Published in final edited form as:

Placenta. 2008 September ; 29(9): 802–808. doi:10.1016/j.placenta.2008.06.010.

Placental Oxidative Stress Alters Expression of Murine Osteogenic Genes and Impairs Fetal Skeletal Formation

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Abstract

Fetal and placental development rely on an intricate balance of nutrients, growth factors, and signaling pathways at precise times in gestation. Disruptions to this balance may result in disease that manifests in adulthood, a situation termed “developmental origins of health and disease.” Diet, exercise, and certain chemical exposures during pregnancy increase oxidative stress (OS), and may alter trajectory of fetal osteogenic regulation in a manner that increases risk of adult bone dysfunction. The present study used gestational methylnitrosourea (MNU), a known inducer of OS, in C57BL/6 mice with or without dietary antioxidant quercetin (Q) supplementation. Several key placental proteins that influence placental development and fetal osteogenesis (Hgf, Kitl, IFN α 4, Ifrd, and IL-1 β) were altered by MNU, and largely normalized by Q. MNU treatment also resulted in small fetuses with disproportionately shortened limbs and distal limb malformations, and caused placental endothelial and trophoblast damage. Q was again protective against these fetal and placental pathologies. An unanticipated finding with Q supplementation was increased interdigital webbing, perhaps due to dose-related effects on apoptosis required for digital sculpting, or prooxidant effects of Q that caused a maturational delay. These results suggest that elevated OS may alter normal placental osteogenic signaling and fetal skeletal formation.

1. INTRODUCTION

Genetic factors, in concert with gestational environmental conditions, influence fetal programming, growth trajectory of offspring, risk of birth defects, and long-term health [1]. This theory, coined “fetal basis of adult disease” or “developmental origins of health and disease” [2], is most significant during times of fetal genetic plasticity, when one genotype may give rise to a range of morphologic states based on prevailing conditions. Resulting phenotypic changes are often not repaired postnatally, and elevate risk of chronic disease, decades later in life [1–3].

Fetal bone is exquisitely sensitive to environmental influences in mid-gestation, when the appendicular skeleton rapidly grows in all dimensions, and in volumetric density. Adverse exposures during this time reduce neonatal size, weight, and growth rate, which influence adult bone mass and elevate long-term risk of fragility fracture [1,3,4]. Reduced bone density and disrupted trabecular architecture are thought to result when the fetus adapts to a suboptimal

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environment, leading to epigenetic changes in endochondral ossification, intrauterine growth restriction (IUGR), and preferential differentiation of mesenchymal stem cells (MSC) into adipocytes rather than osteoblasts [5]. Thus the fetus adopts an alternative “thrifty” phenotype in response to present conditions. However, this adaptation may not benefit long-term health if future environments do not reflect what was experienced prenatally [3].

The link between fetal skeletal programming and chronic bone disease is important because nearly 10 million Americans over age 50 have osteoporosis, with 1.5 million new cases diagnosed annually, and an additional 34 million people thought to be at risk (National Center for Health Statistics, CDC, April 2007). Current therapies for osteoporosis inhibit osteoclastogenesis, are only partially effective, and are associated with significant side effects. Novel alternative therapies that enhance osteoblastic function during childhood or even before birth may more effectively reduce incidence of chronic skeletal disease.

Neonatal bone mass and osteogenesis are positively correlated with placental weight [3,6]. The placenta functions as an immunologic and nutritional intermediary between mother and child, and produces growth hormones, cytokines, and signaling molecules that drive placental and fetal development [3,6]. Low levels of reactive oxygen species mediate cell signaling to support implantation, embryogenesis, and fetal sculpting. However, excessive oxidative stress (OS) modifies lipids, proteins, and DNA leading to placental endothelial dysfunction, ischemia-reperfusion injury, interrupted nutrient (calcium) transfer to fetus, altered osteogenic gene transcription, and fetal death or maldevelopment [1,7–12]. Pilot mouse studies in our laboratory used gestational administration of the teratogenic alkylating agent methylnitrosourea (MNU) to induce OS-mediated fetal and placental pathology [13]. Human exposure to N-nitroso agents such as MNU originates primarily from two sources: tobacco smoke, and endogenous metabolism of nitrosated foods in the gastric acid environment (average daily consumption is 110 µg/day) [14].

