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Original Article

Hylonome

Galnon, a galanin receptor agonist, improves intrinsic cortical bone tissue properties but exacerbates bone loss in an ovariectomised rat model

H.W. McGowan, J.A. Schuijers, B.L. Grills, S.J. McDonald, A.C. McDonald

La Trobe University, Department of Human Biosciences, La Trobe University, Bundoora, Australia

Abstract

Objectives: Previous studies have shown galanin (GAL) injections onto mouse calvaria increased bone thickness and osteoblast number. This study investigated the effects of the GAL receptor agonist galnon on bone loss using the ovariectomised (OVX) rat model. **Methods:** OVX rats were treated with either vehicle or galnon for 6 weeks via mini-osmotic pumps. Plasma osteocalcin concentrations, osseous cell gene expression, morphological and biomechanical properties of the skeleton were compared between the two groups. **Results:** Treatment with galnon increased RANKL:OPG gene ratio ($p<0.001$) plus expression of TNF-α ($p<0.05$) and cathepsin K ($p < 0.05$). μCT analyses revealed galnon-treated OVX animals had reduced trabecular and cortical morphology compared to control animals. Biomechanically, galnon OVX animals required similar peak force to failure to that of control OVX animals although galnon treatment did enhance the mechanical properties of Young's modulus and ultimate tensile stress. **Conclusions:** Our research suggests that galnon, a GAL receptor agonist, may enhance osteoclastic bone resorption in OVX rats. Although galnon reduced bone volume, biomechanical testing revealed that bone of galnon-treated animals was mechanically superior per unit area. Taken together, galnon simultaneously improves the intrinsic quality of cortical bone whilst stimulating osteoclastic activity in the OVX rat model.

Keywords: Galnon, Galanin, Bone, Ovariectomy, Biomechanics

Introduction

Osteoporosis is a debilitating bone disease that is characterised by low bone mass and susceptibility to fractures. It is estimated that 3 million Australians will have osteoporosis by 2021 with associated fractures occurring every 3.5 min. In 2001, the direct costs of osteoporosis in Australia amounted to $1.9 billion (approximately 1.2% of the GDP)[1] and 2010 estimates of the annual medical costs associated with osteoporosis in the United States range from 14 to 20 billion dollars[2]. The ovariectomised rat (OVX) is a common model used to investigate the effects of oestrogen deficiency in bone. The skeletal responses are similar to those in post-menopausal women and therefore OVX rats are considered a gold standard model for evaluating drugs for prevention and reversal of osteoporosis[3-8].

The main cause of osteoporosis in post-menopausal women is decreased sex-hormones, especially oestrogen[9]. A decrease in oestrogen causes changes in various cytokine concentrations in plasma, increasing bone resorbing factors such as interleukin-1 beta (IL-1β), tumour necrosis factor-alpha (TNF-α) and receptor activator of nuclear factor kappa-B ligand (RANKL), while decreasing osteoclastogenesis-inhibiting factors such as osteoprotegerin (OPG)[10,11]. This leads to an increase in activity and number of osteoclasts compared to osteoblast activity, resulting in an overall loss of bone. As plasma concentrations of oestrogen fall, so too do plasma concentrations of galanin (GAL)[12]. GAL is a naturally occurring neuropeptide[13] which inhibits formation of 1L-1β and TNF-α, both of which have been shown to be directly associated with osteoclast formation and the development of osteoporosis. Pre-

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Corresponding author: Aaron McDonald, PhD. La Trobe University, Department of Human Biosciences, Faculty of Health Sciences, Health Sciences Building 2, Room 437, La Trobe University, Bundoora 3086, VIC, Australia
E-mail: A.McDonald@latrobe.edu.au

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vious studies have shown that GAL application causes increased bone thickness and osteoblast number following localised injections onto mouse calvaria[14] and hence may have clinical possibilities for diseases such as osteoporosis; however, a limitation may be its short life span in plasma[15]. Alternatively, galnon, a non-peptide GAL receptor ligand has an advantage over ordinary peptide ligands as it is more stable and can be administered systemically[16]. It is capable of acting at each of the three known GAL receptors, GALR1, GALR2 and GALR3, with an affinity in the micromolar range[16]. GAL expression has been identified in all osseous cells[17] and expression of GAL and its 3 receptors have been identified in mouse calvaria[14]. Immunohistochemistry has also identified GAL and GALR1 proteins in osteoblasts[18]. Therefore it is possible that galnon may act directly on bone cells. In addition, galnon is a small molecule that can cross the blood-brain barrier, and thus access receptors located in the brain with potential for regulating hormone release (eg. growth hormone) and sympathetic outflow[16,19-21]. We are aware of no research linking GAL-signalling and the secretion of regulating hormones with respect to bone metabolism. Although most known effects of galnon are in accordance with those of GAL, there are contradictions which researchers must be aware of. For example, galnon inhibits appetite whereas GAL stimulates it[16]. In addition to galnon's affinity towards the three GAL receptors, evidence has been found to suggest galnon also may have additional routes of action which must be considered when interpreting results[16,22]. As previous findings in this laboratory suggested a positive influence of GAL on osteoblastic activity[14], the aim of this current study was to administer exogenous galnon to OVX rats in order to determine if galnon could improve osteoblast bone formation and offset the bone degeneration which occurs in osteoporosis.

Materials and methods

This project was approved by the La Trobe University Animal Ethics Committee (AEC09-05-H).

