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Comparison between the effects of abrupt and gradual testosterone deficiency on bone quantity and quality in male rats

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ABSTRACT: Changes in bone quantity and quality in abruptly and gradually testosterone-depleted males were less documented and cause complications in osteoporosis treatment. Four-month-old male SD rats were divided into 2 groups: sham-operated (SH) and orchidectomized (ODX) and euthanized at 9 days, 2, 4, 6, and 8 months after operation. Blood sera were collected for testosterone measurements, 4th lumbar vertebra (L4) and right tibia (metaphysis (TM) and diaphysis (TD)) were collected and measured bone quantity (bone mineral content, BMC) and quality (bone area, cortical thickness and circumferences) on total (*total*), trabecular (*trab*) and cortical (*cort*) compartments using peripheral quantitative computed tomography. In the SH rats, testosterone levels were gradually and significantly decreased by 8 months. In the ODX rats, an abrupt, non-detectable testosterone level was evident at 9 days. For bone changes, only TM (*trab*-BMC) had a negative correlation with increasing age in the SH rats. In ODX rats, each bone site showed different loss patterns. Significant decreases were detected in L4 (*total*- and *cort*-BMCs, *cort*-area) and TM (*trab*-BMC). Excluding the effect of aging, age-matched ODX and SH rats were compared. The ODX rats had lower L4's *total*- and *cort*-BMCs and areas, TM's *trab*-BMC, and TD's *total*- and *cort*-BMCs, *cort*-thickness, and periosteal circumference, but larger L4's *trab*-area and thicker TM's *cort*-area and thickness, than the SH rats. Our study indicates that differences in testosterone deficiency patterns convey different bone (mass and quality) changes depending on sites and compartments. This insight could provide information for preventive and therapeutic strategies on bone in males.

KEYWORDS: bone geometry, bone mineral content, computed tomography, lumbar vertebra, osteoporosis

INTRODUCTION

Within these 70 years, the global population has a longer life expectancy of more than 20 years (life expectancy was 73.8 years for women and 68.4 years for men in 2021, and 48.4 years for women and 44.6 years for men in 1950) [1]. Although women had longer life expectancies than men, it was indisputable that men's life expectancies were much longer than 70 years ago. As a consequence of their longevity, humans have undeniably experienced aging and age-related chronic diseases, such as cardiovascular disease, dementia, cancer, diabetes and osteoporosis [2, 3].

Osteoporosis is a skeletal disease characterized by low bone strength, resulting in enhanced bone fragility and, consequently, increased fracture risk [4]. Osteoporosis prevalently occurs in women, majorly during menopause when estrogen levels abruptly decline [5]. In parallel with the high prevalence, the epidemiology, pathophysiology, diagnosis, management, and treatment of osteoporosis were therefore extensively studied in women. Thus, the female animals were used as models to study osteoporosis and apply the knowledge to humans, while the study in males was less extensive than in females [6].

Differing from an abrupt decline of estrogen levels in women, an increasing age in men was associated with a gradual decrease in testosterone levels and a high possibility of osteoporotic fractures [7]. Besides, elderly men substantially encountered the risk of developing prostate cancer, which was commonly treated either by surgical or chemical androgen deprivation therapy which caused abrupt testosterone deficiency in return [8]. The various circumstances of testosterone deficiency in males are expected to influence bone mass (quantity) and structure (quality) in distinct ways, ultimately leading to different patterns of osteoporosis development [9]. Research on bone changes in both animal models and humans has predominantly focused on bone mass, while the structural aspects of bone have received comparatively less attention [10]. Thus, the impact of abrupt versus gradual testosterone deficiency on bone quantity and quality remains poorly understood.

Osteoporosis is an asymptomatic disease. Patients are usually unaware of their disease until they experience bone fractures which makes osteoporosis more challenging to manage [11]. Therefore, the best management strategy for osteoporosis should be early prevention of the deterioration of bone mass and structure. To provide basic information to help design the prevention of osteoporosis from testosterone

deficiency in men, both abrupt and gradual, changes in bone mass and bone geometry were investigated in male rats in this study.

MATERIALS AND METHODS

Animals

One hundred male Sprague-Dawley rats, 2 months old (mo), were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. The rats were housed at Chulalongkorn University Laboratory Animal Center in Thailand. They were kept in pairs in individually ventilated cage systems under controlled light cycles (12:12 h light/dark) and temperature (22 ± 1 °C). The rats were fed with a standard rodent diet (Teklad Global Diets®: ENVIGO Harlans Laboratories, Indianapolis, USA) and had access to water *ad libitum*. The animal use protocol was approved by the Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Science, Chulalongkorn University (Protocol Review Number: 1573004).