MNU administered experimentally at gestation day 9 (GD9) in mice, at the time of limb bud formation and rapid placental development, causes fetal appendicular malformation with placental trophoblast and labyrinthine damage [13,15–18]. Concurrent antioxidant supplementation with butylated hydroxytoluene offers partial placental and fetoprotection [13]; however, precise molecular mechanisms by which MNU and antioxidant supplementation collectively affect bone and placental development remain poorly understood.

Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one; Q) is a common polyphenolic flavonoid found in fruits, vegetables, and nuts; average human consumption of Q is 1g/day. Antioxidant properties of Q alter signaling pathways such as NFκB, JakSTAT, and protein kinase C driving cell proliferation, differentiation, apoptosis, transcription, inflammation, and immunity [19–22]. Q also increases osteogenic differentiation of MSC, elevates diaphyseal calcium, enhances bone tensile strength, upregulates bone matrix formation, and increases trabecular bone density; these beneficial effects of Q are thought to collectively reduce incidence of osteoporosis [23–27]. However, mechanisms linking Q with protection of placental and fetal skeletal formation are poorly understood. Based on previous work in laboratory animals and humans, we hypothesize that clinically appropriate dietary doses of Q in pregnant mice will protect fetal skeletal and placental formation disrupted by MNU, via normalization of key placental growth factor pathways.

2. METHODS

2.1. Mice

Six-week-old male and female C57BL/6N mice (Charles River Laboratories, Portage, MI) were acclimated for 1 week at 22.0 ± 1°C with 40–60% humidity, 12/12 hr light/dark cycle,

and *ad libitum* food (2018; Harlan Teklad, Madison, WI) and fresh water. Male-female pairs were bred overnight, separated, and females checked for presence of vaginal mucous plugs as an indication of breeding. Plug-positive females were designated as GD0. Mice were sacrificed on GD12 or 14 by cervical dislocation. The Virginia Tech Institutional Animal Care and Use Committee reviewed and approved all experiments prior to their initiation. Institutional guidelines were adhered to in the treatment of all animals.

2.2. Experimental Groups

Plug-positive dams ($n \geq 6$ /group) were assigned to 1 of 6 groups in a generalized, randomized complete block design: control, MNU, low-dose Q (QL; 66 mg/kg Q supplemented in rodent chow throughout gestation; approximately 70% of human dose), high-dose Q (QH; 333 mg/kg Q supplemented in rodent chow throughout gestation; approximately 3.5x daily human dose), MNU+QL, or MNU+QH. These two supplementation levels were chosen to minimize potential low-dose prooxidant behavior of Q, and to approximate a physiologically appropriate level of gestational antioxidant supplementation. Experiments were replicated twice for statistical significance. Due to low fetal numbers in QHM litters, additional females were bred and included in the QHM group to more closely approximate total fetal numbers in other treatment groups. Final total pregnant dams per group numbered 17, 16, 16, 17, 18, and 25, respectively. Control mice received IP injection of 100 μ L PBS, and MNU mice received IP injection of 20 mg/kg MNU (Sigma-Aldrich, St. Louis, MO) in 100 μ L PBS on the morning of GD9, which was previously determined to fall early within the window of active appendicular skeletal development, when murine osteogenic genes are optimally plastic, and are highly susceptible to teratogen-induced programming causing distal limb defects [28].

2.3. Fetal Size, Limb Defect, and Limb Length Determinations

Fetal distal limbs were examined at GD14 following results of our prior studies indicating near completion of appendicular sculpting by GD14, which provides meaningful evaluation of limb and digit formation. GD14 crown-to-rump lengths (C-R) were obtained by averaging three measures from top of calvarium to tailhead using traceable digital calipers (Fisher Scientific, Pittsburgh, PA). Morphologic deformities in distal limbs and digits, including syndactyly, oligodactyly, polydactyly, interdigital webbing, and disproportionate distal limb shortening were quantified using an Olympus SZX7 stereomicroscope (Olympus Europa GmbH, Hamburg, Germany). Limbs were measured microscopically by converting pixel number of limb length to mm, using DBX microscope-to-video camera coupler for direct image projection (Diagnostic Instruments, Sterling Heights, MI), Scion digital camera for image acquisition (Scion CFW-1310C 1394; Scion Corporation, Frederick, MD), and Image J software for pixel-to-mm conversion (v1.30, NIH, Bethesda, MD). Forelimbs were measured from olecranon tuber to tip of longest distal phalanx. Hindlimbs were measured from calcaneal tuber to tip of longest distal phalanx.

