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Theobromine upregulates osteogenesis by human mesenchymal stem cells *in vitro* and accelerates bone development in rats

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Abstract

Theobromine (THB) is one of the major xanthine-like alkaloids found in cacao plant and a variety of other foodstuffs such as tea leaves, guarana, and cola nuts. Historically, THB and its derivatives have been utilized to treat cardiac and circulatory disorders, drug-induced nephrotoxicity, proteinuria and as an immune-modulator. Our previous work demonstrated that THB has the capacity to improve the formation of hydroxyl-apatite during tooth development, suggesting that it may also enhance skeletal development. With its excellent safety profile and resistance to pharmacokinetic elimination, we reasoned that it might be an excellent natural osteoanabolic supplement during pregnancy, lactation and early postnatal growth.

To determine whether THB had an effect on human osteoprogenitors, we subjected primary human bone marrow mesenchymal stem cells (hMSCs) to osteogenic assays after exposure to THB *in vitro* and observed that THB exposure increased the rate of osteogenesis and mineralization by hMSCs. Moreover, THB exposure resulted in a list of up-regulated mRNA transcripts that best matched an osteogenic tissue expression signature as compared to other tissue expression signatures archived in several databases. To determine whether oral administration of THB resulted in improved skeletal growth, we provided pregnant rats with chow supplemented with THB during pregnancy and lactation. After weaning, offspring received THB continuously until postnatal day 50 (approximately 10 mg kg⁻¹ day⁻¹). Administration of THB resulted in neonates with larger bones and 50 day old offspring accumulated greater body mass, longer and thicker femora and superior tibial trabecular parameters. The accelerated growth did not adversely affect the strength and resilience of the bones. These results indicate that THB increases the

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osteogenic potential of bone marrow osteoprogenitors, and dietary supplementation of a safe dose of THB to expectant mothers and during the postnatal period could accelerate skeletal development in their offspring.

Keywords

Anabolics; nutrition; pre-clinical studies; stromal/stem cells; theobromine

Introduction

Theobromine (THB) (3,7-dimethyl xanthine, IUPAC: 3,6 dimethyl purine-2,6-dione) [Fig S1] is one of the major xanthine-like alkaloids found in cacao plant and a variety of other foodstuffs such as tea leaves guarana, and cola nuts [1]. It is a member of the xanthine alkaloid family that includes caffeine and theophylline. THB is generally regarded as safe for human consumption [2], and as a natural vasodilator and diuretic, the molecule and synthetic derivatives are utilized to treat a variety of cardiac and circulatory disorders [3–6], ameliorating the effects of drug-induced nephrotoxicity [7], reducing proteinuria [8] and as an immune-modulator [9–12]. The average amount of THB in cocoa is about 1.9% and chocolate about 0.15–0.46% by mass [1, 2, 13, 14]. The toxicity of THB has been determined in rats [15] and anorexia, decrease in body weight and atrophy of the thymus and testes were observed at extremely high doses (0.6% by mass, equivalent to 250–300 mg kg⁻¹ day⁻¹). Extrapolating from these studies, Stavric predicted that toxicity in humans might be achieved by consuming 18 g of THB per day (equivalent to 171 1.05 oz chocolate bars per day), exposure that is unlikely through dietary means [2] and much higher than potential therapeutic doses suggested by the work presented here. In view of its excellent safety profile and prevalence in common foodstuffs, United States Food and Drug Administration (FDA) assigned a “*Generally Recognised as Safe*” (GRAS) notice (GRN 000340) to THB in 2010. Due to our previous data on the effect of THB on apatite-forming-systems [16–18], we further expanded the studies of THB on osteogenic properties. With its excellent safety profile and improved resistance to pharmacokinetic conversion and elimination, we reasoned that it might be an excellent osteo-anabolic supplement during the early postnatal growth.

Some of the first indications that THB may have osteogenic bone forming properties came from observations made during our early studies on the effects of xanthine-like alkaloids on rat tooth development. Maternal dietary administration of about 10 mg kg⁻¹ THB during pregnancy and lactation resulted offspring that had developed teeth with larger hydroxyl-apatite (HAP) crystals that were more resistant to acidic pH challenge than untreated controls [16–18]. *In vitro* HAP formation assays suggested that THB acts on HAP crystallization by directly interacting with the hydroxyl-apatite [19], but a biochemical role for THB in enhancing osteogenesis has not been explored.

To expand our study of THB beyond its role in odontogenesis, we questioned whether THB exposure could enhance the osteogenic capacity of human bone-marrow derived mesenchymal stem cells (hMSCs) and whether oral consumption of THB by rats during pregnancy, lactation and early growth may improve growth and skeletal development of

offspring. The public health significance of this study is highlighted by reports that skeletal growth and development can be significantly inhibited in humans *in utero* through exposure to alcohol [20, 21], nicotine [20, 22], maternal disease [20] and malnutrition [23]. Even when exposure to toxins and nutritional deficits are resolved, reversal of the effects are challenging because administration of anabolic drugs to pregnant mothers and infants can be associated with serious safety concerns [24–26]. With its excellent safety profile [2, 27, 28], GRAS status, natural origin and abundance, excellent oral bioavailability and long plasma half-life [29, 30], THB may serve as a safe and effective natural osteoanabolic supplement.

Materials and Methods

For additional details, please refer to Supplemental Information.

Human mesenchymal stem cells

Cryopreserved vials of hMSCs from 3 human donors were acquired from the Texas A&M Institute for Regenerative Medicine hMSC distribution facility in accordance with an Institutional Review Board approved protocol. For experiments, cells were recovered from frozen aliquots and sub-cultured in complete culture media (CCM) containing alpha minimal essential media, 4 mM supplemental glutamine, penicillin, streptomycin and 20% fetal bovine serum (Atlanta Biologicals, Norcross, GA) as previously described [31].

