UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

RAFAEL ARAUJO

DETERMINAÇÃO DAS CARACTERÍSTICAS MECÂNICAS DO TECIDO ÓSSEO DO FÊMUR DE RATAS WISTAR COM OSTEOPOROSE

DETERMINATION OF THE MECHANICAL CHARACTERISTICS OF FEMUR FROM FEMALE WISTAR RATS WITH OSTEOPOROSIS

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Biologia Buco Dental, na Área de Anatomia.

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Dental Biology, in Anatomy area.

Orientadora: Prof.ª Dr.ª Ana Cláudia Rossi

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RESUMO

O objetivo deste estudo foi comparar o módulo de elasticidade do fêmur de ratos com osteoporose utilizando o teste mecânico e o cálculo computadorizado. Foram utilizadas 6 fêmeas de ratos da linhagem Wistar separadas em 2 grupos: SHAM, grupo controle com 3 ratas submetidas a cirurgia placebo; OVX, grupo com osteoporose induzida com 3 ratas submetidas a cirurgia de ovariectomia. Após a maturação das ratas até idade adulta, estas foram eutanasiadas no período de 120 dias após a cirurgia e o fêmur direito foi removido. O material coletado foi submetido ao exame de imagem realizado no microtomógrafo SkyScan 1174 e posteriormente ao teste mecânico de flexão na diáfise do osso na máquina de teste universal Instron 4411. Foi utilizado o software MIMICS para realizar os cálculos do módulo de elasticidade e densidade. Foi usado o teste t de *Student* para comparar as informações obtidas nos dois grupos. O tecido ósseo acometido por osteoporose possui valores de módulo de elasticidade, densidade mineral muito inferiores em relação ao tecido ósseo sadio, com diferença estatisticamente significativa ($p < 0,05$). O cálculo do módulo de elasticidade pela densidade aparente não apresentou diferença significativa entre os grupos analisados. De forma macroscópica foi notado que o padrão de fratura do tecido ósseo osteoporótico é incompleto em “galho-verde” necessitando uma força muito inferior que o tecido ósseo sadio, o qual possui fratura completa. O osso com osteoporose apresenta uma maior fragilidade, com características mecânicas diferentes do tecido saudável. As informações obtidas podem servir de base para caracterizar o tecido ósseo para realizar experimentos computadorizados.

Palavras-chave: Osteoporose, Fêmur, Módulo de Elasticidade

ABSTRACT

The objective of this study was to compare the elasticity modulus of the femur of rats Wistar with osteoporosis using the mechanical test and the computerized calculation. Six female Wistar rats were separated into two groups: SHAM, control group with 3 rats submitted to placebo surgery; OVX, a group with osteoporosis induced with 3 rats submitted to ovariectomy surgery. After maturation of the rats until adulthood, they were euthanized within 120 days after surgery and the right femur was removed. The collected material was submitted to microtomograph SkyScan 1174 and after it was performed the mechanical flexural test in the bone diaphysis, and to the compression test on the femoral head on the Instron 4411 universal test machine. The MIMICS software was used to perform calculations of the modulus of elasticity and density. Student's t-test was used to compare the information obtained in the two groups. Bone tissue affected by osteoporosis has modulus of elasticity values, mineral density much lower than healthy bone tissue, with a statistically significant difference ($p < 0.05$). The calculation of the modulus of elasticity by the apparent density showed no significant difference between the analyzed groups. Macroscopically it was noted that the fracture pattern of osteoporotic bone tissue is incomplete in "green branch" requiring a much lower force than healthy bone tissue, which has a complete fracture. Bone with osteoporosis presents a greater fragility, with different mechanical characteristics of healthy tissue. The information obtained can serve as a basis for characterizing the bone tissue to perform computerized experiments.

Keywords: Osteoporosis; Femur; Elasticity modulus

SUMÁRIO

1 INTRODUÇÃO

A osteoporose é uma doença degenerativa caracterizada pela redução de massa óssea e a deterioração da microarquitetura dos ossos, o que aumenta a fragilidade dos ossos e aumentam o risco de fratura (1,2). Esta doença tem maior prevalência em mulheres com mais de 50 anos (3).

A degeneração da matriz óssea encontrada na condição do tecido ósseo com osteoporose é resultado da alta atividade dos osteoclastos. Estas células são estimuladas pela ativação dos receptores RANK presente na membrana plasmática das células pelo sinalizador químico RANK-L (4). Mediadores químicos inibidores da produção de RANK-L é o fator de crescimento transformador-β e o hormônio estrógeno(5).

Para a realização de estudos sobre a osteoporose, pesquisadores utilizam o modelo animal para realizar as experimentações e testes de tratamento. Neste modelo, é usualmente utilizado o rato (*Rattus norvegicus*) da linhagem Wistar, onde a osteoporose pode ser induzida cirurgicamente pela ooforectomia (6), ou pela utilização de fármacos (7).

O modelo de indução da osteoporose por ooforectomia se baseia na curta duração do ciclo estral do rato, com média de 4 a 5 dias (8). Sendo assim, após 120 da remoção cirúrgica dos ovários, os animais são considerados portadores de osteoporose (9).

Modelos e simulações utilizando a tecnologia de elementos finitos permite o estudo de condições e situações que não poderiam ser realizados em modelos em vivo, por questões éticas ou logísticas.

Para realizar as simulações, o computador utiliza de propriedade mecânicas dos materiais como por exemplo o módulo de elasticidade (*Young's modulus*), coeficiente de Poisson e módulo de cisalhamento (*Shear Modulus*). Essas propriedades podem ser mensuradas a partir de teste mecânicos específicos como a flexão em 3 pontos, torção e ultrassom (10–12) ou calculadas pela densidade mineral óssea (13,14).

A fim de tentar encontrar uma associação entre os dados obtidos em modelos de ensaio mecânico e computacional, o objetivo deste estudo foi comparar o módulo de elasticidade do fêmur de ratas com osteoporose utilizando o teste mecânico e o cálculo computadorizado.

