The Effect of Long-Term Hypogonadism on Body Composition and Morphometry of Aged Male Wistar Rats

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Summary

Clinical studies show that hypogonadism in the aging male is associated with obesity and osteoporosis. Experimental studies are mostly conducted on relatively young adult animals and the induced hypogonadism lasts for a relatively short time. The present study aimed to describe the effect of long-term hypogonadism beginning in puberty on body composition, morphometry, and bone mineral density in aged male rats. Morphometric measurements and dual-energy X-ray absorptiometry were conducted at the age of 30 months on control and gonadectomized males. Long-term hypogonadism did not affect body weight, but led to a higher fat mass (by 26 %), lower lean mass (by 44 %), shorter body length (by 9 %), and anogenital distance (by 26 %), as well as to lower tail circumference (by 15 %) in comparison to control males. Lower bone mineral density (by 13 %) and bone mineral content (by 15 %) were observed in gonadectomized males. Results showing sarcopenic obesity and osteoporosis in this model of long-term hypogonadism might mimic the situation in aging males better than the widely used short-term hypogonadism induced in young animals. The morphometric analysis could potentially be a useful tool to study normal weight obesity without the need for specific equipment.

Key words

Body Proportion • Bone Metabolism • Castration • Muscle Mass • Old age

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Introduction

In men, aging is associated with a decline of sex steroid hormones, such as testosterone (Harman *et al.* 2001). The prevalence of older men suffering from hypogonadism (Taylor *et al.* 2015) and obesity has an increasing tendency worldwide (Villareal *et al.* 2005, Wannamethee *et al.* 2015). There is an inverse relationship between low testosterone concentration and obesity (Diaz-Arjonilla *et al.* 2009) measured as high body mass index (Zumoff *et al.* 1990, Pasquali *et al.* 1991, Gapstur *et al.* 2002, Svartberg *et al.* 2004, Kaplan *et al.* 2006). In the elderly, low concentrations of androgens are associated with a higher fat mass, lower lean body mass, and muscle strength (Katznelson *et al.* 1996, Torremade-Barreda *et al.* 2013, Moran *et al.* 2015,

Dias *et al.* 2016), even in those with stable normal body weight – the so-called sarcopenic obesity (Lim *et al.* 2010, Choi 2016). Furthermore, long-term androgen deficiency is associated with the loss of bone mineral density (Katznelson *et al.* 1996, Torremade-Barreda *et al.* 2013, Moran *et al.* 2015, Dias *et al.* 2016), leading to osteoporosis (Mellstrom *et al.* 2006, Meier *et al.* 2008).

In humans, the assessment of obesity includes anthropometric measures such as body mass index (BMI) and waist circumference, the latter being more appropriate for the measurement of adiposity than BMI mainly in older patients (Ruff 2000, Ledikwe *et al.* 2003). In addition, morphometric measurements, especially abdominal circumference, are also commonly used in animal studies as an inexpensive, quick, and easy method for the assessment of obesity (Novelli *et al.* 2007, Gerbaix *et al.* 2010, Malafaia *et al.* 2013, Mamikutty *et al.* 2014). However, in humans, for accurate measurement of body composition, especially in sarcopenic obesity, more sophisticated methods allowing simultaneous measurement of fat and lean components are used, such as dual-energy X-ray absorptiometry (DEXA) (Baumgartner 2000, Lee *et al.* 2008). Whether simple morphometric measurements can be used for reliable evaluation of obesity in really aged laboratory animals, have not been elucidated yet.

The commonly used animal model of androgen deficiency is the removal of the primary endogenous androgen source – the testes – *via* gonadectomy. Gonadectomized animals usually show unaltered (Vanderschueren *et al.* 2000, Jiao *et al.* 2009, DeGuire *et al.* 2015, Zügel *et al.* 2016) or lower (Wade *et al.* 1984, Potikanond *et al.* 2016) fat mass, lower lean mass (Vanderschueren *et al.* 2000, Venken *et al.* 2005, Jiao *et al.* 2009, DeGuire *et al.* 2015), lower bone mineral content and density leading to osteoporosis (Vanderschueren *et al.* 2000, Moreau *et al.* 2001, Attardi *et al.* 2011, Potikanond *et al.* 2016, Jayusman *et al.* 2018, Jayusman *et al.* 2018, Li *et al.* 2018). However, the vast majority of the aforementioned studies are focused on the effect of short-term hypogonadism (weeks to months) on the body composition and bone metabolism of adult animals. Data on body composition following long-term androgen depletion are scarce and usually do not focus on the morphometric measurements in aged animals (Borbélyová *et al.* 2017). Whether hypogonadism lasting for more than 2 years alters body composition and bone metabolism in aged rats is unknown. Similarly, the causal relationship between long-term androgen depletion and morphometric variables in aged rats has not been investigated yet.

