ORIGINAL ARTICLE

N-acylated glucosamines for bone and joint disorders: effects of *N*-butyryl glucosamine on ovariectomized rat bone

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The benefit of glucosamine (GlcN) in bone and joint disorders remains controversial. *N*-acetylation and other *N*-acylations of GlcN alter its biological properties fundamentally. We have shown previously that *N*-butyryl glucosamine (GlcNBu) preserved strikingly the subchondral bone structure in a destructive arthritis rat model. Here, we examine whether GlcNBu preserves bone in the ovariectomized (OVX) rat, a model for postmenopausal osteoporosis. Rats were randomized into 4 groups: group 1, sham OVX glucose (Glc) fed; group 2, sham OVX GlcNBu fed; group 3, OVX Glc fed; and group 4, OVX GlcNBu fed. A single, oral, 200-mg/kg dose of GlcNBu or Glc was administered daily for 6 months. Bone mineral content (BMC) and bone mineral density, and biomechanical properties of the femurs and spines were determined by standardized techniques. Two-way analysis of variance with a Bonferroni post hoc test was used for statistical analysis. Ovariectomy in group 3 resulted either in significant or highly significant effects in a number of the tests. For spinal BMCs the interaction between GlcNBu and OVX was significant. For the femurs, this interaction was also seen in energy to failure, and ultimate displacement and ultimate strain tests. In general, ovariectomy was necessary to show significant preventive effects of GlcNBu on mineral content and some biomechanical properties. We conclude that GlcNBu feeding in the OVX rat preserves bone mineral and some biomechanical properties. Translationally, GlcNBu can be positioned between nutriceuticals and pharmaceuticals for the prevention and treatment of osteoporosis. Advantages include low production costs and a favorable safety profile. (Translational Research 2013;■:1–9)

Abbreviations: ANOVA = analysis of variance; BMC = bone mineral content; BMD = bone mineral density; BMP = bone morphogenetic protein; Glc = glucose; GlcN = glucosamine; GlcNAcyl = *N*-acylated GlcN compounds; GlcNBu = *N*-butyryl glucosamine; OVX = ovariectomized; PMMA = polymethyl methacrylate

AT A GLANCE COMMENTARY

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Background

The benefit of glucosamine (GlcN) in bone and joint disorders remains controversial. N-acylation of GlcN alters its biological properties fundamentally. N-butyryl glucosamine (GlcNBu) preserved strikingly the subchondral bone structure in a destructive arthritis rat model. Here, GlcNBu is used in a preventive protocol in the ovariectomized (OVX) rat, a model for postmenopausal osteoporosis.

Translational Significance

Our observation that GlcNBu oral treatment preserves bone mineral density and most biomechanical properties in the OVX model has potential application in human osteoporosis. Also, our translational approach serves as a paradigm for the development of low-toxicity compounds that can be positioned between pharmaceuticals and nutriceuticals.

Chitosan is derived from chitin, the most abundant polymer of the animal kingdom. Glucosamine (GlcN) is derived largely from the chitosan of crustacean exoskeletons. The high prevalence of taking GlcN for joint or bone health was illustrated in a randomly sampled population in the Canadian Multicenter Osteoporosis Study.[1] Beneficial effects of GlcN sulfate salt, primarily for pain in osteoarthritis of weight-bearing joints, has been reported in European randomized clinical trials but have not been confirmed, including a large American randomized clinical trial or in our Cochrane reviews.[2-4] Furthermore, human pharmacokinetic studies of GlcN have indicated that the serum levels of free GlcN, after the ingestion of large amounts of glucosamine sulfate salt, were below limits of detection using an older methodology.[5] A more sensitive method[6] has shown that orally administered GlcN in the rat is absorbed rapidly, cleared, and metabolized in the gut. Also, the levels of free GlcN in the serum and synovial fluid of the horse have also been found to be very low, probably below what would be required for pharmacologic effects.[7]

The hexosamine constituents (including GlcN) of naturally occurring glycoproteins and glycosaminoglycans are almost invariably acetylated, and free N-acetyl GlcN is readily detected in mammalian sera (as described later). The reason for the N-acetylation of GlcN and, more broadly, N-acylation reactions, is not understood. To understand this issue better, we synthesized a number of N-acylated GlcN compounds (GlcNAcyls), including the butyrylated derivative N-butyryl glucosamine (GlcNBu), from the corresponding acyl anhydrides.[8,9] The physical properties we obtained and the formulas of the GlcNAcyls are summarized in Table I.

