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Evaluating Glucocorticoid Administration on Biomechanical Properties of Rats' Tibial Diaphysis

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Background: Osteoporosis is a disease, which causes bone loss and fractures. Although glucocorticoids effectively suppress inflammation, their chronic use is accompanied by bone loss with a tendency toward secondary osteoporosis.
Objectives: This study took into consideration the importance of cortical bone in the entire bone's mechanical competence. Hence, the aim of this study was to assess the effects of different protocols of glucocorticoid administration on the biomechanical properties of tibial bone diaphysis in rats compared to control and low-level laser-treated rats.
Materials and Methods: This experimental study was conducted at Shahid Beheshti University of Medical Sciences, Tehran, Iran. We used systematic random sampling to divide 40 adult male rats into 8 groups with 5 rats in each group. Groups were as follows: 1) control, 2) dexamethasone (7 mg/week), 3) dexamethasone (0.7 mg/week), 4) methylprednisolone (7 mg/kg/week), 5) methylprednisolone (5 mg/kg twice weekly), 6) dexamethasone (7 mg/kg three times per week), 7) dexamethasone (0.7 mg/kg thrice per week), and 8) low-level laser-treated rats. The study periods were 4-7 weeks. At the end of the treatment periods, we examined the mechanical properties of tibial bone diaphysis. Data were analyzed by statistical analyses.
Results: Glucocorticoid-treated rats showed weight loss and considerable mortality (21%). The biomechanical properties (maximum force) of glucocorticoid-treated rats in groups 4 (62 ± 2.9), 6 (63 ± 5.1), and 7 (60 ± 5.3) were comparable with the control (46 ± 1.5) and low-level laser-treated (57 ± 3.2) rats.
Conclusions: In contrast to the findings in humans and certain other species, glucocorticoid administration caused anabolic effect on the cortical bone of tibia diaphysis bone in rats.

Keywords: Glucocorticoids; Biomechanical Phenomena; Rats; Tibia

1. Background

Osteoporosis (OP) is a disease, which causes bone loss and fractures, leading to severe pain, deformities, and in certain cases, secondary complications that result in death (1). Glucocorticoids (GCs) are potent anti-inflammatory and immune-suppressive drugs. Synthetic GCs have been widely used for many decades as treatment for various autoimmune, pulmonary, periodontal, and gastrointestinal disorders (2). Although GCs effectively suppress inflammation, their prolonged use is accompanied by bone loss leading to OP. GC–induced OP is the most common cause of secondary OP (2). Van Staa et al. in their retrospective study reported a 0.5% prevalence for chronic use of oral GCs in the general population of the United Kingdom. The prevalence was higher in women over the age of 55 years (1.7%) and as high as 2.5% in people older than 70 years (3).

Various animal models have been used to investigate the pathogenesis of OP, facilitate preclinical testing, and test new treatment options such as antiresorptive drugs (4). Histomorphometric parameters and biochemical markers of bone metabolism in animal studies only indicate a decrease in bone formation and minimal changes in bone resorption. These parameters are less important with regard to OP-associated fractures and investigations in orthopedic surgery. Furthermore, histological and biochemical studies do not give direct information about the mechanical strength of the bone. The ultimate reason for a bone fracture following minimal trauma is the reduction in mechanical strength (5). Although bone densitometry is often used as a surrogate to evaluate bone fragility, direct biomechanical testing of the bone undoubtedly provides more information in terms of mechanical integrity (6). Small animal models (in particular the rat model) of GC-induced OP have been reported to be relatively resistant to corticosteroid-induced bone damage (7-10). Numerous in vivo studies indicate that GC administration to rats histologically and histomorphometrically inhibit bone formation in the long bones

(11-20), decrease bone strength (21-23) and bone mineral density (BMD) as revealed through BMD and other radiologic imaging techniques (11, 12, 14-17, 21, 22), and result in a decrease in serum biomarkers (12, 14-16, 18). The term low-level laser-therapy (LLLT) is broadly applied to the therapeutic effects of lasers. Mester introduced the earliest clinical application of LLLT in 1968. Previous studies have noted the positive effects of LLLT both on the intact bone and on the bone healing process (24). LLLT can act as a proposed anabolic therapeutic agent on diseased bony tissues.

