Original article

Does *Heteropterys aphrodisiaca* administration and endurance training alter bones of mature rats?

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Abstract

Heteropterys aphrodisiaca infusion, alone or associated with endurance training, was investigated in rat bones in relation to their mechanical properties, collagen content and morphology. Male rats were divided into four groups (n = 8): CS- control sedentary, HS- *H. aphrodisiaca* sedentary, CT–control trained, HT-*H. aphrodisiaca* trained. The training protocol consisted in running on a motorized treadmill, 5 times a week, for 8 weeks, with weekly increase in treadmill velocity and duration. Control groups received water while HS and HT groups received *H. aphrodisiaca* infusion (104 mg/animal) by gavage during the 8 weeks. Tibiae were frozen for collagen dosage and biomechanical analysis or preserved in Karnovsky's fixative, then processed for histomorphological analysis by conventional light microscopy and scanning electron microscopy. The HT group showed significantly higher yield load and yield stress in the tibiae three-point bending test. The maximum load, stiffness, maximum stress and elastic modulus were statistically similar for the experimental groups. The hydroxyproline content, morphometrical and stereological data were not significantly different for the four groups. Scanning electron microscopy showed more lacunae and Havers canals in the bone of trained animals, moreover the osteons were more disorganized, when compared with sedentary groups. These alterations may indicate that the bone of trained animals was being remodeled. However, after 8 weeks of training, it was not possible verify alterations in morphometrical measurements, collagen content, stiffness and modulus of elasticity of the trained and treated animals.

Keywords: biomechanics, hydroxyproline, morphometry, scanning electron microscopy, tibiae.

1 Introduction

The primary function of bone is to provide protection and mechanical support for the body. In addition, bones participate in the regulation of calcium homeostasis (BUCKWALTER, GLIMBCHER, COOPER et al., 1996). Bone consists of a dry weight of 65% mineral and 35% organic matrix (BUCKWALTER, GLIMBCHER, COOPER et al., 1996; JEE, 2000). The collagen network accounts for most of the organic phase of this tissue and provides bone with tensile strength as well as a matrix for mineral deposition, which, in turn, confers rigidity (BAILEY, SIMS, EBBESEN et al., 1999). Bone collagen fibers are predominantly type I, but a small amount of type III and V collagen has been reported to be associated with vasculature and osteocytes, respectively (BAILEY, SIMS, EBBESEN et al., 1999). Type I fibers are composed of parallel aligned, end-over-lapped and quarter staggered molecules. This precise organization of molecules in the fibrils allows head to tail cross-linking of the molecules for strength, and at the same time provides a nucleation site for the deposition of calcium apatite in the gap regions generated in the fiber (LANDIS, HODGENS, ARENA et al., 1996).

It is well accepted that physical exercise improves health (KIVINIEMI, HAUTALA, KINNUNEN, 2007). It strengthens bones, improves cardiorespiratory fitness, speed-strength and lipid profiles (VAINIONPAA, KORPELAINEN, KAIKKONEN et al., 2007). Exercise can potentially increase resistance to skeletal fragility, and it is commonly considered that mechanical stimulation exerts its influence through structural adaptation and the accrual of bone mass (CARTER, VAN DER MEULEN, BEAUPRE, 1996). Rodent models of exercise have shown a link between mechanical loading of bone with increased bone mass, cross-sectional geometric properties, and maintenance or increase in mechanical properties (KODAMA, UMEMURA, NAGASAWA et al., 2000; NOTOMI, OKIMOTO, OKAZAKI et al., 2001). However, the improvement in mechanical properties cannot be fully explained by changes in the size and shape of bones, and mechanical loading can also affect the quality of extracellular matrix (WALLACE, RAJACHAR, ALLEN et al., 2007; KOHN, SAHAR, WALLACE et al., 2009). Physical exercise may change the pace of collagen network

remodeling and therefore significantly affect the generation of collagen cross-links (BRAMA, TEKOPPELE, BANK et al., 2002; KOHN, SAHAR, WALLACE et al., 2009), and consequently, bone mechanical properties.