Experimental design

Male rats were induced an abrupt testosterone deficiency condition by bilateral orchidectomy and induced a gradual decrease in testosterone level condition by following up animals from sexual maturity age (4 mo) to middle-age (1 year old) because the rat has a life span of approximately 2 years old [12]. Rats were randomly divided into 2 groups (50 rats per group): sham-operated (SH) and orchidectomized (ODX). In the SH rats, the testes were kept intact, while in the ODX rats, the testes were removed. After the operation, each group was divided further into 5 subgroups: M0, M2, M4, M6, and M8 (10 rats per group). The SH-M0 and ODX-M0 groups were allowed to recover for 9 days (M0 group) after surgery, before being euthanized, and the data were kept as a baseline. The other 4 groups were euthanized at 2, 4, 6, and 8 months after the surgery (or M2, M4, M6, and M8 group), respectively. Before being euthanized, rats were first measured for their body weight and induced deep anesthesia using carbon dioxide. Their accessory sex organs, including the prostate gland, epididymis, and seminal vesicles, were removed, trimmed free of any remaining fat, and weighed. The relative weights of the accessory sex organs were calculated using the following equation:

$$\text{Relative organ weight} = [\text{Organ weight (g)}/\text{Body weight (g)}] \times 100.$$

The blood samples were collected from euthanized rats and then centrifuged at $1{,}600 \times g$ for 20 min at 4 °C. After centrifugation, sera were separated and stored at −20 °C until use. Serum testosterone levels were measured using the Testosterone ELISA Kit (ab108666, Abcam, MA, USA) following the manufacturer's protocol. The inter- and intra-assay coefficients of variation were 6.52% and 3.57%, respectively. The assay's limit of detection was 0.2 ng/ml.

The fourth lumbar vertebrae (part of the axial skeleton) and right tibia (part of the appendicular skeleton) were obtained for measuring bone quantity (i.e., bone mineral content, BMC) and bone quality (or bone geometry in this study, i.e., bone area, cortical thickness, and periosteal and endosteal circumferences).

Measuring bone quantity and bone geometry by pQCT

Bone quantity and geometry were measured using peripheral Quantitative Computed Tomography (pQCT) (XCT Research SA+, Stratec Medizintechnik GmbH, Pforzheim, Germany) and analyzed using XCT—5.50 E Software (Stratec Medizintechnik GmbH). It was started by performing a scout view scan (SV-scan) at a speed of 20 mm/s to locate the reference line. Then, a computed tomographic scan (CT scan) was performed three times at a speed of 10 mm/s to determine bone quantity and geometric parameters. The bone samples were scanned at three sites: the fourth lumbar vertebra (L4), the tibia metaphysis (TM), and the tibia diaphysis (TD). Each bone sample had a specific scanned location and reference line as described below.

4th Lumbar vertebra: A cross-sectional SV scan was performed along the longitudinal axis of L4 to locate the midpoint of the cranio-caudal axis and use it as a reference line. Then, cross-sectional CT scans were performed at a reference line ±1 mm along the longitudinal axis of L4.

Right tibia: Before indicating the scanned location, tibia length was measured using a vernier caliper. A cross-sectional SV scan was started from the proximal tibia to locate the growth plate. Next, the reference line was set at 2 locations: the growth plate and 50% of the tibia length. These locations were used to measure TM and TD, respectively. For TM, cross-sectional CT scans were performed at 2, 2.5, and 3 mm below the growth plate. For TD, cross-sectional CT scans were performed at 50% of the tibia length ± 1 mm along the longitudinal axis.

In each CT-scanned slice, the voxel size was 0.1 mm^3. The following methods were used: i) Contmode 1 (280 mg/cm^3 threshold) was used to separate soft tissue from the outer edge of bone to obtain total bone, ii) Peelmode 2 (600 mg/cm^3 threshold) was used to obtain trabecular bone, and iii) Cortmode1 (710 mg/cm^3 threshold) was used to define cortical bone. After defining the bone compartments (total, trabecular, and cortical) in a CT-scanned slice, the bone quantity and geometry of each compartment were analyzed using XCT—5.50 E Software (Stratec Medizintechnik GmbH).

Statistical analysis

The data were presented as mean±SEM. The data distribution was assessed for normality using the IMB

SPSS Statistics version 22.0 (IMB, USA). Following the normality test, all bone quantity and geometry data were found to be normally distributed. Then, the interaction between 2 factors; testosterone condition and time, was analyzed using Two-ways analysis of variance (ANOVA). Interaction effect of total BMC, L4's total area, and all cortical parameters reached significance, which was subsequently analyzed using estimated marginal means and simple main effects. TM's and TD's total areas, all trabecular parameters, and both circumferences showed no significant interaction effect. Therefore, those mentioned parameters were analyzed by one-way ANOVA. The multiple comparison test was conducted using Tukey HSD (equal variances assumed) or Dunnett's T3 (equal variances not assumed). The body and relative accessory sex organ weights exhibited non-normal distributions and were analyzed using Kruskal-Wallis one-way ANOVA. When significant differences across samples were detected, multiple pairwise comparisons were performed. A p-value less than 0.05 was considered to indicate a significant difference. Spearman's rank correlation (r) and coefficient of determination (r^2) were calculated using GraphPad Prism version 9.5.0 (GraphPad Software, Boston, MA, USA). All rank correlations were concluded in a single table and visualized by a heatmap.

RESULTS

Testosterone levels in SH and ODX rats

In Table 1, testosterone levels in the SH rats gradually decreased starting at 6 mo (M2 group) and reached a significant difference at 12 mo (M8 group) compared to the baseline (M0 group). In the ODX group, testosterone levels were below the detection limit as early as 9 days after ODX (or in the M0 group).

