Genomics, University of Florida, Gainesville, FL, USA; [2]Dept. Pharmacodynamics, University of Florida, Gainesville, FL, USA.*

Introduction: Parturition is a highly regulated neuroendocrine process that requires maturation of the fetal hypothalamus-pituitary-adrenal (HPA). We previously reported a significant increased expression of genes related to the HPA axis in late gestation. It is not known what other neurological pathways that influence HPA function are turning on in late gestation. Our objective was to examine the ontogeny of the global gene expression in the hypothalamus of ovine fetuses in late gestation.

Methods: mRNA was extracted from the hypothalamus of ovine fetuses at 80, 100, 120, 130, 145 days of gestation and 1 day after delivery (4/group). RNA was purified according the RNA STAT-60 Protocol and analyzed with the Agilent 2100 Bioanalyzer. Microarray was performed according Agilent protocol for 1-color 8x15k microarrays. Data analysis was performed using JMP Genomics 4.1. Functional enrichment analysis identified the ontological biological processes significantly associated with the genes up-regulated at 145 days of gestational age and 1 day of extrauterine life. The analysis was performed by Fisher exact test and the p-value was adjusted by Bonferroni method. Validation of the results from the enrichment analysis was achieved by qRT-PCR using SYBR-Green.

Results: Expression of 6817 genes significantly changed (FDR=0.05; ANOVA) in the latter half of gestation. Within the significant genes were those related with the HPA axis: mineralocorticoid receptor, corticotropin-releasing factor, serum/glucocorticoid regulated kinase 1 and proopiomelanocortin. The expression of these genes coincided with the pattern that we described previously where the ontogeny expression was measured by qRT-PCR. Biological processes that were significantly enriched at 145 days and 1 day of extra uterine life were immune response and antigen processing ($p<0.05$). Immunological genes validated using qRT-PCR were: Interleukins 10 and 18, and Toll-like receptors 2 and 3.

Conclusion: Inflammatory/immune pathways are activated prior to birth in fetal hypothalamus. Activation of anti or pro inflammatory cytokines and immune related genes in the fetal hypothalamus could reflect global gene activation in the placental-fetal unit or could be a critical pathway in fetal neuronal development leading to increased fetal stress responsiveness and/or parturition.

T-068

Maternal (M) Obesity (MO) Programs Female Offspring (OFF) Corticosterone and Anxiety Behavior; Reversal by Dietary Intervention Pre-Gestation (PG- DINT) or in Gestation (G-DINT). Luis A Reyes,[1] Nadia E Moran,[1] Roberto Chavira,[1] Guadalupe L Rodriguez-González,[1] Paola M Martínez-Samayoa,[1] Fernando Larrea,[1] Peter W Nathanielsz,[2] Elena Zambrano.[1] *[1]Reproductive Biology, Instituto Nacional de Ciencias Medicas y Nutricion SZ, Mexico City, DF, Mexico; [2]Center for Pregnancy and Newborn Research, University of Texas Health Sciences, San Antonio, USA.*

Background: Epidemiological and basic studies show that adverse environments in perinatal development predispose to chronic disease (hypertension and endocrine dysfunction), but few address behavioral outcomes. We investigated effects of MO, pre-gestation (PG) and gestation (G) dietary intervention (DINT) on female OFF anxiety related behavior.

Methods: We have reported [1] a new rat model of MO studying three maternal groups fed from weaning either: (i) control (CTR) chow; (ii) high energy, obesogenic diet (HED) to induce MO, and (iii) pre-gestational dietary intervention (PG – DINT; mothers eat HED from weaning to postnatal day (PND) 90 and then chow. We now add a gestational DINT (G-DINT) group – M fed HED until pregnancy and chow during pregnancy and lactation. All M were bred at PND 120 and ate pregnancy diet until their pups were weaned. On pup PND 2 litters were reduced to 10. All OFF ate chow from weaning. Six female OFF, only one from any litter, from each of the four groups were tested during diestrous in an elevated plus maze (EPM), activity recorded by video analysis at PND 110. Serum corticosterone was measured by RIA at 19 days gestation in mothers and PND 110 in female OFF. Data M ± SEM. Statistical analysis by ANOVA with p set < 0.05.

F-113

Maternal Obesity (MO) Produces Marked Changes in Milk Composition Which Are Variably Reversed by Dietary Interventions. Claudia J Bautista,[1] Luis A Reyes,[1] Guadelupe L Rodriguez-González,[1] Paola M Martínez-Samayoa,[1] Fernando Larrea,[1] Peter W Nathanielsz,[2] Elena Zambrano.[1] *[1]Reproductive Biology, Instituto Nacional de la Nutricion SZ, Mexico City, Mexico; [2]Center for Pregnancy and Newborn Research, Dept OB/GYN, The University of Texas Health Science Center at San Antonio, San Antonio, TX, USA.*

Background: Evidence exists in large agricultural animals that poor milk production may result from impaired mammary gland development when overfeeding occurs before puberty in dairy cows (1) and in pig pregnancy (2). There are no data in the commonly studied laboratory rat on mechanisms by which excess body fatness impairs mammary gland development.

Methods: We reported (3) a rat MO model with three maternal groups fed from weaning: (i) control (CTR) chow; (ii) high energy, obesogenic diet (HED) to induce MO or (iii) *pre-gestational* dietary intervention (PG – DINT), mothers fed HED from weaning to postnatal day (PND) 90 and then CTR diet. We now add a gestational DINT (G-DINT) group mothers fed HED until pregnancy and then chow in pregnancy and lactation. All mothers were bred at PND 120 and ate pregnancy diet until their pups were weaned. All litters were adjusted to 10. At pup PND 21 mothers were weighed and pups removed for 4 h after which mothers received oxytocin; milk was expressed 15 min later for water content (gravimetric analysis), protein (Bradford), fat (Folch), CHO's (glucose oxidase) and leptin (RIA). One way ANOVA.

Results:

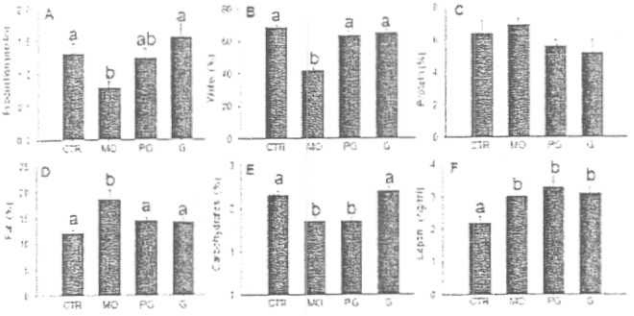


Fig 1. Milk composition A) production, percentage of B) water, C) protein, D) fat, E) CHO's and F) leptin (ng/mL). Mean ± SEM, $p<0.05$ for data not sharing a letter. n= 5-7

Maternal body weight was not different among groups. There were no changes in protein content. Fat content was higher whilst milk production, water and CHO's were lower in MO. Leptin was higher in MO and the DINT groups.

Conclusion: Our results show clear evidence of association of MO obesity and lactation failure and production, partially reversed by DINT. More leptin in the milk of MO may alter OFF appetite.

1) Morrow, J Dairy Sci, 1976; 59:1625. 2) Davison, Equine Sci, 2006; 71:1242. 3) Zambrano, J Physiol, 2010;588:1971.

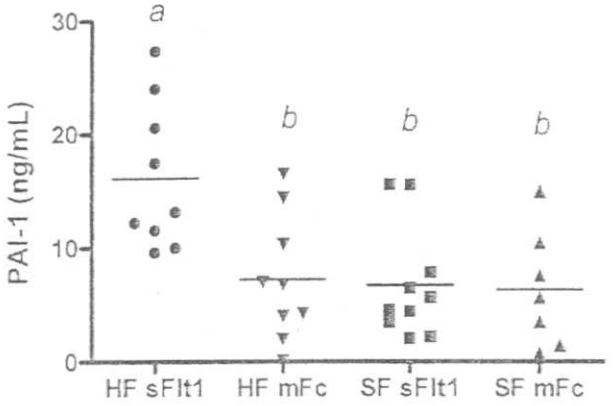
F-114

Prepregnancy Obesity and sFlt1-Induced Preeclampsia: Effect on the Offspring. Egle Bytautiene, Talar Kechichian, Esther Tamayo, Phyllis Gamble, Gary DV Hankins, George Saade. *OB/GYN, UTMB, Galveston, TX, USA.*

OBJECTIVE. We have shown that exposure to maternal prepregnancy obesity and sFlt1-induced preeclampsia during pregnancy alter the offspring's blood pressure and metabolic profile later in life. The objective was to evaluate potential mechanisms focusing on inflammatory and angiogenic pathways.

STUDY DESIGN: CD-1 female mice were placed on either standard fat (SF) or high fat diet (HF) for 3 months before they were bred with SF male. On day 8 of pregnancy, mice were injected with either adenovirus carrying sFlt1 (HF sFlt1 n=6, SF sFlt1 n=6) or adenovirus carrying mFc as virus control (HF mFc n=4, SF mFc n=7). After weaning, all offspring were placed on a SF diet. At 6 months of age, circulating levels of monocyte chemoattractant protein 1 (MCP-1), plasminogen activator inhibitor 1 (PAI-1), resistin, eotaxin and vascular endothelial growth fact (VEGF) were measured using commercially available Luminex assays. One-way ANOVA with Newman-Keuls Multiple Comparison Test were used for statistical analysis (significance: $p<0.05$).

RESULTS: There were no significant differences in resistin ($p=0.5$) levels between the groups of offspring. PAI-1 was significantly elevated in HFsFlt1 offspring ($p=0.002$, Figure) compared to HF mFc and both SF groups, while eotaxin ($p=0.01$) and VEGF ($p=0.03$) levels were significantly higher in HF sFlt1 and HF mFc groups versus both SF groups. The MCP-1 level was also higher in both HF groups compared to SF mice, though not significantly.



CONCLUSION: Exposure to maternal prepregnancy obesity and sFlt1-induced preeclampsia during pregnancy alters inflammatory and angiogenic markers in the offspring. Obesity with or without preeclampsia, but no preeclampsia alone, seems to have the most significant effect.

F-115

Thyroid Hormone Deprivation Disrupts the IGF-I System in the Fetal Heart. Natasha N Chattergoon,[1] Samantha Louey,[1,3] George D Giraud,[1,2,3,4] Kent L Thornburg.[1,2,3] *[1]Heart Research Center, Oregon Health and Science University, Portland, OR; [2]Physiology and Pharmacology, Oregon Health and Science University; [3]Medicine (Cardiovascular Medicine), Oregon Health and Science University; [4]Cardiology, Portland Veterans Affairs Medical Center.*

OBJECTIVES: Published evidence suggests the insulin-like growth factor (IGF-1) system is the dominant stimulant of fetal myocardial proliferation and that triiodothyronine (T_3) drives maturation of the myocardium; T_3 suppresses proliferation and promotes binucleation of fetal cardiomyocytes (CMCs). However, the degree to which circulating T_3 supports the action of IGF-I on the fetal heart has not been investigated. Hypothesis: *Deprivation of circulating T_3 in fetal sheep does not alter cardiomyocyte proliferation if IGF-1 levels are maintained at or above normal concentrations.*

METHODS: Fetal sheep were chronically instrumented at 120 days gestation (dGA, term ≈145 days). One group was thyroidectomized (TX, n= 8) and one served as the non-TX control group (CN, n= 8). At 130d GA, the fetal heart was weighed, a section the left ventricle (LV) removed for molecular studies, and myocytes from each ventricle enzymatically isolated to analyze CMC parameters.

RESULTS: Fetal plasma T_3 concentrations were undetectable 4 days after thyroidectomy; circulating T_3 levels were normal in CN fetuses (TX vs CN: 0 vs 0.1 ng/ml; $p<0.001$). At 130dGA, circulating IGF-I levels were elevated in the TX group (TX vs CN: 105.06 ± 8.94 vs 75.07 ± 6.22 ng/ml; $p<0.05$). IGF-I receptor mRNA levels were 10 times lower in LVs of TX compared to CN ($p<0.001$). Heart weight to body weight ratio was reduced in the TX group (5.9 ± 0.2 vs 6.8 ± 0.3 g/kg; $p<0.05$). CMC cell cycle activity (Ki-67) decreased by 50% and binucleation was depressed in TX fetuses (20% vs 45%, $p<0.05$). TX also resulted in wider binucleated right ventricular CMCs (TX vs CN: 14.3±0.3 vs 16.2±0.6μm; $p<0.05$).

CONCLUSIONS: 1) Loss of plasma T_3 dissociates the fetal IGF-I system leading to increased circulating IGF-1 and decreased myocardial IGF-1 receptor gene expression. 2) The outcome is a smaller heart that is less mature for its gestational age. 3) Increased myocyte width may be a compensation to maintain contractile function in the face of depressed growth. These experiments show for the first time that T_3 is essential for normal cardiomyocyte growth in the fetal heart and suggests that severe fetal hypothyroidism leads to abnormal heart development. Supported by NICHD and NHLBI.

F-116

Enhanced Insulin Secretion in the Fetal Sheep after Chronic Norepinephrine Infusion Suppression. Xiaochuan Chen, Antoni R Macko, Alice S Green, Dustin T Yates, Miranda J Anderson, Amy C Kelly, Aqib Zehri, Sean W Limesand. *Department of Animal Sciences, The University of Arizona, Tucson, AZ, USA.*

Intrauterine growth restriction (IUGR) fetuses have a greater risk of developing glucose intolerance in adulthood, which might reflect perinatal imposed pancreatic β-cell dysfunction. Placental insufficiency-induced IUGR fetuses are

F-288

Within-Patient Variability of Repeat Anti-Müllerian Hormone (AMH) Measurements in the Infertility Population. Vasiliki A Moragianni,[1] Kara Nguyen,[1] Alan S Penzias.[1,2] [1]*Obstetrics & Gynecology, Beth Israel Deaconess Medical Center, Boston, MA;* [2]*Reproductive Endocrinology & Infertility, BostonIVF, Waltham, MA.*

OBJECTIVE

Numerous studies have confirmed anti-müllerian hormone (AMH) to be a robust indicator of ovarian reserve. AMH has been shown to have minimal variation within a menstrual cycle when averaged over groups of individuals, but little has been reported about intra-patient, inter-sample variation and inter-cycle variation, especially in subfertile patients. The objective of this study was to begin to establish estimates of within-patient variability between repeat AMH measurements in the infertility population.

MATERIALS & METHODS

We obtained consecutive AMH measurements from females seeking evaluation and treatment for subfertility. AMH was measured using a commercial assay (Beckman Coulter, Inc.) that has a 7% inter-assay coefficient of variation (CV). Delta was defined as the difference between consecutive AMH measurements.

RESULTS

We analyzed a total of 743 AMH values, measured in 401 subfertile females. On average, patients had AMH levels measured on 2 separate occasions, separated by 79 days (IQR: 29-246 days). Results are summarized in table 1.

Intra-patient variability across AMH values.

AMH-Avg	n	%CV-Avg	Delta-Avg	Delta-SD
0-0.39	121	34	0.11	0.12
0.4-0.79	80	38	0.32	0.26
0.8-1.19	60	31	0.42	0.35
1.2-1.49	20	23	0.44	0.36
1.5-1.99	39	23	0.58	0.44
2.0-2.99	34	22	0.73	0.66
3.0-5.99	39	18	0.97	0.71
6.0-7.87	8	8	0.80	0.83

Avg:average; SD:standard deviation.

CONCLUSION

Amongst a wide range of AMH values, the intra-patient inter-sample CV was similar. The degree of biological variation between samples from the same patient is likely underestimated in current clinical practice. Further study may help better characterize this variation in order to inform clinicians and patients when making clinical decisions with a single AMH value.

F-289

Glycosylated Hemoglobin in the Infertility Population: Is It Worth Checking. Vasiliki A Moragianni,[1] Michele R Hacker,[1] Alan S Penzias.[1,2] [1]*Obstetrics & Gynecology, Beth Israel Deaconess Medical Center, Boston, MA;* [2]*Reproductive Endocrinology & Infertility, BostonIVF, Waltham, MA.*

Objective: To investigate the relationship between glycosylated hemoglobin (HbA1c) values and cycle outcomes in patients undergoing ART.

Design: Retrospective cohort study.

Materials and Methods: We reviewed the charts of all patients who had a HbA1c value measured at our center from June 1, 2004 through April 30, 2010. Patients were included if they underwent infertility treatment in the six months following the HbA1c measurement and cycle data was available. We collected data on age, gravidity, parity, cycle type, cycle number and cycle outcome. Repeated measures log-binomial regression was used to calculate risk ratios (RR) with 95% confidence intervals (CI).

Results: A total of 169 women who underwent 340 cycles met inclusion criteria. The types of cycles included IUI [n=185 (54.4%)], fresh IVF [n=67 (19.7%)], frozen IVF [n=19 (5.6%)] and ovulation induction with relations [n=69 (20.3%)]. Patients were divided in two groups based on their HbA1c value; < 6.0 [n=284 (83.5%), median: 5.4 (interquartile range: 5.2-5.6)] and ≥ 6.0 [n=56 (16.5%), median: 6.4 (interquartile range: 6.1-6.7)]. The two groups were similar with respect to age (p=0.24), gravidity (p=0.46) and parity (p=0.62).

For women with a HbA1c ≥ 6.0 in the 6 months prior to cycle start, the RR of achieving clinical pregnancy was 0.77 (CI: 0.31-1.91) compared to those with values < 6.0. Similar results were noted when adjusting for age [RR: 0.75 (CI: 0.31-1.82)].

Conclusions: This is the largest study to date examining the association between HbA1c levels and cycle outcomes in the infertility population. Our findings failed to demonstrate an association between a HbA1c value within 6 months of cycle start and treatment outcome. Thus, the routine measurement of HbA1c in women undergoing infertility treatment for the purpose of outcome prediction does not appear warranted.

F-290

Leuprolide Acetate Induced-Hypertension in Women Undergoing In–Vitro Fertilization. Ndidiamaka Onwubalili, Kara Goldman, Laura T Goldsmith, David H McCulloh, Peter G McGovern, Aimee Seungdamrong. *Obstetrics, Gynecology and Women's Health, New Jersey Medical School, Newark, NJ, USA.*

Objective:The GnRH agonist leuprolide acetate (LA) is commonly used for downregulation of endogenous gonadotropin production in in-vitro fertilization (IVF). Increased risk of cardiovascular disease in men receiving GnRH agonists for treatment of prostate cancer has been seen. Since we have observed new-onset hypertension after LA therapy in some normotensive patients, we determined which characteristics may be associated with development of hypertension in IVF patients given LA therapy.

Design:A nested case-control study of all patients undergoing controlled ovarian stimulation prior to IVF using the long protocol between Jan 1, 2006-Dec 31, 2008 was performed. We hypothesized that development of hypertension is associated with: increasing age, higher weight/BMI, tobacco use and family history of hypertension.

Materials and Methods:Patients with a preexisting hypertension diagnosis, elevated systolic (SBP) and/or diastolic blood pressure (DBP) at initial visit, and kidney disease were excluded. We identified 203 patients with ≤1yr duration from initial blood pressure (BP) to a long protocol IVF cycle. BP ≥ 140 mmHg systolic and/or ≥ 90 mmHg diastolic was defined as hypertension. Age, weight, BMI, tobacco use and family history of hypertension were assessed for potential correlation with changes in SBP and DBP before and after LA therapy. Continuous variables were assessed by multiple linear regression and Spearman correlation. Fisher's exact test was used for categorical data.

Results:The incidence of new onset hypertension was 9.4% (19/203). In the patients who remained normotensive (N=184), mean initial BP was 118±1/72±1 mmHg (± SEM) and 115±1/72±1mmHg after LA. In the hypertensive group (N=19), mean initial BP was 124±2/77±2 mmHg and 143±3mmHg/87±3mmHg after LA. Weight was a significant predictor of change in SBP in the entire cohort (p=0.036) while in hypertensive patients, weight (p=0.031) and family history of hypertension (p=0.006) were predictive. A significant association between family history of hypertension and hypertension on LA (p<0.055,N=199) was seen in all patients. There was no correlation of SBP or DBP change with age, BMI, family history or smoking in all patients.

Conclusions: Increasing weight and a family history of hypertension may place patients at risk for developing hypertension after LA therapy.

F-291

Maternal Obesity (MO) Impairs Male Offspring (OFF) Reproductive Development: Effects of Pre-Gestation (PG) or during Gestation (G) Dietary Intervention (DINT) in Sexual Development Markers. Guadalupe L Rodriguez-Gonzalez,[1] Luis A Reyes,[1] Peter W Nathanielsz,[2] Fernando Larrea,[1] Elena Zambrano.[1] [1]*Reproductive Biology, Instituto Nacional de Ciencias Medicas y Nutricion SZ, Mexico City, DF, Mexico;* [2]*Center for Pregnancy and Newborn Research, University of Texas Health Sciences Center, San Antonio, TX, USA.*

Objective. Maternal obesity impairs OFF growth and development. There is currently clinical interest in development of effective dietary interventions to reverse adverse outcomes. We have reported [1] a new rat MO model with three maternal groups fed from weaning: (i) control (CTR) chow; (ii) high energy, obesogenic diet (HED) to induce MO or (iii) *pre-gestational* dietary intervention (PG – DINT), mothers eat a HED from weaning to postnatal day (PND) 90 and then CTR diet. Here we add a gestational DINT (G-DINT) group mothers fed HED until pregnancy and then chow in pregnancy and lactation. All mothers were bred at PND 120 and ate pregnancy diet until their pups were weaned. On pup PND 2 all litters were reduced to 10. OFF ate chow from weaning. **We hypothesized** that MO impairs male OFF reproductive development and that DINT recuperates sexual development markers outcomes.

Methods. From PND 21 d male pups were examined daily to determine completed testicular descent and preputial separation. At PND 130 male OFF were mated with unrelated females. Data presented M ± SEM. Analysis by one-way ANOVA and Xi square as appropriate.

fold, p<0.05) and further analysis revealed a number of significantly enriched gene ontologies including vasculature development (p<0.05) and cell-cell signalling (p<0.01). Of these, 3 upregulated genes were chosen for further analysis: CD93 (2.59 fold, p<0.001), STC-1 (2.34 fold, p<0.001) and CXCL10 (2.24 fold, p<0.001). The localisation of these proteins to modified spiral arteries in first trimester decidua was demonstrated by immunohistochemistry.

Conclusion

Using 3-dimensional spheroid co-cultures presents an opportunity to study spiral artery interactions in vitro. EVT conditioned medium promotes changes in gene expression of decidual vascular cells. Expression of these genes may have implications for spiral artery remodelling due to their roles in apoptosis and chemoattraction of trophoblast and immune cells.

S-156

Moderate Inflammatory Stimulation Promotes Prostacyclin Production by Endothelial Cells. Juan Zhao,[1,2] Ruping Fan,[1] Shuang Zhao,[1] Lynn J Groome,[1] Yuping Wang.[1] *[1]Obstetrics and Gynecology, LSUHSC-Shreveport, Shreveport, LA, USA; [2]Cardiovascular Disease, First Hospital of Harbin Medical University, Harbin, China.*

Objective: Normal pregnancy is an inflammatory state compared to the non-pregnant condition. The present study was undertaken to test if moderate inflammatory challenge could affect vasodilator prostacyclin (PGI2) production by endothelial cells (ECs). PGI2 production by NOS inhibition was also examined.

Methods: Cytokine TNFα was selected as an inflammatory stimulator. Confluent ECs were treated with TNFα at concentrations of 10 and 100pg/ml for 24 hours (n=6). EC production of PGI2 was measured by its stable metabolite 6-keto PGF1α in the culture medium by ELISA. We further tested: 1) if TNFα-induced PGI2 production was via activation of PGI synthase. A specific PGI2 synthase inhibitor U51605 (5μg/ml) was used; and 2) if eNOS inhibition could affect PGI2 production. A NOS inhibitor L-NMMA at different concentrations (10-4M, 10-5M, and 10-6M) was used. All samples were assayed in duplicate. Cellular protein concentration was measured. Data was expressed as pg/μg protein and analyzed by ANOVA or paired t-test.

Results: We found that 6-keto PGF1α levels were significantly higher in cells treated with TNFα than the control cells. The treated-to-control ratio of 6-keto PGF1α levels (TNFα 10 pg/ml/control: 1.291±0.117, TNFα 100pg/ml/control: 2.141±0.334) was dose-dependent, p<0.01. Cells treated with U51605 produced significantly less 6-keto PGF1α compared to the control cells, p<0.01. U51605 could also block TNFα-induced PGI2 production, p<0.01. 6-keto PGF1α levels were not different in cells treated with L-NMMA. However, cells treated with TNFα + L-NMMA produced more 6-keto PGF1α than cells treated with TNFα alone.

Conclusions: Cytokine TNFα could promote ECs to produce more PGI2. This stimulatory effect is via activation of PGI synthase. Inhibition of eNOS did not affect EC baseline production of PGI2. However, with moderate inflammatory stimulation, inhibition of eNOS may influence vaso-dilator production by ECs. This data suggests that under moderate inflammatory circumstance, vascular ECs could produce compensatory effects to produce vasodilator PGI2 and even when eNOS was inhibited. (Supported in part by NIH, HL65997 and HD36822).

S-157

TNF-alpha Exposure during Pregnancy Inhibits ATP-Induced $[Ca^{2+}]_i$ and NO Production in Uterine Artery Endothelial Cells by Producing ROS That Mediates Mitochondrial Damage. Fu-Xian Yi, Ronald R Magness, Ian M Bird. *Perinatal Research Labs, University of Wisconsin-Madison, Madison, WI, USA.*

NO plays an important role in maintaining uterine blood flow to the fetus during pregnancy. Preeclampsia is characterized by endothelial dysfunction and increased maternal plasma levels of TNF-a. We wished to examine if acute or prolonged TNF-a exposure could impair $[Ca^{2+}]_i$ signaling and/or eNOS activation in uterine artery endothelium (UA Endo) during pregnancy. TNF-a (50 ng/ml) stimulates ROS production (using H2DCFDA) in UA Endo of late pregnant ewes and in the same cells isolated and maintained in culture (P-UAEC). The origin of ROS was inferred as both mitochondria and NADPH oxidase since rotenone (10 uM, mitochondrial inhibitor) or DPI (20 uM, NADPH oxidase inhibitor) significantly (but not completely) inhibited TNF-a-induced ROS production in P-UAEC, while the combination almost completely blocked ROS production. We have previously reported that ATP (100 uM) continuously induces $[Ca^{2+}]_i$ oscillation and multiple $[Ca^{2+}]_i$ bursts in P-UAEC (at high cell density) over 30 min, and this occurs in a synchronous manner. Herein we further showed that while acute pretreatment with TNF-a (50 ng/ml) for 30 min had no effect on mitochondrial membrane potential (MMP) and ATP-induced $[Ca^{2+}]_i$ responses, prolonged pretreatment of TNF-a for 24 hours resulted in a loss of MMP, and also inhibited ATP-induced the initial $[Ca^{2+}]_i$ peak, the number of $[Ca^{2+}]_i$ bursts, and blocked cell-cell synchronization. N-acetyl cysteine (NAC, 1 mM), an antioxidant, rescued both TNF-a-induced loss of MMP and inhibition of ATP-induced $[Ca^{2+}]_i$. In order to further translate our findings from the UAEC cell model to the in vivo situation, we investigated the effect of TNF-a on ATP-induced Ca^{2+} and NO response in the intact endothelium (P-UA Endo). Simultaneous Fura-2 ($[Ca^{2+}]_i$) and DAF (NO) imaging in individual cells of intact UA Endo showed that acute pretreatment of TNF-a for 30 min again had no effect on the ATP-induced $[Ca^{2+}]_i$ responses, but did acutely inhibit ATP-induced NO production. The reduced NO production was also rescued by the superoxide scavenger PEG-SOD. Conclusion: TNF-a-induced ROS production associated with diseased pregnancy can indeed acutely limit NO availability in UA Endo but also chronic exposure can further independenlty impair $[Ca^{2+}]_i$ signaling (by mitochondrial damage) otherwise necessary for eNOS activation. Funded by NIH HL079020, HD38843, HL49210, *HL50578.*

S-158

Adiposity of Offspring (OFF) of Obese Rats Correlates Wwith Maternal Serum Leptin at Conception in a Pre-Gestation Dietary Intervention (DINT) Model. Elena Zambrano,[1] Paola M Martínez-Samayoa,[1] Carlos Ibañez,[1] Guadelupe L Rodríguez-González,[1] Luis Reyes,[1] Claudia J Bautista,[1] Peter W Nathanielsz.[2] *[1]Reproductive Biology, Instituto Nacional de Ciencias Medicas y Nutricion SZ, Mexico City, DF, Mexico; [2]Center for Pregnancy and Newborn Research, The University of Texas Health Science Center at San Antonio, San Antonio, TX, USA.*

BACKGROUND: Maternal pre-pregnancy weight and body composition are powerful predictors of OFF adverse outcomes. The Institute of Medicine report on maternal obesity (MO) in pregnancy seeks studies to determine optimal dietary and other interventions. We developed (1) a new rat MO model of three maternal groups fed from the time they were weaned: (i) control (CTR) chow throughout; (ii) high energy, obesogenic diet (HED) throughout to induce MO and (iii) pre-gestational DINT – fed HED from weaning to postnatal day (PND) 90 and CTR diet to the end of the study.

METHODS: Rats were bred at PND 120. On pup PND 2 litters were reduced to 10. All OFF ate chow from weaning. Only one M and F from a litter were studied (n=6). OFF were euthanized at PND 110 by decapitation, fat depots removed and weighed. Adiposity index (AI): thoracic and visceral fat weight/body weight * 100. Maternal serum leptin at breeding and OFF at PND 110 by RIA; triglycerides (TG) enzymatically (Synchron CX auto analyser). Analysis - one way ANOVA and Pearson correlations. Data M + SEM, * p < 0.05.

RESULTS:

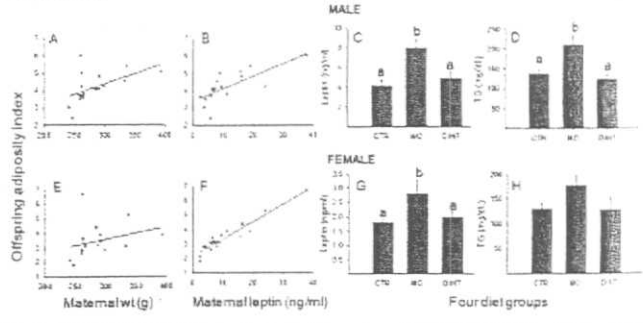


Fig 1. PND 110 OFF AI correlation with maternal weight A) M, (r= 0.5, p=0.03) and E) F (r= 0.3, p=0.2) and leptin at breeding; B) M (r=0.7, p=0.03) and F) F (r= 0.8, p < 0.001). Circles – closed (CTR), open (MO); inverted triangles (DINT). OFF serum leptin in C) M and G) F and TG in D) M and H). M ± SEM, n =6. Data not sharing a letter, p<0.05.

CONCLUSIONS: Maternal leptin at breeding correlated with M and F OFF adult AI but weight only correlated with M AI. DINT recuperated OFF leptin in M and F. MO increase in TG was recuperated in M with no differences between groups in F. This study indicates that OFF MO AI is reversed by pre-gestation gobal DINT with no change in dietary composition. 1) Zambrano 2010; J Physiol 588: 1791.

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RAPID REPORT

Dietary intervention prior to pregnancy reverses metabolic programming in male offspring of obese rats

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Obesity involving women of reproductive years is increasing dramatically in both developing and developed nations. Maternal obesity and accompanying high energy obesogenic dietary (MO) intake prior to and throughout pregnancy and lactation program offspring physiological systems predisposing to altered carbohydrate and lipid metabolism. Whether maternal obesity-induced programming outcomes are reversible by altered dietary intake commencing before conception remains an unanswered question of physiological and clinical importance. We induced pre-pregnancy maternal obesity by feeding female rats with a high fat diet from weaning to breeding 90 days later and through pregnancy and lactation. A dietary intervention group (DINT) of MO females was transferred to normal chow 1 month before mating. Controls received normal chow throughout. Male offspring were studied. Offspring birth weights were similar. At postnatal day 21 fat mass, serum triglycerides, leptin and insulin were elevated in MO offspring and were normalized by DINT. At postnatal day 120 serum glucose, insulin and homeostasis model assessment (HOMA) were increased in MO offspring; glucose was restored, and HOMA partially reversed to normal by DINT. At postnatal day 150 fat mass was increased in MO and partially reversed in DINT. At postnatal day 150, fat cell size was increased by MO. DINT partially reversed these differences in fat cell size. We believe this is the first study showing reversibility of adverse metabolic effects of maternal obesity on offspring metabolic phenotype, and that outcomes and reversibility vary by tissue affected.

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Introduction

Obesity represents an ever increasing epidemic in developing and developed countries that also involves women in their reproductive years. According to WHO the percentage of obese females (BMI greater than 30) in Mexico rose from 21 to 28% between 1999 and 2006 while the increase in USA was even greater, from 19.7 to 33% (World Health Organisation, 2006). Maternal obesity increases maternal obstetric complications (gestational diabetes and preeclampsia) and poor fetal outcomes (macrosomia and stillbirth). Human epidemiological and well-controlled animal research studies provide a clear association between developmental programming of offspring postnatal metabolic, cardiac and endocrine function and maternal obesity during pregnancy and lactation (Barker, 2002; Nathanielsz, 2006; Armitage *et al.* 2008*a*,*b*; Catalano *et al.* 2009). When adult, the offspring of obese rats become obese and hypertensive, presenting a phenotype with insulin resistance and increased plasma leptin (Samuelsson *et al.* 2008; Kirk *et al.* 2009). By 11 years of age, children exposed to maternal obesity during pregnancy are at twice the risk of developing metabolic syndrome (Boney *et al.* 2005).

Whether developmental programming of offspring physiology resulting from maternal obesity and high calorie diets can be reversed by dietary interventions introduced before conception remains an unanswered question of considerable physiological and clinical interest and importance. Although rodent models of offspring metabolic developmental programming by maternal obesity and excessive maternal nutrition have been extensively investigated (Armitage *et al.* 2005), we know of no studies designed to reverse unwanted developmental programming effects by dietary intervention prior to pregnancy. To rectify the lack of information on this

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important question, we induced maternal obesity in non-pregnant female rats by feeding them a high fat diet from weaning through pregnancy and lactation, and determined outcomes in relation to offspring metabolic variables at weaning and 120 days postnatal life, and adipose tissue at weaning and 150 days postnatal life. In a separate group of females, we determined the extent to which dietary intervention by transferring MO rats back to normal chow diet 1 month before mating could reverse the adverse offspring outcomes. An obese maternal phenotype results from multiple and complex interactions between the mother's genetic predisposition and her own programming by factors in her own developmental environment pre- and post-natally. This rich, mechanistic complexity is a major confounding factor in interpretation of human epidemiological data and increases the need for data from well-controlled animal studies. We have embraced this need by rigorously controlling both genetic stock and phenotype of the mothers of the pregnant rats whose male offspring were our study subjects.

We hypothesized that dietary intervention beginning before pregnancy would be able to reverse at least some of the adverse consequences in the offspring of female rats eating a high energy, obesogenic diet from the time they themselves were weaned.

Methods

Care and use of animals

Standardization of phenotype of mothers of females used to produce the pregnancies studied Female albino Wistar rats born and maintained in the colony of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INNSZ), Mexico City, Mexico held in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited light-controlled facility (lights on from 07:00 to 19:00 h at 22–23°C) and fed normal laboratory chow (Zeigler Rodent RQ 22-5, USA) containing 22.0% protein, 5.0% fat, 31.0% polysaccharide, 31.0% simple sugars, 4.0% fibre, 6.0% minerals and 1.0% vitamins (w/w), energy 4.0 kcal g^{-1}. At age between 14 and 16 weeks, when they weighed 220 ± 20 g (mean $\pm$ S.E.M.), females were bred to randomly assigned, non-litter mate, proven male breeders. On postnatal day 2 after delivery (postnatal day 0) litters were culled to 10 pups. At weaning (postnatal day 21), offspring females were randomly assigned to either a control (CTR; $n = 5$) group that received the laboratory chow or to a maternal obesity group (MO; $n = 10$) fed a high energy, obesogenic diet containing 23.5% protein, 20.0% animal lard, 5.0% fat, 20.2% polysaccharide, 20.2% simple sugars, 5.0% fibre, 5.0% mineral mix, 1.0% vitamin mix (w/w), energy 4.9 kcal g^{-1}. Only one female from any one litter was assigned to a group.

At postnatal day 90, 1 month before breeding, five MO females were selected at random for the dietary intervention group (DINT) and placed back on the control diet for the rest of the study, including pregnancy and lactation. The remaining five MO females continued the high fat diet. At postnatal day 120 all three groups were bred and were fed their pre-pregnancy diet throughout pregnancy and lactation. All rats delivered by spontaneous vaginal delivery. Day of delivery was considered as postnatal day 0. Food and water were available *ad libitum*. All procedures were approved by the Animal Experimentation Ethics Committee of the INNSZ, Mexico City. Pregnant and lactating rats were weighed every day through pregnancy and until pups were removed at weaning.

Maintenance of offspring

Litter size and pup weight were recorded at birth. Ano-genital distance, anterior–posterior abdominal distance and head diameter were measured with calipers. Our published data indicate that ano-genital distance is 1.67 ± 0.13 mm ($n = 291$ pups from 43 litters; mean $\pm$ S.E.M.) in female pups and 3.26 ± 0.22 mm ($n = 252$ pups from 43 litters) in males at birth (Zambrano *et al.* 2005). Since a value of 2.5 mm is more than 2 S.D.s from the mean of either group, sex was judged according to whether the ano-genital distance was greater than (male) or less than (female) 2.5 mm. Litters of over 14 were excluded. To ensure homogeneity of offspring evaluated, all litters studied were adjusted to 10 pups per dam except for one MO litter that contained only 9 pups all of which were retained with the mother. The sex ratio was maintained as close to 1:1 as possible. Pups continued to be weighed every week.