Heteropterys aphrodisiaca O. Mach. (Malpighiaceae) is a Brazilian plant found mainly in the "Cerrado" regions of Mato Grosso and Goiás states (PIO CORRÊA, 1984; POTT, A. and POTT, VJ., 1994). It was described by Hoehne in 1920 as a plant with stimulant and aphrodisiac properties, and it is known as "nó-de-cachorro", "nó-de-porco" and "cordão-de-São-Francisco". *H. aphrodisiaca* root infusion is used in popular medicine as a tonic or stimulant, for the treatment of nervous debility, nervous breakdown and for muscle and bone weakness. In previous studies, the association of endurance training with *H. aphrodisiaca* resulted in more organized collagen bundles and more resistant tendons to support high loads from intense muscle contraction (MONTEIRO, GOMES, TOMIOSSO et al., 2009).

In this study, we investigated the effect of the association of endurance training with *H. aphrodisiaca* administration on the size, collagen content, mechanical properties and morphology of tibia of Wistar male adult rats.

2 Material and methods

2.1 Animals

Adult Wistar rats, 90 days old, were obtained from the Multidisciplinary Center for Biological Investigation - CEMIB (State University of Campinas, Campinas, SP, Brazil). The rats were housed, three per cage, under standard conditions with 12hrL:12hrD. Animals were provided with commercial rat food and water ad libitum. The Institutional Committee for Ethics in Animal Care and Use of this University approved the experimental protocol (number 1233-1).

2.2 Medicinal plant

H. aphrodisiaca roots were collected in February 2007, in Mato Grosso State, Brazil. The species was identified by comparison with the voucher herbarium specimen of the plant at the Herbarium of the Federal University of Mato Grosso, Brazil (number 23928). The dried roots were crushed and powdered using a grinding mill. The infusion was routinely prepared by pouring 100 mL of boiling water over 25 g of powdered roots, which were allowed to steep for 4 hours, then filtered using filter paper. The infusion was prepared every four days and stored in the refrigerator (4 °C). The yield was an infusion of 68.66 mg of dry extract (6.866% w/v) and a yield of 6.832% (w/w) in terms of initial crude dry weight of plant material. The doses of *H. aphrodisiaca* were selected according to previous studies (MONTEIRO, PREDES, MATTA et al., 2008).

2.3 Study groups and experimental protocol

Thirty-two male rats were divided into four groups (n = 8/group): control sedentary (CS), *H. aphrodisiaca* sedentary (HS), control trained (CT), *H. aphrodisiaca* trained (HT). The HS and HT received *H. aphrodisiaca* infusion by gavage (104 mg/animal) daily, during the 8 weeks of training or sedentary period, whereas the control groups (CS and CT) received 0.5 mL of distilled water. Trained rats (CT and HT groups) were allowed to adapt to treadmill running for a 3 week period, prior to the beginning of the experimental protocol, which consisted of low to moderate level exercise carried out daily for 5 days a week (Table 1). After adaptation, trained rats were subjected to 8 weeks of intensive aerobic exercise (treadmill running), also on a weekly cycle of 5 consecutive exercising days followed by a two day rest, as shown in Table 1 (adapted from MORASKA, TERRENCE, ROBERT et al., 2000; SMOLKA, ZOPPI, ALVES et al., 2000; DEMIREL, POWERS, ZERGEROGLU et al., 2001; FONTANA, OLIVEIRA, LEONARDO et al., 2008). This program is a form of endurance training and should not be compared with power training (FONTANA, OLIVEIRA, LEONARDO et al., 2008).