2 ARTIGO: DETERMINAÇÃO DO MÓDULO DE ELASTICIDADE DO FÊMUR DE RATAS WISTAR COM OSTEOPOROSE

Artigo submetido ao periódico Journal of Bone and Mineral Metabolism (Anexo 2)

ABSTRACT

The objective of this study was determining the modulus of elasticity of induced osteoporosis femur of rat Wistar. Six female Wistar rats were separated into two groups: SHAM, control group with 3 rats submitted to placebo surgery; OVX, a group with osteoporosis induced with 3 rats submitted to ovariectomy surgery. After maturation of the rats until adulthood, they were euthanized within 6 months after surgery and the right femur was removed. The collected material was submitted to microtomograph SkyScan 1174 and after it was performed the mechanical flexural test in the bone diaphysis, and to the compression test on the femoral head on the Instron 4411 universal test machine. The MIMICS software was used to perform calculations of the modulus of elasticity and density. Student's t-test was used to compare the information obtained in the two groups. Bone tissue affected by osteoporosis has modulus of elasticity values, mineral density much lower than healthy bone tissue, with a statistically significant difference ($p < 0.05$). The calculation of the modulus of elasticity by the apparent density showed no significant difference between the analyzed groups. Macroscopically it was noted that the fracture pattern of osteoporotic bone tissue is incomplete in "green branch" requiring a much lower force than healthy bone tissue, which has a complete fracture. Bone with osteoporosis presents a greater fragility, with different mechanical characteristics of healthy tissue. The information obtained can serve as a basis for characterizing the bone tissue to perform computerized experiments.

Keywords: Osteoporosis, Femur, Elasticity modulus

1 INTRODUCTION

The osteoporosis is a degenerative disease characterized by the reduction bone mass and the deterioration of micro architecture of bone and rises the fragility and fracture risk (1,2). This disease has a higher prevalence in women over 50 years of age (3).

For studies on osteoporosis, researchers use the animal model to perform the trials and treatment trials. In this model, the rat (Rattus norvegicus) from Wistar lineage is used, where osteoporosis can be surgically induced by oophorectomy (6) or by the use of drugs (7).

Models and simulations using finite element technology allow the study of conditions and situations that could not be performed in live models, due to ethical or logistical reasons. To perform the simulations, the computer uses mechanical properties of the materials such as modulus of elasticity (Young's modulus), Poisson's coefficient and shear modulus (Shear modulus). These properties can be measured from specific mechanical tests such as three-point bending, torsion and ultrasound (10–12) or calculated by bone mineral density (13,14).

In order to try to find an association between the data obtained in mechanical and computational test models, the objective of this study was to compare the elasticity modulus of the femur of rats with osteoporosis using the mechanical test and the computerized calculation.

2 MATERIAL AND METHODS

2.1 ANIMAL MODEL

All the procedures were performed in animals was approved (protocol CEUA 4427-1/2016) by the Animal Research Ethics Committee of the State University of Campinas (ANEXO 1). Six female 6 months aged Wistar rats from Biotherm of the Faculty of Dentistry of Araçatuba - FOA / UNESP were used. They were kept in an environment of temperature and controlled humidity (22ºC ± 2 ° C), with light and dark cycle of 12 hours, with free access to water and food.

The animals were divided into 2 groups randomly: OVX group, in which the ovariectomy surgery was performed; and SHAM group in which the ovariectomy surgery is simulated without performing the tubal ligation and removal of the ovaries.

2.2 SURGICAL PROCEDURE

For the ovariectomy procedure the animals were anesthetized with ketamine (Vetaset®, Fort Dodge Saude Animal Ltda, Brazil) at a dose of 50mg / kg body weight in combination with xylazine at a dose of 25mg / kg body weight per injection intraperitoneal. A tricotomy was performed in the lateral region of the abdomino-pelvic cavity and the oblique incision, to perform the tubal ligation and removal of the ovaries. In the SHAM group, the same procedures were performed, except for the tubal ligation and removal of the ovaries. The animals were sutured and received a medical protocol for postoperative pain control. After 6 months, the animals from OVX groups showed the osteoporosis condition, according the FDA guidelines for animal model(9).

All the animals from both groups were euthanized with deepening of the anesthesia. The femur was surgically removed, and soft tissue cleaning was performed.

2.3 MEASUREMENT

The femur diameter and length were measured with a digital caliper (Mitutoyo, Japan).

2.4 MICROTOMOGRAPHY

The femurs were scanned by computerized microtomography with the femur immersed in physiological saline, in the Microtomograph 1174 (Brucker, Belgium) at 50kV and 800mA. Each femur was positioned in the craniocaudal orientation and slices were obtained with a resolution of 15μm³. The images were imported into NRecon software (Skyscan, Belgium) for three-dimensional reconstruction.

2.5 MECHANICAL TEST

The femurs were submitted to the three-point bending mechanical test in the universal test equipment Instron 4411 (Instron Corp. Norwood, MA). The femur was positioned horizontally, and the load was applied on the center on the diaphysis. The force of 5N / s was applied until the moment of the fracture (Figure 1)

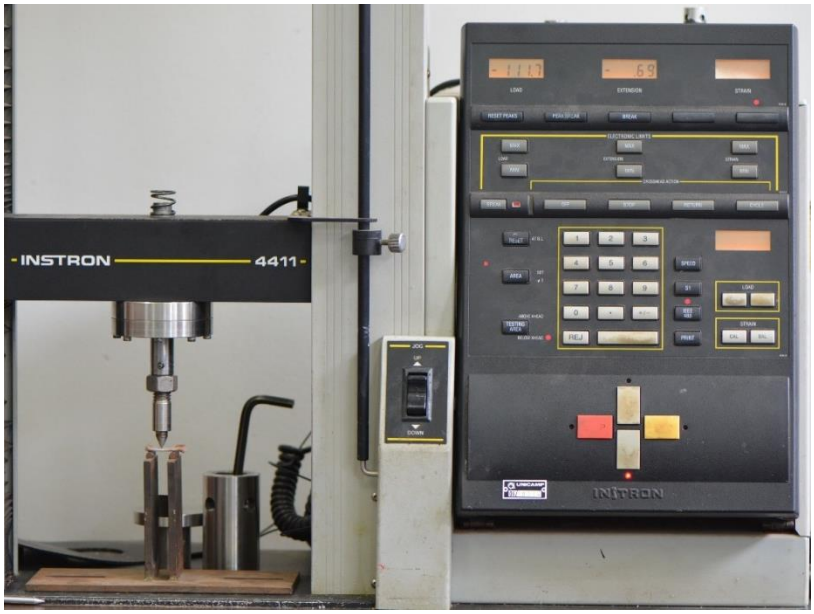

Figure 1. Three-point bending mechanical test on the Instron 4411 universal test machine.

