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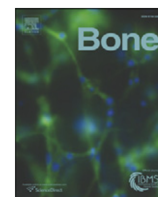

Original Full Length Article

# Pregnant growth restricted female rats have bone gains during late gestation which contributes to second generation adolescent and adult offspring having normal bone health

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ABSTRACT

Low birth weight, due to uteroplacental insufficiency, results in programmed bone deficits in the first generation (F1). These deficits may be passed onto subsequent generations. We characterized the effects of being born small on maternal bone health during pregnancy; and aimed to characterize the contribution of the maternal environment and germ line effects to bone health in F2 offspring from mothers born small.

Bilateral uterine vessel ligation (or sham) surgery was performed on female F0 WKY rats on gestational day 18 (term 22 days) to induce uteroplacental insufficiency and fetal growth restriction. Control and Restricted F1 female offspring were allocated to a non-pregnant or pregnant group. To generate F2 offspring, F1 females were allocated to either non-embryo or embryo transfer groups. Embryo transfer was performed on gestational day 1, where second generation (F2) embryos were gestated (donor-in-recipient) in either a Control (Control-in-Control, Restricted-in-Control) or Restricted (Control-in-Restricted, Restricted-in-Restricted) mother.

Restricted F1 females were born 10–15% lighter than Controls. Restricted non-pregnant females had shorter femurs, reduced trabecular and cortical bone mineral contents, trabecular density and bone geometry measures determined by peripheral quantitative computed tomography (pQCT) compared to non-pregnant Controls. Pregnancy restored the bone deficits that were present in F1 Restricted females.

F2 non-embryo transfer male and female offspring were born of normal weight, while F2 embryo transfer males and females gestated in a Control mother (Control-in-Control, Restricted-in-Control) were heavier at birth compared to offspring gestated in a Restricted mother (Restricted-in-Restricted, Control-in-Restricted). Male F2 Restricted embryo groups (Restricted-in-Control and Restricted-in-Restricted) had accelerated postnatal growth. There was no transmission of bone deficits present at 35 days or 6 months in F2 offspring. Embryo transfer procedure had confounding effects preventing the separation of maternal environment and germ line contribution to outcomes.

Deficits present in F1 non-pregnant Restricted females were absent during late gestation, indicating that pregnant F1 Restricted females experienced gains in bone. These beneficial maternal pregnancy adaptations may have prevented transmission of bone deficits to F2 offspring.

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## Introduction

It is well established that insults during life *in utero* can lead to low birth weight offspring who go on to develop a number of adult metabolic and cardiovascular diseases [1,2]. In addition, low weights at birth and at one year of age have been linked to a range of bone deficits in early and late adulthood, including deficiencies in bone mineral content (BMC), bending strength, and decreased femur length [3–5]. Recently, pQCT measurements of these bone features were shown to correlate with increased fracture risk in low birth weight individuals who had been recruited in an early study in the late 1980s [6].

Uteroplacental insufficiency affects 10% of pregnancies in the Western society [7]. Experimentally, this can be induced by bilateral uterine vessel (artery and vein) ligation in rats resulting in fetal growth restriction and low birth weight [8]. Using this approach, we and others have demonstrated that growth restricted male and female rat offspring have decreased total body, trabecular, and cortical BMC, and femurs that are

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shorter with reduced periosteal and endosteal circumferences and decreased bending strength [9–11]. We subsequently reported that these programmed bone deficits were exacerbated with aging [12]. These studies also highlighted that growth restricted males had more severe bone outcomes compared to females [11,12]. Furthermore, improved maternal calcium delivery during lactation [11] and calcium supplementation from 2 to 6 months of age [10] were not able to rescue these phenotypes.

Debate remains over the exact changes that occur to the maternal skeleton during pregnancy. Some have reported that there is no bone loss nor increased turnover [13,14], while others report significant losses of bone mineral density (BMD) at the proximal femur [15] and lumbar spine, with BMD remaining decreased during the first 6 weeks postpartum [16]. In normal human pregnancy, the maternal skeleton may change in a biphasic manner. In the first two trimesters, markers of bone resorption can be increased, while in the third trimester, markers of formation can be elevated [16,17]. Black and colleagues suggested that maternal bone resorption in the first and second trimester coincides with development of the fetal skeleton and thus a requirement for calcium, which is thought to accumulate in the range of 30–35 g in the fetus [16]. Human studies usually make inference to bone health using blood and urine markers of formation and resorption. While imaging technologies such as dual-energy X-ray absorptiometry (DEXA) and pQCT are useful tools to directly assess bone physiology, the emission of radiation limits their use during pregnancy. There is little known regarding normal bone changes throughout pregnancy in rodents. Using DEXA and pQCT, we have recently reported that healthy pregnant rats experience periods of bone loss, rather than gains, during late gestation while those that underwent surgically-induced uteroplacental insufficiency did not [18]. This indicates that, in rats, late pregnancy may be a critical period of fetal bone formation and that mothers whose pregnancies were compromised by uteroplacental insufficiency failed to provide sufficient amounts of calcium to their developing pups. Indeed, this may explain the deficits in adult bone health in the F1 offspring who were born small [10–12]. Of relevance to the present study, it is not known how F1 females with bone deficits adapt to their own pregnancy and the consequences for skeletal development in the F2.

