Out-of-phase treatment with the synthetic glucocorticoid betamethasone disturbs glucose metabolism in mice

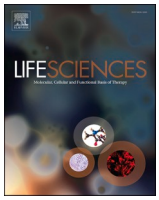
Li, S.; Zhang, S.; Rensen, P.C.N.; Meijer, O.C.; Kooijman, S.; Kroon, J.

Life Sciences 357 (2024) 123080

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie



Out-of-phase treatment with the synthetic glucocorticoid betamethasone disturbs glucose metabolism in mice

Sheng Li, Sen Zhang, Patrick C.N. Rensen, Onno C. Meijer, Sander Kooijman, Jan Kroon *

Department of Medicine, Division of Endocrinology, Leiden University Medical Center, Leiden, the Netherlands
Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, the Netherlands

ARTICLE INFO

Keywords:
Glucocorticoid
Insulin resistance
Betamethasone
Circadian Rhythm

ABSTRACT

Objective: Endogenous glucocorticoid levels display a strong circadian rhythm, which is often not considered when synthetic glucocorticoids are prescribed as anti-inflammatory drugs. In this study we evaluated the effect timing of glucocorticoid administration, i.e. in-phase (administered when endogenous glucocorticoid levels are high) versus out-of-phase (administered when endogenous glucocorticoid levels are low). We investigated the synthetic glucocorticoid betamethasone – which is extensively used in the clinic - and monitored the development of common metabolic side effects in mice upon prolonged treatment, with a particular focus on glucose metabolism.

Methods: Male and female C57BL/6J mice were treated with the synthetic glucocorticoid betamethasone in-phase and out-of-phase, and the development of metabolic side effects was monitored.

Results: We observed that, compared with in-phase treatment, out-of-phase treatment with betamethasone results in hyperinsulinemia in both male and female C57BL/6J mice. We additionally found that out-of-phase betamethasone treatment strongly reduced insulin sensitivity as compared to in-phase administration during morning measurements. Our study shows that the adverse effects of betamethasone are dependent on the time of treatment with generally less side effects on glucose metabolism with in-phase treatment.

Conclusions: This study highlights differences in glucocorticoid outcome based on the time of measurement, advocating that potential circadian variation should be taken into account when studying glucocorticoid biology.

1. Introduction

Glucocorticoids play a crucial role in various physiological processes in the body, including glucose metabolism, immune responses, and the stress response [1–3]. Endogenous glucocorticoid secretion is regulated by the hypothalamic-pituitary-adrenal (HPA) axis, and this hormonal axis is tightly regulated by negative feedback on the level of the pituitary and hypothalamus [4]. The HPA axis is aligned with the light-dark cycle through signals originating from the suprachiasmatic nucleus, which is known as the central pacemaker of the circadian timing system [2,5]. This ensures that endogenous glucocorticoid levels are highest in preparation for the body's daily activities [6]. In humans the peak in cortisol is in the early morning and levels gradually decrease during the active period of the day. As mice are nocturnal animals the glucocorticoid rhythm is reversed with a peak in corticosterone levels at the beginning of the active dark-phase. The circadian rhythm in glucocorticoids appears to be critical for various aspects of lipid and glucose metabolism [7,8] by mediating metabolic activity of several tissues including adipose tissue [9,10], the liver [11] and skeletal muscle [12,13].

Synthetic glucocorticoids are commonly prescribed for anti-inflammatory diseases [14]. It is known that its long term and/or excessive use is associated with pronounced metabolic side effects including osteoporosis, muscle atrophy and altered glucose metabolism [15–19]. Betamethasone is an extensively used synthetic glucocorticoid, with high selectivity for its therapeutic target, the glucocorticoid receptor [20]. Earlier, we evaluated its effects on skeletal muscle [21]. In this study, we evaluated how the timing of betamethasone treatment influences its adverse effects in mice, with a particular focus on glucose metabolism. We found that out-of-phase treatment with betamethasone, when endogenous glucocorticoid levels are low, generally results in more severe adverse effects on glucose metabolism compared to in-

* Corresponding author at: Department of Medicine, Division of Endocrinology, Leiden University Medical Centers, Albinusdreef 2, 2333 ZA Leiden, the Netherlands.
E-mail address: j.kroon@lumc.nl (J. Kroon).

https://doi.org/10.1016/j.lfs.2024.123080
Received 11 July 2024; Received in revised form 11 September 2024; Accepted 23 September 2024
Available online 25 September 2024

Table 1
Timepoints of functional measurements.

	Measurement		Time since last treatment (h)	
	Morning	Evening	Morning	Evening
Body weight and composition	ZT3	ZT11	1 & 16	9 & 3
Grip Strength	ZT4	ZT12	2 & 16	10 & 2
OGTT	ZT7-9	ZT15-17	5 & 21	14 & 5
ITT	ZT7-9	ZT15-17	5 & 21	14 & 5
Plasma biochemistry	ZT7	ZT15	5 & 21	14 & 5
Organ uptake	ZT7-9	ZT15-17	5 & 21	14 & 5

phase treatment, when endogenous glucocorticoid levels are high. We also found that these effects are largely similar between male and female mice after prolonged betamethasone treatment.

2. Methods

2.1. Animals

Animal experiments were approved by the institutional ethics committee of Leiden University Medical Center and executed under a license granted by the central authority for scientific procedures on animals (CCD), in accordance with the Dutch Act on Animal Experimentation and EU Directive 2010/63/EU. Eight to ten week-old male and female C57BL/6J mice were obtained from Charles River Laboratories and group housed in conventional cages. Mice were on a 12-hour light/12-hour dark cycle with clock time 07h00 as lights-on, and 19h00 as lights-off. Mice had ad libitum access to water and RM3 chow diet (Special Diet Services, Essex, UK), unless otherwise specified. We evaluated the effects of treatment timing with betamethasone in two separate experiments, in which we compared out-of-phase with in-phase betamethasone treatment.

In experiment 1, we investigated the effect of out-of-phase versus in-phase betamethasone treatment on the development of glucocorticoid-associated side effects in male ($N = 18$ in total) and female mice (N = 18 in total). Mice were intraperitoneally injected with PBS (vehicle, 100 μl) at zeitgeber time (ZT)-2, with 3.0 mg/kg betamethasone (dissolved in PBS in a volume of 100 μl) at ZT2 ('out-of-phase') or with 3.0 mg/kg betamethasone at ZT10 ('in-phase') by daily injections for a total of 30 days ($N = 6$ mice per group per sex). The dosage of betamethasone was based on a previous study [21].

In experiment 2, we focused on the effect of betamethasone treatment timing on glucose metabolism in male mice ($N = 48$). Mice were intraperitoneally injected with PBS (vehicle) at ZT2, with 3.0 mg/kg/day betamethasone at ZT2 ('out-of-phase'), with PBS (vehicle) at ZT10 or with 3.0 mg/kg/day betamethasone at ZT10 ('in-phase') for a total of 14 days. We included two parallel cohorts of mice to perform functional measurements at two different timepoints ($N = 6$ mice per treatment group for morning-afternoon measurements between ZT2–7 and N = 6 mice per treatment group for evening measurements between ZT8–15. The exact time range of the functional measurements including body mass and composition, grip strength, glucose tolerance, insulin tolerance and lipid/glucose uptake is detailed below and summarized in Table 1).

