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The modified amino sugar N-Butyryl Glucosamine fed to ovariectomized rats preserves bone mineral through increased early mineralization, but does not affect body composition

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ABSTRACT

Background: The toxicities of pharmaceuticals for chronic arthritis and osteoporosis should be of concern to consumers. This partially accounts for the popularity of consumption of the amino sugar glucosamine, in-spite of controversy about its efficacy. We chemically synthesized N-butyryl glucosamine (GlcNBu), which we discovered protected bone and cartilage in an inflammatory arthritis rat model when used as a feed supplement. GlcNBu can also be potentially synthesized biochemically, since we recently demonstrated that human acetyl-CoA: glucosamine-6-phosphate N-acetyltransferase 1 has a relaxed donor specificity and transfers acyl groups of up to four carbons in length, i.e. the butyryl moiety. Oral GlcNBu had no detectable toxicity and also protected against bone loss in ovariectomized (OVX) rats as a model for osteoporosis. However, we demonstrated this only for bones excised at 6 months. Thus, the current study aims to determine when bone mineralization is maximized during daily GlcNBu supplementation, in both OVX and Sham-OVX rats, in addition to the relationship of bone mineralization to body composition.

Methods: Female Sprague-Dawley rats were randomized into 4 groups, containing 8 rats each. The groups consisted of OVX or Sham-OVX rats whose diets were supplemented with either 200 mg/kg/day of GlcNBu or an equimolar amount of glucose. We performed sequential bone

density and body composition measurements, by dual-energy X-ray absorptiometry in the live, anesthetised rats, over a 6-month experimental period, starting at the age of 8 weeks. Results were analyzed by descriptive statistics and 2-way ANOVA.

Results: The major increases in the mineral content and density of the spine and the femur in GlcNBu-supplemented rats occurred early, from the baseline to week 8. Ovariectomy resulted in a number of significant differences in body composition, while feeding GlcNBu had no significant effects on body composition. The significant effects of ovariectomy on body composition initially appeared at 8 weeks, while the GlcNBu effects on increased bone mineral initially appeared at 2 weeks. An interaction between OVX and GlcNBu was seen only at 16 weeks for the bone mineral density of the femoral head.

Conclusions: Supplementation of the diet by GlcNBu in both OVX and Sham-OVX rats increases bone mineral as early as 2 weeks. Ovariectomy but not GlcNBu supplementation had a significant effect on body composition. The effect of GlcNBu occurs independently of changes in body composition, probably as a direct effect of stimulation of bone matrix synthesis which continues to be mineralized. This work represents an important step in the development and commercialization of GlcNBu for the prevention and treatment osteoporosis, where there is now an increasing demand for safe, long term agents.

Keywords: osteoporosis, ovariectomy, N-butyryl glucosamine, bone, mineralization, body composition, dual-energy X-ray absorptiometry

BACKGROUND

Glucosamine (GlcN) in foods and feeds is a component of glycoproteins, proteoglycans, glycolipids, and long-chain polymers such as chitosan. GlcN in the glycoconjugates of animal tissues is almost invariably N-acetylated by glycosyltransferases [1]. The reason for the N-acetylation of GlcN and amino sugars in general in glycoconjugates is not fully understood; however, N-acetylation in chitosan solvation is affected by the electrostatic forces resulting from the degree of acetylation [2].

There is an increasing demand for long term, safe, and naturally occurring functional components of foods for the treatment of chronic arthritis and in the prevention and treatment of osteoporosis. This demand, which is driven mostly by safety concerns about the long-term administration of drugs, has led to the extensive use of GlcN as an oral supplement, mostly for joint and bone conditions. However, scientific evidence for the efficacy of GlcN in these conditions remains unconvincing (see Discussion). GlcN is commercially derived mostly from chitin, which is the most abundant polymer in the animal kingdom. Deacetylation of the insoluble chitin yields partially soluble chitosan, which is hydrolyzed to produce GlcN, primarily as HCl or $S0_4$ salts, which are widely available “over the counter” in North America or often by

prescription in Europe. Chitosan also has diverse biomedical and tissue engineering applications [3].

We demonstrated that the chemically modified glucosamine, N-butyryl glucosamine (GlcNBu), increased type II collagen and aggrecan gene expression in mouse chondrocytes when compared to other N-acylated GlcNs [4]. Pre-treatment with GlcNBu but not with N-acetyl GlcN protected chondrocyte matrix from the decrease of type II collagen mRNA initiated by tumour necrosis factor-α [4]. Additionally, we demonstrated that supplementation by GlcNBu of bovine anchorage-dependant cultures resulted in modest stimulation of cell proliferation and proteoglycan synthesis, while the addition of GlcN resulted in inhibition, seen particularly at high concentrations [5]. Furthermore, supplementation of human chondrocyte cultures by GlcNBu but not GlcN upregulated the expression of a large number of genes [5]. GlcNBu has apparently very low toxicity and is readily accepted by animals. In terms of animal models, we initially observed in an experimental model of inflammatory arthritis in the rat that oral GlcNBu preserved both bone and cartilage, as discussed below. We then reported [6] that daily supplementation of 200 mg/kg/day of GlcNBu of the standard feed of ovariectomized (OVX) rats preserved bone mineral and some biomechanical properties of the spine and femurs. These observations were made at the end of a six-month experimental period and reached significance only in the OVX group fed GlcNBu. We used relatively young rats, weighing approximately 180 g at the start of the experiment, when the daily supplementation of the OVX or Sham-OVX groups with GlcNBu or glucose (Glc) commenced. Additionally, we found that femurs are longer in GlcNBu-fed rats at the end of the six-month period, whether or not they were OVX [6]. Furthermore, the relationship between BMC, BMD, and body composition components, measured by DXA was not determined. These are key issues in the long-term goal of using GlcNBu in novel preventive in addition to treatment strategies in osteoporosis.

GlcNBu is highly water soluble and we developed a high performance liquid chromatography method for determination in plasma with GlcNBu, without a chromophore [7]. Based on this method, pharmacokinetic studies in the rat showed that GlcNBu has rapid but low absorption and is widely distributed and efficiently cleared [8]. The gut rather than liver is mainly responsible for the low bioavailability of GlcNBu. Limited absorption of GlcNBu suggests a transport dependent absorption. Food does not significantly affect the bioavailability of GlcNBu [8]. GlcNBu is very stable and we found no degradation as determined by NMR over 5 years of storage at 8 ℃.

In our previous work [6], the animals were sacrificed at 6 months; it was not clear at what time frame the increases in bone mineral content (BMC) and bone mineral density (BMD), as measured by dual-energy X-ray absorptiometry (DXA), initially occurred. Furthermore, the relationship between BMC, BMD and body composition components, measured by DXA, was not determined. In this study, we exploited sequential DXA measurements for the test compound, N-butyryl glucosamine (GlcNBu), fed to immature rats (entering our facility at 8 weeks of age). We report on the sequentially-determined outputs of BMC and BMD of the spine and femur and on the body composition measurements of Sham-OVX or OVX live rats, during

the once-a-day feeding of either GlcNBu or an equimolar amount of glucose (Glc), over a 20-week period. Since mineralization would presumably follow new bone matrix synthesis during growth, we speculated that by starting the feeding of the GlcNBu and the DXA measurements relatively early in growth, we might more readily observe differential effects on bone mineral of the spine and femurs.