Therefore, the main goal of the present study was to investigate the effect of gonadectomy-induced long-term androgen deficiency on morphometric parameters, bone mineral content and density, and body composition of 30-month-old male Wistar rats. We have hypothesized that gonadectomized males will display higher body weight and fat mass, and lower lean mass compared to control males.

Materials and Methods

Ethical statement

All experimental procedures were approved by the Ethical Committee of the Institute of Molecular Biomedicine, Comenius University, Bratislava, and have been conducted in accordance with the EU Directive 2010/63/EU and Slovak legislation.

Animals and housing conditions

Wistar albino male rats (n=12) at the age of 1 month were purchased from Anlab (Prague, Czech Republic). Animals were group-housed (3-4 per cage) in polycarbonate cages (50×36×19 cm) and kept under standard laboratory conditions (temperature 22±2 °C, humidity 55±10 % and 12:12 light-dark cycle). Access to food (standard diet for rats KKZ-P/M, Dobrá Voda) and water was *ad libitum.*

Surgery

Rats were randomly divided into two groups: control (n=7) and gonadectomized males (n=5). One rat from gonadectomized group did not recover after surgery and one died during the experiment. The post-hoc power analysis with this number of animals showed at p=0.05 power 99.9 % for testosterone concentration, and power >80 % for the rest of the measurements listed below. Gonadectomy was performed on postnatal day 47, under anesthesia with intraperitoneal administration of ketamine (100 mg/kg, Narkamon inj, Bioveta, Czech Republic) and xylazine (10 mg/kg, Xylariem inj, Riemser, Germany). Bilateral gonadectomy was performed through a small incision at the posterior tip of the scrotum, after ligation of vas deferens and spermatic blood vessels with a silk suture. Sham castration was performed on control males. The procedure included displacement of the gonads from the scrotum, followed by their immediate replacement. To minimize postoperative pain, buprenorphine

(0.05 mg/kg) in a volume of 0.1 ml/kg body weight was administered subcutaneously at the end of the surgery, immediately after the incision was closed and also before the animals regained consciousness. Following surgery, rats were group-housed with their previous cage mates until the age of 30 months.

Morphometric measurements in rats

At the age of 30 months, under anesthesia (intraperitoneal administration of ketamine (100 mg/kg) and xylazine (10 mg/kg), rats underwent morphometric measurements according to the protocol of (Aguh *et al.* 2013). Tail length, tail circumference, and head length were measured in the prone position of the rat on the laboratory desk. Body length from nose to anus, anogenital distance, and waist circumference were measured in the supine position of the rat on the laboratory desk. Different instruments (caliper, measuring tape, ruler, scale, and thread) were used to measure these variables.

Tail length (TL, cm) means the distance between the tail base and its tip. Tail circumference (TC, cm) was measured as the perimeter of the tail at its base. Head length (HL, cm) was considered as the distance between the nose tip and back of the ear pinna. Nose-to-anus body length (NABL, cm) was measured as the distance from the nose to the anus. Waist circumference (WC, cm) was measured at the level of the belly button (Schroeder *et al.* 2008) (Fig. 1A). Anogenital distance (AGD, cm) reflects the distance from the base of the genital papilla to the rostral end of the anal opening (Aguh *et al.* 2013) (Fig. 1B). Body mass index (BMI) was calculated as: body weight/body length^2 (g/cm^2).

