REFERÊNCIAS, MÉTODOS E TECNOLOGIAS ATUAIS NA MEDICINA VETERINÁRIA 2

Alécio Matos Pereira
Cledson Gomes de Sá
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(Organizadores)

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Dados Internacionais de Catalogação na Publicação (CIP)

R332 Referências, métodos e tecnologias atuais na medicina veterinária 2 / Organizadores Alécio Matos Pereira, Danrley Martins Bandeira, Cledson Gomes de Sá. – Ponta Grossa - PR: Atena, 2021.

Formato: PDF
Requisitos de sistema: Adobe Acrobat Reader
Modo de acesso: World Wide Web
Inclui bibliografia
ISBN 978-65-5983-379-5
DOI: https://doi.org/10.22533/at.ed.795212008

1. Medicina veterinária. I. Pereira, Alécio Matos (Organizador). II. Bandeira, Danrley Martins (Organizador). III. Sá, Cledson Gomes de (Organizador). IV. Título.
CDD 636

Elaborado por Bibliotecária Janaina Ramos – CRB-8/9166

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APRESENTAÇÃO

Novas tecnologias estão sendo inseridas todos os dias nas diversas profissões, e na medicina veterinária não é diferente, estudantes e profissionais já experientes estão tento que se adequar aos novos tempos, onde a pesquisa realizada pelas universidades e outros centros de pesquisa voltado para medicina veterinária, desenvolve novas técnicas de abordagem aos problemas que sempre existiram, técnicas essas que visam melhorar o tratamento de enfermidades com métodos menos invasivos e mais eficazes no prognósticos dos pacientes.

No entanto o domínio de novas técnicas requer mais especialização dos médicos veterinários, um bom exemplo é a acupuntura que vem garantindo cada vez mais espaço dentro da Medicina veterinária, voltada principalmente para o tratamento de traumas musculares, com o objetivo de minimizar as dores e o sofrimento do animal até sua total recuperação.

Nesse contexto é mais fácil observar a importância do emprego de novas técnicas de abordagem na área clínica, esse capitulo trás dezesseis trabalhos abordando o emprego e a pesquisa de novas técnicas de tratamento das mais diversas patologias na qual os animais são acometidos, fazendo com que profissionais já estabelecidos no mercado de trabalho busquem atualizações e fazendo com que novos médicos saiam da academia cada vez mais especializados.

Alécio Matos Pereira
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SUMÁRIO

CAPÍTULO 2

ASSESSMENT OF BONE TURNOVER MARKERS VARIATIONS ALONG INDUCTION OF OSTEOPOROSIS IN THE GLUCOCORTICOID TREATED OVARIECTOMIZED SHEEP MODEL

Data de aceite: 02/08/2021

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Parte deste estudo foi publicado previamente na revista Anais da Academia Brasileira de Ciências: Coelho C. A., Bordelo J. P., Camassa J. A., Barros V. A., Babo P. S., Gomes M. E., Reis R. L., de Azevedo J. T., Requicha J. P., Faísca P., Carvalho P. P., Viegas C. A., Dias I. R. Evaluation of hematology, general serum biochemistry, bone turnover markers and bone marrow cytology in a glucocorticoid treated ovariectomized sheep model for osteoporosis research. **Anais da Academia Brasileira de Ciências**, v. 92, n. 4, p. 1-16, e20200435, 2020.