We previously reported that, in bovine chondrocyte culture, relatively high concentrations of GlcN (≥ 0.1 mM) inhibited cell proliferation and proteoglycan synthesis.[8] However, these inhibitory effects were reversed by N-acylation of GlcN. The specificity of the N-acylation did not seem to be critical for this reversal of inhibition. However, N-butyrylation of GlcN, which yielded GlcNBu, had a modest stimulatory effect, but the butyryl moiety itself did not account for the different biologic properties. Also, in human osteoarthritis chondrocytes, the addition of GlcNBu led to the upregulation of a number of genes, with only a few downregulated, whereas GlcN addition resulted primarily in the downregulation of a large number of genes.[8] In rat chondrocytes, GlcNBu increased significantly the levels of type II collagen and aggrecan messenger RNA, but GlcNAcyl and N-propionyl GlcN had no significant effect.[9]

The inhibitory effects of chondrocyte proliferation and proteoglycan synthesis by GlcN, which were reversed by N-acylation, were more pronounced under anchorage-dependent conditions compared with anchorage-independent conditions,[10] and GlcN.HCl behaved similarly to the GlcN.S salt. However, differential effects with GlcN sulfate covalently linked isomers were observed, depending on the carbon atom carbon that was substituted.[10]

We studied the pharmacokinetics of GlcNBu by developing a derivatization high-performance liquid chromatographic method[11] applied to the rat. In this animal, the bioavailability of GlcNBu after oral dosing is relatively low, compared with intravenous or intraperitoneal administration, primarily because of low mucosal to serosal transport and not as a result of liver metabolism.[12] Also, there was no significant conversion of GlcNBu to GlcN. In chondrocyte and osteoblast cultures, [^{3}H]GlcN is converted to a number of radiolabeled metabolites, but GlcNBu is metabolized much less actively.[13]

We also determined that GlcNBu had desirable chemical and physical properties, very low toxicity in cell culture and in animals (no toxicity up to 12,000 mg/kg/day in rats), and negative Ames testing. This safety profile of GlcNBu permitted us to proceed to in vivo testing in animal models.

For the testing of orally administered GlcNBu (20 mg/kg/day or 200 mg/kg/day) on cartilage and subchondral bone preservation, we chose a well-characterized animal model: the streptococcal cell wall antigen-induced

Table I. Synthetic *N*-acyl glucosamines

Side chain	Molecular weight, Da	Melting point, °C	Yield, %	Formula
Propionyl	235.2	187.1–188.4	43.7	$C_9H_{17}O_6N$
Butyryl	249.2	205.2–205.8	43.0	$C_{10}H_{16}O_6N$
Pentanyl	263.2	206.3–206.9	41.7	$C_{11}H_{21}O_6N$
Hexanyl	277.3	207.5–207.7	55.5	$C_{12}H_{23}O_6N$
Heptanyl	291.3	209.0–210.4	64.6	$C_{13}H_{25}O_6N$
Lauryl	361.4	198.9–199.3	42.1	$C_{18}H_{35}O_6N$
4-Pentenyl	261.2	208.5–208.9	48.8	$C_{11}H_{19}O_6N$
Benzyl	283.2	186.1–187.5	42.0	$C_{13}H_{17}O_6N$
2-Methylbutyryl	263.2	179.2–179.7	26.3	$C_{11}H_{21}O_6N$

arthritis in the rat.[14] In this model, the chronic phase then leads to rapid cartilage and bone loss and bone remodeling. Strikingly, both the high and low doses of the compound preserved bone mineral density (BMD) and bone connectivity, and prevented further bone loss in a stepwise fashion. We noted that the administration of oral GlcNBu to this model resulted in a greater degree of prevention of subchondral bone loss and connectivity than the relatively modest inhibitory effect on joint inflammation. The critical question then became: Does GlcNBu have positive effects on bone loss *in the absence* of an inflammatory stimulus? To answer this question, we investigated the effect of GlcNBu in the ovariectomized (OVX) rat, a model for postmenopausal osteoporosis that is devoid of inflammatory stimulus.

The OVX rat has been used extensively for drug development in osteoporosis, and the advantages and limitations of this model have been reviewed.[16-18] The OVX rat has been considered the gold standard for drug discovery for postmenopausal osteoporosis[19] because it is characterized by initial high bone turnover and subsequent bone loss, prevented by estrogen replacement. However, there are some limitations to this model,[20,21] as indicated in the Discussion.

