It's about time: Circadian rhythm and metabolism

Schilperoort, M.

Loss of glucocorticoid rhythm induces an osteoporotic phenotype in mice

Maaike Schilperoort, Jan Kroon, Sander Kooijman, Cheraine C.E. Bouten, Ilse Gille, Kathrin Mletzko, Felix N. Schmidt, Hetty C.M. Sips, Leo van Ruijven, Björn Busse, Alberto M. Pereira, Natasha M. Appelman-Dijkstra, Nathalie Bravenboer, Patrick C.N. Rensen, Onno C. Meijer, Elizabeth M. Winter

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Abstract

Glucocorticoid (GC)-induced osteoporosis is a widespread health problem that is accompanied with increased fracture risk. Detrimental effects of GC therapy on bone have been ascribed to the excess in GC exposure, but it is unknown whether disruption of the endogenous GC rhythm inherent to GC therapy also plays a role. To investigate this, we implanted female C57Bl/6J mice with slow-releasing corticosterone (CORT) pellets to blunt the rhythm in CORT levels without inducing hypercortisolism. Flattening of the CORT rhythm for 7 weeks reduced cortical and trabecular bone volume and thickness, as determined by micro-CT analysis. Furthermore, TRAP levels were increased while P1NP levels were decreased in plasma of mice with a flattened CORT rhythm, indicative of a negative balance in bone remodeling. Double calcein labeling of bone *in vivo* revealed a reduced bone formation, as reflected by a reduced mineral apposition rate (MAR), mineralizing surface per bone surface (MS/BS) and bone formation rate per bone surface (BFR/BS). Collectively, these perturbations in bone turnover and structure decreased bone strength and stiffness, as determined by mechanical testing. In conclusion, we demonstrate for the first time that flattening of the GC rhythm results in an osteoporotic phenotype in mice. Our findings indicate that at least part of the fracture risk associated with GC therapy may be the consequence a disturbed GC rhythm, rather than an excess in GC dose alone.

Introduction

Glucocorticoids (GCs) are widely used to treat inflammatory and autoimmune disorders on account of their immunosuppressive properties [1]. Both short and long term use of GCs have been associated with serious side effects, among which the development of osteoporosis [2-6]. It is well described that the negative effects of GCs on bone are dose-dependent [4, 6]. However, the lack of GC rhythm, intrinsic to pharmacological GC administration, could be another potentially contributing factor.

In both mice and humans, circulating levels of GCs show a strong diurnal rhythm, with a peak just before onset of the active phase and a trough at the beginning of the resting phase [7]. This rhythm is regulated by the central circadian clock, also known as the 'biological clock', located in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN modulates the activity of the hypothalamus-pituitary-adrenal (HPA) axis, resulting in the rhythmic release of GCs by the adrenal cortex [7]. By acting through the glucocorticoid receptor (GR), GCs are key mediators of circadian rhythm in peripheral tissues, such as adipose tissue and liver [8, 9]. In addition, recent studies revealed a potential role for GCs in rhythmic bone remodeling. GC treatment induces rhythmic gene expression in osteoblasts and osteoclasts *in vitro* [10, 11]. and depletion of endogenous GCs by adrenalectomy blunts rhythm in osteoclast-related gene expression *in vivo* [10]. suggesting that GC rhythm regulates metabolic activity of bone.

Moreover, bone health is dependent on a regular day-night rhythm, as reflected in the association between chronic circadian disruption through shift work and osteoporosis in humans [12, 13]. We have recently demonstrated that shifting light-dark cycles negatively affects bone health in mice [14], demonstrating a causal relationship between circadian disruption and bone abnormalities. However, it is currently unknown whether a disrupted GC rhythm, which is also observed with GC therapy, could underlie these effects.

In this study we aimed to elucidate the importance of a diurnal rhythm in corticosterone (CORT), the primary GC in mice, for bone quality. We implanted mice with slow-releasing CORT pellets to flatten endogenous CORT rhythm, and demonstrate that a blunted CORT rhythm results in an osteoporotic phenotype.

Materials and methods

Animals

Mice were group housed (n = 4/5 per cage) under standard 12h:12h light:dark conditions (lights on at 7 h clock time), and were fed chow diet (Special Diet Services) *ad libitum*. To examine the effect of a flattened GC rhythm on bone metabolism, twelve-week-old female C57Bl/6J mice (Charles River Laboratories) were randomized to receive either vehicle (n = 10), 4.5 mg CORT (n = 15) or 7.5 mg CORT (n = 10) for a total period of 7 weeks. Vehicle or CORT (Sigma-Aldrich) was administered via subcutaneous implantation of slow-release pellets (100 mg in total, with cholesterol as vehicle. Every two weeks, pellets were replaced to ensure a continuous release of CORT. The continuous release of low dose CORT via pellets flattens endogenous CORT rhythm by providing negative feedback to the HPA axis, as previously described by others [15]. Fluorescent double labeling of bone was applied by injecting mice i.p. with 15 mg/kg calcein (C0875, Sigma-Aldrich) in 0.2% sodium bicarbonate 8 days and 2 days prior to sacrifice. At endpoint, mice were killed around 18 h clock time by CO_2 inhalation and perfused for 5 min with ice-cold PBS through the left ventricle of the heart. Tibiae were collected for gene expression analysis and mechanical testing, and femurs were collected for micro-CT analysis

and evaluation of calcein labeling. All mouse experiments were performed in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals after having received approval from the Central Animal Experiments Committee ('Centrale Commissie Dierproeven', the Netherlands).