2.4. Histopathologic Analysis of Placental Formation

GD14 viable fetuses per litter were counted, and fetal and placental weights were recorded from top-loading balance (Accu-622; Fisher Scientific). Fetuses were fixed in 10% neutral-buffered formalin for 18hr and stored in 70% EtOH. Placentas were preserved in Bouin's fixative for 18hr, washed with 10 volumes ddH₂O, and stored in 70% EtOH. Placentas were transected perpendicular to the long axis of the disc, processed, paraffin-embedded, and stained with hematoxylin-eosin (H&E) for light microscopic evaluation. Ten-400x fields of labyrinthine placenta from different dams in each treatment group were observed for frequency of necrotic endothelial cells vs. controls. Necrotic labyrinthine foci were characterized as areas of cellular swelling, chromatin digestion, disruption of plasma and organelle membranes, mixed (predominantly neutrophilic) inflammation, and hypereosinophilic staining of cellular

remnants. Viable trophoblasts were identified as having intact nuclei with organized chromatin and clearly visible nuclear membranes, with variably distinct cytoplasmic borders that were well-delineated in cytotrophoblastic and glycogen-rich trophoblastic areas, but were relatively inapparent in syncytiotrophoblast. To enumerate viable trophoblasts, intact nuclei per ten-1000x microscopic fields were counted, whereas disrupted, swollen, lysed, or condensed nuclei were considered as nonviable, and were not included in the trophoblast quantification.

2.5. Gene Expression in GD12 Placentas

Mice in gene expression experiments were sacrificed on GD12 based on previous reports suggesting this as the optimal observation period to evaluate gene expression relating to cell differentiation, cell cycle regulation, and RNA metabolism [29], and fetuses were separated from placentas. The placentas were weighed on an Adventurer™ balance (Ohaus, Pine Brook, NJ), placed in RNeasy™ (Ambion, Austin, TX) to reduce mRNA degradation, and stored at -20°C until RNA isolation using RNeasy® Mini Kits (Qiagen, Valencia, CA). Representative segments of placenta from several litters in each treatment group were pooled, and 10 to 30mg total tissue were homogenized with a rotor-stator tissue tearor (Biospec Products Inc, Bartlesville, OK) in guanidine-isothiocyanate buffer and 2-mercaptoethanol (Sigma). Genomic DNA was removed from lysate using a gDNA Eliminator spin column. RNA was purified using an RNeasy spin column, and concentration and quality were assessed via spectrophotometric 260/280 ratio (minimum ultraviolet absorbance was 1.8; BioPhotometer; Eppendorf, Hamburg, Germany) Isolated total RNA was stored at -80°C until cDNA synthesis.

Exploratory pathway-focused gene microarrays (SuperArray Q Series Mouse Common Cytokines) were performed on control and MNU GD12 placenta to identify treatment-induced inflammatory cytokine, growth factor and angiogenic protein changes. Those determined to be significantly altered by MNU in microarray experiments were more closely evaluated for protective effects of QL or QH supplementation, using real-time PCR analysis. The microarrays contained 96 genes from growth and morphogenic protein, interferon, and interleukin functional groups. Housekeeping genes GAPDH, Cyclophilin A, and Rpl13a were used to normalize inter-array variability. Because this is a semi-quantitative gene array system, results were expressed as a ratio of treatment to control expression levels. Total RNA was amplified linearly using the AmpoLabeling-LPR method (SuperArray). Biotin-16-UTP-labeled, amplified cRNA targets were hybridized to nylon membranes with sheared salmon sperm DNA (SuperArray), sodium citrate, and sodium dodecyl sulfate. Detection of the hybridized biotinylated probe was performed on X-ray film (Kodak BioMax XAR Film, New Haven, CT) using alkaline phosphatase-conjugated streptavidin and CDP-star chemiluminescent detection (SuperArray). X-ray time frames ranged from 10 seconds to 2 minutes to optimize signal intensity and maintain consistent saturation of housekeeping genes. Array images were scanned and converted to TIF files for importation into the SuperArray GEArray Analysis Suite. Background correction and data normalization measures were performed and results were exported for statistical analysis. Microarrays of pooled treatment-matched placental samples were replicated twice for statistical evaluation.

