Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Veterinary Medicine
Open Access

Establishing a Murine Osteoporosis Protocol for Biomaterials Testing

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RESEARCH ARTICLE

Abstract

Osteoporosis is one of the most common disorders of bone metabolism, this being a condition that causes pain, frequently causing fractures, as a result of the reduction of bone mass and density. The purpose of this study is the establishment and testing of an experimental protocol to induce osteoporosis in laboratory rats by ovariectomy, at 9 months old. It is desirable that osteoporosis is successfully induced so that various biomaterials for treating pathologic bone healing can be further tested. For this study, 20 Sprague-Dawley rats were split into 2 groups of 10 individuals, which were then operated, while 10 were left as control. Clinical examination was performed each week, but mainly 4 months after surgery, biochemistry and osteodensitometry were determined by CT - scanning to compare the differences between the ovariectomized group and the control group. The rats were humanly euthanasied at 20 weeks post ovariectomy and histological analyses were performed. Biochemistry revealed that progesterone level was decreased by ~50%, calcium values were decreased, alkaline phosphatase was increased and the Ca:P ratio was also altered. Nevertheless, the analysis of bone density (BMD) revealed a decrease in the ovariectomized group compared with the control group and the histological analysis confirmed the induction of osteoporosis pathology. In conclusion, osteoporosis by means of estrogenic deficit, was successfully induced in 9-month-old Sprague-Dawley rats.

Keywords: osteoporosis, ovariectomy, bone density, biochemistry, biomaterials.

Received: 15 September 2023
Accepted: 22 January 2024
Published: 15 May 2024

DOI:
10.15835/buasvmcn-vm:2023.0012

INTRODUCTION

Osteoporosis is a painful condition that affects the bone system, often causing fractures as a result of reduced bone mass and density. There is also the situation in which the bone can be mineralized normally, but the total bone mass is reduced and the clinical manifestations are not observable. In this case the term used is osteopenia. In osteopenia the bone loses its strength and hardness, therefore fractures appear much more easily. If this disorder progresses and the clinical manifestations are visible, it is most often a sign that osteoporosis is setting in (Marcus, 2017). Osteoporosis is characterized by decreased bone mass, the microarchitectonic being already damaged, leading to bone fragility, and increasing the risk of fractures. The World Health Organization defined osteoporosis based on bone mineral density (BMD) measurement obtained by DEXA (dual energy X-ray absorptiometry) - as a score greater than 2.5 standard deviations below normal individuals who are young and healthy at peak bone mass. A low BMD is associated with an increased risk of fracture. Low bone density may be secondary to failure to achieve optimal bone mass, bone loss

caused by increased bone resorption, and inadequate replacement of lost bone as a result of decreased bone formation (Genant et al., 1999; Cummings et al., 1993).

Osteoporosis is a classic age-related disease that affects women more than men. The hypothesis that osteoporosis is a consequence of estrogenic deficiency was proposed in 1941 by Albright and colleagues. The exact mechanism of steroid hormone deficiency in postmenopausal women and elderly men is still being unravelled. Estrogenic deficiency has both a direct and indirect impact on bone metabolism, all of which promote osteoclastogenesis. Osteoporosis is currently defined as a skeletal pathology characterized by compromised bone strength predisposing a person to an increased risk of fracture. Again, the literature is mentioning that bone strength is determined by bone density and the quality of bone tissue (Felsenfeld et al., 2011).

The objectives of this research study were represented by: the choice of the rat line; choosing a different age of rats for the induction of ovariectomy in comparison with the literature; selection of a suitable surgical method for ovariectomy and subsequent verification and evaluation of osteoporosis induced by ovariectomy. Literature references show that *Sprague Dawley* and *Wistar* rats are the most common lines used, moreover responding similarly to ovariectomy (Langdahl et al., 2016). Apparently, the age of 6 months is optimal for the ovariectomy intervention, the rats having the best osteoporotic response compared to those of 3 or 10 months (Francisco et al., 2011). The area of choice for the incision is dorso-lateral and the success of the protocol can be checked 1 - 3 weeks after the intervention. Following the cessation of regular estrous cycles, the decrease of estradiol, progesterone, as well as the volume of the uterus and the increase of LH and FSH levels can be determined. Current data show that the response of trabecular tissue from the proximal tibia, lumbar vertebrae and femur following ovariectomy is similar to that in humans (Yousefzadeh et al., 2020), making this rodent model optimal for testing novel strategies and therapies in veterinary and human medicine.