Body weight and body composition measurements

At baseline and endpoint, body weight was measured with a scale and fat and lean mass were determined with an EchoMRI-100-analyzer.

Plasma biochemistry

To determine plasma CORT levels at indicated time points, blood was collected from the tail vein into capillaries within a time span of 2 min, before CORT levels rise due to stress of animal handling. Plasma CORT concentrations were measured by ELISA according to the manufacturer's protocol (corticosterone EIA, Immunodiagnostics). Additionally, blood was collected to measure plasma concentrations of procollagen type 1 amino-terminal propeptide (P1NP) and tartrate-resistant acidic phosphatase (TRAP) using enzyme immunoassay kits (IDS) according to manufacturer's instructions.

RNA isolation, cDNA synthesis and qRT-PCR

The proximal and distal ends of the tibiae were cut off, bone marrow was flushed out with cold PBS, and tibiae were stored in RNAlater Stabilization Reagent (Qiagen) before RNA isolation. For RNA isolation, tibiae were mechanically homogenised in TRIzol RNA isolation reagent (Roche Diagnostics) by using the Mikro-Dismembrator S (B. Braun Biotech International GmbH) in combination with a 5 mm stainless steel bead (Qiagen), and total RNA was isolated according to the manufacturer's instructions (Roche). 100-500 ng of total RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega), qRT-PCR was performed with a SYBR Green Supermix on a CFX96 PCR machine (Bio-Rad), and expression levels of genes of interest were normalized to expression of the housekeeping gene *β-actin*.

Micro-CT analysis

Micro-CT analysis was performed to evaluate structural changes of the bone. Bones were scanned with a microcomputed tomography system (μCT 40; Scanco Medical AG) using 55 kV, 145 μA, 600 ms integration time and a resolution of 10 μm. Image processing included Gaussian filtering and segmentation with σ=0.8, support 1, threshold 430 mg hydroxyapatite (HA)/cm^3 for trabecular parameters and 600 mg HA/cm^3 for cortical parameters, respectively. For trabecular bone, a total of 150 slides (1.5 mm) starting from 0.1 mm distal to the growth plate were analyzed. For cortical bone, 25 slides above and 25 slides below the exact midpoint of the bone were analyzed (total 0.5 mm). Trabecular and cortical volumes of interest (VOI) were chosen by visual inspection. The morphometry of cortical and trabecular bone was performed using the calibrated micro-CT software uct_evaluation v6.5-3 (Scanco Medical AG).

Calcein double labeling

After sacrifice, femurs were dissected, fixed with formalin, embedded in methyl methacrylate (MMA) and cut into 7 μm sections using a microtome with a tungsten steel D-profile knife. Bone was counterstained using calcein blue (M1255, Sigma-Aldrich), and sections were imaged using a Leica DM6B fluorescence microscope (Leica Microsystems). CalceinHisto open source software [16] was used to determine the mineral apposition rate (MAR), mineralizing surface per bone surface (MS/BS) and bone formation rate per bone surface (BFR/BS) of femoral trabecular bone using consistent thresholds and settings for all images, as determined by visual inspection.

Mechanical testing by three-point bending

Three-point bending tests of tibiae were performed using a material testing device with a 0 N to 200 N force sensor (Z.2.5/TN1S, Zwick/Roell). After rehydration in saline for two days, the tibiae were placed with the posterior surface facing up on two fulcra separated by 7 mm. The distal tibiofibular junction was placed right over one of the fulcra for improved reproducibility. Tibiae were loaded perpendicularly to the shaft of the bone between the two fulcra with a rounded-off indenter that was lowered with a displacement rate of 0.01 mm/s. The response to this load was recorded in force-displacement curves. Maximum load (Fmax) and stiffness were calculated using the testing software (testXpert 10.1, Zwick GmbH & Co).

Statistical analysis

All data are expressed as means ± SEM. Statistical analysis was performed using GraphPad Prism (version 7.02 for Windows). Means were compared using the Student's T-test, one-way ANOVA, or two-way ANOVA as indicated in the figure legends. Pearson correlation analysis was performed to examine potential linear relationships between variables. Differences between groups were considered statistically significant at $P < 0.05$.