Other publications have reviewed the utility of the OVX rat for drug discovery, with an emphasis on the bone-site specific characteristics and on bone modelling or remodeling [9], in addition to the technical aspects of the OVX rat as a model for oestrogen deficiency in osteoporosis research [10]. The effect of OVX on the body composition and bone density of the mature rat by sequential DXA measurements has been communicated [11]. Additionally, it is known that in the rat (Sham-OVX) the vertebrae grow faster than the long bones from 6-20 weeks, and mineralization is significantly higher at 3 and 4 weeks in the vertebrae, peaking at 8 weeks for long bones and at 10 weeks for vertebrae [12]. In our present work, we also examine correlations between body composition and mineralization of the spine and femur in order to determine whether GlcNBu acts through changes in body composition or directly on bone in the OVX and Sham-OVX rat.

METHODS

Animals

Virginal female Sprague-Dawley rats, which were either OVX or not and 8 weeks old (mean weight 175 g), were purchased from Charles River Laboratories. After the ovariecomy and sham OVX animals were randomized into the 4 groups, with 8 animals in each group. The 4 groups were: (1) Sham-OVX, Glc-fed, (2) OVX, Glc-fed, (3) Sham-OVX, GlcNBu-fed, and (4) OVX, GlcNBu-fed. All rats were allowed access to identical feed (rat laboratory chow, Purina 5012), which together with water was permitted *ad libitum.* After accommodation for 5 days, supplementation of the feed with either GlcNBu or glucose (Glc) began. The supplementation consisted of either 200 mg/kg/day GlcNBu or an equimolar amount of Glc, once a day for 6 months, starting at the end of the period of accommodation (day 5). GlcNBu or Glc were administered in approximately 1 g of peanut butter, which was preferentially consumed by the rats generally before their regular Purina rat chow. At that point the rats' mean weight was 177 g for the sham-OVX group and 184 g for the OVX group. The feeding experiment was conducted at the Queen's University Animal Care Facility. The animals were housed at an ambient temperature of 22 ± 1°C, humidity of 50 ± 10%, and a 12 h light/dark cycle for the duration of the experiment. All animal treatments were strictly in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The experimental procedures were approved by the Committee on Animal Care and Use at Queen's University.

Bone Mineral Density and Content

Bone mineral density (BMD) and bone mineral content (BMC) were measured in live animals under anesthesia using a Hologic 4500 dual-energy X-ray absorptiometry machine with a small-animal adapter. The rats were anaesthetized by injecting IP ketamine/xylazine ((75/10)mg/kg).

The anesthetic technique was tested and adapted to our DXA facility using extra rats. The BMD and BMC were then measured at 2, 8, 16, and 20 weeks after commencing the GlcNBu feeding. The number of rats studied was less than 8 at some of the later time points (6 or 7 live animals) because of rat losses during anesthesia. The BMC and BMD were measured for the global spine (L1-5), global femur, and head of femur. The initial scans from each animal were compared to follow-up scans to ensure consistency of positioning.

Body Composition

Body global mass and body global fat were measured in live animals under anesthesia using a Hologic 4500 with a small-animal adapter. The body global mass and body global fat were determined at 2, 8, 16, and 20 weeks after the commencement of GlcNBu feeding. The body global BMC and body global BMC+lean were measured using the Hologic 4500 at 2, 8, 16, and 20 weeks after commencing the GlcNBu feeding. The body global lean was then calculated by subtracting the body global BMC from the body global BMC+lean.

Statistical Analysis

We analyzed data from four groups of live animals: (i) the BMC and BMD in the spine and femur in the Sham-OVX and OVX groups, in rats not fed GlcNBu; (ii) the body composition of the Sham-OVX and OVX groups, in rats not fed GlcNBu; (iii) the BMC and BMD of the spine and femur in the Sham-OVX and OVX groups, in rats fed GlcNBu; (iv) the body composition of the Sham-OVX and OVX, in rats fed GlcNBu. Data were analyzed using IBM SPSS Statistics Version 22. The effect of growth on BMD, BMC, and body composition for each rat was analyzed using paired t-tests. This was preferred over repeated ANOVA as the changes between each point in time were of greater interest than an overall assessment of change. All significant differences in BMD, BMC, and body composition determined using paired t-tests were confirmed with the Sham-parametric Wilcoxon test for small sample sizes. The effect of ovariectomy on BMD, BMC, and body composition was analyzed using unpaired Student t-tests. Results of the unpaired student t-tests were confirmed with the Sham-parametric Mann-Whitney U test due to the relatively small sample.

The effect of both ovariectomy and GlcNBu, on BMD, BMC, and body composition was analyzed using 2-way ANOVA. In the 2-way ANOVA, the first factor was ovariectomy and the second GlcNBu. No adjustment was made for multiple comparisons and $p<0.05$ was used as the criterion for statistical significance.

RESULTS

Effects of growth on the BMC and BMD of the spines and femurs of Sham-OVX and OVX rats, not fed with GlcNBu (fed equimolar amount of Glc)

The results in this section are presented first in Graphs to visually illustrate the type of data generated (in Figures 1a, 1b and 2a, 2b). This is followed by a description of the Results and presentation of the numerical values and statistical significance of the data in Tables 1A, 1B and 2A, 2B. In subsequent sections only the Tables are shown.