Immunophenotyping

MSCs were immunophenotyped using the International Society for Cell Therapy recommended panel as previously described [32].

Adipogenic differentiation assays

Differentiation to adipocytes were performed on confluent monolayer cultures using standard adipogenic media [31]. After 21 days, the cultures were stained with oil red-O solution and visualized using an inverted microscope (Nikon Eclipse, TE200) fitted with a Nikon DXM1200F digital camera.

Chondrogenic differentiation

Chondrogenic differentiation assays were performed by micromass pellet cultures on 200,000 hMSCs using standard chondrogenic media [31]. After 21 days, the chondrogenic pellets were embedded in paraffin, sectioned, and then stained with toluidine blue (Sigma).

Mineralizing (late stage) osteogenic differentiation of MSCs

Confluent cultures of hMSCs were incubated in standard osteo-mineralizing media (OMM) for 14–21 days. Thereafter, calcified monolayers were stained with 40 mM Alizarin Red S (ARS) pH 4.0 (Sigma). In some cases, digital scans of monolayers were densitometrically analyzed using commercial software (Quantity One, Biorad) to generate integrated volume readings of the entire monolayer. For each time-point, values (n=3) were normalized to the mean of the vehicle control, and plotted where the control density was set to = 1.0. Data were statistically analyzed using ANOVA and Tukey's post-test.

Alkaline phosphatase (ALP)

Early osteoblastogenesis was induced on confluent monolayers in 12-well plates by addition of osteo-base media (OBM) consisting of complete culture medium supplemented with 5 mM sodium glycerophosphate, 50 $\mu\text{g mL}^{-1}$ ascorbic acid. THB was also added to the media when required and medium was changed every 2 days. After 8 days, ALP activity as a function of conversion of PNPP to nitrophenolate ($\Delta\text{OD}_{405} \text{ min}^{-1}$) was measured over 10 minutes. The rates were compared against standards with a known concentration of ALP and normalized against the number of cells in the cultures.

Osteoprotegerin (OPG) assays

Two-day conditioned media were recovered from cultures subjected to ALP assays using a commercially sourced enzyme linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN). Media samples were diluted 1:10 with PBS containing 1% (w/v) bovine serum albumin (Sigma) and 0.1% (v/v) Tween 20 (Sigma).

Measurement of cell number

Cells were recovered from each well by trypsinization and their numbers were measured by nucleic acid fluorescence incorporation assay (CyQuant, Invitrogen).

Microarray Transcriptome analysis

Microarray assays were performed using on the HG-U133 Plus 2.0 microarray chips (Affymetrix) by routine procedures. Data were analyzed using dChip software. Expression levels (as functions of arbitrary densitometric values from the array scans) were used to generate lists of up-regulated genes (at least 2-fold). These lists were used to interrogate the tissue expression (Cancer Genome Anatomy Project, CGAP), gene ontology and pathway databases on the Database for Annotation, Visualization and Integrated Discovery (DAVID) website run by the National Institute of Allergy and Infectious Diseases (NIAID), NIH [<http://david.abcc.ncifcrf.gov/>] [33].

Effect of THB on skeletal growth during pregnancy, lactation, and the early growth period

A time-line is provided in Fig 3A. All animal procedures were performed in accordance with a protocol approved by the Texas A&M University Institutional Animal Care and Use Committee. Ten pregnant Sprague Dawley rats (approximate weight, 200 g) were shipped when sperm positive (day 1) (Envigo (previously Harlan) Laboratories). At day 7, dams were divided into 2 groups (n=5 per group) and fed control or experimental diets. THB-containing rat chow (THB-chow) was prepared by finely morselizing standard dry rat food (Rodent 2018, powder meal, Envigo Laboratories) in the presence of powdered THB at a concentration of 0.113 g THB per kg food. This concentration was calculated to maintain an approximate THB dose of 10 mg kg^{-1} maternal body weight per day. Control chow (CTL-chow) was prepared in exactly the same way, but without THB. At day 22 or 23, 4 dams from each group gave birth to live offspring and litters were normalized by size and sex. Eight pups per group were randomly selected at this stage for neonatal bone and mass measurements. The experimental diets were available to the lactating rats throughout suckling and to offspring after weaning until postnatal day 50. At day 44, offspring were

weaned and fed THB-chow or CTL-chow for the remainder of the study. The THB concentration in the chow was modified twice so as to maintain an approximate dose of 10 mg kg⁻¹. At day 72 (50 days old) of the experiment, rats were humanely euthanized.

Digital radiography, micro-computed tomographic (μ CT) scanning and digital histomorphometry

Scans were performed using specimen x-ray imagers (Faxitron M20, Tucson, AZ and Skyscan 1174, Bruker, Contich, Belgium) in accordance with Supplemental Information.

Biomechanical testing

Three point bending tests were performed on an Instron/MTS 1125 ReNew universal mechanical test instrument with a span of 19 mm and crosshead speed of 2 mm min⁻¹. Break sensitivity was set to 90%.

Statistics

For all measurements, data were statistically analyzed by commercially available software (GraphPad Prism version 6.04 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com Prism). In each case, normal distribution of data was tested by D'Agnostino and Pearson omnibus normality test. Multiple comparison statistical analyses were performed by one way analysis of variance (ANOVA) and Tukey or Dunnet post-test where appropriate. Pairwise comparisons were performed by t-test, or in the cases where data were non-parametric (only Fig 4B and 4C), Mann-Whitney tests were performed. Statistical significance was designated at $p < 0.05$.

