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Effect of Growth Hormone (GH) and Resistance Training on the Collagen Properties of Femoral Bone Tissue

Efecto de la Hormona de Crecimiento (HC) y el Entrenamiento de Fuerza en las Propiedades Colágenas del Tejido Óseo Femoral

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SUMMARY: The indiscriminate use of anabolic steroids in gyms has been growing in a generalized way, among which, the most common is growth hormone (GH). In the short term GH may potentiate muscle growth, especially when taken in combination with resistance training. However, the effects of this hormone are not yet fully understood in the literature, especially in relation to collagen properties. The objective of this study was to evaluate the effect of the application of growth hormone (GH) and resistance training (RT) on the collagen properties of femoral bone tissue using Raman Spectroscopy. In this study 40 male rats were randomly distributed into four groups (n=10): control (C), control and GH application (GH), resistance training (T), and resistance training and GH application (GHT). The training consisted of four series of 10 water jumps, performed three times a week, with an overload corresponding to 50 % of body weight and duration of four weeks. GH was applied at a dosage of 0.2 IU/Kg (0.067 mg/kg) to each animal, three times a week, every other day. The animals were euthanized and the right femurs were collected for analysis of bone structure. Raman spectroscopy (RS) was used to observe the following compounds from their respective bands: type I collagen (662 cm^{-1}), amide III (1243 cm^{-1}), proteins including type I collagen (1278 cm^{-1}), woven collagen (1322 cm^{-1}), association of collagen, phospholipids, nucleic acid, and phosphate (1330 cm^{-1}), and collagen and protein deformation (1448 cm^{-1}). The results demonstrated an increase in the collagen properties in all analyzed variables, however, the T group presented a statistically significant difference ($p<0.05$). It is possible to conclude that isolated physical training was shown to be more efficient than when combined with the application of GH to increase the collagen properties of the femoral bone tissue.

KEY WORDS: Physical training; Growth hormone; Bone; Femur; Rats; Collagen.

INTRODUCTION

Collagen is an essential protein that provides increased strength and flexibility in bone mineral tissue. Among several forms of collagen, there is hydrolyzed collagen (HC) which is composed of amino acids and has high levels of glycine and proline (Henrotin *et al*., 2011).

Bone tissue can respond to several training variables such as intensity, volume, specificity, rest time, number of sets and repetitions, and type of muscle contraction (Castoldi *et al*., 2017). In order to promote different adaptations in bone mineral tissue, different training models can be performed, such as resistance, aerobic, and concurrent (Bikle *et al*., 2003; Castoldi *et al*., 2013).

Evidence shows that resistance training (RT) is a potent stimulus to increase the density and remodeling of bone mineral tissue, since it can be used as a regulator of skeletal maturation, maintenance, and strength (Menkes *et al*., 1993). Some theoretical and experimental data suggest that for the training load to generate an increase in bone mass, it must

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be of sufficient magnitude to exceed the minimum effective load and be applied gradually and intermittently (Aguiar *et al*., 2010). However, the absence of load can be determinant for reduction in the bone matrix, and one of the molecular mechanisms responsible for this is the induction of resistance to insulin-like growth factor type 1 (IGF-1) (Bikle *et al*.).

Among the hormones most commonly studied scientifically is growth hormone (GH) (Lima-Silva *et al*., 2006). In its formula, growth hormone (GH) contains 191 amino acids and a weight of 22 kilodaltons (kDa) (Strobl & Thomas, 1994), with approximately 75 % of the total secreted by adenohypophysis somatotrophic cells (Baumann & Gauldie, 1994).

Among the main effects of GH are increased protein synthesis, decreased glucose oxidation, and increased glycogen storage (Hirschberg & Kopple, 1992). The GH regulator in adenohypophysis has a complex mutual relationship between hypothalamic peptides which are responsible for hormonal inhibition or release. In this case, somatostatin "Growth hormone inhibiting hormone" (SRIF) prevents the secretion of GH, while the "Growth hormone releasing hormone" releases it (GHRH) (Ribeiro & Tirapegui, 1995).

Both GHRH and SRIF synthesis are instigated by several neurotransmitters, such as serotonin, dopamine, acetylcholine, and noradrenaline (Wideman *et al*., 2002). There are growth hormone (GH) receptors in various body tissues, such as skeletal muscles, liver, kidneys, pancreas, heart, intestine, lung, and brain. The majority of GH in circulation binds to specific carrier proteins "Growth hormone binding proteins" (GHBP) (Baumann & Gauldie).