2. Objectives

The present study aimed to assess the effects of different GC administration protocols of methylprednisolone (Met) and dexamethasone (Dex) on the biomechanical properties of rats' diaphysis. This assessment included bending stiffness (Young Modulus of elasticity), maximum force, stress high load, energy absorption up to maximum force, and energy absorption up to the breaking point by the use of a 3 point bending biomechanical test. We also intended to compare the data on biomechanical properties of tibial diaphysis in the GC treated-rats to that of LLLT healthy rats.

3. Materials and Methods

3.1. Experimental Animals

We used systematic random sampling in this study. A total of 40 male Wistar rats, aged 4.5 months (Pasteur Institute, Tehran, Iran) were housed individually in a temperature-controlled room (23 ± 1°C) with a 12:12 h light/dark cycle. Animals were provided water ad libitum. The study was performed in Tehran, Iran during 2013 and 2014. All procedures were approved by the Medical Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (protocol no 1391-1-115-1092). Rats' body weights were monitored weekly and the dose of drugs administered was calculated according to the most recent body weights.

3.2. Experimental Protocols

We randomly assigned the rats into the following 8 groups (n = 5 per group) (25, 26):

1) vehicle (intramuscular injections of 100 μL distilled water, twice/week); 2) Dex (Alborz Darou, Tehran, Iran) 7 mg/kg/week for 5 weeks (I) (20); 3) Dex 0.7 mg/kg/week for 5 weeks (I); 4) Met (Alborz Darou,Tehran, Iran) 7 mg/kg/week for 5 weeks (I) (11); 5) Met 5 mg/kg/week, administered as 5 injections during the first week (27) and twice weekly afterwards for a total of 17 injections over 7 weeks. This protocol was revised due to severe weight loss in the rats (II); 6) Dex 7 mg/kg thrice per week for a total of 16 injections over 7 weeks (III); and 7) Dex 0.7 mg/kg thrice per week for a total of 21 injections over 7 weeks (III). Although we observed weight loss in all groups that received GC injections, this weight loss was more severe in rats in groups 2, 4, 5 and 6. In groups 2 and 4, two rats in each group died; in groups 5 and 6, three rats in each group died, which was probably due to unexpected large weight loss. The dead rats were replaced. In group 8, the right tibias of the rats were exposed to a pulsed infrared diode laser (MUSTANG 2000 with LO7 pen [radiating head], Technica Co., Moscow, Russia). Table 1 shows specifications of the laser. In the present study, the surface area of the target tissue (whole length of the tibia) was larger than the pen's spot size; therefore, we used sequential treatments to ensure that every unit area received a similar laser dose (28). LLLT was performed on 4 distinct regions with the laser pen perpendicular to the target tissue from a distance of less than 1 cm. During LLLT, animals were sedated by administration of ketamine hydrochloride (25 mg/kg) injected intramuscularly along with diazepam (25 mg/kg). Animals received LLLT once daily, 6 days per week for a 3-week period.

Table 1. Specifications of the Infrared Laser Used

Parameter	Dose and Unit
Peak power output, W	75
Average power, mW	1.08
Power density, mW/cm^2	1.08
Wave length, nm	890
Pulse frequency, Hz	80
Spot size, cm^2	1
Pulsed duration, μs	180
Duration of exposure for each point, s	900
Energy density, J/cm^2	0.972

3.3. Biomechanical Examination

At the end of the treatment protocols, all animals were sacrificed. Rats' weights (g) were measured using a precision scale (Sartorius TE214S A, Germany). Tibias were extracted and kept moist throughout the testing procedure. Bones were subjected to 3 point bending on a material testing device (Zwick/Roell, Germany) until the occurrence of a fracture, which separated the bone into two pieces. All bones were oriented similarly in the testing machine and the surface area of the bone was also calculated. Two loading points, 19 mm apart, were used to mount each bone and a press head was then activated to compress the midline of the bone shaft until fracture occurred. The compressive loading speed was 0.08 mm/s for all tests. Specimens were loaded uniaxially so that the fracture and complete load-deformation curve could be recorded by transducers coupled to bridges, then sampled in a personal computer by an analog-to-digital convertor (PC-software 27005). Load characteristics were directly plotted on an X-Y chart recorder. From the load-