2.2. Body mass and body composition measurement

In experiment 1, body mass and composition (lean and fat mass) were determined weekly at ZT3 by using an EchoMRI-100 analyzer (EchoMRI, Houston, TX, USA). In experiment 2, body mass and composition were evaluated weekly at ZT3 for the morning measurement and ZT11 for the evening measurement.

2.3. Grip strength test

In experiment 1, grip strength was measured at ZT4. In experiment 2, grip strength was measured weekly at ZT4 for morning measurements and at ZT12 for evening measurements.

2.4. Oral glucose tolerance test

In experiment 2, mice were fasted for 6 h from ZT1-7 (morning measurement) or from ZT9-15 (evening measurement) on day 11 to perform an oral glucose tolerance test (OGTT) at ZT7-9 and ZT15-17, respectively. Blood glucose was measured with an Accu-Chek glucometer (Roche) in blood collected from the tail vein at $t = 0$ and subsequently 2 g/kg of glucose was administered via oral gavage and blood glucose was measured at $t = 15, 30, 60, 120$ min. Data is shown as absolute glucose values, as absolute change from baseline (incremental), and as fold change from the $t = 0$ value (normalized).

2.5. Insulin tolerance test

In experiment 2, mice were fasted for 6 h from ZT1-7 (morning measurement) or from ZT9-15 (evening measurement) on day 14 to perform an insulin tolerance test (ITT) at respectively ZT7-9 and ZT15-17. Blood glucose was measured using an Accu-Chek glucometer (Roche) at $t = 0$ and mice were subsequently intraperitoneally injected with 0.75 U/kg human insulin (Sigma I9278) and blood glucose levels were measured at $t = 15, 30, 60, 120$ min. Mice that became hypoglycemic after insulin injection were rescued by an intraperitoneal injection with 300 μl 10 % glucose solution, and glucose levels of these mice were recorded as the lower limit of 2 mM for the rest of ITT measurement. Data is shown as absolute glucose values and as fold change from the $t = 0$ value (normalized).

2.6. Plasma biochemistry

In experiment 1 mice were fasted for 6 h on day 14 and 30 from ZT1-7 and in experiment 2 mice were fasted for 6 h on day 14 from ZT1-7 (morning measurements) or ZT9-15 (evening measurements). After fasting, blood was collected in heparin-coated capillaries from the tail vein via a small nick and blood was centrifuged 8000 RMP for 5 min to isolate plasma for biochemistry analysis including insulin (Crystal Chem), glucose, triglycerides, cholesterol (colorimetric kits from Roche Diagnostics) and C-peptide (Crystal Chem Catalog #90050). Heart puncture blood was collected in EDTA-coated tubes directly after euthanasia to isolate plasma for measurements of P1NP (Immunodiagnostic Systems) and osteocalcin (Abcam).

2.7. Organ uptake of radiolabeled triglyceride-derived fatty acids and deoxy-glucose

In experiment 2, mice were fasted for 6 h from ZT1-7 (morning measurements) or ZT9-15 (evening measurements). An experiment was performed to evaluate the tissue uptake of [^{3}H]oleate derived from glycerol [^{3}H]oleate-labeled lipoprotein-like emulsion particles (average size 80 nm) and [^{14}C]deoxy-glucose [22], after intravenous injection into the tail vein (in 200 μl PBS per mouse). Mice were euthanized after 15 min using CO_2 asphyxiation and perfused with ice-cold PBS for 5 min. Various tissues were collected and the tissue pieces (max. 50 mg) were dissolved in 500 μl Solvable (Perkin Elmer) overnight at 56 °C. ^{3}H and ^{14}C activity were measured using scintillation counting solution (Ultima Gold XR, Perkin Elmer).

2.8. Gene expression analysis

Total RNA was extracted from snap-frozen tissues using Tripure RNA isolation reagent (Roche). Complementary DNA was generated using M-

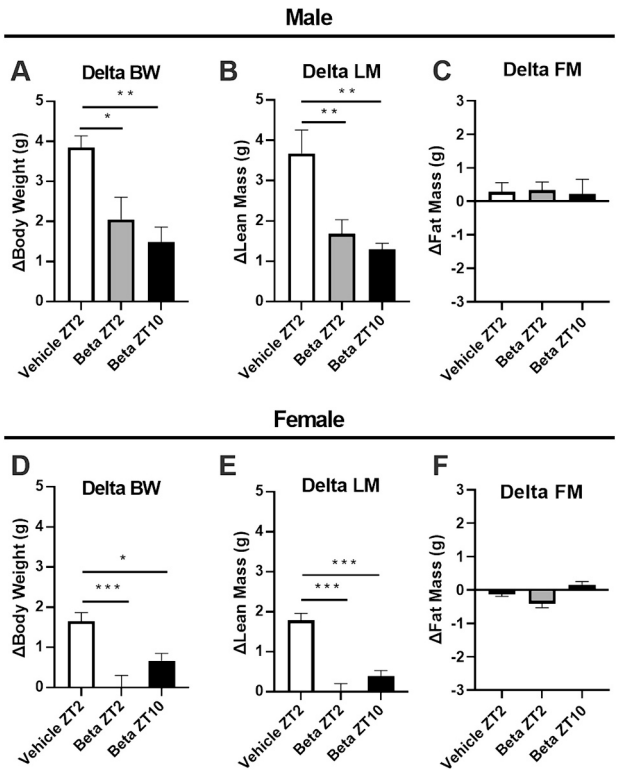


Fig. 1. Out-of-phase and in-phase betamethasone treatment similarly attenuate total body weight and lean mass gain in male and female mice. The effect of treatment with 3.0 mg/kg betamethasone out-of-phase (ZT2) and in-phase (ZT10) on (A) delta body weight (BW), (B) delta lean mass (LM) and (C) delta fat mass (FM) in male mice; and (D) delta BW, (E) delta LM and (F) delta FM in female mice. $N = 6$ mice/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Statistical significance was calculated using a one-way ANOVA.

MLV reverse-transcriptase (Promega). Quantitative reverse transcriptase-PCR was performed on a CFX96 PCR machine (Bio-Rad, Veenendaal, the Netherlands), and expression levels were normalized to the housekeeping gene *Gapdh*. Primer sequences: *Atrogin-1* Fwd: TTCAGCAGCCTGAACTACGA; Rev.: GGATGGCAGTCGAGAAGTCC; *Gapdh* Fwd: GGGGCTGGCATTGCTCTCAA; Rev.: TTGCTCAGTGTCC TTGCTGGGG; *G6pc* Fwd: CTTAAAGAGACTGTGGGCATCAA; Rev.: AT TACGGGCGTTGTCCAAAC; *Hk2* Fwd: GATCGCCGGATTGGAACAGA; Rev.: GTCTAGCTGCTTAGCGTCCC; *MurF-1* Fwd: TGTGCAAGGAACA-GAAGAC; Rev.: CCAGCATGGAGATGCAGTTA; *Pepck* Fwd: ATCTTTGGT GGCCGTAGACCT; Rev.: GCCAGTGGGCCAGGTATTT; *Slc2a4* Fwd: GGCTCTGACGTAAGGATGGG; Rev.: AAACTGAAGGGAGCCAAGCA.