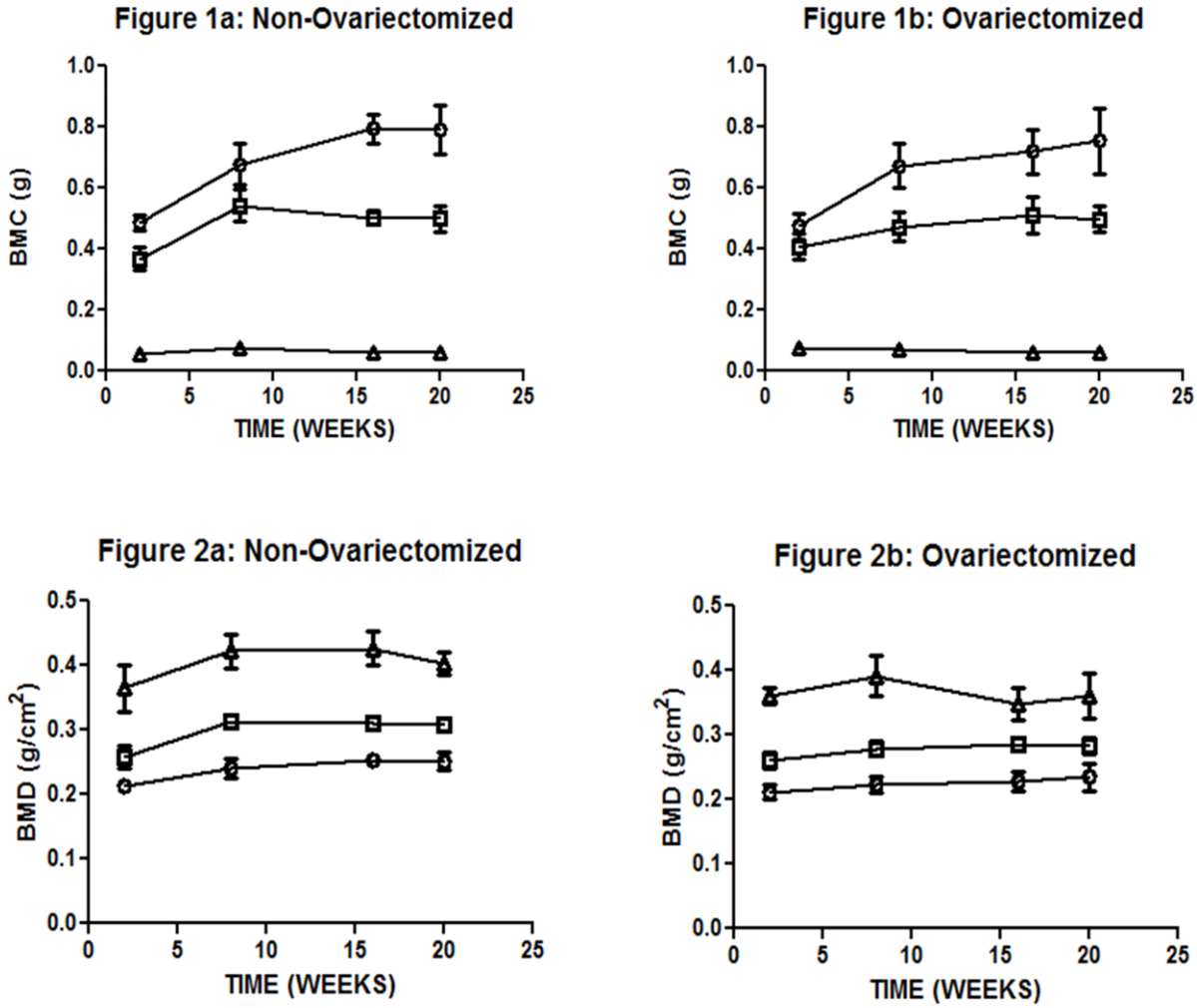


Figure 1 and 2. The Bone Mineral Content (BMC) and Bone Mineral Density (BMD) were measured in the total spine (○), total femur (□) and head of the femur (Δ) of sham-ovariectomized (shown in the Figs as "non-ovarectomized") and ovariectomized rats. Please see "Discussion" for the relative usefulness of the BMC and BMD in the growing rat.

Global spine BMC *(Table 1A)*

The BMC of the spine increased for the Sham-OVX rats from 2-16 weeks, while for the OVX rats the increase appears to continue until 20 weeks. When the BMC measured at each of the time points of this growth period are compared to the base line of week 2, the values are significantly greater.

Global Femur BMC *(Table 1A)*

For the BMC of the global femur for the Sham-OVX rats there is an increase from 2-8 weeks, while for the OVX rats it appears to continue to 16 weeks. When the BMC measured at each of the time points of this growth period are compared to the base line, week 2, the values are

significantly greater ($p<0.01$). The BMC at 8 weeks is significantly greater for Sham-OVX than OVX rats ($p<0.05$).

Head of femur BMC *(Table 1A)*

For the head of the femur, similarly to the global femur, the BMC for the Sham-OVX rats increases from 2-8 weeks. However, for OVX rats, the BMC of this region decreases over this same time period time. The BMC for both the Sham-OVX and OVX rats decreases from 8-16 weeks and is unchanged from week 16-20. When the BMC measured at 2 and 8 weeks are compared for the Sham-OVX rats, the small increase at 8 weeks is significantly greater ($p<0.01$). When the BMC measured at 8 and 16 weeks are compared for the Sham-OVX rats, the value at 8 weeks is greater than that at 16 weeks ($p<0.01$). The BMC of the OVX rats measured at 8, 16, and 20 weeks, compared to the base line of week 2, does not significantly change. The BMC at 2 weeks is greater for Sham-OVX than OVX rats ($p<0.05$).

Global spine BMD *(Table 1B)*

The BMD of the global spine for both the sham-OVX and OVX rats maximizes at 16 weeks and remains maximal at 20 weeks. When the BMD measured at each of the time points of this growth period are compared to the base line of week 2, the values are significantly greater ($p<0.01$ for Sham-OVX and $p<0.05$ for OVX). However, for the Sham-OVX the BMD between weeks 16 and 20 does not change and for the OVX rats, the increase between weeks 16 and 20 is not significant. The BMD values at the 8, 16, and 20 time points for the Sham-OVX rats are higher than for the OVX rats, although statistical significance was reached only at 16 weeks ($p<0.01$).

Global femur BMD *(Table 1B)*

For the BMD of the global femur for the Sham-OVX rats there is an increase from 2-8 weeks, while for the OVX rats it appears to continue to 16 weeks. When the BMD measured at each of the time points of this growth period are compared to the base line, week 2, the values are greater ($p<0.001$ for Sham-OVX and $p<0.01$, for OVX). The BMD at 8, 16, and 20 weeks is greater for Sham-OVX than OVX rats ($p<0.001$, $p<0.05$ and $p<0.01$), respectively.

Head of femur BMD *(Table 1B)*

For the BMD of the Sham-OVX rats there was an increase from 2-16 weeks, while for the OVX rats it only increases to week 8. When the BMD measured at these time points are compared to the base line of week 2, the values are greater ($p<0.05$). The BMD for OVX rats decreases from 8-16 weeks and increases slightly from week 16-20. The decrease in OVX rats BMD from week 8 to 16 is significant ($p<0.05$). The BMD at 16 weeks is highly significant and higher for Sham-OVX than OVX rats ($p<0.001$).

Table 1. BMC (A) and BMD (B), for Glc-fed rats, at different times or treatments for the global spine, global femur and head of femur. Paired t-tests are done at each time point for each bone and each treatment (Sham-ovariectomized (SHAM-OVX) and ovariectomized (OVX) groups) versus 2-week time point and also preceding time point. Unpaired t-tests are done at each time point and bone type for OVX versus-OVX.

(A) Bone Mineral Content (g) for Glc-Fed Rats						
Time	**Global Spine**		**Global Femur**		**Head of Femur**	
(w)	**SHAM-OVX**	**OVX**	**SHAM-OVX**	**OVX**	**SHAM-OVX**	**OVX**
2	0.49±0.03*	0.47±0.04	0.37±0.04	0.41±0.04	0.06±0.01$^{\alpha}$	0.07±0.01
8	0.68±0.07^{b}	0.67±0.07^{c}	0.54±0.05$^{b,\alpha}$	0.47±0.05^{b}	0.07±0.01^{b}	0.07±0.01
16	0.79±0.05^{c,iii}	0.72±0.07^{c,iii}	0.50±0.02^{b}	0.51±0.06^{b}	0.06±0.01^{ii}	0.06±0.02
20	0.79±0.08^{c}	0.75±0.11^{b}	0.50±0.04^{b}	0.50±0.04^{b}	0.06±0.01	0.06±0.01

(B) Bone Mineral Density (g/cm^2) for Glc-Fed Rats						
Time	**Global Spine**		**Global Femur**		**Head of Femur**	
(w)	**SHAM-OVX**	**OVX**	**SHAM-OVX**	**OVX**	**SHAM-OVX**	**OVX**
2	0.21±0.01	0.21±0.01	0.26±0.02	0.26±0.01	0.37±0.04	0.36±0.01
8	0.24±0.01^{b}	0.22±0.01^{a}	0.31±0.01$^{c,\chi}$	0.28±0.01^{b}	0.42±0.02^{a}	0.39±0.03^{a}
16	0.25±0.01$^{c,i,\beta}$	0.23±0.01^{a,i}	0.31±0.01$^{c,\alpha}$	0.29±0.01^{b,i}	0.43±0.03^{a}	0.35±0.03$^{i,\chi}$
20	0.25±0.01^{b}	0.23±0.02^{a}	0.31±0.01$^{c,\beta}$	0.28±0.01^{b}	0.40±0.02	0.36±0.04

*Mean±SD.
Paired t-test versus 2w: $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$.
Paired t-test versus preceding time point: $^{i}p<0.05$, $^{ii}p<0.01$, $^{iii}p<0.001$.
Unpaired t-tests: $^{\alpha}p<0.05$, $^{\beta}p<0.01$, $^{\chi}p<0.001$.