Orchidectomy induced loss in body weights and accessory sex organ weights

In the gradually decreased testosterone-SH rats, body weights continuously increased during the 8-month study period (from 4 mo (M0) to 12 mo (M8) of rats) (Fig. 1) while no significant changes in relative accessory sex organ weights were detected compared to the baseline (M0) ($p > 0.05$).

In the abrupt testosterone-deficient ODX rats, the significant increases in body weights were detected at M4 and M8 compared to the baseline (M0). The weights of the accessory sex organs significantly decreased compared to M0. The prostate gland weights significantly decreased at M4 ($p = 0.043$), M6 ($p = 0.035$), and M8 ($p = 0.006$). Epididymis weights significantly decreased at M4 ($p = 0.016$) and M6 ($p = 0.038$), and seminal vesicle weights significantly decreased at M8 ($p = 0.005$).

To evaluate the effect of testosterone deficiency only, not including the effect of age, the body and accessory sex organ weights in the age-matched SH and ODX rats were compared. The body and accessory sex organ weights in the ODX group were lower than those in the SH group throughout the 8-month study period. Notably, the differences between groups in seminal vesicle and epididymis were observed starting at M0 or 9 days after ODX, which was earlier than observed in the prostate gland, starting at M2.

Table 1 Testosterone levels of sham (SH) and orchidectomized (ODX) rats at 0, 2, 4, 6, and 8 months (M0, M2, M4, M6, and M8) after surgery. The surgery was performed when the rats were 4 months old. * indicates $p < 0.05$ compared to the baseline (M0) in the SH group.

Group	Age	Testosterone levels (ng/ml)	
	(months)	SH	ODX
M0	4 (+9 days)	2.375 ± 0.607	< 0.2
M2	6	2.050 ± 0.625	< 0.2
M4	8	1.282 ± 0.306	< 0.2
M6	10	1.043 ± 0.310	< 0.2
M8	12	$0.660 \pm 0.195^*$	< 0.2

Effects of aging and gradual testosterone depletion on bone quantity and geometry in testes-intact (SH) rats

Bone quantity

At all bone sites of the SH rats, both total and cortical BMC of L4, TM and TD showed positive correlations ($r > 0.4$, $p < 0.05$) with increasing age (Fig. 2), while the L4's and TM's trabecular BMC showed no correlation ($r = 0.211$, $p = 0.154$) and a negative correlation ($r = -0.367$, $p = 0.014$) with increasing age, respectively. Thus, changes of total and cortical BMC of L4, TM and TD in association with the increasing age were examined in detail (Fig. 3).

In contrast to the gradual decrease in serum testosterone level starting at 6 mo, the significant increases in bone mass of SH rats were observed in the following timepoint onwards: 6 mo for TD's total and cortical BMCs, 8 mo for L4's total, cortical BMCs and TM's cortical BMC, and 10 mo for TM's total BMC.

Bone geometry

All bone geometric parameters showed a positive correlation ($r > 0.3$, $p < 0.05$) with increasing age (Fig. 2), except for the TM's trabecular area ($r = 0.109$, $p = 0.476$) and endosteal circumference ($r = 0.128$, $p = 0.397$) which did not exhibit a correlation with increasing age. Thus, changes of total and cortical area of L4, TM and TD, trabecular area of L4, cortical thickness and periosteal circumference of TD in association with the increasing age were examined in detail (Fig. 4).

Following a gradual decrease in serum testosterone levels at 6 mo, significant increases in bone