Because we had found that GlcNBu in culture appeared to have anabolic effects,[8,9] we were interested in testing this compound initially in relatively young OVX rats (with the appropriate controls), which would be expected to have robust cortical osteoblastic function. Also, the impressive effects of increased bone mass and connectivity by GlcNBu in the inflammatory model of arthritis[15] was done in relatively young rats. However, extending the period of feeding the compounds to 6 months would permit us to observe effects of the compounds both during the period of modeling as well as remodeling. In this study, we used a preventive protocol after ovariectomy.

MATERIALS AND METHODS

Virginal female Sprague-Dawley rats, which were either OVX or not at age 8 weeks (mean weight, 175 g),

were purchased from Charles River Laboratories. After accommodation for 5 days, the rats' mean weight was 177 g for the sham OVX group and 184 g for the OVX group. The feeding experiment was done at the Queen's University Animal Care Facility, after the OVX and sham OVX animals were randomized into the 4 groups indicated later (8 animals in each group). Feeding of the compounds was with either 200 mg/kg/day GlcNBu or an equimolar amount of glucose (Glc), once a day for 6 months. GlcNBu or Glc were administered in a small amount of peanut butter, which was preferentially consumed by the rats, generally before their regular Purina rat chow, which was permitted *ad libitum*. BMDs were monitored in live animals under anesthesia using a Hologic 4500 with a small-animal adapter to ensure the ovariectomy resulted in BMD declines—an effect apparent by 2 months (data not shown). The number of rats is less than 8 because of rat losses during anesthesia or during sample processing: Glc-fed sham OVX (group 1), n = 6; GlcNBu-fed sham OVX (group 2), n = 6; Glc-fed OVX (group 3), n = 4; and GlcNBu-fed OVX (group 4), n = 5.

Data were analyzed using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego, California). Two-way analysis of variance (ANOVA) with Bonferroni post hoc test were performed. In the 2-way ANOVA, the first factor was OVX vs sham OVX and the second factor was GlcNBu vs Glc. Student's *t* test was used to compare femur lengths (eg, Glc-fed vs GlcNBu-fed for the sham OVX left femurs). The statistically significant tests by this analysis are shown for the figures on mineralization (Fig 1), and biomechanical properties of the spines and femurs (Figs 2-4).

Bone mineral density and content. At sacrifice (6 months), right and left femurs and spines were dissected and cleaned carefully. For the direct length and weight measurements, the femurs were traced on paper and the lengths determined by a micrometer to 0.1 mm, and weighed on a microbalance. For the bone mineral measurements the BMD, bone mineral content (BMC), and area were determined by dual-energy
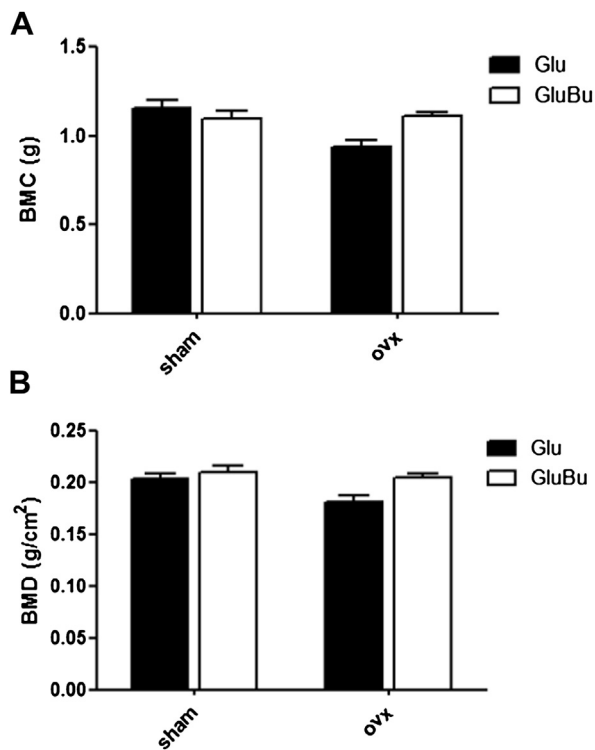
A

B

Fig 1. The effect on bone mineral properties of feeding *N*-butyryl glucosamine (GlcNBu) or glucose (Glc) to sham ovariectomized (OVX) and OVX rats. At sacrifice (6 months), spines were dissected and cleaned carefully. Data were analyzed using GraphPad Prism. The bone mineral density (BMD), bone mineral content (BMC) and area were determined by dual-energy X-ray absorptiometry on the sixth lumbar vertebra (L6), using a Lunar PIXI MUS. Two-way analysis of variance with Bonferroni post hoc test were performed for this and all subsequent figures. (**A**) The effect on spinal BMC. The interaction between the drug (GlcNBu) and OVX was significant (*P* < 0.05). BMC appears to be preserved in group 4. (**B**) The effect on spinal BMD. The interaction between drug and OVX was not significant, but the effects of drug and ovariectomy alone are significant (*P* < 0.05), and BMD appears to be preserved in group 4.