ABSTRACT: Osteoporosis is a skeletal metabolic disorder characterized by a loss of bone mass and architectural microstructure in which bone strength is compromised, increasing the risk of fragility fractures. This study evaluated the variation of serum bone turnover markers (BTMs), selected minerals and 17β-estradiol levels (E_2) before and every 4 weeks until the 24th week after ovariectomy (OVX) and glucocorticoid (GC) administration in sheep model. Six animals have gone OVX and weekly dexamethasone injections along 24 weeks and compared with a sham control group. Osteopenia was confirmed by microcomputed tomography and bone histomorphometry at L4 vertebra. BTMs – total and bone alkaline phosphatases (BALP) activities and intact osteocalcin (OC) concentration showed lower values over time in the GC-treated OVX sheep than in the sham control group, except for intact OC concentration at the 16th post-operative week. Resorption marker C-terminal telopeptides of collagen type I (CTX) concentration always showed higher values in the GC-treated OVX sheep group than the sham control one. Intact OC and CTX values presented statistical difference between both groups ($P<0.001$ and $P<0.01$, respectively). In normal physiologic conditions, the correlations between BALP with Ca (R=0.25, P=0.049) and P (R=-0.5196, P=0.0007), support the involvement of this BTM with the mineralization process. Also, the correlations between BALP with OC (R=0.4184, P=0.0077), BALP with tartrate-resistant acid phosphatase (TRAP) (R=-0.5083, P=0.001) and OC with TRAP (R=-0.3237, P=0.0444) help to confirm their opposite function. From the hemodynamic point of view, these results confirm the reliability of these serum BTMs evaluation in sheep for orthopedic research.
KEYWORDS: Bone histomorphometry; serum bone turnover markers; lumbar vertebral micro-structure; osteoporosis; sheep

RESUMO: A osteoporose é um distúrbio metabólico esquelético caracterizado por perda de massa óssea e microestrutura arquitetônica em que a resistência óssea fica comprometida,

aumentando o risco de fraturas por fragilidade. Este estudo avaliou a variação dos marcadores séricos de remodelação óssea (BTMs), minerais selecionados e níveis de 17β-estradiol (E2) antes e a cada 4 semanas até a 24ª semana após a realização de ovariectomia (OVX) e administração de glicocorticóides (GC) em modelo de ovelha. Seis animais foram submetidos a OVX e a injeções semanais de dexametasona ao longo de 24 semanas e comparados com um grupo de controle simulado. A osteopenia foi confirmada por microtomografia computaorizada e histomorfometria óssea na vértebra L4. Os BTMs - atividades de fosfatases alcalinas total e óssea (BALP) e concentração de osteocalcina (OC) intacta apresentaram valores mais baixos ao longo do tempo nas ovelhas OVX e tratadas com GCs do que no grupo controle, exceto para concentração de OC intacta na 16ª semana pós-operatória. A concentração do marcador de reabsorção - telopeptídeos C-terminal docolágeno tipo I (CTX), apresentou sempre valores mais elevados no grupo de ovelhas OVX e tratadas com GCs do que no grupo controle. Os valores da OC intacta e CTX apresentaram diferença estatística entre os dois grupos ($P<0,001$ e $P<0,01$, respectivamente). Em condições fisiológicas normais, as correlações entre a BALP com Ca (R = 0,25, P = 0,049) e P (R = -0,5196, P = 0,0007), apoiam o envolvimento deste BTM com o processo de mineralização. Além disso, as correlações entre a BALP com OC (R = 0,4184, P = 0,0077), a BALP com fosfatase ácida resistente ao tartarato (TRAP) (R = -0,5083, P = 0,001) e da OC com TRAP (R = -0,3237, P = 0,0444) ajudam a confirmar sua função oposta. Do ponto de vista hemodinâmico, esses resultados confirmam a confiabilidade da avaliação desses BTMs séricos em ovinos para pesquisa ortopédica.
PALAVRAS-CHAVE: Histomorfometria óssea, marcadores do metabolismo ósseo, vértebras lombares, microestrutura, osteoporose, ovelha.

INTRODUCTION

In humans osteoporosis is a skeletal disorder characterized by a loss of bone mass and structure in which bone strength is compromised, thus increasing the risk of fragility fractures. Human osteoporosis is divided in type I, or post-menopausal osteoporosis, mainly characterized by a loss of trabecular bone, increasing fractures of the vertebrae and typically affects women after menopause due to lack of endogenous oestrogens (RIGGS & MELTON 3rd, 1986; RIGGS et al., 2003). Type II, or senile osteoporosis, causes loss of cortical and trabecular bone in both men and women, as the disadvantageous result of ageing and it is characterized by hip, proximal humerus, proximal tibia and pelvis fractures (RIGGS & MELTON 3rd, 1986).