Preliminary histomorphological measurements (not shown) between sham-operated and ovariectomised rats determined that the surgical ovariectomy was responsible for the development of an osteopaenic phenotype. This effect has been thoroughly investigated by other researchers[23,24]. Therefore, the following methods and results compare an ovariectomised-vehicle group to an ovariectomised-galnon treated group.

Rat surgery and tissue collection

Sixteen, 14-week-old female Sprague-Dawley rats were randomly divided into an ovariectomy (OVX) + vehicle (OVXveh) group and an OVX+galnon (OVXgaln) group (n=8 (per group)). The vehicle consisted of 10% DMSO in 0.9% NaCl, whilst the galnon group received 0.2 mg/kg/day galnon (Bachem, Bubendorf, Switzerland) in vehicle. Both vehicle and galnon were delivered over 6 weeks via a single "Alzet 2006" mini-osmotic pump per animal (Durect Corporation, USA) subcutaneously implanted between the shoulder blades on day of surgery. Ovariectomy was performed according to the method described in Waynforth[25].

Following a 42-day treatment regimen plasma was collected at necropsy and stored at -20°C for subsequent ELISA analysis. All soft tissue was removed from the left femur, the bone submerged in RNAlater and stored at 4°C for analysis of gene expression. The right humerus was removed and stored whole in silicone oil at -20°C for biomechanical analysis. The right femur was cleaned, placed in a 4% paraformaldehyde solution for 48 h, washed three times in 0.1 M sodium cacodylate buffer and stored at 4°C in ethanol prior to μCT analysis.

Osteocalcin concentration in plasma

Plasma osteocalcin concentrations were analysed using a rat osteocalcin ELISA kit (Biomedical Technologies Inc. Stoughton, MA, USA) specific for full-length rat osteocalcin, both carboxylated and decarboxylated. Rat plasma was diluted 1:10 with supplied sample buffer and absorbance was measured at 450 nm.

RT-qPCR

Approximately 200 μg of proximal whole femur was snap-frozen in liquid nitrogen, homogenised with PureZOL and RNA extracted using the Bio-Rad Aurum™ Total RNA Fatty and Fibrous Tissue Kit via the supplied protocol (Bio-Rad Laboratories), with purity of RNA validated with $A_{260/280}$ ratios using a NanoDrop 2000 (Thermo Scientific). Resulting samples were then reverse transcribed to cDNA using the iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories). qPCR was run over 55 cycles using the iCycler iQ Multi-Colour Real Time PCR detection system (using SsoFast EvaGreen) with melt-curve analysis performed post-cycling to establish specificity of DNA products. The primer sequences for genes of interest are shown in Table 1. Primer sequences were prepared commercially by GeneWorks Pty Ltd (Adelaide, Australia) and determined using the PubMed genome sequence search (GenBank) and primers designed using the Beacon Designer 2.0 software (Biosoft International, PaloAlto, CA, USA). β-actin was used as an internal reference for each sample. Using the Pfaffl method[26], gene expression was normalised to the β-actin mRNA level and presented as relative expression.

Microcomputed tomography analysis

Three-dimensional (3D) bone images of the distal half of femurs from both rat groups were generated (2234 slices; 9.06 μm/slice) using the microcomputed tomography (μCT) scanner SkyScan 1076 (Kontich, Belgium). The range of interest (ROI) for trabecular bone was 0.77 mm (85 image slices) from the growth plate and was measured for 3 mm. The ROI for cortical bone commenced at 4.05 mm (450 image slices) from growth plate and was measured for 0.5 mm. Scanning was conducted at 70kV and 100 μA (using a 1 mm aluminium filter and rotation step of 0.5 degrees) with a spatial resolution of approximately 9 μm/pixel. Images were reconstructed using NRecon, aligned with DataViewer software and calculation of structural indices performed using a 3D image analysis system,

Primer sequences used in qPCR		
Gene	**Forward Sequence**	**Reverse Sequence**
Genes of interest		
TNF-α	5'-GAACAACCCTACGAGCACCT-3'	5'-TCAGGTCATCACTATCG-3'
IL-1β	5'-CATTGTGGCTGTGGAGAAG-3'	5'-ATCATCCCACGAGTCACAGA-3'
OPG	5'-GCCAACACTGATGGAGCAGAT-3'	5'-TCTTCATTCCCACCAACTGATG-3'
RANKL	5'-GCTCACCTCACCATCAATGCT-3'	5'-GGTACCAAGAGGACAGACTGACTTTA-3'
COL1α1	5'-GCGAAGGCAACAGTCGATTC-3'	5'-CCCAAGTTCCGGTGTGACTC-3'
CTHSPK	5'-TGTCTGAGAACTATGGCTGTGG-3'	5'-ATACGGGTAAGCGTCTTCAGAG-3'
GAL	5'-AGGCAAGAGGGAGTTACCACT-3'	5'-GGTGGCCAAGGGGATG-3'
GALR1	5'-CCCCATCATGTCATCCACCT-3'	5'-ATGGGGTTCACCGAGGAG TT-3'
GALR2	5'-CATCGTGGCGGTGCTTTT-3'	5'-AGCGGGAAGCGACCAAAC-3'
GALR3	5'-CTCATCCTCTGCTTCTGGTAC-3'	5'-GAGTAGACGAGCGGGTTAAG-3'
Reference Gene		
β-actin	5'-ATTGTAACCAACTGGGACG-3'	5'-TCTCCAGGGAGGAAGAGG-3'

Table 1. Primer sequences used in qPCR. Genes of interest were tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), osteoprotegerin (OPG), receptor activator of nuclear factor kappa-B ligand (RANKL), collagen type 1α1 (COL1α1), cathepsin K (CTHSPK), galanin (GAL), galanin receptor 1, 2 and 3 (GALR1, GALR2, GALR3). Reference gene was beta-actin (β-actin).