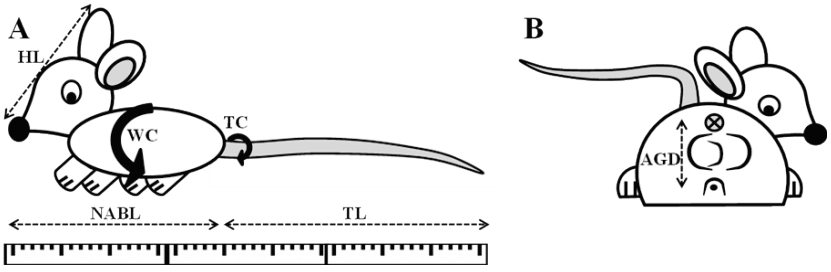

Fig. 1. A schematic representation of morphometric measurements in aged rats including length of the body (nose to anus), head and tail; the circumference of the tail and waist (**A**); and anogenital distance (**B**). TL – tail length, TC – tail circumference, HL – head length, NABL – nose-to-anus body length, WC – waist circumference, AGD – anogenital distance.

Dual-energy X-ray absorptiometry in rats

To evaluate body composition and bone mass, DEXA was used (Grier *et al.* 1996). This non-invasive technique is based on the attenuation of x-ray beams by tissues and enables its application in small animals. The whole-animal DEXA measurement was performed using the LUNAR Prodigy Advance device (GE Medical Systems, Madison, WI, USA) and data were analyzed using Encore TM 2011 software (version 13.60). Calibration was done before the measurements according to the instructions of the manufacturer. The scanner performance was controlled using the quality assurance protocol of the laboratory. Rats were placed on a polystyrene mat on the top of the scanner table. Under anesthesia, rats were straightened and laid flat on the ventral face along the long axis of the scanning area with a complete abduction of the four limbs. Only upper limbs were fixed with textile bands to the polystyrene mat. The tail was bent laterally to reduce scan time. After manually positioning the rat and laser beam for marking the start of a scan, the measurements were performed in a fully automated manner.

Based on the attenuation of the two energy levels, the system provides quantitative data on the bone mineral content (BMC; g), the fat tissue content (g), and the lean tissue content (g). By delineating the projection area of the bones, the software additionally provides the areal bone mineral density (BMD; g/cm^2) (Lochmüller *et al.* 2001). All measurements and analyzes of DEXA images were performed by the same investigator.

Locomotor activity in rats

To assess the locomotor activity of aged rats, the open field test was used. The apparatus consisted of a square arena (100 cm×100 cm) virtually divided into the central zone (40 cm×40 cm) and border zone. Rats were placed into the center of the open field arena and were allowed to freely explore it for 5 min. Total distance moved was observed to evaluate the locomotor activity of rats. The open field arena was cleaned with a damp cloth containing Incidur spray (Ecolab, Dusseldorf, Germany) between tested animals.

Blood collection and hormone assay

To assess plasma testosterone concentrations, blood samples were collected from the tail vein

of 30-month-old rats. Blood samples were centrifuged at 2000× g for 5 min. The measurement of plasma testosterone concentration was conducted using the commercially available ELISA kit (DRG Diagnostic, Marburg, Germany) according to the instructions of the manufacturer. The analytical sensitivity was 0.083 ng/ml. The average intra-assay coefficient of variation for the testosterone assay was below 5 % and the inter-assay coefficient of variation was below 10 %.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics v. 20.0 (IBM, Armonk, NY, USA) and GraphPad Prism version 6 (GraphPad Software, Inc., CA, USA). The normality of data distribution was verified using the D'Agostino test. For comparison of control and gonadectomized rats, an independent-samples Student's *t*-test (two-sided) was used. $p<0.05$ were considered statistically significant. Data are presented as mean plus standard deviation (SD).

Results

Long-term androgen deficiency influences the body composition and morphometric parameters of the aged rats

Androgen deficiency was confirmed by measurement of plasma testosterone concentration in 30-month-old rats. Aged gonadectomized males had significantly lower plasma testosterone concentration compared to control males (2.17±0.46 vs. 5.11±1.43 nmol/l, $p<0.01$).

At the age of 30 months, the body weight of gonadectomized rats did not differ from that of the control males (562±28 g vs. 497±19 g, $p=0.11$, Fig. 2A). There were significant differences in body composition between the groups. Higher amount of fat mass (291.6±30.0 g vs. 217.0±49.6 g, $p<0.05$, Fig. 2B) and conversely, a lower amount of lean mass was detected in gonadectomized males (189.9±26.0 g vs. 338.4±35.9 g, $p<0.001$, Fig. 2C) compared to the control group. Additionally, gonadectomy led to higher fat to tissue ratio (60.6±3.7 vs. 38.8±5.1 %, $p<0.001$, Fig. 2D).