Results

Flattening of corticosterone rhythm reduces lean body mass

Mice implanted with vehicle pellets showed a strong diurnal rhythm in plasma CORT, with an evening peak of around 150 ng/ml (Fig. 1A and 1B). One week after pellet implantation, the 4.5 mg CORT group showed a reduced CORT amplitude, which was due to higher trough levels rather than a reduction in the endogenous CORT peak (Fig. 1A). The 7.5 mg CORT group showed an almost completely blunted CORT rhythm, with increased through values and the absence of a CORT peak at 18 h (i.e. 1 h before onset of the dark active phase). Of note, both the 4.5 and 7.5 mg CORT pellets did not significantly affect the total daily exposure to CORT, as reflected by a similar area under the curve (AUC) of all individual CORT measurements. In week 5 of the study, the CORT pellets similarly affected CORT rhythm as compared to week 1 (Fig. 1B). After 7 weeks of intervention, CORT pellets did not significantly affect total body weight and fat mass, but 7.5 mg CORT pellets did reduce lean body weight, indicating loss of muscle and/or bone (Fig. 1C).

Flattening of corticosterone rhythm results in an osteoporotic phenotype

To investigate whether attenuation of CORT rhythm induces osteoporosis, we evaluated the cortical (Fig. 2A) and trabecular (Fig. 2E) structure of the femoral bone by micro-CT analysis. The cortical bone structure of the 4.5 mg CORT group was not significantly different from the vehicle group after 7 weeks of intervention, while the 7.5 mg CORT group showed a reduced cortical bone volume (-8.1%; Fig. 2B), cortical thickness (-6.9%; Fig. 2C), and cortical BMD (-3.4%; Fig. 2D). Unlike cortical bone structure, the trabecular bone structure was similarly affected by both 4.5 and 7.5 mg CORT pellets, as both CORT groups showed a strong decrease in relative trabecular bone volume (-22.7% and -25.5%, respectively; Fig. 2F). Trabecular number (Fig. 2G) and trabecular thickness (Fig. 2H) also seemed to be reduced in both CORT groups, although this was only significant for trabecular thickness in the 7.5 mg CORT group.