Fig 2. The effect on vertebral energy to fail (normalized data) after feeding *N*-butyryl glucosamine (GlcNBu) or glucose (Glc) to sham ovariectomized (OVX) and OVX rats. At sacrifice (6 months), spines were dissected and cleaned carefully. The mechanical properties of the vertebrae (L6) were determined using an Instron 4465 materials testing machine. The L6 vertebrae were tested in compression. The interaction between drug and OVX was not significant. The effect of the drug alone was not significant, but the effect of the OVX was extremely significant (*P* < 0.0001). Vertebral energy to failure is not fully preserved in group 4.

X-ray absorptiometry on the right femur and the sixth lumbar vertebra (L6) using a Lunar PIXI MUS (dedicated small-animal dual-energy X-ray absorptiometry machine; GE Medical Systems, Canada). Prior to scanning, the machine was first calibrated with an aluminum/Lucite phantom. The femora and L6 were scanned on a polystyrene plate used to simulate soft tissue thickness. Each bone was later analyzed individually for BMC and BMD using Lunar PIXImus software.

Mechanical testing of bone. The mechanical properties of the left femora and vertebrae (L6) were determined using an Instron 4465 materials testing machine (Instron, Norwood, Massachusetts) with a 1-kN load cell and a data acquisition card (National Instruments) interfaced with a Pentium II PC
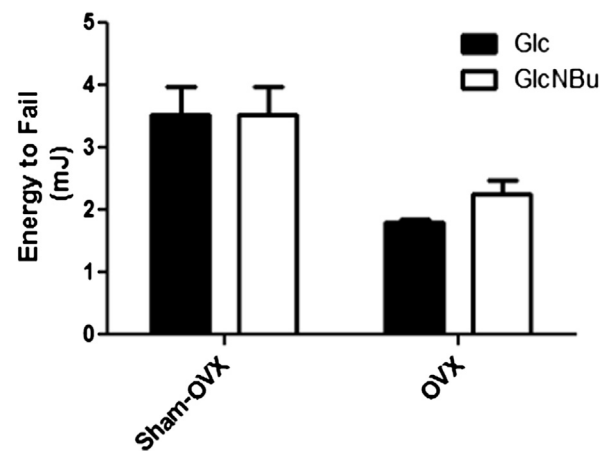
acquiring at 10 Hz. The right femora were tested in torsion using a custom-built device.

The diaphysis of each left femur was tested in 3-point bending according to procedures described previously.[22] Briefly, each femur was positioned posterior side down with a fixed span of 15.6 mm. For our 3-point bending tests, we use a fixed span between the bottom loading points. The span is determined *a priori* by considering the length of the shortest specimens. Therefore, all bones were tested over the same length of diaphysis centered at the mid diaphysis. Each specimen was preloaded to a load of 1 N and deformed at 1 mm/min until complete failure. Stress-strain curves were generated from load deformation curves by accounting for specimen geometry measured from scanning electron microscope images of the cross-sections. From the load deformation curve, ultimate load, failure displacement, energy to failure, and stiffness were determined. From the stress-strain curve, ultimate stress, failure strain, toughness, and modulus were determined.