Effects of growth on the body composition of Sham-OVX and OVX rats, not fed with GlcNBu (fed equimolar amounts of Glc).

Body global mass *(Table 2)*

The body global mass of both Sham-OVX and OVX rats increased over this 20-week experimental period experimental period. When the body global mass measured at each of the time points of this growth period are compared to week 2, the values are greater. For the Sham-OVX rats this difference is highly significant ($p<0.001$) at week 8 and very significant at weeks 16 and 20 ($p<0.01$). For OVX rats the difference is highly significant ($p<0.001$) at all time points. The body global mass measured at weeks 8 and 16 is greater for OVX than Sham-OVX rats ($p<0.01$).

Body global lean *(Table 2)*

The body global lean increased from weeks 2 to 20 for the Sham-OVX rats and only until 16 weeks for the OVX rats. When the body global lean measured at each of the time points of this growth period are compared to week 2, the values are greater ($p<0.001$). The body global lean values are greater for OVX than Sham-OVX rats and reach significance ($p<0.01$) only for the 8-week time point. However, body global lean significantly increases compared to week 2 in both OVX and sham-OVX at all subsequent time points.

Body global fat *(Table 2)*

The body global fat of both Sham-OVX and OVX rats increased over this 20-week growth period. When the body global fat measured at each of the time points of this growth period is compared to week 2, the values are greater, at week 8 $p<0.001$, and at weeks 16 and 20 $p<0.01$. For OVX rats, the difference is highly significant ($p<0.001$) at all time points. The body global fat measured at weeks 8 and 16 is greater for OVX than Sham-OVX rats ($p<0.05$).

Table 2. Body composition (body global fat, body global mass, and body global lean) for Glc-fed rats, at different times or treatments (OVX and SHAM-OVX groups). Paired t-tests are done at each time point for each treatment versus 2-week time point and also preceding time point. Unpaired t-tests are done at each time point for OVX versus SHAM-OVX.

	Body Composition (g) for Glc-Fed Rats					
Time	**Body Global Fat**		**Body Global Mass**		**Body Global Lean**	
(w)	**SHAM-OVX**	**OVX**	**SHAM-OVX**	**OVX**	**SHAM-OVX**	**OVX**
2	33.17±9.20*	33.16±7.67	279.03±10.79	279.21±8.38	237.30±15.18	237.12±12.21
8	61.80±6.70$^{c,\alpha}$	80.90±17.18^{c}	340.37±20.60$^{c,\beta}$	383.15±17.23^{c}	268.11±23.70$^{c,\alpha}$	291.46±6.74^{c}
16	69.18±15.91$^{b,\alpha}$	97.66±23.65^{c,ii}	351.70±30.70$^{b,\beta}$	402.16±22.72^{c,ii}	271.52±22.67^{c,i}	292.87±5.17^{c}
20	74.38±21.94^{b,i}	109.56±28.92^{c,i}	372.86±40.52^{b,ii}	413.93±36.26^{c,i}	286.86±22.80^{c,iii}	292.28±12.88^{c}

*Mean±SD.
Paired t-test versus 2w: $^{b}p<0.01$, $^{c}p<0.001$.
Paired t-test versus preceding time point: $^{i}p<0.05$, $^{ii}p<0.01$, $^{iii}p<0.001$.
Unpaired t-tests: $^{\alpha}p<0.05$, $^{\beta}p<0.01$.

Effects of growth on the BMC and BMD of the spines and femurs of Sham-OVX and OVX rats fed GlcNBu.

Global spine BMC *(Table 3A)*

The BMC of the spine increases for the Sham-OVX and OVX GlcNBu-fed rats from 2-16 weeks. For the Sham-OVX, GlcNBu-fed rats, when the BMC measured at each of the time points of this growth period are compared to week 2, the values are significantly greater ($p<0.001$). For the OVX, GlcNBu-fed rats the values at 8 and 16 weeks are significantly greater than that at 2 weeks ($p<0.001$ and $p<0.01$ respectively).

Global femur BMC *(Table 3A)*

The BMC of the global femur increases for the Sham-OVX and OVX GlcNBu-fed rats from 2-16 weeks. The value measured at 20 weeks is significantly greater ($p<0.05$) than that at 2 weeks for the Sham-OVX, GlcNBu-fed rats. For the OVX, GlcNBu-fed rats the values at 16 and 20 weeks are significantly greater ($p<0.001$ and $p<0.01$ respectively) than that at 2 weeks.

Head of femur BMC *(Table 3A)*

At 8 weeks, ovariectomy has a significant effect on the BMC of the global femur and the head of femur (Table 3). There is no significant change in the BMC of head of femur with time for the Sham-OVX and OVX GlcNBu-fed rats. ***Global spine BMD*** *(Table 3B)*

The BMD of the spine increases for the Sham-OVX and OVX GlcNBu-fed rats from 2-8 weeks. For the Sham-OVX, GlcNBu-fed rats, when the BMD measured at weeks 8, 16, and 20 are compared to the base line, week 2, the values are significantly greater ($P<0.001$, $p<0.01$, and $p<0.05$ respectively). Only the 8-week time point BMD is significantly different to the 2-week time point for the OVX, GlcNBu-fed rats ($p<0.001$).

Global femur BMD *(Table 3B)*

The BMD of the femur increased for the Sham-OVX, GlcNBu-fed rats from week 2-20 and for the OVX, GlcNBu-fed rats until week 16. When the BMD measured at each of the time points of this growth period are compared to week 2, the values are greater. For the Sham-OVX, GlcNBu-fed rats the BMD value is significantly greater ($p<0.01$) at 8 weeks, in addition to at weeks 16 and 20 ($p<0.05$ for both). For the OVX, GlcNBu-fed rats the BMD value is significantly greater ($p<0.05$) at 8 weeks, 16 weeks ($p<0.01$), and 20 weeks ($p<0.01$).

Head of femur BMD *(Table 3B)*

The BMD of the head of femur increases for the Sham-OVX, GlcNBu-fed rats from week 2-8 and 16-20. There is no significant change in the BMD of the head of femur for the OVX, GlcNBu-fed rats over this 20-week growth period. The BMD value at week 8 and week 20 is significantly greater than that at week 2 ($p<0.01$).