deformation curve, the following biomechanical properties were automatically calculated: bending stiffness (N/mm^2), maximum force (N), stress high load (N/mm^2), energy absorption up to maximum force (N.mm), and energy absorption up to the breaking point (N.mm). Briefly, these biomechanical parameters are defined as follows. Bending stiffness is the slope of the linear portion of load-deformation curve, i.e., the ratio of loading to deformation in the elastic region of the curve. Maximum force is the force needed to break the bone microscopically. The stress high load is calculated by dividing the maximum force value by surface area (mm^2) of the bone. Energy absorption up to the maximum force is the amount of energy absorbed by the bone until it is broken microscopically at the point of maximum force; energy absorption up to the breaking point is the amount of energy absorbed by the bone until it is broken macroscopically (29).

3.4. Statistical Analyses

All data are expressed as the mean ± standard error of the mean (SEM). The overall differences of all biomechanical parameters, with the exception of bending stiffness were analyzed using 1-way analysis of variance (ANOVA). Group comparisons were obtained by applying the least significant difference (LSD) method in the analysis. The overall differences of bending stiffness were analyzed using the Kruskal-Wallis test. The group comparisons were obtained by applying the Mann-Whitney U test method in the analysis. P value of less than 0.05 was considered significant for the ANOVA, LSD, and Kruskal-Wallis tests. A P value below 0.007 was considered significant for the Mann-Whitney U test.

4. Results

The rats' weights throughout the study period are shown in Table 2. Results of biomechanical examinations are listed in Table 3 and Figure 1 - 5. Our results showed that GC administration protocols kept the biomechanical properties of rats' tibial diaphysis.

4.1. Bending stiffness

No significant difference was observed between the study groups (Figure 1).

4.2. Maximum Force

According to the ANOVA test, GC rats in groups 4, 6, and 7 showed significant increases compared to control rats (P = 0.007). The same result was observed with the LSD test, where groups 4 (P = 0.010), 6 (P = 0.004), and 7 (P = 0.020) significantly differed from the control rats. As shown in Figure 2, laser-treated rats showed a significant increase compared to control rats (LSD, P = 0.050) and group 5 (LSD, P = 0.038).

Table 2. Comparison of the Mean Values (± SEM) of Rats' Weights at the Beginning and End of the Study in Various Groups; Control Group = (1); 2 = Dex 7mg/kg, I; 3 = Dex 0.7 mg/kg, I; 4 = Met 7mg/kg, I; 5 = Met 5 mg/kg, II; 6 = Dex 7mg/kg, III; 7 = Dex 0.7 mg/kg, III; 8 = Low-Level Laser [a]

	Groups							
Weight, g	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
First	264 ± 4.3	264.4 ± 4.2	257.4 ± 9.8	266.2 ± 7.8	272.2 ± 8.8	272.2 ± 8.8	260.2 ± 8.5	263.2 ± 7.1
Last	263 ± 4.6	216 ± 2.4 [b]	244.4 ±7.5	211.8±4.1 [c]	211.6±3.6 [c]	213±3.7 [b]	253.6±3.7	255.6 ± 6.8

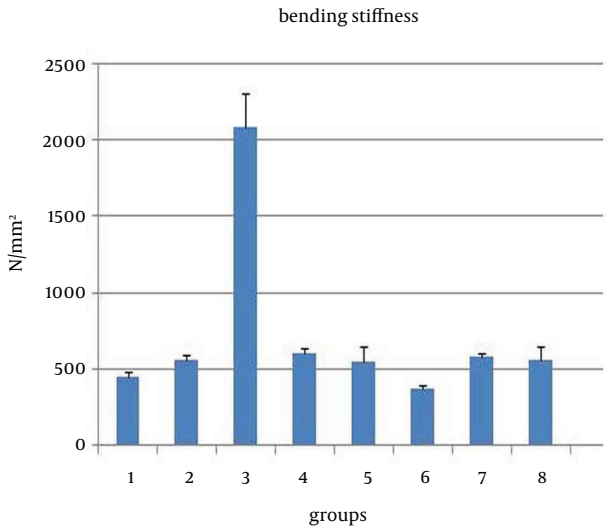
[a] Data are presented as Mean ± SD.
[b] Treated rats < 0.001.
[c] Treated rats < 0.01.