The right femora were tested in torsion using a custom-built torsion testing machine reported previously.[23,24] Prior to testing, a 16-mm gauge length was marked with permanent marker, and the distal and proximal ends were removed with a low-speed bone saw (Isomet, Bueler). The ends were then mounted into holders using polymethyl methacrylate (PMMA) and a jig that ensured concentricity of the bone and the holders, and therefore the axis of rotation of the test machine. After the PMMA was cured, the proximal end of
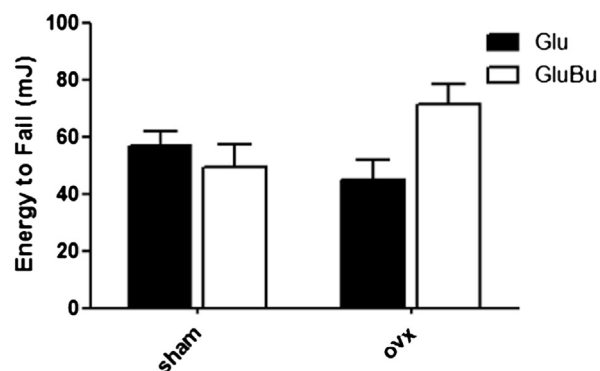
Fig 3. The effect on the energy to failure of femora during 3-point bending (nonnormalized data) after feeding or glucose (Glc) to sham ovariectomized (OVX) and OVX rats. At sacrifice (6 months), femurs were dissected, cleaned carefully, and the biomechanical properties tested. The diaphysis of each femur was tested in 3-point bending, as described in Methods. Based on the load deformation curve, energy to failure was determined. The interaction between drug and OVX was significant ($P < 0.05$), but the effect of either drug or OVX alone was not. Femoral energy to failure in group 4 appears preserved and exceeds values in the other groups.

Fig 4. The effect on femora biomechanical properties during 3-point bending after feeding glucosamine (GlcNBu) or glucose (Glc) to sham ovariectomized (OVX) and OVX rats. At sacrifice (6 months), femurs were processed and tested as in Figure 3 and as described in Methods. Based on the load deformation curve, ultimate load, failure displacement, energy to failure, and stiffness were determined. Based on the stress-strain curve, ultimate stress, failure strain, toughness, and modulus were determined. (**A**) The effect on ultimate displacement (nonnormalized data). The interaction between GlcNBu and OVX was very significant ($P < 0.005$), but the effect of either drug or OVX alone was not. Ultimate displacement in group 4 appears preserved. (**B**) The effect on ultimate strain (normalized data). The interaction between drug and OVX was very significant ($P < 0.005$), but the effect of either GlcNBu or OVX alone was not. Ultimate strain in group 4 appears preserved.

the specimen was mounted to the stationary torque cell (20 in. lb.) and the distal end was attached to the rotary actuator. The tests were run at 0.7 rad/min until failure. Torque-angular displacement data were collected using a data acquisition card and LabView software acquiring at 10 Hz. The data were normalized to shear stress-shear strain data using geometric data from scanning electron microscope images of the failure site. Failure torque, angular displacement, energy to failure, stiffness, failure shear stress, failure shear strain, toughness, and shear modulus were determined and recorded.

Cross-sectional anatomy measures were carried out and include cross-sectional area in square millimeters, anterior-posterior diameter in millimeters, I as the second moment of area in millimeters to the fourth power, and J as the polar moment of area in millimeters to the fourth power. All these measures were done at the mid diaphysis. No statistically significant differences were observed between any of the groups by 2-way ANOVA for these measures.

The L6 vertebrae were tested in compression using a modification of a technique published published.[25] Cross-sections of both proximal and distal ends as well as the smallest observable cross-section were measured optically and recorded. Total lengths of the vertebrae were also measured optically. Bolts with a small well in the top were aligned on a tray to allow for the setting of multiple bones. PMMA was mixed to fill 2 wells at a time. Vertebrae were set immediately in the PMMA using tweezers to ensure attachment. The vertebrae were set approximately 2 mm into the PMMA, enough for the break surface to remain outside the PMMA and for at-
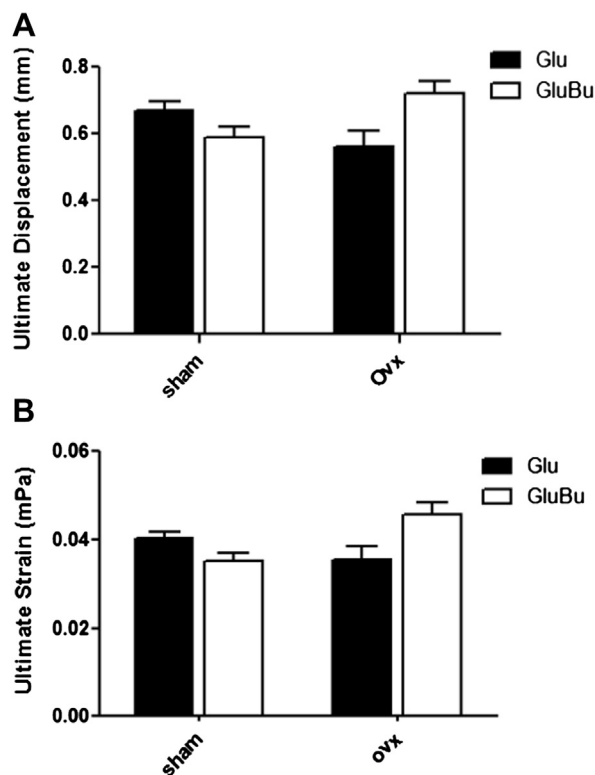
tachment of the bone to be maintained. The gauge length from the PMMA surface to the end of the vertebra was measured. The PMMA was allowed to set for 15 minutes. Vertebral compression was carried out on an Instron 4465 using a 1-kN load cell. Samples were preloaded to 1 N and compressed at a speed of 1 mm/min.