CTAn software.

Analysed tissue included percent of trabecular bone volume per total volume (BV/TV (%)), trabecular thickness (Tb.Th (mm)), trabecular number (Tb.N (1/mm)) and trabecular separation (Tb.Sp (mm)) of the distal femur plus cortical volume (mm^3) and periosteal circumference (mm) of the mid-femur.

Bone biomechanics

The cortical bone strength of the mid-shaft rat humerus (n=8) was tested using a three-point bending apparatus attached to a PC with the Crusher 3-point bending software installed (La Trobe University Technical Support, Australia). Bones were loaded at a rate of 1 mm/sec until fracture occurred or the force lever reached its maximum displacement. Displacement (mm) and force (N) were recorded. Fractured surfaces of both ends of biomechanically-tested bones were imprinted into dental wax. Wax sections were viewed under a Leica Microsystems DM-RBE microscope at 25x magnification attached to a Leica DFC490 camera, with cortical area (mm^2) determined by measurement of outer and inner bone areas under Leica Image Manager software. Young's Modulus, the intrinsic stiffness, determined by $E=(F/Y) \times (\pi L^3/12(A_o^2-A_i^2)^{27}$; ultimate tensile stress, the maximum stress which can be applied to the bone prior to fracture, determined by $\sigma = (F.L.A_t^{1/2})/(\pi^{1/2}(A_o^2-A_i^2))^{27}$; and toughness, the measure of the bone's ability to resist fracture, given by the area under the force displacement curve[28] were all calculated.

Statistical analysis

All values have been expressed as mean ± standard error of the mean (S.E). Two-tailed unpaired t-tests were performed with level of significance set at $p<0.05$.

Results

Plasma osteocalcin concentrations

Analysis using a rat osteocalcin ELISA kit revealed no differences in plasma osteocalcin concentrations between the OVXveh and OVXgaln groups (Figure 1).

qPCR

There was no change in IL-1β expression between the groups (Figure 2A) whereas OVXgaln upregulated TNF-α expression with respect to OVXveh ($p<0.05$) (Figure 2B).

OVXgaln treated animals showed a significant increase in RANKL expression vs OVXveh ($p<0.05$), a significant decrease in OPG ($p<0.01$) and a significant increase in the RANKL: OPG ratio ($p<0.001$) (Figure 3A-C).

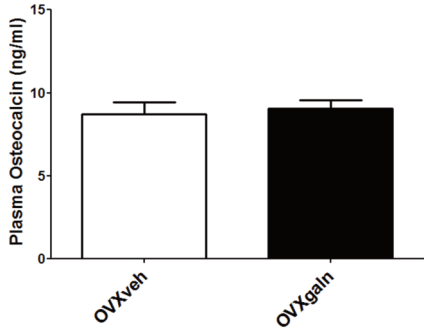

Figure 1. No significant change in plasma osteocalcin concentration between vehicle (OVXveh) and galnon (OVXgaln) treated ovariectomised animals (n=8).

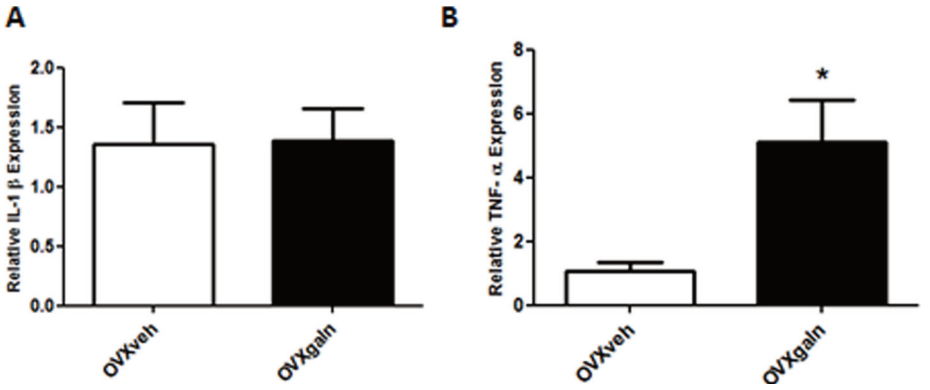

Figure 2. qPCR analyses of proximal femur of vehicle (OVXveh) and galnon (OVXgaln) treated ovariectomised rats. (A) Expression of IL-1β was unchanged with galnon treatment. (B) TNFα expression significantly increased in galnon treated rats relative to vehicle treated rats (*p<0.05). Gene expression relative to reference gene, β-actin (means±S.E.) (n=6).