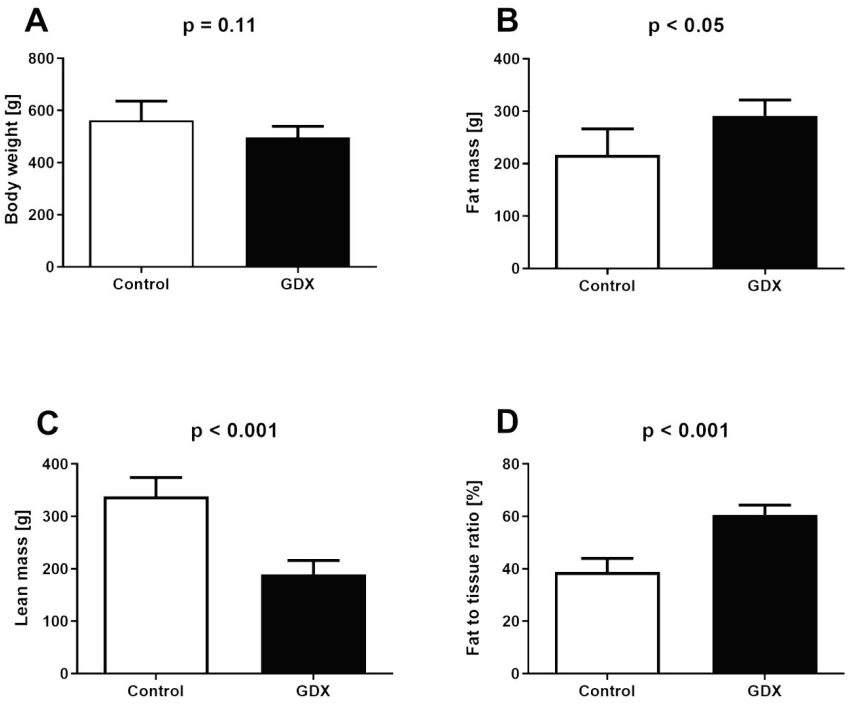

Fig. 2. Body weight and body composition in 30-month-old Wistar rats. Body weight (**A**) did not differ between gonadectomized and control males. More fat mass (**B**) and less lean mass (**C**) was detected in gonadectomized males compared to controls. Following these results, fat to tissue ratio (**D**) was higher in gonadectomized males. Values are expressed as means + SD.

Gonadectomy resulted in a significantly shorter anogenital distance (3.9±0.6 vs. 5.4±0.4 cm, $p<0.001$, Fig. 3A) and tail circumference (2.7±0.1 vs. 3.2±0.2 cm, $p<0.001$, Fig. 3B) compared to control males.

In addition, shorter nose-anus body length (24.1±0.7 vs. 26.4±1.2 cm, $p<0.01$) was observed in gonadectomized males compared to controls (Fig. 4). Tail length (19.8±0.4 cm vs. 19.1±0.3 cm, $p=0.30$) was not affected by long-term androgen deficiency. The values of waist circumference were similar for both groups (20.5±0.4 cm vs. 20.0±0.4 cm, $p=0.49$). The BMI did not significantly differ between the groups (8.3±0.3 g/cm^2 vs. 7.5±0.5 g/cm^2, $p=0.26$).

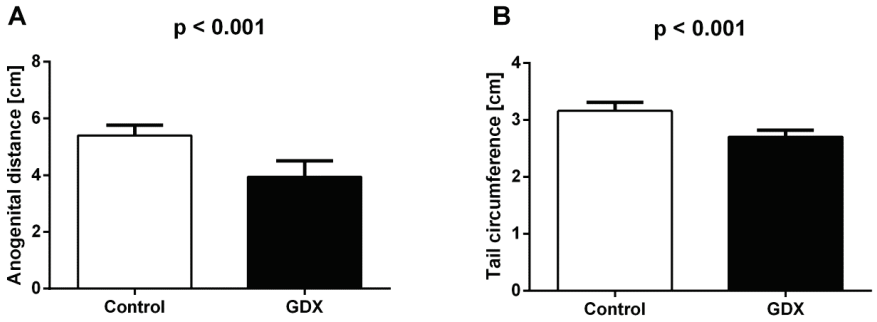

Fig. 3. Anogenital distance and tail circumference of 30-month-old Wistar rats. Aged gonadectomized males had a shorter anogenital distance (**A**) and lower tail circumference (**B**) than control males. Values are expressed as means + SD.