Offspring blood samples and retrieval of organs At postnatal day 21, mothers were removed and pups fasted for 4 h. Two male offspring were chosen at random from each litter and trunk blood samples obtained following rapid decapitation by experienced personnel trained in the procedure using a rodent guillotine (Thomas Scientific, USA). Morphometric measurements were made on the neonates. Subcutaneous fat, the most plentiful site at this stage of development, was scraped clean from the skin and abdominal wall tissue from the axilla to iliac crest, weighed and either frozen in liquid nitrogen or fixed for histology. At postnatal day 120, following an overnight fast, blood was removed from the tail vein of two male rats chosen at random from each litter. At postnatal day 150, following an overnight fast, rats were rapidly killed by decapitation as described above and perigonadal fat was excised.

Quantification of adipocyte size Paraffin sections of fixed fat were stained with haematoxylin and eosin and analysed with a Leica Qwin – 500 W microscope equipped with a digital camera. For each sample, four areas and 10 cells in each area were evaluated. Cell areas were obtained using Leica software for digital imaging processing.

Biochemical analyses

Blood glucose measurement Serum glucose was determined spectrophotometrically using the enzymatic hexokinase method (Beckman Coulter, Co., Fullerton, CA, USA). Intra- and inter-assay coefficients of variations (CV) were <2% and <3%, respectively.

Insulin radioimmunoassay (RIA) Serum insulin concentrations were determined by RIA (Linco Research, Inc., St Charles, MO, USA; Cat. no. RI-13K). The intra- and inter-assay CVs were <4% and <6%, respectively.

Triglycerides and cholesterol measurement Serum triglycerides were determined enzymatically (Synchron CX auto analyzer, Beckman Coulter). Intra- and inter-assay CVs were <6%.

Leptin radioimmunoassay Serum leptin was determined by RIA (Linco Research, Inc., Cat. no. RL-83K). The intra- and inter-assay CVs were <4% and <5%, respectively.

Measurement of food intake at postnatal day 120 to 150 Offspring food intake was measured for 14 consecutive days between 120 to 150 days of age. Two male rats from the same experimental group were housed per cage. Food was provided in the form of large flat biscuits. The amount of food provided each day was weighed as was the amount remaining after 24 h. The amount consumed was averaged between the two rats.

Statistical analysis

Litter sizes were normalized to 10 pups per litter on postnatal day 2 and all measures were made in two randomly selected males per litter, and data from these offspring averaged for analysis to provide an $n = 5$ litters per group. All data are presented as mean ± S.E.M. To conform to common practice we performed the conventional analyses used by ourselves (Zambrano *et al.* 2006) and others (Nivoit *et al.* 2009), in this type of study of equal numbers of subjects per litter in which n refers to the number of litters. The effect of diet before and during pregnancy as well as differences between groups of offspring was assessed by one-way analysis of variance (ANOVA) with the Tukey *post hoc* test where appropriate. $P < 0.05$ was regarded as significant. Confirmation of significant differences was obtained using a mixed linear model to analyse the data with dam as a random effect using data from all the pups rather than litters in which $n = 10$ pups per group. With this method we obtained the same significant changes as with the conventional method. Insulin resistance index (IRI) was assessed by the homeostasis model assessment (HOMA) calculated from the formula IRI = glucose (mmol l^{-1}) × insulin (μU ml^{-1})/22.5 (Nandhini *et al.* 2005).

Results

Maternal phenotype

One month prior to breeding, non-pregnant females on the high energy obesogenic diet were 22% heavier than controls (Fig. 1 and Table 1). When bred at postnatal day 120, the MO group was 16% heavier than the

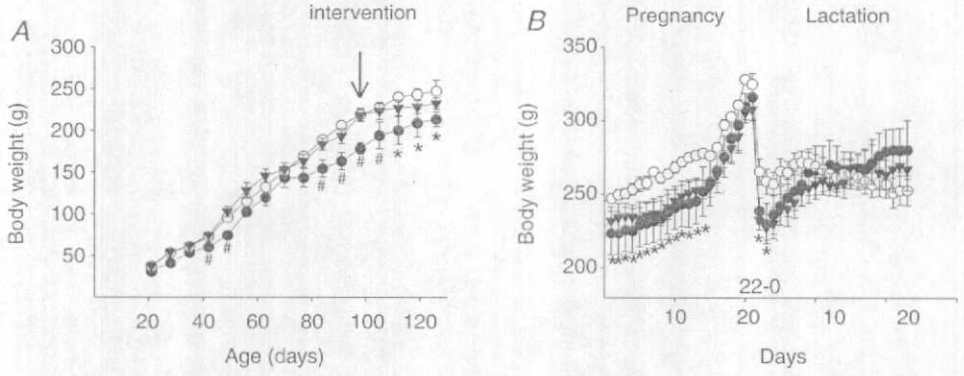

Figure 1. Weight curves for the three groups of mothers
A, pre-pregnancy growth curves from weaning to mating, *B*, maternal weight during pregnancy and lactation. 22-0 means parturition day: 22 end of pregnancy and 0 beginning of lactation. Mothers: control (•), #$P < 0.05$ different from both obese mothers (MO, ○) and dietary intervention mothers (DINT, ▼), *$P < 0.05$ *versus* MO ($n = 5$ per group). Data are mean ± S.E.M.

Table 1. Maternal and Offspring phenotype

	Control	MO	DINT
Maternal phenotype			
Weight 1 month before breeding (g)	178.3 ± 7^a	218.5 ± 8^b	217.8 ± 7^b
Weight at breeding (g)	213 ± 9^a	248 ± 13^b	232 ± 7^{ab}
Weight at the end of pregnancy (g)	316 ± 16	325 ± 1	307 ± 7
Weight at delivery (g)	239 ± 17^a	265 ± 8^b	233 ± 6^a
Weight at weaning (g)	280 ± 19	253 ± 10	267 ± 15
Serum leptin at weaning (ng ml^{-1})	0.8 ± 0.1^a	3.8 ± 0.1^b	1.2 ± 0.1^a
Offspring phenotype			
Weight at birth (g)	6.2 ± 0.1	6.0 ± 0.1	6.0 ± 0.1
Length (mm)	51.1 ± 0.1	51.3 ± 0.4	50.8 ± 0.3
Head diameter (mm)	11.2 ± 0.07	11.3 ± 0.04	11.2 ± 0.08
Abdominal diameter (mm)	12.3 ± 0.05	12.3 ± 0.05	12.4 ± 0.09
Head:abdominal ratio	0.93 ± 0.01	0.92 ± 0.01	0.90 ± 0.01
Ano-genital distance (mm)	3.71 ± 0.05	3.88 ± 0.07	3.72 ± 0.07
Ano-genital distance (mm g^{-1})	0.59 ± 0.02	0.65 ± 0.01	0.62 ± 0.02
Body weight at weaning (g)	40 ± 1.6	42 ± 2.2	37 ± 2.7
Body weight at 120 d (g)	300 ± 10	308 ± 2	313 ± 10
Body weight at 150 d (g)	348 ± 12	354 ± 9	369 ± 10
Food intake (g day^{-1}) between 120 and 150 d	17.9 ± 0.6	17.1 ± 0.7	18.5 ± 0.4

Data are mean ± S.E.M. from $n = 5$ mothers per treatment group. $P < 0.05$ for data not sharing at least one letter. MO: maternal obesity, DINT: dietary intervention.

control females while the dietary intervention group was intermediate in weight and was now only 9% heavier than controls. At postnatal day 21, the time the pups were weaned, maternal serum leptin was higher in the MO group in comparison with the control mothers. Leptin levels in the DINT group were similar to controls (Table 1).

Offspring phenotype at birth

At birth there were no differences in offspring between groups in birth weight or other morphometric variables measured (Table 1).

Offspring phenotype at weaning

There were no differences in body weight in the pups between groups at weaning (Table 1). However, MO pups had more subcutaneous fat tissue, and higher serum triglycerides, leptin and insulin than control. Dietary intervention returned all four measures to control levels (Fig. 2). While serum glucose did not differ between the three groups of offspring, serum insulin was elevated in MO offspring and returned to CTR levels in the DINT group indicating the presence of insulin resistance in the MO offspring.

Insulin resistance index at 120 days postnatal age

At 120 days postnatal age, offspring of MO mothers had elevated fasting serum glucose and insulin and increased insulin resistance when compared with control offspring (Fig. 3). In the dietary intervention offspring, insulin remained elevated above the control group while blood glucose did not differ from either of the two other groups. As a result, recuperation of insulin resistance was intermediate between the control and MO groups and statistically different from both ($P < 0.05$), indicating partial recovery with a degree of persisting insulin resistance.

Adipose tissue characteristics at postnatal day 150

There were no differences in body weight in any of the groups of offspring at postnatal day 150 (Table 1). Offspring of MO mothers had a greater amount of body fat, larger fat cell size and higher leptin concentrations than controls (Fig. 4). Serum leptin in the DINT group was no longer different from controls. However, although the dietary intervention significantly lowered both fat depot mass and fat cell size, these were still significantly higher than controls.

Discussion

A recent review on effects of maternal obesity in pregnancy concludes '*The escalation of obesity amongst women of reproductive age and the complications both short and long term for the mother and child has provided the stimulus for rapid development of an intervention to improve outcomes. To date, none has been validated for clinical use. Undoubtedly, the most successful intervention will*

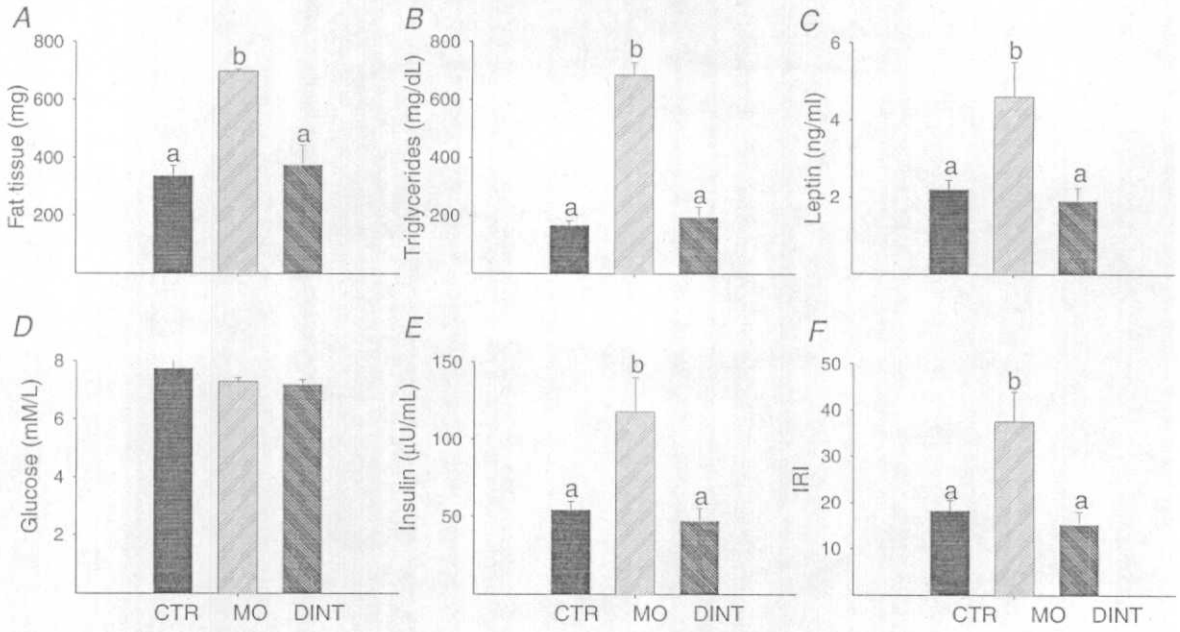

Figure 2. Offspring phenotype on postnatal day 21
A, subcutaneous fat mass; *B*, serum triglycerides; *C*, serum leptin; *D*, glucose; *E*, insulin; *F*, IRI. Data showing different letters are significantly different, $P < 0.05$ between groups. See Methods for description of maternal diets for control (CTR), obese (MO) and dietary intervention (DINT) mothers ($n = 5$ litters per group). Data are mean ± S.E.M.

be that which prevents development of obesity before the reproductive years' (Nelson *et al.* 2010). We sought to determine if effective intervention could be established. We and other investigators have demonstrated that male offspring are more predisposed to the adverse outcomes resulting from exposures to challenges such as poor maternal nutrition and stress on glucose and insulin homeostasis (Desai *et al.* 1997; Sugden & Holness, 2002; Zambrano *et al.* 2006) as well as offspring lipid levels (Lucas *et al.* 1996). This increased susceptibility has been attributed to the faster growth and consequently more critical nutritional need (Lucas *et al.* 1996). Since developmental programming of offspring outcomes by maternal obesity may result from changes in uterine and ovarian function (including egg quality) prior to conception, the optimum intervention should reverse unwanted changes that occur before pregnancy begins.

The increased maternal weight and offspring adipose tissue mass indicates clearly that the experimental diet was able to produce an increase in weight and other maternal characteristics seen in maternal obesity in human pregnancy. If a direct conversion is made from weight to BMI – an extrapolation that must be made with great caution, and control mothers represent a woman with a BMI of 25, the top of the normal range, the obesogenic diet induced an increase to reach a BMI equivalent of 30.8, exceeding the overweight range and entering the lower end of the obese category. Dietary intervention returned the

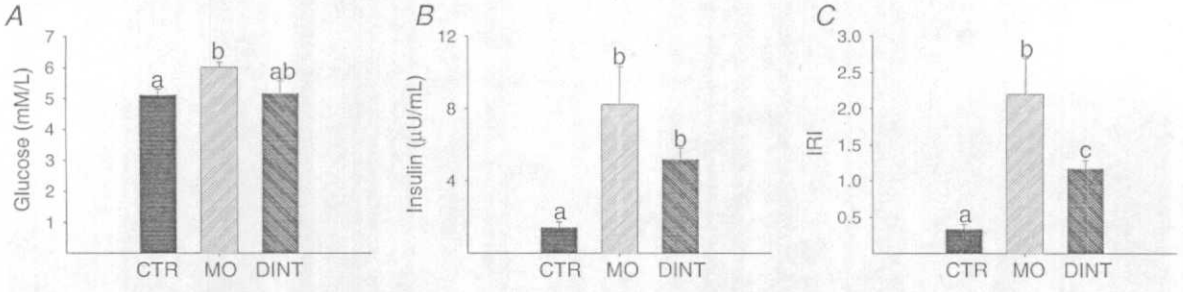

Figure 3. Fasting insulin, glucose and insulin resistance index (IRI) at postnatal day 120
A, fasting glucose; *B*, fasting insulin; *C*, insulin resistance index. Data showing different letters are significantly different, $P < 0.05$. See Methods for description of maternal diets for control (CTR), obese (MO) and dietary intervention (DINT) mothers ($n = 5$ litters per group). Data are mean ± S.E.M.

DINT rats to the equivalent of BMI 27.3 – the middle of the overweight range. Maternal serum leptin was still elevated in the MO group at the end of the weaning period and was completely recuperated by the dietary intervention.

The failure to observe differences in birth weights in the three groups is consistent with findings in many models of effects of maternal diet on developmental programming (Armitage *et al.* 2005, 2008*a*). In one study, birth weight was lower in offspring of mothers with diet-induced obesity due primarily to larger litter sizes of 14.5 pups as against 10.6 per litter in control litters (Nivoit *et al.* 2009). Thus, evaluation of outcomes, especially in the early stages of neonatal life, should be based on body composition rather than weight, which is a poor measure of the quality of the intra-uterine environment.

Studies on the effects of maternal obesity have shown similar altered glucose and insulin-related, as well as fat metabolism changes, in MO offspring outcomes to those shown here (Samuelsson *et al.* 2008; Kirk *et al.* 2009) and thus this model has value in determining ability of the dietary intervention introduced to reverse these basic adverse outcomes. It was not the purpose of this study to determine the mechanisms involved either in the developmental programming observed or its recuperation. The primary purpose was to establish, for the first time, the possibility of reversal of well-established adverse offspring outcomes of maternal obesity and high energy diets.

Although offspring basal fasting glucose levels at weaning were not different in the three groups, insulin levels were raised by maternal obesity, indicating a degree of increased peripheral resistance even at this young age. DINT completely reversed the increased insulin resistance. It is of interest that insulin levels in 21-day-old offspring were 20 times values observed at 120 days of postnatal life. High neonatal serum insulin has been previously reported (Aguayo-Mazzucato *et al.* 2006). By 120 days postnatal life, offspring insulin levels as well as insulin resistance index were significantly elevated in both the MO and DINT groups. Delay in emergence of altered phenotype resulting from challenges during development is a major feature of developmental programming. In addition, while recuperation appeared complete at 21 days of postnatal life, there were persistent metabolic changes since DINT did not return either the serum insulin or the insulin resistance index to control levels at 120 days postnatal life. From the point of view of translation of our observations to programming of human life time health, it is important that these animals were maintained on what is a relatively low fat, low energy normal rodent laboratory chow compared with human diets. It would be important to see if the outcomes differ following high energy dietary challenges such as provided by modern junk food. The explanation of residual effects remain to be determined but it is possible that the initial period of maternal obesity has produced epigenetic changes in the oocyte or other reproductive functions that are not completely reversed in the limited period of dietary intervention imposed.

As with the carbohydrate metabolism variables, dietary intervention completely reversed the increased adipose tissue mass and elevated triglycerides observed in MO offspring at 21 days of life. In contrast, fat cell size at postnatal day 150 was not completely reversed, again showing persistence of adverse effects of maternal obesity in the presence of the dietary intervention.

Maternal obesity resulted in increased offspring leptin concentrations at postnatal days 21 and 150. An extensive literature exists that indicates that leptin is predominantly produced by adipose tissue and acts on arcuate nucleus neurons to inhibit food intake by stimulating secretion of the anorexogenic neuropeptides POMC and CART (Bouret & Simerly, 2007). Neonatal serum leptin in rodents has a characteristic profile that demonstrates a peak between postnatal day 8 and 21 (Elias *et al.* 1998;

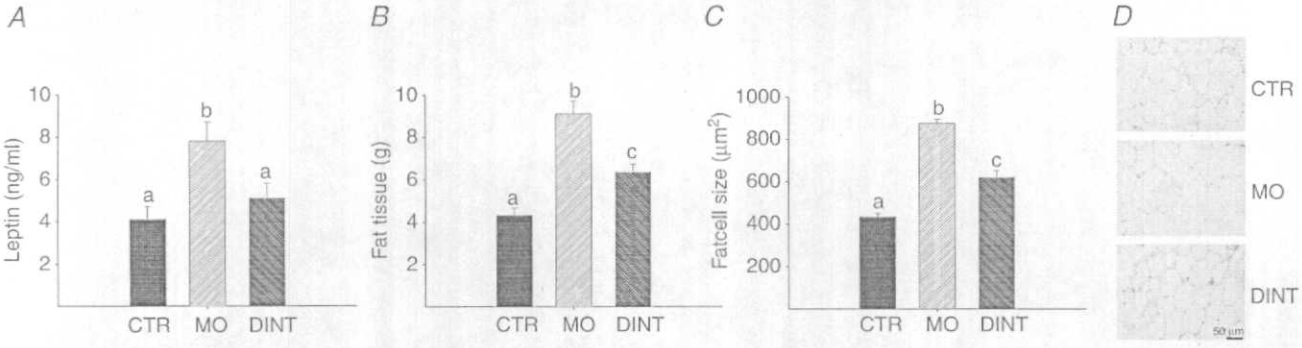

Figure 4. Adipose tissue characteristics at postnatal day 150
A, serum leptin; *B*, perigonadal fat mass, *C*, fat cell size; and *D*, example of haematoxylin and eosin staining of fat cells. The scale bar (50 μm) in the bottom histology panel applies to all. Data are mean ± S.E.M. Data showing different letters are significantly different, $P < 0.05$. See Methods for description of maternal diets for control (CTR), obese (MO) and dietary intervention (DINT) mothers ($n = 5$ litters per group).

Elmquist *et al.* 1998; Proulx *et al.* 2001; Yura *et al.* 2005; Bautista *et al.* 2008; Delahaye *et al.* 2008; Kirk *et al.* 2009). Though the determination of the timing and duration of the leptin peak varies between studies and depends on the precise experimental conditions (e.g. sampling frequency), the existence of a peak is now well established. The postnatal peak differs within rodent species and strains, occuring a couple of days earlier in mice than rats. The timing and trajectory of the postnatal leptin surge in rodents is critical to the development of obesity in later life (Vickers *et al.* 2005, 2008; Bautista *et al.* 2008). One very recent study shows that the leptin peak is amplified and prolonged in offspring of rats made obese by eating a high fat diet. These offspring demonstrated a hyperphagic, obese phenotype in later life (Kirk *et al.* 2009). In the present study, serum leptin was still elevated in offspring of the MO mothers at postnatal day 21 but was recuperated by DINT. However, it will be necessary to examine the whole neonatal profile with more frequent sampling.

Multiple mechanisms are potentially responsible for immediate and delayed offspring outcomes resulting from maternal obesity and over-nutrition. These include increased nutrient delivery to the fetus and newborn, altered growth factor function in mother, placenta and fetus, and inflammatory changes during pregnancy accompanied by obesity (Challier *et al.* 2008). Since an increase in maternal weight depends on increased maternal calorie intake, in both human and animal studies, it is difficult (perhaps impossible) to separate mechanistic pathways resulting from obesity per se from those due to the high calorie diet. Reduction of calorie intake is the simplest and most physiological intervention for reducing maternal weight. However, both maternal weight and diet are reduced simultaneously when dietary intake is reduced. These simultaneous changes make differentiation of outcomes due to the individual components impossible, even in experimental models. This limitation may not be a major concern in obtaining data that translate to human pregnancy since multiple factors operate in pregnant women who reduce calorie intake. Thus, our data support future conduct of similar interventions in women and provide an evidence-based background and rationale for human translation studies. Other potential interventions such as increased exercise and stimulation of calorie utilization may also provide an opportunity to differentiate the effects of weight and diet but they would also introduce other confounds.

There are currently several clinical trials on-going that are attempting to determine potential maternal behavioural and dietary modifications in obese pregnant women to improve outcomes. However, the human situation is complex and involves behavioural modifications that are very individual and the end points to be evaluated are hotly contested – weight gain and/or insulin resistance, for example (Nelson *et al.* 2010). There is a need to determine optimal timing, nature and extent of interventions. We have taken the view that the optimal time for recuperation would be prior to pregnancy and have sought to develop a model to show the ability and extent of the simplest of interventions, reducing global intake, to produce beneficial results. It could be argued that, regardless of the success of any experimental intervention, women will not be willing to take similar, effective action prior to pregnancy. The available evidence indicates that women do not spontaneously alter their dietary patterns when pregnant (Crozier *et al.* 2009). Interventions in pregnancy, as all major health areas, therefore need to be based on firm, reproducible scientific evidence. Evidence to persuade obese women to decrease their BMI either before or during pregnancy must convince them of two things. First that maternal obesity is harmful to mother and offspring in many ways and, second, that appropriately lowering their BMI and food intake will provide significant benefit to themselves and their children. The experience with the efforts that led to a dramatic decrease in cigarette smoking suggests that the strongest of compulsive behaviours can be modified when firm, incontrovertible information on benefit is provided. In the best known of all human epidemiological studies lasting over 50 years, Doll demonstrated the connection between cigarette smoking and lung cancer (Doll *et al.* 2004). One of the most persuasive pieces of evidence in those studies was the demonstration in men born around 1920, that while smoking from early adult life tripled mortality rates, giving up smoking at age 50 halved the risk and stopping at age 30 removed virtually all the risk. The parallel with maternal obesity would be that adjustment of life style with concomitant decrease in obesity would avoid the maternal and offspring hazards. Human studies indicate that maternal pre-pregnancy BMI is a major determinant of adverse offspring metabolic outcomes resulting from maternal obesity (Catalano *et al.* 2009). Therefore, in the absence of any human intervention studies, we chose to begin by recuperating the diet in our rat model before pregnancy and maintaining the recuperation throughout pregnancy and lactation. While this protocol does not allow determination of critical windows, it is an essential first step in demonstrating that recuperation can be achieved by intervention.

In summary, this study is, to our knowledge, the first to attempt to develop a model of dietary recuperation in maternal obesity in an extensively studied rodent model and provides some of the first evidence that unwanted developmental programming effects on offspring that result from maternal obesity are at least partially reversible by dietary intervention prior to pregnancy. We present these data in the hope that they provide a first step in showing the benefits of pre-pregnancy modifications that improve maternal diet and BMI. One of the key findings of this study is that it was not necessary to return the

maternal weight to the level of the controls for benefit to accrue. Further studies comparing different degrees of recuperation of maternal weight will be of interest and importance.

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Maternal obesity in the rat programs male offspring exploratory, learning and motivation behavior: prevention by dietary intervention pre-gestation or in gestation

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ABSTRACT

We studied the effects of maternal high fat diet (HFD, 25% calories from fat administered before and during pregnancy and lactation) and dietary intervention (switching dams from HFD to control diet) at different periconceptional periods on male offspring anxiety related behavior, exploration, learning, and motivation. From weaning at postnatal day (PND) 21, female subjects produced to be the mothers in the study received either control diet (CTR – 5% calories from fat), HFD through pregnancy and lactation (MO), HFD during PNDs 21–90 followed by CTR diet (pre-gestation (PG) intervention) or HFD from PND 21 to 120 followed by CTR diet (gestation and lactation (G) intervention) and bred at PND 120. At 19 days of gestation maternal serum corticosterone was increased in MO and the PG and G dams showed partial recovery with intermediate levels. In offspring, no effects were found in the elevated plus maze test. In the open field test, MO and G offspring showed increase zone entries, displaying less thigmotaxis; PG offspring showed partial recuperation of this behavior. During initial operant conditioning MO, PG and G offspring displayed decreased approach behavior with subsequent learning impairment during the acquisition of FR-1 and FR-5 operant conditioning for sucrose reinforcement. Motivation during the progressive ratio test increased in MO offspring; PG and G intervention recuperated this behavior. We conclude that dietary intervention can reverse negative effects of maternal HFD and offspring outcomes are potentially due to elevated maternal corticosterone.

1. Introduction

Maternal obesity negatively influences maternal, fetal and offspring life-time phenotype including unwanted effects on offspring brain development, behavior, affect and cognition [for review see (Sullivan et al., 2011; Tsoi et al., 2010)]. One prospective clinical study of maternal obesity outcomes reported high inattention scores and a two-fold increase in risk of difficulties with emotional regulation in 5-year-old children (Rodriguez, 2010). In animal models, maternal obesity causes brain developmental abnormalities in offspring hypothalamic and hippocampal areas, and in the serotonergic, dopaminergic and opioid systems which result in increased anxiety, impairment in spatial learning and memory and desensitization of the reward system (Bilbo and Tsang, 2010; Bouret, 2010b; Naef et al., 2008, 2011; Naef and Woodside, 2007; Sullivan et al., 2010; Tozuka et al., 2010; Vucetic et al., 2010; Walker et al., 2008; Wright et al., 2011). Since controlled, experimental dietary manipulation combined with the required intensive behavioral testing of offspring is not possible in humans, it is necessary to use animal models to examine the effects of specific models of maternal over nutrition with and without dietary intervention on offspring development and behavior.

Human epidemiological (Dabelea, 2007; Solomons, 2009; Wadhwa et al., 2009) and animal studies (Bautista et al., 2008; Bouret, 2010a; Han et al., 2004; Nijland et al., 2008; Nuyt and Alexander, 2009; Symonds et al., 2009; Taylor and Poston, 2007; Warner and Ozanne, 2010) demonstrate that the periconceptional, fetal and early post-natal nutritional environments modify the development of offspring physiological systems including cardiovascular, metabolic and endocrine function. These observations have led to the concept of a nutritional basis for the developmental origins of adult disease (Armitage et al., 2004; Warner and Ozanne, 2010). Developmental programming of offspring resulting in metabolic disorders or obesity can occur following either maternal under-nutrition (da Silva et al., 2011; Desai et al., 2007; Hyatt

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et al., 2011; Sebert et al., 2010; Zambrano et al., 2006) or overnutrition (Bayol et al., 2010; Wright et al., 2011; Zambrano et al., 2010). We have recently reported developmental programming effects of pre and/or postnatal protein restriction in rat offspring showing reduced motivation, impaired learning and decreased thigmotaxis at adult age (Reyes-Castro et al., 2011a,b; Torres et al., 2010). However, there a few data on the developmental programming effects of maternal obesity and accompanying excess nutrient intake which are becoming major concerns since more than 60% of childbearing age women in developed countries are overweight (King, 2006).

We recently reported on the potential of dietary intervention to modify offspring metabolic outcomes resulting from maternal obesity and HFD prior to pregnancy (Zambrano et al., 2010). In the present study we wished to determine if dietary intervention, *i.e.* returning the dam from a HFD to a normal diet, at different periconceptional periods would influence offspring behavioral effects. We hypothesized that in male offspring (1) maternal HFD would negatively impact aspects of anxiety related behavior, exploration, learning and motivation behaviors and (2) dietary intervention would ameliorate some of these negative outcomes in a manner dependent on the timing of the dietary recuperation. Four groups of weanling female rats were administered either control diet (CTR – 5% calories from fat), a high fat diet (HFD – 25% calories from fat) from postnatal day (PND) 21 through pregnancy and lactation (MO group), the HFD during PND's 21–90 followed by CTR diet during pregnancy and lactation (pre-gestation (PG) dietary intervention group) and the HFD from PND 21 to 120 followed by CTR diet during pregnancy and lactation (gestation (G) dietary intervention group). Male offspring behavior was assessed to determine HFD effects on anxiety, exploration, learning, and motivation and offspring improvement by maternal dietary intervention.

2. Methods

2.1. Animal care and use

2.1.1. Subjects used to produce the dams for the pregnancies studied

Female albino Wistar rats were born and maintained in the colony of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INNSZ), Mexico City, Mexico: an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International accredited facility. All procedures were approved by the Animal Experimentation Ethics Committee of the INNSZ, Mexico City, Mexico. Subjects were housed in a light-controlled environment (lights on from 07:00 to 19:00 h at 22–23 °C) and fed normal laboratory chow (Zeigler Rodent RQ 22-5, USA) containing 22.0% protein, 5.0% fat, 31.0% polysaccharide, 31.0% simple sugars, 4.0% fiber, 6.0% minerals and 1.0% vitamins (w/w), energy 4.0 kcal g^{-1} (0.2 kcal g^{-1} from fat). Between 14 and 16 weeks of age (average weight 220 ± 20 g), females were bred with a non-litter mate proven male breeder. Dams delivered naturally at term and on postnatal day 2 litters were culled to 10 pups maintaining as near a 1:1 ratio of males and females.

2.2. Experimental dams

At weaning (PND 21), the prospective mothers were randomly assigned to either a control (CTR; $n = 12$) diet that received laboratory chow or a maternal obesity diet (MO; $n = 36$) – a high fat diet containing 23.5% protein, 20.0% animal lard, 5.0% fat, 20.2% polysaccharide, 20.2% simple sugars, 5.0% fiber, 5.0% mineral mix, 1.0% vitamin mix (w/w), energy 4.9 kcal g^{-1} (1.23 kcal g^{-1} from animal lard and fat). Only one female from any one litter was assigned to a group. At PND 90, 1 month before breeding, 12 MO females were assigned at random to the dietary intervention (DINT) pre-gestation group and placed back on CTR diet for the rest of the study, *i.e.* before and during pregnancy and lactation. At PND 120 all groups were bred and the day spermatozoa were present in a vaginal smear designated as day of conception. At this time 12 MO females were assigned at random to the DINT group during gestation (G) and switched to CTR diet for the rest of the study, *i.e.* pregnancy and lactation. The other 3 groups were fed their pre-pregnancy diet throughout pregnancy and lactation (see Table 1 for groups). At 19 days of gestation 6 dams from each of the 4 groups were euthanized to collect serum for corticosterone measurements. All remaining dams (6 per group) delivered spontaneously. The day of delivery was considered as PND 0. Food and water were available *ad libitum*. Pregnant and lactating rats were weighed every day through pregnancy and until pups were removed at weaning.

2.3. Maintenance of offspring

Litter size and pup weight were recorded at birth. Ano-genital distance, anterior–posterior abdominal distance and head diameter were measured with calipers. Our published data indicate that ano-genital distance is 1.67 ± 0.13 mm ($n = 291$ pups from 43 litters; mean ± SEM) in female pups and 3.26 ± 0.22 mm ($n = 252$ pups from 43 litters) in males at birth (Zambrano et al., 2005a). Since a value of 2.5 mm is more than 2 SDs from the mean of either group, sex was judged according to whether the ano-genital distance was >2.5 mm for males. To ensure homogeneity of offspring evaluated, all litters studied were adjusted to 10 pups per dam. The sex ratio was maintained as close to 1:1 as possible. Pups continued to be weighed every week.

2.4. Elevated plus maze (EPM)/open field

Two weeks prior to all behavioral testing, a reverse light cycle was implemented (lights off at 7 a.m. and on at 7 p.m.) with testing occurring during the dark phase. Subjects were assessed 7 days a week at the same time of the dark cycle for each subject between 8 a.m. and 4 p.m. At PND 75 six male unrelated naïve subjects per treatment group were tested. The specifications of data collection, the EPM and the open field apparatus have been described in detail (Reyes-Castro et al., 2011a).

2.5. Operant conditioning

On PND 80 six unrelated male offspring from different litters per diet group were tested in operant chambers (E10-10TC, Coulbourn-Instruments, PA, USA) as previously described (Reyes-Castro et al., 2011b). Two weeks prior to onset of operant training offspring were placed on water deprivation for 23 h/day with 1 h of free access. This continued throughout training and testing with the 1 h of free access immediately following behavioral sessions. For each trial the lever was extended until pressed, after which the subject was allowed 120 s to approach the reward magazine and respond with a nose poke. The registration of the nose poke into the reward magazine by the photocell receptors started the feeding for 10 s. Each trial was followed by an inter-trial interval of 5 s during which the lever was retracted. FR-1 conditioning was complete when subjects earned 20 reinforcements during a 15-min session. After all subjects reached this criterion, they were introduced to a FR-5 schedule with the identical performance criteria as for the FR-1 schedule albeit with 5 responses required per trial.

2.6. Progressive ratio testing

Following operant conditioning, each subject commenced progressive ratio testing for 10 days. In the progressive ratio schedule, an additional lever press is required for all subsequent reinforcements for the first eight reinforcements (progressive ratio + 1). For instance, one press for the first reinforcement and four presses for the fourth reinforcement. Following every eighth reinforcement, the response increment doubles and hence the number of lever presses required to obtain successive sucrose reinforcements was as followed: progressive ratio + 1 = 1, 2, . . ., 8; progressive ratio + 2 = 10, 12, . . ., 24; progressive ratio + 4 = 28, 32, . . ., 56; progressive ratio + 8 = 64, 72, . . ., 120; progressive ratio + 16 = 136, 152, . . . etc. Progressive ratio sessions were 30 min in length.

2.7. Free sucrose consumption

To assess sucrose consumption behavior, subjects were given direct access to bottled sucrose solution (7%) for 30 min in the familiar colony room 1 day after the last progressive ratio session. For this evaluation subjects were single caged and sucrose consumption was calculated by subtraction of the bottle weight at the end of the session from the initial weight. This procedure was performed on 3 consecutive days.

2.8. Corticosterone measurements

In dams at 19 days of gestation and in the male offspring on PND 110, subjects were sacrificed by decapitation and blood samples taken from the neck to determine corticosterone serum levels. Blood samples were centrifuged at 4 °C for 15 min at 3500 rpm to remove red blood cells and serum stored at −20 °C until all samples were analyzed. Corticosterone serum levels were determinate by radioimmunoassay using a commercial rat kit, DPC Coat-a-count (TKRC1) from Diagnostic Products (Los Angeles, CA, USA). Intra- and inter-assay variability was <6% and <7%. The kit was used in accordance with manufacturer's instructions and samples were measured in duplicate.

2.9. Statistical analyses

All data are presented as Mean ± SEM, alpha level was set at 0.05. Behavioral endpoints and corticosterone levels were analyzed by ANOVA with between-subject factor of early life manipulation (maternal diet during the different periods). Post hoc analyses were performed by Tukey test using Sigma Stat 3.5.

Table 1
Experimental groups.

Groups	Maternal diet				Offspring diet
	PND 21–90	PND 90–120	Pregnancy	Lactation	
Control (CTR)	Control	Control	Control	Control	Control
Maternal obesity (MO)	High fat	High fat	High fat	High fat	Control
Pre-gestational dietary intervention (PG)	High fat	Control	Control	Control	Control
Gestational dietary intervention (G)	High fat	High fat	Control	Control	Control

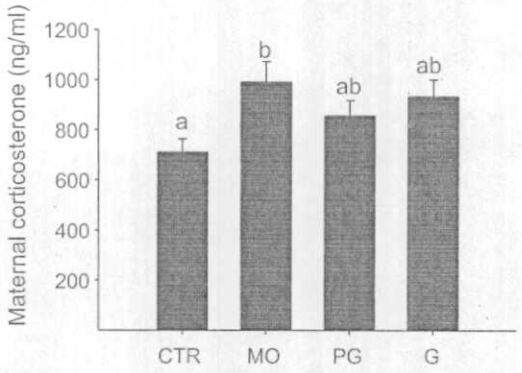

Fig. 1. Maternal serum corticosterone levels at 19 days gestation. Mean ± SEM, $n = 6$ dams. Data not sharing a letter are statistically different, $p < 0.05$.