BMD and mechanical data were analyzed by asking 2 distinct questions: Did OVX have the expected effects compared with sham OVX controls? Did GlcNBu help prevent the effects of OVX?

This work conformed to the relevant ethical guidelines for human and animal research.

RESULTS

All animals gained weight during the 6-month experimental period. Average percentage weight gain for

each group were as follows: group 1, 210%; group 2, 200%; group 3, 229%; and group 4: 243%.

Effect of feeding GlcNBu to OVX and sham OVX rats on bone mineral of spines and femurs. As expected, ovariectomy resulted in lower BMD and BMC of spines (Fig 1). Within the groups of OVX rats, GlcNBu treatment preserved spinal BMC and BMD compared with Glc-treated rats.

For the BMC data, 2-way ANOVA with Bonferroni post hoc test considered the interaction between the GlcNBu and OVX to be significant ($P < 0.05$). In effect, the ovariectomy and drug treatment in combination preserved the BMC (Fig 1, A).

For the BMD data (Fig 1, B), the 2-way ANOVA with Bonferroni post hoc test did not consider the interaction between GlcNBu and OVX to be significant. However, both the effects of drug treatment and ovariectomy were significant ($P < 0.05$) and, as seen from Figure 1, B, BMD appears to be preserved.

For the femurs, the slightly higher values in the OVX GlcNBu-treated groups compared with the OVX Glc-treated group, or other comparisons did not reach statistical significance in the 2-way ANOVA (data not shown).

Effect of feeding GlcNBu to OVX and sham OVX rats on the biomechanical properties of spines and femurs. For the biomechanical properties of the spines, the only results reaching significance were from the vertebral compression in the normalized data (Fig 2). Two-way ANOVA with Bonferroni post hoc test suggested that the interaction between GlcNBu and OVX was not significant. The effect of GlcNBu alone was not significant, but the effect of the OVX was extremely significant ($P < 0.0001$). From Figure 2, it can be seen that, although the drug does not preserve fully the energy to failure in the vertebrae of the OVX rats (group 4), there is a partial effect in that direction.

For Figure 3, energy to failure of the femora during 3-point bending (nonnormalized data) was preserved for the OVX group. Interestingly, the magnitude of the energy to failure for the OVX group resulting from the GlcNBu treatment was even greater than either treatment group of the sham OVX rats. When the data are analyzed by 2-way ANOVA with Bonferroni post hoc test, the interaction between the GlcNBu and OVX was significant ($P < 0.05$), but the effect of either GlcNBu or OVX alone was not.

For femoral ultimate displacement (Fig 4, A), GlcNBu treatment preserved this biomechanical property in the OVX rats (group 4). Analysis of the data by 2-way ANOVA with Bonferroni post hoc test considered the interaction between the drug and OVX to be very significant ($P < 0.005$), but the effect of either GlcNBu or OVX alone was not.
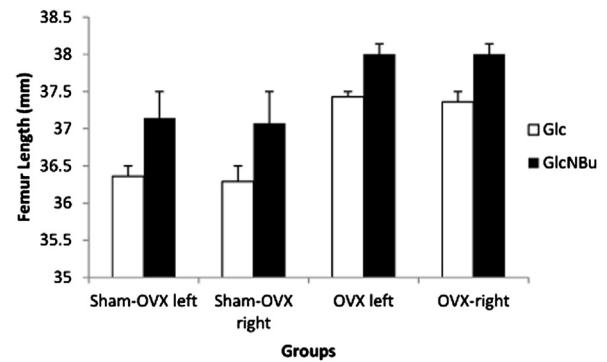

Fig 5. Right and left femoral lengths of ovariectomized (OVX) and sham OVX treatment groups. At sacrifice (6 months), right and left femurs were dissected and cleaned carefully. For the direct length measurements, the femurs were traced on paper and the lengths determined using a micrometer to 0.1 mm. Both drug and ovariectomy appear to have stimulatory effects on femoral length. Glc, glucose; GlcNBu, N-butyryl glucosamine.