2.6 ESTIMATE OF THE ELASTICITY MODULUS BY APPARENT DENSITY

The microtomographic images gotten from the three-dimensional reconstruction imported on software MIMICS v18 (Materialise, Leuven, Belgium) to perform the segmentation and acquisition of apparent density to convert in elasticity modulus (E). The segmentation was realized to landmark all the bone diaphysis structure in which it was obtained by the selection of the pixels included in the range of values in grayscale. From the landmarks the three-dimensional volumetric model was constructed for the mapping of the apparent density and the modulus of elasticity.

To estimate the values of the elasticity modulus from the apparent density was used the following expression described from Lotz, 1991 and Wirtz et al. 2000 (15,16).

$$E^C = 2065 \times \rho^{3.09}$$

where E^C is the elasticity modulus to the cancellous bone and ρ is the apparent density.

It was considered 7 elastic modulus values were considered for each piece, since the number of values corresponded to the number of density variations present in the diaphysis of the sample pieces, such quantity was determined by the software and also demonstrated in a previous study (boularinwa, 2015).

2.7 STATISTICAL ANALYSIS

The sample size was calculated using the software Bioestat 5.0 (Mamimaurá, Brazil), based on the results found by Calero et. to 2000 (11).

The data of mechanical characteristic obtained from the three-point bending test and the obtained from estimated from computed calculation were analised by the software R CRAN. The parametric distribution were analyzed by Shapiro-Wilk test, and the difference between the groups was analized by Student t'test, with significance level of 5%.

3 RESULTS

3.1 THREE-POINT BENDING TEST

According to Figures 2 and 3, there was a noticeable difference in fracture, all the femur of the SHAM group showing a complete fracture (Figure 2), and the femurs from OVX group who presented an incomplete fracture like "green-stick" (Figure 3).

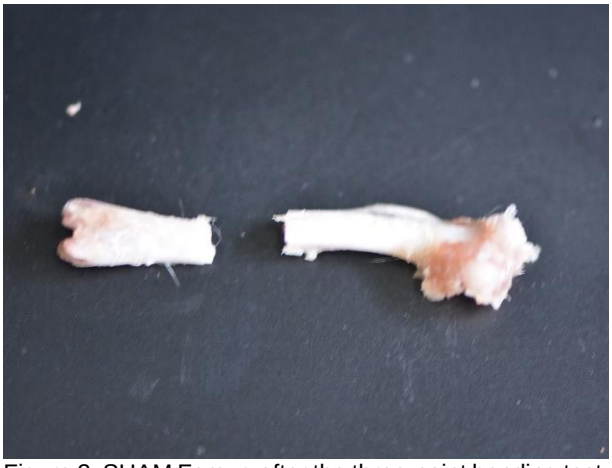

Figure 2. SHAM Femur, after the three-point bending test, showing a complete fracture.

Figura 3. OVX femur after the three-point bending test, showing an incomplete fracture .

Table 1 presents the results of the mechanical test. Each characteristic was compared between the groups by Student's t-test. The maximum flexural load data (figure 4) presented a significant difference greater for the SHAM group in relation to the OVX group ($p = 0.01370$). The maximum flexion extension (figure 5) did not present significant difference between the groups ($p = 0.52942$). The maximum flexion tension (figure 6) was also significantly higher for the SHAM group compared to the OVX group ($p = 0.01168$). The modulus of elasticity (figure 7) was also significantly higher for the SHAM group than for the OVX group ($p = 0.04378$).

Table 1. Result from three-point bending test at femur diaphysis.

	Maximum Flexural Load (N)	Maximum Flexural Extension (mm)	Maximum Flexure Strain (MPa)	Elasticity Modulus (GPa)
	110.5	0.885	104.58	4.29
	110.6	0.869	88.02	3.36
	97.0	0.691	101.57	4.24
SHAM	*106.02 ±7.826*	*0.82 ±0.108*	*98.06 ±8.821*	*3.96 ±0.523*
	78.6	0.921	58.05	1.74
	87.4	1.401	73.58	2.19
	77.3	0.635	70.39	3.25
OVX	*81.08 ±5.469*	*0.99 ±0.387*	*67.34 ±82.02*	*2.93 ±0.775*

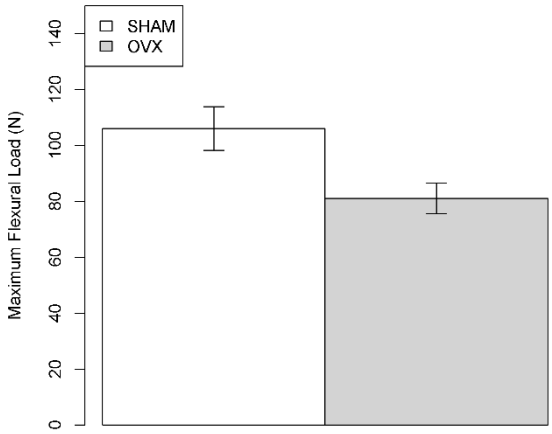

Figure 4. Maximum flexural load, in Newtons (N)

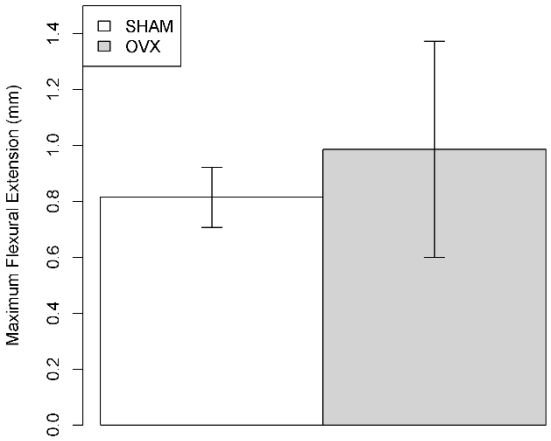

Figure 5. Maximum flexural extension at femur center, in millimeters (mm).