3. Results

3.1. Maternal corticosterone

Maternal corticosterone was higher at 19 days gestation in MO than CTR dams (Fig. 1, $p < 0.05$) with intermediate levels in the PG and G which did not reach significance.

3.2. Elevated plus maze

On PND 75 male offspring were administered the EPM to measure anxiety related behaviors. Offspring displayed no differences in the number of entries, time spent or distance traveled in the open arms, or total distance traveled (Fig. 2A–D).

3.3. Open field

Analyses of behavioral endpoints in the open field test revealed differences in experimental offspring. The MO and G offspring had increased border zone entries compared to CTR offspring (Table 2, $p < 0.05$). The G offspring had increased center zone entries *versus* CTR and increased center zone distance traveled compared to CTR and PG offspring (Table 2, $p < 0.05$). No differences were found for total distance, border zone time, border zone distance, and center zone time (Table 2).

3.4. Operant conditioning and progressive ratio

In offspring initial exposure to the operant chamber revealed differences in the number of sessions before approach and response to the reinforcement contingent lever. The MO, PG and G offspring required more sessions to respond *versus* the CTR offspring (Fig. 3A, $p < 0.05$). For fixed ratio 1 schedule of reinforcement (FR-1), group differences were determined for the number of sessions before

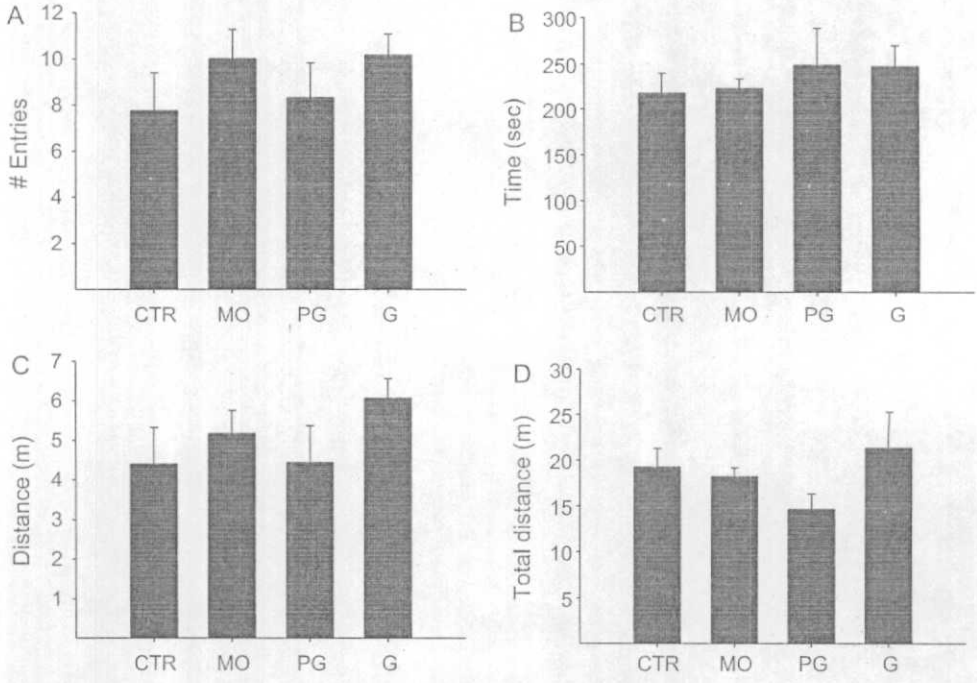

Fig. 2. Elevated plus maze endpoints. (A) Open arm entries, (B) open arm time (s), (C) open arm distance traveled (m), (D) total distance traveled (m). Mean ± SEM, $n = 6$ from different litter. Data not sharing a letter are statistically different, $p < 0.05$.

Table 2
Open field.

Group	Total distance	Border zone entries	Border zone time	Border zone distance	Center zone entries	Center zone time	Center zone distance
CTR	44.3 ± 4.8	12.3 ± 1.7[a]	519 ± 45.7	39.1 ± 4.2	11.6 ± 1.9[a]	81 ± 45.7	5.2 ± 0.9[a]
MO	56.6 ± 10.8	22.3 ± 2.9[b]	539 ± 6.0	48.7 ± 10.2	21.3 ± 2.9[ab]	61 ± 5.9	7.9 ± 0.9[ab]
PG	50.5 ± 5.0	16.3 ± 2.4[ab]	547 ± 9.3	43.8 ± 4.1	15.8 ± 2.5[ab]	53 ± 9.3	6.6 ± 1.2[a]
G	48.5 ± 4.5	25 ± 6.2[b]	461 ± 17.4	36.5 ± 3.2	25.3 ± 2.6[b]	139 ± 17.4	12.0 ± 1.6[b]

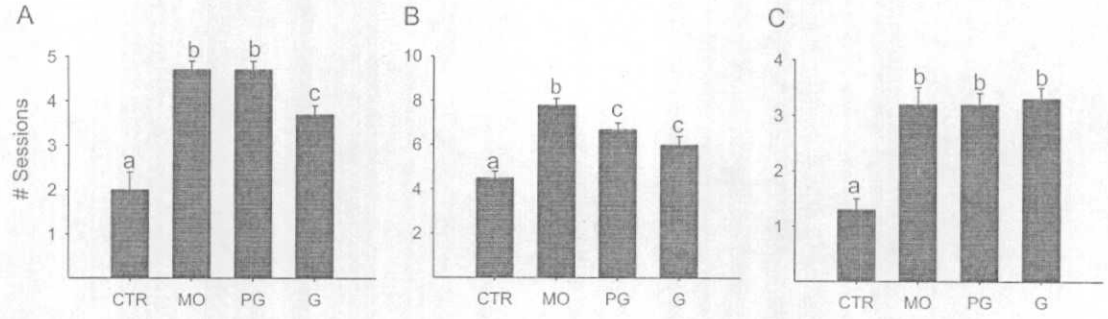

Fig. 3. (A) Number of sessions required for offspring to press the operant lever for initial positive reinforcement. (B) Number of sessions required for offspring to attain FR-1 performance criterion. (C) Number of sessions required for offspring to attain FR-5 performance criterion. Mean ± SEM, $n = 6$ pups. Data not sharing a letter are statistically different, $p < 0.05$.

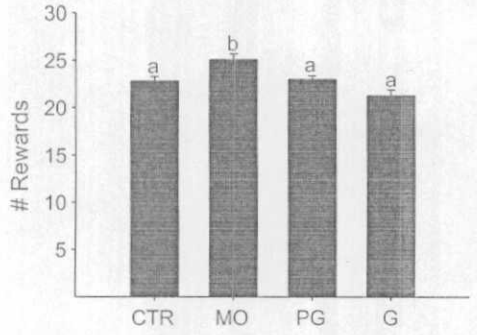

Fig. 4. Number of rewards received during progressive ratio sessions. Mean ± SEM, $n = 6$ pups. Data not sharing a letter are statistically different, $p < 0.05$.

offspring attained criterion. The MO, PG and G offspring all required more sessions to attain performance criterion with the MO group requiring the most sessions (Fig. 3B, $p < 0.05$). Fixed ratio 5 schedule of reinforcement (FR-5) shows MO, PG and G offspring all required more session to reach performance criterion *versus* CTR offspring (Fig. 3C, $p < 0.05$). Effects on motivation as assessed by progressive ratio tasks show increased responding in MO offspring compared to CTR, PG and G groups (Fig. 4, $p < 0.05$).

3.5. Free sucrose consumption

No overall treatment effect was determined for free access to 7% sucrose during three 30 min sessions (Table 3).

3.6. Offspring corticosterone

Corticosterone male serum levels were measured on PND 110. The MO offspring show decreased corticosterone levels compared to the PG and G offspring but not *versus* CTR offspring (Fig. 5, $p < 0.05$).

Table 3
7% sucrose consumption during 30 min (average of 3 days).

	CTR	MO	PG	G	ANOVA
(ml)	18.4 ± 0.3	17.3 ± 0.3	18.5 ± 0.3	18.0 ± 0.3	$P = 0.051$

4. Discussion

Developmental exposure to environmental challenges in offspring can influence various aspects of behavior. In a model of maternal obesity in the rat (Zambrano et al., 2010), we sought to determine the behavioral effects in male offspring born to dams administered a HFD from PND 21 through pregnancy and lactation (maternal obesity, MO group), HFD from PND 21 to 90 and switched to control diet 1 month before mating and during pregnancy and lactation (pre-gestation dietary intervention, PG group) and from PND 21 to 120 but not during pregnancy and lactation (gestational dietary intervention, G group). The different windows of HFD regimens administrated produced physiological differences in the mothers (Zambrano et al., 2010), of particular importance, maternal corticosterone serum levels at 19 days of gestation were increased in the MO group but to a lesser degree in the PG or G groups. Increased maternal corticosterone has been demonstrated in many situations that result in developmental programming of offspring by altered maternal nutrition and may constitute a common feature that explains some of the similarities in outcomes from

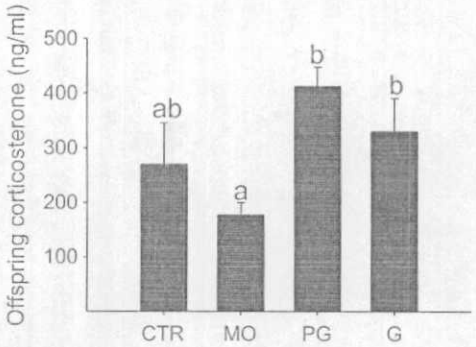

Fig. 5. Offspring corticosterone serum levels. Mean ± SEM, $n = 6$ from different litter. Data not sharing a letter are statistically different, $p < 0.05$.

different challenges (Cottrell and Seckl, 2009; Langley-Evans, 2009; Zambrano et al., 2005b; Guzman et al., 2006).

4.1. Anxiety related behavior and exploratory effects

Consistent with our findings here, anxiolytic effects in offspring following early postnatal overfeeding in male and female offspring (Spencer and Tilbrook, 2009) and following maternal HFD during lactation in male offspring have previously been reported (Wright et al., 2011). Those studies demonstrated that raising rats in small litters (Spencer and Tilbrook, 2009) or dams fed a cafeteria diet during pre-gestation, gestation and/or lactation (Wright et al., 2011) induces obesity in offspring and reduces anxiety in the EPM and the open field. Additionally, chronic consumption of a HFD during pregnancy causes perturbations in the serotonergic system, such as increased expression of tryptophan hydroxylase 2 and serotonin 1 A receptor in the rostral raphe nucleus, and increases anxiety-like behavior in rhesus monkey offspring (Sullivan et al., 2010).

In this study the anxiolytic effects were not apparent in the experimental offspring during EPM but were observed during the open field test. These inconsistent behavioral responses in the EPM and open field tests are not surprising since studies indicate that behavioral tests that rely on unconditioned responses assess different aspects of affect and that emotional reactivity is multidimensional (Ramos, 2008; Ramos et al., 2008; Trullas and Skolnick, 1993; Vendruscolo et al., 2003). A current review contends that the EPM, open field apparatus and the light/dark box tests should be administered concomitantly to more adequately assess affect since time and/or sequence of test administration can influence outcomes (Ramos, 2008). One potential effect of testing sequentially across days is that effects are reduced as testing progresses (Ramos, 2008). However, here we report negative results in the first task administered (EPM) and positive results in the subsequent task (open field) which makes the preceding argument less likely. Alternatively, inconsistent results obtained across tests may be due to construct differences between tests or to uncontrolled, intra-individual fluctuations in behavior (Ramos, 2008).

4.2. Learning effects

During FR-1 and FR-5 operant conditioning MO, PG and G offspring displayed impaired learning. So in experimental offspring, maternal dietary intervention did not prevent these particular cognitive deficiencies. In support of our findings in the MO group, previous studies report hippocampal brain derived neurotropic factor is decreased along with hippocampal neurogenesis and impairment in spatial learning in mice offspring exposed to a HFD during pregnancy and lactation (Tozuka et al., 2009, 2010). It should be noted that maternal dietary intervention at either period, 1 month before pregnancy or at the beginning of pregnancy, did not prevent learning impairment suggesting that maternal diet long-term prior to conception is critical for male development.

We have previously measured elevated corticosterone, estradiol, testosterone (Zambrano et al., 2005b) and progesterone (Guzman et al., 2006), concentrations near term (19 days gestation) in prenatal protein restricted rat dams with subsequent cognitive impairment in offspring (Reyes-Castro et al., 2011b, 2012). In the present study maternal corticosterone levels were increased in MO dams and marginally, though not significantly, increased in the PG and G dams. Maternal steroids can cross the placenta, and such exposure to transplacentally acquired androgens and glucocorticoids in fetal life can result in developmental perturbations which could have a role in the current behavioral findings since human studies report impairment of spatial learning ability in males exposed to excess levels of androgens *in utero* (Meyer-Bahlburg, 2011; Puts et al., 2008). There is also abundant evidence of excess levels of glucocorticoids *in utero* impairing brain development and later behavior in humans and animal models (Antonow-Schlorke et al., 2001, 2003; French et al., 2004; Johnson et al., 1981; Karemaker et al., 2006, 2008; Matthews, 2001; Rodriguez et al., 2011; Seckl, 2008; Szuran et al., 2000; Uno et al., 1990, 1994; Weinstock, 2008) which could explain the cognitive deficits demonstrated in this study.

4.3. Motivation effects

During motivation assessment, MO offspring display increased motivation. In this context, dietary intervention normalized motivation in male PG and G offspring. The increased motivation displayed by the MO offspring is consistent with models showing increased appetitive and/or consummatory drive following maternal or early life over nutrition (Chang et al., 2008; Desai et al., 2007; Sebert et al., 2009). Similar to the MO offspring outcomes in this study, rats born to dams fed a junk food diet during gestation and lactation develop hyperphagia and a preference for fatty, sugary and salty foods over protein-rich foods compared to offspring fed a balanced chow diet prior to weaning or during lactation alone (Bayol et al., 2008). In the present study, effects of maternal HFD on offspring sucrose consumption behavior were measured there was no overall effect of perinatal diet, so the influence of consummatory behavior on progressive ratio (motivation) behavior is not applicable.

5. Conclusions

Maternal HFD administration produces an altered behavioral phenotype in male offspring. Dietary intervention recuperated or ameliorated certain indices in dams and offspring. In dams, corticosterone levels were reduced in the PG and G groups to between CTR and MO levels. However effects on offspring learning could still have been affected by the increased levels of maternal prenatal corticosterone as all experimental offspring displayed learning impairment. Prenatal exposure to increased levels of glucocorticoids changes hypothalamic pituitary adrenal axis function as well as associated receptors expression levels [for review see (Kapoor et al., 2008)]. Normal levels of motivation were restored by PG and G intervention. Additionally during exploratory behaviors, PG intervention prevented the increased exploration behavior displayed by the MO and G offspring in the open field. These findings show the importance of optimizing maternal diet and avoiding the complication of obesity. It also holds out the hope that recuperation of the diet prior to pregnancy can have beneficial long-term effects.

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Interventions designed to prevent adverse programming outcomes resulting from exposure to maternal obesity during development

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Abstract

Maternal obesity is a global epidemic affecting the developed and developing world. Human and animal studies indicate that maternal obesity programs development predisposing offspring to later-life chronic diseases. Several mechanisms act together to produce these adverse health problems. There is a need for effective interventions that prevent these outcomes and guide management in human pregnancy. We report here dietary and exercise intervention studies in both altricial and precocial species, rats and sheep, designed to prevent adverse offspring outcomes. Both interventions present exciting opportunities to at least in part prevent adverse metabolic and other outcomes in mother and offspring.

INTRODUCTION

Worldwide nearly 1.5 billion people are overweight (body mass index – BMI - greater than 25 kg/m^2) or obese (BMI greater than 30 kg/m^2) [1]. Almost every country is affected: Mexico,32% of women of reproductive years are obese [2, 3]; USA, 35% of women of reproductive years are obese [4]; Brazil 50% of the population is overweight or obese [5, 6]; United Kingdom 33% of pregnant women are overweight or obese [7]; India 26% of women of reproductive years are overweight and 8% obese [8]; China 16% of women are overweight or obese [9]; Ghana 64.7% of women are either overweight or obese[10]. The WHO (www.who.int/nut/obs.htm) has declared obesity one of the top ten adverse health risk conditions in the world and one of the top five in developed nations.

As pointed out in our previous review "Animal Studies that Reveal Mechanisms of Programming of Offspring Outcomes of Maternal Obesity", maternal obesity programs

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offspring predisposition to a wide variety of chronic, later-life diseases. A better understanding of developmental programming requires integration of factors involved in challenges, mechanisms and outcomes involved. Reports of experimental interventions in animal models in the setting of obesity are scarce [11, 12]. Using nutritional or targeted therapeutic interventions during windows of developmental programming studies in some experimental animal models have shown potential reversibility of unwanted offspring metabolic outcomes [12]. For example, leptin treatment of neonatal female rats born to undernourished mothers prevents development of metabolic compromise in adulthood [13]; maternal antioxidant supplementation in rats fed with the Western diet partially prevents offspring adiposity and normalizes glucose tolerance [14]. In other studies it has been shown that genistein supplementation in mice during gestation protects offspring susceptibility to obesity [15]. This chapter will focus on animal studies designed to illuminate mechanisms by which interventions involving a change in maternal diet or level of exercise may act to improve maternal and offspring outcomes.

There are many reasons why animal intervention studies are needed. Importantly, animal studies are much more controllable than human clinical interventions which is the parallel human approach to hypothesis driven animal research. In addition a greater depth of mechanistic interrogation is possible resulting from tissue retrieval and multiple testing in animal studies and results are obtained much more quickly to guide management in human pregnancy. Reproducibility and independent confirmation, the indispensible requirements of scientific certainty, are also generally easier to achieve in animal studies. Carefully designed clinical trials to determine effects of interventions and improve maternal health in pregnancy and offspring outcomes are now in progress [16]. However, in addition to the length of time needed to obtain the required data, clinical trials have to contend with multiple confounds related to the mother's socioeconomic status and pre-pregnancy health that not only make their analysis and interpretation difficult but also may limit their usefulness in determining mechanisms. There is a pressing need, as the recent IOM report indicates [17], for the development of evidence-based interventions that inform and motivate pregnant women to adopt a healthy lifestyle before and during pregnancy. Currently there is much interest in both maternal diet and exercise as potentially modifiable factors to use as interventions [18-20]. The optimal timing and extent to which adverse effects of the maternal metabolic phenotype resulting from maternal obesity and associated high calorie diets can be prevented and/or possibly reversed by these interventions remain unanswered questions of considerable physiological interest and importance in clinical obstetric management. Most authorities believe that interventions introduced before conception will have the best results. It should always be born in mind that poor maternal nutrition also programs adverse offspring outcomes [21-23] and sudden and excessive restriction of maternal and fetal nutrient availability may well introduce new dangers. Thus firm scientific data are needed to guide interventions.

When considering the goals of specific interventions to beneficially impact developmental programming outcomes, a distinction must always be made between interventions designed to prevent negative offspring outcomes and interventions conducted at later stages of an offspring's life to reverse adverse health outcomes. Clearly prevention is a better strategy

than to try to reverse problems. The present review focuses on maternal intervention to prevent negative offspring outcomes by maternal obesity.

STUDIES ON PRE-PREGNANCY DIETARY INTERVENTION IN THE OBESE RAT

Investigation of programming of offspring by maternal obesity requires that investigators ensure initial phenotypic homogeneity of the different groups of mothers studied - controls, obese mothers and mothers in which interventions are introduced. Care is necessary to achieve this important goal when females who will be the study mothers are purchased from commercial vendors without information on their background. Specific information should be obtained as to the lineage of all females to avoid inclusion of sibling females in the same sub-group. To avoid this and related problems we maintain our own colony of non-pregnant females which are bred to deliver the female pups that will be recruited as the mothers in our studies. All rats are maintained on the same laboratory chow unless exposed to an experimental diet [24]. At delivery (postnatal day – PND- 0) litters are culled to 10 pups, each litter containing at least four female pups. This standardization is important since programming effects have been shown in offspring according to different litter sizes reared by mothers during lactation [25].

At weaning (PND 21) female offspring are randomly assigned to either a control (C) group fed normal laboratory chow or to a maternal obesity group (MO) fed a high-energy, obesogenic diet containing 23.5% protein, 20% animal lard, 5% fat, 20.2% polysaccharide, 20.2% simple sugars, 5% fiber, 5% mineral mix, 1% vitamin mix (w/w), energy 4.9 Kcal/g. Only one female from any one litter is assigned to any study group. At PND 90, one month before breeding, half of the obese females are selected at random for the dietary intervention (DINT) group and placed back on the C diet for the rest of the study including pregnancy and lactation. The remaining obese females continue on the high fat diet during pregnancy and lactation. We have chosen to breed females at 120 days as we have shown that at younger breeding ages the mothers are still growing – albeit not as fast as earlier in life - and offspring outcomes of key factors such as growth, triglycerides and leptin are affected by maternal age as well as the nutritional challenge (unpublished data). At PND 120 all three groups C, MO and DINT are bred and fed their pre-pregnancy diet throughout pregnancy and lactation. All mothers deliver by spontaneous vaginal delivery. Day of delivery is considered as post natal day 0 [24, 26] (Figure 1).

Changes in maternal and offspring phenotypes resulting from MO and DINT

At breeding MO females were 16% heavier than controls, equivalent to a pregnant women increasing her BMI from 25 (the top of the normal BMI range) to 30.5 (the lower end of the obese range). DINT females were 9 percent heavier than controls (equivalent to BMI in the mid-overweight range) at breeding. Maternal serum leptin at the end of lactation was higher in MO than C. Leptin levels in the DINT group were similar to controls [27].

The effects of MO and DINT have only been reported in male offspring [27]. No differences in body weight were seen between pups at birth and at weaning. At weaning MO offspring

had more subcutaneous fat tissue, and higher serum triglycerides and leptin than C offspring – showing dysregulation of lipid metabolism; pre-pregnancy maternal dietary intervention prevented these increases offspring measures. This important finding shows the limitations of weight alone at any age as an assessment of outcomes. Body composition is much more important in predicting future offspring health. Serum glucose did not differ between the three offspring groups, but offspring serum insulin was elevated in MO and returned to C levels in DINT offspring indicating the presence of insulin resistance in MO offspring [27].

At PND 120, male MO offspring had elevated resting serum glucose and insulin and increased insulin resistance compared with C offspring. Insulin remained elevated in DINT compared with C offspring while blood glucose did not differ from either of the two other groups [27]. It is important to note that all offspring were on the same post-weaning diet. Thus there was no opportunity for effects of an increase in offspring dietary intake acting as a *"second hit"* on the background of developmental programming that had already occurred.

STUDIES ON PRE-PREGNANCY EXERCISE INTERVENTION IN THE OBESE RAT

We have recently completed a study of effects of an exercise intervention on mother and offspring of obese and control rats. General management of the pregnancies and lactation were as described above for the dietary intervention. At PND 90, one month before breeding, one half of the group of C non-pregnant females and half of the MO female groups were selected at random to continue on their diet and begin wheel-running exercise (C exercised – CEx; MO exercised - MOEx) (Figure 2). Mothers continued to be placed in the wheel through pregnancy. All females continued on their respective diets. CEx and MOEx rats were trained to wheel run on two separate days the week before they reach PND 90. A training session lasted 15 min which we established was the optimum running schedule that was always completed, followed by a 15 minute rest period and a second 15 minute run. Rats were allowed two days rest every seven days. Before pregnancy, all rats completed the 30 minutes running while during pregnancy rats were placed in the wheel for only one 15 minute session per day and the amount of voluntary exercise completed varied between animals especially in late gestation. During lactation mothers nursed their pups and did not exercise. Therefore, lactating mothers were maintained on their pregnancy diet and not placed in the wheel.

Changes in maternal phenotype and fertility resulting from maternal exercise prior to and during pregnancy

Calorie and food intake per day were similar in all four groups and exercise did not affect calorie or food intake in either group. Exercise had no effect on maternal weight at any stage except for the CEx group in which exercise decreased body weight in comparison with C at parturition. There were no differences between groups in average distance run per session before pregnancy or in the first 15 days of pregnancy but interestingly MO rats that were exercised ran further than control rats in the last few days of pregnancy. We hypothesize that this difference was a result of their lower circulating estrogen levels since a negative correlation has been shown to exist between estrogen levels and physical activity [28].

As in the DINT study mentioned above, at the end of lactation maternal insulin, glucose, HOMA, leptin, triglycerides and cholesterol were all elevated in MO mothers. Exercise did not alter these variables in C mothers but prevented the changes in all these variables except leptin in MO.

Effect of maternal obesity and exercise on male offspring metabolism at postnatal day 36

We evaluated offspring outcomes since the goal of interventions in MO pregnancy is to improve both maternal and offspring outcomes. At offspring PND 36, one male offspring from each litter (n= 8) was chosen at random, fasted for 4 hours and euthanized by decapitation, trunk blood samples obtained and fat depots excised and weighed. Litter size, litter weight individual pup body weight or sex ratio at birth was not affected by either MO or exercise. In male offspring body weight, cholesterol and insulin were not different at PND 36 between the four groups. Maternal obesity increased offspring leptin, triglycerides and fat mass in males. Exercise in MO prevented the male offspring MO leptin increase and partially prevented the increased triglycerides. Maternal exercise in C reduced male offspring glucose and male HOMA. The importance of paying attention to the phenotype of study mothers is shown by the interesting observation that maternal exercise decreased weight and cholesterol in control offspring indicating that even animals recruited as controls may be affected by experimental protocols.

Maternal voluntary exercise intervention has been previously reported in lean pregnant rats in two different models. Male, but not female, offspring of exercised mothers show increased percent lean mass and decreased fat mass percent compared to male offspring from controls, showing that maternal exercise can affect offspring [29, 30]. These effects on offspring metabolic phenotype show similarities to the effects with our present study in control mothers. In another study normal, lean, pregnant rats performed voluntary exercise [31, 32] training from 42 days before pregnancy and continued on to day 19 of gestation with the result that maternal plasma antioxidant status was improved. Both of these studies provide important data for design of studies in obese mothers.

Comparison of DINT and Exercise models

One of the important differences between the DINT and the exercise intervention model is the maternal weight. In the DINT model [27] the increased maternal body weight of the DINT group at breeding was partially prevented, and there were no differences in maternal body weight during pregnancy and lactation between DINT and C. In contrast, maternal exercise intervention did not modify the maternal body weight at any stage. The weight of MOEx mothers was the same as MO before and during pregnancy as well as during lactation. However, for both models, mothers undertaking the intervention presented a better metabolic and hormonal maternal environment than MO with regards to offspring outcomes.

In a completely independent study we compared all groups with both interventions (maternal dietary and exercise interventions. Corticosterone was increased in serum of MO mothers prior to breeding. Maternal DINT and exercise intervention before pregnancy decreased maternal corticosterone concentration but MOEx values were not returned to those in C (Fig 3 A). A similar picture was seen at the end of lactation (Fig 3 B) and in the

neonate and young adult male offspring (Fig 3 C and D). These changes may be protective mechanisms of future metabolic problems in the offspring by MO.

Both maternal interventions improved the adverse offspring metabolism outcomes produced by MO, but the improvement was different in the two models. Maternal DINT partially prevents glucose, insulin, HOMA, fat and fat cell size, and completely prevents leptin increases offspring by MO. Maternal exercise intervention partially prevents fat and insulin and completely glucose, HOMA and fat cell size increase in offspring by MO.

For both models the data indicate that there were different changes in metabolism in various tissues since no differences in food intake and body weight were found in the young adult offspring. The two intervention models appear to benefit offspring metabolism in diverse ways suggesting dissimilar maternal mechanisms. Excessive gestational weight gain has been associated with adverse maternal pregnancy outcomes. Catalano has clearly indicated that maternal pre-pregnancy BMI is a major factor in determining maternal and offspring outcome [33]. One important finding of the exercise intervention study was that the adverse offspring outcomes produced by MO are the result of maternal metabolic changes and/or in increased maternal corticosterone and not due to maternal body weight since MOEx prevented many of the MO offspring outcomes offspring without any change in maternal calorie intake or body weight. This finding again shows that outcomes are more related to body composition rather than body weight. The overwhelming evidence in favor of the importance of body composition clearly indicates that the most successful interventions will at least contain a component of intervention prior to pregnancy. Addition of interventions during pregnancy will further build on the interventions prior to pregnancy.

In our report of effects of maternal dietary intervention on offspring outcomes in the setting of obesity we wrote: *"There is a need to determine optimal timing, nature and extent of interventions. We have taken the view – as others have done - that the optimal time for recuperation would be prior to pregnancy and have sought to develop a model to show the ability and extent of the simplest of interventions, reducing global intake, to produce beneficial results. ... The available evidence indicates that women do not spontaneously alter their dietary patterns when they discover they are pregnant* [34]. *Interventions in pregnancy, as in any other major health area, therefore need to be based on firm, reproducible scientific evidence"* [27].

Obese women contemplating pregnancy need to be provided with firm information as to the benefits that accrue from decreasing their BMI both before and during pregnancy for at least two reasons. First, they need to be aware of the biological reasons that maternal obesity is harmful to themselves and their baby in many ways. Secondly they need to be confident that appropriately lowering their BMI and food intake will provide significant benefit to themselves and their children.

Influence of interventions on offspring Aging

Maternal low protein diets accelerate aging in rat offspring [35]. We have shown increased adiposity index, leptin, and triglycerides (TG) in male OFF of MO mothers in young adult life (postnatal day110) [27] with no changes in body weight. However by 650 days MO results

in a more rapid aging of some metabolic indices such as body weight (Figure 4, 5 and 6), fat and adiposity index, increases which were prevented by maternal dietary and exercise intervention (Figure 4).

One good example of positive features of programming is seen in the offspring of CEx mothers which, at this early stage of aging (PND 650), had a better metabolic phenotype than the rest of the groups, including the control group.

STUDIES ON DIETARY INTERVENTION IN OBESE PREGNANT SHEEP

As discussed in our earlier review of developmental programming ("Animal Studies that Reveal Mechanisms of Programming of Offspring Outcomes of Maternal Obesity", there are differences between pregnancy in altricial, polytocous mammals such as the rat, and precocial, monotocous species such as humans, sheep and nonhuman primates. The pregnant sheep has been extensively investigated to determine the impacts of decreased maternal nutrition but fewer studies have been conducted on effects of maternal overnutrition/obesity on fetuses and offspring in this important, precocial, experimental species [36-44]. Although there are differences in some capabilities – locomotion for example - both sheep and pregnant women produce well-developed, precocial offspring, exhibit similar newborn to maternal weight ratios, and temporal pattern of fetal tissue and organ development. Further, investigators worldwide have utilized the fetal sheep as a biomedical model to design studies on human pregnancy such as fetal behavior, heart rate and sleep states [45-48].

Our studies on the impact of maternal overnutrition/obesity in the ewe on fetal growth and development and offspring health are conducted with animals from a well-characterized, closed flock at the Center for the Study of Fetal Programming, University of Wyoming. Ewes of similar, size and breeding are maintained in the source flock developed from lambs born within the flock whose mothers were fed National Research Council (NRC) feed requirements throughout pregnancy and lactation. The ewe lambs are then maintained on the same diet and are used as the mothers in all studies and are housed together and fed only to NRC requirements from weaning to maturity. This management policy provides assurance that animals have not been exposed to highly variable environments prior to any investigation and thus limits the chance of markedly different environmental (epigenetic) influences on study results, and other influences such as sibships within groups.

We have developed and characterized a model of maternal overnutrition/obesity (MO) where ewes are fed a highly palatable pelleted diet at 150% of requirements from 60 days before conception through pregnancy. On this diet, ewes become obese by the time they are bred and continue to gain additional weight throughout pregnancy and fetuses show a definitive endophenotype [36-39, 43, 44, 49, 50]. Overweight and obesity at conception in pregnant women has been shown to have the greatest impact on increasing adiposity of infants at birth, leading to insulin resistance and exhibit obesity in later life. In our model of diet–induced MO, lambs are born with increased adiposity, and by 19 months of age they exhibit hyperphagia, glucose and insulin dysregulation and increased adiposity compared to offspring of ewes fed only to requirements [38]. Previous studies in our laboratory demonstrated that maternal undernutrition (50% global undernutrition) starting at day 28 of

gestation resulted in delivery of offspring who exhibited metabolic disturbances (i.e. they were hyperphagic, insulin resistant, and were obese) as adults [51]. We therefore hypothesized that a dietary intervention in which the obesogenic diet is reduced from 150% to 100% of NRC requirements (MO intervention, MOI) beginning on day 28 of gestation would be early enough to at least in part prevent the negative impacts of maternal overnutrition/obesity on the fetus and resulting offspring. Further, day 28 in the sheep is equivalent to ~day 50 in human pregnancy, which is about the time when women confirm they are pregnant and early enough for their obstetrician to provide overweight/obese women with a corrective dietary regimen if deemed necessary.

MOI eliminated MO-induced fetal macrosomia at mid-gestation, and either reduced (right ventricular weight, liver weight) or prevented MO-induced increases in organ weight (left ventricular weight, total kidney weight, pancreatic weight, and total perirenal fat weight). At day 135, while fetal weight was similar between CON, MO and MOI fetuses, MO fetuses exhibited greater left ventricular weights and thicknesses, right ventricular thicknesses, total kidney weight, and total perirenal fat, and reduced pancreatic weight than CON fetuses. Weights and thicknesses of these organs and tissues were returned to CON levels in the MOI fetuses. The data provide the first indication that alterations in fetal organ and tissue growth as well as endocrine changes (see below) can at least in part, be prevented by early pregnancy MOI in the face of maternal obesity.

To date we have only evaluated the cortisol changes in the MOI model in order to observe any similarities with our findings in the obese rat model described above (Fig 3). MO increased both maternal and fetal cortisol at 0.9G and this increase was prevented by MOI at both ages. Similar results were obtained at 0.9G. Interestingly, while the maternal increases in cortisol were accompanied by increased ACTH, this was not so in the fetus where cortisol but not ACTH, was increased above CON in MO at both ages. We hypothesize two possible mechanisms for this finding. First, MO may change adrenal sensitivity to ACTH. The second hypothesis is that much of the fetal cortisol in the setting of maternal obesity is produced in peripheral fetal tissues by increased activity of 11BHSD1, converting inactive cortisone to active cortisol. We have shown that the 11BHSD1 system is up-regulated in fetal female perirenal fat and fetal male liver in the setting of maternal under-nutrition supporting a potential role for increased 11BSD1 activity in response to maternal dietary challenges [52]. Importantly, it remains to be seen to what extent inhibition of the increase in fetal cortisol will prevent adverse side effects of MO on offspring. These findings illustrate clearly the value of the combining studies in precocial and altricial species. We are currently evaluating CON, MO and MOI offspring to determine whether reducing maternal nutrition to recommended levels in early pregnancy of overnourished/obese ewes prevents endocrine and metabolic disturbances in offspring in adult life.

How do we get the message of the need for interventions to the general public? Lessons from the anti-smoking campaign

Improved women's health and especially the institution of effective corrective measures is vitally important to obtain the optimal obstetric outcomes in the face of the current epidemic of obesity in women of reproductive years. There may be lessons that can be learned from

the success in the developed world in decreasing the incidence of smoking. The anti-smoking campaign has met with considerable success in the developed world at least. This success suggests that even the strongest of compulsive behaviors can be modified when firm, incontrovertible information on benefit is provided. It has taken over 50 years since Sir Richard Bradford Doll demonstrated the connection between cigarette smoking and lung cancer [53]. Changing this self-destructive behavior has taken decades but the decrease in smoking has saved thousands of lives. One of the most persuasive pieces of scientific evidence in the antismoking campaign was the demonstration in that while smoking from early adult life tripled mortality rates, giving up smoking at age 50 halved the risk and stopping at age 30 removed virtually all the risk. Human studies indicate that maternal pre-pregnancy BMI in women is a major determinant of adverse offspring metabolic outcomes resulting from maternal obesity [54]. The parallel between smoking and the adverse effects of MO would be the suggestion from the evidence given here that life-style adjustment of life-style aimed at reducing obesity would have the potential to avoid the maternal and offspring hazards.

OVERALL CONCLUSIONS

There are epidemiological studies that shown that once a woman knows is pregnant, she does not modify her life style [34]. Clearly the earlier the intervention the better the outcome. As the important IOM report and reviews by several clinical and basic science leaders indicate that studies such as those reported here are essential to the determination of mechanistic targets in the mother to develop predictive and clinical tools in human pregnancy [55]. Other potential interventions remain to be investigated to permit evidence-based changes in clinical management. These include supplemented diets with polyunsaturated fatty acids or anti-oxidants. Different interventions to improve outcomes may act through different mechanisms. If so, a combination of approaches may lead to even better results for the mother and the offspring.

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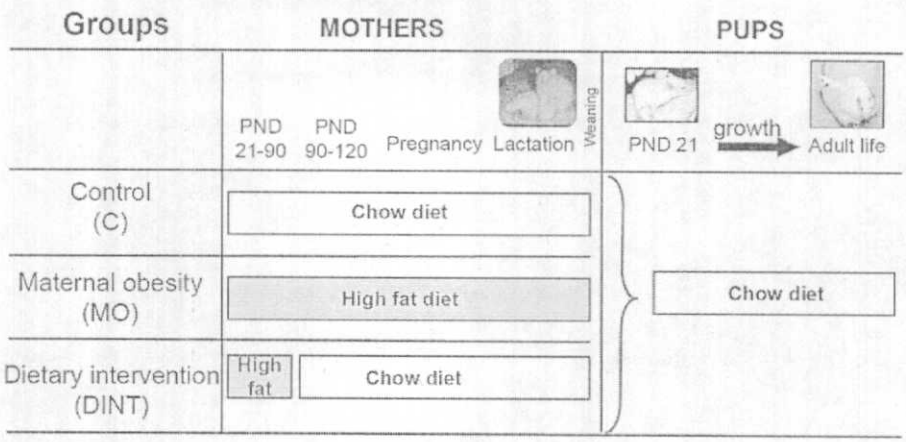

Figure 1.
Time line of the dietary intervention studies in maternal obesity.