To further explore MNU-altered expression of genes identified by microarray analysis, and to quantify protective effects of quercetin against MNU-induced placental gene dysregulation, real-time-PCR was performed on those genes significantly altered by MNU from microarray experiments. The same placental RNA from microarray analysis was used to synthesize cDNA using SuperArray ReactionReady First Strand cDNA Synthesis kit, RT2 PCR primers, and SYBR® Green and ROX reference dyes. Two-step PCR cycling was run on an ABI 7300 (95°C for 10 minutes for HotStart DNA polymerase activation and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, at which time the SYBR Green fluorescence was detected) followed by melting point dissociation analysis (ABI, Foster City, CA). Relative quantification ($\Delta\Delta Ct$

method) was used for the comparison of all treatment groups to the control treatment group calibrator (RQ Software, ABI, Foster City, CA).

2.6. Statistics

For fetal C–R and placental cell count data, one-way ANOVA and Tukey-Kramer honestly significant difference were used. ANOVA compared means for the placental and fetal weights and for limb length with the MIXED procedure of SAS System (Version 9.12; SAS Institute, Cary, NC). The factorial treatment structure was analyzed with contrasts constructed to test for interaction effects of MNU with Q. Significant interactions were further investigated using tests of simple main effects. Model adequacy was assessed using standardized residual plots. Data were determined to be statistically different when $p < 0.05$. Data described as different in this report were significantly different. Because of the relative infrequency of fetal distal limb malformation, categorical data were utilized from the numeric incidence of findings instead of statistical analysis in order to avoid erroneous conclusions. Background adjusted raw probe intensities were extracted from SuperArray image processing software and exported into the open source Bioconductor (<http://www.bioconductor.org/>) collection of packages for further processing and statistical analysis. Data were normalized, and a generalized logarithmic transformation was applied using the VSN package. To obtain differentially expressed genes, the Bioconductor package limma was used to fit a linear model to the data (with treatments as factors), followed by pair-wise contrasts. For every contrast, the top ten most differentially expressed genes were selected using empirical Bayes moderated t-statistic while controlling for the false discovery rate. False discovery rate and level of statistical significance were set at 5%.

3. RESULTS

Daily monitoring of food consumption throughout the duration of the study determined that neither standard rodent chow, nor rodent chow supplemented with QL or QH was consumed with greater frequency (3.98 ± 0.08 mg/day SD, 4.29 ± 0.11 mg/day QL, and 4.07 ± 0.09 mg/day QH per dam, respectively). Average GD14 fetal weight was diminished following MNU (214 ± 16.4 mg in MNU vs. 252 ± 16.4 mg in controls, $P < 0.05$). QL or QH alone did not alter GD14 fetal weight (292 ± 16.4 mg and 246 ± 18.9 mg, respectively). QL did not return MNU fetuses to control weight (201 ± 16.4 mg); however, QH did return fetal weight to near-control values (223 ± 14.6 mg). MNU also decreased GD14 C–R by 9.2%, and both QL and QH restored control-level C–R. QL and QH alone increased fetal C–R (12.24 ± 0.09 mm and 12.09 ± 0.17 mm, respectively) (Table 1a).