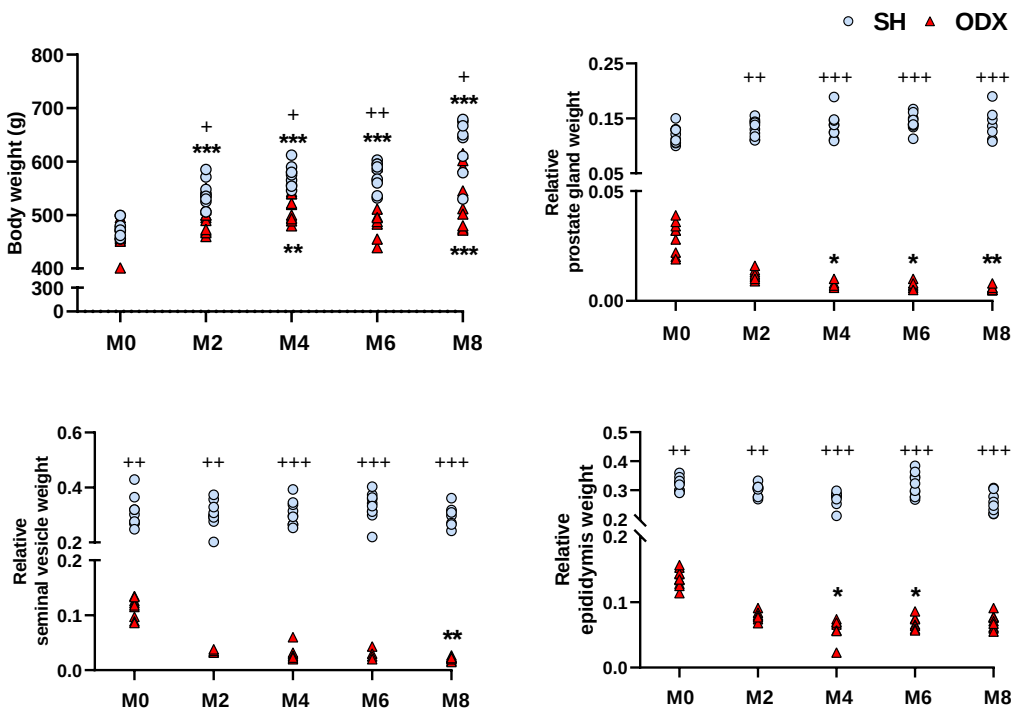

Fig. 1 Body and relative accessory sex organ weights in the SH and ODX rats. The data from each rat is presented as an aligned dot (SH) or triangle (ODX). *, **, and *** indicate $p < 0.05$, 0.01, and 0.001 compared to M0 in the SH (above the dot symbol) or ODX rats (below and above the triangle symbols in body weight and accessory sex organ weight graphs, respectively). +, ++, and +++ indicate $p < 0.05$, 0.01, and 0.001 compared between the SH and ODX rats in the same month.

geometry of SH rats were observed at a subsequent timepoint onward: 6 mo in L4's total and trabecular areas and TD's cortical area, 8 mo in TD's total area, cortical thickness, and periosteal circumference, and 10 mo in TM's cortical area and thickness.

Effects of aging and abrupt testosterone deficiency on bone quantity and geometry in ODX rats

Each bone site and compartment of bone quantity and geometry in the ODX rats showed a distinct response to an abrupt decrease in testosterone level (Fig. 2). These bone responses were categorized into three groups: increase, decrease, and unchanged, regardless of age or androgen-deficient condition.

Bone quantity

There were two patterns of changes in bone mass of the ODX rats: increase and decrease (Fig. 2). Positive correlation (increase) was seen in L4's trabecular BMC, TM's and TD's total and cortical BMCs. Negative correlation (decrease) was seen in L4's total and cortical BMCs and TM's trabecular BMC. Thus, changes of those parameters in association with the increasing age were examined in detail (Fig. 5).

The increase in TM's and TD's BMCs (total and cortical) was observed as early as 6 mo, while the increase in L4's trabecular BMC was detected only at 10 mo. The significant decreases in L4's total (12 mo only) and cortical BMCs (10 to 12 mo) and the TM's trabecular BMC (8 mo only), when the androgen-deficient rat aged, varied.

Bone geometry

After inducing an abrupt androgen-deficient condition, the measurement of bone area in the ODX rats (Fig. 2) showed positive correlation for L4's trabecular area, TM's cortical area and cortical thickness, TD's total and cortical areas, and TD's periosteal and endosteal circumferences, but showed negative correlation for L4's cortical area. Thus, changes in those parameters in association with the increasing age were examined in detail (Fig. 6).

It was observed that when an androgen-deficient condition was induced in the ODX rats, there were increases in L4's trabecular area (6 mo), the TM's cortical area (6 mo), as well as the TD's total and cortical areas (8 and 12 mo). Additionally, there was an increase in TM's cortical thickness (6 mo) and the TD's periosteal (8 and 12 mo) and endosteal (12 mo)

Bone sites		Bone parameters	Correlation coefficient (r) SH	ODX
L4	BMC	Total	**0.451 ****	**-0.453 ****
		Trabecular	0.211	**0.352 ***
		Cortical	**0.423 ****	**-0.589 *****
	Area	Total	**0.688 *****	0.152
		Trabecular	**0.679 *****	**0.689 *****
		Cortical	**0.329 ****	**-0.613 *****
TM	BMC	Total	**0.501 *****	**0.516 *****
		Trabecular	**-0.367 ***	**-0.340 ***
		Cortical	**0.749 *****	**0.708 *****
	Area	Total	**0.325 ***	0.285
		Trabecular	0.109	0.007
		Cortical	**0.618 *****	**0.606 *****
	Geometry	Cortical thickness	**0.467 ****	**0.556 *****
		Periosteal circumferences	**0.309 ***	0.283
		Endosteal circumferences	0.128	0.039
TD	BMC	Total	**0.770 *****	**0.422 ****
		Cortical	**0.807 *****	**0.408 ****
	Area	Total	**0.727 *****	**0.591 *****
		Cortical	**0.768 *****	**0.412 ****
	Geometry	Cortical thickness	**0.691 *****	-0.084
		Periosteal circumferences	**0.727 *****	**0.588 *****
		Endosteal circumferences	**0.435 ****	**0.580 *****

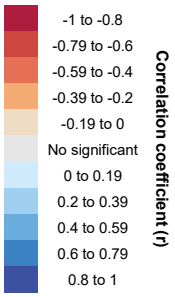

Fig. 2 Heatmap of correlations between rat ages (4 to 12 months old) and bone parameters, including bone mineral content and area of each bone compartment (total, trabecular and cortical), cortical thickness, periosteal and endosteal circumferences in the SH and ODX rats. Negative and positive correlation levels are displayed as an increase in light greyish orange to red and light greyish blue to blue, respectively. Bold correlation coefficient (r) means its p-value is statistically significant. In addition, *, **, and *** indicate $p < 0.05$, 0.01, and 0.001. Light grey indicates no significance in the correlation coefficient p-value.

circumferences. The L4's cortical area was the only parameter that decreased with increasing age, and a significant decrease was pronounced starting from 10 mo.