Table 3. Mean ± SEM Biomechanical Parameters of Tibial Diaphysis in the Study Groups; Control Group = (1); 2 = Dex 7mg/kg, I; 3 = Dex 0.7 mg/kg I, 4 = Met 7mg/kg, I; 5 = Met 5 mg/kg, II; 6 = Dex 7mg/kg, III; 7 = Dex 0.7 mg/kg, III; 8 = Low-Level Laser-Treated Rats [a, b]

	Groups							
Parameter	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Bending stiffness(N/mm^2)	456 ± 27	565 ± 29	2088 ± 213	611 ± 23	547 ± 102	369 ± 23	585 ± 24	563 ± 86
Maximum force (MF)(N)	46 ± 1.5	54 ± 3.6	46 ± 1.2	62 ± 2.9	45 ± 6.1	63 ± 5.1	60 ± 5.3	57 ± 3.2
stress high load (SHL) (N/mm^2)	8.9 ± 0.19	10.1 ± 0.54	27 ± 3.2	13.3 ± 0.41	11.6 ± 1.6	10.2 ± 1.1	27.1 ± 0.71	11.3 ± 1.4
Energy absorption up to MF(Nmm)	31.8 ± 4.5	27.4 ± 2.2	26.1 ± 2.7	33.6 ± 5.7	26.3 ± 2	39.9 ± 3	36.4 ± 3.2	38.8 ± 3.6
Energy absorption up to SHL(Nmm)	51 ± 4.5	50.4 ± 5.7	36.1 ± 3.7	62.4 ± 1.4	35.7 ± 1.9	50.5 ± 3.1	46.6 ± 7.3	62.3 ± 9.8

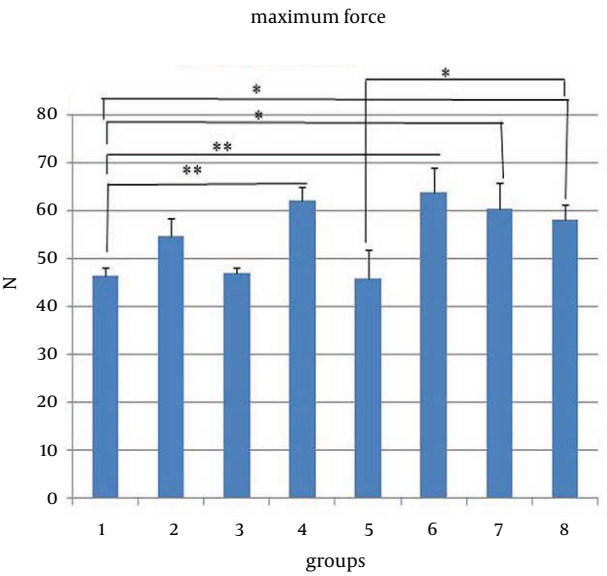
[a] Data are presented as Mean ± SD.
[b] Abbreviations: Maximum force, MF; Stress high load, SHL.

Figure 1. Bending Stiffness (N/mm^2) of Tibial Diaphysis in the Study



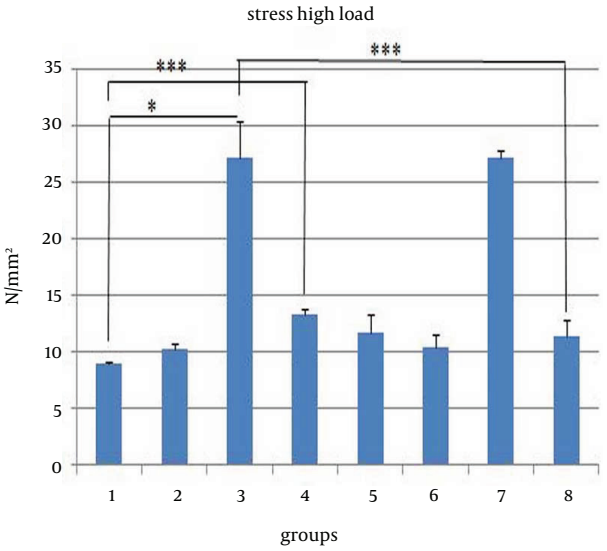
Values are mean ± SEM for 5 animals per group. Statistical comparisons made against control rats (Kruscall wallis and Mann Whitney U tests). Control group = Control (1); 2 = Dex 7 mg/kg, I; 3 = Dex 0.7 mg/kg I, 4 = Met 7mg/kg, I; 5 = Met 5 mg/kg, II; 6 = Dex 7mg/kg, III; 7 = Dex 0.7 mg/kg, III; 8 = low-level laser-treated rats; *P < 0.05, ** P< 0.01; *** P< 0.001.