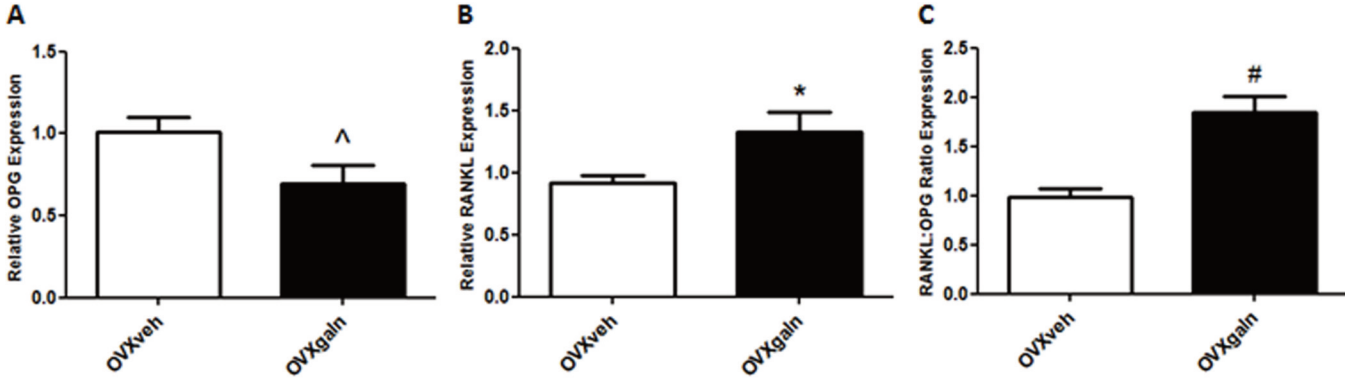

Figure 3. qPCR analyses of proximal femur of vehicle (OVXveh) and galnon (OVXgaln) treated ovariectomised rats. (A) RANKL expression significantly increased with galnon treatment (*p<0.05) whereas (B) expression of OPG significantly decreased in galnon treated rats relative to vehicle treated rats (^p<0.01). Therefore, the (C) RANKL:OPG ratio was significantly higher in galnon treated rats (#p<0.001). Gene expression relative to reference gene, β-actin (means±S.E.) (n=6).

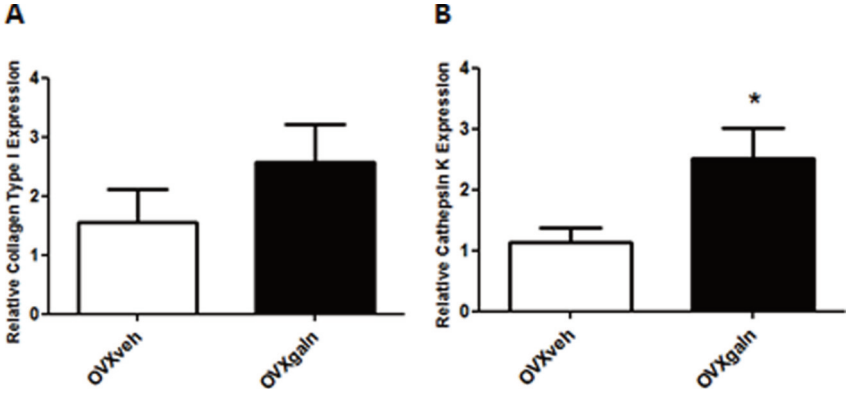

Figure 4. qPCR analyses of proximal femur of vehicle (OVXveh) and galnon (OVXgaln) treated ovariectomised rats. (A) Expression of collagen type 1α1 (osteoblast marker) was greater in the galnon treated rats relative to vehicle treated rats but this was not statistically significant whereas (B) Cathepsin K expression (osteoclast marker) significantly increased with galnon treatment (*p<0.05). Gene expression relative to reference gene, β-actin (means±S.E.) (n=6).

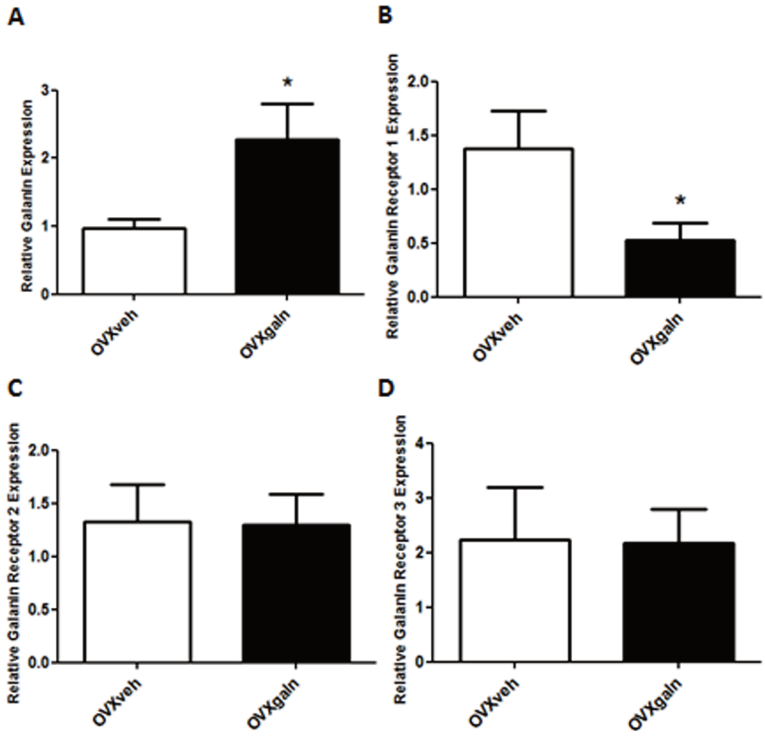

Figure 5. qPCR analyses of proximal femur of vehicle (OVXveh) and galnon (OVXgaln) treated ovariectomised rats. (A) Expression of galanin significantly increased in galnon treated rats relative to vehicle treated rats (*p<0.05). (B) Galanin receptor 1 expression significantly decreased with galnon treatment (*p<0.05) however expression of galanin receptors 2 (C) and 3 (D) I did not vary between galnon treated rats relative to vehicle treated rats. Gene expression relative to reference gene, β-actin (means±S.E.) (n=6).