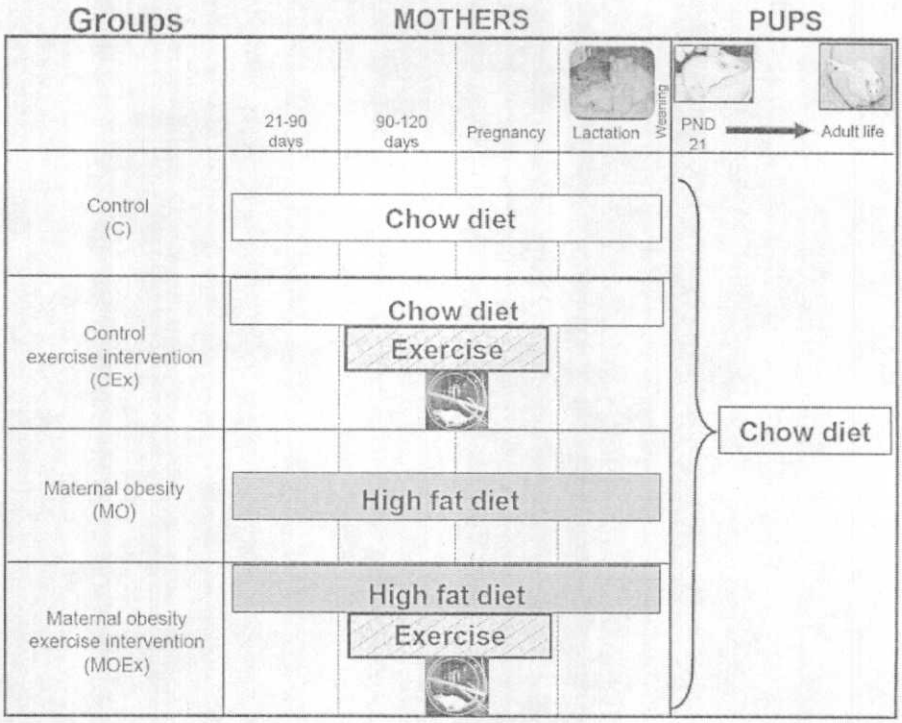

Figure 2.
Time line study of the exercise intervention studies in maternal obesity.

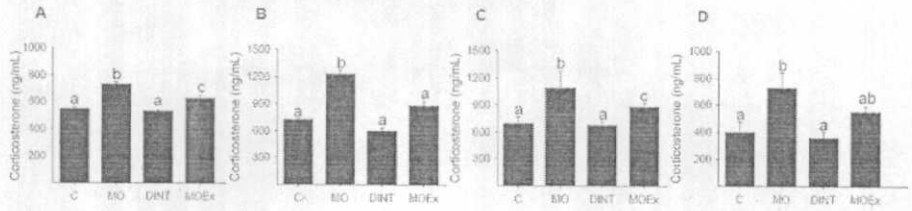

Figure 3.

Rat serum corticosterone levels: (A) maternal pre-pregnant, (B) maternal end of lactation (C) 2 days offspring male and female combined neonate and (D) 110 d young adult male. Mean ± SEM; n = 6. C control diet, MO maternal obesity, DINT maternal dietary intervention, MOEx maternal obesity exercise intervention; $p<0.05$ for groups not sharing at least one letter.

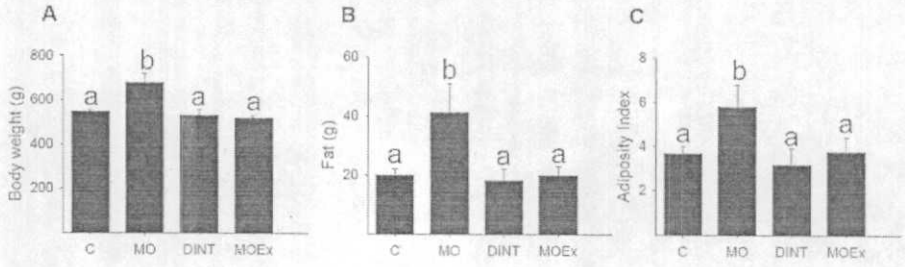

Figure 4.

Rat male offspring (A) body weight (B) fat and (C) adiposity index at 650 days. Mean ± SEM; n = 6. C control diet, MO maternal obesity, DINT maternal dietary intervention, MOEx, maternal obesity exercise intervention; $p<0.05$ for groups not sharing at least one letter.

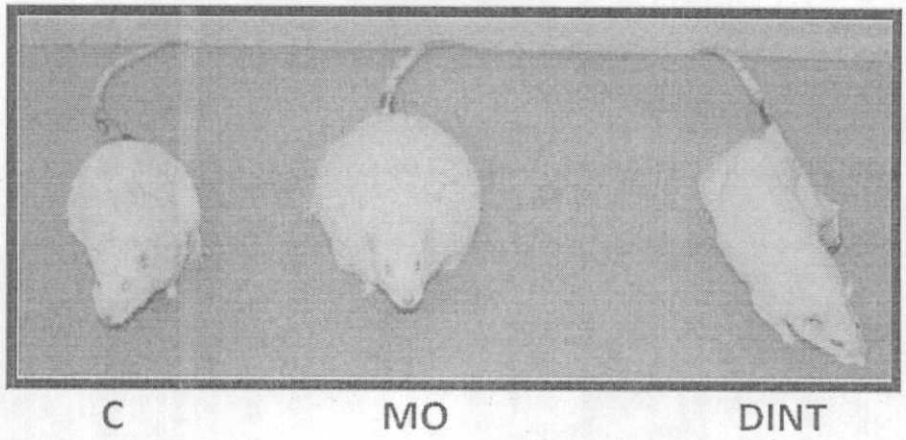

Figure 5.

Representative pictures of male offspring at postnatal day 650. C control diet, MO maternal obesity and DINT maternal dietary intervention.

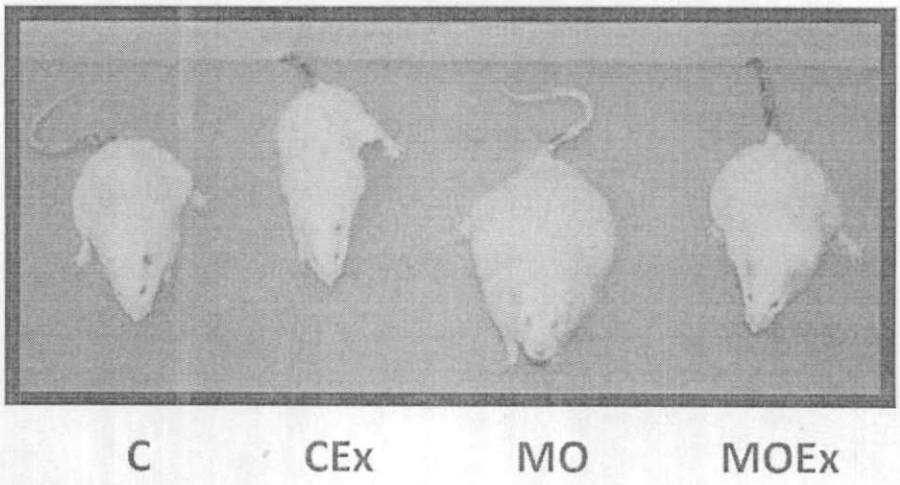

Figure 6.

Representative pictures of male offspring at postnatal day 650. C control diet, CEx control diet + maternal exercise intervention, MO maternal obesity and MOEx maternal obesity + maternal exercise intervention.

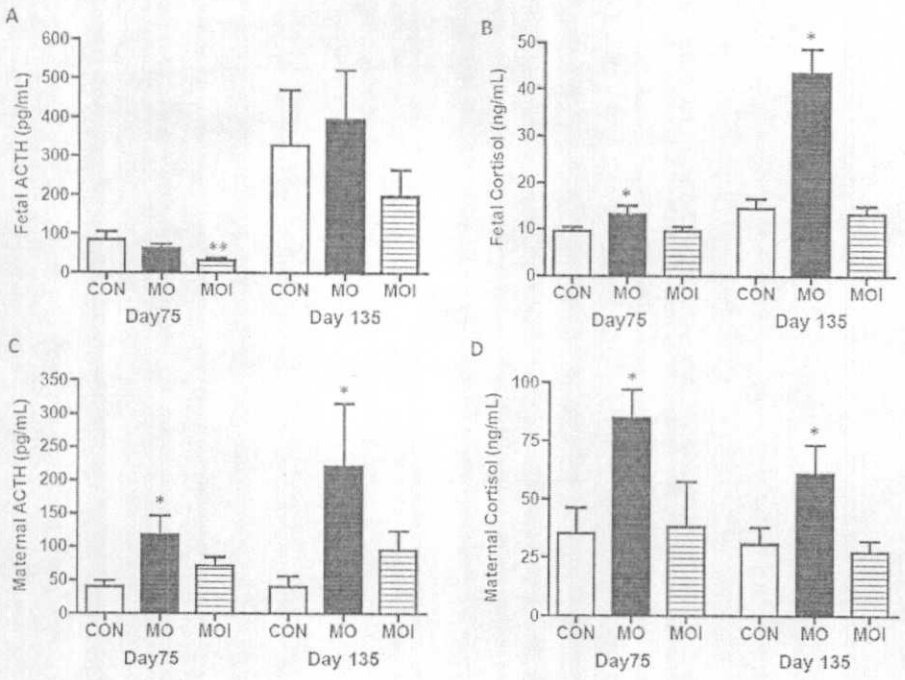

Figure 7.

Sheep fetal A) ACTH and B) cortisol, and maternal C) ACTH and D) cortisol at 75 and 135 days gestation (0.5 and 0.9G; Term 150 days gestation). Control (CON - open), maternal obesity (MO - solid), maternal obesity dietary intervention (MOI - striped); Mean ± SEM; n = 6; $p<0.05$ ** vs CON and MO, * vs CON and MOI.

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ORIGINAL ARTICLE

Maternal obesity and overnutrition increase oxidative stress in male rat offspring reproductive system and decrease fertility

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PURPOSE: Increasing evidence exists that maternal obesity (MO) and overnutrition during pregnancy and lactation have long-lasting consequences for progeny metabolism, cardiovascular and endocrine function. Data on effects of MO on offspring reproduction are limited. We hypothesized that MO during pregnancy and lactation in founder F_0 rat mothers would increase testicular and sperm oxidative stress (OS) and adversely impact male fertility in their F_1 offspring.

METHODS: We induced pre-pregnancy MO by feeding F_0 females a high-fat diet from weaning through pregnancy and lactation. After weaning, all F_1 rats ate control (C) diet. We determined serum testosterone, malondialdehyde (MDA), reactive oxygen species (ROS) and superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity in F_1 testes and sperm at postnatal days (PNDs) 110, 450 and 650.

RESULTS: At PNDs 450 and 650, MO offspring had lower luteinizing hormone while testosterone levels were lower at all ages. Testicular MDA and ROS concentrations and SOD and GPx activity were higher in MO F_1 at all ages. Nitrotyrosine immunostaining was higher at all ages in MO F_1 testes than C F_1. At PNDs 450 and 650, MO F_1 spermatozoa showed higher MDA concentrations and lower SOD and GPx activity with reduced sperm concentration, viability and motility, and more sperm abnormalities. Fertility rate was not affected at PND 110 but was lower in MO F_1 at PNDs 450 and 650.

CONCLUSIONS: We conclude that MO during pregnancy and lactation increases F_1 testicular and sperm OS leading to premature aging of reproductive capacity.

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INTRODUCTION

The worldwide prevalence of obesity has risen markedly over the past few decades, an increase also reflected in women of reproductive age.[1,2] Adverse environments *in utero* including both maternal under- or overnutrition may disturb the process of cell proliferation and differentiation leading to changes in organogenesis and developmental programming. The resultant changes in phenotype can enhance susceptibility to diseases in adult offspring[3,4] such as type 2 diabetes,[5] hypertension,[6] obesity[7,8] and alterations in reproductive function.[9,10] Male rat offspring of mothers that were protein restricted during pregnancy are overweight and have increased serum triglycerides, insulin and leptin, as well as a lower sperm count and decreased fertility.[10–12] At the other extreme, in humans[7,13] and experimental animal models, offspring of obese mothers[14,15] are obese and exhibit metabolic changes as well as alterations in anxiety, associative learning and motivation.[16,17]

The recent growth of interest in maternal obesity (MO) and developmental programming has generally focused on complications in the cardiovascular system, especially hypertension and predisposition to metabolic dysfunction, mainly obesity and diabetes. In contrast, programming of offspring reproductive capacity by MO is not well documented. In rats, female offspring of mothers who fed a high-fat diet during pregnancy and lactation enter puberty earlier,[18] have higher serum leptin and insulin levels,[19] and an increased incidence of prolonged or persistent estrus, which may affect reproductive function.[20] In humans, MO is related to earlier timing of pubertal milestones in male offspring.[21] Increased maternal body mass index is associated with decreased inhibin B in serum of male offspring.[22]

In recent years, obesity and male infertility have increased in parallel.[23] Studies in humans[24,25] and in animals[26,27] have shown that obese males have lower sperm quality and that their sperm have more morphological defects, more DNA damage and increased oxidative stress (OS). The causes of male infertility are multifactorial but among these causes, OS is involved in normal and accelerated age-related male infertility.[28] However, a correlation between MO and male offspring infertility associated with OS during active reproductive life and aging has not been established. We hypothesized that MO before and during pregnancy increases OS in offspring testes and sperm, which then leads to premature aging of offspring reproductive function. We evaluated the relationship between MO during pregnancy and OS, as well as reproductive performance of their male offspring.

METHODS

Standardization of females used to produce the mothers (F_0) in this study

Female albino Wistar rats were born and maintained in the animal facility of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, Mexico accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Rats were

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maintained under controlled lights (lights on from 0700 to 1900 hours at 22–23 °C) and fed normal laboratory chow (Zeigler Rodent RQ22-5, Gardner, PA, USA) containing 22.0% protein, 5.0% fat, 31.0% polysaccharide, 31.0% simple sugars, 4.0% fiber, 6.0% minerals and 1.0% vitamins (w/w), energy 4.0 kcal g^{-1}. At age between 16 and 17 weeks, when they weighed between 200 and 240 g, females were bred to randomly assigned, non-litter mate, proven male breeders. At delivery on day 0, litters that provided Founder Generation (F_0) mothers were culled to 10 pups, each containing at least four females. At weaning (day 21), one female F_0 pup from each litter was randomly assigned to either a control (C; $n=6$) group fed laboratory chow or to a MO group (MO; $n=6$) fed a high energy, obesogenic diet containing 23.5% protein, 20.0% animal lard, 5.0% fat, 20.2% polysaccharide, 20.2% simple sugars, 5.0% fiber, 5.0% mineral mix, 1.0% vitamin mix (w/w), energy 4.9 kcal g^{-1}. Thus, each F_0 group had only one female from any litter, and F_0 females in different groups, but not within groups, were sisters, providing homogeneity in F_0 mothers' own developmental programming and genetics.

F_1 offspring

F_0 female rats were placed with proven male breeders on day 120 and conceived during the next cycle. Lactating mothers were maintained on their pregnancy diet. Litter size and pup weight were recorded at birth. Ano-genital distance was measured to identify males and females.[11] Litters with >14 pups were excluded. To ensure F_1 (first generation) offspring homogeneity, on postnatal day (PND) 2 all litters studied were adjusted to 10 pups with equal numbers of males and females wherever possible. Offspring were weaned at PND 21, housed five per cage and fed chow diet throughout the study. Only male offspring were used for the study. At PNDs 110, 450 and 650, after 6 h of fasting, male rats were decapitated using a rodent guillotine (Thomas Scientific, Swedesboro, NJ, USA) by trained personnel experienced in the procedure. F_1 males evaluated at the three ages were siblings. F1 males were decapitated between 1200 and 1400 hours. For each age group, trunk blood was collected and serum was separated. Both testes were dissected, cleaned from surrounding fat and weighed. One testis was frozen and stored at −70 °C until analyzed. The second testis was fixed in 4% paraformaldehyde and was paraffin embedded. The sternal, pancreatic, retroperitoneal and gonadal fat pads were dissected and weighed individually. At the three PND studied, the cauda epididymis and vas deferens were rapidly removed and placed in saline at 37 °C. Sperm were removed by clearing the vas deferens with tweezers and chopping the cauda epididymis with scissors.

Tissue and sample preparation

Testes were homogenized in saline at 4 °C and aliquots were obtained and frozen at −70 °C for later protein quantification using the Bradford method and for determination of biomarkers of OS (reactive oxygen species (ROS) and antioxidant enzymes). Lipid peroxidation was determined at the time of testicular homogenization and sperm release. All determinations were performed in duplicate and were averaged for statistical analysis.

Sperm aliquots containing 5×10^6 and 10×10^6 spermatozoa underwent six thermal shock cycles from −70 to 45 °C, sonicated for 2 min with six intervals of 20 s each, and were then placed in ice and stored frozen at −70 °C until quantified for antioxidant enzymes.

Hormone measurements

Serum follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels were determined by double antibody radioimmunoassay using standard and specific anti-rat LH antibodies from the National Institute of Diabetes, Digestive Kidney Diseases. FSH and LH were iodinated by the chloramine-T method, following separation of protein-bound and free [^{125}I] by Sephadex G-100. Results are expressed as NIDDK-rat-FSH-RP2 and NIDDK-rat-LH-RP3. The intra- and interassay coefficients of variation were <4 and <7.9 and <5.1 and <6.5, respectively.[10]

Serum testosterone was determined using a commercial rat kit, DPC Coat—a—Count (TKTT1) obtained from Diagnostic Products (Los Angeles, CA, USA). The intra-and inter assay variability were <8.17 and <8.83, respectively.[10]

Lipid peroxidation assay

Lipid peroxidation was determined the same day on which testes were homogenized and sperm were obtained. Aliquots containing 5 million sperm were adjusted with saline to 100 µl at room temperature without thermal shock or sonication. Lipid peroxidation was determined in 100 µl aliquots of either homogenized testes or 5×10^6 sperm by measuring malondialdehyde (MDA) by the thiobarbituric acid-reactive substances assay. All samples were read in a plate at 532 nm in a Perkin-Elmer LS50-B luminescence spectrometer (Waltham, MA, USA). Results were expressed as nmol MDA per mg of protein[15] or nmol MDA per 5 million sperm.[29] Intra- and interassay coefficients of variation were <6% and <8%, respectively.

ROS assay

Five microliters of homogenized testes were used to determine ROS. A standard curve was obtained using increasing concentrations of 2′,7′-dichlorofluorescein (DFC) and incubated in parallel with the samples (37 °C for 60 min). At the end of the incubation period, fluorescent signals at an excitation wavelength of 488 nm and an emission wavelength of 525 nm were recorded in a Perkin-Elmer LS50-B luminescence spectrometer. Results were expressed as nmol of DFC formed per mg of protein per minute.[15,29]

Superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined in 10 µl aliquots of homogenized testes or sperm with a RANSOD kit (RANDOX laboratories limited, Crumlin, Co Antrim, UK). A standard curve was obtained according to the manufacturer's instructions. All samples were read in a plate at 505 nm in a Perkin-Elmer LS50-B luminescence spectrometer at 0 and 30 s and 3 min at 37 °C. Results were expressed as units of activity per mg of protein[15] units of activity per 5 million sperm.[29]

Glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was determined in 10 µl aliquot of homogenized testes or sperm with the RANSEL kit (RANDOX Laboratories Limited, UK). All samples were read in a plate at 304 nm in a Perkin-Elmer LS50-B luminescence spectrometer at baseline 1, 2 and 3 min at 37 °C. Results were expressed as milliunits per mg of protein[15] or milliunits per 10 million sperm.[29]

Nitrotyrosine histology

Paraffin sections (5 µm) of the testis were immunostained with affinity-purified mouse monoclonal antibody anti-nitrotyrosine MAB5404 (Millipore, Billerica, MA, USA) at 1:200 using an ABC elite kit (Vector Laboratories, Burlingame, CA, USA) and visualized using 2.5% nickel sulfate with 0.02% chromogen in 0.175 M sodium acetate.[15]

Histological evaluation of the testes

Tissue was mounted on slides with the tubules cut transversally to evaluate seminiferous tubule diameter and area and number of spermatogonia and spermatocytes. From one tissue section, at least 30–40 transverse seminiferous tubules per animal were photographed at ×40 with an Olympus BX51 light microscope (Melville, NY, USA) using image analysis software (Image-Pro Plus Version 3.1, Media Cybernetics, Inc., Rockville, MD, USA). Spermatogonia and spermatocytes were counted according to their hematoxylin and eosin staining (spermatogonia stained purple and spermatocytes pink) using a digital counter. The seminiferous tubule diameter and area (μm^2) were measured by planimetry (AxioVision Rel. 4.0 USA, Media Cybernetics, Inc.) in 40–50 transverse seminiferous tubules per animal that were photographed at ×10.[30,31] All histological measurements were performed by two independent observers and results were averaged.

Sperm measurements

Sperm viability was assessed by mixing 10 ml of saline (0.90% w/v of NaCl) containing spermatozoa with 10 ml of eosin. Live spermatozoa were identified by membrane integrity that prevented staining with eosin. Dead sperm were easily identified after staining as previously described.[29] Two hundred sperm were counted under a light microscope. Results were expressed as the percentage of live cells. Sperm concentration and motility were evaluated with a computerized sperm analyzer (Sperm Quality Analyzer). For sperm morphology, 10 µl of sample was taken smeared and stained using a quick staining kit for sperm morphology (FertiMexico S.A de C.V, Mexico City, Mexico). Abnormal sperm morphology was classified as defects in the sperm head, midpiece or tail. Head defects included large, small, tapered, pyriform, round and amorphous heads, heads with a small acrosomal area and double heads. Midpiece defects included bent neck,

asymmetrical insertion of the midpiece into the head, a thick or irregular midpiece and an abnormally thin midpiece. Tail defects included short, multiple, hairpin, broken or bent tails, tails of irregular width and coiled tails. For analysis of sperm abnormalities, 200 sperm were counted under light microscope at ×40.

Evaluation of fertility rate

At PNDs 110, 450 and 650, one experimental male was placed for 1 week with two nonexperimental group virgin females aged 4 months. Males were then separated from the females who were kept individually until day 15 of gestation.[29] The male was considered fertile when at least one of the two females became pregnant. Results were expressed as the percentages of fertile males and pregnant females.

Statistical analysis

All data are presented as mean ± s.e.m., $n = 5–6$ per group from different litter. We evaluated the differences between groups at the same age and within the same groups at different ages. Data were analyzed using two-way multiple analysis of variance followed by Holm–Sidak test. Fertility rate was analyzed using a χ^2-test. $P < 0.05$ was considered significant.

RESULTS

Offspring (F_1) body, testicular, epididymal fat and total fat weight

At PND 110, group body weights were similar, but at PNDs 450 and 650 MO F_1 were heavier than C pups (Figure 1a). Testicular weight was not different between F_1 C and MO at any age (Figure 1b). In contrast, gonadal and total fat were both higher in F_1 MO at PNDs 110 and 450 than those from the C (Figures 1c and d). Body weight and gonadal fat increased at PND 450 compared with to PND 110 in both groups; total fat in both groups did not change with age. Testicular weight did not change with age (Figure 1).

F_1 hormone concentrations

F_1 FSH serum levels were similar between groups at all ages (Figure 2a), whereas LH serum levels at PNDs 450 and 650 were lower in MO offspring compared with C offspring (Figure 2b). Testosterone serum levels were lower in MO F_1 pups than C at all ages (Figure 2c). When hormone concentrations were analyzed within the same groups at different ages, FSH did not change with age in both groups, whereas LH and testosterone serum levels were decreased at PNDs 450 and 650 (Figure 2).

F_1 testicular OS biomarkers

MDA and ROS concentrations were higher at all ages in the testes of F_1 MO (Figures 3a and b) as were the antioxidant enzymes SOD and GPx (Figures 3c and d). When OS biomarkers were analyzed within groups at different ages MDA concentration, SOD and GPx activity did not change owing to the age, while the increase in ROS levels in the MO were much higher at PND 650 in comparison with PNDs 110 and 650 (Figure 3). At all ages, testes of F_1 MO demonstrated higher nitrotyrosine immune reactivity than C (Figure 4a). No differences were observed by age (Figure 4a).

F_1 testicular histology

At all ages, the seminiferous tubule area (Figure 4b) as well as the number of spermatogonia per transversal tubule (Figure 4c) were lower in F_1 MO compared with C offspring. In F_1 MO males, the number of spermatocytes per transversal tubule was lower than C offspring at PNDs 110 and 650 (PND 110 = C: 155 ± 5a, MO: 120 ± 5a*; PND 450 = C: 120b ± 8, MO: 115 ± 13a; PND 650 = C: 131 ± 4b, MO: 65 ± 4b*). In both C and MO offsprings, the seminiferous tubule area did not change with age (Figure 4b), but spermatogonia (Figure 4c) and spermatocyte number per seminiferous tubule decreased with age in both groups.

F_1 sperm OS biomarkers

At PNDs 450 and 650, MDA levels were higher and SOD and GPx activity lower in sperm of F_1 MO compared with F_1 C. (Figures 5a–c). While age increased MDA and decreased GPx activity, the changes occurred earlier, PND 450, in F_1 MO compared with C (5A and C). SOD decreased with age in MO but not in the F_1 C (Figure 5b).

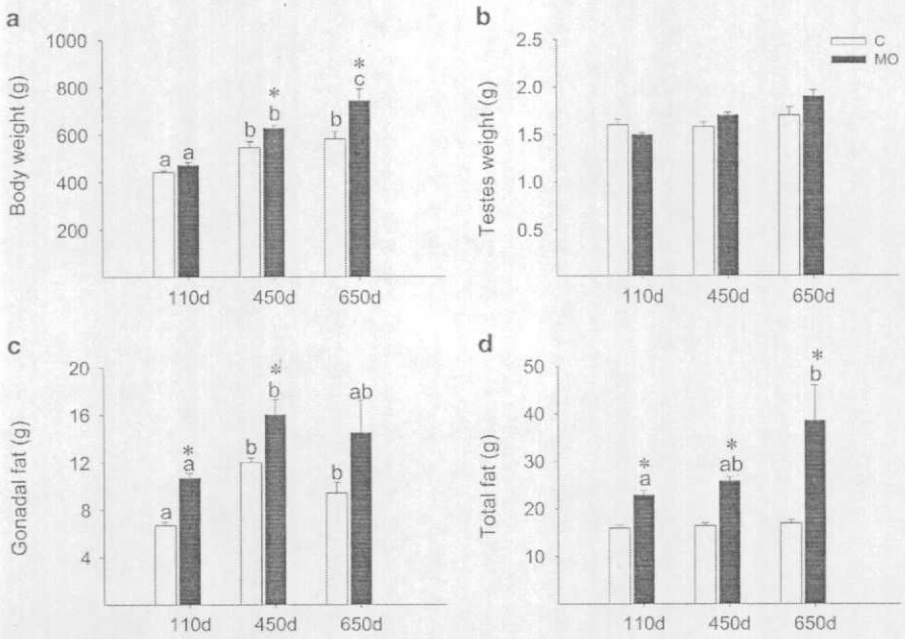

Figure 1. Weights at different ages in F_1 control (C) and maternal obesity (MO) males. (**a**) Body weight, (**b**) testicular weight, (**c**) gonadal fat and (**d**) total fat. Mean ± s.e.m., $n = 5–6$ rats from different litters. $P < 0.05$ for data not sharing at least one letter at the same maternal diet; *$P < 0.05$ vs C.

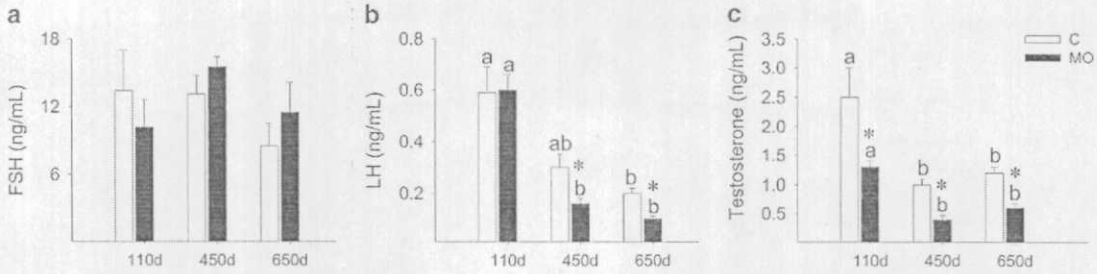

Figure 2. Hormone concentrations at different ages in F_1 control (C) and maternal obesity (MO) males. (**a**) FSH, (**b**) LH and (**c**) testosterone. Mean ± s.e.m., $n = 5–6$ rats from different litters. $P < 0.05$ for data not sharing at least one letter at the same maternal diet, $^*P < 0.05$ vs C.

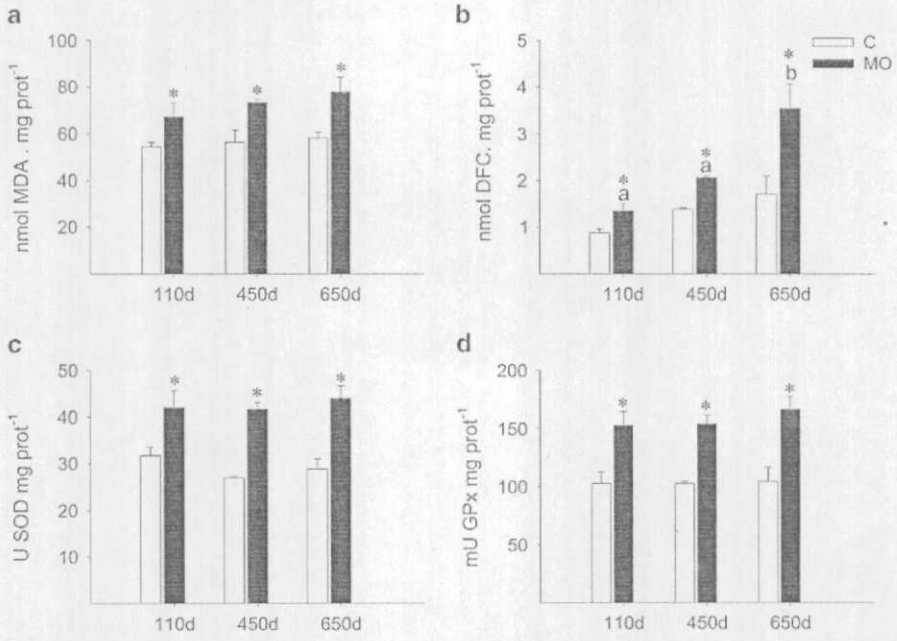

Figure 3. Testicular OS biomarkers at different PNDs in F_1 control (C) and maternal obesity (MO) males. (**a**) MDA, (**b**) ROS, (**c**) SOD and (**d**) GPx. Mean ± s.e.m., $n = 5–6$ rats from different litters. $P < 0.05$ for data not sharing at least one letter at the same maternal diet, $^*P < 0.05$ vs C.

F_1 sperm parameters

Sperm viability, motility, concentration and morphology at PND 110 were similar in the two groups. However, at PNDs 450 and 650, all three measures were decreased in F_1 MO compared with C (Figures 5d–f). The percentage of normal sperm was reduced in the F_1 MO compared with F_1 C at PND 450 (Figures 6a and b).

F_1 fertility rate

Fertility rate expressed as fertile males and the number of females successfully bred was similar in both groups at PND 110. At PND 450, the percentage of pregnant females was lower in the F_1 MO than in C (Figure 6d). At PND 650, the percentage of fertile males and pregnant females was lower in the F_1 MO than in C (Figures 6c and d). Both evaluations of fertility decreased with age in MO offspring but was unchanged in C offspring (Figures 6c and d).

DISCUSSION

Epidemiological[32] and animal studies[14,33] have shown that MO during gestation is an important determinant of offspring body weight. In several previous studies, we have reported that F_1 males from F_0 obese mothers exhibit increased body weight, fat, adiposity index, serum triglycerides, leptin, insulin as well as insulin resistance.[14,15,34] This occurs even when the F_1 males from F_0 obese mothers are fed the normal C laboratory diet. In the present study, the MO F_1 were heavier than C and had more gonadal and total fat accumulation, demonstrating the consistency of the model. Abdominal obesity is not only associated with metabolic abnormalities[35] but also with a decline in testosterone levels that becomes more pronounced with aging.[36] Testosterone production depends on normal function of the hypothalamic–pituitary–gonadal axis.[37] Obesity decreases LH concentration.[38] In the present study, LH and testosterone serum levels were decreased in the male MO F_1. However, the decline was much more pronounced as they aged. This may be owing to a primary and/or a secondary testicular failure as suggested by other authors[39] additional to damage caused by OS resulting from the imbalance between the production of ROS and the antioxidant enzymes responsible for their removal.[40]

Obesity *per se* has been associated with OS and high levels of ROS.[41] Prenatal hypoxia, maternal under- or overnutrition and overexposure to glucocorticoids can lead to OS.[42,43] In previous studies, we reported that MO increased F_0 maternal insulin, glucose, homeostasis model assessment, leptin, triglycerides, cholesterol and retroperitoneal fat, and also that the maternal liver showed increased MDA and ROS concentrations and SOD and GPx activity.[15] In addition, several studies have shown that

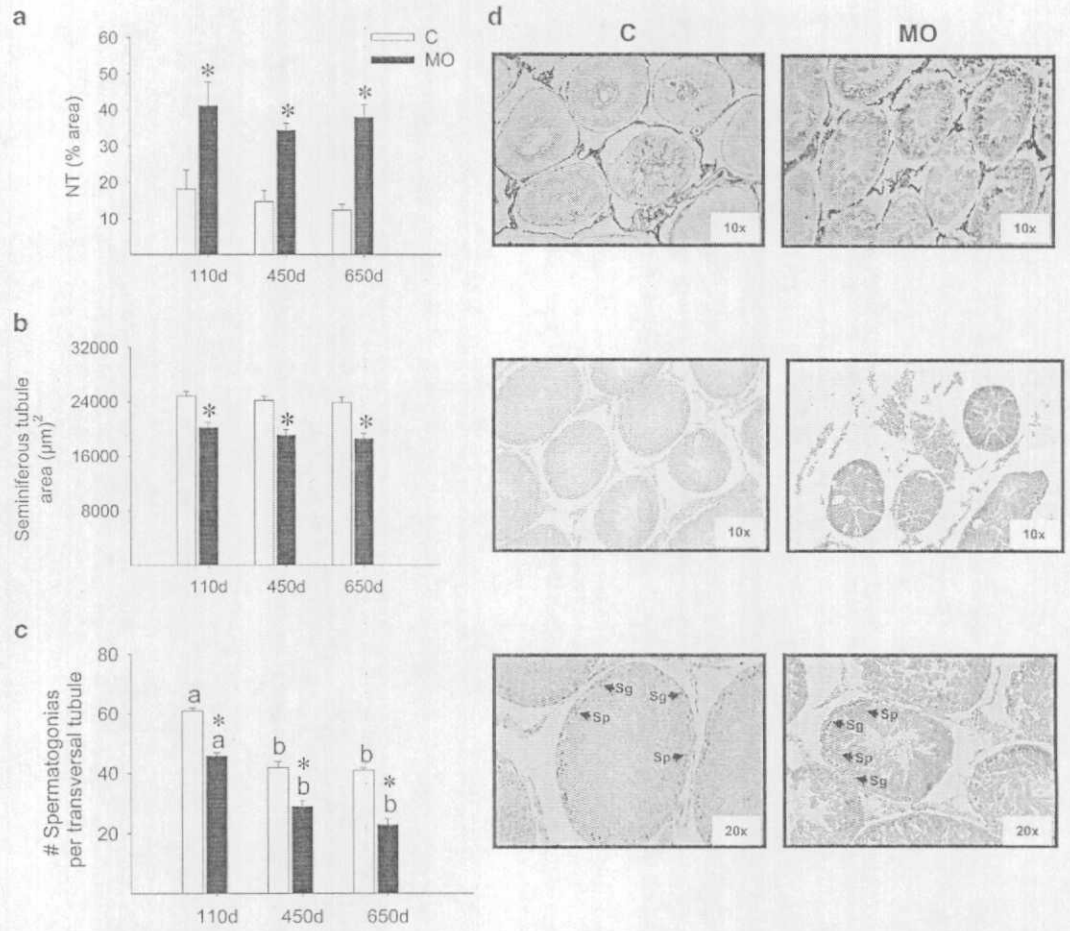

Figure 4. Testicular nitrotyrosine (NT) fraction stained and histology at different PNDs in F_1 control (C) and maternal obesity (MO) males. (**a**) % Area immunostained, (**b**) seminiferous tubule area, (**c**) number of spermatogonias per tranversal and (**d**) representative pictures of seminiferous tubules at PND 110; arrows show the location of spermatogonias (Sg) and spermatocytes (Sp). Mean ± s.e.m., $n = 5–6$ rats from different litters. $P < 0.05$ for data not sharing at least one letter at the same maternal diet, $^*P < 0.05$ vs C.