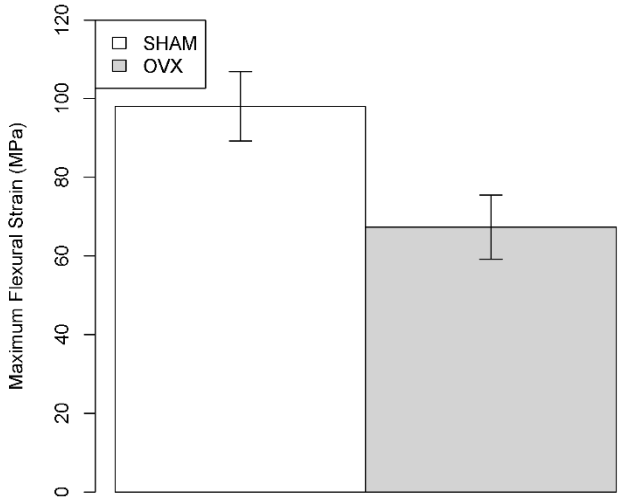

Figure 6. Maximum flexural strain, in Mega-Pascal (MPa).

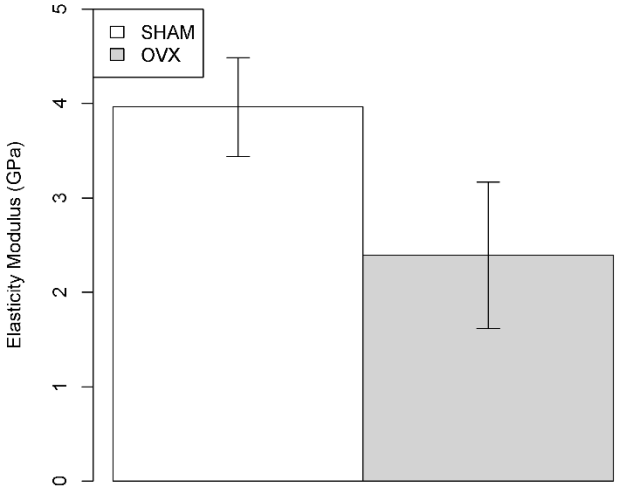

Figure 7. Elasticity modulus, in Giga-Pascal (GPa).

3.2 ESTIMATION FROM APPARENT DENSITY

The simulated tests in the MIMICS software for calculating the density-based modulus of elasticity showed close absolute values (table2), with no significant difference in apparent density (p = 0.2475256) (figure 8, 9, 10) or in the modulus of elasticity (p = 0.2456867) in relation to SHAM groups (figure 11) and OVX group (figure 12).

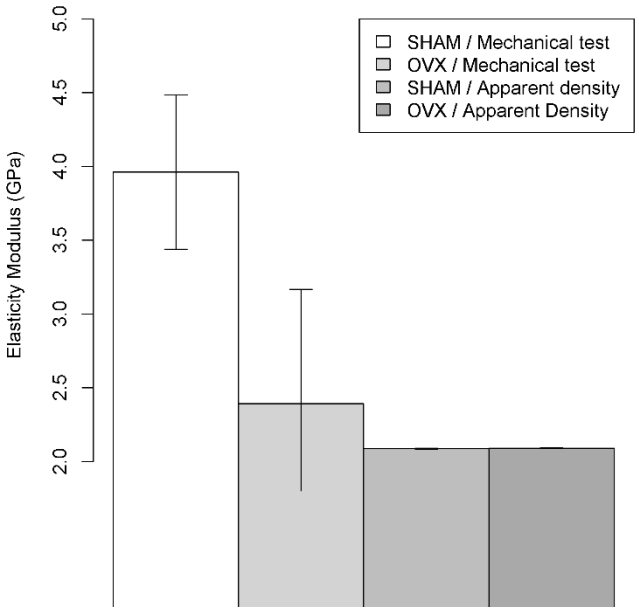

Figure 8. Comparative graph of the elastic modulus obtained by the mechanical test and computerized estimation.

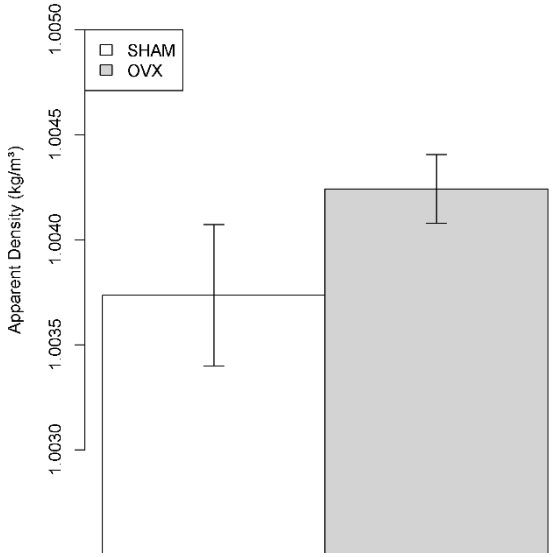

Figure 9. Apparent density calculated by reconstructed microgram image in gray scale.

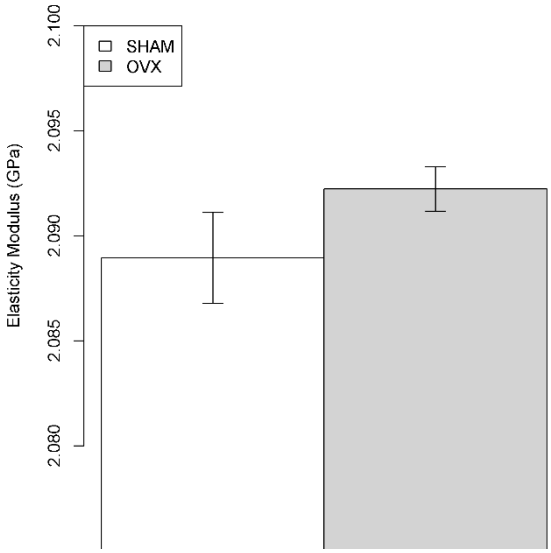

Figure 10. modulus of elasticity estimated by the apparent density formula.