The indiscriminate use of GH can cause damage to the health of the individual, with harmful side effects such as cardiac instability, hypertension, insulin resistance, and acromegaly (Rennie, 2003). However, few studies have attempted to identify the effects of GH on bone tissue, mainly used in conjunction with RT. Thus, the objective of this study was to investigate the alterations induced in the collagen properties of bone tissue by RT and the application of growth hormone (GH), using Raman spectroscopy for the measurement parameters.

MATERIAL METHOD

Animals. A total of 40 male rats, 60 days of age, of the Wistar lineage were used. The animals were separated into groups of 5 animals and kept in plastic boxes (polyethylene) with dimensions of 41x34x17,5 cm, and controlled temperature (20 to 30 ºC), brightness (light/dark cycle of twelve hours), and humidity (55±15 %), from 7:00 am to 7:00 p.m., with free access to water and Supralab® feed (Supra, Empresa Alisul, Brazil). The present study was approved by the local ethics committee, under approval number (CEUA - 2626).

Experimental Protocols: The animals were divided into four experimental groups: control (C) [n=10], control with the application of GH (GHC) [n=10], muscle strength training (T) [n=10], and muscle strength training with the application of GH (GHT) [n=10]. The training period consisted of four weeks.

The animals were submitted to a one week period of adaptation to the liquid medium and equipment (1x10 jumps, 2x10 jumps, 3x10 jumps), with progressively increased overload and duration, according to the method proposed by Manchado *et al*. (2006).

Experimental Groups:

C Group: the animals remained free in their boxes with unrestricted access to feed and water. They received the same volume of physiological solution (0.9 % sodium chloride in water) as the groups that received GH.

GHC Group: the animals remained free in their boxes with unrestricted access to feed and water. In addition, 0.2 IU/Kg (0.067 mg/Kg) of GH was administered to each animal three times a week on alternate days.

T Group: performed four series of 10 jumps, executed three times a week, in a cylindrical PVC container, specially modified for water jumping, with a depth appropriate to the length of the animals (38 cm). An interval of 1 minute was established between each of the series of jumps. The overload used corresponded to 50 % of the body weight of each animal and was corrected weekly. The overload was accommodated in the anterior region of the thorax using a vest, as proposed by (De Mello Malheiro *et al*., 2009) and used by (Castoldi *et al*., 2015). In addition, the animals received physiological solution (0.9 % sodium chloride in water) in equal volume to the GH-treated animals (Fig. 1). GHT Group: composed in the same way as the protocol mentioned above. However, as in the case of the GHC group, 0.2 IU/Kg (0.067 mg/Kg) of GH, rather than physiological solution, was administered to each animal. Immediately after application, the animals performed the RT protocol.

Bone Tissue: After euthanasia, the right femur was removed by longitudinal incision to separate the skin and soft

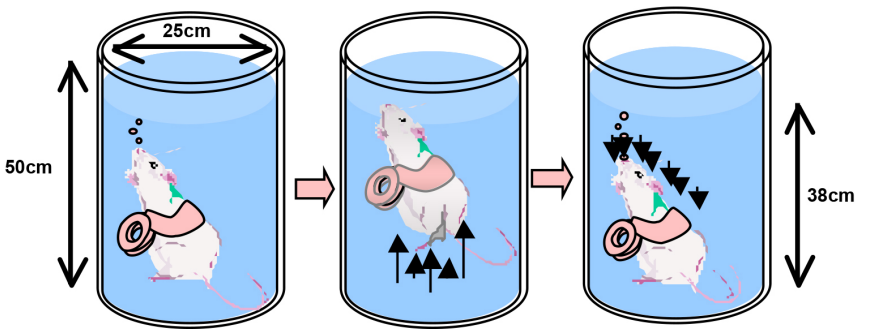

Fig. 1. Demonstration of the form of training used in the present study.