Osteoporosis in rats is verified by the determination of bone mineral density, a lower number of trabeculae, thinning but also greater intratrabecular separation, changes that are observed post ovariectomy firstly at the level of the proximal tibia, then lumbar vertebrae and femur (Wu et al., 2015; Yousefzadeh et al., 2020; Li et al., 1997).

The aim of the present study was to test an osteoporotic Sprague Dawley rat model for biomaterials testing. Our study consisted in creating and inducing an osteoporosis protocol by ovariectomy. It was desired to create a delayed bone healing environment to evaluate the therapeutic potential of a specific biomaterial designed by our research cluster. Their role and beneficial effects have been demonstrated in other previous studies, (Dreanca et al., 2021; Dreanca et al., 2023).

MATERIALS AND METHODS

This study has the potential to give accurate surgical details for the possibility of remaking completely the protocol, therefore following the ethics guidelines in laboratory animal research. Moreover, a few number of biomarkers were selected in order to see if these are sufficient for determining an early diagnosis and therefore confirming osteoporosis. Clinical pathology parameters consisting in hormonal and biochemical dosages and pathologic changes in the microstructure of the bones in the selected rats were evaluated by comparison between the ovariectomized group and the control one.

Experimental protocol

10 ovariectomized rats and 10 intact (control group) rats, aged 9-month-old, females of the *Sprague Dawley* line with an average weight of 290 g were selected. The animals used in this experiment were procured from an accredited institution. In the week prior to the experiment, the animals were left to acclimate to the new conditions. Their environment was stable in terms of temperature and humidity, with a 12-hour light/dark cycle. The rats received a standardized diet (Cantacuzino Institute, Bucharest, Romania) and fresh water ad libitum. The experimental protocol is in accordance with European and Romanian directives set in place, with the approval number 309/10.05.2022.

Anesthetic and surgery protocol

The preoperative preparation consisted in restraining the rats and performing intraperitoneal anaesthesia with a cocktail of Xylazine (XYLAZIN BIO 2%, Bioveta, Czech Republic; 20mg/ml) in a dose of 8mg/kg, Ketamine (Narkamon Bio 10%, Bioveta, Czech Republic; 100mg/ml) in a dose of 80mg/kg. Trimming of the elected area was carried out, being framed in a rectangle, the cranial limit being the last 3 ribs, caudal the sacral area, and latero-laterally the line that joins the hypochondrium area with the thigh. Antisepsis was performed with solution of chlorhexidine 2% (Lifo-Scrub, Bbraun, Germany; 4%), solution of betadine (Betadine, Egis Pharmaceuticals; 100mg/ml) and alcohol 70% (Saniblu, Romania; 70%). Ophthalmic gel (Xailin Gel, Visufarma, Carbomer 0.2% m/m, sodium perforates, electuary) was applied to the surface of the eyeballs to avoid corneal desiccation. Each

rat was positioned in sternoabdominal recumbency, the area of interest being isolated with sterile fields (Figure 1A).

Two dorso-lateral incisions involving the skin were made using a 15-gauge scalpel blade, midway between the last rib and the thigh, and 1-2 cm ventral to the spine, in the flank region. This was followed by incision of the musculature and peritoneum using Metzenbaum fine tissue dissecting scissors. After the peritoneal cavity was accessed, the ovary was highlighted, being surrounded by a variable mass of adipose tissue (Figure 1B). Two ligations were performed on the ovarian pedicle using a 4-0 monofilament polydioxanone thread (BioSintex, Ilfov, Romania), and one ligation on the uterine horn. Incisions were made above the ligatures and haemostasis was checked. The skin suture was made in simple interrupted pattern (Figure 1C).