2.4 Surgical procedures

Forty-eight hours after the last training, the rats were anesthetized with xylazine chloride (Anasedan, Vetbrands, São Paulo, Brazil) and ketamine chloride (Cetamin, Syntec, Cotia, Brazil) (5 and 80 mg.kg^{-1} body weight, respectively). The left and right tibiae were harvested. All soft tissues were removed from the bones. Right tibiae lengths were measured according Lammers, German, Lightfoot et al. (1998), they were wrapped in aluminum foil and kept at –20 °C for future biomechanical testing and hydroxyproline analysis. The left tibiae were preserved in Karnovsky's fixative for morphological analysis

2.5 Scanning electron microscopy

Decalcified cross-sectioned bone samples (n = 4/group) were rinsed three times with 0.1 M sodium phosphate buffer, pH 7.2, and then dehydrated in an ascending ethanol series prior to critical point drying. The specimens were mounted on stubs, sputter-coated with gold and examined with the scanning electron microscope (Jeol-JMS 560).

2.6 Biomechanical test

A three-point bending model test was adopted for measuring the mechanical properties of bone tissues using a material testing system (MTS, model Teststar II). The span of the two support points was 21 mm, and the deformation rate was 3 mm/min. Load-displacement data were transported to a computer and acquired by software. Original data were used to calculate the structural properties: yield load, maximum load and stiffness. Stiffness was computed as the slope of the linear portion of the load-displacement curve. After testing the specimens in three-point bending test, the failure sites of all bone specimens were photographed together with a standard measurement, using a high-resolution digital camera at a standard distance, according to Huang, Lin, Chang et al. (2003). Cross-sectional parameters, including

Table 1. Exercise protocol in treadmill running.

Event	Week	Velocity (m/minute)	Duration (minutes)
Treadmill adaptation	1	10.68	5
	2	12.42	7.5
	3	14.16	10
Training	1	14.16	20
	2	19.62	30
	3	19.62	40
	4-8	22.92	45

cortical bone thickness and cross-sectional area (CSA) of cortical bone, were measured from the photographs using the software Image Pro Plus (v6.1, Media Cybernetics). The cross-sectional moment of inertia (I) was calculated under the assumption that the cross sections were elliptical (Equation 1):

$$I = \pi/64[ab^3-(a-2t) \times (b-2t)^3] \quad (1)$$

where I is the cross-sectional moment of inertia, a is the width of the cross section in the mediolateral direction, b is the width of the cross section in the anteroposterior direction, and t is the average of the cortical thickness (TURNER and BURR, 1993). Data of load displacement were transferred to a stress-strain curve using the following equations: $\sigma = F.L.c/4I$ and $E = Stiffness.L^3/48I$, where σ is stress, c is half the value of b (described above), F is the applied load (N), E is elastic modulus, and L is the span between the two supporting points of the bending fixture (mm). Then, the material properties (yield stress, maximum stress and elastic modulus) were measured. Yield load and yield stress were determined following the 0.2%-offset method (TURNER and BURR, 1993). A line 0.002-strain offset and parallel to the linear part of the stress–strain curve was constructed. The intersection point of this 0.002-strain offset line and the stress–strain curve was called the yield stress. The original loading value of this point was called the yield load. The yield point (yield load and yield stress) is the imaginary point that divides the elastic region of the plastic region. It is defined as the highest force that the material can withstand, without leaving any permanent deformation when unloaded.

2.7 Hydroxyproline analysis

To quantify hydroxyproline, the same tibia samples used for biomechanical analysis (n = 4) were dehydrated in acetone for 48 hours and, subsequently, for another 24 hours in a mixture of chloroform and ethanol, at a ratio of 2:1. The tibia fragments were then hydrolyzed in 6N HCl (10 mg of tissue/mL), for 18 hours at 120 °C, and the hydrolysate was neutralized with 6N NaOH. After, the samples were treated with chloramine T solution and perchloric acid/aldehyde, as described by Stegemann and Stalder (1967). After incubation for 15 minutes at 60 °C, the material was cooled and the absorbance was measured at 550 nm in a spectrophotometer, Ultrospec 2100 (Pro Amersham Biosciences, England). The amount of hydroxyproline in the sample was calculated by comparison with a standard curve of hydroxyproline, and expressed as $mg.g^{-1}$ of wet tissue.