Results

Effect of THB on osteogenesis by hMSCs

Upon recovery from cryopreservation, the hMSCs proliferated with a doubling time of 12–16 hours, retaining a spindle-shaped fibroblastic morphology. After expansion through approximately 25 population doublings, hMSCs were subjected to an immunophenotypic panel by flow cytometry. The cells were found to be positive for mesenchymal and non-hematopoietic markers CD73, CD90, CD105 and negative for hematopoietic markers CD11b, CD14, CD19, CD79a, CD34 and CD45 and MHC class II antigens (HLA-DP, DQ, DR) [Fig 1A]. When subjected to osteogenic conditions, the hMSCs generated ARS-stained calcified monolayers demonstrating the capacity to differentiate into osteoblast-like cells [Fig 1B]. Upon exposure to adipogenic media the hMSCs generated adipocyte-like cells characterized by the presence of lipid-filled oil-red O-positive adipocytes [Fig 1C]. Micro-mass culture of the hMSCs in the presence of chondrogenic media resulted in the formation of cartilaginous pellets that stained purple with toluidine blue-borate, confirming the presence of cartilage proteoglycans [Fig 1D]. Together these data confirmed that the cells satisfy the currently accepted definition for hMSCs in that they have the expected immunophenotype and undergo tri-lineage differentiation under classical conditions [34].

We next questioned whether THB had the capacity to accelerate differentiation of hMSCs to early osteoblast progenitors. To achieve sufficient concentrations in the media, THB was

dissolved in alkaline conditions followed by dilution in sterile distilled H₂O prior to use in assays. Confluent cultures of hMSCs were induced to differentiate into early osteoprogenitors by exposure to OBM media containing 1–100 μ M THB or an appropriate volume of vehicle. Media and THB was replaced every 2 days, and after 8 days of culture, intact monolayers were subjected to colorimetric assays of membrane-localized ALP activity [35]. ALP activity, a marker of early to mid-stage osteogenesis, was elevated in cultures that received 25 μ M THB and reached a maximal level of stimulation at 50–100 μ M THB [Fig 2A]. Similarly, we observed that OPG levels were increased in cultures that received greater than 25 μ M THB but unlike ALP, this did not appear to increase with greater concentrations [Fig 2B]. Cell yields were not significantly affected by THB treatment, but a slight negative trend suggested that concentrations in excess of 100 μ M may inhibit proliferation and/or viability [Fig 2C].

We next tested whether THB could affect the terminal or mineralizing stages of osteoblast differentiation. For this purpose, confluent cultures of hMSCs were exposed to THB-supplemented OBM for 8 days, followed by THB-supplemented OMM media for up to 14 additional days. After 7 days of exposure to THB-supplemented OMM, 50 μ M THB caused accelerated mineralization of the monolayers as compared to lower doses and the vehicle when visualized by ARS staining and densitometric scanning of the stained monolayers [Fig 2D,E]. Microscopy confirmed the presence of raised, ARS-dense nodules, suggestive of monolayer biomineralization [Fig 2F]. After 14 additional days, cultures containing lower THB doses and the control underwent mineralization, but densitometric scanning indicated that the 5 and 50 μ M THB-exposed cultures remained more densely stained with ARS than the vehicle control [Fig 2D,E].

The data indicated that THB has the capacity to upregulate a selection of common osteogenic markers expressed by hMSCs but to confirm this hypothesis, we performed transcriptomic analyses on THB treated and untreated cultures. Monolayer cultures from 2 donors were subjected to 8 days of exposure to OBM supplemented with 50 μ M THB. Total RNA was recovered from the cultures and analyzed by Affimetrix HG-U133 Plus 2.0 microarray chips. A total of 562 and 1029 transcripts were identified as upregulated more than 2-fold by treatment with THB. When the upregulated lists were analysed by the DAVID database, we found that both corresponded to bone tissue expression signatures with the greatest statistical significance as compared to other tissues [Table S1]. When the lists were analysed for associated gene-ontology terms, there was a prevalence of gene clusters associated with extracellular matrix-integrin interactions and focal adhesion signalling consistent with the formation of collagen rich osteoid-like tissue [Table S2]. The 2 donors tested also shared upregulation of bone morphogenic protein/transforming growth factor- β (BMP/TGF β), focal adhesion pathway-related genes and some *wingless* (Wnt) pathway related transcripts. Similar results were observed when monolayers were cultured with 100 μ M THB, but the association with bone differentiation became less apparent with 25 μ M treatment. High dose THB treatment (100 μ M) resulted in downregulation of cyclin 1 and 2, and cyclin dependent kinases (CDK) 2 and 6, but there was no significant upregulation of apoptotic transcripts, suggesting that THB affected proliferation rather than apoptosis in the assays presented in Fig 2C.

Effect of dietary THB exposure on postnatal development of rodent offspring

To test whether THB had a skeletogenic effect *in vivo*, pregnant rats were exposed to dietary THB (about 10 mg kg⁻¹) in chow for the duration of their pregnancy and during lactation, and offspring were exposed to THB during the postnatal growth period up to day 50 [Fig 3A]. The rationale for this strategy was to experimentally mimic a situation where THB might be used to enhance the skeletal development and size of offspring. Rats gave birth at day 22 resulting in 51 live pups in the THB-chow group and 44 in the control (CTL)-chow group. The viability and sex distribution of offspring in each group was normal [Table S3].