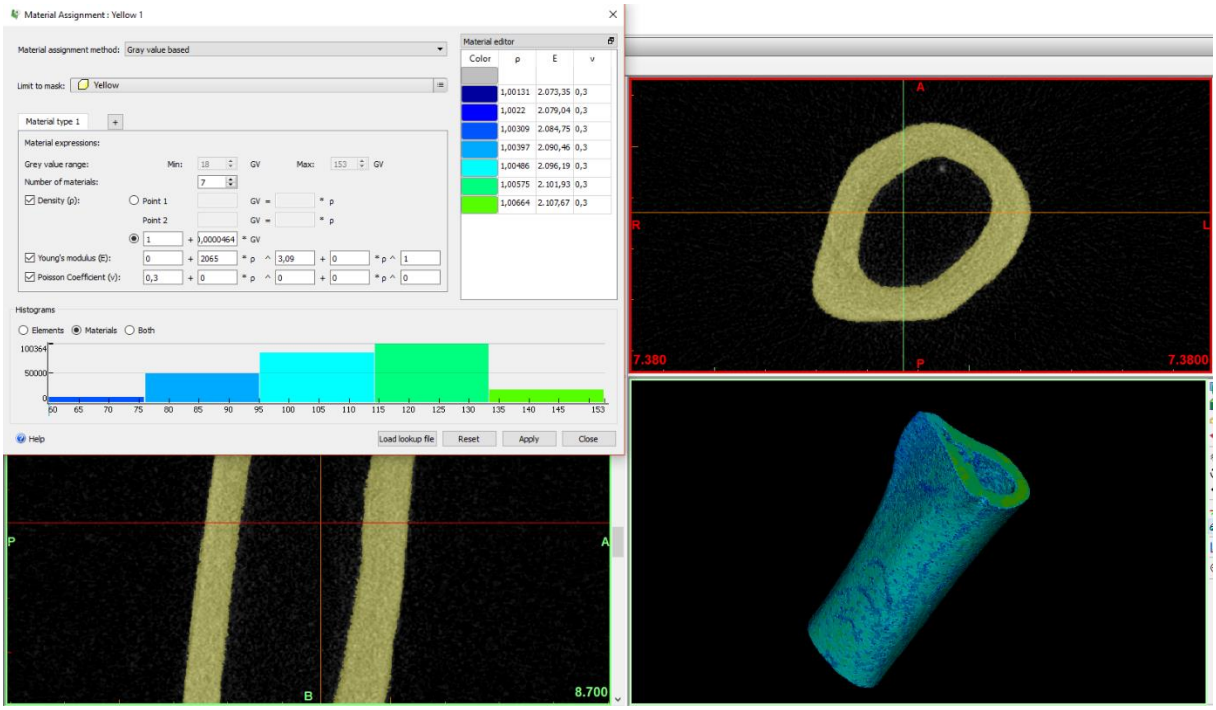

Figure 11. Calculation of the modulus of elasticity by the apparent density of the SHAM group in the MIMICS software..

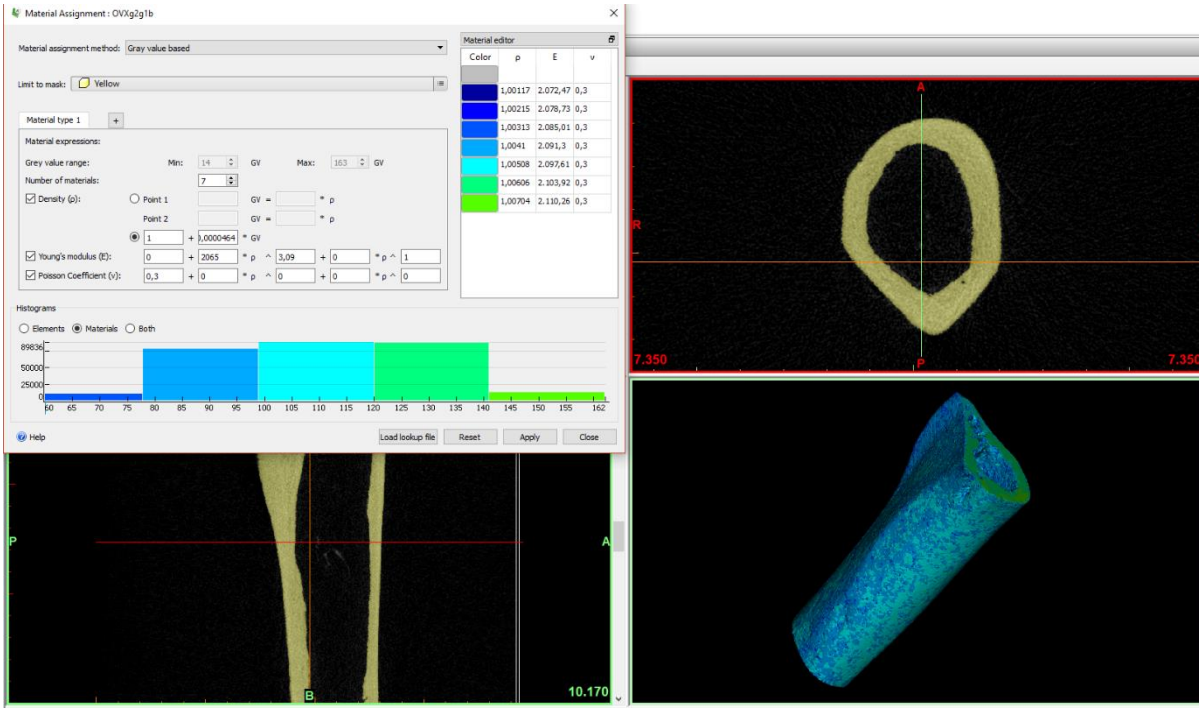

Figure 12. Calculation of the modulus of elasticity by the apparent density of the OVX group in the MIMICS software.

Table 2. Results from calculation of the elasticity modulus by apparent density.

	SHAM	OVX	p value
Apparent Density	1.00374 ± 0.0003	1.00424 ± 0.0002	0.2475256
Elasticity Moduli (GPa)	2.08895 ± 0.0022	2.09223 ± 0.0011	0.2456867

4 DISCUSSION

The stiffness of the bone tissues can be studied through the modulus of elasticity. This parameter is related to the mineral density of the bone tissue. Bone tissue with osteoporosis has a much lower bone mineral density than healthy tissue, which gives it a lower stiffness, and therefore a lower modulus of elasticity(17).

Associated with the low mineral density, the deterioration of the bone microarchitecture with osteoporosis is one of the main factors that increases the fragility of the bone(12). This evidence is noted by the fact that bone tissue with osteoporosis requires a smaller force to cause a fracture as can be observed in flexural tests on the diaphysis of long bones(18)

Although the flexion data at the center of the femur did not present a significant difference, a large variance was observed within the OVX group, and its mean is numerically higher in relation to the SHAM group. This can be explained by the fact that the disease does not act evenly on the deterioration of the bone architecture. As it has been observed that the modulus of elasticity is about 40% lower in bone tissue with osteoporosis, which consequently confers a lower flexural stiffness thus allowing a greater deformation of the femur.