2.8 Statistical analyses

The Statistica software (v 8.0) (Tulsa, OK, USA) was used for the statistical analysis. All data were presented as mean ± SD and a value of $p < 0.05$ was considered significant. The statistical comparison was determined using one-way ANOVA followed by the post hoc Duncan test.

3 Results

There was an increase of body mass in all groups during treatment, but the trained groups of animals gained less mass than those of sedentary animals (Figure 1).

3.1 Morphometry analyses

There were no statistically significant differences in tibia length, proximal width, cortical bone thickness and cross-sectional moment of inertia between the sedentary and trained groups. The distal width of the tibiae of animals of the HT group was significantly greater than in the CS group (Table 2).

3.2 Scanning electron microscopy analysis

Scanning electron microscopy analysis of the bones of trained animals showed apparent increase in lacunae, which indicated the increase in osteocyte number in the cortical bone. The number of Havers canals also increased, suggesting an increase in bone vascularization of trained animals. The bones of trained animals showed more disorganized osteons, which may indicate that these bones were in the process remodeling. However, no morphological difference in the compact bone of control and treated trained rats could be identified (Figure 2).

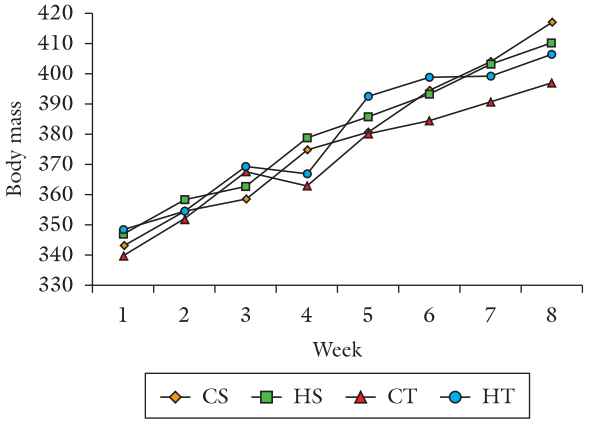

Figure 1. Body mass (g) of sedentary and trained Wistar male rats and/or treated with *H. aphrodisiaca* infusion.

Table 2. Morphometric data for the tibiae.

	CS	HS	CT	HT
Length (mm)	42.80 ± 1.28	42.02 ± 1.01	41.39 ± 1.07	41.77 ± 0.63
Proximal width (mm)	7.80 ± 0.25	7.03 ± 0.44	7.24 ± 0.35	7.25 ± 0.20
Distal width (mm)	4.61 ± 0.4	4.79 ± 0.38	4.88 ± 0.28	5.18 ± 0.24*
Cortical bone thickness (mm)	0.63 ± 0.03	0.60 ± 0.05	0.60 ± 0.04	0.62 ± 0.08
Cross-sectional moment of inertia (mm^4)	1.18 ± 0.21	1.24 ± 0.26	1.14 ± 0.3	1.39 ± 0.53

CS - control sedentary, HS - *H. aphrodisiaca* sedentary, CT - control trained, HT - *H. aphrodisiaca* trained.

The values are the mean ± SD. * $p < 0.05$ compared with CS group by Duncan test.