2.9. Statistical analysis

Statistical analyses were performed with SPSS (version 27) and GraphPad Prism version 10.0.3. ANOVA with Turkey multi-comparison was used according to number of variables i.e. including 1-way ANOVA for one variable and 2-way ANOVA for two variables. 2-way ANOVA interaction terms are shown in Supplementary Table 1. All data are presented as means ± SEM.

3. Results

3.1. Out-of-phase and in-phase betamethasone treatment similarly restrain gain of body weight and lean mass in both male and female mice

We evaluated the metabolic effects of out-of-phase (ZT2) versus in-phase (ZT10) treatment with the synthetic glucocorticoid betamethasone by daily intraperitoneal injection of male and female C57BL/6J mice with 3.0 mg/kg betamethasone for 30 consecutive days. We found that both ZT2 and ZT10 betamethasone treatment similarly attenuated the gain in body weight and lean mass in both male and female mice (Fig. 1A-F, Supplementary Fig. 1). Total fat mass was not significantly altered in male nor female mice upon ZT2 and ZT10 betamethasone treatment (Fig. 1D and F). When evaluating the wet weight of tissues, we did not observe any significant effect of ZT2 or ZT10 betamethasone treatment on the total weight of the liver, gonadal white adipose tissue weight (gWAT) or interscapular brown adipose tissue (iBAT) weight in either sex (Supplementary Fig. 2A-F).

3.2. Out-of-phase betamethasone treatment increases plasma insulin levels in both male and female mice

To evaluate the effect of timing of betamethasone administration on glucose metabolism, we measured plasma insulin and glucose levels after a 6 h fast from ZT1-7, during which the ZT2 treatment was given. Daily betamethasone administration at ZT2 (out-of-phase) but not at ZT10 (in-phase) significantly increased plasma insulin levels in male and female mice at day 14, while plasma glucose was significantly lowered in male but not female mice upon ZT2 betamethasone treatment (Fig. 2A-D). The effect of ZT2 betamethasone administration on plasma insulin was slightly less pronounced after 30 days of treatment, although still significantly elevated in female mice (Supplementary Fig. 3A-D). We measured C-peptide levels as a readout for pancreatic insulin release, which were elevated upon ZT2 treatment in both male and female mice (Fig. 2E and G). C-peptide levels mirrored the effects on insulin in both male and female mice and both at 14 and 30 days, with the exception of ZT10 betamethasone treatment in female mice at day 30 (Supplementary Fig. 3E and G). Consistent with the elevated insulin levels, ZT2 but not ZT10 betamethasone treatment resulted in a higher HOMA-IR in male and female mice at day 14 (Fig. 2F and H) and in female mice at day 30 (Supplementary Fig. 3F and H). To investigate how morning betamethasone administration causes hyperinsulinemia, we measured expression levels in tissues collected at ZT3–5 of genes involved in glucose metabolism including *Pepck* and *G6pc* in liver and *Hk2* and *Slc2a4* (encoding Glut4) in skeletal muscle. Neither ZT2 nor ZT10 betamethasone treatment affected the expression of these genes in either male or female mice (Supplementary Fig. 4A-H). We additionally measured markers for other synthetic glucocorticoid-related side effects. With regard to lipid metabolism, we did not observe changes in plasma triglyceride or total cholesterol levels after a 6 h fast from ZT1-7 on day 14 (Supplementary Fig. 5A-D). Similarly, we did not observe any effects of ZT2 or ZT10 betamethasone treatment on bone turnover markers P1NP and osteocalcin on day 30 (Supplementary Fig. 5E-H). Daily treatment with betamethasone at ZT2 or ZT10 also did not influence the weight of the triceps, gastrocnemius, soleus and tibialis anterior (TA) muscles at day 30, and we did not observe any major effects on forelimb grip strength and muscle atrophy-related genes *Atrogin-1* and *Murf-1* in gastrocnemius and triceps muscle (Supplementary Fig. 6A-R). Altogether we show that out-of-phase treatment with 3.0 mg/kg betamethasone at ZT2 markedly influences glucose metabolism but no other markers for glucocorticoid-associated side effects.

3.3. Out-of-phase and in-phase betamethasone treatment similarly restrain total body weight and lean mass gain independent on time of measurement

In a subsequent experiment we further focused on the effects of out-of-phase (ZT2) and in-phase (ZT10) treatment with betamethasone on glucose metabolism. We investigated a 14 day treatment regimen in two parallel cohorts to be able to perform functional measurements on two different times (morning measurement and evening measurement; Table 1). This was done in order to establish if effects of betamethasone treatment are dependent on time of measurement. In line with our previous experiment, we found that both ZT2 and ZT10 betamethasone