Table 3. BMC (A) and BMD (B), for GlcNBu-fed rats, at different times or treatments for the global spine, global femur and head of femur. Paired t-tests are done at each time point for each bone, for Sham-ovariectomized (SHAM-OVX) and ovariectomized (OVX), versus the 2-week time point in addition to the preceding time point.

(A) Bone Mineral Content (g) for GlcNBu-Fed Rats

Time	Global Spine		Global Femur		Head of Femur	
(w)	SHAM-OVX	OVX	SHAM-OVX	OVX	SHAM-OVX	OVX
2	0.52±0.04*	0.53±0.05	0.46±0.07	0.42±0.05	0.10±0.02	0.06±0.01
8	0.76±0.05^{c}	0.73±0.06^{c}	0.51±0.04	0.47±0.03	0.08±0.01^{a}	0.07±0.01
16	0.84±0.04^{c,ii}	0.82±0.11^{b}	0.58±0.08^{i}	0.59±0.05^{c,ii}	0.08±0.01	0.08±0.01
20	0.83±0.08^{c}	0.78±0.11	0.58±0.07^{a}	0.58±0.06^{b}	0.09±0.01	0.07±0.01

(B) Bone Mineral Density (g/cm^2) for GlcNBu-Fed Rats

Time	Global Spine		Global Femur		Head of Femur	
(w)	SHAM-OVX	OVX	SHAM-OVX	OVX	SHAM-OVX	OVX
2	0.22±0.01	0.22±0.01	0.27±0.02	0.27±0.02	0.37±0.02	0.37±0.02
8	0.25±0.01^{c}	0.23±0.01^{c}	0.30±0.02^{b}	0.28±0.01^{a}	0.42±0.02^{b}	0.36±0.02
16	0.26±0.01^{b}	0.24±0.02	0.31±0.02^{a}	0.31±0.02^{b,i}	0.41±0.03	0.40±0.04
20	0.26±0.02^{a}	0.24±0.02	0.32±0.02^{a}	0.30±0.01^{b}	0.45±0.04^{b,i}	0.45±0.03^{b}

*Mean±SD.

Paired t-test versus 2w: $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$.

Paired t-test versus preceding time point: $^{i}p<0.05$, $^{ii}p<0.01$, $^{iii}p<0.001$.

Effects of growth on the body composition of Sham-OVX and OVX rats fed with GlcNBu.

Body global mass *(Table 4)*

Body global mass of both Sham-OVX and OVX GlcNBu-fed rats increases over this 20 week growth period. When the body global mass measured at each of the time points of this growth period are compared to week 2, the values are greater. For the Sham-OVX, GlcNBu-fed rats this difference is significant at weeks 8 ($p<0.001$), 16 ($p<0.01$), and 20 ($p<0.01$). For OVX, GlcNBu-fed rats the difference is highly significant ($p<0.001$) at all time points.

Body global lean *(Table 4)*

The body global lean increases from weeks 2 to 8 for the Sham-OVX and OVX GlcNBu-fed rats. When the body global lean measured at weeks 8, 16, and 20 are compared to week 2, the values are greater. For both the Sham-OVX and OVX GlcNBu-fed rats the differences at week 8 are highly significant ($p<0.001$) and remain significant at weeks 16 and 20 ($p<0.01$ for both).

Body global fat *(Table 4)*

The body global fat of both Sham-OVX and OVX GlcNBu-fed rats increases over this 20 week growth period. When the body global fat measured at each of the time points of this growth period are compared to week 2, the values are greater. For the Sham-OVX rats this difference is highly significant ($p<0.001$) at week 8 and 20 and significant at week 16 ($p<0.01$). For OVX rats the difference is highly significant ($p<0.001$) at week 8 and 16 and very significant ($p<0.01$) at week 20.

Table 4. Body composition (body global fat, body global mass and body global lean), for GlcNBu-fed rats, at different times or treatments (OVX and SHAM-OVX groups). Paired t-tests are done at each time point for each treatment versus the 2-week time point and also preceding time point.

	Body Composition (g) for GlcNBu-Fed Rats					
Time	**Body Global Fat**		**Body Global Mass**		**Body Global Lean**	
(w)	**SHAM-OVX**	**OVX**	**SHAM-OVX**	**OVX**	**SHAM-OVX**	**OVX**
2	35.77±6.79[*]	37.38±6.21	280.16±7.93	290.65±8.65	235.64±11.35	243.94±10.60
8	54.34±13.08[b]	72.60±19.52[c]	332.89±13.42[c]	396.16±19.17[c]	267.81±9.05[c]	312.21±25.85[c]
16	66.87±20.42[a]	104.60±23.30[c]	345.03±20.73[b]	430.56±22.54[c,ii]	267.03±12.39[b]	317.78±30.77[b]
20	74.57±23.29[b,i]	126.08±38.41[b]	355.10±22.11[b,ii]	446.40±37.79[c]	269.02±11.94[b]	307.52±31.73[b]

[*]Mean±SD.

Paired t-test versus 2w: [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$.

Paired t-test versus preceding time point: [i]$p<0.05$, [ii]$p<0.01$, [iii]$p<0.001$.

Effects of ovariectomy and GlcNBu feeding on the BMC and BMD of the spines and femurs of rats analyzed by 2-way ANOVA *(Table 5).*

Analysis by 2-way ANOVA indicates GlcNBu feeding has significant effects on BMD and BMC that can be seen at different time points. From the descriptive statistics, these effects are in the positive direction. The significant effects of GlcNBu on global spine, global femur, and head of femur on the BMC of Sham-OVX rats can be seen starting at 2 weeks. At 16 weeks, GlcNBu continues to have a significant effect on the BMC of all bones, but the interaction of GlcNBu

with OVX is seen only at 16 weeks for the BMD of the head of the femur (Table 5). At 20 weeks, there is a significant effect of GlcNBu on both the BMC and BMD of the global femur and the head of femur measurements.

As expected, there are significant effects of ovariectomy on BMD and BMC bone by 2-way ANOVA (Table 5), which are in the negative direction. At 8 weeks, ovariectomy has a significant effect on the BMC of the global femur and head of femur, and on the BMD of all the types of bone. At 16 weeks, there are significant effects are on the BMD of the global spine, global femur, and head of femur. At 20 weeks, there is a significant effect of ovariectomy on the BMD of the global spine and global femur.

Additionally, there is an interaction between GlcNBu and ovariectomy that significantly affects BMD at 16 weeks (Table 5).

Table 5: Significant differences by 2-way ANOVA in BMC and BMD, assessed by DXA, for global spine, global femur and head of femur at sequential time points: At each time point and bone, 2-way ANOVAS are used to see if there is a significant difference in the mean BMC or BMD of the 4 different treatment groups (1. Sham-OVX, 2. OVX, 3. GlcNBu, Sham-OVX, 4. GlcNBu, OVX) and whether ovariectomy or GlcNBu has a significant effect on the BMD or BMC value. The 2-way ANOVA was done at each time point and for each bone but only the significant effects are shown.