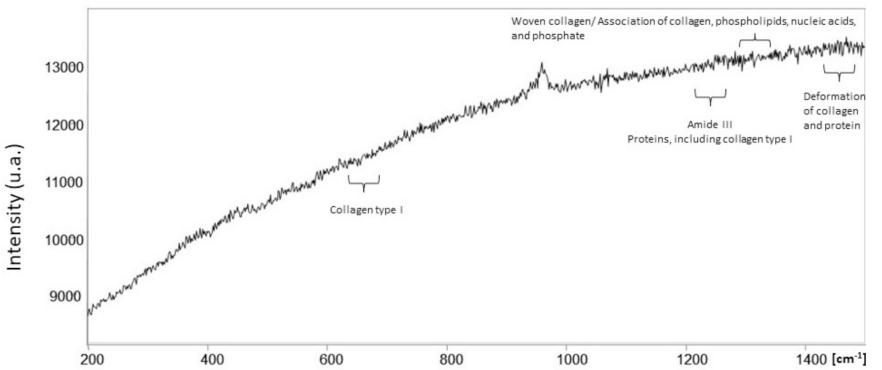

Fig. 2. Spectrum model and definition of analyzed bands.

Table I. Raman spectrum band assignments of bone tissue.

_ [cm^{-1}]	Band Assignment
662	Collagen type I
1243	Amide III
1278	Proteins, including collagen type I
1322	Woven collagen
1330	Association of collagen, phospholipids, nucleic acids, and phosphate
1448	Deformation of collagen and protein

(References: Shafer-Peltier *et al*., 2002; Gazi *et al*., 2003; Cheng *et al*., 2005; Malini *et al*., 2006).

tissue. After this procedure, the femur was immersed in physiological solution and stored at -20°C for further analysis.

Raman Microscopy: Analysis of the bone structure (bone cells) was performed using the Raman microscope "Renishaw-branded Raman microscope, model in-Via" surface-enhanced Raman scattering. In this case, the alterations in the bands corresponding to the following compounds were measured: collagen type I (662 cm^{-1}), amide III (1243 cm^{-1}), proteins including collagen type I (1278 cm^{-1}), woven collagen (1322 cm^{-1}), association of collagen, phospholipids, nucleic acids, and phosphate (1330 cm^{-1}), and collagen and protein deformation (1448 cm^{-1}), according to the standardization previously published in the literature (Movasaghi *et al*., 2007) (Fig. 2).

The Raman spectroscopy measurements were obtained using a micro-Raman spectrograph, Renishaw in-Via model. A laser with a wavelength of 633 nm, and sample power of the microwatt order (mW) was used and the diffraction grating was 1800 lines per mm. The exposure time was 10 s with three accumulations.

Optical microscopy was obtained using an optical microscope of the Leica brand (DMLM series), coupled to the spectrograph with an objective of 50x magnification, providing spatial resolution in the order of 1.00 μm^2, a Peltier CCD detector (cooled to -70 °C), and motorized platform XYZ (stepper motor - 0.10 mm) in which the samples were placed. Three spectra were collected from each sample, totaling nine spectra per group (Table I).

Statistical Analysis. After obtaining the data, the normality was verified by the Shapiro-Wilk test and then univariate ANOVA analysis of variance analysis was used for comparison between means, with Tukey's post test. All procedures adopted a significance of 5 % ($p<0.05$) and were performed using SPSS 22.0 Software.

RESULTS

After analyzing the data, it was verified that the group of animals that performed RT alone (group T) presented higher values for all variables analyzed ($p<0.05$). In addition, although the GHC and GHT groups presented increases, these were not statistically significant in comparison to group C ($p>0.05$).

Similar behavior was observed for all the variables analyzed in the present study (collagen type I, amide III, proteins including collagen type I, woven collagen, association of collagen, phospholipids, nucleic acids, and phosphate, and finally, collagen and protein deformation) (Fig. 3).