MNU reduced R and L forelimb lengths by 24 and 23% respectively, and reduced R and L hindlimb lengths 13 and 10%, respectively. Supplementation with QL or QH partially corrected MNU-induced R fore- and R hindlimb lengths (Table 1a), but neither was effective in correcting L fore- or L hindlimb reductions associated with MNU. QL and QH alone increased L hindlimb lengths, as compared to controls. To evaluate whether limb lengths were proportionately reduced with respect to smaller body length, a ratio of average limb length to C–R was calculated (Table 1b). These data demonstrated that MNU disproportionately reduced distal limb length, and Q variably restored limbs to near-control lengths. More specifically, QL alone moderately reduced corrected forelimb lengths, and more markedly reduced corrected L hindlimb lengths, whereas QH alone actually increased corrected hindlimb length. When administered concurrently, QL partially protected MNU-induced corrected forelimb reduction but did not protect hind limbs from MNU length reduction. QH partially protected forelimbs from MNU-induced reduction, and fully protected against corrected hindlimb reduction associated with MNU.

GD14 digit malformations were numerically increased following GD9 MNU exposure, with syndactyly and interdigital webbing being the most prominent malformations; oligodactyly and polydactyly occurred less frequently (Table 2); statistical analysis was not performed due to low-level occurrence of distal limb defects. The C57BL/6N mouse strain was highly sensitive to fetal distal limb effects of MNU, and responded exquisitely to the protective effects of QL, as concurrent exposure to QL resulted in marked reduction in MNU-associated syndactyly, oligodactyly, and polydactyly. QH was less effective in preventing distal limb malformation, as evidenced by minimal protection against some malformations and exacerbation of others. Unexpectedly, Q alone resulted in numerically higher incidence of forelimb syndactyly and hindlimb interdigital webbing.

Given the vital role of the placenta in fetal development, the present study compared changes in placental weight and cellularity to fetal formation following MNU $\pm$ Q. GD9 MNU reduced GD14 placental weight vs. control or Q alone (80 ± 7.6 mg in MNU group vs. 124 ± 6.6 mg controls, 103 ± 5.9 mg QL, and 100 ± 7.6 mg QH); concurrent QL or QH did not protect against MNU-induced reduced placental weight (89 ± 6.6 mg QL and 93 ± 5.3 mg QH). However, trophoblast numbers were reduced in number per high power field by MNU. Labyrinthine cell necrosis with concurrent fibrin deposition, mixed inflammatory infiltrate, and multifocal hemorrhage were increased following MNU exposure and were largely prevented with concurrent QL or QH (Fig 1–Fig 3). Arterial modifications were not observed to be altered by MNU, QL, or QH, and spiral arteries of the implantation sites appeared morphologically similar to controls.

Several key placental genes associated with placental angiogenesis, signaling, and prenatal bone formation were altered in GD12 placenta following GD9 MNU, and returned towards control expression levels with concurrent QL or QH (Table 3); these changes closely mirrored skeletal changes. MNU reduced expression of hepatocyte growth factor (Hgf), kit ligand (Kitl), and interferon- $\alpha 4$ (Ifn $\alpha 4$), and increased expression of interferon-related developmental regulator-1 (Ifrd) and interleukin-1 β (IL-1 β). While changes in angiogenic gene expression such as placental growth factor or hypoxia inducible factor following gestational MNU exposure were anticipated, exploratory microarray analysis did not yield significant differences vs. controls, and thus further examination of these genes was not performed. RT-PCR validated gene array data and demonstrated variably protective effects of concurrent QL or QH against MNU-induced gene dysregulation.

4. DISCUSSION

The present study demonstrates complex effects of Q on murine placental and fetal skeletal development following MNU. Placenta was exquisitely sensitive to MNU and Q, as was fetal C–R. Distal limbs responded to chemical insult asymmetrically, both laterally and rostrocaudally. Vertebrate L–R symmetry is typically established early in embryogenesis, and therefore was not an expected observation following teratogenic insult at GD9. Further examination of L–R asymmetry in this model of distal limb teratogenesis will be required to more fully understand the significance of this observation.

Changes in fore- and hindlimbs were more straightforward. Murine forelimb buds typically appear at GD9.5, and hindlimbs begin to develop one-half day later, at GD10 [30]. This sequential distal limb patterning may explain why GD9 MNU more severely affected forelimbs than hindlimbs. MNU is rapidly metabolized and eliminated, and as such, specifically affects tissues that are differentiating at the time of exposure. Corrected forelimb lengths were reduced by 17% with MNU, but reduced by only 5% in hindlimbs. Q was also less effective in returning forelimb lengths towards control-levels, emphasizing the narrow teratogenic or therapeutic window of limb development. An interesting and unexpected finding in this study was that Q

increased interdigital webbing. These data suggest dose-related effects on the apoptotic pathway that directs digital sculpting, and emphasize necessity of a low-level of reactive oxygen metabolites for proper digital formation. Alternatively, this observation could represent prooxidant effects of Q and subsequent maturational delay.