The osteoporosis disease has an effect mainly on the epiphyses altering the microarchitecture of the bone marrow(2). Although the modulus of elasticity is dependent on the degree of mineralization of bone tissue(17) it is also dependent on bone porosity (19). Considering the anisotropy of the bone, these two conditions were not determinant in the calculation of the apparent density in the application of perpendicular forces in the diaphysis of the long bone.

The diaphysis because it consists largely of cortical bone does not suffer as much the degenerative effects of osteoporosis as it does in the epiphyses. When comparing the computerized assay with the mechanical test (figure 9), we noticed that the modulus of elasticity values obtained by calculating the apparent density did not have a significant difference between the SHAM and OVX groups.

The osteoporosis disease affects the diaphysis making the structure of the cortical bone thinner. Studies have shown that this lower thickness of the cortical bone with osteoporosis is one of the main factors that increases the risk of bone fracture(20,21).

We observed that the three-point bending test uses diaphragm diameter and shape data to calculate the force distribution and obtain the modulus of elasticity, that is, the module found corresponds to the location and force application. The computational method considers the apparent density distributed throughout the diaphysis, so the method of calculating the modulus of elasticity by apparent density should take into account the bone thickness.

5 CONCLUSION

The mechanical test showed a modulus of elasticity significantly lower for osteoporotic bone tissue, in the order of 2.93 GPa. This characteristic gave the bone a lower stiffness that allowed an incomplete fracture with a lower applied force. The thickness of the bone at the site of force application is a determining condition for bone stiffness, and consequently the calculation of the modulus of elasticity.

Thus, we conclude that the computational method was not able to identify the difference of the moduli of elasticity observed by the mechanical test.

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3 CONCLUSÃO

O teste mecânico demonstrou que o osso osteoporótico possui menos rigidez em relação ao tecido ósseo saudável. O módulo de elasticidade encontrado para a diáfise do fêmur com osteoporose foi de 2,93 GPa, significantemente menor (p=0,0032) enquanto a diáfise do fêmur do grupo saudável foi de 3,96 GPa.

Pelo cálculo da densidade aparente não foi possível verificar a diferença do módulo de elasticidade entre os dois grupos.

O teste mecânico apresentou de forma macroscópica que o padrão de fratura do tecido ósseo osteoporótico é incompleto em “galho-verde” necessitando uma força muito inferior que o tecido ósseo sadio, o qual possui fratura completa.

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* De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors - Vancouver Group. Abreviatura dos periódicos em conformidade com o PubMed.

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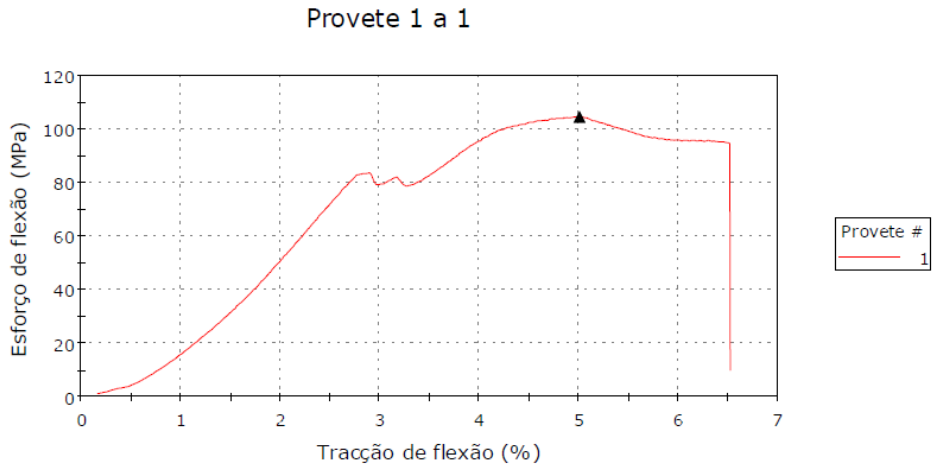
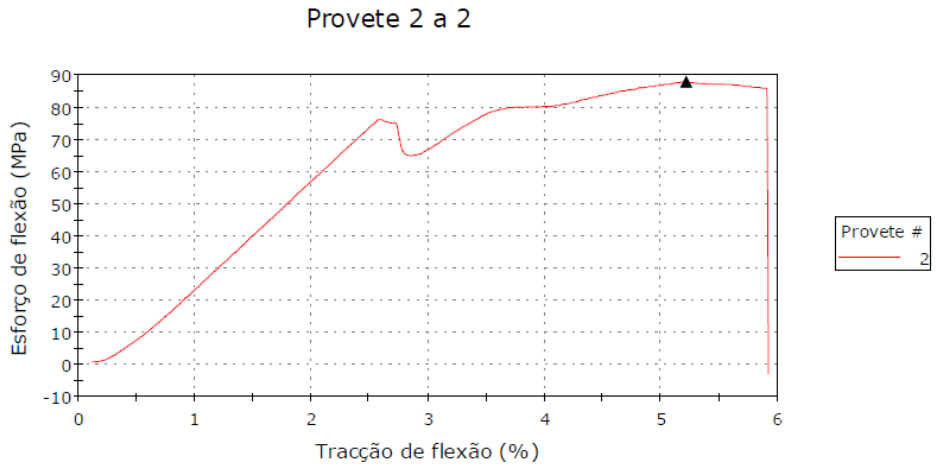
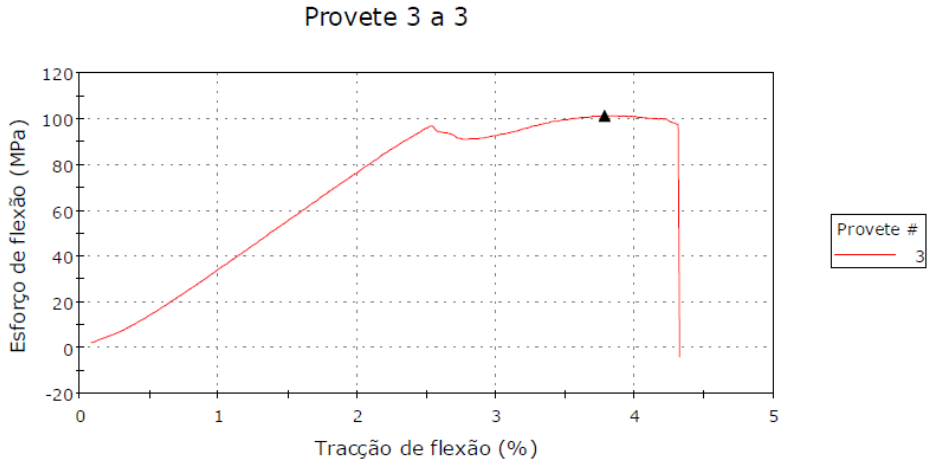
APÊNDICES