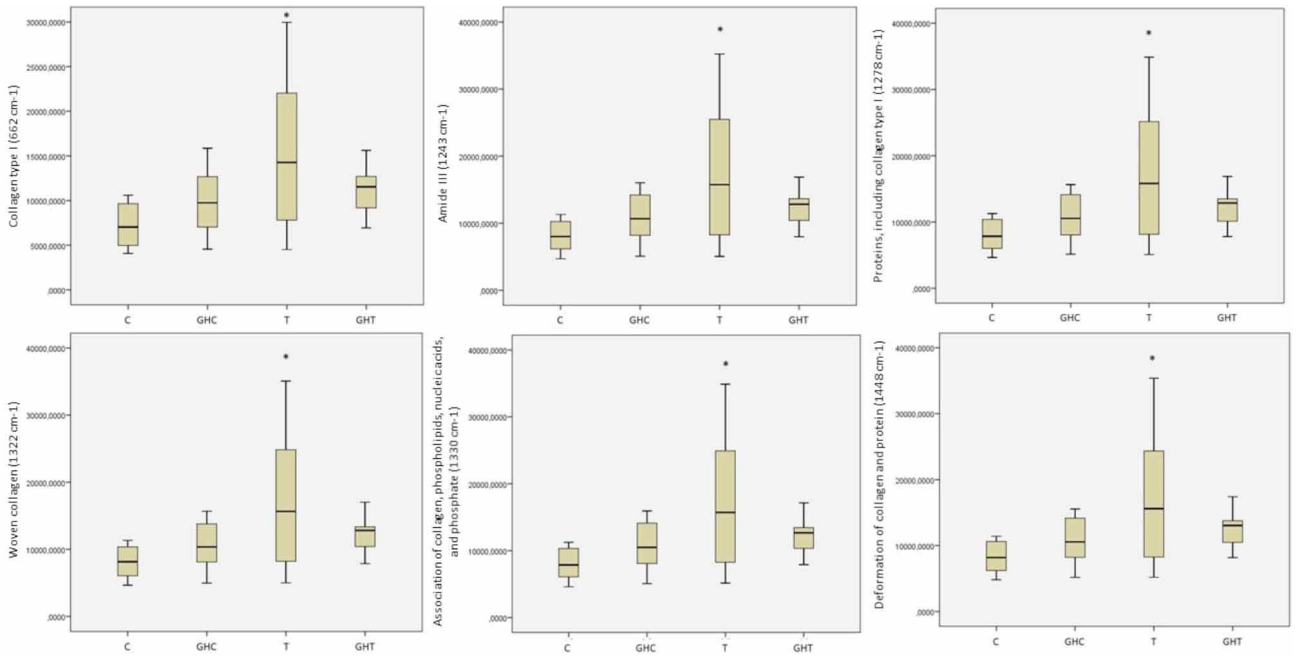

Fig. 3. Comparison of intensity values among different groups of animals. (*): Analysis of variance test - ANOVA unidirectional with Tukey post test with significance of 5 % ($p<0.05$). Bands corresponding to compounds of collagen type I (662 cm^{-1}); amide III (1243 cm^{-1}); proteins, including collagen type I (1278 cm^{-1}); woven collagen (1322 cm^{-1}); association of collagen, phospholipids, nucleic acids, and phosphate (1330 cm^{-1}); collagen and protein deformation (1448 cm^{-1}). (C): Control, (GHC): Control with the application of GH, (T): Resistance training, and (GHT): Resistance training with the application of GH.

DISCUSSION

The main objective of the present study was to verify the effects of the application of growth hormone (GH) and the performance of a resistance training protocol (RT) on the collagen properties of femoral bone tissue. It was verified that the group of animals that performed RT alone presented higher values for the variables collagen type 1 (662 cm^{-1}), amide III (1243 cm^{-1}), proteins including collagen type l (1278 cm^{-1}), woven collagen (1322 cm^{-1}), association of collagen, phospholipids, nucleic acids, and phosphate (1330 cm^{-1}) and collagen and protein deformation (1448 cm^{-1}) ($p<0.05$).

The standardization of the bands was elaborated by Movasaghi *et al*. Evidence shows that RT is a potent stimulus able to promote increased density and remodeling of bone mineral tissue (Menkes *et al.*).

It has been observed that RT can contribute to the increase in the mineral components of bone tissue, so the absence of the load can be a determinant for the reduction in the bone matrix, being an important mechanism responsible for the inhibition of resistance to IGF-1 (Bikle *et al.*). The increase in mechanical load generates a tension force, important for regulation of the maturation and maintenance of bone tissue (Craig *et al*., 1989; Aguiar *et al.*).

In the case of the present study, it was observed that the RT protocol (4 sets of 10 jumps) was adequate to increase the concentration of the collagen properties analyzed. Training programs have been widely used as part of the prevention and treatment of diseases such as osteoporosis, since physical training induces an increase in mechanical load, which acts on the bone tissue due to external forces and muscle contractions. The increased mechanical load generates a tensile force, which promotes bone remodeling and bone mass increase (Mottini *et al*., 2008).