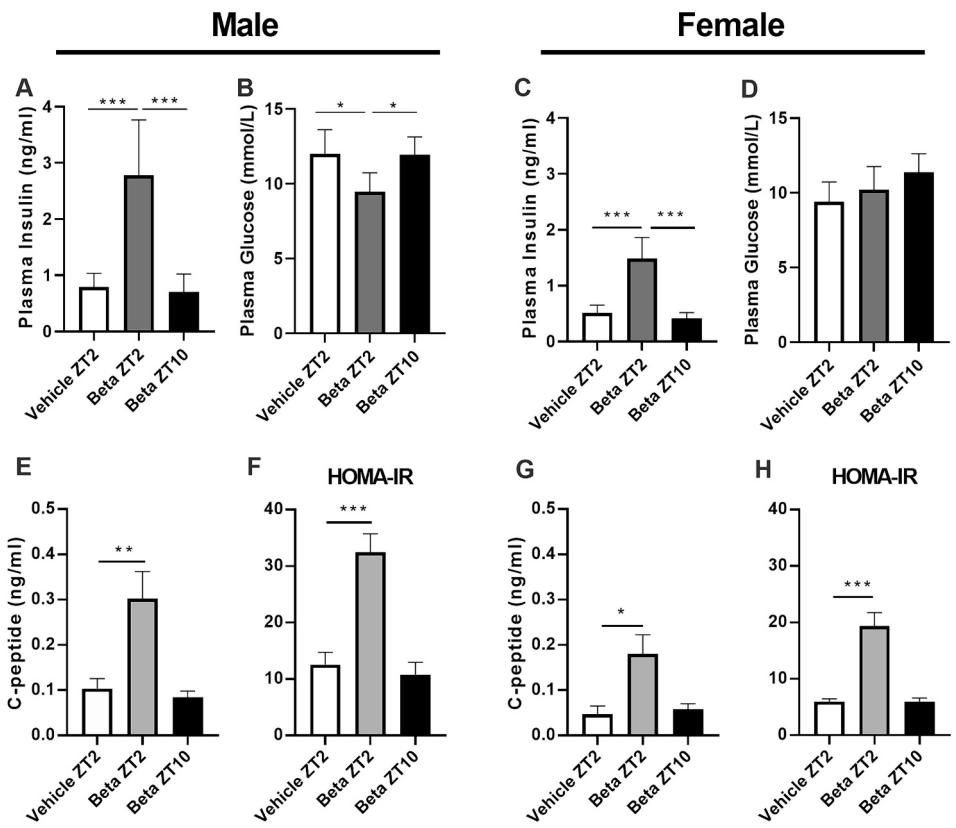


Fig. 2. Out-of-phase but not in-phase betamethasone treatment increases plasma insulin levels in both male and female mice. Plasma biochemistry measured at ZT7 upon treatment with 3.0 mg/kg betamethasone out-of-phase (ZT2) and in-phase (ZT10) after a 6 h fast on day 14. (A) Insulin and (B) glucose in male mice, (C) insulin and (D) glucose in female mice, (E) plasma c-peptide and (F) HOMA-IR in male mice, (G) plasma c-peptide, and (H) HOMA-IR in female mice. $N = 6$ mice/group. *p < 0.05, **p < 0.01, ***p < 0.001. Statistical significance was calculated using a one-way ANOVA.

treatment similarly attenuated total body weight gain and reduced lean mass, and we observed that these effects were independent of the time of measurement (Fig. 3A-F, Supplementary Fig. 7). On fat mass we did not observe significant effects in the morning measurement while we found minor effects of betamethasone treatment in the evening measurement (Fig. 3C and F, Supplementary Fig. 7). In both morning and evening measurements, we did not observe any effect of ZT2 or ZT10 betamethasone treatment on the wet weight of the liver, gWAT, subcutaneous white adipose tissue (sWAT) and iBAT (Supplementary Fig. 8A-H). Consistent with our previous experiment, treatment with betamethasone at ZT2 or ZT10 did not alter forelimb grip strength during either morning or evening measurement (Supplementary Fig. 8I-J).

3.4. Out-of-phase and in-phase betamethasone treatment differentially influence insulin sensitivity, which is dependent on time of measurement

To further investigate effects on glucose metabolism, we performed an OGTT at day 11. The absolute glucose levels and corresponding area under the curve (AUC) are shown in Supplementary Fig. 9A-D. When evaluating incremental and normalized glucose levels, we observed a nonsignificant increase in glucose and the total glucose exposure (AUC) upon ZT2 betamethasone treatment in the morning measurement (Fig. 4A-B, Supplementary Fig. 9E-F). These measures were strongly dependent on time of measurement, as evening OGTT measurement showed a sharp reduction in total glucose exposure with betamethasone treatment independent of the time of administration (Fig. 4C-D, Supplementary Fig. 9G-H). On day 14, we performed an ITT for which the absolute glucose levels and corresponding AUC are shown in Supplementary Fig. 9I-L. After insulin injection, we observed that ZT2 and ZT10 vehicle-treated mice were highly insulin sensitive, with declining blood glucose levels during both morning and evening measurements (Fig. 4E-H, Supplementary Fig. 9I-L). In sharp contrast, ZT2 betamethasone treatment completely abolished insulin sensitivity in the morning, with no meaningful reduction in blood glucose during the morning ITT measurement, while ZT10 treatment only modestly reduced insulin sensitivity (Fig. 4E-F). During the evening ITT measurement, both ZT2 and ZT10 betamethasone similarly diminished insulin sensitivity (Fig. 4G-H). On day 15, we evaluated plasma biochemistry after a 6 h fast and found that ZT2 betamethasone treatment reduced plasma glucose in both morning and evening measurements while ZT10 administration only influenced evening glucose levels (Supplementary Fig. 10A-B). We observed a modest and nonsignificant increase in plasma insulin upon ZT2 betamethasone administration in the morning measurement with no effects in the evening measurements (Supplementary Fig. 10C-D).

3.5. Out-of-phase and in-phase betamethasone treatment similarly increase deoxyglucose uptake in the liver and gWAT but differentially affect sWAT and iBAT

To investigate whether the timing of betamethasone treatment influences lipid and glucose uptake we intravenously injected triglyceride-rich lipoprotein-like emulsion particles labeled with glycerol tri[^{3}H] oleate, and [^{14}C]deoxyglucose added to the emulsion. We observed that both ZT2 and ZT10 betamethasone treatment enhanced the uptake of triglyceride-derived fatty acids and [^{14}C]deoxyglucose by liver and gWAT specifically in the evening measurement, potentially explaining the rapid reduction in blood glucose during the OGTT at this timepoint (Fig. 5A-D; Supplementary Fig. 11A-D). We did not observe significant effects in the uptake of triglyceride-derived fatty acids and [^{14}C]deoxyglucose by liver and gWAT in the morning measurement (Fig. 5A-D; Supplementary Fig. 11A-D). In sWAT, we found both ZT2 and ZT10

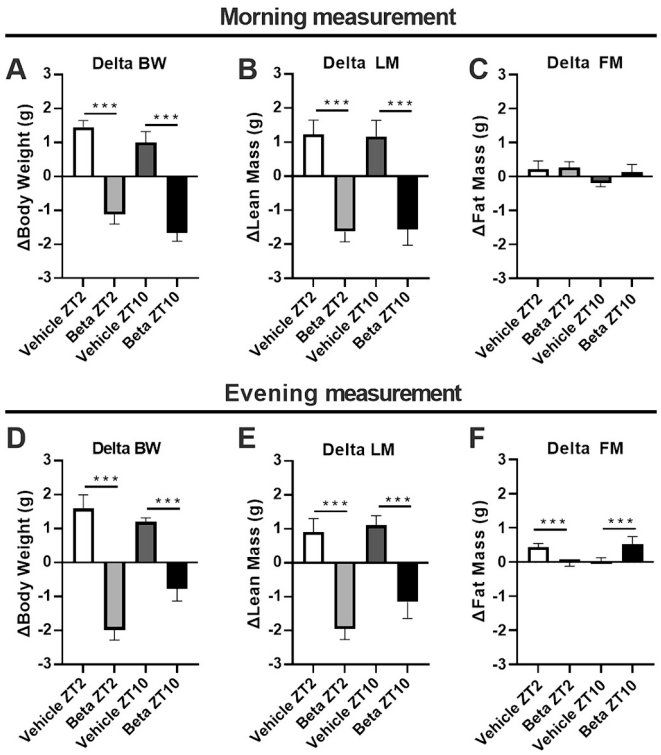


Fig. 3. Out-of-phase and in-phase betamethasone treatment similarly attenuate total body weight and lean mass gain independent on time of measurement. The effect of treatment with 3.0 mg/kg betamethasone out-of-phase (ZT2) and in-phase (ZT10) on (A) delta body weight (BW), (B) delta lean mass (LM) and (C) delta fat mass (FM) in male mice during the morning measurement. (D) Delta BW, (E) delta LM and (F) delta FM during evening measurement. N = 6 mice/group. ***$p < 0.001$. Statistical significance was calculated using a one-way ANOVA.

betamethasone treatment reduced [^{14}C]deoxyglucose uptake in the morning, while only ZT10 treatment reduced [^{14}C]deoxyglucose uptake in the evening measurement (Fig. 5E-F). We did not observe any effects of ZT2 or ZT10 betamethasone treatment on [^{3}H]oleate updateby sWAT (Supplementary Fig. 11E-F). In iBAT, we observed treatment timing-specific effects, as ZT10 treatment increased [^{14}C]deoxyglucose uptake in the morning measurement while ZT2 treatment decreased this in the evening measurement (Fig. 5G-H). ZT10 betamethasone treatment also reduced triglyceride-derived fatty acid uptake in iBAT in the evening measurement but not in the morning measurement (Supplementary Fig. 11G-H).