Post-operatively, the rats were given a thick litter made of sawdust, and an infrared lamp. Antibiotic therapy with Amoxicillin + clavulanic acid (Synulox 175mg/ml, Zoetis, Romania) in a dose of 20mg/kg was administered subcutaneously (SC). Fluid therapy NaCl 9% solution + Glucose 5% solution (1ml:1ml) SC, Tramadol (Tramadol 50mg/ml, Krka, Romania) in a dose of 15mg/kg SC and Atipamezole (Antisedan, Orion Pharma, Vetequinol, Hungary & Finland) 0.1 ml/rat SC was implemented as postoperative treatment further conferring analgesia and intensive care.

10 animals from the ovariectomy group were humanly sacrificed post-surgery by prolonged narcosis. Bone tissue samples were harvested for histopathological evaluation.

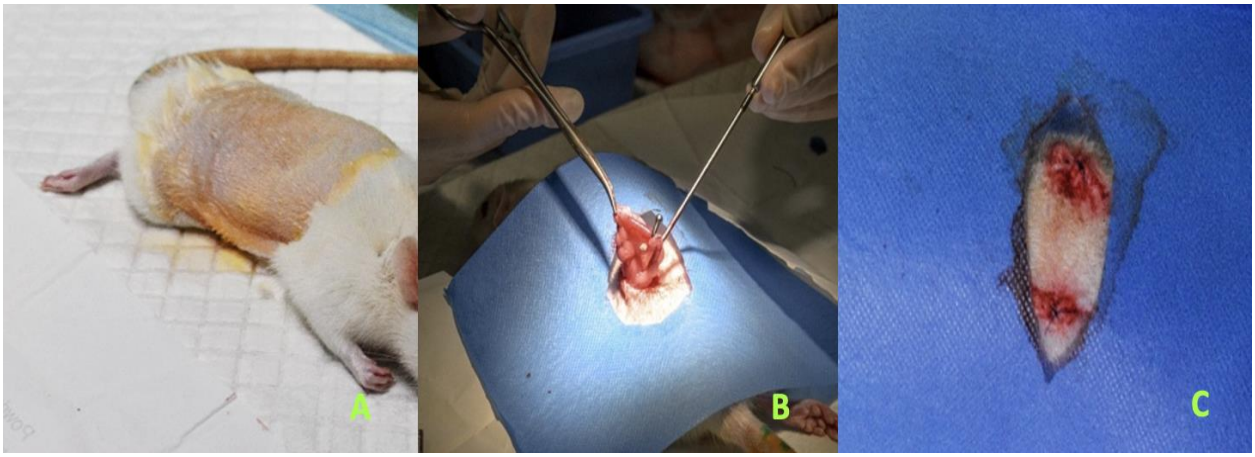


Figure 1. (A) Preoperative preparation, (B) Highlighting in the operative wound of the ovary and the adjacent uterine horn, (C) Two dorso-lateral incisions. Two sutures at simple interrupted pattern.

Confirmation of the induction of osteoporosis in rats

Biochemistry analyses

For the confirmation of the induction of osteoporosis, at 8 weeks after the ovariectomy, blood samples were taken, obtaining serum and plasma to measure progesterone (P4), alkaline phosphatase (ALP), calcium (Ca) and phosphorus (P). The samples were obtained from the orbital sinus level after the induction with Xylazine and Ketamine. The samples were collected in vacutainers with "clot activator" and vacutainers with heparin and centrifuged at 4000 rpm for 5 minutes to obtain serum for progesterone dosing and plasma for biochemical analyses. Biochemical analyses were performed by spectrophotometry using the Touch UV-VIS Screen analyzer. Progesterone was dosed from serum samples using the Healvet device, which is a quantitative immunofluorescence analyzer.

Imaging Computer Tomograph scanning

The medical imaging regarding the Computer Tomograph (CT) analysis was carried out with the help of the Siemens Somatom Scope device, 4 months after the ovariectomy. The anatomical portion of interest comprised the hindquarters of rats, with the bones of interest being the femur and tibia. At the time of the scan, the rats were alive under general anaesthesia according to the protocol mentioned above. The limbs were scanned axially with a thickness of 1 mm and the recording images were saved in DICOM (Digital Imaging and Communications in Medicine) format on the Siemens workstation in the PACS server. Bone density analysis to confirm the success of osteoporosis induction in the femur and tibia was carried out using the Syngo Somaris 5 CT VC 28 program.