Figure 2. Maximum Force (N) of Tibial Diaphysis in the Study



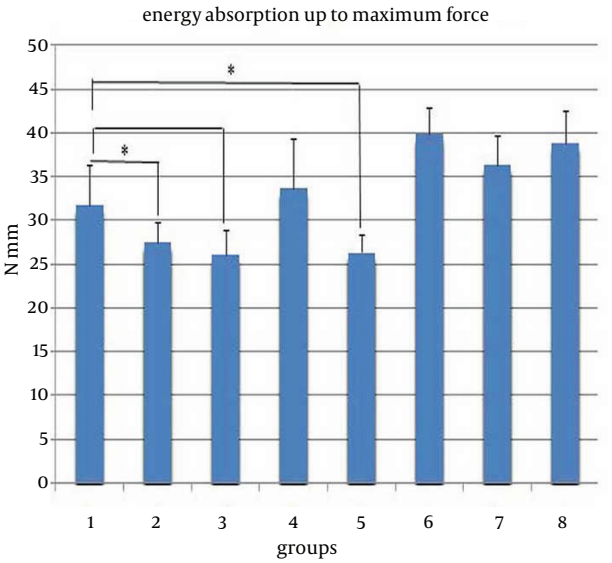
Values are mean ± SEM for five animals per group. Statistical comparisons made against control rats (ANOVA). Control group = control (1); 2 = Dex 7mg/kg, I; 3 = Dex 0.7 mg/kg, I; 4 = Met 7 mg/kg, I; 5 = Met 5mg/kg, II; 6 = Dex 7mg/kg, III; 7 = Dex 0.7 mg/kg, III; 8 = Laser-treated rats; *P < 0.05, **P < 0.01; ***P < 0.001.

Figure 3. Stress High Load (N/mm^2) of Tibial Diaphysis From the Groups Studied



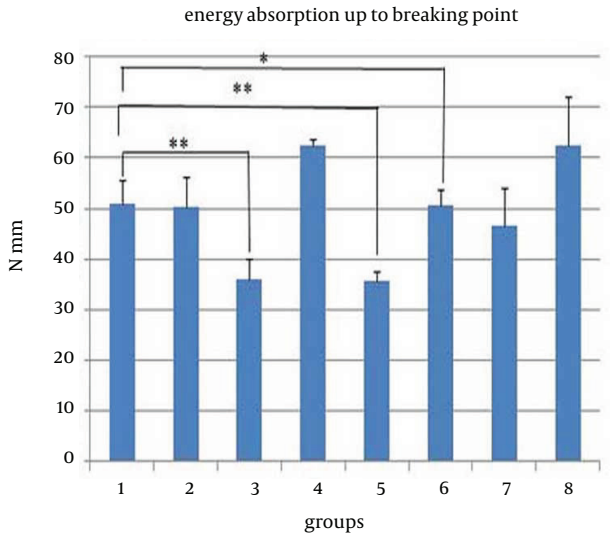
Values are mean ± SEM for five animals per group. Statistical comparison made against control rats (ANOVA). Control group = control (1); 2 = Dex 7 mg/kg, I; 3 = Dex 0.7 mg/kg, I; 4 = Met 7 mg/kg, I; 5 = Met 5 mg/kg, II; 6 = Dex 7 mg/kg, III; 7 = Dex 0.7 mg/kg, III; 8 = Laser-treated rats; * P< 0.05; ** P<0.01; *** P< 0.001.