4. Discussion

Synthetic glucocorticoid treatment is widely used in the clinic for a range of applications including inflammatory diseases and autoimmune diseases and as an antiemetic during cancer chemotherapy. In our study we found that out-of-phase treatment with the synthetic glucocorticoid betamethasone causes a clear disturbance of glucose metabolism, while the effects of in-phase betamethasone treatment are less pronounced.

The effects of glucocorticoids are influenced by time of day. It is known that the endogenous steroids cortisol and corticosterone exhibit a strong diurnal rhythm and glucocorticoid replacement treatment for adrenal insufficiency often intends to mimic this circadian fluctuation in glucocorticoid exposure. Evening treatment (in rodents) is thus aligned with the circadian rhythm of endogenous glucocorticoids [23,24], and this might lead to fewer side effects [25,26]. Many treatment regimens do not necessarily take circadian variation in glucocorticoid levels into account and are typically only given at one time of the day, albeit more often in the morning during the endogenous glucocorticoid peak. For example in rheumatoid arthritis symptoms like stiffness and pain are more severe in the morning and glucocorticoid is therefore administered during this period [27,28]. It is however unclear if the side effects that develop as a consequence of glucocorticoid therapy are also dependent on timing. We therefore compared the effect of time of treatment with the potent synthetic glucocorticoid betamethasone to evaluate how this influences various side effects, with a particular focus on glucose metabolism.

We first evaluated timing effects of betamethasone with a particular focus on potential sex differences in glucocorticoid effects, given the different responses between males and females previously reported by others and ourselves [21,29–31]. We compared 'out-of-phase' betamethasone treatment at ZT2 (during the endogenous corticosterone trough in mice) with 'in-phase' treatment at ZT10 (during the endogenous corticosterone peak in mice). Despite baseline differences between male and female mice in total body weight and lean mass, the reduction in body weight gain and lean mass in response to ZT2 and ZT10 betamethasone treatment was largely similar between sexes. We did not find effects of either ZT2 or ZT10 betamethasone treatment on many other known glucocorticoid-associated side effects, including muscle function, adiposity, lipid metabolism and bone turnover. This is not fully consistent with a previous study in which 3.0 mg/kg daily betamethasone 'in-phase' (ZT9) resulted in modest effects on body composition and muscle function [21]. It is also worthwhile to note that in the current experiment we only observed modest sex differences in glucocorticoid response, while other studies show more pronounced sex differences in response to excess corticosterone [29,31].

A significant effect on fasted plasma insulin levels was observed after 14 days of ZT2 betamethasone treatment in both sexes albeit more pronounced in male as compared to female mice. This hyperinsulinemia was explained by enhanced pancreatic insulin release as evidenced by elevated C-peptide levels. Given the robust effects on plasma insulin (release) in this study, our second experiment was designed to study glucose metabolism specifically. The effects of ZT2 and ZT10 betamethasone on total body weight and fat mass were consistent with the previous experiment and with literature [32,33], and the effects were independent on the time of measurement (morning vs evening measurement; Table 1). We included three functional tests to measure glucose metabolism, namely an OGTT, an ITT and the uptake of radiolabeled deoxy-glucose. For the OGTT, the effects of betamethasone were highly dependent on the time of measurement, with a possible increase in glucose excursion the morning after ZT2 betamethasone treatment, but with a very steep decrease in glucose excursion upon ZT2 and ZT10 betamethasone treatment. This reduction during the evening is potentially explained by betamethasone-induced uptake of deoxy-glucose by liver and gWAT. It is worthwhile to note that there were baseline differences in blood glucose between morning and evening measurements in vehicle-treated mice, skewing incremental glucose values. We observed decreased plasma glucose after 14 days of ZT2 betamethasone treatment, which is counterintuitive but suggests that these mice are in transition towards overt insulin resistance and are still partially responsive to (increased) insulin.

It is known that glucocorticoids influence pancreas β-cell function and insulin secretion [34], and we therefore performed an ITT in the morning and evening [35,36] and again observed differences between time of measurements. During the morning measurement, we found that betamethasone markedly decreased insulin sensitivity but that this effect was much more pronounced upon ZT2 treatment compared to ZT10 treatment. During the evening measurement we found that betamethasone treatment strongly decreased insulin sensitivity independent of timing of treatment. In the second experiment the effect of ZT2 betamethasone treatment on fasted insulin was much less pronounced as compared to the previous experiment, but it has to be noted that these results may be confounded by the ITT test that was performed shortly