To evaluate the effect of THB on skeletal growth that may have occurred *in utero*, 8 male neonates were randomly selected from the THB-treated and CTRL-treated groups and euthanized by decapitation. Qualitative examination of the offspring indicated that the bodies of THB treated rats were larger than controls and no external deformities in either group were evident [Fig S2]. Skulls were scanned by μ CT and the total volume of bone was found to be greater in the THB-chow group [Fig 3B]. The cranial diameter in the horizontal direction at the midpoint between the anterior and posterior extremity of the cranium was also found to be larger in the THB treated group [Fig 3C]. Humeri were also longer in THB-treated neonates than in controls [Fig 3D]. While not statistically significant based on the parameters set by this study, the data also displayed a strong trend in favour of longer spinal columns in the THB-chow treated neonates ($p=0.0513$, Fig S3). The overall masses of THB-treated neonates were also higher in the THB-treated group [Fig 3E].

To examine the effects of THB on further postnatal development, litter sizes and gender distribution were normalized between dams to minimize the effects of competition between offspring during feeding and to ensure an adequate number of specimens for analyses. For technical reasons, assays were restricted to females. After birth, dams continued to receive THB-chow or CTL-chow for 22 days during lactation and weaned offspring received THB-chow or CTL-chow thereafter. Offspring were weighed at postnatal day 26, 33 and 40 (4, 11 and 18 days post wean) and at each time-point, those receiving THB-chow were consistently heavier than controls [Fig 3F]. The difference in mass between THB-chow and CTL-chow groups was +9.66 (SD 0.57%), +9.18% (SD 0.47%) and +9.24% (SD 0.44%) for day 26, 33 and 40 respectively. At postnatal day 50 (28 days post wean) rats were then humanely euthanized.

Hind-limbs were dissected from the offspring and μ CT was performed on the trabecular structures of the tibiae. While there was no statistically detectable difference in trabecular thickness (Tb.Th.) [Fig 4A], we observed that trabecular spacing (Tb.Sp.) was reduced [Fig 4B] and trabecular number (Tb.N.) [Fig 4C] was substantially increased in the THB-chow group as compared to control specimens. As expected, this was associated with an increase in trabecular surface area [Fig 4D]. The data suggested that THB causes an increase in the frequency but not size of trabecular structures, an observation that could be confirmed qualitatively when axial cross-sections were inspected [Fig 4E]. We also observed that the trabecular structures of the tibiae were deeper in the THB treated group, extending distally to one third of the length of the tibia as compared to about one quarter in the controls [Fig 4F,G].

We next examined the structure and biomechanical characteristics of femora. In contrast with the tibiae, we found that the femora were longer in THB-chow treated offspring than controls [Fig 5A]. We also observed that the mean cortical thickness at mid-diaphysis was significantly greater [Fig 5B] associated with an increased polar moment of inertia (J) [Fig 5C]. Surprisingly, 3-point biomechanical testing of the femora indicated that the energy to break [Fig 5D] and energy to yield [Fig 5E] did not attain statistical significance in either direction, but the slightly increased energy to break values and reduced energy to yield values in the THB treated bones suggests increased resilience to fracture, but with slightly less resistance to deformation.

Discussion

In this study, we found that THB has the biological capacity to upregulate osteogenesis by hMSCs. We initially observed that in the presence of osteogenic supplements, THB upregulated early osteogenic markers and induced mineralization when cultured in mineralizing osteogenic media. The effect of THB in inducing monolayer mineralization is more likely to be due to biological activity rather than enhancement of HAP crystal formation because the slightly acidic conditions of tissue culture favor the formation of simpler calcium phosphate salts such as brushite rather than HAP [32, 36]. While the data strongly suggested that THB enhanced osteogenesis by hMSCs, a broader, more objective analysis was performed where monolayers were incubated in THB followed by analysis of over 47,000 mRNA transcripts. THB treatment resulted in a transcriptomic signature that best matched bone tissue and analysis of gene ontology terms demonstrated a strong enrichment of transcripts involved with ECM attachment, ECM secretion and focal adhesion kinase signaling. This observation was expected given the enhanced formation of ECM usually associated with metabolically active osteoblasts. While a single, definitive molecular mechanism for THB in accelerating hMSC osteogenesis was not clear from the transcriptomic study, there was a robust upregulation of genes involved in TGF β /BMP (SMAD) and cWnt signaling. Both of these pathways are key positive regulators of osteogenesis, functioning synergistically to drive differentiation through upregulation of the master regulator of osteogenic commitment, runt-related transcription factor 2 (Runx2) [37–40]. A direct role for THB in enhancing cWnt and SMAD signaling has not yet been reported in the literature, but a THB derivative can induce ECM deposition through cWnt and SMAD signaling in a model of pulmonary fibrosis [41].

A recent report by Jang *et al.* has described an inhibitory role for THB on adipogenesis by the murine stromal adipocyte-progenitor cell line 3T3-L1 [42]. This is interesting in the context of the results presented here because the pathways that regulate adipogenesis and osteogenesis in mammalian cells are antagonistic to one another [32, 43–48]. In the report, Jang *et al.* describe the reduction of PPAR γ protein with several other adipogenic markers and an overall reduction in lipid accumulation. The collective results of Jung *et al.* and those from our study suggest that THB stimulates osteogenic pathways at the expense of adipogenesis in cultured multipotent progenitor cells, but further work is required to confirm this hypothesis. Jiang *et al.* also reported that THB had the capacity to arrest 3T3/L1 cells in the G₀/G₁ phase of the cell cycle and regulated the expression of CDK2, and we observed a

similar effect on hMSCs with high doses reducing cell yields and inhibiting cell-cycle transcripts.

The *in vitro* data performed on hMSCs strongly suggests that THB stimulates osteoprogenitor stem cells to undergo osteoblast differentiation but it was unclear whether THB has an equivalent effect *in vivo*. To model the complex series of events that contribute to skeletogenesis and growth, we chose to orally administer THB to rats during pregnancy, lactation and to offspring during the postnatal growth period followed by measurements of hind-limb long bone growth. This developmental model was chosen in part because we have previously utilized the approach to demonstrate a positive effect of THB in strengthening tooth hydroxyl-apatite [16–18] and our familiarity with the protocols would ensure the best chance of demonstrating proof-of-concept. The experimental approach was also designed to incorporate the processes of *de novo* bone development, growth and maintenance. This is particularly important to note because we do not yet know to what degree THB affects these individual processes.