In healthy skeleton, constant bone remodelling occurs in which mature bone tissue is removed through a bone resorption process, and new tissue is formed in order to maintain bone strength and mineral homeostasis in continuum with a strict coordination between their phases (ANDERSEN et al., 2009). The bone remodelling cycle has three phases: the initiation, the transition (reversal) and the termination of bone formation as thoroughly described in literature (MATSUO & IRIE, 2008; ANDERSEN et al., 2009; BONEWALD 2011). The process of bone remodelling involves the osteoprotegerin (OPG)/receptor

activator of nuclear factor NF-κB ligand (RANKL)/its membrane-bound receptor (RANK) system on osteoblasts and osteoclasts with OPG and RANKL constituting a ligand-receptor system that directly regulates osteoclast differentiation, and OPG acting as an inhibitor of osteoclastogenesis by competing with RANKL for the membrane receptor (MEIKLE, 2006; TEITELBAUM, 2007; LIU et al., 2015; IKEDA & TAKESHITA, 2016; KAPASA et al., 2017). The abnormality in the bone remodeling process, which conducts to osteoporosis, depends on the miss-coordination of multiple communication pathways between the osteoblast and the osteoclast lineages (FENG & MCDONALD, 2011). These cells activities are controlled by a variety of cytokines and hormones, in particular the lack of oestrogen induces an unbalanced remodelling, increasing bone loss and risk of osteoporosis (LERNER, 2006).

Ewes have been among widely used large animals for preclinical testing of new anti-osteoporotic pharmacologic strategies, research in biomaterials and in the development of orthopedic implants and prostheses for fixation of fragility fractures and joints replacement in the osteopenic/osteoporotic skeleton. The sheep subjected to bilateral ovariectomy (OVX) with 12 months postoperatively (or more), or by the combined treatment of OVX sheep associated to glucocorticoid (GC) applications for 6 months and, in some protocols, a calcium/vitamin-D deficient diet, have been used frequently as an animal model for this purpose (DIAS et al., 2018). The GCs cause bone and muscle loss and weakness (KLEIN, 2015). ZHANG et al. (2016) describes in detail the underlying mechanism of osteoporosis induced by GCs in OVX animals. ANDREASEN et al. (2015) concluded that GC-treated OVX aged sheep induced a significant bone loss, promoted by an arrest of the reversal bone remodeling phase, resulting in an uncoupling of bone formation and resorption, as demonstrated in postmenopausal women with GC-induced osteoporosis (ANDERSEN et al., 2013; JENSEN et al., 2011). Another very recent study elucidates the osteocyte regulation of OPG/RANKL system in the sheep model of osteoporosis, concluding that in the late progressive phase of the osteoporosis induced by steroids, the RANKL expression is stimulated in osteocytes (EL KHASSAWNA et al., 2017).

In small ruminants, clinical conditions of osteoporosis are also described as being in goats associated with phosphorus and calcium deficiency (BRAUN et al., 2009, ROSA et al., 2013a, 2013b; BARBOSA et al., 2018), osteopenia in goats by copper deficiency (BUCK et al., 2012) and natural occurring osteopenia in the old sheep (MAENZ et al., 2020). MAENZ et al. (2020) even state that old sheep may represent a suitable model of senile osteopenia, since it presents a markedly diminished bone structure and formation, and substantially augmented bone erosion, which could be representative of the human condition. Also, osteoporosis is enumerated as one of the most common metabolic bone diseases in grazing sheep by THOMPSON et al. (2008) due to imbalances or deficiencies in nutrients such as vitamin D, calcium and phosphorus, also reported as associated to 1,25-dihydroxyvitamin D-3 deficiency in sheep by WILKENS et al. (2010).

This study intends to evaluate the variation pattern of an analytical panel composed