Since IUGR caused by gestational exposures is associated with low infant bone mass and adult skeletal fragility, improvement of maternal environment (diet, exercise, chemical exposures) should improve perinatal skeletal formation and reduce future risk of osteoporotic fracture in adulthood [3]. Poor gestational environment modulates set points for basal activities of HPA and IGF, changes intrauterine bone mineral acquisition, reduces birth size, slows childhood growth, and increases risk of adult-onset hip fractures in humans [4]. The present study is the first to highlight an association between MNU alteration of placental genes and dysregulation of placental and prenatal bone formation. Hgf and its receptor c-met are secreted by cytotrophoblasts and mesenchymal cells in placental villi [31]. Hgf mediates angiogenesis and extension of chorioallantoic villous branching [32,33]. Reduced Hgf causes placental labyrinthine thinning by GD10.5, placental vasculopathy that impairs fetal-maternal circulation via ischemia-reperfusion injury, and abnormal fetal development [31,34]. In the present study, MNU reduced Hgf, thinned labyrinthine placenta, and increased endothelial cell necrosis at the time when the placenta should be reaching developmental maturity [34]. Physiologically relevant Q normalized Hgf, protected placental vasculature, and regulated fetal skeleton, suggesting the importance of appropriate placental ROS levels in regulation of growth factors and skeletal formation. Additionally, MNU may also directly alter fetal growth by traversing placental barriers, damaging fetal DNA damage (formation of adducts, point mutations, etc.), or causing protein nitration via increased peroxynitrite production [35]. Future studies will include evaluation of MNU and OS in fetal tissues, and their ability to directly influence appendicular growth.

GD 12 placental *Kitl* was also reduced following MNU, and returned to control-level expression with concurrent Q. *Kitl* is thought to protect against OS-mediated apoptosis [36], and influence bone mass, density, and trabecular development; genetic deletion of *Kitl* leads to osteopenia, impaired osteoblast development, decreased endochondral ossification, and reduced longitudinal bone growth [37]. These reports concur with findings of the present study suggesting that reduced expression of *Kitl* is associated with fetal skeletal dysmorphogenesis caused by MNU and elevated OS. Concurrent dietary Q normalized *Kitl* expression and restored placental and bone development to near control levels, which supports prior reports that offer Q as a potentially effective preventive or restorative treatment for osteoporosis by stimulation of bone formation and proliferation, differentiation, and mineralization [38].

Gestational MNU also appears to have immunological effects on placenta, as IFN α 4, *Ifrd*, and IL-1 β were altered in the present study. IFN α 4 is a type-I interferon that modulates gestational Th1/Th2 balance and influences fetal development via Jak/STAT pathway [39,40]. Diminished IFN α 4 suggests imbalanced fetal-maternal immunity, however the significance of this finding is unknown. *Ifrd* expression was also modulated following MNU in association with altered placental cellular pathology. In health, *Ifrd* is highly expressed in midgestation, relating to targeted apoptosis in organ sculpting [41]; however, pathologic upregulation of proprotein convertase genes such as *Ifrd*, as in this study and others, may dysregulate apoptosis, cause placental or fetal maldevelopment, and increase fetal death [42]. The inflammatory cytokine IL-1 β was likewise upregulated following MNU. A recent study linked OS-mediated alteration in conceptus IL-1 β with enhanced lipid peroxidation, protein carbonylation, intrauterine hypoxia, and altered NF κ B/MapK signaling [43], which was reversed with antioxidants [15]. Additional study of relationships between placental immune modulation and fetal skeletogenesis is currently underway to more completely elucidate complex interactions between placenta and fetus during mid-gestational fetal formation.