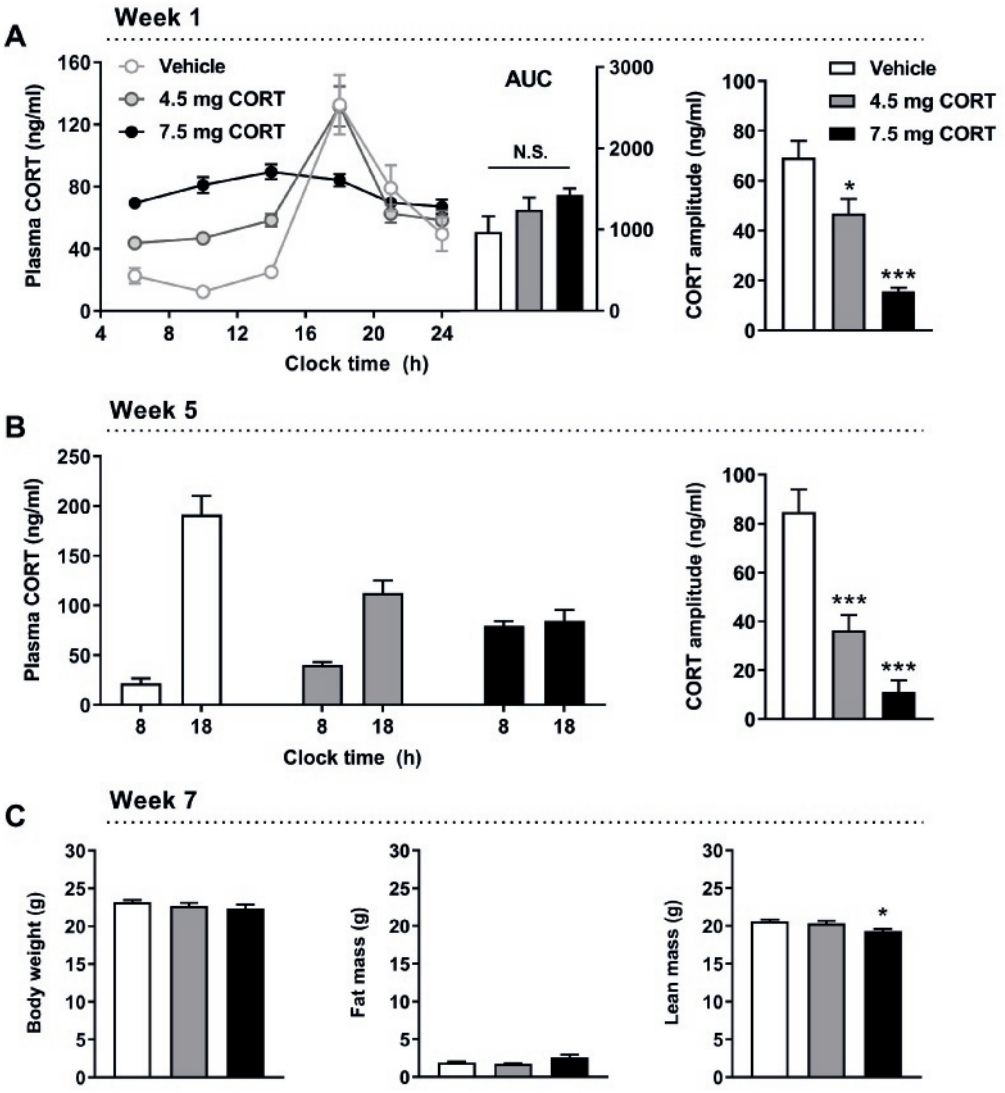

Figure 1. Corticosterone pellets flatten the rhythm in plasma corticosterone and reduce lean body mass. (A) Plasma corticosterone (CORT) levels were measured in mice 1 week after implantation of a vehicle (n = 10), 4.5 mg CORT (n = 15) or 7.5 mg CORT pellet (n = 10), at regular intervals throughout the day and night. An area under the curve (AUC) of all individual plasma corticosterone (CORT) measurements was calculated to determine total CORT exposure. For each mouse, a CORT amplitude was calculated by subtracting the lowest value in plasma CORT from the peak value, and dividing this by 2. (B) After 5 weeks of pellet implantation, morning and evening plasma CORT levels were measured and used to calculate the amplitude in CORT rhythm. (C) At endpoint, after 7 weeks of pellet implantation, body weight, fat mass and lean mass were measured. Data represent means ± SEM. *$P < 0.05$, ***$P < 0.001$ compared to the vehicle control group, according to one-way ANOVA with Dunnett's post hoc test. N.S., non-significant.