Dependent Variable	Factor	Significance
Global Spine BMC 2 weeks	GlcNBu	$p<0.01$
Global Femur BMC 2 weeks	GlcNBu	$p<0.05$
Head of Femur BMC 2 weeks	GlcNBu	$p<0.01$
Global Spine BMC 8 weeks	GlcNBu	$p<0.05$
Global Spine BMD 8 weeks	Ovariectomy	$p<0.01$
Global Femur BMC 8 weeks	Ovariectomy	$p<0.01$
Global Femur BMD 8 weeks	Ovariectomy	$p<0.001$
Head of Femur BMC 8 weeks	Ovariectomy	$p<0.01$
Head of Femur BMD 8 weeks	Ovariectomy	$p<0.001$
Global Spine BMC 16 weeks	GlcNBu	$p<0.05$
Global Spine BMD 16 weeks	Ovariectomy	$p<0.01$
Global Femur BMC 16 weeks	GlcNBu	$p<0.01$
Global Femur BMD 16 weeks	Ovariectomy	$p<0.05$
Head of Femur BMC 16 weeks	GlcNBu	$p<0.001$
Head of Femur BMD 16 weeks	Ovariectomy	$p<0.01$
Head of Femur BMD 16 weeks	Ovariectomy/GlcNBu	$p<0.05$
Global Spine BMD 20 weeks	Ovariectomy	$p<0.05$
Global Femur BMC 20 weeks	GlcNBu	$p<0.01$
Global Femur BMD 20 weeks	GlcNBu	$p<0.05$
Global Femur BMD 20 weeks	Ovariectomy	$p<0.01$
Head of Femur BMC 20 weeks	GlcNBu	$p<0.01$
Head oF Femur BMD 20 weeks	GlcNBu	$p<0.001$

Effects of Growth, ovariectomy and GlcNBu feeding on the body composition of rats

When ovariectomy and GlcNBu feeding are considered together, using a 2-way ANOVA, there is no significant effect of GlcNBu on body composition at any time point. Table 6 demonstrates

how there is a significant effect of ovariectomy on body composition at week 8, 16, and 20 time points. There is no significant effect of ovariectomy on body composition at 2 weeks after commencing the feeding experiment. There is no interaction between GlcNBu and ovariectomy that significantly effects the body composition at any time point (Table 6). For the descriptive statistics, the body global fat, mass and lean is greater for the OVX than the Sham-OVX rats, whether they were fed GlcNBu or not (Tables 2 and 4).

Table 6. Significant differences in body composition (body global fat, body global mass and body global lean) assessed by dual-energy X-ray absorptiometry at sequential time points: At each time point, 2 way ANOVAS are used to see if there is any significant difference in the mean body global mass, fat or lean of the 4 different treatment groups (1. Sham-OVX, 2. OVX, 3. GlcNBu, Sham-OVX, 4. GlcNBu, OVX) and whether ovariectomy or GlcNBu has a significant effect on the body global mass, fat or lean. At each time point, the 2-way ANOVA was done, but this table shows only the significant effects.

Dependent Variable	Factor	Significance
Body Global Fat 8 weeks	Ovariectomy	$p<0.01$
Body Global Mass 8 weeks	Ovariectomy	$p<0.001$
Body Global Lean 8 weeks	Ovariectomy	$p<0.001$
Body Global Fat 16 weeks	Ovariectomy	$p<0.01$
Body Global Mass 16 weeks	Ovariectomy	$p<0.001$
Body Global Lean 16 weeks	Ovariectomy	$p<0.001$
Body Global Fat 20 weeks	Ovariectomy	$p<0.01$
Body Global Mass 20 weeks	Ovariectomy	$p<0.001$
Body Global Lean 20 weeks	Ovariectomy	$p<0.05$

DISCUSSION

In this study, we determined the effects of the combination of ovariectomy and GlcNBu on bone mineral and body composition in rats over time. At the start of the study, the rat age was 8 weeks and a Sham-OVX, Glc-fed control group was included; accordingly, gain of bone mass attributable to age could be differentiated from changes due to ovariectomy or GlcNBu feeding [6]. There are several considerations [13-15] related to our model: the skeleton of a rat reaches maturity after 10 months. In older, intact female rats age-related decreases in cortical BMD have been reported to start in the lumbar vertebrae at the age of 15 months. Moreover, while long bones reach a peak dry mass at 20 weeks of age, the dry mass of vertebrae was shown to continue to increase. Ovariectomized immature rats also tended to demonstrate a more sensitive skeletal response compared to aged female rats.

Our data, Table 1A, illustrates that the change of the BMC with time of the spine and global femur increases sharply from base line to week 8 for the Sham-OVX, glc-fed controls and then the increases become less steep. The increases in BMD, including the initial period from base line to week 8, are relatively much smaller than the BMC increases and the changes of BMD with time are generally flatter (Table 2B). This is likely the result of calcification which follows closely matrix synthesis and growth, with a peak at about 8 weeks. Additionally, [^{3}H]tetracycline labelling using a histological technique demonstrated a peak of calcification occurring at 8 weeks

for long bones and at 10 weeks for vertebrae in the rat, which is likely related to the age of closure of the growth plates [12]. However, the relation between cancellous and cortical bone mineralization during growth periods in young animals undergoing an experimental intervention can be complex. For example, mild calcium deficiency in young, growing, female rats increased osteoclastic recruitment, which was followed by increased osteoblastic recruitment in cancellous bone, suppressing mineralization; furthermore, cancellous bone loss was counteracted through the redistribution of calcium from cortical bone to cancellous bone [16].

Within the time frame of 8-10 weeks, the most rapid elaboration of matrix would occur by active osteoblasts and could constitute an early and suitable time frame for studying an anabolic agent, such as may be the case for GlcNBu. Additionally, choosing an early time frame could be helpful if one wished to screen a number of potentially anabolic compounds inexpensively. Relatively small numbers of animals would be required, using paired t-test statistics in sequential testing, as each animal would serve as its own control starting with a defined baseline.

For the OVX rats, the pattern of the changes in BMC and BMD shows some important differences compared to the Sham-OVX animals (Table 1A and 1B). However, for the global spine BMC of the Sham-OVX and OVX rats, the Sham-OVX values are higher at all time points, but the differences did not reach significance (Table 2A). Further, for the Sham-OVX the BMC between weeks 16 and 20 does not change and for the OVX rats, the increase between weeks 16 and 20 is not significant (Table 1A).

Generally, in models using growing animals, serial measurements of the BMC provide more useful information than serial measurements of the BMD. This is because the BMC, which is expressed in grams, reflects the absolute increase in bone mass for any particular bone or region of interest during serial time points. On the other hand, BMD, which is expressed in grams/cm^2, does not give direct information on increases in bone mass during growth but provides the ratio of the amount of mineral in a fixed unit of area (reflecting a fixed volume) in a region of interest. For example, in order to compare the effect of GlcNBu with the Glc control in the OVX rats, we can compare the BMC values of the spine and the femur of the OVX rats. This data was included in Tables 1A and 3A, which is also abstracted in Table 7. The p values of the comparison have been calculated by unpaired t-tests. For the OVX rats, GlcNBu feeding results in higher or equal BMC values, compared to Glc feeding. For the global spine a statistically significant difference is seen early at 2 weeks, while for the global femur a statistically significant difference is not seen until the end of the experiment at 20 weeks. Likely, a larger sample size would have resulted in significant differences also at other time points.