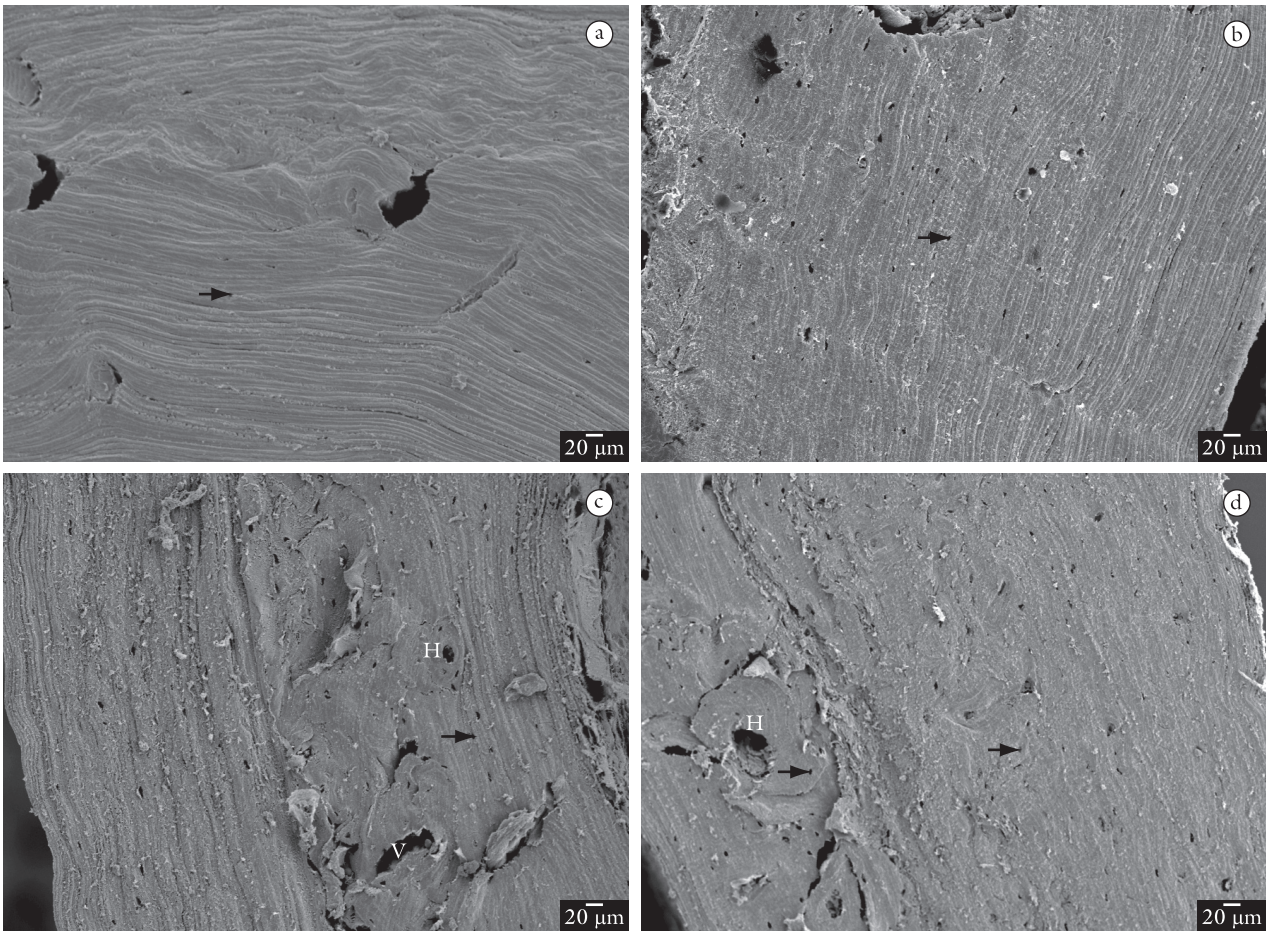

Figure 2. Scanning electron microscopy of cross-section of the tibia. a) Control sedentary; b) *H. aphrodisiaca* sedentary; c) Control trained; d) *H. aphrodisiaca* trained; Arrow - lacuna; H - Havers canal; v - Volkmann canal; Bar = 20 µm.