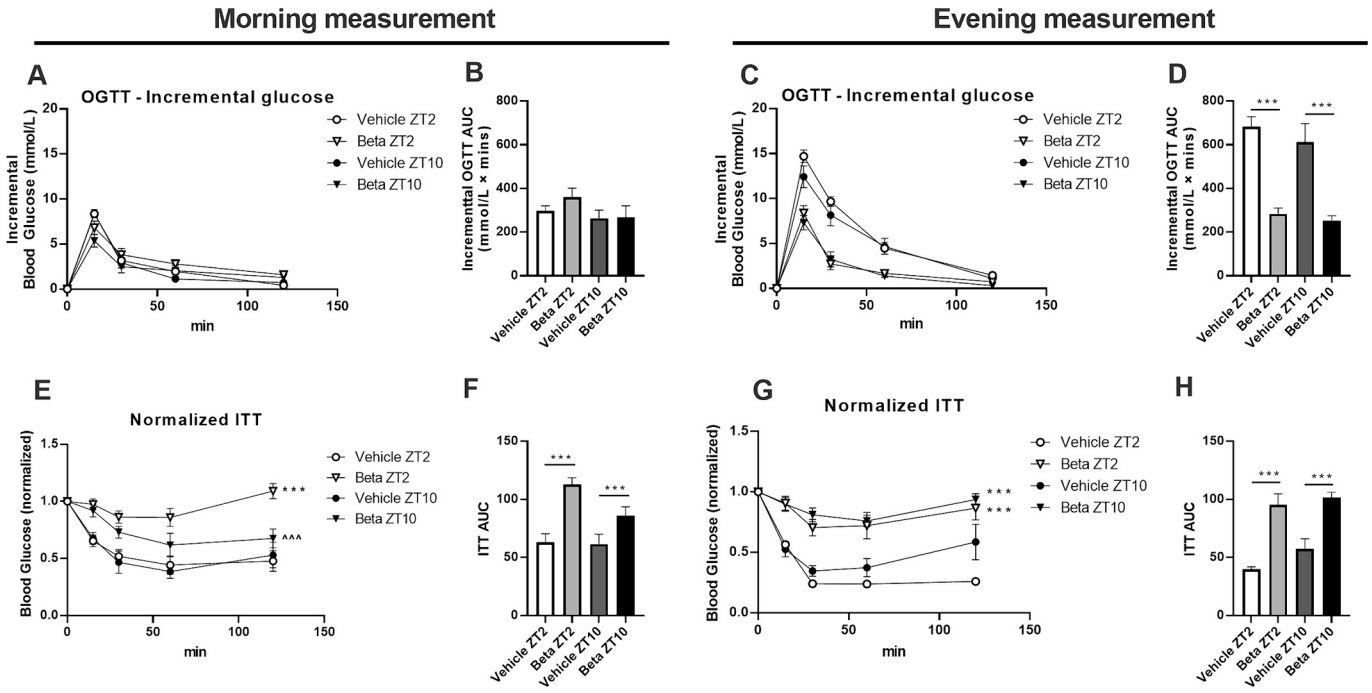
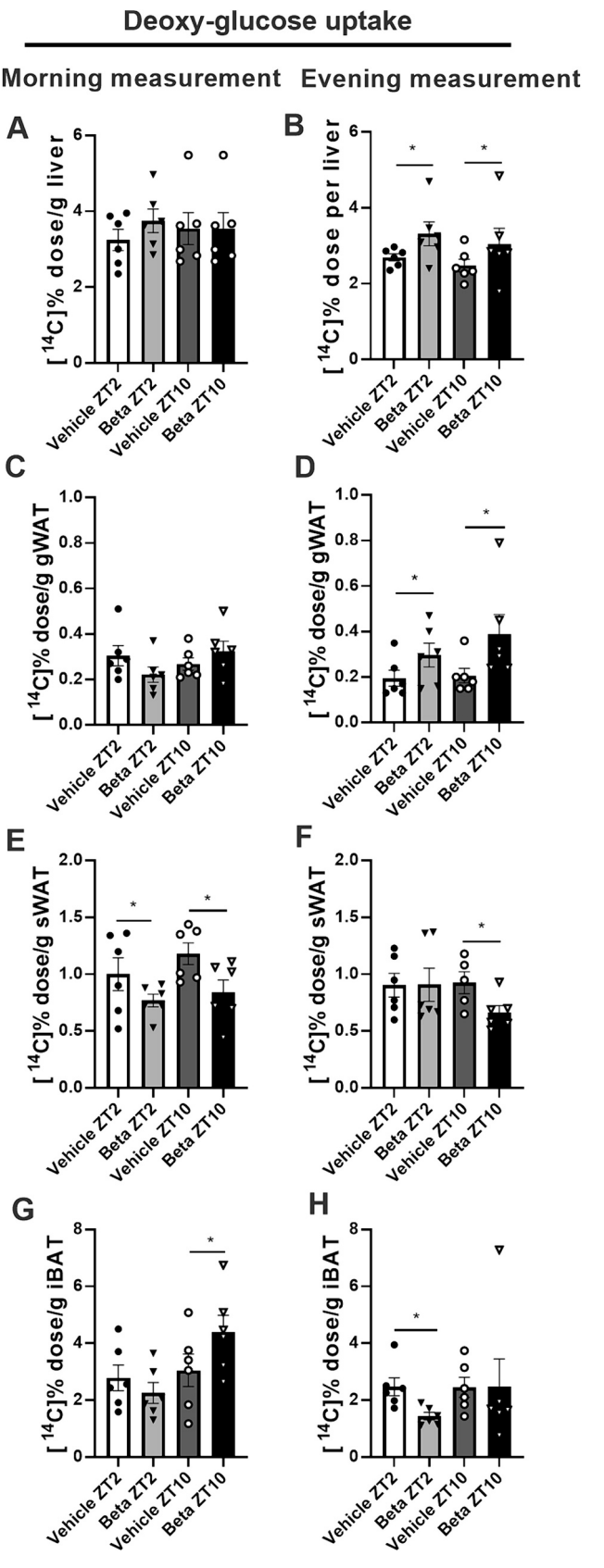


Fig. 4. Out-of-phase betamethasone treatment impairs glucose tolerance and insulin sensitivity which is dependent on time of measurement. The effect of treatment with 3.0 mg/kg betamethasone out-of-phase (ZT2) and in-phase (ZT10) on glucose metabolism in male mice. (A) Incremental glucose levels during an oral glucose tolerance test (OGTT) in the morning (ZT7–9) and (B) area under the curve (AUC), and (C) during an OGTT in the evening (ZT15–17) and (D) AUC. (E) Normalized glucose levels during an insulin tolerance test (ITT) in the morning (ZT7–9) and (F) AUC, and (G) during an ITT in the evening (ZT15–17), and (H) AUC. N = 6 mice/group. *** and ^^^ $p < 0.001$ vs. respective vehicle groups. Statistical significance was calculated using a two-way ANOVA.



(caption on next column)

Fig. 5. Out-of-phase and in-phase betamethasone treatment similarly increase deoxyglucose uptake in the liver and gWAT but differentially affect sWAT and iBAT. The effect of treatment with 3.0 mg/kg betamethasone out-of-phase (ZT2) and in-phase (ZT10) on [^{14}C]deoxyglucose uptake in (A-B) liver, (C-D) gonadal white adipose tissue (gWAT), (E-F) subcutaneous white adipose tissue (sWAT) and (G-H) interscapular brown adipose tissue (iBAT) in the morning (ZT7–9) and evening (ZT15–17) after a 6 h fast. N = 6 mice/group. *p < 0.05. Statistical significance was calculated using a two-way ANOVA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

before blood collection for plasma biochemistry measurements. When evaluating deoxy-glucose uptake by different tissues we again found differences between time of measurement, e.g. with an increase of glucose uptake in the liver and gWAT during the evening but not morning measurement. For brown adipose tissue we found an increase upon ZT10 betamethasone in the morning and a decrease in glucose uptake upon ZT2 treatment measured during the evening. Taken all those findings together, we can conclude the effect of glucocorticoid treatment on glucose metabolism is dynamic and that outcome differs throughout the time of day.