Table 7. Comparison of Bone Mineral Content of Spine and femur for Glc-treated and GlcNBu-treated OVX Rats (BMC values are from Tables 1A and 3A)

Time (months)	**Global Spine**			**Global femur**		
	Glc - treated	GlcNBu-treated	p-value	Glc-treated	GlcNBu-treated	p-value
2	0.47±0.04	0.53±0.05	0.033	0.41±0.04	0.42±0.05	0.581
8	0.67±0.07	0.73±0.06	0.110	0.47±0.05	0.47±0.03	0.990
16	0.72±0.07	0.82±0.11	0.076	0.51±0.06	0.59±0.05	0.054
20	0.75±0.11	0.78±0.11	0.623	0.50±0.04	0.58±0.06	0.033

The changes of BMD of the head of the femur with OVX are specifically of interest for modeling for human estrogen deficiency, as this is the only significant decline for the femoral head ($p<0.05$, Table 1B). The decline in the BMD of the femoral head after OVX is highly significant compared to the BMD of the femoral head of the Sham-OVX rats ($p<0.001$, Table 1B). Furthermore, the BMD of the spine and global femur are significantly higher in the Sham-OVX compared to the OVX rats at various other time points as described in the Results. Thus, time points around 16 weeks should have general utility for relatively inexpensive screening of compounds under conditions of estrogen deficiency when one wishes to model for risk of femoral head fractures. This would apply whether or not the test compound works through stimulation of osteoblastic stimulation (anabolic effect) or inhibition of osteoclastic activity (anti-catabolic effect). In the case of GlcNBu, an interaction between this compound and ovariectomy was demonstrated only for the BMD of the femoral head at the 16 week time point, by 2-way ANOVA (Table 5).

The body composition measurements, in the Glc-fed rats (Table 2), show a similar pattern to the BMC (Table 1A), in that there is an increase also in body global mass, body global fat ,and body global lean over this 20 week growth period for all groups. For GlcNBu fed OVX and Sham-OVX rats analyzed by the t-tests, a number of significant differences, compared to week 2, were also observed for body global mass, lean, and fat (Table 4). However, "lean" body mass does not measure only muscle but also includes gut and internal organs with this type of whole body DXA measurement. This is an important limitation if one is attempting to relate bone to muscle, through the effects of an intervention such as feeding GlcNBu or ovariectomy.

The two-way ANOVA shows that there was a significant positive difference of GlcNBu feeding on the dependent variables of the BMC of the global spine, the global femur, and the head of the femur, which occurs very early in the experiment, i.e., by 2 weeks (Table 5). At this time point, the GlcNBu-fed Group would have ingested this compound for only 9 days, after the accommodation period (see Materials and Methods). For GlcNBu fed rats, Global spine BMC is significantly increased at 8, 16, and 20 weeks, while global femur and head of femur significantly increased at 16 and 20 weeks.

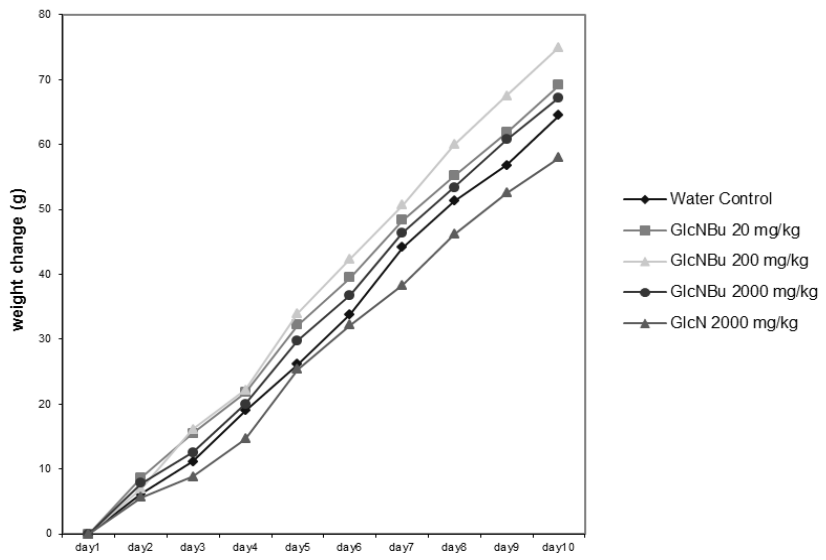
It appears that GlcNBu has a stimulatory effect on the mineralization of bones, as evidenced by the accelerated BMC of the bones throughout the experimental period. This is presumably largely a result of stimulation of bone matrix synthesis which continues to be mineralized. It is not until 20 weeks that the BMD of the GlcNBu-fed group becomes significantly increased, suggesting some additional increase in mineralization by GlcNBu. However, the compound did not show any significant effects on body composition by 2-way ANOVA (Table 6). This suggests a direct effect of GlcNBu on bone, rather than a secondary mechanism mediated through changes in global body mass, lean, or fat. In contrast, ovariectomy resulted in a number of significant differences of the dependent variables of body composition (Table 6). Furthermore, the significant effects, by 2-way ANOVA, of ovariectomy on body composition appear first at 8 weeks, while the GlcNBu effects on BMC appear first at 2 weeks (compare Tables 5 and 6).

In our initial work with N-acylated glucosamines, we worked with chondrocyte culture systems in order to investigate chondroprotective properties of this class of compounds (see Introduction). Overall, it appears that N-acylation abolishes the inhibitory effect of GlcN seen in

high concentrations in culture. However, the effects of GlcN in culture are highly dependent on the GlcN concentration and the cell state (e.g. monolayer, pellet type of culture) [5].

In a rat model of a destructive streptococcal cell wall-induced destructive arthritis of cartilage and bone, oral administration of GlcNBu at either 20 or 200 mg/kg/day had strong protective effects in both cartilage and subchondral bone, with a number of quantitative parameters being preserved in the latter in a dose-dependent fashion [17]. It was this effect of GlcNBu on bone that led us to investigate this compound as a dietary supplement in the ovarectomized rat model for increased bone loss at the dose of 200 mg/kg/day [6].

Figure 1 Supplementary



Legend of Figure 1
(Supplementary).

Average weight gained by rats gavaged with GlcN or GlcNBu. Thirty Sprague Dawley male rats were adapted for one week. Body weights at the onset of treatment were approximately 185 grams. Treatment Groups were: water (control), 20mg/kg GlcNBu, 200 mg/kg GlcNBu, 2000 mg/kg GlcNBu of 2000 mg/kg GlcN, with 6 animals in each Group. All animals were fed Purina Rat Chow and water *ad libitum*, weighed daily and observed for indications of toxicity. The compounds were administered by gavage in water, daily for nine days, at half doses, at 08:00 and at 17:00. (From US patent Application 20040152665 A1. T. Anastassiades.)