Long-term androgen deficiency affects the bone mineral density and content in aged rats

Densitometric measurements revealed lower bone mineral density (0.202±0.01 vs. 0.232±0.00 g/cm^2, p<0.001, Fig. 5A) as well as bone mineral content (17.68±1.32 vs. 20.70±1.45 g, p<0.01, Fig. 5B) in gonadectomized males compared to controls. Bone area values did not differ significantly between gonadectomized (86.6±3.0 cm^2) and control rats (88.2±2.8 cm^2).

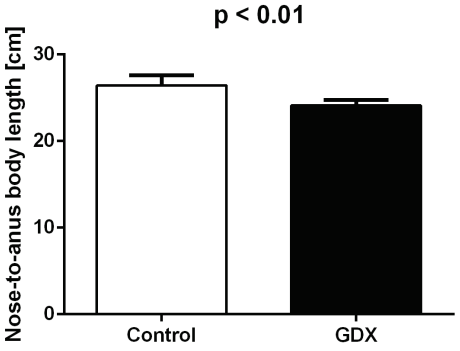

Fig. 4. Nose-to-anus body length in 30-month-old Wistar rats. Gonadectomized males had shorter nose-anus body length than control males. Values are expressed as means + SD.

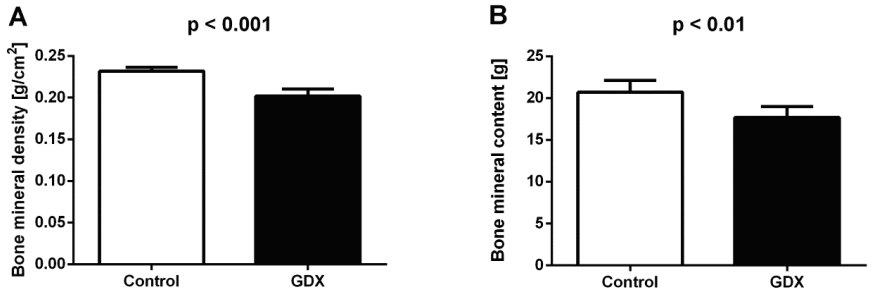

Fig. 5. Bone mineral density and content in 30-month-old Wistar rats. Long-term androgen deficiency resulted in a reduction of both, bone mineral density (**A**) and content (**B**) in aged rats. Values are expressed as means + SD.

To assess whether lower muscle mass is caused by lower locomotor activity in the gonadectomized group, rats were tested in the open field test (Fig. 6). No significant differences were observed in locomotor activity of rats measured as total distance moved in the open field test (p=0.54), suggesting that lower bone mass in GDX rats is probably the result of long-term androgen deficiency.

Discussion

The link between hypogonadism and alterations in body composition and bone metabolism has been proved by both, clinical (Katznelson *et al.* 1996, Torremade-Barreda *et al.* 2013, Moran *et al.* 2015, Dias *et al.* 2016) and experimental studies (Wade *et al.* 1984, Vanderschueren *et al.* 2000, Moreau *et al.* 2001, Venken *et al.* 2005, Jiao *et al.* 2009, Attardi *et al.* 2011, DeGuire *et al.* 2015, Potikanond *et al.* 2016, Jayusman *et al.* 2018, Jayusman *et al.* 2018, Li *et al.* 2018).

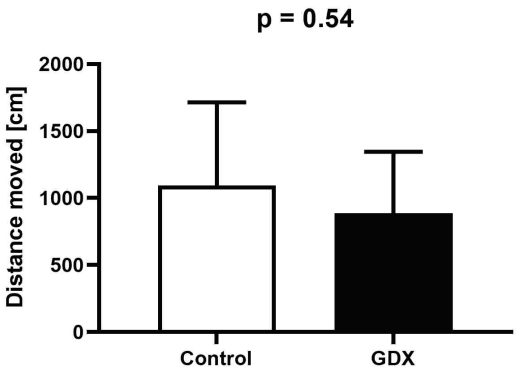

Fig. 6. Locomotor activity of 30-month-old Wistar rats. Control and GDX rats displayed similar locomotor activity in the open field test. Values are expressed as means + SD.