For our study, bone densitometry is among the primary goals in determining the efficacy of ovariectomy to induce osteoporosis in rats. By performing this analysis, we were able to track the occurrence of osteoporosis per groups. In order to visualize and interpret the images obtained by the computer tomograph, the Biotronics 3D viewer program was also used, being an application used to display and process the images obtained by the computer tomograph.

Histopathological examination

For the histological examination, the femoral bones were collected, and the adjacent soft tissues were removed. Following a 48-hour fixation period in 10% neutral buffered formalin with one fresh solution change, the bones were decalcified for 24 hours with Thermo ScientificTM Richard-Allan ScientificTM Decalcifying Solution. Following a complete 24-hour wash in running tap water, the samples were cut into smaller pieces and subjected to standard histology procedure. In a nutshell, the samples were dehydrated using alcohols in varying concentrations (70, 95, and 100%), clarified in xylene, and embedded in high-melting-temperature paraffin wax. Hematoxylin and eosin (H&E) staining was done on the paraffin blocks after they were manually cut into slices that were 2-3 m thick using a Thermo ScientificTM HM 325 Rotary Microtome. The slides were evaluated using Olympus BX41 and Zeiss. Scope A1 microscopes. The photomicrographs were taken using both Olympus SP 350 digital camera with Stream Basic imaging software (Olympus Corporation, Japan) and AxioCam 208 Color digital camera connected to ZEN Core 3.0 software (Carl Zeiss Microscopy GmbH, Germany).

Statistical analysis

All data reported for clinical evaluation, biochemical profile parameters and bone density are as the mean ± SD. The values were analysed by two-way analysis of variance ANOVA, followed by the Bonferroni post-test. Statistical significance was at $P \leq 0.05$ in all cases. Statistical values were obtained using GraphPad Prism 8.0 software.

RESULTS AND DISCUSSIONS

Regarding the clinical evaluation, no significant statistical differences were identified in terms of the body mass of the rats from the ovariectomized groups compared to the control group, but during the experiment the rats from the experimental group gained approximately 80 grams in weight (Table 1).

The obtained results from the biochemical profile highlights the decrease of serum progesterone values by ~50% in ovariectomized animals compared to the non-operated control group (Table 2). Luckily, at an interval of 8 weeks after ovariectomy, biochemical analyses successfully showed the decrease in progesterone levels by more than 40%, which indicates, according to (Yousefzadeh et al., 2020), the occurrence of osteoporosis.

Table 1. Evaluation of the body mass

Groups	Day 0	Week 10	Week 15	Week 20
Control	302.5±23.76	352.4±39.77	344.4±34.52	380.6±32.95
Group 1	306.1±10.06	364.4±45.63	364.8±40.82	388.4±42.50