The analysis of collagen type I expression as an osteoblast activity marker yielded no significant differences between the two groups. The expression of the osteoclastic marker cathepsin K was, however, significantly increased in OVX mice treated with galnon relative to OVXveh treated mice (p<0.05) (Figure 4A-B).

There are three known GAL receptors (GALR1, 2 & 3) and galnon is capable of binding to all three. The action of each receptor varies dramatically, therefore the expression of GAL and all three of its receptors were analysed using qPCR. Although no change in expression of the GALR2 and GALR3 receptors was recorded, GAL expression increased significantly and GALR1 expression decreased significantly in OVXgaln treated rats compared to OVXveh rats (p<0.05 for both) (Figure 5A-D).

Microcomputed tomography analysis

Figure 6A-E shows results of μCT analysis of the distal femur of OVXveh and OVXgaln treated rats. OVXveh values equate with other μCT measurements undertaken on ovariectomised rats over a similar treatment period[29,30]. As indicated, there is a significant (p<0.001) decrease in trabecular bone volume in OVXgaln treated rats (7.8 %BV/TV±1.6 %BV/TV) compared to OVXveh rats (13.7 %BV/TV±0.7%BV/TV). Galnon treatment also significantly (p<0.01) decreased trabecular number (1.1 1/mm±0.2 1/mm vs 1.9 1/mm±0.1 1/mm). Trabecular thickness values were approaching significance (p=0.058) with galnon treatment decreasing thickness compared to OVXveh treated rats (0.068 mm±0.001 mm vs 0.071 mm±0.001 mm) however no statistically significant values were recorded for trabecular separation.

Figure 7A-C represents μCT analysis of the cortical bone mid-femur. Cortical volume was significantly decreased (p<0.001) in OVXgaln treated rats versus OVXveh rats (5.6 mm^3±0.08 mm^3 vs 6.2 mm^3±0.09 mm^3). This decrease in cortical volume is most likely a result of a decrease in the periosteal circumference of the mid femur (11.19 mm±0.13 mm vs 11.77 mm±0.08 mm; p<0.005) as the endosteal surface area was unchanged (data not shown).

Biomechanics

The biomechanical measurements that characterise bone fragility can be used to determine both the extrinsic and intrinsic properties of bone (Figure 8A-F). The extrinsic measurement of bone brittleness is measured by ultimate displacement. As the ultimate displacement of the galnon-treated OVX bone is significantly higher than OVXveh (0.51 mm±0.03 mm vs 0.40 mm±0.01 mm; p>0.005) the bone can be considered to be less brittle. The peak force to failure, however, did not vary between groups. The intrinsic stiffness (Young's modulus) of the

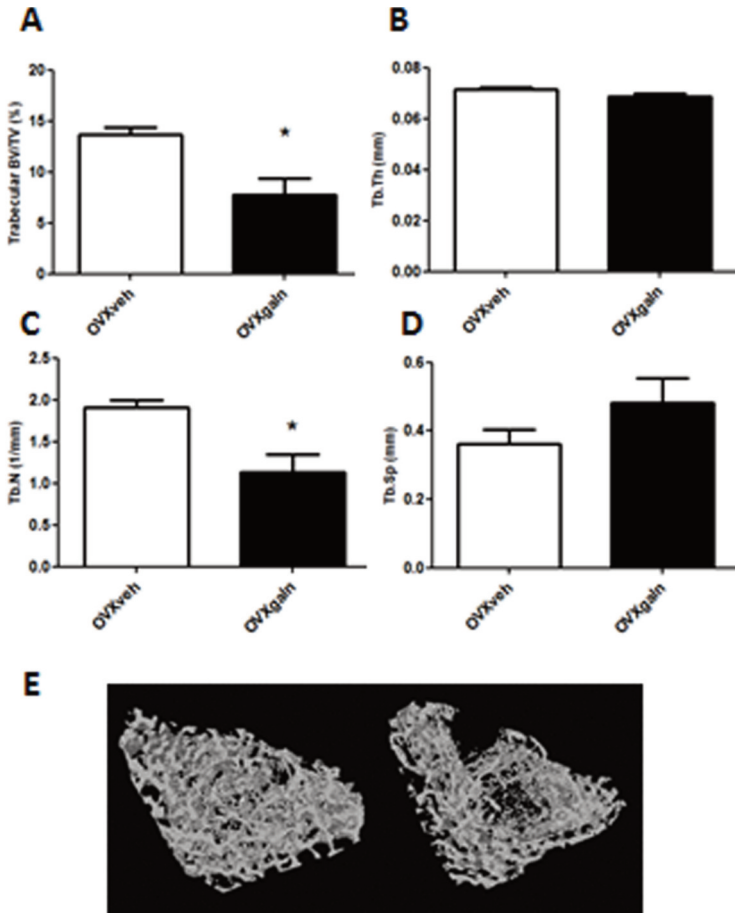

Figure 6. μCT analysis of distal femur of vehicle (OVXveh) and galnon (OVXgaln) treated ovariectomised rats. Results indicate a decrease in (A) trabecular bone volume/total volume (trabecular BV/TV) (*p<0.01). Trabecular thickness (Tb.Th; B) was approaching significant difference (p=0.058) and (C) trabecular number (Tb.N) decreased in OVXgaln treated animals (*p<0.01). There was no difference evident in trabecular separation (Tb.Sp; D) (n=5). (E) Representative μCT photos of distal femur of vehicle (OVXveh) and galnon (OVXgaln) treated ovariectomised rats.