Effect of testosterone deficiency on bone quantity and geometry in male rats

To assess the impact of testosterone deficiency on bone quantity and geometry, age-matched comparisons were conducted between the SH and ODX rats. Only the differences detected between these two groups are presented here.

Bone quantity

Compared to the SH rats, the ODX rats showed lower L4' in total and cortical BMC, TM's trabecular BMC, and TD's total and cortical BMC levels (Fig. 7). The significantly lower BMC levels were observed starting from M4, except for the trabecular TM which was detected only at M2 and M4. Notably, the TM's cortical BMCs in the ODX rats were higher than those of the SH rats at M2 and M4.

Bone geometry

When comparing bone areas, the ODX rats showed lower L4's total and cortical areas compared to the SH rats, as well as lower TD's total and cortical areas (Fig. 7). The L4's and TD's cortical area differences were observed since M4. The lowered L4's and TD's total areas were detected in M8 and M6 to M8, respectively. Additionally, the L4's trabecular area and TM's cortical area in the ODX rats were higher than in the SH rats, which was observed only at M6 and M2 to M4, respectively.

In Fig. 7, the TM's cortical thickness in ODX rats was higher than the SH rats at M2 to M4. TD's cortical thickness and periosteal circumference in ODX rats were lower than the SH rats at M4 to M8, and only at M6, respectively.

DISCUSSION

It was reported that testosterone levels gradually decreased when age was advanced [13] which was first detected in 6 mo young adulthood (or M2-SH) rats

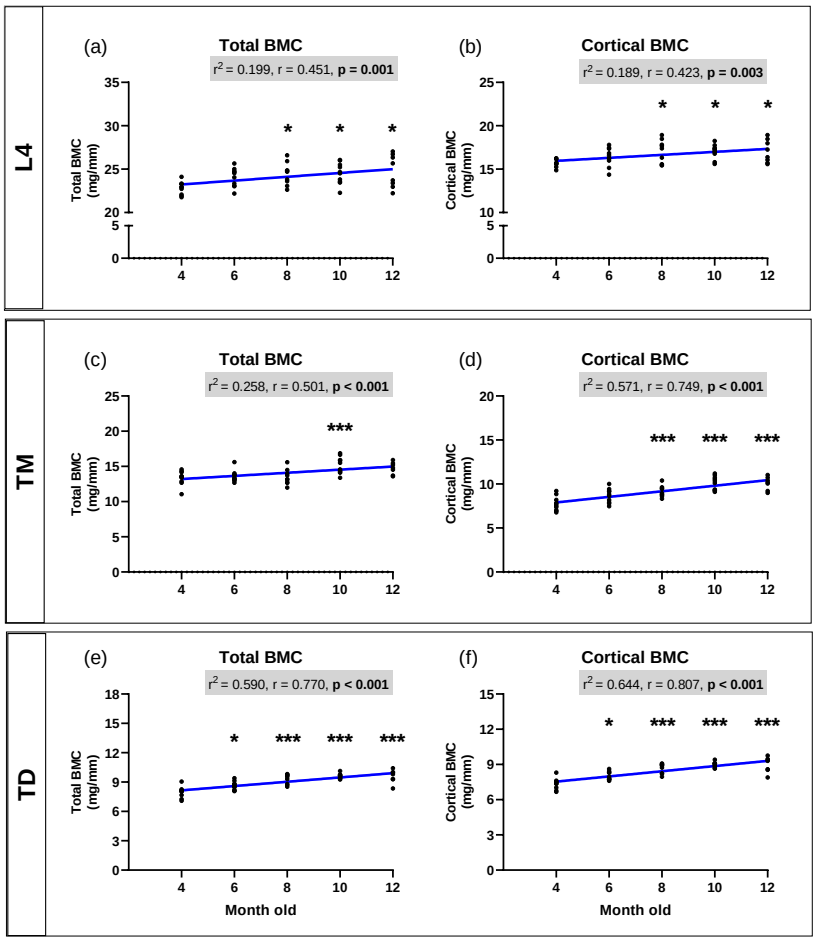

Fig. 3 Bone quality (bone mineral content) of the 4th lumbar (L4, a and b), tibia metaphysis (TM, c and d), and tibia diaphysis (TD, e and f) in the SH rats. The data from each rat is presented as an aligned dot. *, **, and *** indicate $p < 0.05$, 0.01, and 0.001 compared to 4 months old rats, respectively. The grey highlight indicates r^2, r, and p values of the correlation coefficient, respectively. Bold p-value means $p < 0.05$. This statistical description is identical in Fig. 3 to Fig. 6. To avoid redundancy, only Fig. 3 is described in detail.

and reached significance in 12 mo middle-aged (or M8-SH) rats. This age-related decrease in testosterone levels was partly from a reduction in the efficiency of testosterone production and its regulatory system [14]. A previous study showed that testosterone levels in the ODX rats were lower than the detection limit within one day after orchidectomy [15], thus, it was logically undetectable in M0-ODX rats in this study because the sera were collected at 9 days after orchidectomy. Taken together, the results of testosterone determinations in the SH and ODX rats confirm gradual and abrupt testosterone-deficient conditions in those animals, respectively.