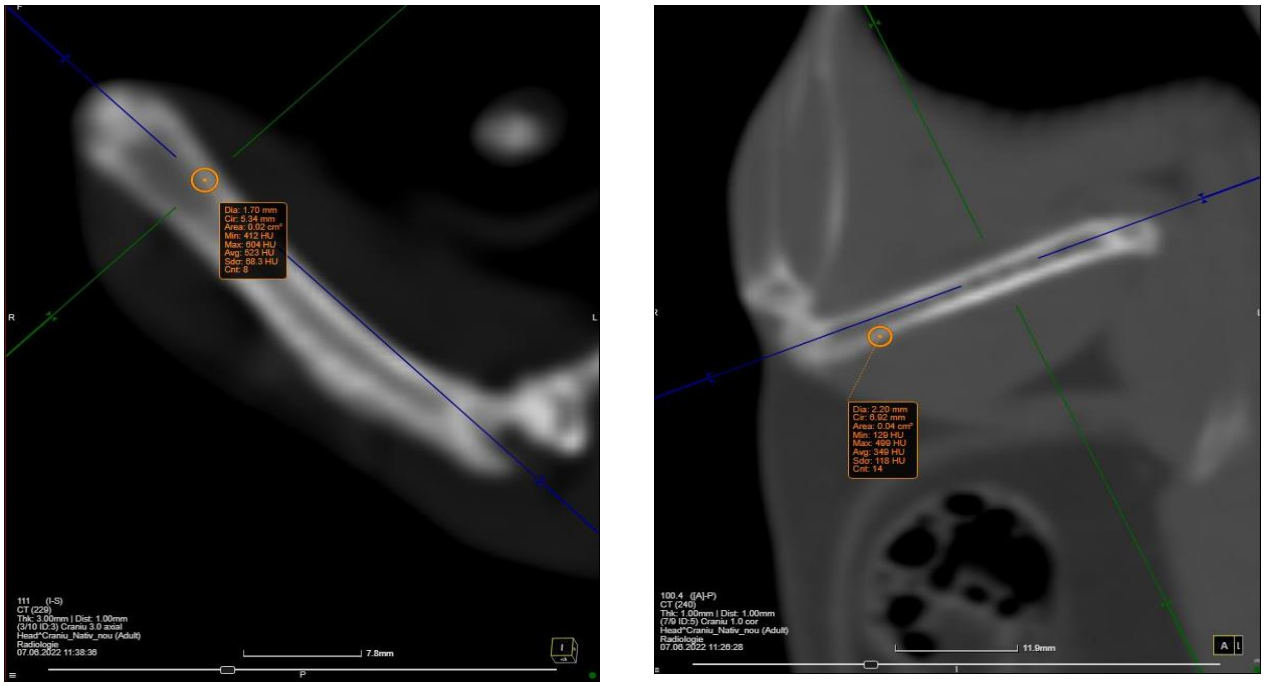
Serum calcium in most rats decreased from the physiological value of 9.7–11 mg/dl, which further induced increased secretion of parathyroid hormone (PTH) secreted by the parathyroid glands, a hormone that stimulates osteoclasts for bone resorption by release of calcium into the systemic circulation (Synevo, 2009, Yousefzadeh et al., 2020; Khan et al., 2023). Alkaline phosphatase values increased compared to the physiological value (26-147 U/L), ALP being most likely of bone origin being released from bone tissue through the process of bone necrosis and remodelling (Khan et al., 2023). The calcium-phosphorus ratio in the ovariectomized group compared to the control was 2:1, in favour of phosphorus whose physiological value is 5-10 mg/dl. Interestingly, its values increased as a compensatory mechanism for the decrease in serum calcium.

Table 2. Biochemical analysis of the experimental groups

Groups	Progesteron (ng/ml)	Ca (mg/dl)	ALP (U/L)	P (mg/dl)
Control	14.75±6.69	10.1±0.63	134.83±36.25	4.036±0.047
Group 1	7.44±1.12*	7.65±0.45***	244.03±13.85*	5.673±0.15**

Note: Ca- calcium, ALP-alkaline phosphatase, P-phosphorus, *- p≤ 0.05, **- p≤0.01, ***- p≤0.001

After obtaining the CT images, the reconstruction of the CTs was done with the help of the Biotronics 3D program, whose area of interest was the posterior train. The femur was identified, at it's level the marker was positioned on 5 zones of the selected area (Figure 2A, Figure 2B) in the bone tissue (trabecular and cortical) to obtain the average of these measurements. Bone density was measured in the control and ovariectomized groups to compare them (Table 3).



(a) **(b)**

Figure 2. (a) Image from the Biotronics 3D program with the control group; (b) Image from the Biotronics 3D program with the ovariectomized group.