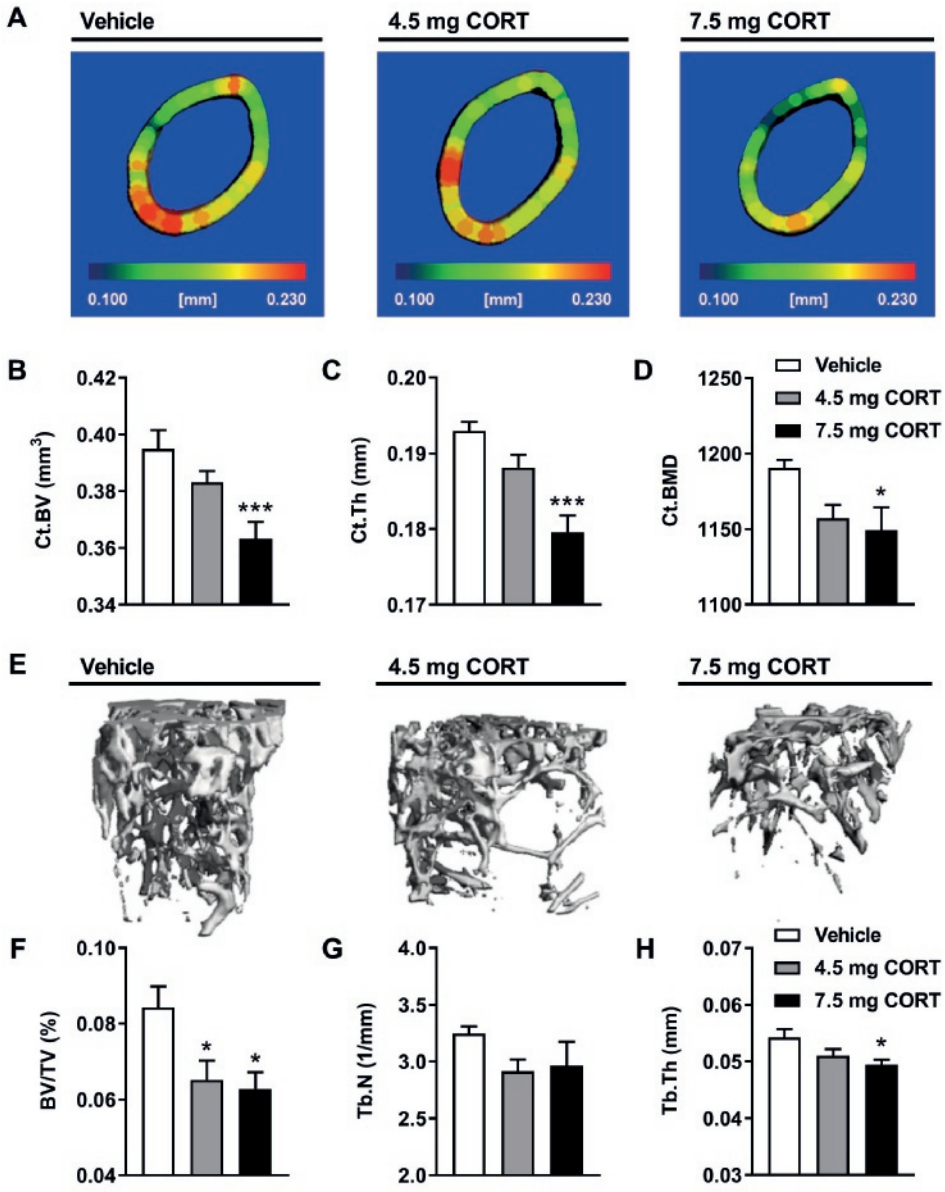

Figure 2. Flattening of corticosterone rhythm reduces both cortical and trabecular bone volume. (A) Representative structural images of the cortical bone in all groups, indicating cortical thickness in mm by color codes. (B-D) Micro-CT analysis was used to assess cortical bone volume (Ct.BV; B), cortical thickness (Ct.Th; C) and cortical bone mineral density (Ct.BMD; D). (E) Representative structural images of the trabecular bone in all groups. (F-H) Micro-CT analysis of the relative trabecular bone volume (BV/TV; F), trabecular number (Tb.N; G) and trabecular thickness (Tb.Th; H). Data represent means ± SEM. *P $<$ 0.05, ***P $<$ 0.001 compared to the vehicle control group, according to one-way ANOVA with Dunnett's post hoc test.

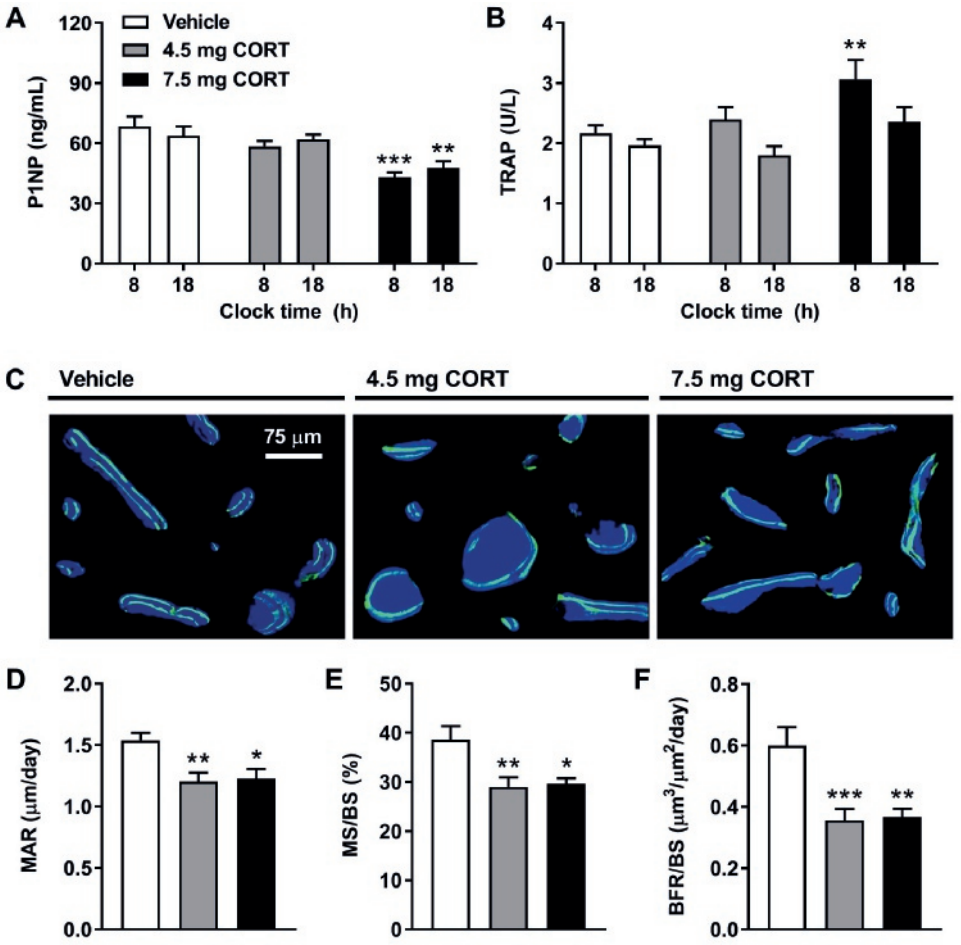

Figure 3. Flattening of corticosterone rhythm modulates bone turnover markers and reduces bone formation. (A, B) Plasma levels of procollagen type 1 amino-terminal propeptide (P1NP; A) and of tartrate-resistant acidic phosphatase (TRAP) were evaluated in the morning and evening after 5 weeks of pellet intervention. (C) Representative images of femoral trabecular bones double labeled with calcein (green), and counterstained with calcein blue (blue). (D-F) Calcein labeling was used to determine the mineral apposition rate (MAR; D), mineralizing surface per bone surface (MS/BS; E) and bone formation rate per bone surface (BFR/BS; F). Data represent means ± SEM. *P $<$ 0.05, **P$<$ 0.01, ***P $<$ 0.001 compared to the vehicle control group, according to two-way ANOVA (A,B) or one-way ANOVA with Dunnett's post hoc test (D-F).