Similarly, for ultimate strain (Fig 4, B), GlcNBu treatment preserved this biomechanical property in the OVX rats (group 4). Analysis of the data by 2-way ANOVA with Bonferroni post hoc test considered the interaction between GlcNBu and OVX to be very significant ($P < 0.005$), but the effect of either GlcNBu or OVX alone was not.

To understand more completely the effects of GlcNBu treatment on femoral size of both the sham OVX and OVX groups, we compared the femoral lengths of the 4 groups obtained by direct measurement of the excised bones (Fig 5). The ovariectomy itself resulted in increased femoral length. Nevertheless, feeding GlcNBu resulted in increased femoral length in both the OVX and sham OVX animals. The weights of the femurs demonstrated a similar pattern to the lengths (t test trends, data not shown).

DISCUSSION

The effect of GlcNBu in preserving bone mineral and some of the biomechanical properties of bone becomes significant only in the OVX animals (group 4). Thus, it appears that the protective effects of GlcNBu on osteoporotic bone are expressed, or at least magnified, by ovariectomy. The biomechanical properties in the OVX GlcNBu-treated vs the OVX Glc-treated groups showed better preservation in both the vertebrae (increase in energy to fail) as well as the femurs in the 3-point bending experiments (energy to failure, ultimate strain, and ultimate displacement). Thus, the effect of GlcNBu during ovariectomy on both vertebral, mostly cancellous, and femoral, mostly cortical, bone suggests that GlcNBu extends protection to both types of bone.

The relatively high performance of GlcNBu in the OVX group 3-point bending experiments (eg, energy

to fail) is particularly interesting. Here, the protective effect of this compound reaches or exceeds the properties of the sham OVX controls (Figs 3 and 4). It is possible that this high performance of the femurs may be related to a growth stimulatory and, in general, anabolic effect of GlcNBu. This type of model, with a 6-month experimental period, includes time frames when the animal is younger as well as time frames when it is more mature. An anabolic compound may demonstrate a growth stimulatory effect to a relatively greater extent during earlier time frames, which may be advantageous, because the effect during earlier time frames would be included in measurements done when the animal is sacrificed at 6 months and is less likely to be missed. An anabolic effect of GlcNBu is supported by previous experiments with this compound. A large number of genes are upregulated by GlcNBu in a chondrocyte system,[8] and we have shown that young rats fed GlcNBu had a positive, dose-dependent effect on growth, compared with Glc, whereas equimolar amounts of GlcN were growth inhibitory (unpubl.). The growth stimulatory effect of GlcNBu on femurs is illustrated clearly in Figure 5, in which direct measurement of femoral length is increased by GlcNBu. However, these measurements show increases in femoral length in both the sham OVX group as well as the OVX group, although the absolute increases in length are greatest in the OVX group.

To understand these findings better, one needs to first consider that certain bones in the rat skeleton retain lifelong growth and, in general, bone modeling (growth) dominates early after OVX.[20] This is followed later by remodeling, depending on the site.[20] However, in later stages of estrogen deficiency, impaired osteoblast function may be a problem. In general in the rat skeleton, cortical bone gain is in the periosteum and cortical loss is in the endosteum. For example, relatively young rats (240 g in weight) were used to demonstrate parathyroid hormone effects on cortical bone.[21] These relatively young OVX rats also demonstrated increased cortical bone mass in vehicle-treated controls, which was the result of a marked increase in periosteal bone formation.