Table 3. Bone density statistics (BMD)

Rats	Control group	OVX groups
4 months post-ovariectomy	482.8±62.17 HU	383.8±50.04* HU

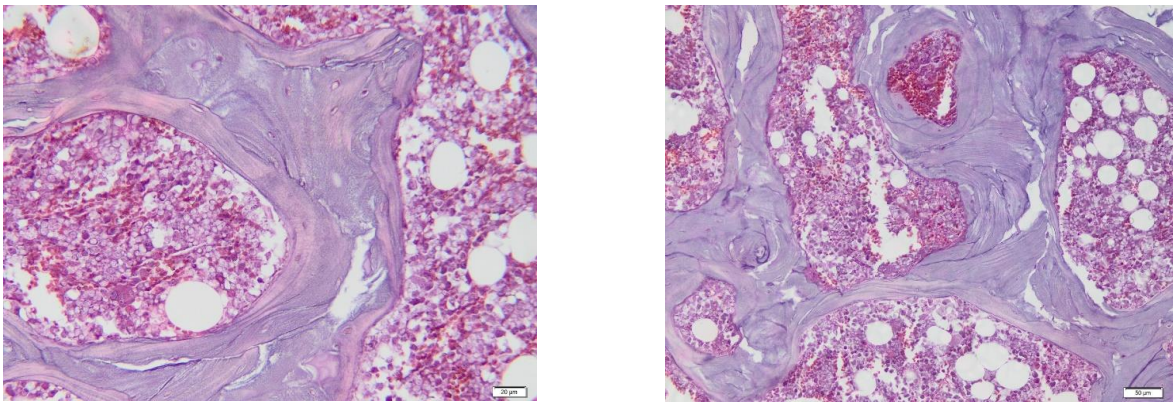
Note: Control – non-ovariectomized group, OVX- ovariectomized group; * P≤ 0.05, HU- Hounsfield units

Following the interpretation of the obtained results, a decrease in bone density is observed in the ovariectomized group compared to the non-operated control group, which demonstrates the occurrence of osteoporosis pathology.

In addition, during the necropsy, the accumulation of adipose tissue was observed in the abdominal cavity, especially at the retroperitoneal level. Also, the histopathological examination showed the appearance of multiple cementation lines or spaces, suggesting a reactive response of the bone to metabolic stressors. At the same time, one can also observe the presentation spaces in the trabeculae denoting osteolysis and the decrease in trabecular thickness (Figure 3, a and b). The results are consistent with Torok-Oance et al. (2014).

In our view osteoporosis was successfully induced, even though the rats were 9 months old. At an interval of 8 weeks after ovariectomy, biochemical analyses successfully diagnosed the decrease in progesterone levels by more than 50%, which indicates, according to (Yousefzadeh et al., 2020), the occurrence of osteoporosis. Moreover, calcium values decreased, the results being consistent with the literature that says that calcium decreases when osteoporosis is induced, due to the estrogen deficiency that stimulates the excretion of calcium at the renal level and the decrease in its absorption at the intestinal level (Heaney et al., 1978; Gennari et al., 1990). Bone-type alkaline phosphatase increases in pathologies such as hyperparathyroidism, bone metastases, bone tumors, osteomalacia, rickets or the evolution of multiple fractures and, last but not least, osteoporosis (Synevo, 2009). Together with calcium ions, phosphate ions give rise to calcium phosphate which finally converts to hydroxyapatite, with an important role in bone remodelling. Our results also showed an increase in alkaline

phosphatase, being consistent with the literature. Phosphorus values increased as a compensatory mechanism for decreased calcium. We also recorded results in relation to body mass change. The rats gained weight by accumulating abundant stores of adipose tissue due to the estrogenic deficiency resulting from ovariectomy (Meli et al., 2004).



(a) **(b)**

Figure 3. Microscopic image of femur with osteoporosis. In the image you can see both the bone marrow with hematogenous cells (a) and a section through the femur (b), bone trabeculae characterized by the presence of cementation lines and spaces that are considered strictly pathological and specific to osteoporosis. Hematoxylin-Eosin stainin

At 4 months after ovariectomy, the bone densimetry analysis clearly revealed that the femoral bone density in the ovariectomized rats decreased compared to those in the control group, thus demonstrating that the experiment was successful and we obtained the desired results, which are similar to the results from the article written by (Hejazi et al., 2021), where the authors followed a protocol similar to ours, but used Wistar rats to induce osteoporosis in order to test biomaterials. They also observed decreased bone density in the ovariectomized group compared to the control group following CT analysis, but at a 3-month post-operative interval. At the same time, the histopathological analysis succeeds in conferring once again the induction of osteoporosis in the animals used in the experiment. (Xu et al., 2019; Shaheen et al., 2021).