Flattening of corticosterone rhythm disturbs the balance in bone turnover by favoring bone resorption

To investigate potential mechanisms underlying the effect of a flat CORT rhythm on bone structure, we measured a variety of markers of bone formation and resorption. Although our intervention modulated gene expression markers of GR activation and the circadian clock (*Gilz* and *Per1*, respectively) in tibia bone samples, we did not observe any changes in the expression

of genes involved in bone formation (i.e. *Opg, Runx2, Blap, Col1a1, cFos*) or bone resorption (i.e. *Rank, Rankl, Trap, Ctsk, Nfatc*) (Suppl. Fig. 1). We did observe effects of a flattened CORT rhythm on circulating bone turnover markers. Plasma levels of the bone formation marker P1NP were reduced in the 7.5 mg CORT group, while plasma levels of the bone resorption marker TRAP were increased (Fig. 3B). To evaluate whether the reduced P1NP levels reflect a decrease in bone formation, we performed histological analyses of femoral trabecular bone double labeled with calcein *in vivo* (Fig. 3C). This revealed a reduction in MAR (Fig. 3D), MS/BS (Fig. 3E) and BFR/BS (Fig. 3F), all indices of bone formation, in both the 4.5 and 7.5 mg CORT groups. Collectively these results demonstrate that flattening of the corticosterone rhythm disturbs the balance in bone turnover by favoring bone resorption over bone formation.

Flattening of corticosterone rhythm decreases bone strength

Next, we investigated whether the observed alterations in bone turnover and structure could affect the mechanical properties of bone. To this end, three-point bending tests were performed to evaluate cortical bone strength and stiffness of the tibia. Consistent with the fact that cortical bone structure was not significantly altered in the 4.5 mg CORT group, we did not find a significant difference in the Fmax, or highest load that the bone can withstand, in this group (Fig. 4A). We did observe a substantial decrease in Fmax in the 7.5 mg CORT group (-14.7%), indicating a reduced bone strength. Fmax correlated positively with the femoral cortical bone volume (Fig. 4B) and cortical thickness (Fig. 4C), demonstrating that the variation in bone strength can be partly explained by differences in cortical bone structure. Bone stiffness was also reduced in the 7.5 mg CORT group (-11.1%; Fig. 4D), and like Fmax correlated positively to both femoral cortical bone volume (Fig. 4E) and cortical thickness (Fig. 4F). Together, these results indicate that a flattened CORT rhythm induces weak and fracture prone bones.

Discussion

In this study, we evaluated the importance of GC rhythm for bone health. We demonstrate that flattening of the GC rhythm leads to a situation where bone resorption prevails over bone formation, resulting in an osteoporotic phenotype. Our data indicate that disruption of the GC rhythm may underlie the association between chronic circadian disruption through shift work and osteoporosis, and could in itself contribute to the negative effects of pharmacological GC administration on bone.

Previous studies show that endogenous GCs are important for physiological bone remodeling. Rats that lack endogenous GC as a result of adrenalectomy show a decreased bone mass [17]. This is likely the result of a diminished osteoblast activity, as osteoblast-specific glucocorticoid receptor (GR)-deficient mice show a comparable reduction in bone mass [18]. Similar findings were observed in mice with osteoblast-specific overexpression of 11-β-hydroxysteroid dehydrogenase 2 (11β-HSD2), an enzyme that inactivates GCs [19, 20]. Osteoclast-specific overexpression of 11β-HSD2 does not affect skeletal development [21], suggesting that endogenous GCs are primarily important for osteoblast function and bone formation. In our study, we demonstrate that flattening of the GC rhythm reduces bone formation, similar to the effects observed with diminished GC levels. This suggests that a lack of GCs may affect bone turnover via a lack of rhythmic input, rather than an actual absence of GC signaling.