Age plays a critical role in the biomechanics of long bones. A study of the mechanical properties of cortical bone (mid shaft of femur) in children and adults[26] found that, although the young bone specimens were weaker and less stiff, they deflected more and absorbed more energy before failure than the adult specimens. In a more recent direct comparison of child and adult cortical bone tissue (femoral shaft from cancer patients), it was found that the cortical bone from children had higher compressive ultimate strain (+24%) than adult cortical bone.[27] Our results on the preservation of the mechanical properties of the 3-point bending of the femoral shaft in the OVX GlcNBu-treated group are reminiscent of this property in childhood bone. However, we found no difference in the modulus of elasticity among the groups in the femoral shafts of our rat model. Also, a recent study on the mineral-to-matrix ratio in rats age 6 weeks–36 weeks (approximately corresponding to our experimental model) showed progressive increases in the ultimate load to failure of the femurs, which correlated with increases in this ratio with maturation.[28]

From a molecular perspective, bone morphogenetic protein (BMP) and its receptor systems play a key role in matrix synthesis. Synergistic effects of different type I BMP receptors on alkaline phosphatase induction have been reported,[29] and autogenous stromal bone marrow cells transfected with human BMP-2 promote bone formation in osteoporotic rats.[30]

In a detailed histologic study of the effect of 17β-estradiol directly on the distal femoral diaphyseal trabecular bone was evaluated[31] using OVX and control rats of a similar age to ours. A large increase in osteoblast number and osteoid surface of the trabecular bone of femurs was found as a result of the ovariectomy. For our system, bone appositional rate studies (tetracycline labeling) and similar histomorphometric studies, too,[31] are needed at different times during the 6-month experimental period. Also, a comparative study with older animals using both preventive and curative (established osteoporosis) protocols would be useful in clarifying whether the effects we observed with GlcNBu are related to animal age. This type of model, with older animals, may also be of interest in exploring the relationship of subchondral bone and cartilage in osteoarthritis, based on what we have observed for the inflammatory arthritis model.[15]

It has not been determined whether one should classify the synthetic N-acyl glucosamines, which have acyl chains other than acetyl, as pharmaceuticals or nutriceuticals. The potential for biologic synthesis of some of the GlcNAcyls exists through the GCN5-related N-acetyltransferases superfamily, which contains more than 10,000 members.[32] So, it may be also possible to consider GlcNBu, and other GlcNAcyl with naturally occurring acyl-coenzymes A as their substrates, as being types of nutriceuticals. Nevertheless, the compounds shown in Table I are, in general, not known to occur in nature as free monosaccharides.

GlcNBu has also been manufactured commercially and we achieved proof-of-concept to current good manufacturing standards. Cost of manufacturing is relatively low and is competitive with other glucosamines.

The safety profile GlcNBu appears to be very good because we have not seen toxicity in rodents or dogs. Although, very large doses of GlcNBu fed acutely to

rats showed no toxicity, formal subchronic dosing toxicology studies in animals have not been completed, and GlcNBu has not been fed to humans. We anticipate both veterinary and human applications for GlcNBu and related compounds.

The mechanism of action of GlcNBu in preserving cartilage and bone, and upregulating certain genes has not been elucidated. In terms of a translational strategy, our approach was to test our compound in animal models relatively early in its development and then look for a mechanistic "target." This is also a relatively inexpensive strategy suited to a university laboratory. It is greatly facilitated by a straightforward synthesis of the GlcNAcyls (Table I), an inexpensive, naturally occurring parent compound (GlcN), which can be purchased in high purity in bulk. This is quite a different strategy than usual pharmaceutical drug development. It appears that consumers currently are increasingly treating themselves with naturally sourced supplements, largely as a result of the mistrust of medications,[33] similar to what we found earlier for the use of glucosamine in bone health.[1]

Speculations. The biological properties of GlcN are altered fundamentally if the *N* group is acetylated naturally or chemically to yield GlcNAcyl. However, the *N* group of GlcN can be also modified chemically by other *N*-acylations to give new compounds. One of these, the butyrylated derivative GlcNBu, protects bone from becoming osteoporotic in the OVX rat model. This likely occurs, at least in part, by an anabolic effect on bone matrix, which is linked closely to matrix mineralization. We suggest that a number of these synthetic GlcNAcyls can be positioned between pharmaceuticals and nutriceuticals, and could have potential applications either in bone health (prevention) or treatment of osteoporosis. These compounds would be selected by having the potential of being synthesized biologically, through their cognate donor acyl-coenzyme A substrates. This implies the development of a class of compounds with possibly low toxicity, as appears to be the case for GlcNBu. Furthermore, we develop a paradigm, in translational research, in which specific animal models would be used relatively early during the development of such a class of compounds. This is possible because toxicity of these selected GlcNAcyls is likely to be low. Also, their chemical synthesis is straightforward and the naturally occurring parent compound (GlcN) is inexpensive and available in bulk quantities in high purity. In this paradigm, the elucidation of specific mechanisms of action (targets) can follow, establishing proof-of-principle for the animal model in question.

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