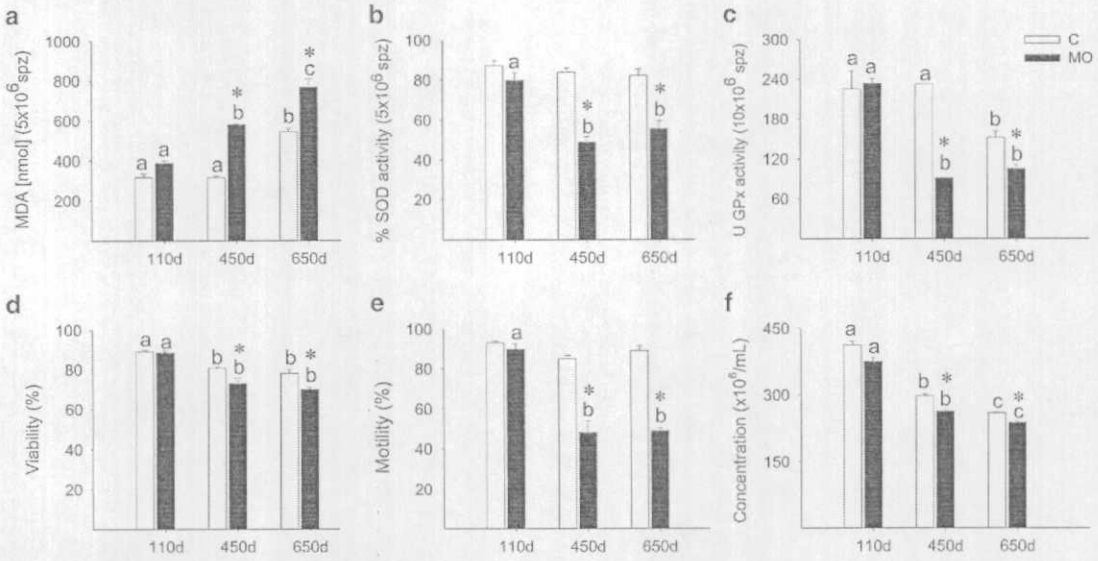

Figure 5. Sperm (spz) OS biomarkers and sperm quality at different PNDs in F_1 control (C) and maternal obesity (MO) males. (**a**) MDA, (**b**) SOD, (**c**) GPx, (**d**) viability, (**e**) motility and (**f**) sperm concentration. Mean ± s.e.m., $n = 5–6$ rats from different litters. $P < 0.05$ for data not sharing at least one letter at the same maternal diet, $^*P < 0.05$ vs C.

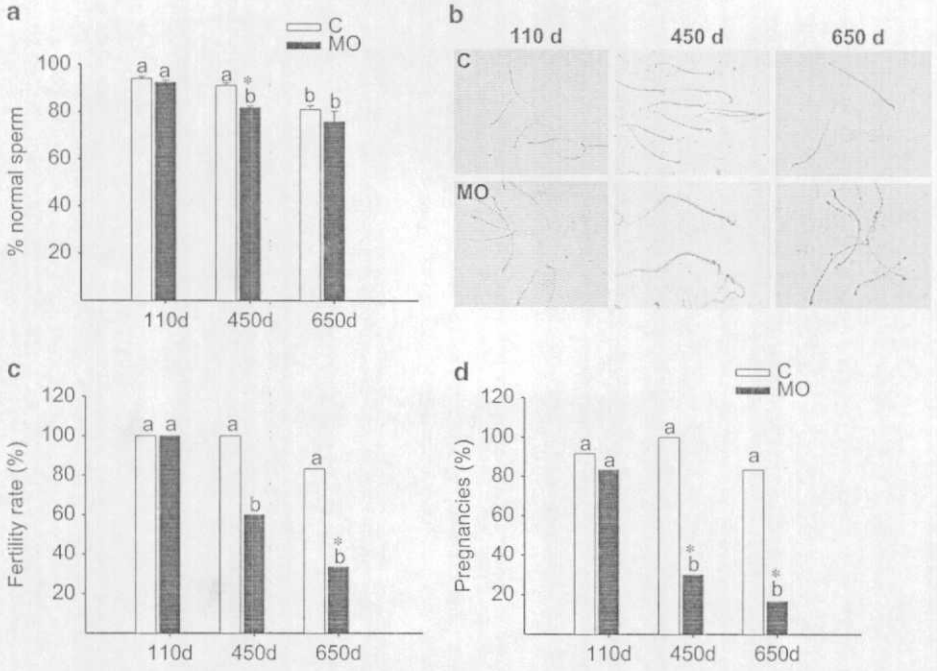

Figure 6. Sperm morphology and fertility rate at different PNDs in F_1 control (C) and maternal obesity (MO) males. (**a**) Normal sperm, (**b**) representative photographs of sperm morphology at PND 650, (**c**) fertile males and (**d**) pregnant females. Mean ± s.e.m., $n = 5–6$ rats from different litters. $P < 0.05$ for data not sharing at least one letter at the same maternal diet, $^*P < 0.05$ vs C.

MO is associated with altered levels of plasma interleukin-6 and 10,[44] augmented OS in maternal oocytes before pregnancy[45] and in the placenta, all of which may have harmful effects on the embryos and fetuses.[46]

Studies in experimental animals support the hypothesis that OS in the offspring induces programmed phenotypes.[43,47] Maternal protein restriction in the rat increases pancreatic OS and impairs offspring pancreatic antioxidant enzyme function.[48] At the other extreme, MO in the rat negatively affects female offspring reproductive function.[18,49]

Testicular OS appears to be a common feature of the male infertility that results from decreased spermatogenesis and sperm damage.[50] In the present study, we found that OS was increased (MDA and ROS) in the MO F_1 testes from the MO. Oxidative damage can also be caused by an increase in reactive nitrogen species (RNS), which have also been implicated in producing testicular dysfunction and abnormal semen.[51] Nitrotyrosine is a product of tyrosine nitration and is an indicator of cell damage.[52] We observed that the testes from the MO offspring showed higher nitrotyrosine immunoreactivity. To maintain physiological levels of ROS and RNS, all tissues including reproductive tissues possess different antioxidant enzymes that work in synergy to reduce cytotoxic effects of free radicals.[53] Our data show increased activity of SOD and GPx in the MO F_1 testes accompanied by increased production of markers of OS. We interpret these findings as showing compensation by antioxidant systems that was, however, inadequate to prevent the rise in OS. This dysregulation in the redox balance can have potentially harmful effects on germ cells and sperm.[54] Aging is characterized by a progressive decline in cellular function owing to the accumulation of ROS, which eventually will lead to the dysfunction and failure of physiological functions.[55]

Lipoperoxidation and its products (such as MDA) are toxic to germ cells damaging proteins and DNA and increasing apoptosis.[28,56] All these can affect the process of spermatogenesis that transforms spermatogonia to spermactocytes, spermatids and spermatozoa.[57] Our histological analysis demonstrated that MO decreases offspring spermatogonia and spermatocyte numbers, a potential cause of the observed reduction in sperm concentration. Sperm are especially susceptible to ROS because they lack cytoplasmic antioxidant enzymes and have high levels of membrane polyunsaturated fatty acids.[58] OS in sperm can increase DNA damage and apoptosis and affect sperm quality;[40] the latter can be used as an index of fertility.[57] In this context, we found that sperm from MO offspring had increased OS and decreased sperm quality and fertility. Evidence indicates that obesity can directly negatively impact male fertility[59] through different mechanisms such as decreased sex hormone secretion,[24,60] hyperinsulinemia or due to the direct action of leptin on testicular function.[60] We propose that in addition to these direct effects of obesity, the observed changes in MO F_1 offspring are also due to developmental programming by the challenge of MO to the F_1 during their development. Further studies will be needed to determine the role played by each of these mechanisms. What our study does establish, for the first time, is that there are F_1 negative reproductive outcomes consequent on MO.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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SALVADOR ZUBIRÁN

Ciudad de México a 26 de abril de 2017

Dra. Norma Bobadilla Sandoval
Coordinadora de la Comisión de la Investigación en Animales
Presente.

A continuación, encontrará el informe final del CINVA 271. En el 2015, se concluyó con la parte experimental relacionada a la caracterización fenotípica y metabólica de las madres controles, obesas y las obesas intervenidas nutricionalmente un mes antes del embarazo. Se analizaron los efectos en las crías al nacimiento, al destete, alrededor de la pubertad y en edad adulta joven. Los resultados mostraron que las crías de madres obesas (al igual que sus madres), presentan daños metabólicos, hormonales y aumento en el daño oxidativo, lo cual repercute negativamente en términos no sólo metabólicos sino de memoria y aprendizaje, así como en el desarrollo sexual y la función reproductiva. Las crías de madres obesas, intervenidas nutricionalmente un mes antes del embarazo, no presentaron tantos daños metabólicos. La intervención materna logró prevenir por completo y en algunos casos parcialmente los daños debidos a la obesidad materna. A pesar de que el proyecto únicamente planteaba los efectos metabólicos a nivel de glucosa e insulina, el estudio pudo extenderse a la cuantificación de otros sistemas afectados. Los resultados obtenidossirvieron para la titulación de alumnos a nivel licenciatura y para la publicación de artículos en revistas indezadas.

Tesis:

Carlos Ibáñez Chávez (2011)Grado: Licenciatura
Obesidad materna de la rata y predisposición de la progenie a alteraciones metabólicas: ventajas de la intervención nutricional.

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Adriana Ramírez Rodríguez (2013)Grado: Licenciatura
Intervención nutricional en la obesidad materna de la rata: beneficios en la conducta y aprendizaje de la progenie (machos)

Laura Nalleli Garrido Castillo (2013)Grado: Licenciatura
La obesidad materna de la rata tiene impacto negativo sobre el aprendizaje y motivación de la cría hembra

Artículos:

Dietary intervention prior to pregnancy reverses metabolic programming in male offspring of obese rats. Zambrano E, Martínez-Samayoa PM, Rodríguez-González GL, Nathanielsz PW.J Physiol. 2010 May 15;588(Pt 10):1791-9. doi:10.1113/jphysiol.2010.190033. Epub 2010 Mar 29.

Maternal obesity in the rat programs male offspring exploratory, learning and motivation behavior: prevention by dietary intervention pre-gestation or in gestation. Rodriguez JS, Rodríguez-González GL, Reyes-Castro LA, Ibáñez C, Ramírez A, Chavira R, Larrea F, Nathanielsz PW, Zambrano E.Int J Dev Neurosci. 2012 Apr;30(2):75-81. doi: 10.1016/j.ijdevneu.2011.12.012. Epub 2012 Jan 5.

Interventions designed to prevent adverse programming outcomes resulting from exposure to maternal obesity during development . Nathanielsz PW, Ford SP, Long NM, Vega CC, Reyes-Castro LA, Zambrano E.Nutr Rev. 2013 Oct;71 Suppl 1:S78-87. doi: 10.1111/nure.12062. Review.

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Maternal obesity and overnutrition increase oxidative stress in male rat offspring reproductive system and decrease fertility. Rodríguez-González GL, Vega CC, Boeck L, Vázquez M, Bautista CJ, Reyes-Castro LA, Saldaña O, Lovera D, Nathanielsz PW, Zambrano E.Int J Obes (Lond). 2015 Apr;39(4):549-56. doi: 10.1038/ijo.2014.209. Epub 2014 Dec 15.

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Dra. Elena Zambrano González

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Ciudad de México a 26 de abril de 2017

Dra. Norma Bobadilla Sandoval
Coordinadora de la Comisión de la Investigación en Animales
Presente.

Estimada Dra. Bobadilla

Por este conducto me permito solicitar el cierre del protocolo "***Efectos de la obesidad materna de la rata sobre el metabolismo de las crías"*** con número de registro ***CINVA: 271,*** en el 2012 se solicitó la extensión del mismo y el cambio de nombre a"**Intervención nutricional en la obesidad materna de la rata: beneficio en el metabolismo de las crías"** debido a que fue financiado por CONACyT con el ***número de proyecto 155166***. El proyecto antes mencionado concluyó en el 2015.

Sin otro particular por el momento, quedo de usted.

Atentamente

Dra. Elena Zambrano González
Investigador en Ciencias Médicas F
Biología de la Reproducción

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INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN SALVADOR ZUBIRÁN

Recibí
Silvia Carrera E
10/02/2016

se modifica fecha de cierre al 26 de abril 2017
Nayeli Ortega Mtz.
3-JUL-17

México, D.F. a 10 de Febrero de 2016

Dra. Elena Zambrano González
Depto. Biología de la Reproducción
Presente

Estimada Dra. Zambrano:

Por este conducto me permito solicitar el cierre del Protocolo: "**INTERVENCIÓN NUTRICIONAL EN LA OBESIDAD MATERNA DE LA RATA: BENEFICIO EN EL METABOLISMO DE LAS CRÍAS.**", con registro **CINVA 271.**, debido a que el periodo de realización y la prórroga correspondiente autorizada por la CINVA ha concluido. Favor de llenar el formato de cierre del protocolo que se anexa a la presente. De no recibir el formato de su parte en el plazo de 30 días, el protocolo se dará por cerrado.

Nayeli Ortega Mtz.
Mary
Cerrado
10-marzo
2016

Sin otro particular por el momento, quedo de usted.

Atentamente,

INS. NACIONAL DE CIENCIAS MEDICAS Y NUTRICIÓN "SALVADOR ZUBIRÁN"
10 FEB 2016
DIRECCIÓN DE INVESTIGACIÓN

Dra. Norma A. Bobadilla Sandoval
Coordinadora de la Comisión de Investigación en Animales

703

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c.c.p. Dr. Gerardo Gamba Ayala, Director de Investigación
MVZ Mariela Contreras Escamilla, Jefa del DIEB

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12 MAR 2012

México, D.F., a 02 de marzo del 2012

DRA. ELENA ZAMBRANO GONZÁLEZ
INVESTIGADORA EN CIENCIAS MÉDICAS E
DEPARTAMENTO DE BIOLOGÍA DE LA REPRODUCCIÓN

Estimada Dra. Zambrano,

En respuesta a su atenta comunicación referente a extender la vigencia del proyecto de investigación: **Efecto de la obesidad materna de la rata sobre el metabolismo de las crías** con **Registro CINVA: 271.**

Con base en la información proporcionada, le informo que **no existe inconveniente para extender la vigencia del proyecto hasta el 30 de diciembre del 2015** como usted lo solicita.

Por otra parte, en referencia a su solicitud de cambiar el nombre registrado ante CINVA de este proyecto por el de: **"Intervención nutricional en la obesidad materna de la rata: beneficio en el metabolismo de las crías"** ante la necesidad de cambiarlo para poder acceder a los fondos otorgados por **CONACyT** bajo el **número de proyecto: 155166**. Le informo que es procedente su solicitud y debe realizar los trámites correspondientes ante la Dirección de Investigación y el Departamento de Control de Fondos Especiales para Investigación (CFEI).

Sin otro particular y en espera de contar con su valioso apoyo, me despido de usted con un afectuoso saludo.

Recibí comunicado 05/02/12

ATENTAMENTE

M.V.Z, M.Sc., Cert. L.A.M. RAFAEL HERNÁNDEZ GONZÁLEZ
COORDINADOR DE LA COMISIÓN DE INVESTIGACIÓN EN ANIMALES

c.c.p. Dr. Rubén Lisker Y. Director de Investigación
C.P. Martha Arredondo Urzúa. Jefe del Departamento C.F.E. I.

Recibió
Alma Pérez
5-3-12

México, D.F., a 02 de marzo del 2012

INNSZ

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
SALVADOR ZUBIRÁN

DRA. ELENA ZAMBRANO GONZÁLEZ
INVESTIGADORA EN CIENCIAS MÉDICAS E
DEPARTAMENTO DE BIOLOGÍA DE LA REPRODUCCIÓN

Estimada Dra. Zambrano,

En respuesta a su atenta comunicación referente a extender la vigencia del proyecto de investigación: **Efecto de la obesidad materna de la rata sobre el metabolismo de las crías** con **Registro CINVA: 271.**

Con base en la información proporcionada, le informo que **no existe inconveniente para extender la vigencia del proyecto hasta el 30 de diciembre del 2015** como usted lo solicita.

Por otra parte, en referencia a su solicitud de cambiar el nombre registrado ante CINVA de este proyecto por el de: **"Intervención nutricional en la obesidad materna de la rata: beneficio en el metabolismo de las crías"** ante la necesidad de cambiarlo para poder acceder a los fondos otorgados por **CONACyT** bajo el **número de proyecto: 155166**. Le informo que es procedente su solicitud y debe realizar los trámites correspondientes ante la Dirección de Investigación y el Departamento de Control de Fondos Especiales para Investigación (CFEI).

Sin otro particular y en espera de contar con su valioso apoyo, me despido de usted con un afectuoso saludo.

ATENTAMENTE

M.V.Z, M.Sc., Cert. L.A.M. RAFAEL HERNÁNDEZ GONZÁLEZ
COORDINADOR DE LA COMISIÓN DE INVESTIGACIÓN EN ANIMALES

c.c.p. Dr. Rubén Lisker Y. Director de Investigación
C.P. Martha Arredondo Urzúa. Jefe del Departamento C.F.E. I.

• Vasco de Quiroga 15,
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• C. P. 14000 México, D. F.
• Tel. 54-87-09-00

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INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
SALVADOR ZUBIRÁN

México, D.F. a 02 de Marzo del 2012

M. Sc Rafael Hernández González
Jefe del Depto. Investigación Experimental y Bioterio y
Coordinador de la Comisión de Investigación en Animales

Mediante la presente me permito informarle que el pasado 28 de Julio del 2009, la Comisión de Investigación en Animales (CINVA) me notificó la aprobación del proyecto "***Efectos de la obesidad materna de la rata sobre el metabolismo de las crías***" con número de registro ***CINVA: 271***. Cabe mencionar que este proyecto fue sometido al CONACyT para su posible financiamiento bajo el siguiente título: ***"Intervención nutricional en la obesidad materna de la rata: beneficio en el metabolismo de las crías"***. Afortunadamente la propuesta fue aprobada y apoyada con el ***número de proyecto 155166*** de la convocatoria de Investigación Científica Básica 2010 – 2012 (CB-2010-01). Por tal motivo someto a su consideración la posibilidad de cambiar en la carta del CINVA el título anterior por el correspondiente proyecto aprobado por el CONACyT con el fin de cubrir los requisitos para el depósito y disposición de los recursos financieros del mismo. Así mismo me permito informarle que me encuentro realizando los trámites necesarios para extender dicho proyecto el cual está próximo a vencer

Como se puede observar en los documentos adjuntos, el proyecto aprobado por el CONACyT representa al proyecto aprobado previamente por el CINVA.

Agradeciendo la atención que le sirva dar a la presente, me despido de usted enviándole un cordial saludo

Atentamente

Dra. Elena Zambrano González
Investigadora en Ciencias Médicas E
Biología de la Reproducción
Ext. 2417

ccp. Dr. Rubén Lisker Y, Director de Investigación
C.P. Martha Arredondo Urzúa, Jefe del Departamento C.F.E.I

Investigación

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INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
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México, D.F. a 02 de Marzo del 2012

M. Sc Rafael Hernández González
Jefe del Depto. Investigación Experimental y Bioterio y
Coordinador de la Comisión de Investigación en Animales (CINVA)

Me fue notificado que el proyecto "***Efectos de la obesidad materna de la rata sobre el metabolismo de las crías"*** con número de registro ***CINVA: 271*** está por vencer en el mes de julio del presente año. Cabe mencionar que dicho proyecto fue sometido al CONACyT para su posible financiamiento y afortunadamente la propuesta fue aprobada y apoyada con el ***número de proyecto 155166*** de la convocatoria de Investigación Científica Básica 2010 – 2012 (CB-2010-01). Por tal motivo le solicito a usted de la manera más atenta la prórroga para la extensión del proyecto hasta el año 2015, solicitando para el mismo 280 ratas de la cepa Wistar por año.

Como se puede observar en los documentos adjuntos, el proyecto registrado ante la CINVA ha generado la publicación de artículos en revistas arbitradas así como la presentación en congresos nacionales e internacionales.

Agradeciendo la atención que le sirva dar a la presente, me despido de usted enviándole un cordial saludo.

Atentamente

Dra. Elena Zambrano González
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INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
SALVADOR ZUBIRÁN

Febrero 27, 2012

Claudia Janet Bautista Carbajal
Investigador en Ciencias Médicas "B"
Departamento de Biología de la Reproducción
P r e s e n t e .

Con referencia al proyecto de investigación: **"Efectos de la Obesidad Materna de la Rata Sobre el Metabolismo de las Crías".**

Registro CINVA: **271**

Le informo que este proyecto fue aprobado el 28 de julio de 2009 y sigue vigente.

A t e n t a m e n t e

MVZ., M. Sc. Cert. L.A.M. Rafael Hernández González
Coordinador del Comité de Investigación en Animales

ccp. Dra. Elena Zambrano González. Departamento de Biología de la Reproducción

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INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
SALVADOR ZUBIRÁN

14 de febrero del 2012

DRA. ELENA ZAMBRANO GONZÁLEZ
DEPARTAMENTO DE BIOLOGÍA DE LA REPRODUCCIÓN
P R E S E N T E

Estimada Dra. Zambrano.

Por este conducto le informo que el proyecto activo que lleva por nombre: **Efectos de la Obesidad Materna de la Rata Sobre el Metabolismo de las Crías** con registro CINVA 271 finaliza el mes de julio del año en curso. Le solicito de la manera más atenta me haga saber si el proyecto requerirá una prorroga. En caso afirmativo le solicitaré envié a la CINVA la justificación correspondiente.

Sin otro particular por el momento.

Atentamente.

P. P.

Dr. Rafael Hernández González
Coordinador de la CINVA

Recibí original
Luis A. Reyes
29/02/12

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INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
SALVADOR ZUBIRÁN

28 de Julio, 2009

Dra. Elena Zambrano González
Departamento de Biología de la Reproducción
P r e s e n t e .

En referencia con el proyecto de investigación: "**Efectos de la Obesidad Materna de la Rata Sobre el Metabolismo de las Crías".**

Registro CINVA: **271**

El Comité de Investigación en Animales ha revisado su respuesta a las observaciones emitidas por este Comité y decidió **APROBARLO**.

A t e n t a m e n t e

MVZ., M. Sc. Rafael Hernández González
Coordinador del Comité de Investigación en Animales

INSTITUTO NACIONAL DE CIENCIAS MEDICA Y NUTRICION INNSZ "SALVADOR ZUBIRAN"
4 AGO 2009
DIRECCIÓN DE INVESTIGACIÓN

RECIBI FORMA ORIGINAL MARGARITA ROMERO G.

ccp. Dr. Rubén Lisker Y. Director de Investigación
MVZ., M.en C. Octavio Villanueva Sánchez. Secretario CINVA
Dr. Patricio Santillán Doherty. Comité de Investigación en Animales.
Dr. Gerardo Gamba Ayala. Comité de Investigación en Animales.
MVZ. M.enC. Ma.de la Luz Streber J.. Comité de Investigación en Animales
MVZ. Griselda Salmerón Estrada. Comité de Investigación en Animales.
Dra. Nimbe Torres y Torres. Comité de Investigación en Animales.

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INSTITUTO NACIONAL DE
CIENCIAS MÉDICAS
Y NUTRICIÓN
SALVADOR ZUBIRÁN

Ciudad de México a 21 de junio de 2017

Dra. Norma Bobadilla Sandoval
Coordinadora de la Comisión de la Investigación en Animales
Presente.

En respuesta a las observaciones plasmadas al proyecto titulado "Efecto de la obesidad materna de la rata sobre el metabolismo de las crías" con número de CINVA 271, nos permitimos contestar a los señalamientos plasmados.

1. Explicar cómo se determina el tamaño de muestra.

El cálculo se realizó según lo reportado en la ILAR J 2002; 43:207-13. Para asegurar un 95% de probabilidad de generar obesidad, se plantea que el 70% (0.7) de los animales no presentan obesidad.

2. La anestesia con éter debe de ser profunda.

Gracias por la observación. Se incluirá en el procedimiento.

3. No se menciona el número de muestras que se tomarán del plexo orbitario.

En el escrito se incluyó que las muestras de sangre del plexo orbitario se tomaran a tiempo 0, 30, 60 y 120 minutos, lo que dará un total de 4 muestras.

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4. Mencionar el volumen de medicamento que será administrado

En el escrito se incluyó que el volumen del medicamento que se administrará estará en función del peso de la rata.

5. Se recomienda utilizar el término estirpe para describir a la rata Wistar ya que no es una cepa (strain).

Gracias por la observación. Se cambió el término cepar por estirpe.

6. En la descripción de las condiciones ambientales no se menciona los parámetros de humedad.

La humedad relativa será del 75-80%. Esta información ya fue incluida en el escrito.

7. El uso de lavado vaginal para la observación de espermatozoides puede producir pseudogestación e infecciones vaginales que disminuyen la fertilidad, ¿Por qué no utilizar, la presencia del tapón vaginal como indicador del día uno?

Este es un punto importante, sin embargo, con base a nuestra experiencia, el tapón vaginal no siempre es visible por lo cual sería difícil establecer el día en que inició la gestación. La presencia de los espermatozoides se realizará mediante frotis vaginal que es un método menos invasivo.

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8. Mencionar que se utilizan dietas semipurificadas y dietas comerciales para evitar confusión con el término "normal" que se utiliza a veces como control. Describir que es "dieta chow de bioterio". Aparentemente esa dieta no existe en el DIEB.

Se cambió el término "normal" por dietas purificadas. Al decir "dieta chow de bioterio" nos referimos a la dieta comercial, lamentamos la confusión. Esto ya fue corregido.

9. Se recomienda utilizar el término científico gestante en lugar del lenguaje coloquial preñada.

La palabra preñada fue sustituida por gestante.

10. Eliminar la frase en "en caso de ser menor o mayor a 14, los animales serán excluidos del experimento". Ya que se presta a confusión.

Se eliminó la frase.

11. El procedimiento de desecho de los animales en bolsas amarillas para incineración es innecesario ya que representa un elevado costo para la institución y los animales no son inoculados con agentes infecciosos, radioactivos o tóxicos para la salud humana y de otros animales. Se sugiere usar bolsas de basura municipal (transparentes) para los procedimientos que se indican.

Agradecemos la observación. Se cambió en el texto que las ratas serán desechadas en bolsas para basura municipal.

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12. Sustituir el término "carcasas" por cadáveres o canales.
En el punto 14. no se proporciona evidencia de que los estudiantes estén capacitados para hacer todos los procedimientos descritos (cruzamiento, lavado vaginal, obtención de sangre, eutanasia, etc.).

Carcasa se define como el conjunto íntegro, sin descuartizar la carne y huesos con el vaciado de sus vísceras u órganos internos toráxicos y abdominales. Por lo que consideramos que el termino carcasa en el presente proyecto es bien utilizado.
Con respecto al punto 14. Nuestro grupo de trabajo cuenta con personal entrenado para realizar los procedimientos descritos y ellos son quienes se encargan de capacitar a los alumnos de nuevo ingreso, también cuando es posible los alumnos son enviados a algún curso de manejo de animales. Los estudiantes nunca realizan ningún procedimiento sin previa capacitación.

13. En los objetivos mencionan que van a medir SREBP-1, PPARg, FAS, CPT, en hígado y PPARg en tejido adiposo y no mencionan que van a medir el gen de la insulina. Sin embargo, en la metodología solo mencionan que van a medir el gen de la insulina por Northerblotya que casi no se usa (consultar PCR de tiempo real) y no mencionan como van a medir los otros genes.

Agradecemos la observación. Estos errores ya fueron corregidos en el proyecto se menciona que la determinación de los genes mencionados se realizará por PCR tiempo real.

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SALVADOR ZUBIRÁN

14. El requerimiento de grasa para animales en crecimiento y embarazo es del 7% de aceite de soya (consultar AIN 93) y no del 5%.

Agradecemos la observación, este cambio ya se incluyó en la tabla de la composición de las dietas.

15. El requerimiento de proteína es del 20% cuando la pureza es >85% (consultar AIN 93) y no del 23.11%.

Agradecemos la observación, este cambio ya se incluyó en la tabla de la composición de las dietas.

Atentamente

Dra. Elena Zambrano González

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México D.F., a 16 de julio de 2007

Dra. Elena Zambrano
Departamento Biología de la Reproducción

En relación con la revisión de protocolo remitido por usted para su revisión por el Comité de Investigación en Animales
Registro CINVA 271
"Efectos de la obesidad materna de la rata sobre el metabolismo de las crías"

El Comité requiere de la siguiente información para emitir su dictamen.

Observaciones:
1. Explicar como determina el tamaño de muestra.
2. La anestesia con éter debe ser profunda.
3. No se menciona el número de muestras que se tomarán del plexo orbitario.
3. Mencionar el volumen de medicamento que será administrado.
4. Se recomienda utilizar el término estirpe para describir a la rata Wistar ya que no es una cepa (strain).
5. En la descripción de las condiciones ambientales no menciona los parámetros de humedad.
6. El uso de lavado vaginal para la observación de espermatozoides puede producir pseudogestación e infecciones vaginales que diminuyen la fertilidad, ¿Porque no utilizar, la presencia de tapón vaginal como indicador del día uno?.
7. Mencionar que se utilizan dietas semipurificadas y dietas comerciales para evitar confusión con el término "normal" que se utiliza a veces como control. Describir que es dieta "Chow de bioterio". Aparentemente esa dieta no existe en el DIEB.
8. Se recomienda utilizar el término científico rata gestante en lugar del lenguaje coloquial preñada.
9. Eliminar la frase: "En caso de ser menor o mayor a 14, los animales se serán excluidos del experimento". Ya que se presta a confusión.

Recibí original
Paola Martínez S.
17/07/09

• Vasco de Quiroga 15,
• Delegación Tlalpan
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• Tel. 54-87-09-00

10 El procedimiento de desecho de los animales en bolsas amarillas para incineración es innecesario ya que representa un elevado costo para institución y los animales no son inoculados con agentes infecciosos, radioactivos o tóxicos para la salud humana y de otros animales. Se sugiere usar bolsas de basura municipal (transparentes) para los procedimientos que indican.

11. Sustituir el término "carcasas" por cadáveres o canales.

En el punto 14. No se proporciona evidencia de que los estudiantes estén capacitados para hacer todos los procedimientos descritos (cruzamiento, lavado vaginal, obtención de sangre, eutanasia, etc.).

12. En los objetivos mencionan que van a medir SREBP-1,PPARg, FAS, CPT en hígado y PPARg en tejido adiposo y no mencionan que van a medir el gen de la insulina. Sin embargo, en la metodología solo mencionan que van a medir el gen de la insulina por el método de Northern blot que ya que casi no se usa (consultar PCR de tiempo real) y no mencionan como van a medir los otros genes.

13. El requerimiento de grasa para animales en crecimiento y embarazo es del 7% de aceite de soya (consultar AIN 93) y no del 5%

14. El requerimiento de proteína es del 20% cuando la pureza es >85% (consultar AIN 93) y no del 23.11%

Sin otro particular

Atentamente

M.V.Z., M.Sc. Rafael Hernández González
Coordinador del CINVA

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INCMNSZ
DIRECCIÓN DE NUTRICIÓN

- 2 JUL 2009

FECHA DE CLASIFICACIÓN: 30 Jun / 2009
ÁREA: INV EXP. Y BIOTERIO
CONFIDENCIAL: ☐
RESERVADA: ☒ TOTAL ☐ PARCIAL ☐
PERIODO DE RESERVA: 5 AÑOS
PARTES O SECCIONES: completa
FUNDAMENTO LEGAL. Art. 13, 14 y 18 de la L.F.T.A.I.P.G.
FIRMA DEL TITULAR DEL ÁREA:

INCMNSZ

COMITÉ INSTITUCIONAL DE INVESTIGACIÓN EN ANIMALES

FORMATO DE EVALUACIÓN DE PROYECTO DE INVESTIGACIÓN

FISIOLOGÍA DE LA NUTRICIÓN

Colocar cursor dentro del área blanca para llenar la información (NO utilizar áreas sombreadas)

No. de registro CINVA: 271

1. Título del proyecto

Efectos de la obesidad materna de la rata sobre el metabolismo de las crías

2. Investigadores

2a. Identificación

Nombre, firma y adscripción de cada uno de los investigadores participantes. El investigador principal deberá ser un profesional adscrito a la Institución (médico de base o investigador) y no un alumno de curso, residente, pasante, interno, etc.).

INVESTIGADOR	Posición institucional	Posición en el proyecto	Teléfono (ext.)	Correo-E
Dra. Elena Zambrano González	Investigador en Ciencias Médicas "E"	Investigador principal	55 54 87 09 00 (ext. 2417)	zamgon@laneta.apc.org
QA. Paola Martínez Samayoa	Estudiante	Apoyo en investigación	55 54 87 09 00 (ext. 2417)	[redacted]
QFB. Guadalupe Rodríguez González	Estudiante	Apoyo en investigación	55 54 87 09 00 (ext. 2417)	[redacted]

Articulo 113Fracción I de LFT AIT eleminado correo electronico por tratrarse de un dato personal

2b. Pertinencia del grupo de investigadores con respecto del proyecto

Brevemente describa las calificaciones del grupo investigador con respecto del proceso de investigación científica en general y con respecto del proyecto presentado (v.gr.: grado académico, experiencia laboral, miembro del sistema de investigadores de los INS, del SNI, etc).

Grado académico: Doctorado en Ciencias Biológicas
Miembro del SNI: SNI II
Experiencia laboral: Labora como investigadora en ciencias médicas del departamento de Biología de la Reproducción del INCMNSZ desde 1989 hasta la fecha.

3. Instituciones participantes

Nombre y dirección de la o las instituciones participantes y breve descripción de en qué consistirá su participación. Para estudios multicéntricos añadir los datos del centro coordinador.

4. Patrocinio

4a. Organismos patrocinadores

Nombre, dirección y teléfono de la o las organizaciones, instituciones o laboratorios que aportarán recursos.

4b. Especificar si los investigadores reciben pago (monetario o en especie) por su participación específica en la investigación.

En caso afirmativo, describir.

5. Marco teórico

Explicar detalladamente los fundamentos disponibles a la fecha en los que se basa el estudio que se propone (sentido biológico, datos de experimentos en animales o en humanos):
a) antecedentes,
b) definición del problema,
c) justificación

a) Antecedentes

La desnutrición y la malnutrición son un problema actual de salud pública. Los cambios en los patrones de alimentación y de actividad física han incrementado el desarrollo de obesidad (1). Dentro de los sectores de la población afectados por el desarrollo de obesidad en México, se encuentran las mujeres en edad joven y reproductiva y los niños en edad escolar. En el primer grupo, la

prevalencia de obesidad ha aumentado de 9 a 32 %, de 1988 al 2006; mientras que en el sector infantil, la prevalencia ha incrementado de 11 a 18 %, de 1999 al 2006, de acuerdo a la Encuesta Nacional de Nutrición (2). El desarrollo de obesidad infantil puede ser resultado, no solo de las condiciones de sedentarismo, estilos de vida y alimentación, sino también de las condiciones nutricionales y metabólicas de la madre. Esto se debe a que el desarrollo de obesidad materna durante la gestación y la lactancia, puede ser una condición de estrés que predisponga al feto al desarrollo de enfermedades metabólicas desde la niñez y en mayor grado, en la vida adulta (3). Dentro de estos efectos adversos que la obesidad en el embarazo y lactancia ocasiona en la progenie, se encuentran características del síndrome metabólico como resistencia a la insulina, aumento de tejido adiposo abdominal e hipertensión (4).

Investigaciones científicas tanto en humanos como en animales de experimentación, han demostrado que la salud está íntimamente relacionada con la calidad de vida en las primeras etapas del crecimiento. El fenotipo del adulto es la suma de los factores genéticos, así como de la influencia del ambiente fetal y postnatal. El ambiente perinatal subóptimo puede programar la predisposición para el desarrollo de enfermedades crónicas, incluyendo la alteración del metabolismo de hidratos de carbono (5-9). Los hallazgos realizados en 1986 por el Dr. David Barker de la Universidad de Southampton, Inglaterra, en donde encuentra una correlación entre el bajo peso al nacimiento y el riesgo a enfermedades coronarias en la vida adulta (10) fueron el inicio de varios estudios epidemiológicos encaminados a identificar la relación que existe entre la talla y peso del neonato con la hipertensión arterial, enfermedades cardiovasculares y cerebrovasculares, obesidad, dislipidemias e intolerancia a la glucosa (11-13).

La hipótesis de los orígenes del desarrollo de la salud y la enfermedad (*DOHaD, por sus siglas en inglés*), antes conocida como "programación del desarrollo", propone que la fisiología y metabolismo fetal y neonatal pueden ser alterados por cambios durante periodos críticos del desarrollo, como la gestación y la lactancia. Estas alteraciones generan una respuesta fisiológica permanente en el feto que se asocia con el desarrollo de enfermedades en el adulto (14). Estudios con animales de experimentación han utilizado diferentes modelos para evaluar el efecto de la programación del desarrollo en el metabolismo (15,16). Algunos de estos modelos son la restricción nutricional en la dieta de la madre gestante y durante la lactancia o la diabetes gestacional. Se ha demostrado que el ambiente intrauterino de madres desnutridas durante la gestación predispone a padecer diabetes y obesidad en la vida adulta. La alteración del desarrollo del páncreas, tejido adiposo, músculo e hígado fetales, entre otros, puede tener consecuencias en la predisposición de enfermedades metabólicas a largo plazo.

Sin embargo, actualmente son pocos los estudios que recientemente se han enfocado en los efectos de la programación del desarrollo por una dieta hipercalórica, como una dieta materna alta en grasa. El feto metabólicamente programado presenta modificaciones permanentes en la estructura y fisiología de órganos, así como en la expresión de genes involucrados en su propio metabolismo (17). Por lo que los factores ambientales como la obesidad materna pueden alteran permanentemente el metabolismo de tejidos y sistemas del organismo.

Existen estudios que han reportado los efectos de una dieta hiperlipídica durante la gestación y/o la lactancia en la salud de la descendencia en edad adulta. En un estudio realizado con babuinos, con dieta materna hiperlipídica durante la gestación, se demostró que el aumento de peso corporal y de concentraciones séricas de triglicéridos en la madre puede relacionarse con el incremento del crecimiento fetal (18). Esto sugiere que la madre podría presentar alteraciones metabólicas como altas concentraciones de insulina y leptina, cambios en el perfil lipídico e incremento de tejido adiposo que se relacionen con el desarrollo de obesidad en las crías (18).