While endogenous GCs are thus important for bone formation, excess GCs leads to osteoporosis by inducing bone loss [22]. This is the result of an initial increase in bone resorption, followed by a more progressive reduction in bone formation. High-dose GCs have been shown to extend the lifespan of osteoclasts in a GR-dependent manner, by promoting osteoclastogenesis and

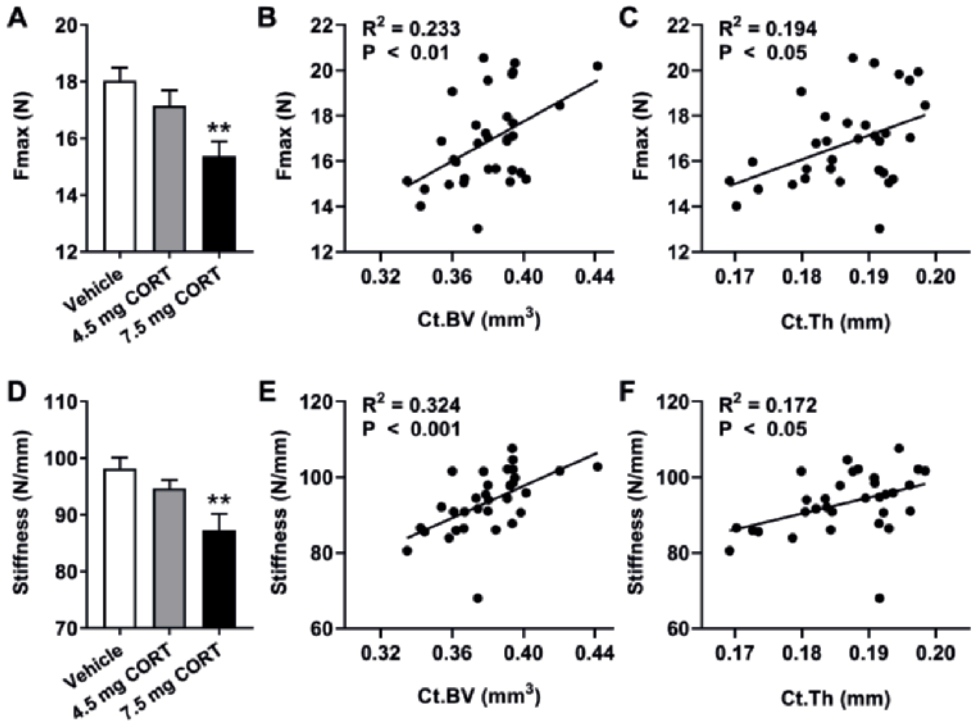

Figure 4. Flattening of the corticosterone rhythm reduces bone strength and stiffness. (A) Three-point bending tests of tibiae were performed to determine the maximum load (Fmax). (B, C) The relationship between Fmax and femoral cortical bone volume (B) and cortical thickness (C) was evaluated by Pearson correlation analysis. (D) Bone stiffness was evaluated through three-point bending tests. (E, F) Bone stiffness was correlated to femoral cortical bone volume (E) and cortical thickness (F). Data represent means ± SEM. $^{**}P < 0.01$ compared to the vehicle control group, according to one-way ANOVA with Dunnett's post hoc test.

osteoclast survival [21, 23, 24]. GCs also promote the production of RANKL by osteoblasts [25], thereby further stimulating osteoclast number and activity. Prolonged treatment with GCs suppresses differentiation and induces apoptosis of osteoblasts. This diminishes activation of osteoclasts through the RANK-RANKL pathway while at the same time reducing bone formation [18, 26]. The synthetic GC prednisolone induces an osteoporotic phenotype in mice dependent on GR signaling in osteoclasts and osteoblasts [18, 21, 26], demonstrating an essential role of both cell types in GC-induced osteoporosis.

The above-mentioned negative effects of GC administration on bone have often been ascribed to the excess in GC exposure. However, not only high but also low dose GC administration induces osteoporosis in humans [4, 27]. In both conditions, physiological GC rhythm is disrupted, which could contribute to deleterious effects of GCs on bone health. This is supported by our results, showing that complete flattening of GC rhythm in mice resulted in an osteoporotic phenotype, as reflected by a reduced cortical and trabecular bone volume and a diminished bone strength. This is likely the consequence of an increased bone resorption in combination with a decreased bone formation, demonstrated by increased TRAP and decreased P1NP levels, respectively.