by serum bone turnover markers (BTMs) all along the bone loss induction process in the GC-treated OVX sheep model. Conventionally, the BTMs are divided into bone formation and bone resorption markers. The markers of bone formation are synthetized by the osteoblasts, during the different phases of their development and differentiation, reflecting different cell functions and the bone formation process. They include the osteoblast enzymes total alkaline phosphatase (ALP) and its bone isoform (BALP), the matrix protein osteocalcin (OC) and the by-products of collagen synthesis – C-terminal and N-terminal (PINP) propeptides of type 1 collagen (LEEMING et al., 2006; CREMERS et al., 2008; SOUSA et al., 2015; SHETTY et al., 2016). The markers of bone resorption are synthetized during the bone resorption process of bone remodelling and these include the products of collagen breakdown – C-terminal (CTX) and N-terminal telopeptides of collagen type I, CTX-matrix metalloproteinase, hydroxyproline and the collagen cross-links pyridinoline and deoxypyridinoline and also the non-collagenous protein bone sialoprotein and the enzymes secreted by the osteoclasts – tartrate-resistant acid phosphatase (TRAP) 5b isoform and cathepsin K (LEEMING et al., 2006; CREMERS et al., 2008; SOUSA et al., 2015; SHETTY et al., 2016). Which concern the novel osteocyte activity markers – RANKL and OPG, these ones reflect bone microenvironment, and more recently the Dickkopf-related protein 1 and sclerostin (LEEMING et al., 2006; CREMERS et al., 2008; SOUSA et al., 2015; SHETTY et al., 2016). In this way the BTMs have been evaluated as potential tool for predicting impaired quality of bone and monitoring treatment of osteoporosis, such as undercarboxylated OC (VERGNAUD et al., 1997), PINP and CTX (VASIKARAN et al., 2011). The OC has also been found as a useful biomarker in steroid-induced osteoporosis (CLEMENS & KARSENTY, 2001).

So, the present study aimed to contribute for the evaluation of bone loss during the first 24 weeks after GC-treated OVX sheep model induction through the interpretation of the pathophysiological significance of the serum variation pattern of a complete panel of serum BTMs – ALP, BALP and intact OC, CTX and TRAP, with the originality of assessing their correlations with the serum minerals – total calcium (Ca), phosphorus (P) and magnesium (Mg), and the hormone 17β-estradiol (E_2). Additionally, an evaluation of micro-architectural characteristics and bone mineral density (BMD) of L4 vertebra, that were acquired by micro-computed tomography (μ-CT) and bone histomorphometry, was performed to prove the effects of OVX and GC administration on bone tissue.

MATERIAL AND METHODS

Animals, housing and anesthetic and surgical protocols

Twelve ewes (Portuguese Serra-da-Estrela breed), 3 to 4-years-old, 55.9±4.5kg weight, were acclimatized for 4 weeks. The animals were housed indoors in boxes and feed with grass hay and food pellets (0.250kg/animal/day). Diet was offered at an estimated 1.20

x energy maintenance requirements according to the NCR (1985) recommendations for sheep nutrition. The animals were divided in a sham control group (n=6) and a GC-treated OVX sheep group (n=6). For OVX realization, the anesthetic protocol was composed by acepromazine maleate (0.1mg/kg EV, Calmivet; Univete, Lisbon, Portugal), butorphanol tartrate (0.06mg/kg EV, Torbugesic; Fort Dodge Veterinaria, S.A., Vall de Vianya, Girona, Spain) and propofol 2% (3mg/kg EV, Propofol-Lipuro; B.Braun, Melsungen, Germany) and maintained with 1.5% isoflurane in oxygen. Analgesia was accomplished by flunixin meglumine (1mg/kg, IM, q24h, Finadyne; Vetlima, Lisbon, Portugal) for 72 hours. Amoxicillin (15mg/kg, IM, q48h, Clamoxyl LA; Laboratórios Pfizer, Lda, Barreiro, Portugal) was also administrated during the first week. The OVX sheep received 1mg/kg dexamethasone weekly injections (0.6mg/kg IM, Dexafort; MSD Animal Health, Portugal and 0.4mg/kg IM, Oradexon, N.V. Organon, The Netherlands), as described by ZARRINKALAM et al. (2009). During the last four weeks the gradual tapering of steroids was performed to prevent symptoms associated with abrupt withdrawal of GCs. Animals were euthanized at the 24th postoperative week with EV pentobarbital sodium (Eutasil; Sanofi Veterinária, Miraflores, Algés, Portugal). The experiments on animals followed Directive 2010/63/EU and of the local Council on the protection of animals used for scientific purposes (authorization DGAV 0420/000/000/09).

Collection of blood samples

Blood samples were collected preoperatively and every 4 weeks until the 24th postoperative weeks (T0 to T6). The blood samples were drawn from the jugular vein into serological tubes (S-Monovette®, SARSTEDT, Nümbrecht, Germany). Samplings were performed between 9:00a.m. and 10:00a.m. and immediately centrifuged at 3000rpm for 10min. Then the serum were stored in Eppendorf tubes at -20°C for serum total ALP, TRAP, E_2 and mineral analyses and at -80°C for the other BTMs analyses.