Plant phenolics are highly effective antioxidant free radical scavengers that protect cell viability by reducing DNA adduct formation, lipid peroxidation, protein carbonylation, and mitochondrial dysfunction [11]. Flavonoid potency is directly proportional to number of hydroxyl groups present on the phenyl ring; Q has two, making it an effective antioxidant. However, Q also exhibits prooxidant activity at low concentrations (<300 μ M) and in the presence of a free radical source, which can exacerbate rather than protect against OS damage [44]. Another study discusses a “Q paradox” in which Q behaves as an antioxidant in reduced form, but reacts with protein thiols in oxidized form, producing adducts that trigger cytotoxicity and apoptosis [45]. Evidence of prooxidant damage to placenta and fetus may explain QL-associated elevation in interdigital webbing. Alternatively, increased interdigital webbing with Q may suggest dose-related effects on the apoptotic pathway. Follow-up experiments to evaluate degree of oxidative lipid peroxidation, through placental malondialdehyde quantification, are currently underway in our laboratory to more fully characterize placental OS and altered fetal skeletal development. Future studies to quantify MNU-induced DNA damage, reduced DNA adduct formation by Q, and altered topoisomerase catalytic activity, and to determine their relationship to placental and fetal skeletal formation, are planned to validate and forward pilot work in the present study.

In conclusion, this study suggests that MNU-altered gene expression results in placental pathological as well as fetal skeletal developmental changes. Genes altered by MNU have previously been associated with regulation of placental and prenatal bone formation. However, this study is the first to demonstrate that placental gene alterations related to MNU administration and elevated placental OS may influence fetal skeletal formation. This study also highlighted the importance of dietary antioxidant supplementation to reduce adverse effects of gestational OS on placenta and fetus. Additional studies are currently underway to quantify late gestational total bone volume, bone density, and long bone trabecular architectural development following maternal MNU exposure in the mouse model, and to follow these changes through adulthood, to determine whether dysregulated prenatal osteogenic signaling is repaired postnatally, or alternatively, if chemical-induced prenatal genetic and phenotypic alterations persist into adulthood and increase risk of chronic disease. This knowledge will further elucidate associations between dysregulated gene expression involved in bony formation and long-term consequences of prenatal alterations in osteogenesis.

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ACKNOWLEDGEMENTS

Supported by: NIH-K01RR17018

Figure 1. Histopathologic examination of GD14 placenta

Bouin's-fixed, H&E-stained GD14 MNU (B) placenta revealed thinned syncytiotrophoblast (ST) and glycogen-rich trophoblast (GRT) layers, and multifocal labyrinthine (LL) fibrinonecrosis, hemorrhage, and mixed inflammation vs. controls (A), QL (D), or QH (E). Incidence of MNU-induced placental pathology was mitigated with concurrent Q (QLM-E; QHM-F).

Figure 2. Low-magnification examination of GD14 placenta

Low magnification of Bouin's-fixed, H&E-stained GD14 control (A) MNU (B) MNU+QL (C), and MNU+QH (D) placenta demonstrated magnitude of placental changes caused by MNU, and placental protection offered by Q.

Figure 3.

Figure 3a: Quercetin protects against placental trophoblast loss. Histopathologic examination of Bouin's-fixed, H&E-stained GD14 syncytiotrophoblast placenta following GD9 MNU exposure demonstrated significant trophoblast loss, and restoration of control-level trophoblast density with concurrent Q ($P<0.05$).

Figure 3b: Quercetin protects against placental labyrinthine necrosis. Histopathologic examination of Bouin's-fixed, H&E stained GD14 labyrinthine placenta following GD9 MNU exposure demonstrated significantly increased endothelial cell necrosis, multifocal fibrinous inflammation and hemorrhage ($P<0.05$). Near control-level necrotic endothelial foci within labyrinthine placenta were observed with concurrent Q.