Thus, a flattened GC rhythm affects both osteoclast and osteoblast function, closely resembling the phenotype observed with pharmacologic GC administration in humans.
While mice implanted with a 4.5 mg CORT pellet did show a reduced trabecular bone formation and volume, cortical bone volume and bone strength were not significantly affected. Although we cannot exclude a lack of power, bone health could be partly maintained in the 4.5 mg CORT group due to the CORT peak that was still present, as well as the higher rhythm amplitude as compared to the 7.5 mg group. Restoring the amplitude in GC rhythm by reintroducing a daily GC peak could therefore be an interesting strategy to reduce the negative effects of GC administration on bone. However, a natural through in GC rhythm may be equally important for bone health. Subtle increases in through levels may not yet affect GR occupancy, but could already result in overactivity the mineralocorticoid receptor (MR), that has a higher affinity for GCs as compared to the GR. Like the GR, the MR is expressed in bone and has been suggested to play a role in GC-induced osteoporosis [28, 29]. Therefore, in addition to reintroducing a peak in GCs, it may be important to block GC action by administering an MR antagonist at the right time of day, to mimic a through in GC activity. Future research is warranted to investigate whether these novel strategies could rescue part of the osteoporotic phenotype associated with GC therapy.
Another way in which our results could be integrated into clinical applications is by promoting healthy ageing. Ageing not only increases the risk of osteoporotic fractures [30], but is also associated with profound changes in the circadian timing system [31]. With increasing age, circadian output of the SCN declines [32], thereby reducing the amplitude of cortisol rhythm in humans [33]. Our findings suggest that a dampened GC rhythm may contribute to the age-related risk of osteoporosis. Restoring the amplitude in cortisol rhythm could thus be an interesting therapeutic strategy to reduce fracture risk in the elderly, and to promote healthy ageing.
A limitation of our study setup is that there was a minor dose-dependent effect of the CORT pellets on total CORT plasma levels. Although levels were still far lower as those observed with pharmacologic GC administration, we cannot fully exclude the possibility that modest increases in CORT plasma levels influenced our results. Nevertheless, a recent study showed that disruption of GC rhythm in mice results in metabolic disturbances, while introduction of a supraphysiological peak in GCs by injection of CORT at the right time of day (i.e. at the natural peak of GCs) does not [34]. Thus, a mild excess in GCs does not necessarily induce adverse effects, as long as the GC rhythm is maintained.
In conclusion, disruption of GC rhythm without hypercortisolism induces an osteoporotic phenotype in mice, similar to the phenotype observed with pharmacologic GC administration in humans. It is estimated that 1-2% of the population receives long-term GC therapy, which corresponds to approximately 10 million people worldwide. Depending on the dose and duration of GC therapy, the associated fracture risk is ~2-5% [35]. This means that ten thousands of individuals are suffering from the symptoms of GC-induced osteoporosis. Our findings indicate that at least part of the effects of GC therapy on bone could be attributed to a disturbed GC rhythm, rather than an excess in GC dose alone. Future studies should investigate whether reintroducing a trough and/or peak in GCs at the right time could prevent or reduce GC-induced osteoporosis and the associated fracture risk.

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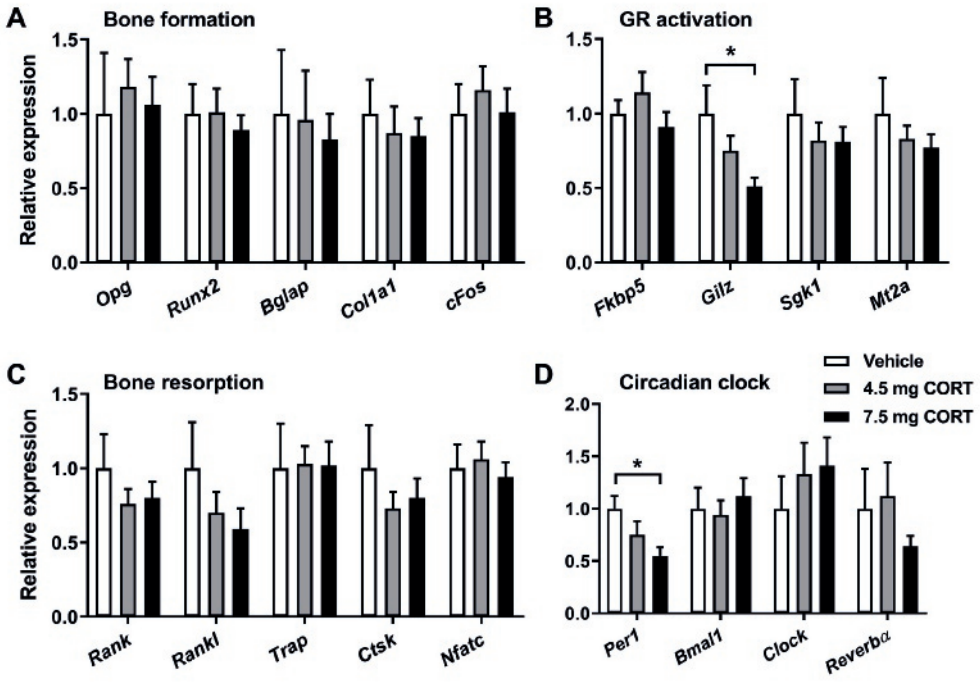
Appendix



Supplementary Figure 1. Corticosterone pellets do not affect the expression of genes involved in bone formation and bone resorption. (A-D) Expression of genes involved in bone formation (A), markers of glucocorticoid receptor (GR) activity (B), genes involved in bone resorption (C) and circadian clock genes (D) was measured in tibia bone samples. Data represent means ± SEM. *P $<$ 0.05 according to one-way ANOVA with Dunnett's post hoc test.