Testosterone plays a crucial role in the maintenance of body weight gain and accessory sex organ structure and function [16]. Despite the gradually depleted testosterone levels, a decrease in relative accessory sex organ weights was not detected in the 12 mo (M8-SH) rats. According to a previous study, a certain level of androgens was required to maintain accessory sex organ weights [17]. Therefore, testosterone levels of 0.660 ± 0.195 ng/ml observed in the M8-SH rats in this study might be sufficient to maintain accessory sex organ weight. On the other hand, if the testosterone level was lower than 0.2 ng/ml (detection limit) for 9 days following the orchidectomy the relative accessory sex organ weights were significantly lower than the age-matched SH rats. Interestingly, the accessory sex organ weights continued to decrease from the baseline (M0) level when the animals were older, suggesting the additional effect of aging on adrenal-derived androgen production [18].

Age-match comparison between groups showed that body weights in ODX rats were lower than those in the SH rats, which was consistent with the previous study that reported a decrease in body weight gain [19]

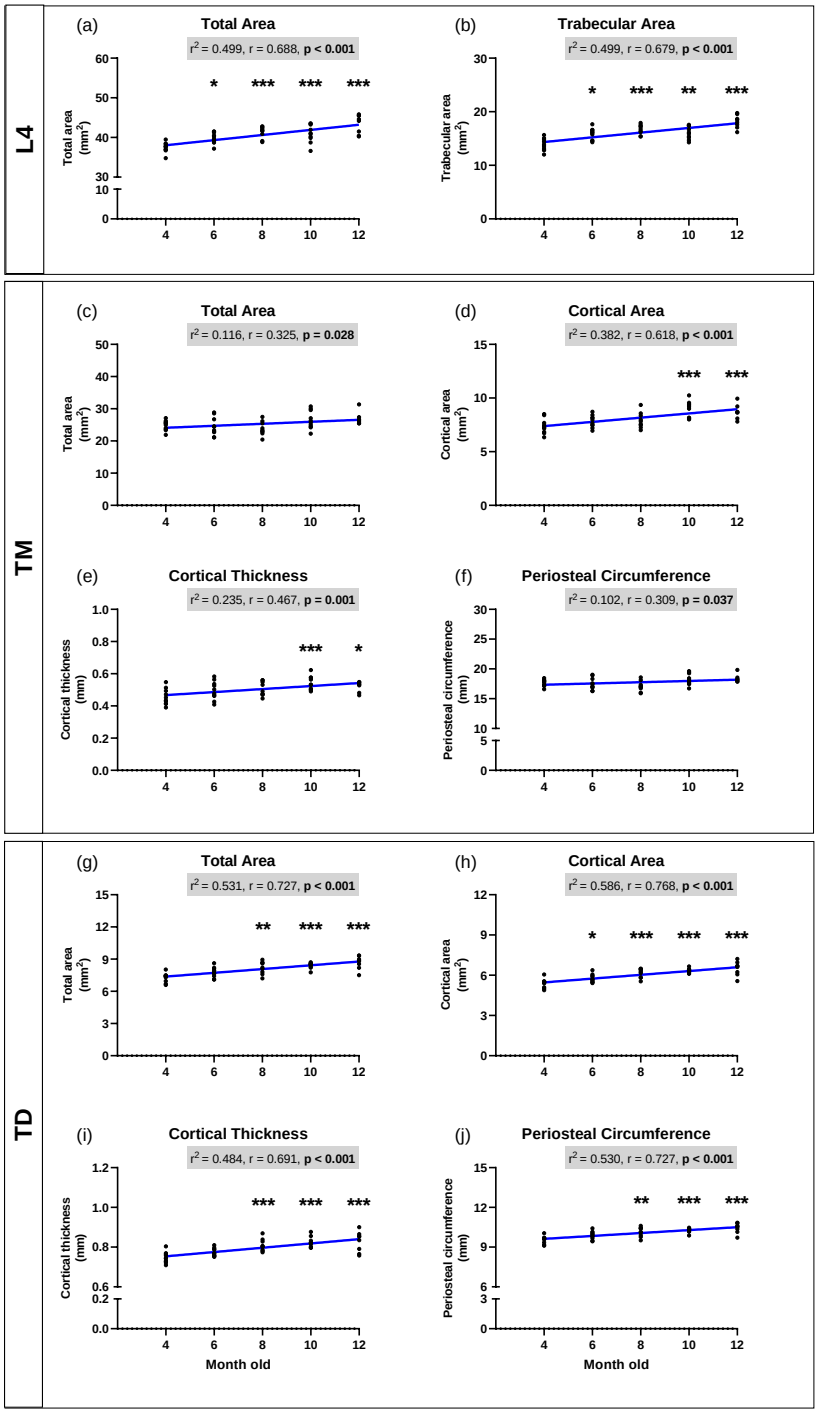

Fig. 4 Bone geometry of 4th lumbar (a and b), tibia metaphysis (c–f), and tibia diaphysis (g–j) in SH rats.