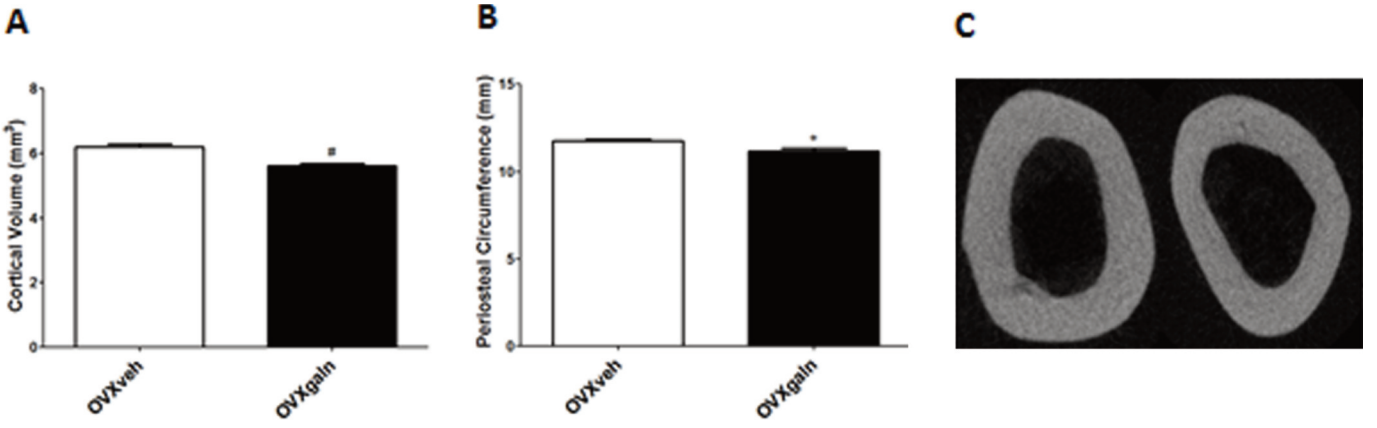

Figure 7. μCT analysis of mid-femur of vehicle (OVXveh) and galnon (OVXgaln) treated ovariectomised rats. Results indicate a decrease in (A) cortical volume and (B) periosteal circumference in OVXgaln treated animals compared to OVXveh rats (#p<0.001; +p<0.005) (n=5). (C) Representative μCT photos of mid-femur of vehicle (OVXveh) and galnon (OVXgaln) treated ovariectomised rats.