With regard to metabolic and cardiovascular health, we have reported that F2 offspring born to mothers of low birth weight have reduced nephron number in fetal life, blunted first-phase insulin response and gender-specific alterations to pancreatic morphology and blood pressure [19,20]. Mechanisms for transmission of programmed diseases, in the absence of a subsequent adverse exposure, have been described previously [7]. Briefly, this may occur *via* the germ line when a pregnant female (initial generation F0) is exposed to an insult and the germ cells of the developing F1 embryo are also directly exposed (multigenerational effects), and/or there are epigenetic changes in the F1 that are transmitted to the F2 (intergenerational effects) [7]. Alternatively, the F1 female may experience inadequate pregnancy adaptations that impact on the F2 as a *de novo* insult, which occurs regardless of, or in addition to, germ line effects [7].

The aims of this study were to characterize the bone phenotype of pregnant F1 females who were born growth restricted as a result of uteroplacental insufficiency, and to determine whether bone deficits were transmitted to F2 offspring. In an attempt to discern the mode of transmission of bone deficits to the F2, embryo transfer techniques were employed. It was hypothesized that growth restricted pregnant females would have adverse skeletal adaptations during pregnancy and that, *via* the germ line and/or poor maternal adaptations, her F2 offspring would exhibit adverse bone outcomes.

## Materials and methods

### *Animals*

Prior to commencement of animal experimental work, approval was granted by The University of Melbourne Animal Ethics Committee complying with the accepted standard of animal care (AEEC number 0707367.4). Wistar–Kyoto rats from at least 9 weeks of age were housed in an environmentally-controlled room (22 °C) with a 12 h light–dark cycle and provided with access to standard rat chow (0.46% calcium; consumed by all generations) and tap water *ad libitum*. Female (F0, initial generation) rats were mated with normal males and underwent either uteroplacental insufficiency (offspring termed Restricted) or sham (offspring termed Control) surgery on day 18 of gestation [8,21–23]. The F0 females delivered naturally at term on gestational day 22. As previously published uteroplacental insufficiency reduced the number of viable pups in the F1 generation but not the F2 generation; with no differences in sex ratios [24,25]. Litter size was not standardized or reduced in any generation due to the known affects that this has on maternal mammary gland morphology and milk quality and quantity [22,26,27]. Birth weights of F1 offspring were recorded and pups remained with their mothers until weaning at postnatal day 35 (PN35). Weaning was performed at PN35 to ensure that natural weaning of both Control and Restricted pups had occurred. At 12 weeks of age, F1 Control and Restricted females were randomly allocated to either a pregnant or non-pregnant group. Females allocated to the pregnancy group were mated with normal males and underwent post-mortem on gestational day 20. The same post-mortem protocol was carried out in aged-matched non-pregnant females when in estrous. An additional cohort of F1 females (Control and Restricted) were mated with either normal or vasectomized males with embryo transfer performed on gestational day 1. We have previously published that at 4 months of age the pregnancy success rate was 100% for both Control and Restricted F1 females [28]. Furthermore, mating age (18.9 to 21.7 weeks) was not different between groups consistent with previously published work [25]. Non-embryo transfer F2 offspring were simultaneously generated using F1 Control and Restricted females. We have previously reported that embryo transfer resulted in reduced F2 litter size when compared to non-embryo transfer offspring [25].

### *Embryo transfer procedure*

Embryo transfer was performed as previously published [25]. Briefly, Control and Restricted F1 females in pro-estrous were randomly allocated to one of two groups, embryo donor or embryo recipient. Embryo donor females were mated with control males while embryo recipients were mated with vasectomized males (to elicit pseudo-pregnancy) simultaneously. Day 1 of pregnancy in donor females was confirmed by the presence of sperm in a vaginal smear. An intraperitoneal injection of Ketamine (Parnell Laboratories, Alexandria, NSW, Australia; 100 mg·kg$^{-1}$ body weight) and Ilium Xylazil-20 (Troy Laboratories, Smithfield, NSW, Australia; 30 mg·kg$^{-1}$ body weight) was administered to euthanize donor females allowing for embryos to be harvested from the oviducts. Both oviducts were exposed to allow embryos to be flushed out from surrounding cumulus cells. To ensure viability, embryos were placed into a 50 μl drop of culture medium (GMOPS, Vitrolife AB, Goteborg, Sweden) and kept on a warming plate, at 37 °C, for 20 min. The recipient females were anesthetized with isoflurane. By inserting a glass pipette into the ampular opening, the recovered embryos were transferred into one oviduct. No more than 12 embryos were transferred into an oviduct of a recipient female. Recipient females were housed individually with minimal handling and delivered naturally at term on day 22, with pups remaining with their mother until weaning at PN35. As a result of embryo transfer, 4 groups were generated (donor-in-recipient): Control-in-Control, Control-in-Restricted, Restricted-in-Control and Restricted-in-Restricted. In addition, there were two F2 non-embryo transfer groups (termed F2 Control and Restricted) which were used as controls for embryo transfer. Male and female offspring from the 6 abovementioned groups were studied at PN35 and 6 months of age (1 male and 1 female per litter allocated to each study age; $n = 8$–15 per group).

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