APÊNDICE 1 – TESTE DE FLEXÃO EM 3 PONTOS NA INSTRON – GRUPO SHAM





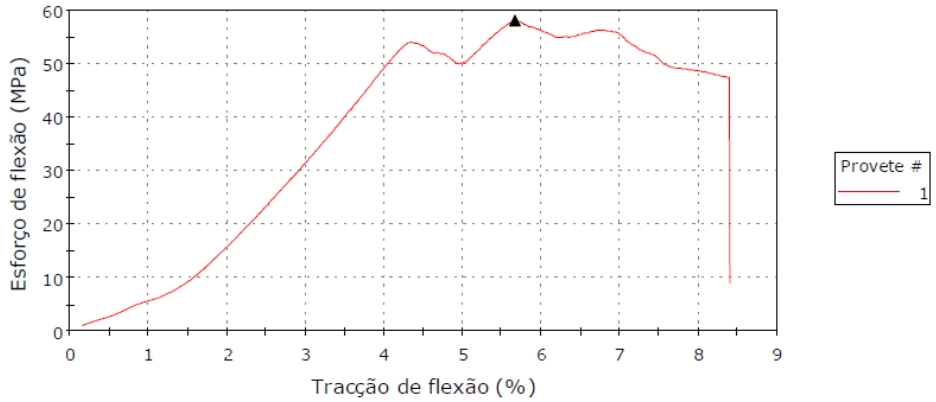


	Carga de FlexãoemMaximum Flexure stress (N)	Carga de FlexãoemMaximum Flexure load (kgf)	Extensão da flexãoemMaximum Flexure load (mm)
1	110,47	11,26	0,885
2	110,60	11,28	0,869
3	96,98	9,89	0,691
Média	106,02	10,81	0,815

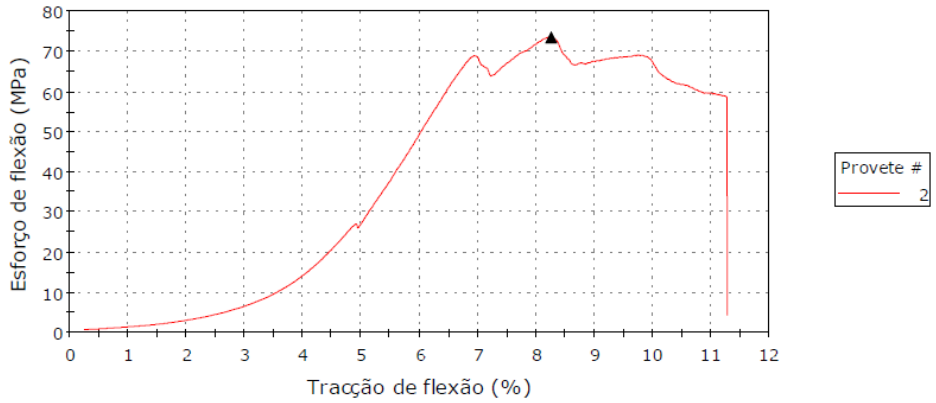
	Esforço de flexãoemMaximum Flexure stress (MPa)	Módulo (Automático) (GPa)
1	104,58	4,29
2	88,02	3,36
3	101,57	4,24
Média	98,06	3,96