in ODX rats compared to testosterone-intact SH rats. Despite delay changes (at least 9 days after orchidectomy), the decreased relative accessory sex organs and body weights in male rats could be a key indicator reflecting a testosterone-deficient condition. Noted, the lowered seminal vesicle and epididymis weights were pronounced after orchidectomy for only 9 days, the earliest time point of our detection, while the lowered prostate gland weights could be detected later at 2 months after orchidectomy, indicating that seminal

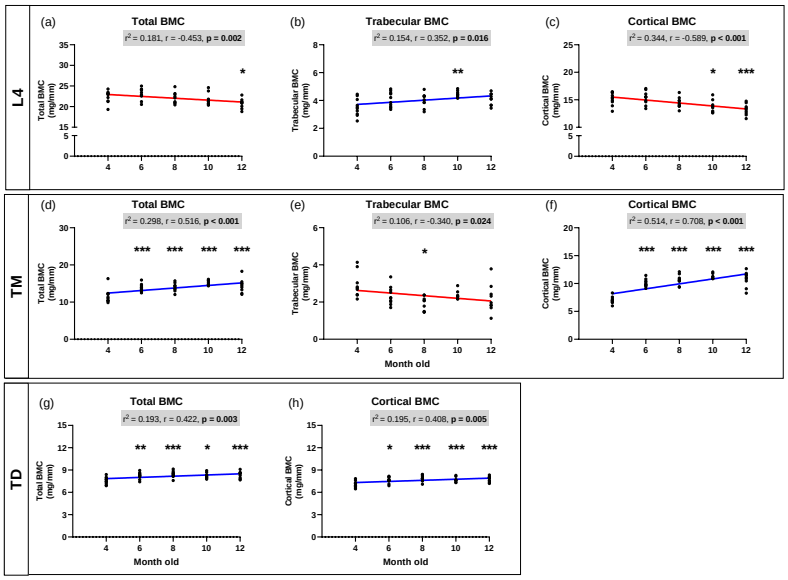

Fig. 5 Bone mineral content of 4th lumbar (a–c), tibia metaphysis (d–f), and tibia diaphysis (g and h) in the ODX rats.

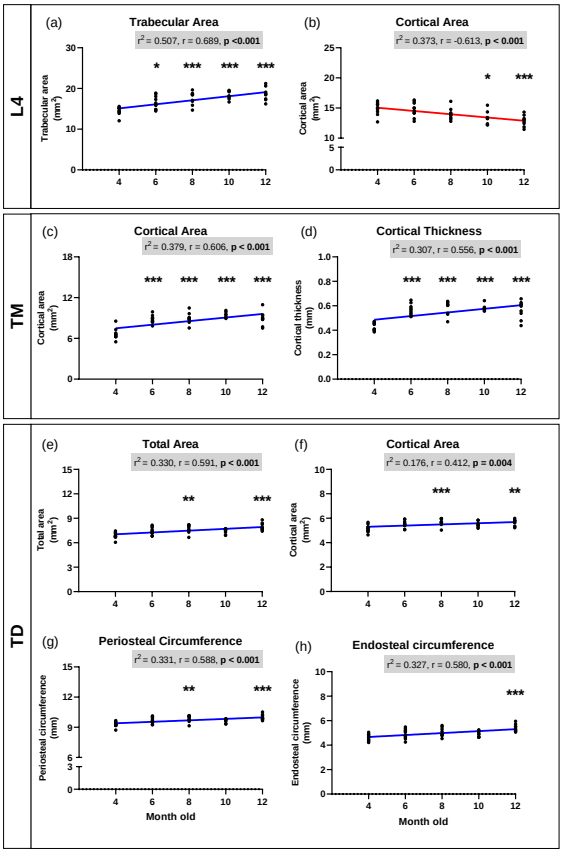

Fig. 6 Bone geometry of 4th lumbar (a and b), tibia metaphysis (c–d), and tibia diaphysis (e–h) in ODX rats.

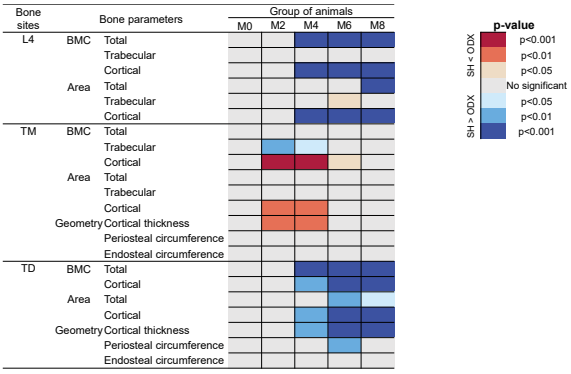

Fig. 7 Heatmap of changes of bone parameters of aged match comparison between SH and ODX rats. Blue and red shaded colors indicate that ODX rats had lower and greater bone values than the SH rats, respectively. Darker shading colors indicate higher significant values. Light grey indicates no significance between the SH rats and the ODX rats.

vesicle and epididymis were sensitive to testosterone-deprived condition.