In addition, the GHC group that received only GH application presented lower values of the analyzed variables. This finding may reflect health risks due to the indiscriminate use of anabolics in bodybuilding gyms, a behavior that has been growing, especially among young people (Macedo *et al*., 1998).

The release of GH into the bloodstream seems to be linked to exertion above anaerobic threshold levels, as is the case with resistance training (Gomes *et al*., 2003). In addition, studies have shown a decreased secretion response during rest and after training sessions (Nicklas *et al*., 1995; Bell *et al*., 2000).

The motive for people to use GH is mainly due to its anabolic effects, which can take up to nine months to take effect after application. For this reason, consumption of anabolic steroids is a problem in pre-adolescence and adolescence (Macedo *et al*.).

GH is secreted by adenohypophysis, known as somatotropin (Baumann & Gauldie). In addition, GH alters the flow, oxidation, and metabolism of almost all nutrients in circulation, as it has an anabolic action, stimulating tissue growth and metabolic rate (Strobl & Thomas). The indirect effects of GH are related to the modulation of the synthesis of Insulin-like Growth Factors 1 (IGF-1), being a mediator of the anabolic effects of GH, which is related to human growth (Ribeiro & Tirapegui).

Thus, the present study collaborates with the literature in identifying the effects of RT and GH application on the collagen properties of bone tissue. However, some limitations should be considered, such as the training protocol and the hormonal dosage used (0.2 IU/Kg or 0.067 mg/Kg). Future studies that seek to investigate different forms of training, hormonal dosages, supplementation, and other forms of analysis may contribute to the findings presented in the current study.

CONCLUSION

It is possible to conclude that RT alone was able to promote an increase in the collagen properties of femoral bone tissue when compared to the other groups of animals. In addition, the GHT and GH groups, although presenting higher values than the C group, did not demonstrate statistically significant differences.

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RESUMEN: El uso indiscriminado de anabolizantes en los gimnasios ha aumentado de forma generalizada, entre éstos la hormona de crecimiento (HC) es una de las más utilizadas, y a corto plazo puede potencializar el crecimiento muscular, principalmente cuando es realizado en combinación con el entrenamiento de fuerza. Sin embargo, los efectos de esta hormona aún no están totalmente esclarecidos en la literatura, especialmente en relación a las propiedades colágenas. El objetivo del estudio fue evaluar el efecto de la aplicación del HC y entrenamiento de fuerza (E) en las propiedades colágenas del tejido óseo femoral a partir de la utilización de la espectroscopía Raman. Se usaron 40 ratas Wistar distribuidos en cuatro grupos (n=10): control (C), control y aplicación del HC (HCC), entrenamiento de fuerza (E) y entrenamiento de fuerza y aplicación del HC (THC). El entrenamiento fue compuesto por cuatro series de 10 saltos acuáticos, realizados tres veces por semana, con sobrecarga correspondiente a 50 % del peso corporal y duración de cuatro semanas. El HC fue aplicado en una dosificación de 0,2 UI/Kg (0,067 mg/kg) en cada animal, tres veces por semana, en días no consecutivos. Los animales fueran eutanasiados y se retiró el fémur derecho para realización del análisis de la estructura ósea. La espectroscopía Raman (ER) fue utilizada para observar los siguientes compuestos a partir de las respectivas bandas: colágeno tipo I (662 cm^{-1}), amida III (1243 cm-1), proteínas, incluido colágeno tipo I (1278 cm^{-1}), colágeno retorcido (1322 cm^{-1}), asociación de colágeno, fosfolípidos, ácidos nucleicos y fosfato (1330 cm^{-1}), deformación de colágeno y proteína (1448 cm^{-1}). Hubo aumento en las propiedades colágenas en todas las variables analizadas, sin embargo, solamente el grupo E demostró una diferencia estadísticamente significativa ($p<0,05$). En conclusión, para el aumento de las propiedades colágenas del tejido óseo femoral, el entrenamiento físico aislado es más eficiente que el entrenamiento combinado con el uso de HC.

PALABRAS CLAVE: Entrenamiento físico; Hormona del crecimiento; Hueso; Fémur; Ratas; Colágeno.

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