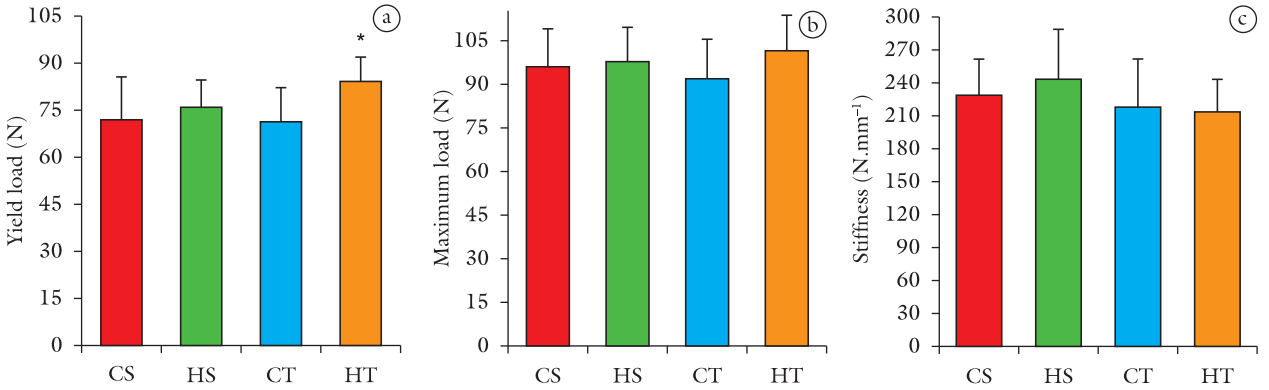

Figure 3. Structural biomechanical properties estimated by three-point bending test of tibiae from control and *H. aphrodisiaca* sedentary and trained rats. a) Yield load; b) Maximum load; c) Stiffness; CS - control sedentary; HS - *H. aphrodisiaca* sedentary; CT - control trained; HT - *H. aphrodisiaca* trained. The columns are the mean + SD. * Differences were significant for $p < 0.05$ (ANOVA) compared with CS and CT groups, by the Duncan test.

3.3 Biomechanical parameters

The structural and material mechanical properties of bone tissue were measured in this study. The animals trained and treated with *H. aphrodisiaca* (HT) showed significantly higher yield load and yield stress (Figures 3a and 4a, respectively) in the tibiae three-point bending test. The maximum load (Figure 3b), stiffness (Figure 3c), maximum stress (Figure 4b) and elastic modulus (Figure 4c) were not significantly different among the four groups.