La expansión del tejido adiposo involucra tanto la hipertrofia como la hiperplasia de adipocitos. Los estadios tempranos de la vida son periodos muy vulnerables y sensibles a factores nutricionales y hormonales, que modulan la multiplicación y diferenciación de los precursores de células adiposas (19). La desnutrición durante la gestación genera hipotrofia de los adipositos (20), mientras que la sobrealimentación durante la lactancia, asociada con altas actividades de enzimas lipogénicas (21), genera hipertrofia de los adipocitos.

Por otro lado, el aumento de tejido adiposo genera el incremento de la liberación de ácidos grasos libres a la circulación (22). La acumulación de ácidos grasos intracelulares en órganos no adiposos conocido como lipotoxicidad, es regulado por el metabolismo de lípidos el cual a su vez, es controlado por una familia de factores de transcripción conocida como PPARs (Proliferator Peroxisome Activator Receptor), que están involucrados en la activación de genes involucrados en el transporte y oxidación de lípidos (22,23). Uno de estos factores de transcripción es PPARγ (receptor activado por proliferadores de peroxisomas γ), el cual se expresa principalmente en tejido adiposo y controla el almacenamiento de triglicéridos mediante la diferenciación de adipocitos y la esterificación de ácidos grasos en los adipocitos maduros, regulando la expresión de genes involucrados como la expresión de la enzima sintasa de ácidos grasos (FAS) (23). La acumulación de lípidos en tejidos no adiposos está relacionada con la reducción del contenido de PPARγ. Otra isoforma de los PPARs y que se expresa principalmente en el hígado, es PPARα. Este factor de transcripción juega un papel importante en la oxidación de ácidos grasos regulando la expresión de genes como la enzima carnitin palmitoil CoA transferasa (CPT) (24). La familia de las SREBPs (Sterol Regulatory Element Binding Protein), formada por tres miembros SREBP-1a,1c y 2, también participa en el metabolismo de lípidos. Estos factores de trascripción controlan la homeostasis de lípidos al activar de manera coordinada, la expresión de genes involucrados en la glucólisis, así como el metabolismo de colesterol y ácidos grasos y el de lipoproteínas (24). Específicamente SREBP-1 se une a elementos de respuesta a esteroles de genes que participan en el metabolismo de lípidos (24). La participación de SREBPs y PPARs en el desarrollo de lipotoxicidad del hígado, ha comenzado a investigarse y dentro del área de la programación del desarrollo, es necesario el estudio de mecanismos moleculares que ayuden a explicar la relación entre la calidad de vida prenatal y el riesgo de padecer enfermedades metabólicas en la vida adulta.

El desarrollo de obesidad materna, debido al aumento en el consumo de dietas ricas en lípidos y carbohidratos, también puede alterar otros parámetros metabólicos en la descendencia como son la distribución de grasa corporal, la función cardiovascular y el metabolismo de glucosa (25). En este trabajo se plantea la hipótesis de que dichas alteraciones tienen su origen en la programación del balance de energía, la cadena se señalización de insulina, en la expresión de factores de transcripción relacionados con la regulación del metabolismo de lípidos o en la expresión de receptores en el hipotálamo involucrados en el control de los centros de apetito y saciedad, como el receptor OB-Rb de la leptina, hormona relacionada con el balance energético y secretada por el tejido adiposo (26). Se ha demostrado que ratas de experimentación sobrealimentadas durante su vida temprana postnatal presentan un estado de resistencia a leptina, mediada por la regulación negativa del receptor hipotalámico OB-Rb (27).

b) Definición del problema

El propósito del presente estudio es determinar cómo es que el desarrollo de obesidad en ratas hembras, dado por el consumo de dieta hiperlipídica desde el destete hasta la edad adulto joven y durante el embarazo y la lactancia, programa el metabolismo de glucosa y lípidos de sus crías, así como alteraciones en la ingesta de alimento y desarrollo de adiposidad, de tal forma que estas serán más susceptibles a desarrollar enfermedades metabólicas en la vida adulta a pesar de haber sido alimentadas normalmente desde el destete, a diferencia de un grupo de crías de madres control.

Los estudios preliminares realizados con un modelo de obesidad han mostrado aumento de tejido adiposo y aumento en las concentraciones séricas de triglicéridos en las crías de madres alimentadas con dieta hipercalórica en el periodo perinatal, por lo que esperamos encontrar alteraciones a nivel bioquímico y molecular en el tejido adiposo, muscular, cerebro y páncreas que permitan explicar el mecanismo por el que se desencadenan los efectos metabólicos de la programación del desarrollo en las crías.

c) Justificación

Algunos estudios previos realizados con ratas, han demostrado que las modificaciones en el contenido de carbohidratos y de proteínas materna durante el inicio del embarazo ya sea generado por diabetes, por la administración materna de glucocorticoides, por la restricción proteínica materna o por la restricción global de calorías alteran el metabolismo de las crías (28-32). Con respecto al modelo de obesidad materna que se plantea en este trabajo, nos proponemos determinar cómo es que el fenotipo de la rata madre que presenta obesidad durante la gestación y la lactancia, puede contribuir al desarrollo de obesidad y problemas metabólicos de las crías. En este estudio se utilizará como modelo animal a la rata, dado que con éste se pueden evaluar diferentes etapas del crecimiento, desde el destete hasta la vida adulta en corto tiempo y con los resultados obtenidos se espera conocer mecanismos que expliquen la importancia que puede tener el consumo de dieta hipercalórica en la programación del feto.

En el presente estudio se plantea conocer la importancia del adecuado ambiente metabólico durante la vida intrauterina que incide en la programación desde la vida fetal en diferentes funciones y mecanismos de adaptación durante la vida postnatal. Los resultados de este estudio serán importantes para conocer el impacto de la calidad de vida embrionaria y postnatal sobre la funcionalidad de sistemas complejos y hormonalmente regulados.

6a. Hipótesis

Definido como un enunciado comprobable acerca de la relación entre una variable dependiente y una variable independiente.
(recordar que los conceptos de "hipótesis nula", "hipótesis alterna" se relacionan al análisis estadístico por lo que NO deben incluirse en este rubro)

La obesidad materna programa el metabolismo del feto lo cual aumenta el riesgo de desarrollar enfermedades metabólicas en la vida adulta.

6b. Objetivos.

Aquellos que se esperan obtener puntualmente en el estudio y especificados como objetivo general y objetivos específicos

Objetivo general:

Estudiar los efectos de la obesidad materna durante el periodo perinatal de la rata para la programación de enfermedades crónico degenerativas y su asociación con desórdenes metabólicos en las crías de ambos sexos.

Objetivos específicos:

Estudiar a la madre durante el crecimiento y la gestación para poder esclarecer el ambiente intrauterino metabólico y endócrino al que estarán sometidas las crías. Para esto se determinará el peso corporal, la ingesta de alimentos y el perfil lipídico de las ratas madre durante la gestación y la lactancia, así como la composición química de la leche al término de la lactancia.

Evaluar el registro de peso corporal y de ingesta de alimento de las crías en diferentes etapas del desarrollo.

Analizar a la leptina y su receptor en hipotálamo en las crías neonatas y su correlación con modificaciones en el apetito, tejido

adíposo y peso corporal.

Analizar la concentración sérica de leptina, insulina, glucosa y del perfil lipídico, así como la composición corporal de las crías en diferentes etapas del desarrollo.

Determinar la concentración y nivel de expresión de los factores de transcripción SREBP-1 y PPARα, así como de las enzimas FAS y CPT en hígado y PPARγ en tejido adiposo, de las crías en edad adulta.

Comparar las diferencias de género de los efectos metabólicos por el impacto de la obesidad materna.

7. Metodología: Diseño general.

Describir el diseño general del estudio y, si es pertinente, especificar los siguientes puntos:
a) Diseño del estudio: describir si es aleatorio/no aleatorio, controlado, de cohorte, tipo de cegamiento (doble-ciego, simple), tipo de controles (placebo, medicamento activo), periodo de lavado..
b) Descripción de la maniobra o intervención
c) Tamaño de muestra (# de pacientes a incluir; justificar el cálculo)
d) Mecanismo de asignación del tratamiento (aleatorio/abierto)
e) Grupos de tratamiento y
f) Duración del seguimiento individual

Modelo biológico

Se utilizarán ratas hembras albinas de la cepa Wistar recién destetadas. Los animales serán mantenidos en el bioterio del Instituto Nacional de Ciencias Médicas y Nutrición SZ bajo condiciones controladas de luz-obscuridad (de 7:00 a 19:00 h), así como de humedad y temperatura (22–23°C). Todos los procedimientos fueron previamente aprobados por el comité de ética de experimentación animal del INCMNSZ. Cuando las ratas alcancen un peso de 240 ± 20 g y entre 10 y 12 semanas de edad, serán apareadas con machos y el día en el cual se encuentren espermatozoides en la vagina se designará como el inicio de la gestación. Durante los diferentes tiempos que dure el experimento, las ratas, tanto las madres como las crías serán pesadas diariamente.

Se utilizarán dos dietas con diferente contenido grasa. Dieta control (C) con 5% de grasa y dieta hiperlipídica (H) con 25% de grasa. La dieta hiperlipídica será compensada en su valor energético con la adición de carbohidratos (Tabla 1). La dieta utilizada es la recomendada por el Instituto Americano de Nutrición para roedores en las fases de embarazo, lactancia y crecimiento (33).

Tabla 1. Composición de las dietas experimentales

	DIETA CONTROL (%)	DIETA HIPERLIPÍDICA (%)
Caseía	23.11	23.11
L-Cistina	0.3	0.3
Colina	0.165	0.165
Vitaminas	1	1
Minerales	5	5
Celulosa	5	5
Aceite	5	5
Manteca de cerdo	----	20
Hidratos de carbono		
Almidón	30.21	20.59
Dextrosa	30.21	20.59
	4 Kcal/g dieta	5 Kcal/g dieta

Las ratas preñadas y lactantes serán pesadas todos los días durante el embarazo y hasta el destete. También la ingesta de alimento será cuantificada todos los días. Las ratas nacerán por parto natural. La distancia ano-genital al nacimiento será medida con regla Vernier para la determinación del sexo. Nuestros datos (34) indican que las crías hembras al nacimiento tienen una distancia ano genital de 1.67 ± 0.128 mm (n=291 crías de 43 diferentes camadas) y los machos 3.26 ± 0.22 mm (n=252 crías de 43 camadas). De tal forma que 2.5 mm es más de 2 SD's de la media de cada grupo. Por tanto, el sexo será determinado de acuerdo a la distancia ano-genital > (machos) o < (hembras) 2.5 mm.
Para asegurar homogeneidad en el estudio, camadas de 10 a 14 crías serán ajustadas al nacimiento a 10 crías por madre tratando de mantener una relación de sexo de 1:1. En caso de ser menor a 10 o mayor a 14, los animales serán excluidos del experimento.
Para cada experimento se utilizarán 50 ratas a preñar por grupo. De acuerdo a nuestra experiencia en proyectos anteriores, se estima

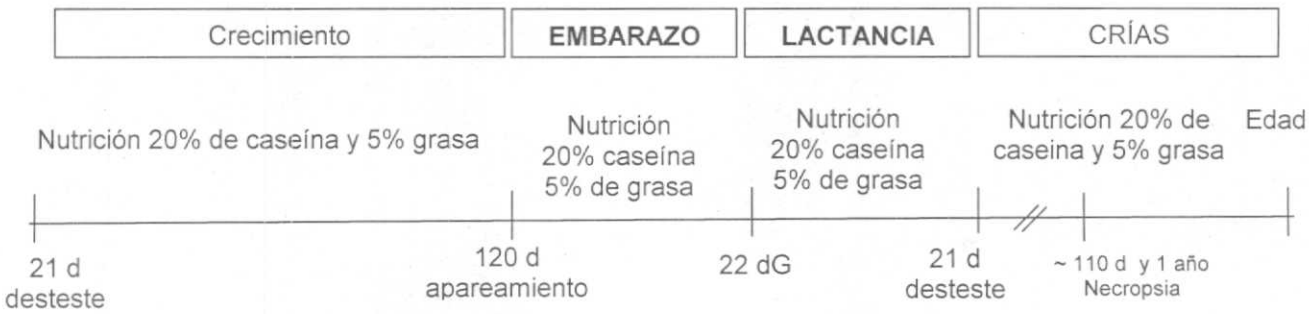
que al finalizar el experimento quedará una "n" aproximada de 6 madres por grupo, dado que la tasa de fertilidad de las ratas del Bioterio del INCMNSZN es del 80% y que se eliminarán animales por tener camadas grandes o pequeñas.
Las diferentes dietas experimentales serán administradas a las ratas desde el destete y en el embarazo y la lactancia quedando 2 grupos experimentales:

1) Grupo Control (C). Nutrición normal a base de 20% de caseína y con 5 % de grasa a partir del destete y durante el embarazo y la lactancia.
2) Grupo Hiperlipídico (H). Nutrición hiperlipídica a base de 25% de grasa durante a partir del destete y durante el embarazo y la lactancia.

Al destete todas las crías de ambos grupos serán alimentadas con dieta normal hasta el momento del sacrificio. Es importante resaltar que el consumo de dietas será a partir del destete y no únicamente durante el embarazo y/o lactancia. Las crías y la ingesta de alimento serán pesadas todos los días hasta el término del estudio.

GRUPOS EXPERIMENTALES:

Grupo I. Control (C).

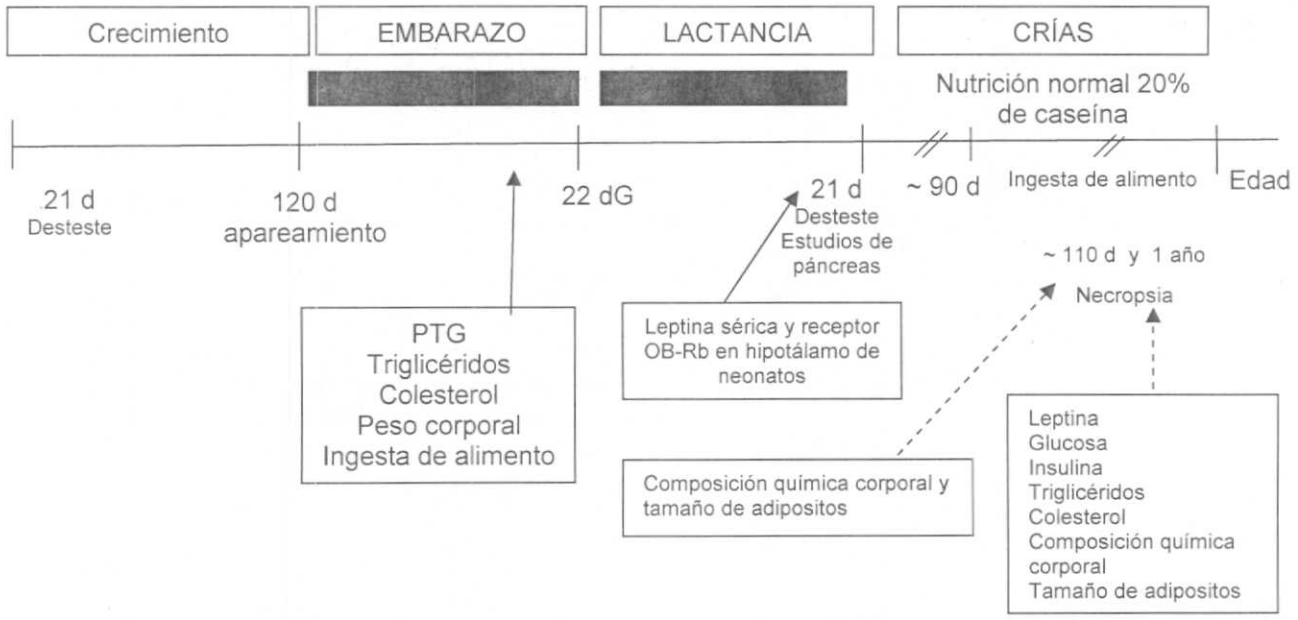


Grupo II. Hiperlipídico (H)

dG = días gestacionales; d = días.

Diseño experimental

Línea de tiempo



METODOLOGÍA ESPECÍFICA CON RESPECTO AL MANEJO DE ANIMALES

El presente estudio requiere de la utilización de ratas hembras de la cepa Wistar recién destetadas y con un peso aproximado de 60g. Así mismo se utilizarán ratas machos de la misma cepa con fertilidad probada para el apareamiento con las hembras.

Nutrición

Se proponen diversos tipos de dietas:
Dieta Chow de bioterio y agua *ad limitum* para alimentar a las ratas madre de las que se obtendrán las ratas destetadas para conformar los grupos experimentales.
Dietas control e hiperlipídica con diferentes proporciones de grasa (como se indica en la tabla 1, páginas 6 y 7).

Administración de medicamentos

Para la prueba de tolerancia a la glucosa, la rata será puesta en ayuno durante la noche previa. Un gramo/Kg de peso de D-glucosa será administrado vía intraperitoneal como está descrito en la página 9.

Obtención de muestras de sangre

Las muestras de sangre se obtendrán por punción del plexo retro-orbital. El animal será anestesiado ligeramente en una cámara de éter para la realización de dicho procedimiento. Se utilizará éter por sus bajos o nulos efectos en las concentraciones de hormonas gonadotrópicas, receptores esteroideos y expresión de genes metabólicos (36,37). Posteriormente se sujetará al animal y se colocará con el tórax hacia arriba. Se introducirá un tubo capilar en el ángulo externo del ojo hasta encontrar el seno venoso. La sangre fluirá inmediatamente por capilaridad hacia el interior del tubo. Se colectarán muestras entre 400 y 600 µl de sangre. Al finalizar se limpiará el ojo con un algodón humedecido con agua estéril y se aplicará una gota de solución de antibiótico-antimicótico.

Obtención de tejidos

El día de la necropsia se obtendrán todos los órganos del animal y se pesarán, para su normalización con respecto al peso corporal.

Eutanasia

Los animales serán sometidos a eutanasia por decapitación sin anestesia previa, utilizando una guillotina para roedores (Thomas Scientific, EU). La razón por la cual no utilizaremos ningún tipo de anestésico es que en nuestra experiencia (28,33) y la de otros (36,38) hemos observado que la previa manipulación incrementa el grado de estrés de la rata, lo cual, además de afectar los valores de algunas hormonas, como la corticosterona (39), genera mayor sufrimiento al animal.

Cabe aclarar que para evitar estrés adicional en los animales a ser sacrificados, el proceso se llevará a cabo en un lugar separado al resto de las ratas, y entre animal y animal, se lavará la guillotina y los guantes del personal involucrado.

Los restos de los animales serán colocados en las bolsas amarillas (asignadas para residuos peligros biológico infecciosos: RPBI), posteriormente se congelarán a -20°C (en el depósito de cadáveres de animales para necropsia que el Bioterio del Instituto tiene asignado para tal propósito). Una compañía privada contratada por el INCMNSZ se encargará de la posterior incineración de los animales.

Características de los animales, condiciones de alojamiento y maniobras experimentales que requiere el proyecto

Especie	Raza,cepa	Condición microb.	No. Hembras	Distribu–ción	Alojamiento	Densidad	Nivel Bioseg.*	Nivel de afectación**	Destino	Eutanasia
Rata	Wistar	Ninguna	250	50 c/6 meses	Caja de policarbo–nato de piso sólido	4/ caja	Nivel I	Categoría B	Eutanasia	Decapitación

*Nivel de bioseguridad I, trabajo con agentes químicos, físicos o biológicos que no producen enfermedad y no son un riesgo para la salud de personas sanas y el medio ambiente.

**Nivel de afectación, Categoría B, experimentos que causen molestia o estrés mínimo (inyección no dolorosa, restricción de movimiento, marcado o aretado de orejas).

Ubicación de los animales: cuarto No. 6 y 9
Lugar de realización de los procedimientos Laboratorio y cuarto de procedimientos

Nivel de habilidad y experiencia del personal :
El personal que trabajará directamente con los animales cuenta con la habilidad, entrenamiento y experiencia para realizar las siguientes maniobras experimentales que se realizarán en el proyecto:

- Inmovilización
- Anestesia
- Toma de sangre
- Eutanasia

8. Metodología: Criterios de selección

a) Criterios de inclusión (considerar que no participen en otras investigaciones y anticoncepción en caso necesario)

Las ratas preñadas y lactantes serán pesadas todos los días durante el embarazo y hasta el destete. También la ingesta de alimento será cuantificada todos los días. Las ratas nacerán por parto natural. La distancia ano-genital al nacimiento será medida con regla Vernier para la determinación del sexo. Nuestros datos (34) indican que las crías hembras al nacimiento tienen una distancia ano genital de 1.67 ± 0.128 mm (n=291 crías de 43 diferentes camadas) y los machos 3.26 ± 0.22 mm (n=252 crías de 43 camadas). De tal forma que 2.5 mm es más de 2 SD's de la media de cada grupo. Por tanto, el sexo será determinado de acuerdo a la distancia ano-genital > (machos) o < (hembras) 2.5 mm.

Para asegurar homogeneidad en el estudio, camadas de 10 a 14 crías serán ajustadas al nacimiento a 10 crías por madre tratando de mantener una relación de sexo de 1:1. En caso de ser menor a 10 o mayor a 14, los animales serán excluidos del experimento.

Para cada experimento se utilizarán 50 ratas a preñar por grupo. De acuerdo a nuestra experiencia en proyectos anteriores, se estima que al finalizar el experimento quedará una "n" aproximada de 6 madres por grupo, dado que la tasa de fertilidad de las ratas del Bioterio del INCMNSZN es del 80% y que se eliminarán animales por tener camadas grandes o pequeñas.

b) Criterios de exclusión

Criterios para dar por terminado el experimento en caso de que los animales presenten signos de sufrimiento

Los animales que en el transcurso del experimento presenten algún tipo de sufrimiento (no generado por el diseño experimental), serán sometidos a eutanasia. Los criterios son (www.ahc.umn.edu/rar/euthanasia.html):

1. Pérdida de peso mayor al 20-25%.
2. Pérdida del apetito: completa anorexia por 24 h o anorexia parcial (50% del la ingesta calórica) durante 3 días.
3. Debilidad o inhabilidad para obtener su alimento y agua.
4. Estado moribundo: signos de depresión o la falta de respuesta a estímulos.
5. Presencia de alguna infección.
6. Signos de disfunción severa de algún órgano o sistema
7. Presentación de alguna anormalidad física (tumores).

Para los siguientes procedimientos: maniobras conductuales, modificaciones ambientales, restricción física y ejercicio, inmunizaciones, inoculación de agentes biológicos, sustancias peligrosas, radiaciones, trauma, cirugía, NO APLICA.

c) Criterios de eliminación (considerar embarazo en caso necesario).

9. Metodología: Desenlaces y variables

a) Variables/desenlaces principales a medir
b) Variables/desenlaces secundarios a medir
b) Frecuencia de las mediciones,
c) Criterios de éxito y falla, en caso necesario y
d) Estrategia de análisis estadístico.
Cuando corresponda deben especificarse y fundamentarse las técnicas, aparatos y/o instrumentos que se utilizarán en la medición (esto incluye equipos mecánicos/electrónicos/cibernéticos especiales, formatos de evaluación, cuestionarios, tablas de cotejo, etc.), señalando los criterios de validez, reproducibilidad y controles de calidad que se tengan de los mismos.

Cuantificación de la ingesta de alimento en las crías a los 110 d de edad

Se pondrán en la misma caja dos ratas del mismo grupo experimental y mismo sexo. La comida se proveerá en forma de galletas. Se pesará diariamente tanto la cantidad de comida que se proporcione cada día así como la parte restante después de 24 horas. La cantidad de alimento consumido será promediado entre las dos ratas.
Las crías serán pesadas todos los días. Sin embargo, dado que las concentraciones de leptina de la cría adulta se obtendrán alrededor de los 110 días de edad, para la obtención de la correlación entre las diferentes variables: peso, ingesta de alimento, contenido de grasa corporal y concentraciones séricas de leptina, se utilizarán los datos obtenidos a los 110 días de edad.

Composición química de las carcasas

Después del sacrificio las ratas serán congeladas en pequeñas bolsas de plástico hasta su análisis. El abdomen se abrirá y las vísceras serán removidas y desechadas. Se pesará el animal, cantidad que será designada como peso húmedo, después se pondrá a secar la carcasa a 60°C hasta peso constante. El peso perdido se considerará como cantidad de agua en el cuerpo. La carcasa seca será molida y se realizarán alícuotas para la determinación de la cantidad de grasa por el método de Soxhlet (35) y la cantidad de nitrógeno total (proporcional a la cantidad de proteína) por el método de Kjeldahl (35).

Apareamiento de hembras

A los 120 días de edad las hembras alimentadas con dieta control o hiperlipídica, serán apareadas con machos (no incluidos previamente en el estudio). Durante el embarazo todas las hembras serán alimentadas con las dietas experimentales y las ratas tendrán partos naturales. Se obtendrá el tamaño de la camada y el peso corporal de las crías al nacimiento. La distancia ano-genital se medirá al nacimiento para la determinación del sexo. Las camadas serán ajustadas a 10 crías/madre manteniendo una relación de 1:1 hembras:macho, en lo posible. Al destete (día 21) las crías serán divididas por sexo y acomodadas en diferentes cajas por grupo.

Prueba de tolerancia a la glucosa (PTG) en las madres preñadas de la generación F1

Un experimento aparte será realizado para el estudio de las PTG de las crías hembras preñadas, de tal forma que el estrés generado por las tomas de muestra no afecte en los resultados obtenidos de las crías.
Una rata hembra de cada camada preñada en día 17 de gestación, será puesta en ayuno durante la noche. Un gramo/Kg de peso de D-Glucosa será administrado vía ip. La sangre será obtenida por punción retro-orbital al tiempo 0, 30, 60 y 120 min. La sangre será colectada en tubos de polietileno y centrifugada. El suero obtenido será almacenado a -20°C hasta el momento de su uso.

Análisis Bioquímicos

Cuantificación de glucosa en sangre
Las concentraciones de glucosa en suero serán determinadas espectrofotométricamente utilizando el método enzimático de la hexocinasa (Beckman Coulter, Co Fullerton, CA).

Radioinmunoensayo de Insulina
Las concentraciones de insulina en suero serán determinadas por RIA utilizando un estuche comercial de Linco Research INC #Cat RI-13K.

Cuantificación de triglicéridos y colesterol
Las concentraciones de triglicéridos y colesterol en suero se determinarán enzimáticamente con el autoanalizador Sycnchron CX (Beckman coulter, Co, Fullerton, CA).

Radioinmunoensayo de Leptina
Las concentraciones de leptina en suero serán determinadas por RIA utilizando un estuche comercial de Linco Research, Inc #Cat RL-83K.

Toma de tejidos

De las crías de ambos grupos experimentales se disecará el tejido adiposo retro-peritoneal, hipotálamo y páncreas para su estudio a nivel celular y molecular en las diferentes edades requeridas.

Tamaño de las células adiposas

Células adiposas provenientes de la zona retroperitoneal serán fijadas como lo describe Etherthon et al. Brevemente, 100 a 150 mg de tejido adiposo retroperitoneal será cortado y lavado a 37°C en 0.15 M de NaCl. Los cortes de tejido adiposo serán fijados en tetróxido de osmio al 3% en buffer de Colidina-HCl (50 mM, pH 7.4) por 72-96 h. Las células fijadas serán lavadas en NaCl al 0.15 M por 24 h, se eliminará el tejido conectivo incubando en 8 M de urea por 24-48h. El tamaño de las células será medido utilizando un microscopio óptico.

Inmunohistoquímica del receptor de leptina (OB-Rb) en hipotálamo

Se realizarán cortes seriados del hipotálamo y serán fijados a 60°C por 20 min. Posteriormente se realizarán lavados seriados con xyleno, etanol al 100% y al 95%. Se agregará solución de peroxidasa endógena en metanol/ H_2O_2 por 30 minutos. Se incubará con albúmina bovina y se agregará el anticuerpo primario (anti-OB-Rb) por una hora. Como segundo anticuerpo se utilizará suero de conejo anti-cerdo (DAKO # E0353), posteriormente streptavidina por 30 minutos (DAKO #P0397) y al final DAB por 5 minutos. Las laminillas serán teñidas con verde de metileno por 1 minuto y deshidratadas con 95% y 100% de etanol y xileno.

Estudios Celulares y Moleculares a nivel del páncreas

La cuantificación de la insulina y glucagon, así como la expresión de sus genes a nivel del páncreas, será de utilidad para indagar si las modificaciones en el metabolismo de la glucosa están mediadas en parte por la regulación y equilibrio entre estas dos hormonas.

Extracción de RNA y análisis de la expresión del gen de la insulina

El páncreas será removido inmediatamente después del sacrificio de cada animal. El RNA total será extraído, utilizando 2 ml de TRIzol (Gibco BRL). Una vez obtenido, el RNA se cuantificará por espectrofotometría.
Un total de 20 µg de RNA de tejido de cada rata, se separará en geles desnaturalizantes de agarosa al 1% y 2.2 M de formaldehído. Después de la electroforesis, el gel será transferido por capilaridad a una membrana de nitrocelulosa (Genescreen, NEN Research Products, Dupon) con SSC 10X (citrato de sodio 150 mM, NaCl 1.5 M) por toda la noche. Las membranas se prehibridarán en 0.2 ml/cm^2 de la solución A (formamida 50%, SDS 0.2%, EDTA 10mM, SSC 2X, fosfato de sodio 120mM, pH 6.8 y 50 µg/ml de

DNA de esperma de salmón) por 24 horas a 42°C. Después de este tiempo, las membranas serán hibridizadas con un fragmento PstI de 360 pares de bases del DNA complementario (DNAc) de la insulina humana, el cual se marcará radiactivamente con ^{32}P con el método de "random primer". Las membranas se incubarán en 0.1 ml/cm^2 de la solución A, en presencia de la sonda radiactiva, a 42°C durante toda la noche. Después de la hibridización, las membranas se lavarán en condiciones de alta astringencia, 2 veces a temperatura ambiente en una solución de SSC 2X y 2 veces a 50°C en una solución de SSC 0.1X, SDS 0.1% y se expondrán a placas autorradiográficas Kodak X-OMAT por 24 horas a –70°C, usando pantallas intensificadoras. Los autoradiogramas se analizarán en un densitómetro de imagen (Eagle eye II, Stratagene). Las membranas se lavarán y rehibridarán con el DNAc de la actina como control de expresión constitutiva. La expresión del gen de insulina será referido como la expresión del gen de insulina sobre la expresión del gen constitutivo (actina).

Procesamiento del tejido por métodos histológicos

Para obtener el páncreas de los animales de los diferentes grupos considerados, se inyectará a la rata con 300 µl de pentobarbital intraperitoneal. Una vez anestesiada se disecará el páncreas y se fijará por inmersión en 10 ml de solución fría de paraformaldehido 4% en amortiguador de fosfatos 100mM pH 7.4 por 18-20 horas a 4°C. El páncreas será tratado por métodos histológicos de rutina, después de lavarlo con agua corriente por 2 horas se deshidratará sumergiéndolo de manera sucesiva en alcohol etílico a diferentes concentraciones (25, 50, 60, 75, 80, 90 y 96%) además de xileno-etanol absoluto (1:1) y xileno 100% para después ser incluido en parafina. Se realizarán cortes de 5 µm de grosor para procesarlos por inmumohistoquímica y TUNEL (terminal nucleotidyl transferase-mediated dUTP nick end labeling-fluorescein conjugated)

Inmunohistoquímica de insulina y glucagon

Para conocer si el contenido de insulina y glucagon se modifica por efecto de la obesidad materna en los animales de estudio, se analizará la insulina y glucagón en los cortes de páncreas de los animales de estudio por inmunohistoquímica. Después de desparafinar las laminillas y re-hidratarlas en concentraciones crecientes de etanol (25,50,75,100%), los cortes se incubarán con suero normal de cabra al 2% por 15 minutos, después de este tiempo se incubarán con el anticuerpo primario correspondiente (anti-insulina o anti-glucagón de ratón) por 2 horas y después de lavarlos con PBS 100 mM pH 7.4, los cortes se incubarán con anticuerpo secundario (anti-IgG de ratón) conjugado con rodamina o fluoresceína, durante 2h. Las laminillas serán preparadas con medio para fluorescencia y posteriormente serán analizadas por microscopía de fluorescencia. Se utilizarán al menos 100 islotes por páncreas.

Análisis estadístico

Para la prueba de la tolerancia a la glucosa, el área bajo la curva será calculada. El índice de resistencia a la insulina será determinado con la fórmula IRI = Glucosa x Insulina / 22.5. Todos los datos serán expresados como la media ± EE. Se realizará ANOVA de una vía seguido de la prueba de Dunnett para la comparación entre los grupos y ANOVA de 2 vías para la determinación de la interacción entre sexo y grupos experimentales. La correlación entre las diferentes variables (concentraciones de leptina, ingesta de alimento, grasa y peso corporal) serán calculadas utilizando la Correlación de Pearson; $p \leq 0.05$ será considerado como significativo.

10. Riesgos y beneficios del estudio

a) Molestias generadas por el estudio (en caso de tomas de sangre, anotar el número total de punciones, la cantidad de sangre por punción y/o total y la frecuencia de las punciones.)
b) Riesgos potenciales (presencia de complicaciones o efectos adversos, considerar interacciones medicamentosas, considerar efectos psicológicos de los métodos de evaluación, v.gr.: encuestas sobre temas sensibles),
c) Métodos de detección de los riesgos anticipados.
d) Medidas de seguridad para el diagnóstico oportuno y prevención de los riesgos..
e) Procedimientos a seguir para resolver los riesgos en caso de que se presenten.
f) Beneficios directos esperados.
g) Beneficios indirectos esperados.
h) Ponderación general de riesgos contra beneficios del estudio propuesto.

11. Costos

a) Especificar costos (directos/indirectos, monetarios, en tiempo de participación, visitas/traslados) que la investigación genere para los sujetos del estudio.
b) Definición sobre quienes de los participantes (departamentos, instituciones, etc.) va a cubrir dichos costos.

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13. Aplicación del principio de las 3 "Rs"

El principio de las tres erres: Reemplazo, Reducción y Refinamiento propuesto por Russell y Burchs (The Principles of Humane Experimental Technique, W.M.S. Russell and R.L.Burch, 1959) es ampliamente reconocido a nivel internacional como el lineamiento ético que rige la investigación con animales y forma parte de la mayor parte de las legislaciones, regulaciones y normatividades referentes al uso de animales de laboratorio. La NOM-062-ZOO-1999 que rige en México el uso de los animales también lo incorpora.

Se entiende por Reemplazo: ***aquellas acciones que promueven la sustitución del uso de animales vivos en la investigación biomédica por métodos alternos, siempre que esto sea posible.***
Se entiende por Reducción: ***aquellas acciones que promueven la disminución en la cantidad de animales utilizados en un experimento hasta el nivel estrictamente necesario para obtener resultados válidos y estadísticamente confiables.***
Se entiende por Refinamiento: ***aquellas acciones que promueven la disminución o eliminación del sufrimiento, dolor, agonía innecesarios de los animales en un experimento o investigación biomédica.***
El investigador principal es responsable de la aplicación de este principio a las investigaciones que realice con animales y se compromete a revisar exhaustivamente la literatura actual sobre la aplicación de estos principios a su proyecto de investigación y el cumplimiento del mismo por los ejecutores del procedimiento experimental. El investigador deberá especificar la forma en que identificará los niveles de dolor o sufrimiento de los animales y especificará claramente los puntos de terminación del estudio o aplicación de eutanasia a los animales, de presentarse dolor extremo o sufrimiento durante el estudio. En caso de no encontrarse los investigadores presentes o no poder localizarles (horario nocturno, fines de semana, vacaciones, etc.) el médico veterinario responsable determinará la condición más apropiada para los animales que se encuentren en sufrimiento o agonía.
No se considera apropiada la condición de agonía como punto terminal o parte del procedimiento experimental. Se deberá evitar siempre que el animal alcance esta condición.

14. Declaración de los investigadores y aplicación del principio de las tres "erres" a su proyecto de investigación

Copiar e imprimir esta declaración en hoja con membrete del Instituto.

Abrir archivo **CINVA 03 DECLARACION DE INVESTIGADORES** para IMPRIMIRLA y que sea firmada por todos y cada uno de los participantes en el proyecto propuesto. Anexar dicha hoja al Formato de Evaluación impreso entregado.

15. Resolución del Comité

Esta sección es únicamente para conocimiento de los investigadores: Los proyectos serán revisados por cada uno de los miembros del Comité. La evaluación formal y su resolución serán realizadas por el Comité en sesión plenaria. La discusión que se dé podrá generar Observaciones y/u Objeciones mismas que redundarán en el Dictamen el cual podrá ser Aprobatorio, No Aprobatorio o Pendiente.

15a. Observaciones

Son puntos detectados en el proyecto que generan duda o ameritan aclaración pero que se considera NO afectan la estructura científica/ética del mismo.

15b. Objeciones

Son puntos detectados en el proyecto que generan duda o ameritan aclaración y se considera que SÍ afectan la estructura científica/ética del mismo y ameritan explicación, contestación, aclaración, modificación y/o justificación para continuar su evaluación y llegar a una resolución

15c. Dictámen

Aprobado Se entregará Carta de Aprobación de Proyecto el cual procederá a terminar su registro institucional y podrá ser iniciado. En caso de existir Observaciones deberán ser contestadas por el investigador principal.		
No aprobado El proyecto presenta Objeciones formales de carácter científico o ético que impiden su aprobación. Podrá ser modificado y vuelto a presentar mediante una nueva Solicitud de Revisión.		
Pendiente o en proceso El Comité no llegó a un Dictamen definido ya que el proyecto presenta Observaciones/Objeciones que ameritan explicación, contestación, aclaración, modificación y/o justificación para continuar su evaluación y llegar a una resolución. Se entregará una carta con las Observaciones/Objeciones y se continuará la evaluación en cuanto sean contestadas.		