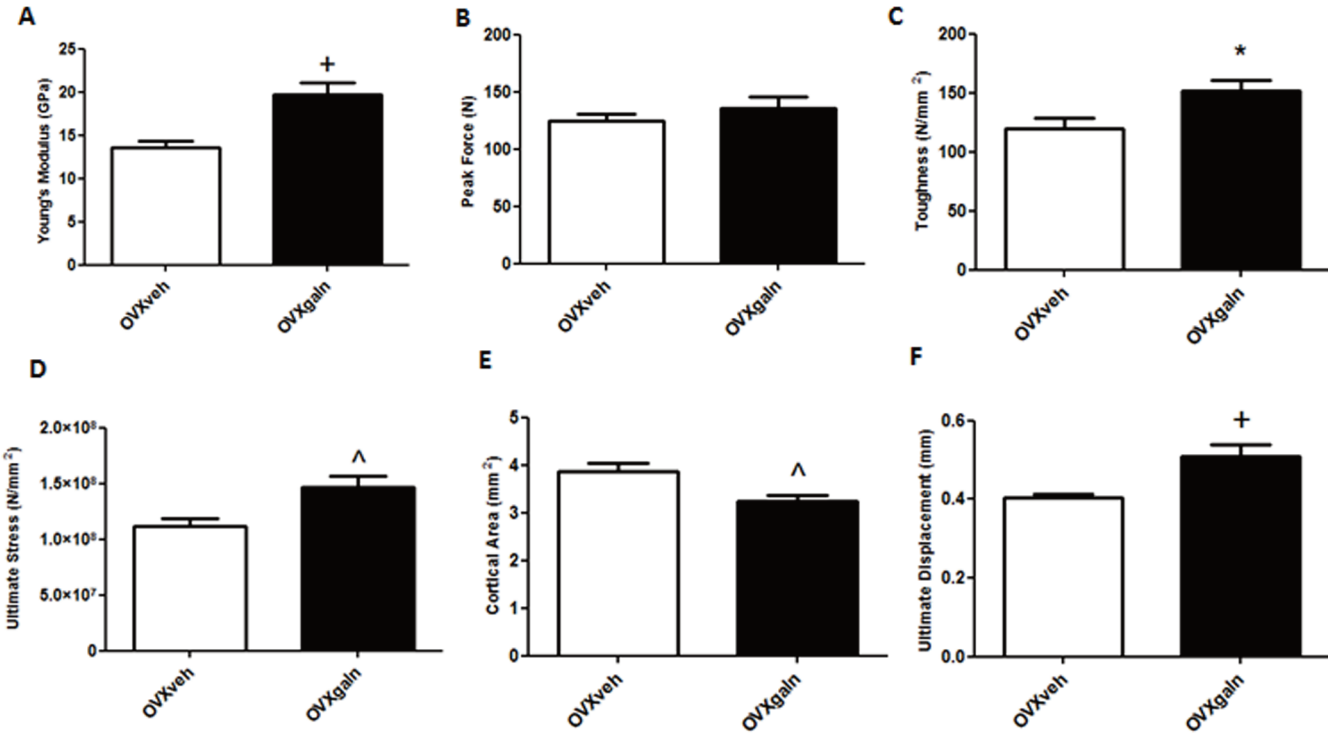

Figure 8. Biomechanical analysis of humerus of vehicle (OVXveh) and galnon (OVXgaln) treated ovariectomised rats. Results indicate an increase in (A) Young's modulus, (C) toughness (D) ultimate stress and (F) ultimate displacement plus a decrease in (E) cortical area (+$p<0.005$; *$p<0.05$; ^$p<0.01$). No significance was recorded for peak force (B) between the groups. (n=8).

galnon treated OVX bone was significantly higher (19.7 GPa±1.36 GPa vs 13.6 GPa±0.68 GPa; $p>0.005$) and the ultimate stress (intrinsic strength) of the bone was also significantly higher in the OVXgaln treated rat humerus relative to the OVXveh bone (1.50×10^8 N/m^2±9.2×10^6 N/m^2 vs 1.12×10^8 N/m^2±7.7×10^6 N/m^2; $p>0.01$). Although there was no change in peak force the intrinsic resistance to fracture, indicated by toughness[31], was significantly greater (152 J/m^2±9.1 J/m^2 vs 119 J/m^2±9.2 J/m^2; $p>0.05$). Although these results indicate a less brittle and tougher bone with galnon treatment, the actual amount of cortical bone (cortical area) was significantly smaller (3.24 mm^2±0.13 mm^2 vs 3.88 mm^2±018 mm^2; $p>0.01$).

Discussion

Previous research performed by this lab indicated that GAL injections onto mouse calvaria stimulated osteoblast number and activity[14]. The non-peptide GAL receptor agonist galnon is of interest as an alternative to GAL due to its stability and ability to cross the blood-brain barrier[16]. Our lab therefore considered galnon as a potential intervention for disorders such as osteoporosis, where osteoclastic activity is greater than osteoblastic activity, resulting in bone loss. However, results from this research indicate that systemically administered galnon (0.2 mg/kg/day for 6 weeks) may enhance osteoclastic activity, resulting in further loss of bone in the ovariectomised rat.

The OVX rat is a recommended and most frequently used animal model for studying human postmenopausal osteoporosis because of many similarities in their pathophysiological mechanisms[5,32-34]. The currently accepted mechanism for osteoporosis is an imbalance in bone turnover, with bone resorption surpassing bone formation. Although bone formation markers such as osteocalcin and alkaline phosphatase increase in concentration in ovariectomised mice[34], bone resorption markers (such as collagen type I CTX) also increase with a consequential loss in cortical and cancellous bone[5,34].

Osteocalcin assays can be used as indicators of both osteoblastic activity (full-length osteocalcin)[35] and osteoclastic activity (by measuring the fragments released from bone during resorption)[36]. As the osteocalcin assay kit used in this experiment measured full-length osteocalcin, by utilising the N-terminal and C-terminal of the osteocalcin in a sandwich assay, only intact osteocalcin was measured thus measuring osteoblastic, rather than osteoclastic, activity. Similar to other research[37], our previous experiments have found a significant increase in plasma osteocalcin concentrations in OVXveh compared to non-OVX rats (data not shown). However treatment with galnon did not influence osteocalcin concentrations relative to untreated OVX rats. While there have been no studies on the effect of GAL or galnon on osteocalcin levels, a localised injection of 20 ng GAL/day for 14 days increased osteoblast size and number and activity[14]. The absence of a

significant change in the plasma concentrations of osteocalcin and collagen I gene expression between the vehicle and galnon treated OVX rats suggest that 0.2 mg/kg/day galnon for 6 weeks had no effect on osteoblastic bone formation.

A number of genes have been shown to be responsible for mediating bone turnover. Increases in IL-1β, TNF-α and RANKL correlate with an increase in bone resorption[38,39], while increases in OPG inhibit bone resorption[40]. Galnon treatment of OVX rats showed an increased expression of TNF-α in bone compared to vehicle treated groups, seemingly inconsistent with our previous study which shows that GAL decreased TNF-α and IL-1β following subcutaneous injection onto mouse calvaria[14]. OPG and RANKL are derived from osteoblasts and are responsible for regulation of bone mass; RANKL stimulates bone resorption by activating osteoclasts, while OPG is a natural decoy-receptor for RANKL and works to prevent resorption[41,42]. The RANKL:OPG ratio of galnon treated OVX rats significantly increased indicating an increase in osteoclast activity. qPCR results in this experiment also indicate galnon treatment of OVX rats decreased GALR1 gene expression but had no influence on GALR2 and GALR 3 expression. GALR1 activation inhibits adenylate cyclase activity[43]. The isotype adenylate cyclase 3 has been shown to inactivate the osteoclastogenic transcription factor NFATc1[44]; the inhibition of GALR1 expression in OVXgaln treated rats may have resulted in enhanced osteoclastogenesis in these experiments. Overall, the increase in TNF-α, RANKL:OPG ratio and the osteoclastic marker cathepsin K suggest galnon stimulated osteoclastic bone resorption in the OVX rat. These findings also correlate with the μCT results.