It would be important to be able to easily reproduce the protocol described above, because we would create an environment in which delayed bone healing takes place, so we can test various biomaterials that would improve the results and treat various types of fractures produced under such conditions. It is important to study delayed bone regeneration, which is a common problem among both humans and animals, but at the same time it is necessary to respect the ethics of laboratory animals when breeding this essential condition. The protocol described above is reported in detail, because it was applied and confirmed to rats of the Sprague-Dawaley breed aged 9 months. The same protocol was described and reproduced in other conditions at the age of 6 months, aspect from which it shows the originality of this study demonstrating the opposite, it can be reproduced at another age than that described in other studies (Yousefzadeh, et al., 2020). The limitations of this study are represented by the lack of micro-CT analysis, which is performed post-mortem to confirm the onset of osteoporosis in the bone, aspect considered for a future study where we will induce osteoporosis according to the protocol described above and we will test the application of biomaterials, thus avoiding the use of a large number of laboratory animals. The choice of age was based on the impossibility of acquiring rats at 6 months of age, and reproducing them would have meant too much cost for the experiment to raise and maintain them during this time. The limitations described above are rendered by biobases in Romania. The limitations of the study regarding the murine species, especially rats, are of an anatomical order, respectively rats do not show Haversian modeling and remodeling at the level of cortical bone, which in humans causes extreme porosity. Osteoporosis promotes decreased porosity, leading more obviously and over time at bone franctures . To note, an important aspect in rats, bone loss in osteoporosis occurs especially at the level of the endosteum, where there is trabecular bone, from where bone remodeling begins, forming spaces and decreasing porosity. However, the international literature accepts ovariectomy as the main method of inducing a pathological condition similar to menopause, which through estrogen deficiency, bone loss through metabolic and molecular mechanisms is induced and, thus the pathology is identical to the onset of osteoporosis during menopause. At the same time, there are numerous other models of inducing osteoporosis in rats (low calcium diet, parathyroidectomy, chemical pharmaceutical induction, immobilization) according to Lelovas et al. (2008) which are considered very difficult and expensive.

The protocole performed by us being much more well tolerated by the scientific community, although there is that anatomical limitation. (Turner et al., 2001). The degree of osteopenia determined by ovariectomy in rats

implies the same pathological behavior of the bone as that found in humans.

CONCLUSIONS

Osteoporosis was successfully induced by ovariectomy in 9-month-old Sprague-Dawley rats. Progesterone is a reliable biomarker for establishing cessation of the menstrual cycle in female rats and induction of osteoporosis. Densitometric analysis and histopathological examination are an aid in confirming the success of the experimental osteoporosis induction protocol in the rat. This experimental protocol can be used for testing different treatments and therapeutic protocols, being similar in a major proportion to post-menopausal osteoporosis in humans, representing an ideal translational model. This work is of importance for the animal laboratory welfare community in Romania, adding to the improvement of animal testing in experimental research.

Author Contributions:

A.N.A. Wrote the paper, collected the data, performed the surgery; A.D. Conceived and designed the analysis, collected the data, contributed data or analysis tools; T.M. collected the data, contributed data or analysis tools; S.G.B. Performed the surgery; C.D. Performed the surgery; S.M. Performed the analysis, Contributed data or analysis tools; R.A.P. Performed the analysis, Contributed data or analysis tools; A.I. Performed the analysis, Contributed data or analysis tools; L.S.J. Collected the data; C.T. collected the data, contributed data or analysis tools; C.P. Preformed the anaesthesia. K.M. Wrote the paper, conceived and designed the analysis; L.O. Conceived and designed the analysis;

Funding Source: This work was supported by a Grant of the Ministry of Research and Innovation, CNCS-UEFISCDI, Project No. PN-III-P1-1.1-TE-2019-1138, within PNCDI III.

Acknowledgments

This research was support of the Research Grant of the University of Agricultural Science and Veterinary Medicine, Cluj-Napoca, No 21864/04.10.2021.

Conflicts of Interest

The authors declare that they do not have any conflict of interest.

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