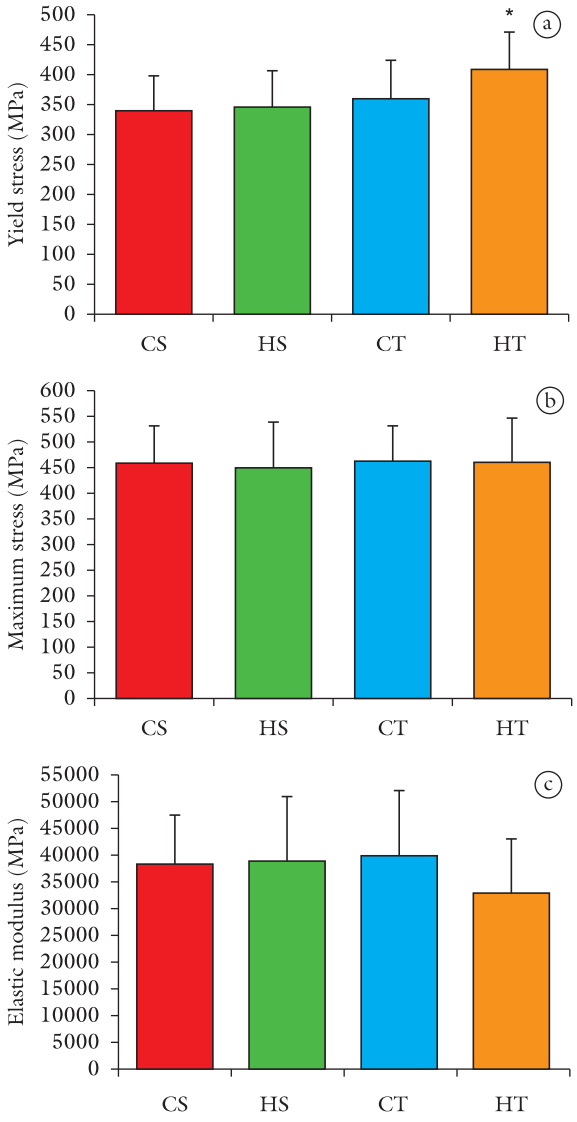

Figure 4. Material biomechanical properties estimated by three-point bending test of tibiae from control and *H. aphrodisiaca* sedentary and trained rats. a) Yield stress; b) Maximum stress; c) Elastic modulus; CS - control sedentary; HS - *H. aphrodisiaca* sedentary; CT - control trained; HT - *H. aphrodisiaca* trained. The columns are the mean + SD. * Differences were significant for $p \leq 0.05$ (ANOVA) compared with CS group, by the Duncan test.

3.4 Hydroxyproline dosage

Hydroxyproline is an indicator of collagen concentration in tissues. The hydroxyproline content was similar in all groups (Figure 5).

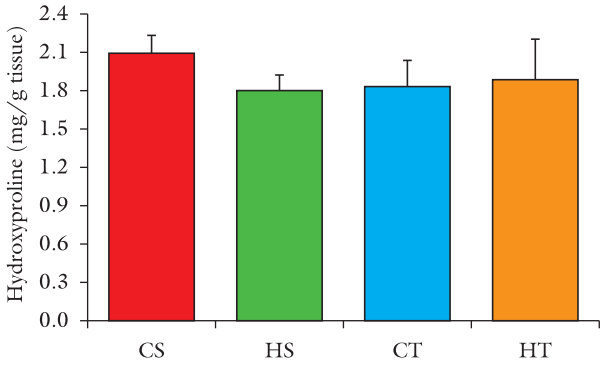

Figure 5. Hydroxyproline dosage of tibiae from control and *H. aphrodisiaca* sedentary and trained rats. CS- control sedentary, HS- *H. aphrodisiaca* sedentary, CT- control trained, HT- *H. aphrodisiaca* trained. The columns are the mean ± SD.

4 Conclusion

Many previous studies have used either young, old or surgically osteopenic rodents to determine effectiveness of exercise-induced loading on the skeleton. This may be justified by the importance of achieving peak bone mass and minimizing bone loss, but these models increase confounding factors due to accelerated bone growth or loss due to age alone. The study of skeletally mature, intact young adult rodents more directly address the normal adult skeletal adaption to mechanical loading (WARNER, SHEA, MILER et al., 2006). As such, we studied 90-day-old male rats, and this discussion mostly focuses on previous studies which have included the effects of endurance exercises in the skeletally mature rats. We used the *Heteropterys aphrodisiaca* infusion to evaluate whether this plant, alone or associated with endurance training, exerts some effect on the mechanical properties and collagen content of rat bone, as was observed in tendons (MONTEIRO, GOMES, TOMIOSSO et al., 2009).

Changes in body mass can influence the mechanical properties of bone. The present study, however, showed no significant difference in body mass between control and trained groups. The present results, therefore, were not dependent on body mass, and the differences shown in bone properties were likely a consequence of the exercise and/or of the treatment with *H. aphrodisiaca*.

Exercise has been shown to change bone morphometry in experimental animals. Cortical bone area increased following an exercise program in swine (RAAB, CRENSHAW, KIMMEL et al., 1991) and rats (NEWHALL, RODNICK, VAN DER MEULEN et al., 1991; WHEELER, GRAVES, MILLER et al., 1995). However, other studies with rats and mice showed that the endurance exercise did not alter the morphometric measurements of bone of exercised animals (FORWOOD and PARKER, 1991; WARNER, SHEA, MILER et al., 2006; ISAKSSON, TOLVANE, FINNILÃ et al., 2009). In our study, the length, proximal width and cortical bone thickness displayed no statistical differences between the sedentary and trained groups. The statistically greater distal width of tibiae of the HT group was not considered important because this point does not support any major muscle.