One limitation of our study is that time of measurements was not symmetric for all functional measurements, with different time between ZT2 and ZT10 betamethasone treatment and functional measurements (Table 1). We expect that this may influence some of the analyses, although the OGTT, ITT and organ uptake study were all performed with comparable time after ZT2 treatment until morning measurement and ZT10 treatment until evening measurement. It is reassuring that some of the timed glucocorticoid effects like the effect of ZT2 betamethasone treatment on insulin sensitivity during the ITT were observed during both the morning and evening test, excluding that the time since last treatment is a confounding factor for this readout. The glucocorticoid effects that we observed may be mediated via disturbance endogenous glucocorticoid levels as well as overexposure to synthetic glucocorticoids [37,38]. Our study setup does not allow us to dissect the exact contribution, but both scenarios share the aspect of GR activation at the wrong time of day. Finally, it should be noted that species differences (e.g. between rodents and humans) exist for glucocorticoids, so it is unclear to what extent these findings translate to humans [39].

In addition to glucose metabolism, we also measured other possible glucocorticoid-associated side effects. We did not observe strong effects on muscle mass or function, besides a transient reduction of muscle strength in female mice. We previously found that female mice were more sensitive to betamethasone-induced muscle dysfunction after 14 days of treatment [21], but we did not observe strong effects in this study (up to 30 days). We also monitored markers for glucocorticoid-induced osteoporosis [40], including plasma osteocalcin and P1NP, but these were not significantly influenced by neither ZT2 nor ZT10 betamethasone treatment in both male and female mice. Hyperinsulinemia can be caused by different processes in various tissues [41–43]. We could not attribute the effects of betamethasone on one tissue per se, as we did not find transcriptional changes in the liver and skeletal muscle. It is noted that we performed expression analysis after 30 days and that the effect on plasma insulin was attenuated as compared to after 14 days of treatment.

In summary, our study compared the effects of morning and evening daily betamethasone treatment on the development of glucocorticoid-associated side effects. Even if the magnitude of side effects in our study was overall modest, we found that out-of-phase betamethasone administration generally caused more adverse effects on glucose metabolism. Given the differences we observed between different times of measurement we urge the importance to carefully consider the time of measurements in studies focusing on glucocorticoid-induced side effects, at least in relation to glucose metabolism.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2024.123080.

Funding

SL and SZ were supported by a Chinese Scholarship Council grant.

CRediT authorship contribution statement

Sheng Li: Writing – original draft, Project administration, Investigation, Formal analysis, Data curation. **Sen Zhang:** Investigation. **Patrick C.N. Rensen:** Writing – review & editing, Resources, Methodology. **Onno C. Meijer:** Writing – review & editing, Supervision. **Sander Kooijman:** Writing – review & editing, Conceptualization. **Jan Kroon:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

OM received research funding from Corcept Therapeutics. JK is seconded to Corcept Therapeutics.

Acknowledgements

We thank Amanda Pronk and Trea Streefland for their excellent technical support.