However, the vast majority of experimental studies have focused on the effect of relatively short-term hypogonadism lasting either 1-2 months (Kakolewski *et al.* 1968, Vanderschueren *et al.* 1992, Borst *et al.* 2006, Christoffersen *et al.* 2006, Jiao *et al.* 2009, Chin *et al.* 2016, Starcevic *et al.* 2017, Jayusman *et al.* 2018, Jayusman *et al.* 2018, Doulamis *et al.* 2019) or 3-4 months in adult (Wade *et al.* 1984, Moreau *et al.* 2001, Attardi *et al.* 2011, Harada *et al.* 2016, Potikanond *et al.* 2016, Zügel *et al.* 2016, Saki *et al.* 2018) or aged animals (Vanderschueren *et al.* 1992, Vanderschueren *et al.* 2000, Venken *et al.* 2005, DeGuire *et al.* 2015) on body composition and bone metabolism. Although the effect of long-term androgen deficiency is clinically more relevant for investigation of the effect of hypogonadism on body composition, morphometry, and bone metabolism in humans, experimental studies evaluating the impact of such long-term androgen depletion are lacking. Therefore, the present study aimed to describe the effect of long-term hypogonadism beginning in puberty on body composition, morphometry, and bone mineral density in aged, 30-month-old male rats. In the current study, long-term androgen deficiency led to lower lean mass and higher fat mass, with no changes in body weight in aged rats mimicking sarcopenic obesity in humans. These changes were not caused by altered locomotor activity. Aged gonadectomized rats had lower bone mineral density and bone mineral content compared to controls that imitate osteoporosis in the elderly. Moreover, morphometric analysis in aged rats revealed a shortening of body length, anogenital distance, and lowering of tail circumference following androgen deficiency lasting more than two years compared to that of control males.

In the present study, long-term androgen depletion starting at puberty did not affect the body weight of 30-month-old gonadectomized animals. The only study, with a similar duration of androgen deprivation (18-month-long), however, not focused on body composition, morphometry, and bone metabolism is our previous study, where we also did not find differences in body weight of aged rats following long-term androgen deficiency when compared to controls (Borbélyová *et al.* 2017). Previous studies have shown that short-term (lasting 1-2 months) androgen deficiency in adulthood results in either lower (Kakolewski *et al.* 1968, Borst *et al.* 2006, Jiao *et al.* 2009) or no changes in body weight (Christoffersen *et al.* 2006, Chin *et al.* 2016, Starcevic *et al.* 2017, Doulamis *et al.* 2019) of rats compared to control males. The majority of experimental studies have reported lower body weight of adult mice (Harada *et al.* 2016) and rats (Moreau *et al.* 2001, Attardi *et al.* 2011, Potikanond *et al.* 2016, Saki *et al.* 2018) following 3-4-month-long androgen depletion. Contradictory findings reporting no changes in body weight following similar duration of androgen deficiency in adult hamsters (Wade *et al.* 1984) and rats (Zügel *et al.* 2016, Li *et al.* 2018) have been also shown. On the contrary, it has been demonstrated that androgen deficiency (3-4-month-long) induced in aged guinea pigs (DeGuire *et al.* 2015) and aged rats results in lower (Vanderschueren *et al.* 1992, Venken *et al.* 2005) or similar body weight (Vanderschueren *et al.* 2000) compared to control males. According to these results, it seems that androgen depletion induced either in adulthood or during aging could have body weight-lowering effects as well as could result in no changes in body weight. In the current study, however, we did not focus on the dynamics of the effects of long-term gonadectomy on body composition and morphometry, thus, the changes in body weight could be observable earlier during the aging of animals.

The main difference between the current and previous experimental studies dealing with the effect of androgen depletion on body composition and bone metabolism besides the species used (mice, guinea pigs, hamsters vs. rats) and age of animals at androgen deficiency initiation is mainly the duration of hypogonadism. The present study demonstrates that long-term androgen deprivation leads to lower muscle mass that is more pronounced than the increase of fat mass in gonadectomized male rats in comparison to controls. Thus, it seems, that long-term androgen depletion in aged animals affects more the muscle mass compared to fat mass, that is supported by a study by DeGuire *et al.* (2015) (DeGuire *et al.* 2015) reporting reduced lean mass already 2 weeks following gonadectomy (and also during a 4-month-long observing period) in aged, 18-month-old guinea pigs. On the contrary, the study of (Brown *et al.* 2001) showed no changes in muscle mass 2 weeks after gonadectomy in adult (6-month-old) rats. In addition, no changes in lean mass even following 3-month-long androgen depletion have been shown in adult rats (Zügel *et al.* 2016). Based on these results, younger adult rats may be more resistant to gonadectomy-induced muscle loss than aged animals.