Figure 4. Energy Absorption up to Maximum Force (Nmm) of Tibial Diaphysis in the Study



Values are mean ± SEM for five animals per group. Statistical comparison made against control rats (ANOVA). Control group = control (1); 2 = Dex 7 mg/kg, I; 3 = Dex 0.7 mg/kg, I; 4 = Met 7 mg/kg, I; 5 = Met 5 mg/kg, II; 6 = Dex 7 mg/kg, III; 7 = Dex 0.7 mg/kg, III; 8 = Laser-treated rats; *P < 0.05; **P <0.01; ***P < 0.001.

Figure 5. Energy Absorption up to Breaking Point (N.mm) of Tibial Diaphysis



Values are mean ± SEM for 5 animals per group. Statistical comparison made against control rats (ANOVA). Control group = control (1), 2 = Dex 7 mg/kg, I; 3 = Dex 0.7 mg/kg, I; 4 = Met 7mg/kg, I; 5 = Met 5 mg/kg, III, 8 = Laser-treated rats; *P < 0.05; **P <0.01; ***P < 0.001.

4.3. Stress High Load

GC-treated rats from groups 3 and 4 showed significant increases compared to the control group (ANOVA, P < 0.000). According to the LSD test, groups 3 (P = 0.043) and 4 (P < 0.000) also showed significant differences with the control group rats. GC-treated rats from group 3 showed a significant increase compared to laser-treated rats (LSD, P < 0.000, Figure 3).

4.4. Energy Absorption up to Maximum Force

Laser-treated rats showed a significant increase compared to GC- treated rats from groups 2, 3, and 5 (ANOVA, P = 0.044). According to the LSD test, the laser-treated rats also showed a significant increase compared to groups 2 (P = 0.032), 3 (P = 0.018), and 5 (P = 0.019) (Figure 4).

4.5. Energy Absorption up to Breaking Point

There was a significant increase in laser-treated rats compared to GC-treated rats in groups 3, 5, and 7 (ANOVA, P = 0.007). According to the LSD test, laser-treated rats also showed a significant increase compared to rats from groups 3 (P = 0.002), 5 (P = 0.001), and 7 (P = 0.049, Figure 5).

5. Discussion

Excess GC administration is usually associated with excessive bone loss and a subsequent increase in bone fractures (1). Based on previously published rat models of GC administration, we established different rat models to investigate the adverse effects of GC administration on the biomechanical properties of rats' tibial diaphysis. Although weight loss was observed in all groups that received GC, this loss was more severe in groups 5 and 6. Rats from group 5 received 17 injections of Met 5 mg/kg for 7 weeks, and rats from group 6 received 16 injections of Dex 7 mg/kg for 7 weeks. We observed a considerable mortality rate (21%) in the GC-treated rats, which was not reported in previous studies. We also observed that the biomechanical properties of the tibial diaphysis of GC-treated rats were comparable to those of both the control and laser-treated rats. LLLT is an anabolic agent for bones (24). The LLLT protocol used in the present study has shown a positive effect in complex medical situations in previous studies (30, 31).

We concluded that these findings might be attributed to the anabolic effects of GC administration at the level of the cortical bone. The results of the biomechanical examination of GC-treated rats were accompanied by body weight loss and a relatively high rate of mortality. These data were markedly different from findings in patients with supraphysiologic GC administration, which showed bone loss (1) in some animal model studies (21-23). A summary of related articles are shown in Table 4. Administration of Dex 7 mg/kg/day for 13 days in 30-day-old rats significantly decreased the femoral load-bearing capacity associated with impairment in bone geometric properties and body weight gain (21). Four weeks administration of prednisolone 10 mg/kg/day in 6-month-old rats has been reported to decrease femoral bone strength without any change in BMD. This was accompanied by a decrease in the content of enzymatic cross-links (22). A 12-week treatment period with prednisolone 3.5 mg/kg/day in 6-month-old rats significantly decreased femoral strength and BMD (23). Supraphysiologic doses of GCs also cause weight gain in patients (32).

The anabolic effects of GC administration on the tibia reported in the present study support the results of previous studies (7-10, 33). Treatment with cortisol for 14 days in 24-day-old rats significantly increased the relative integrated radiographic density of the tibias when compared with control animals. Yasumura concluded that this effect (likely due to a decreased rate of bone resorption) could not be excluded as a contributing factor (33). Ortoft et al. reported that treatment of rats with Met 5 mg/kg for 30 days had no significant effect on cortical bone (7). Binz et al. investigated the influence of IGF-I on the bone of male rats treated with a high dose of Dex (1 mg/L) in drinking water. Dex-treated rats lost weight. Histomorphometric analysis revealed no difference in vertebral trabecular bone density between the study groups. In contrast, mean trabecular bone density in tibial metaphysis increased markedly after treatment with Dex (8). Bone volume significantly increased in 24-day-old rats treated for 19 days with Dex at a constant rate of 16.25 μg/day.