Trabecular bone is readily resorbed in osteoporosis[45] and altered bone volumes can be easily identified between healthy and osteoporotic patients[46]. OVX reduces trabecular number and trabecular thickness and increases trabecular separation[47-49]. OVX reduces trabecular bone volume by up to 18% in proximal tibia eight weeks post-OVX[50], and previous experiments in this laboratory have found, via histological analysis, BV/TV (%) decreased after 6 weeks, as a result of the OVX surgery, by 9.3% ($p<0.05$) (data not shown). Consistent with the gene expression results, μCT measurements showed that trabecular bone volume, number and thickness plus cortical volume and periosteal circumference all decreased in galnon treated OVX rats compared to OVX vehicle treated rats.

Other studies have reported no discernible difference between biomechanical properties of cortical bone in rats at 6-weeks post-OVX[51]. Significant changes in our 6-week study were, however, observed in the rat humerus between OVXveh and OVXgaln groups. There are at least three ways to improve the strength of bone: (1) an increased bone mass, (2) a redistribution of bone mass more effectively and (3) an improvement of the material properties of the bone tissue. Depending on the various types of treatments for osteoporosis, the improvement in bone mass may be offset by detrimental effects to bone quality and architecture[52]. The extrinsic property (peak force) of the rat humerii was not significantly different between the groups, indicating both groups were able to withstand a similar force prior to fracture. The galnon-treated OVX bone was, however, found to have increased Young's modulus, toughness and ultimate stress properties indicating a greater intrinsic strength and improved tissue properties of the cortical bone. Although cortical bone area had decreased, most likely due to increased osteoclastic activity, the improved intrinsic biomechanical properties of galnon treated OVX bones suggests multiple actions of galnon on osseous cells. For a better understanding of how galnon improves the intrinsic properties of the cortical bone, *in vitro* OB research measuring the sub-nanostructures of bone (such as mineral, collagen, and non-collagenous organic proteins[53]) needs to be performed.

Previous studies utilising GAL applied the drug locally in a series of regular injections[14,54-56] whereas application of galnon in our experiments was introduced systemically and continuously. The continuous administration of galnon via miniosmotic pumps may have produced a different skeletal effect to intermittent, daily injections. This has previously been noted with parathyroid hormone which has been indicated as an anabolic agent following daily subcutaneous injections but catabolic following continuous administration via miniosmotic pumps[57,58].

Other possible explanations for the improved tissue properties of cortical bone with enhanced osteoclastic activity include GAL having been shown to have varying effects depending on whether it acts upon a specific GAL receptor (GALR1, GALR2 or GALR3; activating a variety of second messenger pathways) or a specific cell type[59,60]. For example, GALR1 and GALR3 have analgesic effects on nociception, while activation of GALR2 has a pro-nociceptive role[61]. GAL increases proliferation of lactotrophs in the anterior pituitary[62] but increases apoptosis of immature rat thymocytes[63]. To date only GAL and GALR1[18] proteins have been identified in osteoblasts, however GAL gene expression has been identified in all osseous cells[17] and expression of GAL and its 3 receptors have been identified in mouse calvaria[14]. All three GAL receptor subtypes are members of the G-protein coupled receptor superfamily. Each subtype has substantial signalling differences resulting in the diverse physiological effects of GAL. It is possible that this multiple signalling may be associated with bone cells. For example, GALR1 stimulates mitogen-activated protein kinase (MAPK)[64]. MAPK is shown to stimulate OB differentiation[65]; GALR2 mediates the release of Ca2+ into the cytoplasm[64]. Intracellular Ca^{2+} signaling is important for osteoclast differentiation[66]; GALR3 activates an inward K^+ current[64]. Inwardly rectifying K^+ current helps maintain membrane potential in osteoclasts[67]. These are all speculations and a number of experiments are required to provide evidence of how GAL influences osseous cells. Our current research into identifying the remaining GAL receptor proteins on osteoclasts and osteoblasts cultured from bone marrow, plus the effects of galnon on these cells *in vitro*, could distinguish which GAL receptor is activated and for which cell type.

In addition to the varying effects of specific GAL receptor activation, the actions of GAL and galnon have proven similar in various settings but contradictory in others, indicating galnon

may activate signalling systems additional and distant to GAL signalling systems[20,68]. For example, galnon is capable of binding to: a) serotonin receptors which are found on monocyte/macrophage cells[69], b) alpha-1 adrenergic receptors which are known to regulate osteoclast activity[70] and c) NPY receptors in which stimulation by NPY has an inhibitory effect on osteoclastogenesis[71]. Therefore further research into the effects of galnon on bone cells and specific receptor activation is needed.

Conclusion

In conclusion, this research indicates that the non-peptide galanin receptor agonist galnon may influence osteoclast activity during ovariectomy-induced osteoporosis. This may be indirect via stimulation of osteoblasts to increase RANKL expression, or a direct effect on osteoclasts. The loss in overall bone mass may, however, be overshadowed by the positive influence of galnon on the intrinsic biomechanical properties of cortical bone. Knowledge of the mechanisms by which galnon seems to increases osteoclastic activity and enhance the intrinsic cortical bone tissue properties may be better understood following selective bone cell culturing experiments and treatments with varying concentrations of galnon.

Contribution of authors

Heath McGowan and Aaron McDonald were the main contributors in all procedures undertaken and manuscript preparation. Stuart McDonald contributed to the μCT and biomechanical analyses. John Schuijers contributed to the qPCR analysis and rat surgery. Brian Grills contributed to the rat surgery.

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