It has been shown that short-term androgen deficiency (lasting 2-4 months) in adult rats led to lower

lean mass (Moreau *et al.* 2001, Jiao *et al.* 2009) without any change in fat mass compared to control males. Similarly, short-term androgen depletion (lasting 3-4 months) in either aged guinea pigs (DeGuire *et al.* 2015) or aged rats (Vanderschueren *et al.* 2000, Venken *et al.* 2005) resulted in lower lean mass with unchanged fat mass compared to control animals. These results together with the results of the present study suggest that compared to muscles, the manifestation of changes in the amount of fat mass requires a longer time. Thus, in the current experiment, androgen deficiency lasting more than two years resulted in a serious loss of muscle mass and slower fat mass gain during the aging of rats, and in turn, the body weight of gonadectomized rats remained stable mimicking sarcopenic obesity of humans.

Numerous experimental studies have shown a decrease in bone mass (measured as bone mineral density and bone mineral content) in adult rats following a short-term, either 1-2-month-long androgen deficiency (Jayusman *et al.* 2018, Jayusman *et al.* 2018) or 3-4-month-long androgen depletion (Moreau *et al.* 2001, Attardi *et al.* 2011, Potikanond *et al.* 2016, Li *et al.* 2018, Saki *et al.* 2018). Similarly, aged rats (Vanderschueren *et al.* 1992, Vanderschueren *et al.* 2000, Venken *et al.* 2005, Potikanond *et al.* 2016, Saki *et al.* 2018) and guinea pigs (DeGuire *et al.* 2015) following a short-term, 3-4-month-long androgen depletion, display lower bone mineral content and lower bone mineral density in comparison to control animals. Therefore, it could be argued that androgen depletion leads to a reduction of bone mineral density and bone mineral content independently of the age at gonadectomy induction. Although, (Vanderschueren *et al.* 1992) have reported that androgen deficient aged male rats had a lower bone mass following 4 months, but not 1 month after gonadectomy, suggesting that in aged animals bone loss occurs apparently slower than in younger adult animals (Vanderschueren *et al.* 1992), which may be related to a physiological decrease in bone turnover during aging (Demontiero *et al.* 2012). Therefore, our findings are in line with the previously published data as we have found lower bone mineral content and bone mineral density in 30-month-old rats following long-term androgen depletion, suggesting the presence of osteoporosis in aged rats.

To the best of our knowledge and in comparison to other studies, the current study is the first one investigating the effect of long-term hypogonadism lasting for more than 2 years on body composition and bone metabolism in 30-month-old Wistar rats. In addition, in the present study, the most widely used model of male osteoporosis, surgical castration by orchiectomy, was used. In comparison to other experimental studies, this study is the first that analyzes the effect of long-term androgen depletion on the whole palette of morphometric measurements in 30-month-old Wistar rats. However, it is also affected by several limitations. All measurements were conducted only once, at the age of 30-months, and we have not analyzed the dynamics of changes in body weight, morphometric parameters, body composition, and bone metabolism during the aging process. A missing group of gonadectomized rats with testosterone supplementation will provide evidence of the key role of testosterone in the shaping of body composition and bone metabolism during the aging of rats. Another limitation of our study is that calcium metabolism, crucial for bone formation, was not analyzed. Testosterone or the lack of it affects calcium *via* modulating calcium absorption in the gut, but also *via* affecting reabsorption in the kidney. This should be taken into account in future studies.

Conclusions

In conclusion, our data show that long-term hypogonadism results in sarcopenic obesity and osteoporosis in aged rats. This model of long-term hypogonadism might be clinically more relevant than currently used experimental models of short-term hypogonadism in young adult animals. Further studies are needed to uncover the chronology of manifestation of observed changes in body morphology and composition, and also bone metabolism together with monitoring of calcium concentrations following gonadectomy throughout aging. If the present findings from morphometric analysis will be reproducible in other models of hypogonadism it could be used as a possible tool for diagnosis of sarcopenic obesity without the need for specific equipment as DEXA.

Conflict of Interest

There is no conflict of interest.

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