Rats gave birth at day 22 with no effect of THB on the viability of offspring or sex distribution. This observation concurs with previous studies on the peri-natal, post-natal and teratogenicity of THB [49]. Neonates originating from THB-chow fed dams appeared larger and were heavier than control pups. At least some of the increased body mass could be attributable to bone tissue given that the total volume and cranial diameter of skulls and humerus length was significantly greater in the THB-chow group as compared to controls. Spinal columns also appeared longer in the THB-chow treated neonates, but the data did not meet the significance threshold for this study. Collectively, these observations indicated that THB has the capacity to accelerate skeletal growth *in utero*. At 22 days old, offspring were weaned and continuously provided THB or CTL-chow for the duration of the experiment. The CTL-chow group exhibited a normal accumulation of body mass, but we observed an increase in the masses of THB-chow group which remained constant at approximately +10% of the control group. While it was not possible to definitively attribute the increased body mass to skeletal development, the rats did not appear to have accumulated excess body fat and food intake was comparable between the groups. Of note, THB administration to rats on a fatty diet has been reported to actually reduce overall body fat [50].

Micro-CT scans and histomorphometric measurements of the trabecular structures of the proximal tibiae demonstrated increased trabecular number and reduced spacing, but not thickness in the THB-chow group compared to the controls. These results suggested that dietary supplementation of THB affected the *de novo* generation of the trabecular structures, rather than the homeostatic mechanisms that control trabecular thickness. Longitudinal scans of tibiae indicated that the trabecular structures penetrated deeper into the diaphysis in THB-chow treated rats. Given that *de novo* trabecular bone is generally synthesized by endochondral ossification, contributing to bone elongation, it is possible that THB acts primarily on endochondral bone growth [51].

Femora in the THB-chow group were longer and exhibited significantly thicker cortical bone, predicting greater strength, but when subjected to biomechanical testing the energies required to break or deform the THB-treated and control bones were statistically equivalent

between THB-treated and control groups. However, trends in the data suggested that THB-treated femora are generally more deformable yet more resistant to fracture. This is a trait that corresponds well with rapidly growing bone tissue and normal fluctuations in bone density that occur in the bones of developing mammals, including humans. Rapid phases of growth are frequently accompanied by transient reductions in bone density that occur due to a phase lag between the rate of bone growth and the attainment of peak bone mass [51, 52]. One would therefore expect a transient reduction in the hardness of bone during a period of THB-induced acceleration of bone growth, but not so as to significantly increase the risk of fracture. Bone density does recover as the rate of growth slows [52], and the bone that contributed to extra trabecular structures and femoral length is predicted to strengthen with age. Therefore, benefits of enhanced bone growth attributed to THB may indeed persist until adulthood in humans.

In the current study, the offspring rats received THB *in utero*, during lactation, and in food until early sexual maturity. These experiments have convincingly demonstrated the efficacy of THB in accelerating bone growth and development, but the protocol employed combines the processes of growth and development with homeostatic bone maintenance without the capacity to distinguish between the individual processes. In future studies, it may be necessary to determine how long the effects of THB persist in offspring if withdrawn at birth or shortly after lactation and whether THB can affect homeostatic bone turnover in adult rats.

To determine whether THB had a direct osteoanabolic effect on human osteoprogenitor cells, we performed *in vitro* molecular assays on hMSCs. In future studies, it will be necessary compare the mechanism behind THB-mediated upregulation of osteogenic processes in hMSCs with the mechanisms that cause THB-mediated enhancement of osteogenesis developing rats. A major goal of this work will be to elucidate, at the molecular level, how and at what stage THB enhances bone formation. THB appears to be effective for enhancement of growth and development as is shown in the present study, and therefore might serve as a therapeutic for enhancement of skeletal growth and acceleration of bone healing. It is also possible that THB might be an effective natural treatment for chronic bone loss associated with diseases such as osteoporosis and diabetes. The data presented here therefore clearly justify further investigation of THB mechanism in human cells and animal models of bone trauma and systemic bone loss. It is noteworthy to add that the upregulation of OPG by MSCs during exposure to THB could indicate a role in the modulation of bone homeostasis given that OPG is an inhibitor of receptor activator of NFkappaB ligand, a key activator of osteoclast activity [53], but in the current study we did not enumerate osteoclasts in the experimental rats.

Collectively these data demonstrate that THB has a potent osteogenic effect on human osteoprogenitor cells *in vitro* and when THB is fed to rats during pregnancy, lactation and during the postnatal growth period, the offspring exhibit robust signs of accelerated skeletal growth *in utero* that persists after weaning. These findings are particularly relevant to cases of intrauterine growth retardation in humans caused by exposure to malnutrition, nicotine, drugs or alcohol [20–23]. These cases are difficult to treat and administration of bone modifying drugs to pregnant mothers and infants is associated with a high degree of risk