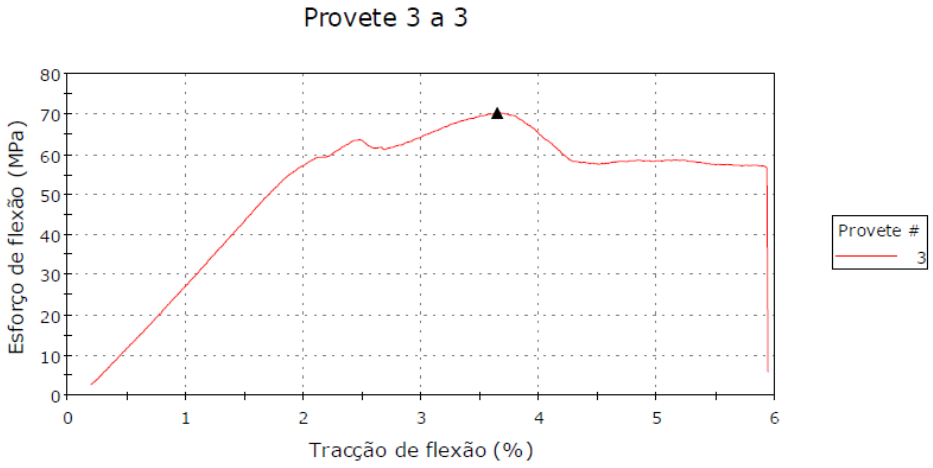
APÊNDICE 2 – TESTE DE FLEXÃO EM 3 PONTOS NA INSTRON – GRUPO OVX

Provete 1 a 1



Provete 2 a 2

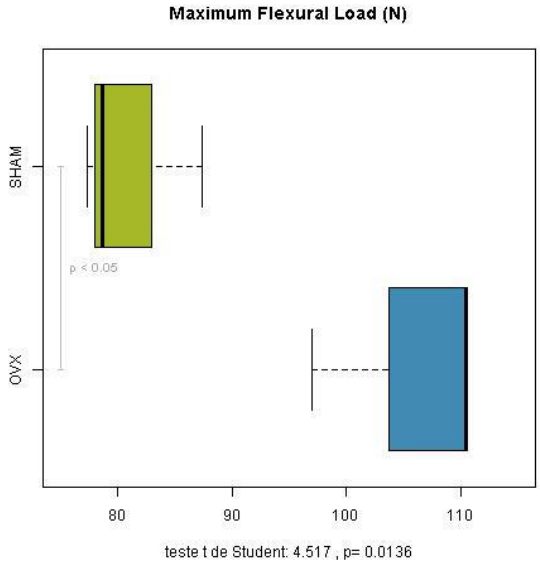




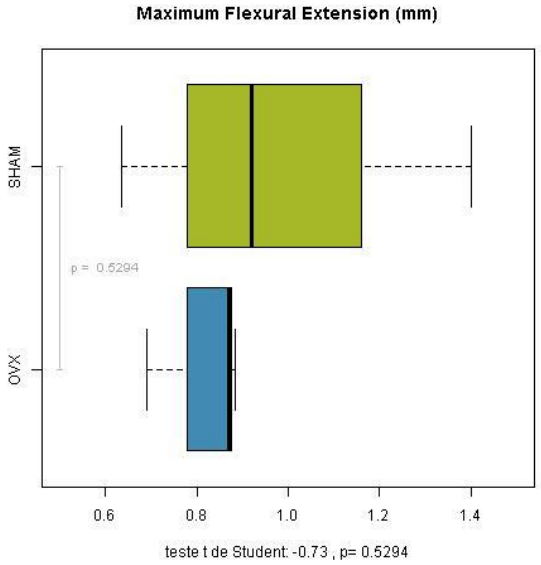
	Carga de FlexãoemMaximum Flexure stress (N)	Carga de FlexãoemMaximum Flexure load (kgf)	Extensão da flexãoemMaximum Flexure load (mm)
1	78,55	8,01	0,921
2	87,36	8,91	1,401
3	77,34	7,89	0,635
Média	81,08	8,27	0,986

	Esforço de flexãoemMaximum Flexure stress (MPa)	Módulo (Automático) (GPa)
1	58,05	1,74
2	73,58	2,19
3	70,39	3,25
Média	67,34	2,39

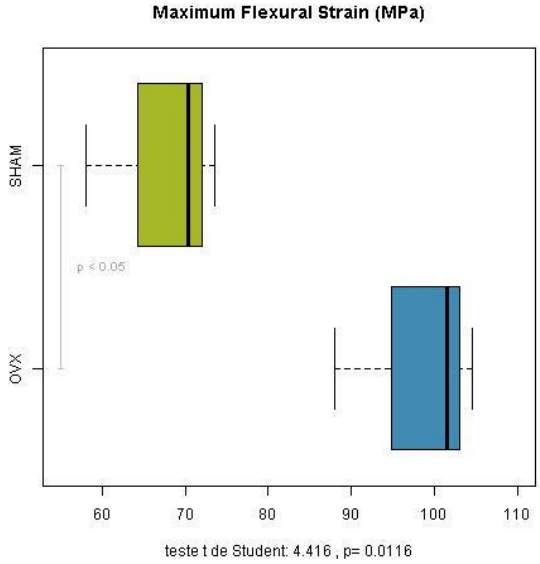
APÊNDICE 3 – GRÁFICOS DE DISTRIBUIÇÃO *BOXPLOT*



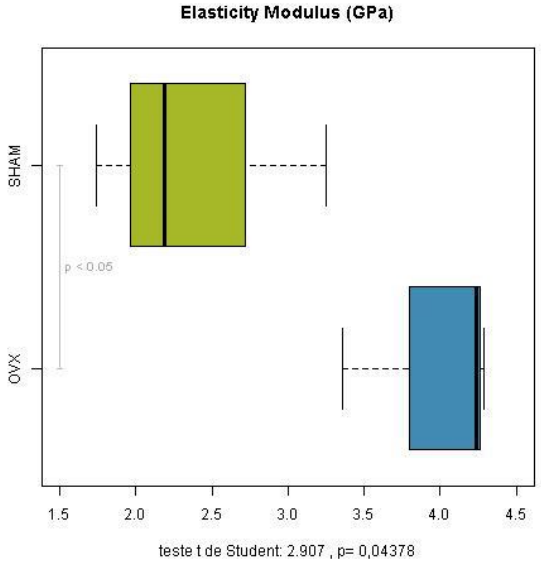
Força máxima de Carga (N)



Deflexão no centro do fêmur (mm)



Tensão de Flexão máxima (MPa)



Módulo de Elasticidade (GPa)

ANEXO 1 – CERTIFICADO DO COMITÊ DE ÉTICA EM PESQUISA EM ANIMAIS

UNICAMP

CEUA/UNICAMP

CERTIFICADO

Certificamos que a proposta intitulada **Caracterização das propriedades mecânicas do tecido ósseo do fêmur de ratos induzidos à condição de osteoporose**, registrada com o nº **4427-1**, sob a responsabilidade de **Profa. Dra. Ana Claudia Rossi e Rafael Araujo**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal** (**CONCEA**), **tendo sido** aprovada pela **Comissão de Ética no Uso de Animais** da **Universidade Estadual de Campinas - CEUA/UNICAMP,** em **19 de dezembro de 2016**.

Finalidade:	**( ) Ensino (X) Pesquisa Científica**
Vigência do projeto:	**01/02/2017-01/02/2018**
Vigência da autorização para manipulação animal:	**01/02/2017-01/02/2018**
Espécie / linhagem/ raça:	**Rato heterogênico / HanUnib: WH (Wistar)**
No. de animais:	**16**
Peso / Idade:	**06 meses / 400g**
Sexo:	**fêmeas**
Origem:	**CEMIB/UNICAMP**

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio** e é **restrita** a protocolos desenvolvidos em biotérios e laboratórios da Universidade Estadual de Campinas.

Campinas, 19 de dezembro de 2016.

Profa. Dra. Liana Maria Cardoso Verinaud
Presidente

Fátima Alonso
Secretária Executiva

IMPORTANTE: Pedimos atenção ao prazo para envio do relatório final de atividades referente a este protocolo: até 30 dias após o encerramento de sua vigência. O formulário encontra-se disponível na página da CEUA/UNICAMP, área do pesquisador responsável. A não apresentação de relatório no prazo estabelecido impedirá que novos protocolos sejam submetidos.

ANEXO 2 – COMPROVANTE DE SUBMISSÃO DO ARTIGO AO PERIÓDICO