The discrepancy in results described in the literature for morphometrical data could be due to differences in the exercise protocol, animal age and species, and the overall length or intensity of the exercise study. Moreover, the difference of these results could be explained by the proximo-distal location of the functional adaptations of limb bone to mechanical loading. Femoral rates and amounts of bone formation were significantly greater than those of the tibiae in exercised mice (PLOCHOCKI, RIVERA, ZHANG

et al., 2008). Ontogenetically constrained bone formation in distal limb elements may be an evolutionary adaptation to conserve bone mass and maintain energetic efficiency during high stride frequency locomotion (PLOCHOCKI, RIVERA, ZHANG et al., 2008). However, there may also be a biomechanical explanation for proximodistal differences in bone growth in mammals with tapered limbs. Muscular contractions exert larger loads on bone than body mass during running activities because muscles work as poor lever arms and require great force (FROST, 1999). In rodents, mammals with tapered limbs, the muscle mass is concentrated proximally on the limb. Because bone growth is, in part, regulated by mechanical stress, heavier muscle proximal skeletal elements can be expected to experience greater loading and consequently greater modeling than distal skeletal elements under conditions of intense running (PLOCHOCKI, RIVERA, ZHANG et al., 2008).

The effects of exercise on the biomechanical properties of bone using experimental animals have yielded controversial results. Wheeler, Graves, Miller et al. (1995) showed that tibial stiffness, the energy absorbed and the angle of twist at failure were affected by exercise. Conversely, Warner, Shea, Miler et al. (2006) and Isaksson, Tolvane, Finnilã et al. (2009) and collaborators showed that treadmill exercise did not alter the mechanical properties of bone of the adult mice and rats, respectively. Our results were in agreement with the last results cited, since we showed that treadmill running alone did not alter the structural and material properties of tibiae bone. However, the difference of results might be due to the duration and intensity of exercise used in each study. Wheeler, Graves, Miller et al. (1995) explained that the duration of exercise may affect the bone mineralization more strongly than the intensity of exercise in rats.

In this study, the association of endurance exercise and the plant treatment determined tibiae with higher yield load and yield stress, when compared with control trained and sedentary animals. Having the significantly higher yield load and yield stress implies that the HT group bones sustained more elastic deformation or strain. These alterations in biomechanical properties could be related to bone mineral density and the collagen organization in the bone. According to Isaksson, Tolvane, Finnilã et al. (2009), exercise speeded up the rate of reorientation of the collagen structure, e.g, arrangement of fibrils especially in the longitudinal direction, rather than increased collagen formation. This could be the case in our study, since the quantity of collagen did not increase, as did the yield load and the yield stress of the tibiae.

Contrary to the findings for tibiae, a previous study associating the treatment with this plant and endurance exercise showed that the animal's tendons had a significant increase in mechanical properties and of collagen content, resulting in stronger tendons, able to support intense muscular contraction. Moreover, the tendons analyzed by polarized microscopy showed brighter collagen fibers, possibly indicating highly compacted bundles (MONTEIRO, GOMES, TOMIOSSO et al., 2009).

Scanning electron microscopy analysis of the bone showed an increase in lacunae and Havers canals for trained animals. Moreover, the osteons of trained animals were more disorganized than in the sedentary groups, which suggest that the bone of trained animals were being remodeled.

Therefore, 8 weeks of training did not show alterations in morphometrical measurements, hydroxyproline content and some mechanical properties (stiffness and modulus of elasticity) of the bone of trained and treated animals, as was found in the tendons of animals that received *H. aphrodisiaca* and endurance training. Studies with longer training periods should be undertaken to determine whether alterations will ensue with further exercise.

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