[24–26]. It has also been reported that 10–30% of growth restricted infants do not exhibit catch-up growth and can suffer from a variety of serious health problems in later life [54, 55]. While careful safety and efficacy studies are necessary to proceed to clinical translation, the excellent safety profile and natural origins of THB suggest that it could represent a safe, natural and effective method for improving the skeletal growth of neonates and infants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Smit HJ. Theobromine and the pharmacology of cocoa. *Handb Exp Pharmacol*. 2011;201–234.
2. Stavric B. Methylxanthines: toxicity to humans. 3. Theobromine, paraxanthine and the combined effects of methylxanthines. *Food Chem Toxicol*. 1988; 26:725–733. [PubMed: 3058562]
3. Champion S, Lapidus N, Cherié G, Spagnoli V, Oliary J, Solal AC. Pentoxifylline in heart failure: a meta-analysis of clinical trials. *Cardiovasc Ther*. 2014; 32:159–162. [PubMed: 24758396]
4. Kaur J, Sodhi GS. Diuretic activity of organomercury (II) complexes of theophylline and theobromine. *J Inorg Biochem*. 1992; 48:305–310. [PubMed: 1479363]
5. Skudicky D, Bergemann A, Sliwa K, Candy G, Sareli P. Beneficial effects of pentoxifylline in patients with idiopathic dilated cardiomyopathy treated with angiotensin-converting enzyme inhibitors and carvedilol: results of a randomized study. *Circulation*. 2001; 103:1083–1088. [PubMed: 11222470]
6. Sliwa K, Skudicky D, Candy G, Bergemann A, Hopley M, Sareli P. The addition of pentoxifylline to conventional therapy improves outcome in patients with peripartum cardiomyopathy. *Eur J Heart Fail*. 2002; 4:305–309. [PubMed: 12034156]
7. Nasiri-Toosi Z, Dashti-Khavidaki S, Khalili H, Lessan-Pezeshki M. A review of the potential protective effects of pentoxifylline against drug-induced nephrotoxicity. *Eur J Clin Pharmacol*. 2013; 69:1057–1073. [PubMed: 23179178]
8. Badri S, Dashti-Khavidaki S, Lessan-Pezeshki M, Abdollahi M. A review of the potential benefits of pentoxifylline in diabetic and non-diabetic proteinuria. *J Pharm Pharm Sci*. 2011; 14:128–137. [PubMed: 21501559]
9. Bell SG. Immunomodulation, part I: pentoxifylline. *Neonatal Netw*. 2005; 24:45–48. [PubMed: 16117244]
10. Noel C, Copin MC, Hazzan M, Labalette M, Susen S, Lelievre G, Dessaint JP. Immunomodulatory effect of pentoxifylline during human allograft rejection: involvement of tumor necrosis factor- α and adhesion molecules. *Transplantation*. 2000; 69:1102–1107. [PubMed: 10762214]
11. Costantini TW, Deree J, Peterson CY, Putnam JG, Woon T, Loomis WH, Bansal V, Coimbra R. Pentoxifylline modulates p47phox activation and downregulates neutrophil oxidative burst through PKA-dependent and -independent mechanisms. *Immunopharmacol Immunotoxicol*. 2010; 32:82–91. [PubMed: 19839729]
12. Rieneck K, Diamant M, Haahr PM, Schönharting M, Bendtzen K. In vitro immunomodulatory effects of pentoxifylline. *Immunol Lett*. 1993; 37:131–138. [PubMed: 7505000]
13. Zoumas BL, Kreiser WR, Martin RA. Theobromine and caffeine content in chocolate products. *Journal of Food Science*. 1980; 45:314.

14. Craig WJ, Nguyen TT. Caffeine and theobromine levels in cocoa and cocoa products. *Journal of Food Science*. 1984; 49:302.
15. Tarka SM Jr, Zoumas BL, Gans JH. Short-term effects of graded levels of theobromine in laboratory rodents. *Toxicology and applied pharmacology*. 1979; 49:127–149. [PubMed: 473198]
16. Nakamoto, T., Simmons, WB., Falster, AU. Products of apatite-forming-systems. US Patent. US5919426A. 1999.
17. Nakamoto, T., Simmons, WB., Falster, AU. Apatite-forming-systems: Methods and products. US Patent. US 6183711. 2001.
18. Nakamoto T, Falster AU, Simmons WB. Theobromine: a safe and effective alternative for fluoride in dentifrices. *The Journal of Caffeine Research*. 2016 Feb.;1–9.
19. Amaechi BT, Porteous N, Ramalingam K, Mensinkai PK, Ccahuana Vasquez RA, Sadeghpour A, Nakamoto T. Remineralization of artificial enamel lesions by theobromine. *Caries Res*. 2013; 47:399–405. [PubMed: 23615395]
20. Namgung R, Tsang RC. Factors affecting newborn bone mineral content: in utero effects on newborn bone mineralization. *Proc Nutr Soc*. 2000; 59:55–63. [PubMed: 10828174]
21. Habbick BF, Blakley PM, Houston CS, Snyder RE, Senthilselvan A, Nanson JL. Bone age and growth in fetal alcohol syndrome. *Alcohol Clin Exp Res*. 1998; 22:1312–1316. [PubMed: 9756047]
22. Godfrey K, Walker-Bone K, Robinson S, Taylor P, Shore S, Wheeler T, Cooper C. Neonatal bone mass: influence of parental birthweight, maternal smoking, body composition, and activity during pregnancy. *J Bone Miner Res*. 2001; 16:1694–1703. [PubMed: 11547840]
23. Curtis EM, Moon RJ, Dennison EM, Harvey NC. Prenatal calcium and vitamin D intake, and bone mass in later life. *Current osteoporosis reports*. 2014; 12:194–204. [PubMed: 24740166]
24. Sanz-Salvador L, Garcia-Perez MA, Tarin JJ, Cano A. Bone metabolic changes during pregnancy: a period of vulnerability to osteoporosis and fracture. *Eur J Endocrinol*. 2015; 172:R53–R65. [PubMed: 25209679]
25. Gerin M, Jambon G, Fouque-Aubert A, Raybaud C, Cochat P, Claris O, Bacchetta J. Neonatal transient hypophosphatemic hypercalciuric rickets in dizygous twins: A role for maternal alendronate therapy before pregnancy or antireflux medications? *Arch Pediatr*. 2016; 23:957–962. [PubMed: 27150561]
26. French AE, Kaplan N, Lishner M, Koren G. Taking bisphosphonates during pregnancy. *Can Fam Physician*. 2003; 49:1281–1282. [PubMed: 14594094]
27. Rosenkranz HS, Ennever FK. Evaluation of the genotoxicity of theobromine and caffeine. *Food Chem Toxicol*. 1987; 25:247–251. [PubMed: 3106176]
28. Shively CA, White DM, Blauch JL, Tarka SM Jr. Dominant lethal testing of theobromine in rats. *Toxicology letters*. 1984; 20:325–329. [PubMed: 6701919]
29. Drouillard DD, Vesell ES, Dvorchik BH. Studies on theobromine disposition in normal subjects. Alterations induced by dietary abstention from or exposure to methylxanthines. *Clinical pharmacology and therapeutics*. 1978; 23:296–302. [PubMed: 627135]
30. Shively CA, Tarka SM Jr, Arnaud MJ, Dvorchik BH, Passananti GT, Vesell ES. High levels of methylxanthines in chocolate do not alter theobromine disposition. *Clinical pharmacology and therapeutics*. 1985; 37:415–424. [PubMed: 3979003]
31. Gregory, CA., Prockop, DJ. Fundamentals of Culture and Characterization of Mesenchymal Stem/Progenitor Cells from Bone Marrow Stroma. In: Freshney, RIS.G, N., Auerbach, JM., editors. *Culture of Human Stem Cells*. Hoboken NJ: Wiley-Liss; 2007. p. 208
32. Zeitouni S, Krause U, Clough BH, Halderman H, Falster A, Blalock DT, Chaput CD, Sampson HW, Gregory CA. Human mesenchymal stem cell-derived matrices for enhanced osteoregeneration. *Sci Transl Med*. 2012; 4:132ra155.
33. Dennis G, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol*. 2003; 4:P3. [PubMed: 12734009]
34. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells.