In this study, the supplementation of feed with GlcNBu was also conducted with a dose of 200 mg/kg/day. It is possible that higher doses of GlcNBu would have larger effects on body composition. However, we carried out a preliminary feeding experiment with different doses of GlcNBu (Supplementary Fig 1). In this experiment, male rats were gavaged with 20, 200, or 2,000 mg/kg GlcNBu or 2,000 mg/kg glucosamine (GlcN) or water (control) for nine days and the daily weights of the rats recorded, as explained in the legend of Supplementary Fig 1. GlcN at 2000 mg/kg/day demonstrated the lowest rate of growth during this experimental period, which was lower than the water-fed rats and thereby suppressive of growth. The highest rate of weight gain was seen in animals fed GlcNBu at 200 mg/kg/day, followed by 20 and 2000 mg/kg/day and the water (control) respectively. Consequently, we considered the dose of 200 mg/kg/day to have the optimal stimulatory growth effect and this concentration was used in the bone study reported here and previously [6, 17]. In another experiment (data not shown) radiometric analysis of growth plates of long bones after ^{35}S intraperitoneal injection indicated a more rapid turnover of the GlcNBu-fed animals compared to GlcN-fed or Glc-fed controls, suggesting an effect of GlcNBu on epiphyseal growth. Moreover, we demonstrated that femurs

are longer in GlcNBu-fed rats at the end of a six month period, whether or not they were OVX [6]. These experiments support the notion that the oral administration GlcNBu has a stimulatory effect on bone growth in live rodents over a certain dose range. In conjunction with the data presented in the Results, particularly Table 5, it appears that GlcNBu fed to rodents likely results in an anabolic effect on bone seen increased mineralization through DXA measurements at an early stage of growth of the animal. In these experiments, the increased BMC was observed by 2 weeks of supplementation by GlcNBu in the female rats, at age 8 weeks and with mean body weight of 175 g.

The mechanism of action of the GlcNBu on bone has not been elucidated and there is no putative receptor of this glycan monomer. In an attempt to understand a mechanism of action, we studied the butyrylation of glucosamine moieties of the polymer hyaluronic acid, whose cell surface receptors have been identified and are dependent on the size of this polymer. When we chemically substituted butyryl moieties, replacing some of the acetyl moieties on the glucosamines of low molecular mass hyaluronic acid, we demonstrated anti-inflammatory cytokine activity of these mixed GlcNBu polymers on cultured macrophages [18]. These anti-inflammatory effects are likely mediated through the TLR-4 receptor system [13] and could represent a mechanism of action for GlcNBu, if it were to be efficiently incorporated into newly synthesized hyaluronic acid. Additionally, we have recently demonstrated that human acetyl-CoA: glucosamine-6-phosphate N-acetyltransferase 1 has a relaxed donor specificity and transfers acyl groups up to the butyryl moiety (i.e. up to four carbons in length) [19]. Currently, we do not know to what extent GlcNBu fed to animals may be incorporated into the hyaluronic acid or into other abundant glycoconjugates of the bone matrix. We have not tested for anti-inflammatory cytokine effects of GlcNBu in the current rat model, but GlcN itself is much less active than GlcNBu in preserving bone in this model, as evidenced by a micro-CT study (unpublished), suggesting a functional role of the butyryl group in GlcNBu.

In order to assess the acceptability of GlcNBu in bone and joint disorders in humans and in domestic animals, it is useful to examine the current usage of the parent molecule, GlcN. The amino-sugar GlcN is commercially derived mostly from the chitin of crustacean shells. By de-acetylating the insoluble chitin to produce partially soluble chitosan, followed by acid hydrolysis, large amounts of inexpensive, highly purified GlcN can be obtained generally as the hydrochloride or sulfate salts. These GlcN salts have found great acceptability in human animal health, where they are used primarily for osteoarthritis. Approximately 47% of consumers associated GlcN use with joint health and 20% with bone health (data base of the National Marketing Institute). To a large extent, this acceptance appears be related to the belief that GlcN is relatively safe, at least compared to non-steroidal ant inflammatory drugs. However, the results of the large numbers of human randomized clinical trials (RTC) which have been conducted to assess the effect of GlcN on joint pain related to osteoarthritis remain controversial and unconvincing. Rigorous domestic animal trials are quite limited. We had undertaken a Cochrane Data Review (updated) [20], which demonstrated that allocation concealment is an important consideration, even in "blinded" RTCs with standardized outcomes of pain, stiffness, and function of human osteoarthritis. This review found GlcN was not superior compared to placebo, when studies with allocation concealment in the protocols were considered in the analysis. A

large, NIH-supported RTC, "GAIT" [21], without commercial sponsor support found that GlcN, when compared with a placebo, did not achieve a clinically important difference, using a standardized pain measurement for osteoarthritis of the knee as the primary outcome. Furthermore, this 2-year RTC revealed that approximately 60% of the improvement in outcome measures occurred within the first three visits (18 weeks) for all comparator groups (GlcN, chondroitin, combination, placebo, and celecoxib). The magnitude of improvement, including the placebo, did not differ significantly by treatment group [21], which underlines the importance of the placebo (global) effect in these types of RTCs in osteoarthritis using standardized pain scale measurements. In general, RTCs have supported the safety of oral glucosamine at doses of approximately of 1,500 mg/day and it is generally considered to be as safe as placebo. However, a number of animal studies suggest that GlcN can adversely impact glucose metabolism, and clinical studies have provided mixed evidence about the effect of exogenous GlcN on glucose metabolism in humans [22].

In regards osteoporosis, we found that GlcN use increased substantially over 5 years in the Canadian Multicenter Osteoporosis Study, a random, population-based sample of 9,423 Canadians [23]. The increase was from baseline to year 5 of the study and the use of GlcN was associated with a number of factors, including as a preventive measure to maintain health. In the same study, we found a very sizeable osteoporosis "care gap". Annual data collection over a 10-year period of this multicenter study showed that 42–56% of women [24] and approximately 90% of men [25] with a clinical fragility fracture were not treated with an osteoporosis medication.

As recently pointed out [26], the search for newer therapeutics for osteoporosis continues because: (i) None of the available drug classes eliminates the risk of fragility fractures, (ii) the side effects of all classes remain a major concern as we also noted [24, 25], and that (iii) no one drug restores the micro architectural deterioration caused by this disease. We discovered GlcNBu to be safe in very high doses in animal studies (unpublished), and we have also observed no toxicity with high concentrations in vitro [4, 5], although formal toxicology has not been conducted. Additional studies would also need to be performed, with GlcNBu alone or in combination with other therapeutic agents, to assess its usefulness in the treatment of diseases of bone loss, with respect to points (i) and (iii) [26].

CONCLUSION

1. Supplementation of the diet by GlcNBu, in both OVX and Sham-OVX rats increases bone mineral, an effect that is first observed at 2 weeks, during the experimental period of 6 months.
2. OVX rather than GlcNBu supplementation had a significant effect on body composition, which was first observed at 8 weeks.
3. The effect of GlcNBu on mineralization occurs independently of changes in body composition. The GlcNBu effect on mineralization likely reflects stimulation of bone matrix synthesis which then continues to be mineralized.
4. This work has significant potential applications in bone health, particularly in osteoporosis where there is now an increasing demand for safe, long-term agents.

List of Abbreviations: ANOVA, analysis of variance; BMD, bone mineral content; BMC, bone mineral density; GlcNBu, N-butyryl glucosamine; OVX, ovariectomized; GlcN, glucosamine.

Authors' Contributions: TA is the principal investigator and responsible for the overall concept and experimental animal design. KRM assisted in the experimental design and the organization of the data in the Tables and performed a number of the statistical analysis. WH was primarily responsible for the statistical approach and analyses.

Competing Interests: Tassos Anastassiades (TA) holds patents on the applications of N-butyryl glucosamine, licensed to Queen's University (PARTEQ Innovations); Karen Rees-Milton (KRM) and Wilma Hopman (WH) have no competing interests.

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