Regarding bone measurement, our study determined 2 types of bone parameters, including the bone quantity (BMC) and bone geometry (area, thickness, and circumference), and the changing of testosterone levels affected bone mass and structures, respectively. During the 8-month study in the SH group, all bone quantities and qualities at all bone sites (L4, TM and TD) increased with age, except the L4's and TM's trabecular BMCs, and TM's trabecular area and endosteal circumference. Age-related increases in bone mass in SH rats in this study were consistent with the

previous reports [20, 21]. Considering TM's trabecular BMC, the only bone quantity parameter that decreased with age in the SH rats, this suggests that increasing age with gradually decreased testosterone levels marginally expanded TM's trabecular area, by enlarging periosteal circumference but kept consistency on endosteal circumference. However, this expansion was unable to compensate for bone loss in the original area. Thus, TM's trabecular BMC could be used as an early warning indicator for preparing to manage bone loss. A similar pattern was also seen in men, where the bone mineral density of trabecular bone at the distal tibia and lumbar spine (L1, L2, and L3) decreased at a young age and continued throughout life. They experienced a 42% loss of trabecular bone over their lifetime before age 50, and this significant loss during sex steroid sufficiency was unexplained [22].

Typically, the anabolic effect of testosterone on bone was mediated via the androgen receptor (AR) and estrogen receptors (ERs), which were expressed in osteoblast, osteoclast, and osteocyte [23]. Although the localization of AR was reported either on cortical or trabecular bone [24], AR inactivation in the AR knockout mice caused bone loss only in trabecular bone [25]. This might explain why only lowered trabecular BMC was detected in middle-aged SH rats which experienced a gradual decline in testosterone levels. A low conversion of testosterone to estradiol, which affects mediated ERs, was another noteworthy aspect for consideration which was not determined here [26].

When age advanced, unlike SH rats, a decrease in bone mass in ODX rats was observed, particularly at L4, indicating that bone changes from increasing age after an induced-abrupt testosterone deficiency differed from those in a gradual testosterone decline. This also underscored that L4's bone mass and geometry were regulated by testosterone. Interestingly, bone-gaining patterns in bone mass and geometry (except cortical thickness) of the ODX rats could be clearly detected at the TD. A previous report indicated that gaining bone geometry after induced androgen deficiency was attributed to the influence of growth hormone [27]. Not only different bone sites that responded differently to an abrupt testosterone deficiency, but different bone compartments within a single bone site also responded differently. Comparing cortical and trabecular bone compartments within L4 site, while bone loss occurred at cortical compartment (BMC and area), bone gain was observed at trabecular compartment without affected total bone size, suggesting that increasing age under an abrupt testosterone deficiency increased trabecularization in L4. Therefore, different bone sites have different responses after increasing age with testosterone deficiency, and bone loss remarkably occurs at axial skeleton. Although testosterone is a primary sex steroid hormone in males, numerous studies have reported that 17β-estradiol, which is converted from testosterone by the aromatase enzyme, plays a crucial role in bone health in men by acting mainly through ER alpha [28]. As ERs are expressed in both cortical and trabecular sites of long bones (femur and tibia) and vertebrae, the effects of lowered estradiol levels on L4 and other bone sites should be explored further.

Considering only the effect of abrupt testosterone deficiency, age-matched comparisons between ODX and SH groups showed that ODX rats had lower bone mass and deteriorated bone geometry than the SH rats which is consistent with other age-matched comparison studies [29, 30]. Bone loss after induced testosterone deficiency could result from increased bone remodeling, in which bone resorption exceeds bone formation [30]. The loss in bone mass of our rats emerged before most geometric deterioration, which aligned with previous studies [32]. Our study supports the conclusion that a hierarchical organization of bone [33], in which bone remodeling alters the nanoscale (bone mineral content) before an alteration on the macroscale (bone geometry) emerges. Although changes in bone geometry were detected after bone mass, this can significantly affect bone strength [34]. Therefore, to apply in humans, both bone mass and bone geometry are recommended for diagnosing osteoporosis.

Different patterns of changes between axial (L4) and appendicular (TM and TD) sites, and between trabecular and cortical compartments indicated that testosterone deficiency caused bone loss in various patterns depending on bone sites and compartments. This might be possibly caused by the differences in bone cell number between sites and compartments [35], the arrangement of osteon and Haversian canals [36, 37], cortex-to-trabecular ratio [9], and mechanical loading modes [38]. Varied changing patterns may affect mechanical properties, such as the loading intensity (stress) and the deformation (strain) at different bone sites [38]. Therefore, this change in mechanical properties was likely related to the occurrence of bone fractures, which emerged at different frequencies on different bone sites [39].

In conclusion, our longitudinal study in male rats indicates that gradual and abrupt testosterone deficiency induces distinct patterns of changes in bone mass and geometry depending on the bone compartment and bone site. This knowledge should give a clue on designing any interventions for the prevention or treatment of bone loss or bone fracture in males. A gradual decrease in testosterone levels in the SH rats exerts bone loss mainly at TM's trabecular bone, both quantity and quality, suggesting close monitoring of this bone site before middle age. For an abrupt testosterone deficiency, a representative of androgen deprivation therapy in men, the management and treatment should be immediately executed at L4 because severe loss and deterioration in bone

mass and structure could be detected. Although the bone structure and remodeling in male rats do not particularly resemble those of humans, particularly the absence of the Haversian system (secondary osteon) in rats [40], they can be a promising model for studying osteoporosis in testosterone-deficient conditions, and a selection of specific bone sites for experimentation could provide insights on bone change that are later applicable to humans.

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