The International Society for Cellular Therapy position statement. *Cytotherapy*. 2006; 8:315–317. [PubMed: 16923606]

35. Krause U, Seckinger A, Gregory CA. Assays of osteogenic differentiation by cultured human mesenchymal stem cells. *Methods Mol Biol*. 2011; 698:215–230. [PubMed: 21431522]
36. Johnsson MS, Nancollas GH. The role of brushite and octacalcium phosphate in apatite formation. *Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists*. 1992; 3:61–82.
37. Gaur T, Lengner CJ, Hovhannisyan H, Bhat RA, Bodine PV, Komm BS, Javed A, van Wijnen AJ, Stein JL, Stein GS, Lian JB. Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. *J Biol Chem*. 2005; 280:33132–33140. [PubMed: 16043491]
38. Dong YF, Soung dY, Schwarz EM, O'Keefe RJ, Drissi H. Wnt induction of chondrocyte hypertrophy through the Runx2 transcription factor. *J Cell Physiol*. 2006; 208:77–86. [PubMed: 16575901]
39. Zhang R, Oyajobi BO, Harris SE, Chen D, Tsao C, Deng HW, Zhao M. Wnt/ β -catenin signaling activates bone morphogenetic protein 2 expression in osteoblasts. *Bone*. 2013; 52:145–156. [PubMed: 23032104]
40. Kugimiya F, Kawaguchi H, Ohba S, Kawamura N, Hirata M, Chikuda H, Azuma Y, Woodgett JR, Nakamura K, Chung UI. GSK-3 β controls osteogenesis through regulating Runx2 activity. *PLoS One*. 2007; 2:e837. [PubMed: 17786208]
41. Wang Z, Zhang JN, Hu XF, Chen XL, Wang XR, Zhao TT, Peng MJ, Zou P. Effects of pentoxifylline on Wnt/ β -catenin signaling in mice chronically exposed to cigarette smoke. *Chinese medical journal*. 2010; 123:2688–2694. [PubMed: 21034654]
42. Jang YJ, Koo HJ, Sohn EH, Kang SC, Rhee DK, Pyo S. Theobromine inhibits differentiation of 3T3-L1 cells during the early stage of adipogenesis via AMPK and MAPK signaling pathways. *Food & function*. 2015; 6:2365–2374. [PubMed: 26085110]
43. Krause U, Harris S, Green A, Ylostalo J, Zeitouni S, Lee N, Gregory CA. Pharmaceutical modulation of canonical Wnt signaling in multipotent stromal cells for improved osteoinductive therapy. *Proc Natl Acad Sci U S A*. 2010; 107:4147–4152. [PubMed: 20150512]
44. Bennett CN, Ross SE, Longo KA, Bajnok L, Hemati N, Johnson KW, Harrison SD, MacDougald OA. Regulation of Wnt signaling during adipogenesis. *J Biol Chem*. 2002; 277:30998–31004. [PubMed: 12055200]
45. Farmer SR. Regulation of PPAR γ activity during adipogenesis. *Int J Obes (Lond)*. 2005; 29(Suppl 1):S13–S16. [PubMed: 15711576]
46. Moldes M, Zuo Y, Morrison RF, Silva D, Park BH, Liu J, Farmer SR. Peroxisome-proliferator-activated receptor gamma suppresses Wnt/ β -catenin signalling during adipogenesis. *Biochem J*. 2003; 376:607–613. [PubMed: 12954078]
47. Xu C, Wang J, Zhu T, Shen Y, Tang X, Fang L, Xu Y. Cross-Talking between PPAR and WNT Signaling and Its Regulation in Mesenchymal Stem Cell Differentiation. *Curr Stem Cell Res Ther*. 2015
48. Takada I, Kouzmenko AP, Kato S. Wnt and PPAR γ signaling in osteoblastogenesis and adipogenesis. *Nat Rev Rheumatol*. 2009; 5:442–447. [PubMed: 19581903]
49. Tarka SM Jr, Applebaum RS, Borzelleca JF. Evaluation of the perinatal, postnatal and teratogenic effects of cocoa powder and theobromine in Sprague-Dawley/CD rats. *Food Chem Toxicol*. 1986; 24:375–382. [PubMed: 3744190]
50. Inoue H, Kobayashi-Hattori K, Horiuchi Y, Oishi Y, Arai S, Takita T. Regulation of the body fat percentage in developmental-stage rats by methylxanthine derivatives in a high-fat diet. *Bioscience, biotechnology, and biochemistry*. 2006; 70:1134–1139.
51. Parfitt AM. The two faces of growth: benefits and risks to bone integrity. *Osteoporos Int*. 1994; 4:382–398. [PubMed: 7696836]
52. Heaney RP, Weaver CM. Newer perspectives on calcium nutrition and bone quality. *J Am Coll Nutr*. 2005; 24:574S–581S. [PubMed: 16373957]
53. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinoshita M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor

and is identical to TRANCE/RANKL. *Proc Natl Acad Sci U S A*. 1998; 95:3597–3602. [PubMed: 9520411]

54. Seminara S, Rapisardi G, La Cauza F, Mattei P, Donzelli G. Catch-up growth in short-at-birth NICU graduates. *Horm Res*. 2000; 53:139–143. [PubMed: 11044794]
55. van der Steen M, Hokken-Koelega AC. Growth and Metabolism in Children Born Small for Gestational Age. *Endocrinol Metab Clin North Am*. 2016; 45:283–294. [PubMed: 27241965]

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Figure 1. Effect of THB on osteogenic capacity of hMSCs

Panel A: Flow cytometric profiling of surface markers on one of the hMSC preparations used in the study. The horizontal line on the plots represents gating of positively stained cells as defined by isotype controls. **Panel B–D:** *In vitro* osteogenic (**panel B**), adipogenic (**panel C**) and chondrogenic (**panel D**) differentiation of a representative hMSC preparation using classical culture conditions. Osteogenic monolayers are stained with the calcium binding dye alizarin red S, adipogenic monolayers are stained with the lipid binding dye oil red O and histological sections of chondrogenic micromasses are stained with toluidine blue, resulting in purple coloration in the presence of cartilage. For the osteogenic and adipogenic

assays, control experiments in the absence of differentiation factors (control media) are presented.

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Figure 2. Effect of THB on osteogenic capacity of hMSCs

Panel A: Colorimetric ALP assays on intact monolayers demonstrate a dose-dependent upregulation of activity in the presence of THB. **Panel B:** ELISA for OPG in culture media demonstrates a dose-dependent upregulation of OPG secretion in the presence of THB.

Panel C: Cell recoveries from THB treated cultures. **Panel D:** Monolayers were treated for 8 days in the presence of OBM containing THB, then transferred to THB-containing OMM for 14 additional days. High doses of THB accelerate mineralization when detected by ARS staining at 7 and 14 days. **Panel E:** Densitometry profiles of the stained monolayers are presented below each plate, internally normalized to intensity of untreated controls. **Panel F:** Micrographs of control (*above*) and 50 μM THB-treated monolayers (*below*) after 7 days

after ARS staining. Fifty- μ M THB treatment results in generation of raised, calcium rich-nodules. **Statistics for panel B–E:** Data presented as means and standard deviations (n=3) tested by one-sided ANOVA and Dunnett's or Tukey's post-test. Comparisons are between the vehicle and THB doses, $p<0.05$ *, $p<0.01$ **, $p<0.005$ ***.

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Figure 3. Effect of exposure to THB on cranial growth at birth and on body-masses of offspring throughout lactation

Panel A: Scheme describing the experimental timeline. **Panel B:** The volume of skulls from new-born male pups as measured by μ CT. **Panel C:** The cranial diameter at the midpoint between the anterior and posterior cranial extremity in new-born male pups as measured by μ CT. **Panel D:** Mean humerus length. **Panel E:** Neonatal body mass. For box and whisker plots in Panels B–E, boxes represent 25–75 percentile, horizontal line represents median, plus sign represents mean and error bars represent range of data. **Panel F:** Post-natal body

mass of offspring. Data presented as means and standard deviations (n=20–25) with Student's t-tests between treated and appropriate control group, $p<0.005$ ***.

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Figure 4. Effect of exposure to THB on trabecular parameters of the proximal tibia in 50 day-old offspring

Panel A: Trabecular thickness. **Panel B:** Trabecular spacing. **Panel C:** Trabecular number. **Panel D:** Trabecular surface area. **Panel E:** Representative axial image of trabecular structures in control and THB treated tibiae. While the thickness of trabecular structures are equivalent, THB treated tibiae possess a greater frequency. **Panel F:** Depth of trabecular structures presented as a fraction of the entire length of the bone. **Panel G:** Representative longitudinal image of trabecular structures in control and THB treated tibiae. For Panel A–D

and F, data are presented as box and whisker plots (n=5–6). P values are calculated by Student's t-test (D–F) or Mann-Whitney test (B,C).

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Figure 5. Effect of exposure to THB on femoral length, cortical thickness and biomechanical strength in 50 day old offspring

Panel A: Femoral length. **Panel B:** Average cortical thickness at midpoint of diaphysis.

Panel C: Polar moment of inertia at midpoint of diaphysis. **Panel D:** Energy required to break. **Panel E:** Energy required until yield. Data are presented as box and whisker plots (n=10). P values are calculated by Student's t-test.