Table 1

Table 1a. GD14 Limb Lengths following GD9 Maternal MNU ± Low-Dose Dietary Quercetin (QL, 66 mg/kg) or High-Dose Quercetin (QH, 333 mg/kg) in C57BL/6N Mice (within columns, a shared superscript letter indicates no statistically significant difference, P<0.05)						
Treatment	Fetal C-R Length (mm)	Limb Measurements (mm)				L Hindlimb
		R Forelimb	L Forelimb	R Hindlimb	L Hindlimb	
Control	11.29±0.19 ^a	3.80±0.06 ^a	3.77±0.07 ^a	3.82±0.06 ^c	3.64±0.06 ^c	
MNU	10.41±0.11 ^b	2.92±0.06 ^b	2.92±0.06 ^b	3.31±0.06 ^b	3.30±0.06 ^b	
QL	12.24±0.09 ^c	3.73±0.05 ^a	3.74±0.05 ^a	3.85±0.05 ^a	3.84±0.05 ^c	
QH	12.09±0.17 ^c	3.98±0.08 ^a	3.93±0.06 ^a	4.04±0.09 ^a	4.08±0.08 ^c	
MNU + QL	10.88±0.09 ^a	3.25±0.05 ^c	3.16±0.05 ^b	3.48±0.05 ^c	3.36±0.04 ^b	
MNU + QH	10.86±0.10 ^{a,b}	3.26±0.05 ^c	3.20±0.06 ^b	3.56±0.06 ^c	3.41±0.05 ^b	
Table 1b. Ratio of GD14 Limb Length to Body Length following GD9 Maternal MNU ± QL or QH Supplementation in C57BL/6N Mice (within columns, a shared superscript letter indicates no statistically significant difference, P<0.05)						
Treatment	Ratio of Limb Length to Body Length	Ratio of Limb Length/Body Length				L Hindlimb
		R Forelimb	L Forelimb	R Hindlimb	L Hindlimb	
Control	0.336 ^a	0.334 ^a	0.338 ^a	0.322 ^a	0.317 ^a	
MNU	0.280 ^b	0.280 ^b	0.317 ^b	0.314 ^{a,b}	0.337 ^c	
QL	0.305 ^c	0.306 ^c	0.315 ^b	0.308 ^b	0.313 ^{a,b}	
QH	0.329 ^a	0.325 ^a	0.334 ^a			
MNU + QL	0.298 ^c	0.290 ^c	0.319 ^b			
MNU + QH	0.300 ^c	0.294 ^c	0.327 ^{a,b}			

Table 2
Incidence (and % prevalence in live fetuses) of GD14 Distal Limb and Digital Anomalies with GD9 MNU +/- QL or QH Supplementation in C57BL/6N Mice

Malformation	Control (17 litters) (137 fetuses)	MNU (16 litters) (115 fetuses)	QL (16 litters) (174 fetuses)	QH (17 litters) (110 fetuses)	QLM (18 litters) (87 fetuses)	QHM (25 litters) (118 fetuses)
Front Limbs						
Syndactyly	1 (0.7%)	37 (32.2%)	10 (5.7%)	2 (1.8%)	13 (14.9%)	36 (30.5%)
Oligodactyly	0	19 (16.5%)	0	0	6 (6.9%)	24 (20.3%)
Polydactyly	0	7 (6.1%)	0	0	0	4 (3.4%)
Webbing	1 (0.7%)	9 (7.8%)	0	0	2 (2.3%)	4 (3.4%)
Hind Limbs						
Syndactyly	8 (5.8%)	38 (33.0%)	2 (1.1%)	7 (6.4%)	10 (11.5%)	48 (40.7%)
Oligodactyly	0	34 (29.6%)	0	0	17 (19.5%)	29 (24.6%)
Polydactyly	0	3 (2.6%)	0	0	6 (6.9%)	6 (5.1%)
Webbing	15 (10.9%)	42 (36.5%)	28 (16.1%)	20 (18.2%)	48 (55.2%)	46 (39.0%)

Table 3

Altered GD12 Placental Growth Factor Gene Expression Following GD9 MNU +/- Dietary Supplementation with 66 mg/kg (QL) or 333 mg/kg (QH) (data expressed as ratio of treatment/control expression)

Gene Expression	Microarray	MNU	MNU+QL	PCR
	MNU			MNU+QH
Hgf	0.687	0.659	1.042	1.141
Kitl	0.554	0.578	0.926	0.870
IFN α 4	0.773	ND	1.474	1.777
Ifrd	1.705	1.231	0.841	0.659
IL-1 β	1.777	2.000	0.939	1.057

ND (not determined)