References

[1] T. Kuo, A. McQueen, T.C. Chen, J.C. Wang, Regulation of glucose homeostasis by glucocorticoids, Adv. Exp. Med. Biol. 872 (2015) 99–126.
[2] D.W. Cain, J.A. Cidlowski, Immune regulation by glucocorticoids, Nat. Rev. Immunol. 17 (4) (2017) 233–247.
[3] A.J. Rose, A. Vegiopoulos, S. Herzig, Role of glucocorticoids and the glucocorticoid receptor in metabolism: insights from genetic manipulations, J. Steroid Biochem. Mol. Biol. 122 (1–3) (2010) 10–20.
[4] F. Spiga, J.J. Walker, J.R. Terry, S.L. Lightman, HPA axis-rhythms, Compr. Physiol. 4 (3) (2014) 1273–1298.
[5] D.P. Macfarlane, S. Forbes, B.R. Walker, Glucocorticoids and fatty acid metabolism in humans: fuelling fat redistribution in the metabolic syndrome, J. Endocrinol. 197 (2) (2008) 189–204.
[6] S. Chung, G.H. Son, K. Kim, Circadian rhythm of adrenal glucocorticoid: its regulation and clinical implications, Biochim. Biophys. Acta 1812 (5) (2011) 581–591.
[7] J. Qian, C. Dalla Man, C.J. Morris, C. Cobelli, F. Scheer, Differential effects of the circadian system and circadian misalignment on insulin sensitivity and insulin secretion in humans, Diabetes Obes. Metab. 20 (10) (2018) 2481–2485.
[8] C.J. Morris, J.N. Yang, J.I. Garcia, S. Myers, I. Bozzi, W. Wang, O.M. Buxton, S. A. Shea, F.A. Scheer, Endogenous circadian system and circadian misalignment impact glucose tolerance via separate mechanisms in humans, Proc. Natl. Acad. Sci. U. S. A. 112 (17) (2015) E2225–E2234.
[9] I.J. Bujalska, S. Kumar, M. Hewison, P.M. Stewart, Differentiation of adipose stromal cells: the roles of glucocorticoids and 11beta-hydroxysteroid dehydrogenase, Endocrinology 140 (7) (1999) 3188–3196.
[10] H. Hauner, G. Entenmann, M. Wabitsch, D. Gaillard, G. Ailhaud, R. Negrel, E. F. Pfeiffer, Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium, J. Clin. Invest. 84 (5) (1989) 1663–1670.
[11] R.M. O'Brien, P.C. Lucas, C.D. Forest, M.A. Magnuson, D.K. Granner, Identification of a sequence in the PEPCK gene that mediates a negative effect of insulin on transcription, Science 249 (4968) (1990) 533–537.
[12] M.J. Charron, F.C. Brosius 3rd, S.L. Alper, H.F. Lodish, A glucose transport protein expressed predominately in insulin-responsive tissues, Proc. Natl. Acad. Sci. U. S. A. 86 (8) (1989) 2535–2539.
[13] M.J. Birnbaum, Identification of a novel gene encoding an insulin-responsive glucose transporter protein, Cell 57 (2) (1989) 305–315.
[14] A. Gupta, Y. Gupta, Glucocorticoid-induced myopathy: pathophysiology, diagnosis, and treatment, Indian J. Endocrinol. Metab. 17 (5) (2013) 913–916.
[15] P.T. Donnan, G. Libby, A.C. Boyter, P. Thompson, The population risk of fractures attributable to oral corticosteroids, Pharmacoepidemiol. Drug Saf. 14 (3) (2005) 177–186.
[16] R.S. Weinstein, Glucocorticoid-induced osteonecrosis, Endocrine 41 (2) (2012) 183–190.
[17] O. Schakman, H. Gilson, J.P. Thissen, Mechanisms of glucocorticoid-induced myopathy, J. Endocrinol. 197 (1) (2008) 1–10.
[18] M. McMahon, J. Gerich, R. Rizza, Effects of glucocorticoids on carbohydrate metabolism, Diabetes Metab. Rev. 4 (1) (1988) 17–30.
[19] G. Arnaldi, V.M. Scandali, L. Trementino, M. Cardinaletti, G. Appolloni, M. Boscaro, Pathophy- siology of dyslipidemia in Cushing's syndrome, Neuroendocrinology 92 (Suppl. 1) (2010) 86–90.
[20] O.C. Meijer, A.M. Pereira, Three percent annually on systemic glucocorticoids: facts, worries and perspectives, Eur. J. Endocrinol. 181 (6) (2019) C23–C28.
[21] S. Li, M. Schonke, J.C. Buurstede, T.J.A. Moll, M. Gentenaar, M. Schilperoort, J. A. Visser, K. Kaikaew, D. van de Vijver, T. Abbassi-Daloii, V. Raz, A. Aartsma-Rus, M. van Putten, O.C. Meijer, J. Kroon, Sexual dimorphism in transcriptional and functional glucocorticoid effects on mouse skeletal muscle, Front. Endocrinol. (Lausanne). 13 (2022) 907908.
[22] P.P. Khedoe, G. Hoeke, S. Kooijman, W. Dijk, J.T. Buijs, S. Kersten, L.M. Havekes, P.S. Hiemstra, J.F. Berbee, M.R. Boon, P.C. Rensen, Brown adipose tissue takes up plasma triglycerides mostly after lipolysis, J. Lipid Res. 56 (1) (2015) 51–59.
[23] L. Zhang, D.A. Prosdocimo, X. Bai, C. Fu, R. Zhang, F. Campbell, X. Liao, J. Coller, M.K. Jain, KLF15 establishes the landscape of diurnal expression in the heart, Cell Rep. 13 (11) (2015) 2368–2375.
[24] A. Balsalobre, S.A. Brown, L. Marcacci, F. Tronche, C. Kellendonk, H.M. Reichardt, G. Schutz, U. Schibler, Resetting of circadian time in peripheral tissues by glucocorticoid signaling, Science 289 (5488) (2000) 2344–2347.
[25] T. Wu, J. Jiang, L. Yang, H. Li, W. Zhang, Y. Chen, B. Zhao, B. Kong, P. Lu, Z. Zhao, J. Zhu, Z. Fu, Timing of glucocorticoid administration determines severity of lipid metabolism and behavioral effects in rats, Chronobiol. Int. 34 (1) (2017) 78–92.
[26] S. Tholen, R. Patel, A. Agas, K.M. Kovary, A. Rabiee, H.T. Nicholls, E. Bielczyk-Maczynska, W. Yang, F.B. Kraemer, M.N. Teruel, Flattening of circadian glucocorticoid oscillations drives acute hyperinsulinemia and adipocyte hypertrophy, Cell Rep. 39 (13) (2022) 111018.
[27] F. Buttgereit, J.S. Smolen, A.N. Coogan, C. Cajochen, Clocking in: chronobiology in rheumatoid arthritis, Nat. Rev. Rheumatol. 11 (6) (2015) 349–356.
[28] N.G. Arvidson, B. Gudbjornsson, A. Larsson, R. Hallgren, The timing of glucocorticoid administration in rheumatoid arthritis, Ann. Rheum. Dis. 56 (1) (1997) 27–31.
[29] S.J. Gasparini, M.M. Swarbrick, S. Kim, L.J. Thai, H. Henneicke, L.L. Cavanagh, J. Tu, M.C. Weber, H. Zhou, M.J. Seibel, Androgens sensitise mice to glucocorticoid-induced insulin resistance and fat accumulation, Diabetologia 62 (8) (2019) 1463–1477.
[30] J. Kroon, A.M. Pereira, O.C. Meijer, Glucocorticoid sexual dimorphism in metabolism: dissecting the role of sex hormones, Trends Endocrinol. Metab. 31 (5) (2020) 357–367.
[31] K. Kaikaew, J. Steenbergen, T.H. van Dijk, A. Grefhorst, J.A. Visser, Sex difference in Corticos- terone-induced insulin resistance in mice, Endocrinology 160 (10) (2019) 2367–2387.
[32] H. Ozdemir, T. Guvenal, M. Cetin, T. Kaya, A. Cetin, A placebo-controlled comparison of effects of repetitive doses of betamethasone and dexamethasone on lung maturation and lung, liver, and body weights of mouse pups, Pediatr. Res. 53 (1) (2003) 98–103.
[33] M. Quattrocelli, D.Y. Barefield, J.L. Warner, A.H. Vo, M. Hadhazy, J.U. Earley, A. R. Demonbreun, E.M. McNally, Intermittent glucocorticoid steroid dosing enhances muscle repair without eliciting muscle atrophy, J. Clin. Invest. 127 (6) (2017) 2418–2432.
[34] N.H.F. Fine, C.L. Doig, Y.S. Elhassan, N.C. Vierra, P. Marchetti, M. Bugliani, R. Nano, L. Piemonti, G.A. Rutter, D.A. Jacobson, G.G. Lavery, D.J. Hodson, Glucocorticoids reprogram beta-cell signaling to preserve insulin secretion, Diabetes 67 (2) (2018) 278–290.
[35] B.E. Corkey, Banting lecture 2011: hyperinsulinemia: cause or consequence? Diabetes 61 (1) (2012) 4–13.
[36] B.B. Kahn, J.S. Flier, Obesity and insulin resistance, J. Clin. Invest. 106 (4) (2000) 473–481.
[37] B.G. Slavin, J.M. Ong, P.A. Kern, Hormonal regulation of hormone-sensitive lipase activity and mRNA levels in isolated rat adipocytes, J. Lipid Res. 35 (9) (1994) 1535–1541.
[38] Z. Gao, X. Zhang, A. Zuberi, D. Hwang, M.J. Quon, M. Lefevre, J. Ye, Inhibition of insulin sensitivity by free fatty acids requires activation of multiple serine kinases in 3T3-L1 adipocytes, Mol. Endocrinol. 18 (8) (2004) 2024–2034.
[39] L.E. Ramage, M. Akyol, A.M. Fletcher, J. Forsythe, M. Nixon, R.N. Carter, E.J. van Beek, N.M. Morton, B.R. Walker, R.H. Stimson, Glucocorticoids acutely increase Brown adipose tissue activity in humans, revealing species-specific differences in UCP-1 regulation, Cell Metab. 24 (1) (2016) 130–141.
[40] I.R. Reid, Glucocorticoid osteoporosis–mechanisms and management, Eur. J. Endocrinol. 137 (3) (1997) 209–217.
[41] M.C. Moore, M.S. Smith, B. Farmer, K.C. Coate, G. Kraft, M. Shiota, P.E. Williams, A.D. Cherrington, Morning hyperinsulinemia primes the liver for glucose uptake and glycogen storage later in the day, Diabetes 67 (7) (2018) 1237–1245.
[42] P.J. Rozance, A.K. Jones, S.L. Bourque, A. D'Alessandro, W.W. Hay Jr., L.D. Brown, S.R. Wesolowski, Effects of chronic hyperinsulinemia on metabolic pathways and insulin signaling in the fetal liver, Am. J. Physiol. Endocrinol. Metab. 319 (4) (2020) E721–E733.
[43] M.C. Turner, N.R.W. Martin, D.J. Player, R.A. Ferguson, P. Wheeler, C.J. Green, E. C. Akam, M.P. Lewis, Characterising hyperinsulinemia-induced insulin resistance in human skeletal muscle cells, J. Mol. Endocrinol. 64 (3) (2020) 125–132.