INNSZ

INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN
SALVADOR ZUBIRÁN

Miércoles, 24 de Junio de 2009

DECLARACIÓN DE LOS INVESTIGADORES

TÍTULO DEL PROYECTO: **Efectos de la obesidad materna de la rata sobre el metabolismo de las crías**
Número de Registro CINVA:

Los investigadores que participamos en el proyecto arriba mencionado sometemos voluntariamente a evaluación dicho proyecto ante el Comité de Investigación en Animales y libremente declaramos:

- ***Que conocemos todos los aspectos del estudio y contamos con la capacidad de llevarlo a buen término.***
- ***Que la revisión minuciosa de los antecedentes científicos del proyecto justifican su realización y nos comprometemos a mantener un estándar científico elevado que permita obtener información útil para la sociedad.***
- ***Que conocemos los riegos potenciales a los que exponemos a los animales de experimentación.***
- ***Que pondremos la seguridad de los animales de investigación por encima de cualquier otro objetivo.***
- ***Que nos conduciremos de acuerdo a las especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio que contiene la Norma Oficial Mexicana NOM-062-ZOO-1999, publicada por SAGARPA en el Diario oficial de la Federación el miércoles 22 de agosto del 2001, y los lineamientos nacionales e internacionales para el buen uso de los animales de experimentación.***

Nombre del investigador	Firma
Dra. Elena Zambrano González	
Q.A. Paola Martínez Samayoa	
Q.F.B. Guadalupe Rodríguez González	

• Vasco de Quiroga 15,
• Delegación Tlalpan
• C. P. 14000 México, D. F.
• Tel. 54-87-09-00

INCMNSZ

Dirección de Investigación

FORMA ÚNICA PARA REGISTRO DE PROYECTOS

No invada las zonas sombreadas

EI C - BRE 112-12-15-1

CLAVE: BRE - 112 - 12 / 12 - 1

FECHA DE RECEPCIÓN : _____

TÍTULO: INTERVENCION NUTRICIONAL EN LA OBESIDAD MATERNA DE LA RATA: BENEFICIO EN EL METABOLISMO DE LAS CRIAS.

INVESTIGADOR RESPONSABLE: DRA. ELENA ZAMBRANO GONZALEZ

DEPARTAMENTO O SERVICIO: BIOLOGIA DE LA REPRODUCCION

TIPO DE INVESTIGACIÓN:

	1. Investigación Clínica	(incluye seres humanos o sus productos biológicos)
X	2. Investigación Experimental	(incluye animales de investigación o sus productos biológicos)
	3. Investigación Documental	(revisión de expedientes, revisión bibliográfica, informe de casos, etc.)
	4. Desarrollo Tecnológico	(instrumental, equipo, metodos diagnósticos, drogas nuevas, etc)
	5. Investigación Epidemiológica	(estudios en poblaciones, en comunidad o en hospital)
	6. Otros	(organización de eventos, asistencia a reuniones, donativos, etc)

PATROCINADORES:

	Cantidad:
CONACyT	769,000.00
	TOTAL
	Fondo de Apoyo

PERÍODO DE UTILIZACIÓN DE LOS RECURSOS: *de mes:* 01 *año:* 2012 *a mes:* 01 *año:* 2015

FORMA EN LA QUE SE RECIBIRÁN LOS FONDOS:

	primer trimestre	segundo trimestre	tercer trimestre	cuarto trimestre
Primer año:				
Segundo año:				
Tercer año:				
Cuarto año:				
Quinto año:				

COSTOS TOTALES DE LA INVESTIGACIÓN (ver instrucciones al reverso)	
1. Personal: (sueldos y sobresueldos al personal)	43,000.00
2. Equipos: (de laboratorio, cómputo, transporte, etc.)	125,000.00
3. Materiales: (reactivos, consumibles, desechables, etc.)	240,000.00
4. Animales: (adquisición, cuidado, procedimientos, etc.)	150,000.00
5. Estudios: (de laboratorio, gabinete, especiales, etc.)	
6. Viáticos: (reuniones científicas y trabajo de campo)	128,000.00
7. Publicaciones: (costos directos de publicación, sobretiros)	30,000.00
8. Suscripción: (libros, revistas, software, periódico, etc.)	53,000.00
9. Varios: (teléfono, fax, fotocopias, mensajería, etc.)	
10. Fondo de Apoyo:	

INSTITUCIONES PARTICIPANTES

FIRMAS

Investigador Responsable

Jefe del Departamento

Comité de Investigación en Humanos

Comité de Investigación en Animales

Director de Investigación

Director General

3/05/2012

EFECTOS DE LA OBESIDAD MATERNA DE LA RATA SOBRE EL METABOLISMO DE LAS CRIAS

Antecedentes

La desnutrición y la malnutrición son un problema actual de salud pública. Los cambios en los patrones de alimentación y de actividad física han incrementado el desarrollo de obesidad (1), un ejemplo de malnutrición. Dentro de los sectores de la población afectados por el desarrollo de obesidad en México, se encuentran las mujeres en edad joven y reproductiva y los niños en edad escolar. En el primer grupo, la prevalencia de obesidad ha aumentado de 9 a 32 %, de 1988 al 2006; mientras que en el sector infantil, la prevalencia ha incrementado de 11 a 18 %, de 1999 al 2006, de acuerdo a la Encuesta Nacional de Nutrición (2). El desarrollo de obesidad infantil puede ser resultado, no solo de las condiciones de sedentarismo, estilos de vida y alimentación, sino también de las condiciones nutricionales y metabólicas de la madre. Esto se debe a que el desarrollo de obesidad materna durante la gestación y la lactancia, puede ser una condición de estrés que predisponga al feto al desarrollo de enfermedades metabólicas desde la niñez y en mayor grado, en la vida adulta (3). Dentro de estos efectos adversos que la obesidad en el embarazo y lactancia ocasiona en la progenie, se encuentran características del síndrome metabólico como resistencia a la insulina, aumento de tejido adiposo abdominal e hipertensión (4).

Investigaciones científicas tanto en humanos como en animales de experimentación, han demostrado que la salud está íntimamente relacionada con la calidad de vida en las primeras etapas del crecimiento. El fenotipo del adulto es la suma de los factores genéticos, así como de la influencia del ambiente fetal y postnatal. El ambiente perinatal subóptimo puede programar la predisposición para el desarrollo de enfermedades crónicas, incluyendo la alteración del metabolismo de hidratos de carbono (5-9). Los hallazgos realizados en 1986 por el Dr. David Barker de la Universidad de Southampton, Inglaterra, en donde encuentra una correlación entre el bajo peso al nacimiento y el riesgo a enfermedades coronarias en la vida adulta (10) fueron el inicio de varios estudios epidemiológicos encaminados a identificar la relación que existe entre la talla y peso del neonato con la hipertensión arterial, enfermedades cardiovasculares y cerebrovasculares, obesidad, dislipidemias e intolerancia a la glucosa (11-13).

La hipótesis de los orígenes del desarrollo de la salud y la enfermedad (*DOHaD, por sus siglas en inglés*), antes conocida como "programación del desarrollo", propone que la fisiología y metabolismo fetal y neonatal pueden ser alterados por cambios durante periodos críticos del desarrollo, como la gestación y la lactancia. Estas alteraciones generan una respuesta fisiológica permanente en el feto que se asocia con el desarrollo de enfermedades en el adulto (14). Estudios con animales de experimentación han utilizado diferentes modelos para evaluar el efecto de la programación del desarrollo en el metabolismo (15,16). Algunos de estos modelos

son la restricción nutricional en la dieta de la madre gestante y durante la lactancia o la diabetes gestacional. Se ha demostrado que el ambiente intrauterino de madres desnutridas durante la gestación predispone a padecer diabetes y obesidad en la vida adulta. La alteración del desarrollo del páncreas, tejido adiposo, músculo e hígado fetales, entre otros, puede tener consecuencias en la predisposición de enfermedades metabólicas a largo plazo.

Sin embargo, actualmente son pocos los estudios que recientemente se han enfocado en los efectos de la programación del desarrollo por una dieta hipercalórica, como una dieta materna alta en grasa. El feto metabólicamente programado presenta modificaciones permanentes en la estructura y fisiología de órganos, así como en la expresión de genes involucrados en su propio metabolismo (17). Por lo que los factores ambientales como la obesidad materna pueden alteran permanentemente el metabolismo de tejidos y sistemas del organismo.

Existen estudios que han reportado los efectos de una dieta hiperlipídica durante la gestación y/o la lactancia en la salud de la descendencia en edad adulta. En un estudio realizado con babuinos, con dieta materna hiperlipídica durante la gestación, se demostró que el aumento de peso corporal y de concentraciones séricas de triglicéridos en la madre puede relacionarse con el incremento del crecimiento fetal (18). Esto sugiere que la madre podría presentar alteraciones metabólicas como altas concentraciones de insulina y leptina, cambios en el perfil lipídico e incremento de tejido adiposo que se relacionen con el desarrollo de obesidad en las crías (18).

La expansión del tejido adiposo involucra tanto la hipertrofia como la hiperplasia de adipocitos. Los estadios tempranos de la vida son periodos muy vulnerables y sensibles a factores nutricionales y hormonales, que modulan la multiplicación y diferenciación de los precursores de células adiposas (19). La desnutrición durante la gestación genera hipotrofia de los adipositos (20), mientras que la sobrealimentación durante la lactancia, asociada con altas actividades de enzimas lipogénicas (21), genera hipertrofia de los adipocitos.

Por otro lado, el aumento de tejido adiposo genera el incremento de la liberación de ácidos grasos libres a la circulación (22). La acumulación de ácidos grasos intracelulares en órganos no adiposos conocido como lipotoxicidad, es regulado por el metabolismo de lípidos el cual a su vez, es controlado por una familia de factores de transcripción conocida como PPARs (Proliferator Peroxisome Activator Receptor), que están involucrados en la activación de genes involucrados en el transporte y oxidación de lípidos (22,23). Uno de estos factores de transcripción es PPARγ (receptor activado por proliferadores de peroxisomas γ), el cual se expresa principalmente en tejido adiposo y controla el almacenamiento de triglicéridos mediante la diferenciación de adipocitos y la esterificación de ácidos grasos en los adipocitos maduros, regulando la expresión de genes involucrados como la expresión de la enzima sintasa de ácidos grasos (FAS) (23). La acumulación de lípidos en tejidos no adiposos está relacionada con la reducción del contenido de PPARγ. Otra isoforma de los PPARs y que se expresa principalmente en el

hígado, es PPARα. Este factor de transcripción juega un papel importante en la oxidación de ácidos grasos regulando la expresión de genes como la enzima carnitin palmitoil CoA transferasa (CPT) (24). La familia de las SREBPs (Sterol Regulatory Element Binding Protein), formada por tres miembros SREBP-1a,1c y 2, también participa en el metabolismo de lípidos. Estos factores de trascripción controlan la homeostasis de lípidos al activar de manera coordinada, la expresión de genes involucrados en la glucólisis, así como el metabolismo de colesterol y ácidos grasos y el de lipoproteínas (24). Específicamente SREBP-1 se une a elementos de respuesta a esteroles de genes que participan en el metabolismo de lípidos (24). La participación de SREBPs y PPARs en el desarrollo de lipotoxicidad del hígado, ha comenzado a investigarse y dentro del área de la programación del desarrollo, es necesario el estudio de mecanismos moleculares que ayuden a explicar la relación entre la calidad de vida prenatal y el riesgo de padecer enfermedades metabólicas en la vida adulta.

El desarrollo de obesidad materna, debido al aumento en el consumo de dietas ricas en lípidos y carbohidratos, también puede alterar otros parámetros metabólicos en la descendencia como son la distribución de grasa corporal, la función cardiovascular y el metabolismo de glucosa (25). En este trabajo se plantea la hipótesis de que dichas alteraciones tienen su origen en la programación del balance de energía, la cadena se señalización de insulina, en la expresión de factores de transcripción relacionados con la regulación del metabolismo de lípidos o en la expresión de receptores en el hipotálamo involucrados en el control de los centros de apetito y saciedad, como el receptor OB-Rb de la leptina, hormona relacionada con el balance energético y secretada por el tejido adiposo (26). Se ha demostrado que ratas de experimentación sobrealimentadas durante su vida temprana postnatal presentan un estado de resistencia a leptina, mediada por la regulación negativa del receptor hipotalámico OB-Rb (27).

El propósito del presente estudio es determinar cómo es que el desarrollo de obesidad en ratas hembras, dado por el consumo de dieta hiperlipídica desde el destete hasta la edad adulto joven y durante el embarazo y la lactancia, programa el metabolismo de glucosa y lípidos de sus crías, así como alteraciones en la ingesta de alimento y desarrollo de adiposidad, de tal forma que estas serán más susceptibles a desarrollar enfermedades metabólicas en la vida adulta a pesar de haber sido alimentadas normalmente desde el destete, a diferencia de un grupo de crías de madres control.

Los estudios preliminares realizados con un modelo de obesidad han mostrado aumento de tejido adiposo y aumento en las concentraciones séricas de triglicéridos en las crías de madres alimentadas con dieta hipercalórica en el periodo perinatal, por lo que esperamos encontrar alteraciones a nivel bioquímico y molecular en el tejido adiposo, muscular, cerebro y páncreas que permitan

explicar el mecanismo por el que se desencadenan los efectos metabólicos de la programación del desarrollo en las crías.

Se utilizará a la rata como modelo experimental. Ratas serán alimentadas con dieta control o dieta hiperlipídica con 20% de grasa saturada, desde el destete y durante el embarazo y/o lactancia. Después del destete todas las crías durante su crecimiento comerán dieta control hasta la vida adulta. Se cuantificará glucosa, insulina y triglicéridos séricos en las ratas madres. En los neonatos se determinará leptina sérica y receptores de leptina en el hipotálamo. Se realizará una prueba de tolerancia a la glucosa (PTG) en las crías, así como la concentración de glucosa e insulina basal, triglicéridos, colesterol y el análisis químico corporal. Se realizarán correlaciones entre algunas variables como peso, ingesta de alimento y leptina. Además se realizarán estudios de morfología y del páncreas y tejido adiposo, que permitan identificar si la modificación en el metabolismo de glucosa y lípidos se debe a alteraciones generadas en el desarrollo del páncreas o aumento del tejido adiposo durante la vida fetal. En hígado se determinará la concentración de los factores de transcripción PPARα y SREBP-1, y en tejido adiposo del factor de transcipción PPARγ, para evaluar alteraciones en el metabolismo de lípidos en las crías. Debido a que los efectos de la programación del desarrollo normalmente se manifiestan en etapas tardías de la vida (adulto maduro), las crías serán estudiadas en dos periodos en la vida joven y adulta.

Originalidad

Algunos estudios previos realizados con ratas, han demostrado que las modificaciones en el contenido de carbohidratos y de proteínas materna durante el inicio del embarazo ya sea generado por diabetes, por la administración materna de glucocorticoides, por la restricción proteínica materna o por la restricción global de calorías alteran el metabolismo de las crías (28-32). Con respecto al modelo de obesidad materna que se plantea en este trabajo, nos proponemos determinar cómo es que el fenotipo de la rata madre que presenta obesidad durante la gestación y la lactancia, puede contribuir al desarrollo de obesidad y problemas metabólicos de las crías. En este estudio se utilizará como modelo animal a la rata, dado que con éste se pueden evaluar diferentes etapas del crecimiento, desde el destete hasta la vida adulta en corto tiempo y con los resultados obtenidos se espera conocer mecanismos que expliquen la importancia que puede tener el consumo de dieta hipercalórica en la programación del feto.

En el presente estudio se plantea conocer la importancia del adecuado ambiente metabólico durante la vida intrauterina que incide en la programación desde la vida fetal en diferentes funciones y mecanismos de adaptación durante la vida postnatal. Los resultados de este estudio serán importantes para conocer el impacto de la calidad de vida embrionaria y postnatal sobre la funcionalidad de sistemas complejos y hormonalmente regulados.

Objetivos

Objetivo General

Estudiar los efectos de la obesidad materna durante el periodo perinatal de la rata para la programación de enfermedades crónico degenerativas y su asociación con desórdenes metabólicos en las crías de ambos sexos.

Objetivos Particulares

Estudiar a la madre durante el crecimiento y la gestación para poder esclarecer el ambiente intrauterino metabólico y endócrino al que estarán sometidas las crías. Para esto se determinará el peso corporal, la ingesta de alimentos y el perfil lipídico de las ratas madre durante la gestación y la lactancia, así como la composición química de la leche al término de la lactancia.

Evaluar el registro de peso corporal y de ingesta de alimento de las crías en diferentes etapas del desarrollo.

Analizar a la leptina y su receptor en hipotálamo en las crías neonatas y su correlación con modificaciones en el apetito, tejido adiposo y peso corporal.

Analizar la concentración sérica de leptina, insulina, glucosa y del perfil lipídico, así como la composición corporal de las crías en diferentes etapas del desarrollo.

Determinar la concentración y nivel de expresión de los factores de transcripción SREBP-1 y PPARα, así como de las enzimas FAS y CPT en hígado y PPARγ en tejido adiposo, de las crías en edad adulta.

Comparar las diferencias de género de los efectos metabólicos por el impacto de la obesidad materna.

Metodología científica.

Modelo biológico

Se utilizarán ratas hembras albinas de la cepa Wistar recién destetadas. Los animales serán mantenidos en el bioterio del Instituto Nacional de Ciencias Médicas y Nutrición SZ bajo condiciones controladas de luz-obscuridad (de 7:00 a 19:00 h), así como de humedad y temperatura (22–23°C). Todos los procedimientos fueron previamente aprobados por el comité de ética de experimentación animal del INCMNSZ. Cuando las ratas alcancen un peso de 240 ± 20 g y entre 10 y 12 semanas de edad, serán apareadas con machos y el día en

el cual se encuentren espermatozoides en la vagina se designará como el inicio de la gestación. Durante los diferentes tiempos que dure el experimento, las ratas, tanto las madres como las crías serán pesadas diariamente.

Se utilizarán dos dietas con diferente contenido grasa. Dieta control (C) con 5% de grasa y dieta hiperlipídica (H) con 25% de grasa. La dieta hiperlipídica será compensada en su valor energético con la adición de carbohidratos (Tabla 1). La dieta utilizada es la recomendada por el Instituto Americano de Nutrición para roedores en las fases de embarazo, lactancia y crecimiento (33).

Tabla 1. Composición de las dietas experimentales

	DIETA CONTROL (%)	DIETA HIPERLIPÍDICA (%)
Caseía	23.11	23.11
L-Cistina	0.3	0.3
Colina	0.165	0.165
Vitaminas	1	1
Minerales	5	5
Celulosa	5	5
Aceite	5	5
Manteca de cerdo	----	20
Hidratos de carbono		
Almidón	30.21	20.59
Dextrosa	30.21	20.59
	4 Kcal/g dieta	5 Kcal/g dieta

Las ratas preñadas y lactantes serán pesadas todos los días durante el embarazo y hasta el destete. También la ingesta de alimento será cuantificada todos los días. Las ratas nacerán por parto natural. La distancia ano-genital al nacimiento será medida con regla Vernier para la determinación del sexo. Nuestros datos (34) indican que las crías hembras al nacimiento tienen una distancia ano genital de 1.67 ± 0.128 mm (n=291 crías de 43 diferentes camadas) y los machos 3.26 ± 0.22 mm (n=252 crías de 43 camadas). De tal forma que 2.5 mm es más de 2 SD's de la media de cada grupo. Por tanto, el sexo será determinado de acuerdo a la distancia ano-genital > (machos) o < (hembras) 2.5 mm.

Para asegurar homogeneidad en el estudio, camadas de 10 a 14 crías serán ajustadas al nacimiento a 10 crías por madre tratando de mantener una relación de sexo de 1:1. En caso de ser menor a 10 o mayor a 14, los animales serán excluidos del experimento.

Para cada experimento se utilizarán 10 ratas a preñar por grupo. De acuerdo a nuestra experiencia en proyectos anteriores, se estima que al finalizar el experimento quedará una "n" aproximada de 6 madres por grupo, dado que la tasa

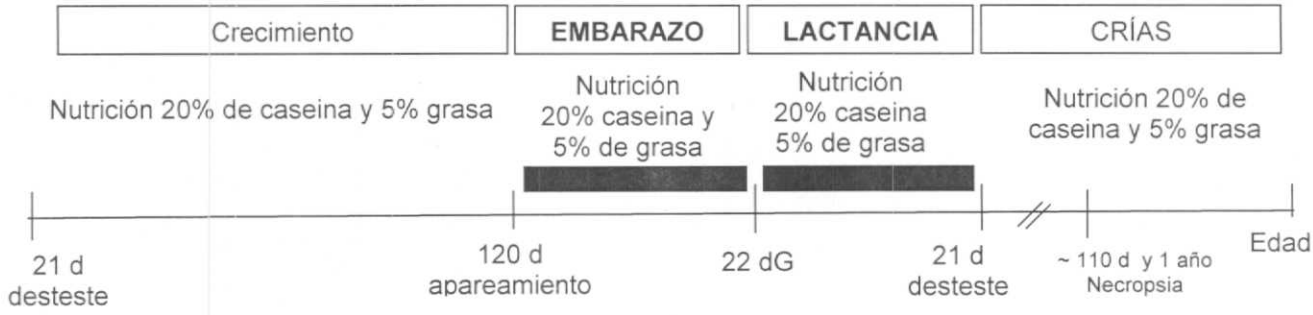
de fertilidad de las ratas del Bioterio del INCMNSZN es del 80% y que se eliminarán animales por tener camadas grandes o pequeñas.
Las diferentes dietas experimentales serán administradas a las ratas desde el destete y en el embarazo y la lactancia quedando 2 grupos experimentales:

1) Grupo Control (C). Nutrición normal a base de 20% de caseína y con 5 % de grasa a partir del destete y durante el embarazo y la lactancia.
2) Grupo Hiperlipídico (H). Nutrición hiperlipídica a base de 25% de grasa durante a partir del destete y durante el embarazo y la lactancia.

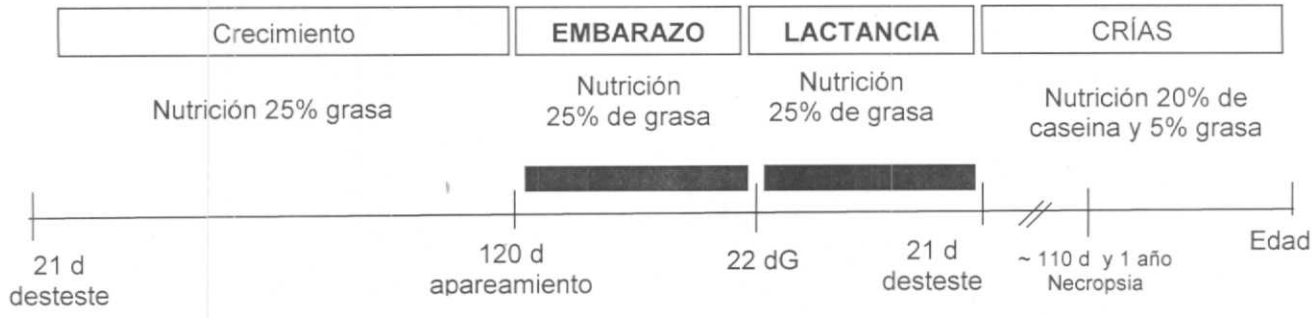
Al destete todas las crías de ambos grupos serán alimentadas con dieta normal hasta el momento del sacrificio. Es importante resaltar que el consumo de dietas será a partir del destete y no únicamente durante el embarazo y/o lactancia. Las crías y la ingesta de alimento serán pesadas todos los días hasta el término del estudio.

GRUPOS EXPERIMENTALES:

Grupo I. Control (C).



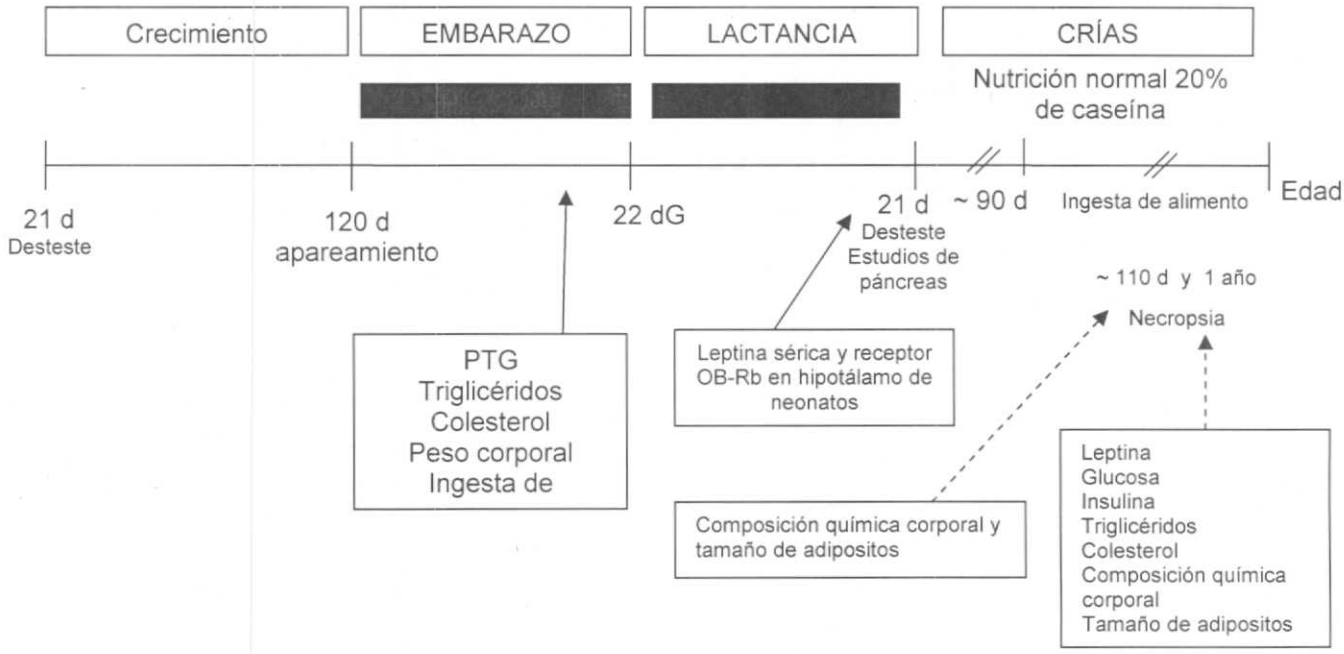
Grupo II. Hiperlipídico (H)



dG = días gestacionales; d = días.

Diseño experimental

Línea de tiempo



Cuantificación de la ingesta de alimento en las crías a los 110 d de edad

Se pondrán en la misma caja dos ratas del mismo grupo experimental y mismo sexo. La comida se proveerá en forma de galletas. Se pesará diariamente tanto la cantidad de comida que se proporcione cada día así como la parte restante después de 24 horas. La cantidad de alimento consumido será promediado entre las dos ratas.

Las crías serán pesadas todos los días. Sin embargo, dado que las concentraciones de leptina de la cría adulta se obtendrán alrededor de los 110 días de edad, para la obtención de la correlación entre las diferentes variables: peso, ingesta de alimento, contenido de grasa corporal y concentraciones séricas de leptina, se utilizarán los datos obtenidos a los 110 días de edad.

Composición química de las carcasas

Después del sacrificio las ratas serán congeladas en pequeñas bolsas de plástico hasta su análisis. El abdomen se abrirá y las vísceras serán removidas y desechadas. Se pesará el animal, cantidad que será designada como peso húmedo, después se pondrá a secar la carcasa a 60°C hasta peso constante. El peso perdido se considerará como cantidad de agua en el cuerpo. La carcasa seca será molida y se realizarán alícuotas para la determinación de la cantidad de

grasa por el método de Soxhlet (35) y la cantidad de nitrógeno total (proporcional a la cantidad de proteína) por el método de Kjeldahl (35).

Apareamiento de hembras

A los 120 días de edad las hembras alimentadas con dieta control o hiperlipídica, serán apareadas con machos (no incluidos previamente en el estudio). Durante el embarazo todas las hembras serán alimentadas con las dietas experimentales y las ratas tendrán partos naturales. Se obtendrá el tamaño de la camada y el peso corporal de las crías al nacimiento. La distancia ano-genital se medirá al nacimiento para la determinación del sexo. Las camadas serán ajustadas a 10 crías/madre manteniendo una relación de 1:1 hembras:macho, en lo posible. Al destete (día 21) las crías serán divididas por sexo y acomodadas en diferentes cajas por grupo.

Prueba de tolerancia a la glucosa (PTG) en las madres preñadas de la generación F1

Un experimento aparte será realizado para el estudio de las PTG de las crías hembras preñadas, de tal forma que el estrés generado por las tomas de muestra no afecte en los resultados obtenidos de las crías.

Una rata hembra de cada camada preñada en día 17 de gestación, será puesta en ayuno durante la noche. Un gramo/Kg de peso de D-Glucosa será administrado vía ip. La sangre será obtenida por punción retro-orbital al tiempo 0, 30, 60 y 120 min. La sangre será colectada en tubos de polietileno y centrifugada. El suero obtenido será almacenado a -20°C hasta el momento de su uso.

Análisis Bioquímicos

Cuantificación de glucosa en sangre

Las concentraciones de glucosa en suero serán determinadas espectrofotométricamente utilizando el método enzimático de la hexocinasa (Beckman Coulter, Co Fullerton, CA).

Radioinmunoensayo de Insulina

Las concentraciones de insulina en suero serán determinadas por RIA utilizando un estuche comercial de Linco Research INC #Cat RI-13K.

Cuantificación de triglicéridos y colesterol

Las concentraciones de triglicéridos y colesterol en suero se determinarán enzimáticamente con el autoanalizador Sycnchron CX (Beckman coulter, Co, Fullerton, CA).

Radioinmunoensayo de Leptina

Las concentraciones de leptina en suero serán determinadas por RIA utilizando un estuche comercial de Linco Research, Inc #Cat RL-83K.

Toma de tejidos

De las crías de ambos grupos experimentales se disecará el tejido adiposo retro-peritoneal, hipotálamo y páncreas para su estudio a nivel celular y molecular en las diferentes edades requeridas.

Tamaño de las células adiposas

Células adiposas provenientes de la zona retroperitoneal serán fijadas como lo describe Etherthon et al. Brevemente, 100 a 150 mg de tejido adiposo retroperitoneal será cortado y lavado a 37°C en 0.15 M de NaCl. Los cortes de tejido adiposo serán fijados en tetróxido de osmio al 3% en buffer de Colidina-HCl (50 mM, pH 7.4) por 72-96 h. Las células fijadas serán lavadas en NaCl al 0.15 M por 24 h, se eliminará el tejido conectivo incubando en 8 M de urea por 24-48h. El tamaño de las células será medido utilizando un microscopio óptico.

Inmunohistoquímica del receptor de leptina (OB-Rb) en hipotálamo

Se realizarán cortes seriados del hipotálamo y serán fijados a 60°C por 20 min. Posteriormente se realizarán lavados seriados con xyleno, etanol al 100% y al 95%. Se agregará solución de peroxidasa endógena en metanol/ H_2O_2 por 30 minutos. Se incubará con albúmina bovina y se agregará el anticuerpo primario (anti-OB-Rb) por una hora. Como segundo anticuerpo se utilizará suero de conejo anti-cerdo (DAKO # E0353), posteriormente streptavidina por 30 minutos (DAKO #P0397) y al final DAB por 5 minutos. Las laminillas serán teñidas con verde de metileno por 1 minuto y deshidratadas con 95% y 100% de etanol y xileno.

Estudios Celulares y Moleculares a nivel del páncreas

La cuantificación de la insulina y glucagon, así como la expresión de sus genes a nivel del páncreas, será de utilidad para indagar si las modificaciones en el metabolismo de la glucosa están mediadas en parte por la regulación y equilibrio entre estas dos hormonas.

Extracción de RNA y análisis de la expresión del gen de la insulina

El páncreas será removido inmediatamente después del sacrificio de cada animal. El RNA total será extraído, utilizando 2 ml de TRIzol (Gibco BRL). Una vez obtenido, el RNA se cuantificará por espectrofotometría.
Un total de 20 μg de RNA de cada rata, se separará en geles desnaturalizantes de agarosa al 1% y 2.2 M de formaldehído. Después de la electroforesis, el gel será transferido por capilaridad a una membrana de nitrocelulosa (Genescreen, NEN

Research Products, Dupon) con SSC 10X (citrato de sodio 150 mM, NaCl 1.5 M) por toda la noche. Las membranas se prehibridarán en 0.2 ml/cm^2 de la solución A (formamida 50%, SDS 0.2%, EDTA 10mM, SSC 2X, fosfato de sodio 120mM, pH 6.8 y 50 μg/ml de DNA de esperma de salmón) por 24 horas a 42°C. Después de este tiempo, las membranas serán hibridizadas con un fragmento PstI de 360 pares de bases del DNA complementario (DNAc) de la insulina humana, el cual se marcará radiactivamente con ^{32}P con el método de "random primer". Las membranas se incubarán en 0.1 ml/cm^2 de la solución A, en presencia de la sonda radiactiva, a 42°C durante toda la noche. Después de la hibridización, las membranas se lavarán en condiciones de alta astringencia, 2 veces a temperatura ambiente en una solución de SSC 2X y 2 veces a 50°C en una solución de SSC 0.1X, SDS 0.1% y se expondrán a placas autorradiográficas Kodak X-OMAT por 24 horas a –70°C, usando pantallas intensificadoras. Los autoradiogramas se analizarán en un densitómetro de imagen (Eagle eye II, Stratagene). Las membranas se lavarán y rehibridarán con el DNAc de la actina como control de expresión constitutiva. La expresión del gen de insulina será referido como la expresión del gen de insulina sobre la expresión del gen constitutivo (actina).

Procesamiento del tejido por métodos histológicos

Para obtener el páncreas de los animales de los diferentes grupos considerados, se inyectará a la rata con 300 μl de pentobarbital intraperitoneal. Una vez anestesiada se disecará el páncreas y se fijará por inmersión en 10 ml de solución fría de paraformaldehido 4% en amortiguador de fosfatos 100mM pH 7.4 por 18-20 horas a 4°C. El páncreas será tratado por métodos histológicos de rutina, después de lavarlo con agua corriente por 2 horas se deshidratará sumergiéndolo de manera sucesiva en alcohol etílico a diferentes concentraciones (25, 50, 60, 75, 80, 90 y 96%) además de xileno-etanol absoluto (1:1) y xileno 100% para después ser incluido en parafina. Se realizarán cortes de 5 μm de grosor para procesarlos por inmumohistoquímica y TUNEL (terminal nucleotidyl transferase-mediated dUTP nick end labeling-fluorescein conjugated)

Inmunohistoquímica de insulina y glucagon

Para conocer si el contenido de insulina y glucagon se modifica por efecto de la obesidad materna en los animales de estudio, se analizará la insulina y glucagón en los cortes de páncreas de los animales de estudio por inmunohistoquímica. Después de desparafinar las laminillas y re-hidratarlas en concentraciones crecientes de etanol (25,50,75,100%), los cortes se incubarán con suero normal de cabra al 2% por 15 minutos, después de este tiempo se incubarán con el anticuerpo primario correspondiente (anti-insulina o anti-glucagón de ratón) por 2 horas y después de lavarlos con PBS 100 mM pH 7.4, los cortes se incubarán con anticuerpo secundario (anti-IgG de ratón) conjugado con rodamina o fluoresceína, durante 2h. Las laminillas serán preparadas con medio para fluorescencia y

posteriormente serán analizadas por microscopía de fluorescencia. Se utilizarán al menos 100 islotes por páncreas.

METODOLOGÍA ESPECÍFICA CON RESPECTO AL MANEJO DE ANIMALES

El presente estudio requiere de la utilización de ratas hembras de la cepa Wistar recién destetadas y con un peso aproximado de 60g. Así mismo se utilizarán ratas machos de la misma cepa con fertilidad probada para el apareamiento con las hembras.

Nutrición

Se proponen diversos tipos de dietas:
Dieta Chow de bioterio y agua *ad limitum* para alimentar a las ratas madre de las que se obtendrán las ratas destetadas para conformar los grupos experimentales.
Dietas control e hiperlipídica con diferentes proporciones de grasa (como se indica en la tabla 1, páginas 6 y 7).

Administración de medicamentos

Para la prueba de tolerancia a la glucosa, la rata será puesta en ayuno durante la noche previa. Un gramo/Kg de peso de D-glucosa será administrado vía intraperitoneal como está descrito en la página 9.

Obtención de muestras de sangre

Las muestras de sangre se obtendrán por punción del plexo retro-orbital. El animal será anestesiado ligeramente en una cámara de éter para la realización de dicho procedimiento. Se utilizará éter por sus bajos o nulos efectos en las concentraciones de hormonas gonadotrópicas, receptores esteroideos y expresión de genes metabólicos (36,37). Posteriormente se sujetará al animal y se colocará con el tórax hacia arriba. Se introducirá un tubo capilar en el ángulo externo del ojo hasta encontrar el seno venoso. La sangre fluirá inmediatamente por capilaridad hacia el interior del tubo. Se colectarán muestras entre 400 y 600 µl de sangre. Al finalizar se limpiará el ojo con un algodón humedecido con agua estéril y se aplicará una gota de solución de antibiótico-antimicótico.

Obtención de tejidos

El día de la necropsia se obtendrán todos los órganos del animal y se pesarán, para su normalización con respecto al peso corporal.