Table 4. Report of Articles Regarding the Effect of Glucocorticoids (GC) on Bone

Order	Reference Number	Investigator (s)	Animal and Drug	Duration of drug Treatment	Result (on bone)
1	(21)	Ferretti et al., 1995	Rat, Dexamethasone	13 days	Femoral impairment
2	(22)	Saito et al., 2011	Rat, Prednisolone	4 weeks	Decreased femoral strength
3	(23)	Cui et al., 2012	Rat, Prednisolone	12 weeks	Decreased femoral strength
4	(7)	Ortoft et al., 1992	Rat, Methylprednisolone	30 days	No significant effect on bone
5	(8)	Binz et al., 1994	Rat, Dexamethasone	30 days	Increased mean trabecular density
6	(9)	King et al., 1996	Rat, Prednisolone	Four weeks	Protective effect on skeleton
7	(10)	Henneicke et al. , 2011	Mice, Corticosterone	Four weeks	Decrease bone resorption
8	(33)	Yasmura, 1976	Rat, Adrenocortical steroids	Two weeks	Inhibition of tibial bone resorption
9	(34)	Ortoft and Oxlund, 1998	Rat, Methylprednisolone	5 - 90 days	No difference in short use
10	(35)	Li et al, 1996	Rat, Corticosterone	3 weeks	Inhibits tibial growth and turnover

This was accompanied by a significant gain in femoral weight and calcium content. King et al. concluded that in marked contrast to findings in humans and certain other species, Dex treatment increased bone mass in rats. This might partly be due to a relatively greater suppression of resorption compared to formation (8). Four weeks of prednisolone treatment alone or in combination with estrogen, dietary calcium deficiency, or immobilization in 6-month-old rats significantly inhibited bone formation, but did not induce bone loss in mature rats. King et al. concluded that unlike the effects observed in humans treated with GCs, treatment of rats with prednisolone not only did not result in bone loss but might also exert a protective effect on the skeleton through the inhibition of bone resorption (9). Eight-week-old male transgenic (tg) and wild-type (WT) mice were treated with either corticosterone (CS) 1.5 mg or placebo for a 4-week period. Their Tibiae bones were analyzed by micro-CT and histomorphometry at the end of the treatment period (10). The effect of CS on cortical bone differed by site. At the endosteal surface, exposure to CS significantly increased bone resorption and reduced bone formation. In contrast, at the pericortical surface bone resorption significantly decreased. Tg mice were partially protected from the effects of exogenous CS, both at the cellular and structural levels (10). Our observation that the cortical bone area of rats treated with GCs for 4-7 weeks remained at the levels of control rats agreed with the previous findings by Henneicke et al. (10), Ortoft and Oxlund (34), and Li et al. (35) that neither the cortical bending stress and BMD nor the cortical bone mass decreased with 3 to 4 weeks of GC treatment in rats compared to rats treated with GC for 90 days. The cellular mechanisms underlying the anabolic effects of GC administration on tibial diaphysis observed in this study remain unclear.

The strong points of this study include the assessment of different GC medication administration (Dex and Met) effects on cortical bone (tibia) strength in vivo and the use of evaluating methods that directly report bone strength. The weak points of this study include the medication side effects of weight loss and death. Thus, we were obligated to administer the medications over shorter time periods. Further elucidation of our findings may shed light over the physiology of cortical bone formation and how GC affects this process in rats. A clear understanding of the molecular and cellular mechanisms underlying this observation can possibly have therapeutic applications. We conclude that in marked contrast to the findings in human and certain other species, GC administration causes severe weight loss and a considerable mortality rate in rats, accompanied by an increase in the biomechanical properties of rats' tibial diaphysis, which was comparable to those of the control group and the laser-treated group. This result may reflect the anabolic effect of GC administration on the cortical bony tissue of rats' tibial diaphysis.

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