Eutanasia

Los animales serán sometidos a eutanasia por decapitación sin anestesia previa, utilizando una guillotina para roedores (Thomas Scientific, EU). La razón por la cual no utilizaremos ningún tipo de anestésico es que en nuestra experiencia (28,33) y la de otros (36,38) hemos observado que la previa manipulación incrementa el grado de estrés de la rata, lo cual, además de afectar los valores de algunas hormonas, como la corticosterona (39), genera mayor sufrimiento al animal.
Cabe aclarar que para evitar estrés adicional en los animales a ser sacrificados, el proceso se llevará a cabo en un lugar separado al resto de las ratas, y entre animal y animal, se lavará la guillotina y los guantes del personal involucrado.
Los restos de los animales serán colocados en las bolsas amarillas (asignadas para residuos peligros biológico infecciosos: RPBI), posteriormente se congelarán a -20°C (en el depósito de cadáveres de animales para necropsia que el Bioterio del Instituto tiene asignado para tal propósito). Una compañía privada contratada por el INCMNSZ se encargará de la posterior incineración de los animales.

Criterios para dar por terminado el experimento en caso de que los animales presenten signos de sufrimiento

Los animales que en el transcurso del experimento presenten algún tipo de sufrimiento (no generado por el diseño experimental), serán sometidos a eutanasia. Los criterios son (www.ahc.umn.edu/rar/euthanasia.html):

1. Pérdida de peso mayor al 20-25%.
2. Pérdida del apetito: completa anorexia por 24 h o anorexia parcial (50% del la ingesta calórica) durante 3 días.
3. Debilidad o inhabilidad para obtener su alimento y agua.
4. Estado moribundo: signos de depresión o la falta de respuesta a estímulos.
5. Presencia de alguna infección.
6. Signos de disfunción severa de algún órgano o sistema
7. Presentación de alguna anormalidad física (tumores).

Para los siguientes procedimientos: maniobras conductuales, modificaciones ambientales, restricción física y ejercicio, inmunizaciones, inoculación de agentes biológicos, sustancias peligrosas, radiaciones, trauma, cirugía, NO APLICA.

Análisis estadístico

Para la prueba de la tolerancia a la glucosa, el área bajo la curva será calculada. El índice de resistencia a la insulina será determinado con la fórmula IRI = Glucosa x Insulina / 22.5. Todos los datos serán expresados como la media ± EE. Se realizará ANOVA de una vía seguido de la prueba de Dunnett para la comparación entre los grupos y ANOVA de 2 vías para la determinación de la interacción entre sexo y grupos experimentales. La correlación entre las diferentes variables

(concentraciones de leptina, ingesta de alimento, grasa y peso corporal) serán calculadas utilizando la Correlación de Pearson; $p \leq 0.05$ será considerado como significativo

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Intervención nutricional en la obesidad materna de la rata: beneficio en el metabolismo de las crías

ANTECEDENTES

Los cambios en los patrones de alimentación y de actividad física han incrementado el desarrollo de obesidad (1). Dentro de los sectores de la población afectados por el desarrollo de obesidad en México, se encuentran las mujeres en edad joven y reproductiva y los niños en edad escolar. En el primer grupo, la prevalencia de obesidad ha aumentado de 9 a 32 %, de 1988 al 2006; mientras que en el sector infantil, la prevalencia ha incrementado de 11 a 18 %, de 1999 al 2006, de acuerdo a la Encuesta Nacional de Nutrición (2). El desarrollo de obesidad infantil puede ser resultado, no sólo de las condiciones de sedentarismo, estilos de vida y alimentación, sino también de las condiciones nutricionales y metabólicas de la madre. Esto se debe a que el desarrollo de obesidad materna durante la gestación y la lactancia, es una condición de estrés que predispone al feto en crecimiento al desarrollo de enfermedades metabólicas desde la niñez y en mayor grado, en la vida adulta (3).

Investigaciones científicas tanto en humanos como en animales de experimentación, han demostrado que la salud está íntimamente relacionada con la calidad de vida en las primeras etapas del crecimiento. El ambiente perinatal subóptimo puede programar la predisposición para el desarrollo de enfermedades crónicas, incluyendo la alteración del metabolismo de hidratos de carbono (5-9). Los hallazgos realizados en 1986 por el Dr. David Barker de la Universidad de Southampton, Inglaterra, en donde encuentra una correlación entre el bajo peso al nacimiento y el riesgo a enfermedades coronarias en la vida adulta, (10) fueron el inicio de varios estudios epidemiológicos encaminados a identificar la relación que existe entre la talla y peso del neonato con la hipertensión arterial, enfermedades cardiovasculares y cerebrovasculares, obesidad, dislipidemias e intolerancia a la glucosa (11-13).

La hipótesis de los orígenes del desarrollo de la salud y la enfermedad (*DOHaD, por sus siglas en inglés*), antes conocida como "programación del desarrollo", propone que la fisiología y metabolismo fetal y neonatal pueden ser alterados por cambios durante una ventana de tiempo crítica del desarrollo, como la gestación y la lactancia. Estas alteraciones generan una respuesta fisiológica permanente en el feto que se asocia con el desarrollo de enfermedades en el adulto (16,24,26,27). El feto metabólicamente programado presenta modificaciones permanentes en la estructura y fisiología de órganos, así como en la expresión de genes involucrados en su propio metabolismo (17). Por lo que el fenotipo del adulto es la suma de los factores genéticos, así como de la influencia del ambiente fetal y postnatal.

Estudios con animales de experimentación han utilizado diferentes modelos para evaluar el efecto de la programación del desarrollo en el metabolismo (15,16). Algunos de estos modelos son la restricción nutricional en la dieta de la madre gestante y durante la lactancia, y la diabetes gestacional. En los últimos años se

ha empezado a explorar también los efectos de la obesidad materna y la ingesta de dietas hipercalóricas de la madre gestante (4,18,23).
La obesidad materna está relacionada tanto con el retardo en el crecimiento intrauterino como con el aumento en el tamaño del feto para su edad gestacional (28,29). Recientes estudios epidemiológicos y con animales de experimentación (3,4,14,19,25,30,31) han reportado que el desarrollo de obesidad previa y durante el embarazo es un importante factor responsable de los efectos adversos de la programación del desarrollo en la progenie, tales como predisposición a la diabetes, aumento de tejido adiposo abdominal, obesidad y enfermedades cardiovasculares.
Algunos de los estudios que se han reportado son los relacionados con los efectos de dietas hiperlipídicas durante la gestación y/o la lactancia en la salud de la descendencia en edad adulta. En un estudio realizado con babuinos, que fueron alimentados con dieta materna hiperlipídica durante la gestación, se demostró que el aumento de peso corporal y de concentraciones séricas de triglicéridos en la madre puede relacionarse con el incremento del crecimiento fetal (18). Esto sugiere que la madre podría presentar alteraciones metabólicas como altas concentraciones de insulina y leptina, cambios en el perfil lipídico e incremento de tejido adiposo que se relacionen con el desarrollo de obesidad en las crías (18).

A pesar de que actualmente existe un gran número de estudios relacionados con la obesidad materna y la programación del desarrollo, no se ha explorado si los cambios en la alimentación previa al embarazo de la madre obesa ayudan a prevenir algunos de los efectos metabólicos negativos de la programación. En un artículo recientemente publicado por nuestro grupo de trabajo, se demostró que la intervención nutricional un mes previo a la gestación de ratas con obesidad, revierte algunos de los efectos metabólicos de la programación en las crías (27). Algunos de los resultados obtenidos son:

Las ratas madres del grupo de intervención nutricional previa a la gestación (MI), mostraron menor peso corporal al apareamiento con respecto a el grupo de ratas madres alimentadas con dieta hiperlipídica (MO). Este último grupo tuvo mayor peso corporal (16%) en comparación con el grupo control (MC).

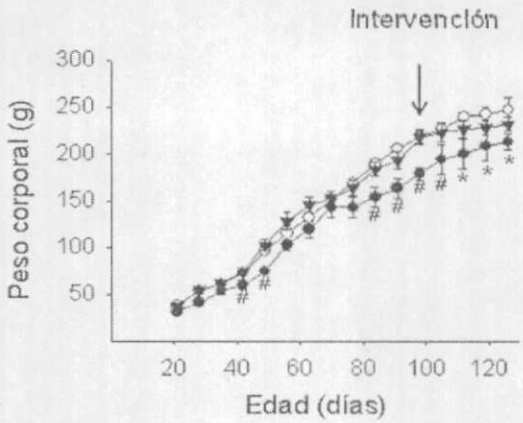


Figura 1. Registro de peso corporal desde el destete hasta el día del apareamiento de los tres grupos experimentales. # $p<0.05$ para madres con obesidad (MO - ○) y madres con intervención nutricional (MI - ▼) versus madres control (MC - ●), * $p<0.05$ MI vs MO, (n = 5 ratas por grupo). Los datos se presentan como la media ± EE.

Con respecto a las crías a los 21 días de edad, se observó mayor cantidad de tejido adiposo, triglicéridos, leptina e insulina en suero tanto en hembras como en machos del grupo MO y todos estos parámetros fueron recuperados en las crías del grupo MI. A los 120 días de edad las crías machos del grupo MO tuvieron mayor concentración sérica de glucosa, insulina así como en el índice de resistencia a la insulina (IRI); las crías de las MI tuvieron una recuperación en la concentración de glucosa y parcialmente en el IRI. No hubo diferencias significativas en ninguno de estos parámetros en las crías hembras. A los 150 días de edad, tanto en hembras como en machos la concentración de leptina fue mayor en el grupo MO, mientras que no hubo diferencias en los grupos MI con respecto al MC. El tamaño de las células de los adipocitos (para ambos sexos) fue mayor en el grupo MO, intermedio en el MI y menor en el grupo control (27).

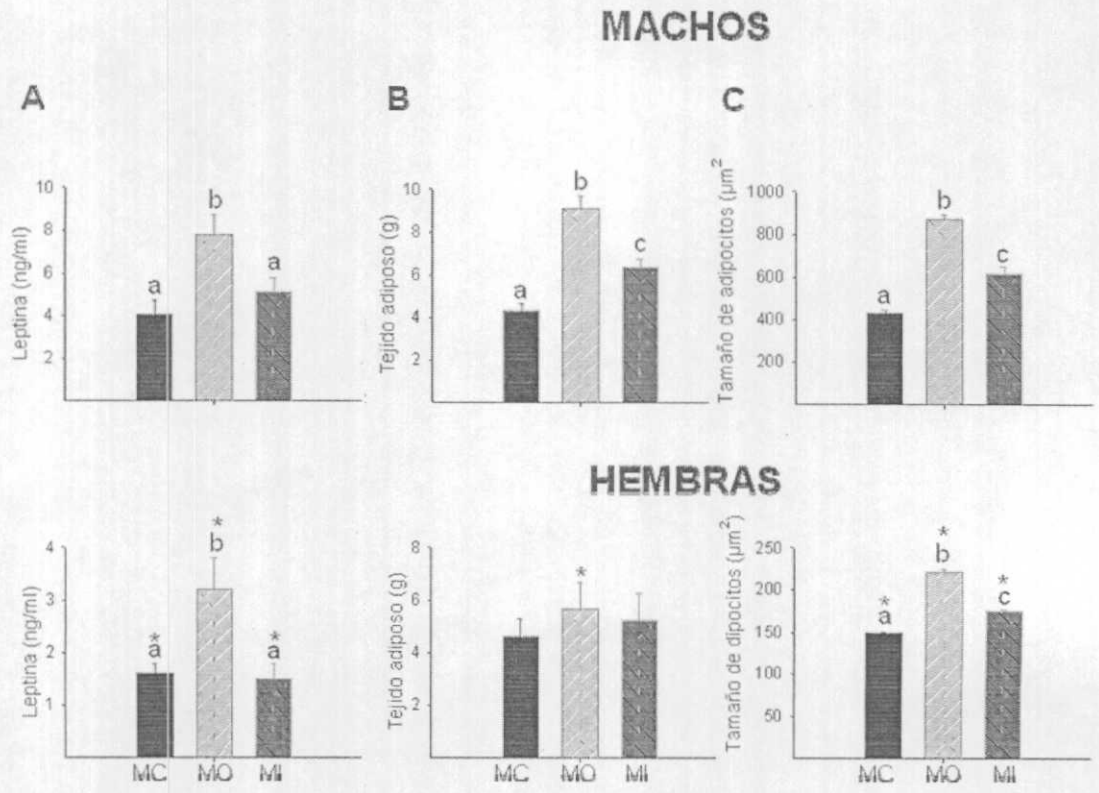


Figura 2. **A)** Leptina sérica; **B)** tejido adiposo y **C)** tamaño de adipocitos de crías machos y hembras a los 150 días de edad. Datos con letras diferentes son significativamente diferentes, $p < 0.05$ para grupos con letras diferentes, * $p<0.05$

vs machos. MC: madres control, MO: madres con obesidad y MI: ratas madres con intervención nutricional. (n = 5 camadas por grupo). Los datos se presentan como la media ± EE.

Estos datos demuestran que la intervención nutricional materna previa al embarazo pueden revertir parcialmente o totalmente algunos de los efectos adversos de la programación del desarrollo generados por la obesidad materna

El desarrollo de obesidad desencadena una serie de alteraciones relacionadas con el metabolismo energético, afectando a órganos como hígado y tejido adiposo, y en este proyecto se plantea que la intervención nutricional previa al embarazo puede ayudar a prevenir alteraciones en el metabolismo de lípidos de las crías. El metabolismo de lípidos involucra una serie de factores de transcripción que a continuación se describen.

El desarrollo de obesidad y el aumento de tejido adiposo incrementan la liberación de ácidos grasos libres a la circulación (20). La acumulación de ácidos grasos intracelulares en órganos no adiposos conocido como lipotoxicidad, es regulado por el metabolismo de lípidos el cual a su vez, es controlado por una familia de factores de transcripción conocida como PPARs (Proliferator Peroxisome Activator Receptor), que están involucrados en la activación de genes relacionados en el transporte y oxidación de lípidos (20,21). Uno de estos factores de transcripción es PPARγ (receptor activado por proliferadores de peroxisomas γ), el cual se expresa principalmente en tejido adiposo y controla el almacenamiento de triglicéridos mediante la diferenciación de adipocitos y la esterificación de ácidos grasos en los adipocitos maduros, regulando la expresión de genes involucrados como la expresión de la enzima sintasa de ácidos grasos (FAS) (21). La acumulación de lípidos en tejidos no adiposos está relacionada con la reducción del contenido de PPARγ. Otra isoforma de los PPARs y que se expresa principalmente en el hígado, es PPARα. Este factor de transcripción juega un papel importante en la oxidación de ácidos grasos regulando la expresión de genes como la enzima carnitin palmitoil CoA transferasa (CPT) (22). La familia de las SREBPs (Sterol Regulatory Element Binding Protein), formada por tres miembros SREBP-1a,1c y 2, también participa en el metabolismo de lípidos. Estos factores de trascripción controlan la homeostasis de lípidos al activar de manera coordinada, la expresión de genes involucrados en la glucólisis, así como el metabolismo de colesterol y ácidos grasos y el de lipoproteínas (22). Específicamente SREBP-1 se une a elementos de respuesta a esteroles de genes que participan en el metabolismo de lípidos (22). La participación de SREBPs y PPARs en el desarrollo de lipotoxicidad del hígado, ha comenzado a investigarse y dentro del área de la programación del desarrollo, es necesario el estudio de mecanismos moleculares que ayuden a explicar la relación entre la calidad de vida prenatal y el riesgo de padecer enfermedades metabólicas en la vida adulta.

El desarrollo de obesidad materna, debido al aumento en el consumo de dietas ricas en lípidos y carbohidratos, también puede alterar otros parámetros

metabólicos en la descendencia como son la distribución de grasa corporal, la función cardiovascular y el metabolismo de glucosa (23). En este trabajo se plantea que dichas alteraciones tienen su origen en la programación del balance de energía y/o en la expresión de factores de transcripción relacionados con la regulación del metabolismo de lípidos.

Los estudios preliminares realizados con un modelo de obesidad han mostrado aumento de tejido adiposo e incremento de las concentraciones séricas de triglicéridos en las crías de madres alimentadas con dieta hipercalórica en el periodo perinatal, por lo que esperamos encontrar alteraciones a nivel bioquímico y molecular en el tejido adiposo, hígado y cerebro que permitan explicar el mecanismo por el que se desencadenan los efectos metabólicos de la programación del desarrollo en las crías.

En el presente trabajo se pretende estudiar mecanismos en el hígado y el tejido adiposo por lo que el grupo intervenido nutricionalmente previo a la gestación de la rata con obesidad, previene los efectos metabólicos adversos observados en las crías en la vida postnatal. Dado que se ha reportado que los efectos de la programación del desarrollo son diferentes en las hembras y los machos (16,32), nosotros esperamos que los efectos de la intervención nutricional sean diferentes en ambos sexos.

La obesidad materna genera cambios metabólicos en la progenie, el presente trabajo pretende estudiar algunos de los mecanismos involucrados en estas modificaciones metabólicas a nivel del hígado y del tejido adiposo. Se estudiarán variables bioquímicas en el suero (leptina, insulina, etc), histológicas en el tejido adiposo e hígado, composición bioquímica del hígado, estudios moleculares de la expresión y cantidad" de enzimas involucradas en el metabolismo de los lípidos. Así mismo se pretende estudiar las modificaciones de los parámetros anteriormente mencionados en las crías de madres obesas previamente intervenidas. Dado que se ha reportado que los efectos de la programación del desarrollo son diferentes en las hembras y los machos (16,32), nosotros esperamos que los efectos de la intervención nutricional sean diferentes en ambos sexos.

HIPOTESIS

La obesidad materna desencadena en la progenie cambios adversos en el metabolismo de lípidos de las crías.

La intervención nutricional de la rata con obesidad previa a la gestación, previene parcial o totalmente, las alteraciones en el metabolismo de lípidos en hígado y tejido adiposo de las crías. Esto conlleva a la prevención del desarrollo de enfermedades metabólicas de la descendencia en la vida adulta.

OBJETIVOS GENERALES

Estudiar los efectos de la obesidad materna durante el periodo perinatal de la rata en la programación de desórdenes metabólicos de lípidos de las crías en la vida adulta.

Estudiar los efectos de la intervención nutricional de la obesidad materna previa al periodo de gestación de la rata para la programación de desórdenes en el metabolismo de lípidos en hígado y tejido adiposo en las crías de ambos sexos.

OBJETIVOS PARTICULARES

Madres

Estudiar a la madre durante el crecimiento y la gestación para poder esclarecer el ambiente intrauterino metabólico al que estarán sometidas las crías. Para esto se determinará el peso corporal, la ingesta de alimento, prueba de tolerancia a la glucosa y el perfil lipídico de las ratas madre previo y durante la gestación y la lactancia, así como la composición corporal al término de la lactancia.

Crías

Evaluar el registro de peso corporal y de ingesta de alimento de las crías en diferentes etapas del desarrollo.

Analizar a la leptina en las crías neonatas y su correlación con modificaciones en el apetito, tejido adiposo y peso corporal.

Analizar la concentración sérica de leptina, insulina, glucosa y del perfil lipídico, así como la composición corporal de las crías en diferentes etapas del desarrollo.

Determinar la concentración y nivel de expresión de los factores de transcripción SREBP-1 y PPARα, así como de las enzimas FAS y CPT en hígado y PPARγ en tejido adiposo, de las madres y de las crías.

Comparar las diferencias de género de los efectos metabólicos por el impacto de la obesidad materna así como de la intervención nutricional previa a la gestación de la rata.

METODOLOGIA CIENTIFICA

Modelo biológico

Se utilizarán ratas hembras albinas de la estirpe Wistar recién destetadas. Los animales serán mantenidos en el bioterio del Instituto Nacional de Ciencias Médicas y Nutrición SZ bajo condiciones controladas de luz-obscuridad (de 7:00 a 19:00 h), así como de humedad relativa (75-80%) y temperatura (22–23°C). Todos los procedimientos fueron previamente aprobados por el comité de ética de experimentación animal del INCMNSZ (ver archivos adjuntos). Cuando las ratas alcancen entre 10 y 12 semanas de edad, serán apareadas con machos y el día en el cual se observe la presencia de tapón vaginal, se designará como el inicio de la gestación. Durante los diferentes tiempos que dure el experimento, las ratas, tanto las madres como las crías serán pesadas diariamente.

Se utilizará una dieta comercial y otra semipurificada con diferente contenido grasa: la dieta control (C) con 5% de grasa y la dieta hiperlipídica (H) con 25% de grasa. (Tabla 1). La dieta control utilizada es la recomendada por el Instituto Americano de Nutrición para roedores en las fases de embarazo, lactancia y crecimiento (33).

Tabla 1. Composición de las dietas experimentales

	DIETA CONTROL (%)	DIETA HIPERLIPÍDICA (%)
Caseía	20	20
L-Cistina	0.3	0.3
Colina	0.165	0.165
Vitaminas	1	1
Minerales	5	5
Celulosa	5	5
Aceite	7	7
Manteca de cerdo	----	20
Hidratos de carbono		
Almidón	33.24	23.62
Dextrosa	33.24	23.62
	4 Kcal/g dieta	5 Kcal/g dieta

Las ratas gestantes y lactantes serán pesadas todos los días durante el embarazo y hasta el destete. También la ingesta de alimento será cuantificada todos los días. Las ratas nacerán por parto natural. La distancia ano-genital al nacimiento será medida con regla Vernier para la determinación del sexo. Nuestros datos (32) indican que las crías hembras al nacimiento tienen una distancia ano genital de 1.67 ± 0.128 mm (n=291 crías de 43 diferentes camadas) y los machos 3.26 ±

0.22 mm (n=252 crías de 43 camadas). De tal forma que 2.5 mm es más de 2 SD's de la media de cada grupo. Por tanto, el sexo será determinado de acuerdo a la distancia ano-genital > (machos) o < (hembras) 2.5 mm.
Para asegurar homogeneidad en el estudio, camadas de 10 a 14 crías serán ajustadas al nacimiento a 10 crías por madre tratando de mantener una relación de sexo de 1:1.

Para cada experimento se iniciará el estudio con 10 ratas a preñar para el grupo control, 20 del grupo intervenido y 20 de obesidad. Se utilizará este número de muestra tomando en cuenta las posibles ratas que serán excluidas del proyecto por diversas razones como un número de camada mayor de 14 o menor de 10, o que no queden preñadas. De acuerdo a nuestra experiencia en proyectos anteriores, la tasa de fertilidad de las ratas del Bioterio del INCMNSZ es del 80% y de las ratas obesas del 40% por lo que se espera que al menos 8 ratas queden preñadas por grupo y que al menos 6 puedan ser incluidas en el estudio, por lo que se estima que al finalizar el experimento quedará una "n" de 6-8 madres por grupo.

Las diferentes dietas experimentales serán administradas a las ratas desde el destete y en el embarazo y la lactancia quedando 3 grupos.

GRUPOS EXPERIMENTALES:

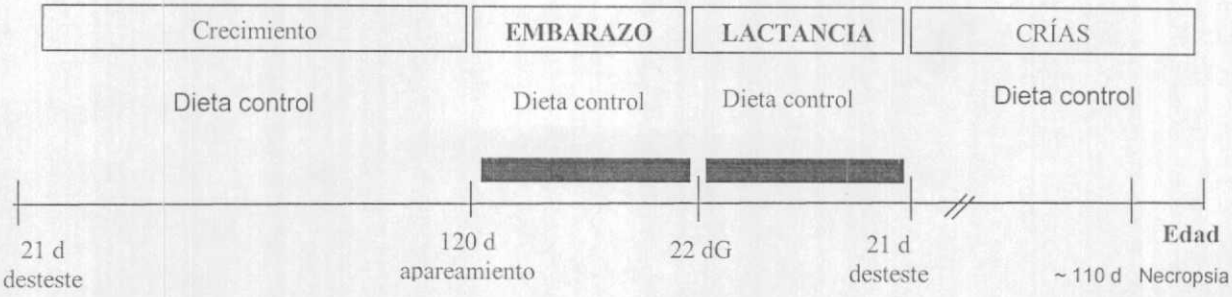
Madres
Se formarán tres grupos experimentales con base al consumo de las dietas experimentales:

1. Grupo Control (MC): las ratas serán alimentadas con dieta control a partir del destete y durante la gestación y la lactancia.
2. Grupo Hiperlipídico o madres con obesidad (MO): las ratas serán alimentadas con dieta hiperlipídica desde el destete y durante la gestación y la lactancia.
3. Grupo Intervenido nutricionalmente previo al embarazo (MI): las ratas serán alimentadas con dieta hiperlipídica desde el destete y hasta un mes (90 días de edad) previo al apareamiento (120 días). La alimentación de las ratas será sustituida por dieta control y continuarán con esta dieta durante la gestación y la lactancia.

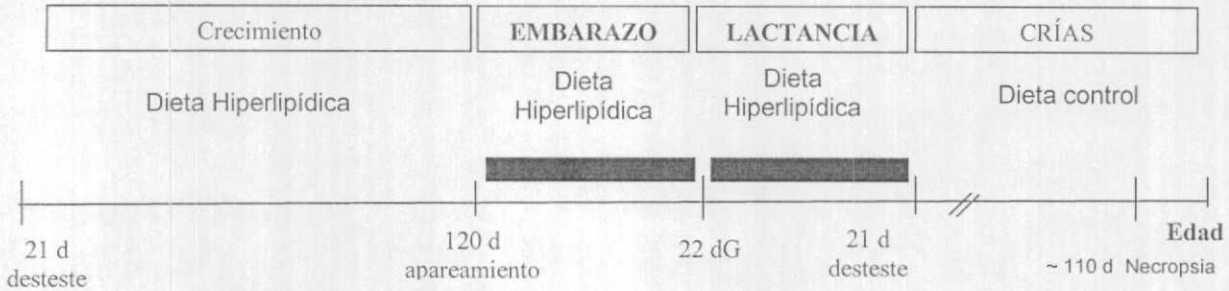
Crías
Al destete todas las crías de los diferentes grupos experimentales serán alimentadas con dieta control hasta el momento del sacrificio. Las crías y la ingesta de alimento serán pesadas todos los días hasta el término del estudio.

GRUPOS EXPERIMENTALES

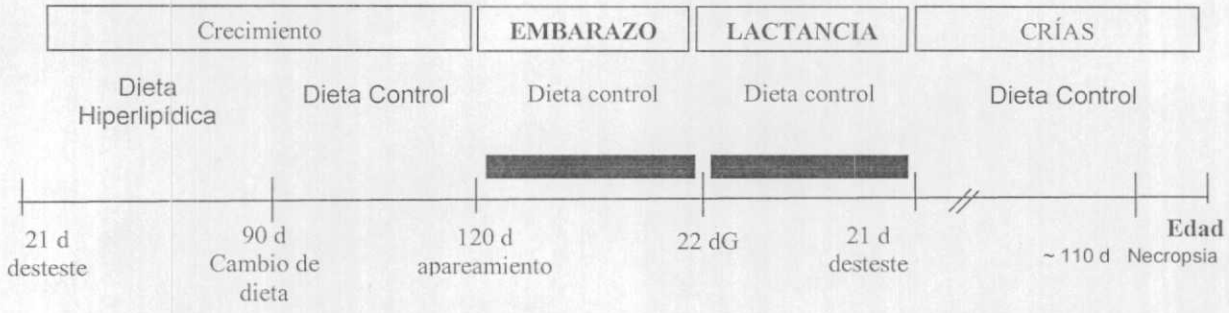
Grupo I. Madres Control (MC).



Grupo II. Madres Obesas (MO)



Grupo III. Madres Intervenidas nutricionalmente previo al embarazo (MI)



dG = días gestacionales; d = días.

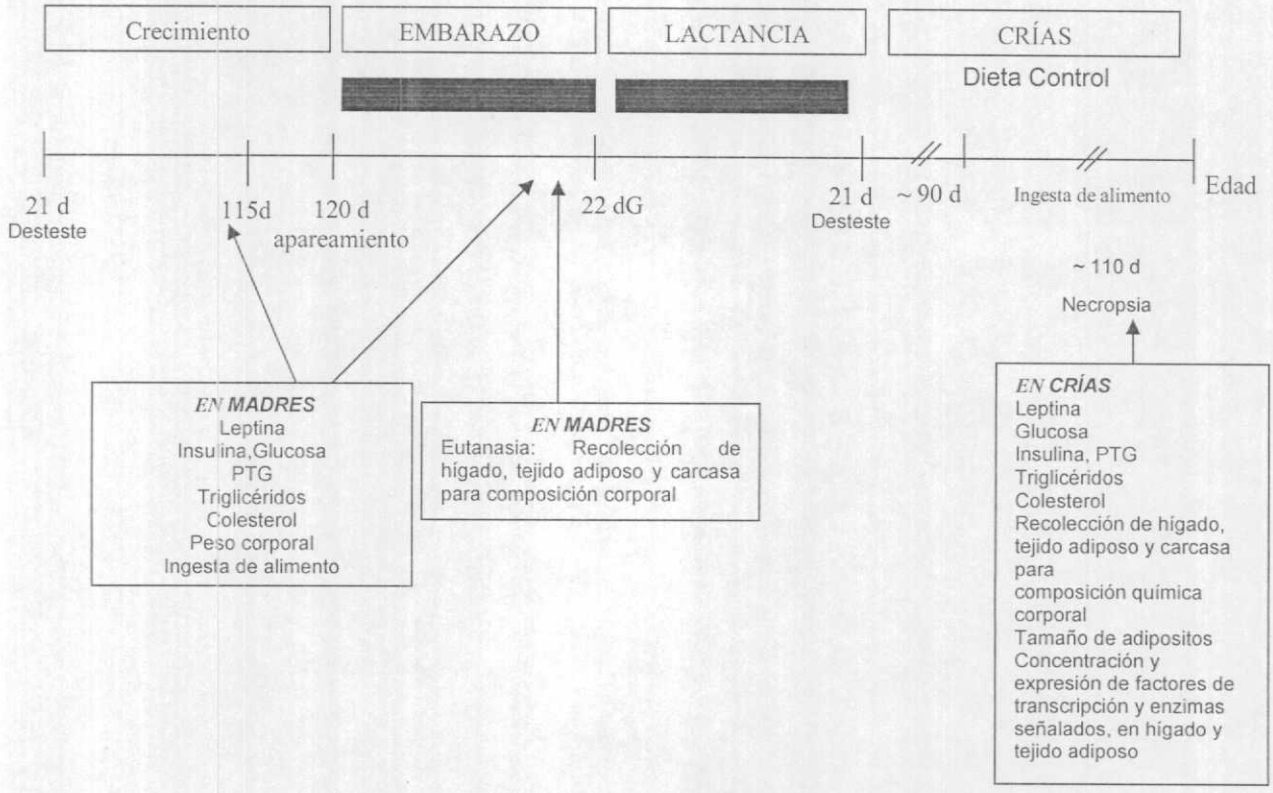
Diseño experimental

Variables experimentales a determinar en la línea de tiempo

Las variables experimentales a estudiar, tanto en ratas madres como en crías, son las siguientes:

- Concentración sérica de insulina
- Concentración sérica de glucosa
- Concentración sérica de colesterol
- Concentración sérica de triglicéridos
- Concentración sérica de leptina
- Peso corporal
- Ingesta de alimento
- Peso del hígado
- Peso de tejido adiposo
- Tamaño de los adipocitos
- Prueba de tolerancia a la glucosa (PTG)
- Contenido de lípidos en la carcaza de los animales
- Contenido de proteína en la carcaza de los animales
- Concentración y expresión de SREBP-1 en hígado
- Concentración y expresión de FAS en hígado
- Concentración y expresión de PPARα en hígado
- Concentración y expresión de CPT-1 en hígado
- Concentración y expresión de PPARγ en tejido adiposo

Línea de tiempo



MADRES

Apareamiento de hembras

A los 120 días de edad los tres grupos experimentales (MC, MO y MI) serán apareadas con machos (no incluidos previamente en el estudio). Las ratas tendrán

partos naturales. Se obtendrá el tamaño de la camada y el peso corporal de las crías al nacimiento. La distancia ano-genital se medirá al nacimiento para la determinación del sexo. Las camadas serán ajustadas a 10 crías/madre manteniendo una relación de 1:1 hembras:macho, en lo posible. Al destete (día 21) las crías serán divididas por sexo y acomodadas en diferentes cajas por grupo.

Prueba de tolerancia a la glucosa (PTG), cuantificación sérica de leptina, triglicéridos y colesterol al día 19 de gestación (dG) en las ratas madres

Un experimento aparte será realizado para el estudio de las PTG de las madres hembras preñadas, de tal forma que el estrés generado por las tomas de muestra no afecte en los resultados obtenidos de las crías (n= 8 ratas gestantes por grupo).
Al día 18 de gestación, las ratas serán puestas en ayuno durante la noche. Al día siguiente, un gramo/Kg de peso de D-Glucosa será administrado vía ip. Se utilizarán jeringas de insulina de 1mL. La sangre será obtenida por punción retro-orbital al tiempo 0, 30, 60 y 120 min. La sangre será colectada en tubos de polietileno y centrifugada. El suero obtenido será almacenado a -20°C hasta el momento de su uso. De la muestra basal se cuantificará las concentraciones de glucosa, insulina (se calculará el índice de resistencia a la insulina), leptina y el perfil lipídico.

CRÍAS

Las crías serán estudiadas

1) Nacimiento: características antropométricas.
2) Destete (21 d): peso, cuantificación de grasa corporal, concentraciones séricas de leptina.
3) Adulto joven, 110 días de edad: peso, ingesta de alimento, concentraciones séricas de leptina, insulina glucosa, GTT, perfil lipídico, composición corporal y cuantificaciones de expresión en hígado y tejido adiposo de factores de transcripción relacionados con el metabolismo de lípidos. Medición del tamaño de los adipocitos.

En cada edad de estudio (21 d y 110 d) se utilizará por camada una cría por sexo por grupo/camada. De tener de 6 a 8 madres por grupo, se tendrá un total de 18 a 24 crías por cada sexo/edad. Este número de animales ha sido previamente manejado por nuestro grupo sin problema alguno, el bioterio del Instituto nos ofrece las facilidades para experimentos a largo plazo.

Metodología utilizada para madres y crías.

Cuantificación de la ingesta de alimento.

Se pondrán en la misma caja dos ratas del mismo grupo experimental y mismo sexo. La comida se proveerá en forma de galletas. Se pesará diariamente tanto la cantidad de comida que se proporcione cada día así como la parte restante después de 24 horas. La cantidad de alimento consumido será promediado entre las dos ratas, este procedimiento lo hemos publicado previamente (16).

Composición química de las carcazas

Después del sacrificio las ratas serán congeladas en pequeñas bolsas de plástico hasta su análisis. El abdomen se abrirá y las vísceras serán removidas y desechadas. Se pesará el animal, cantidad que será designada como peso húmedo, después se pondrá a secar la carcasa a 60°C hasta peso constante. El peso perdido se considerará como cantidad de agua en el cuerpo. La carcaza seca será molida y se realizarán alícuotas para la determinación de la cantidad de grasa por el método de Soxhlet (25) y la cantidad de nitrógeno total (proporcional a la cantidad de proteína) por el método de Kjeldahl (25).

Análisis Bioquímicos

Cuantificación de glucosa en sangre

Las concentraciones de glucosa en suero serán determinadas espectrofotométricamente utilizando el método enzimático de la hexocinasa (Beckman Coulter, Co Fullerton, CA).

Radioinmunoensayo de Insulina

Las concentraciones de insulina en suero serán determinadas por RIA utilizando un estuche comercial de Linco Research INC #Cat RI-13K.

Cuantificación de triglicéridos y colesterol

Las concentraciones de triglicéridos y colesterol en suero se determinarán enzimáticamente con el autoanalizador Sycnchron CX (Beckman coulter, Co, Fullerton, CA).

Radioinmunoensayo de Leptina

Las concentraciones de leptina en suero serán determinadas por RIA utilizando un estuche comercial de Linco Research, Inc #Cat RL-83K.

Tamaño de las células adiposas

Células adiposas provenientes de la zona gonadal serán fijadas como lo describe Etherthon et al. Brevemente, 100 a 150 mg de tejido adiposo gonadal será cortado y lavado a 37°C en 0.15 M de NaCl. Los cortes de tejido adiposo serán fijados en tetróxido de osmio al 3% en buffer de Colidina-HCl (50 mM, pH 7.4) por 72-96 h. Las células fijadas serán lavadas en NaCl al 0.15 M por 24 h, se eliminará el tejido conectivo incubando en 8 M de urea por 24-48h. El tamaño de las células será medido utilizando un microscopio óptico.

Determinación de la concentración y expresión de SREBP-1, PPARα, PPARγ, FAS y CPT

Posterior al sacrificio se congelará en nitrógeno líquido una porción de hígado y tejido adiposo. Para determinar la concentración de SREBP-1, PPARα, PPARγ, FAS y CPT se utilizará el método de Western blot. Para determinar la expresión de los genes se realizará una extracción de RNA total de tejido adiposo e hígado y se cuantificará la concentración relativa de RNAm de SREBP-1, PPARα, PPARγ, FAS y CPT por PCR tiempo real.

Análisis estadístico

Para la prueba de la tolerancia a la glucosa, el área bajo la curva será calculada. El índice de resistencia a la insulina será determinado con la fórmula IRI = Glucosa x Insulina / 22.5. Todos los datos serán expresados como la media ± EE. Se realizará ANOVA de una vía seguido de la prueba de Dunnett para la comparación entre los grupos y ANOVA de 2 vías para la determinación de la interacción entre sexo y grupos experimentales. La correlación entre las diferentes variables (concentraciones de leptina, ingesta de alimento, grasa y peso corporal) serán calculadas utilizando la Correlación de Pearson; $p \leq 0.05$ será considerado como significativo. En cada edad de estudio se utilizará por camada una cría por sexo por grupo. De tener de 6 a 8 madres por grupo, se tendrá un total de 18 a 24 crías por cada sexo.

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