Biochemical analysis

Commercially available kits from QUIDEL Corporation (Santa Clara, CA, USA) were used for ELISA determinations of serum BALP activity (Ref. 4660; EIA kit) and intact OC levels (Ref. 8002; EIA kit). The CTX levels were also measured with a commercial kit (ACP, Ref. O2F1; ELISA kit; IDS, Boldons, UK). The TRAP was performed via an enzymatic method and molecular absorption spectrophotometry using a commercial kit (ACP, Ref. 17304; Sentinel Diagnostics, Milan, Italy). Serum ALP activity (Ref. 6004), total Ca (Ref. 60117), inorganic P (Ref. 6122) and Mg (Ref. 6189) were determined by Beckman Coulter (CA, USA) commercial kits, via chemical method and molecular absorption spectrophotometry (Olympus AU400 automated biochemistry analyzer, Olympus America Inc., PA, USA). Serum E_2 levels (eE2 Ref. 10490889; ADVIA Centaur-Siemens Healthcare Diagnostics, Frimley, UK) were determined by automated direct competitive chemiluminescent immunoassay.

The manufacturer's protocol was followed as described and samples were assayed in duplicates. The sensitivity of this assay was 19pg/mL, and intra- and inter-assay coefficients of variation were 2.3%-11.1% and 0.9%-2.6%, respectively.

X-ray μ-CT

Samples from the body of the 4th lumbar vertebra (L4) (6mm diameter) were scanned using X-ray scan micrograph (μ-CT; SkyScan 1272; Bruecker, Kontich, Belgium). The samples were maintained in wet conditions by wrapping them with filter paper soaked in saline. Series of two dimensional projections, with a resolution of 7µm were acquired over a rotation range of 180°, with a rotation step of 0.45°, by cone-beam acquisition and using a 0.35mm copper + 0.15mm aluminium filter. The data were reconstructed using the software NRecon (Version: 1.6.6.0, Skyscan) and analysed in a CT analyser (Version: 1.17.0.0, Skyscan). The region of interest (ROI) was defined as a 4.5mm diameter circle centred over the specimen. By auto-interpolation of manually-defined ROI with the inner and outer limits of trabecular bone, it was yielded a volume of interest (VOI) with a shape of a cylinder representative of the sample, which was the essential basis for the quantitative analyses. The BMD (g/cm^3) of each sample was determined using the 8mm phantom calibrators of 0.25 and 0.75g/cm^3. The bone volume fraction (BV/TV; %), trabecular thickness (Tb.Th; µm), specific bone surface (BS/BV; %), trabecular number (Tb.N; 1/mm) and trabecular spacing (Tb.Sp; μm), closed porosity (Po(cl); %), open porosity (Po(op); %), and total porosity (Po(tot); %) were calculated using the Batman tool of CT analyser software. For the three-dimensional (3D) analysis, the bone region of each section was automatically defined (Ridler-Calvard method) and the resulting binarised image despeckled to remove the background (for bright speckles <40 voxels). The 3D reconstructions were produced using the CTVOX software.

Bone histomorphometry

Identical L4 vertebra biopsies were fixed in formalin 10% (NBF-neutral buffered formalin, Thermo Scientific, USA) and stored at 4ºC. For histological preparations, the bone samples were decalcified by incubation in a solution of TBD-2 (Thermo Scientific, USA) with mechanical stirring during 7 days. The decalcification end-point was defined as two consecutive days with negative tests for the presence of calcium in the decalcification solution supernatant. In brief, to 0.5mL of supernatant were added 1.0mL of citrate-phosphate buffer (0.20M citric acid and 0.16M dibasic potassium phosphate, pH 3.2-3.6) and 2.5mL of saturated ammonium oxalate. After 20min a calcium precipitate in the test tube is formed when the decalcification is still occurring. The decalcification was further confirmed by puncturing the decalcified bone biopsies with a needle to test the resistance. The decalcified bone samples were then dehydrated in ascending alcohol concentrations before embedding the specimens in paraffin. Sections of 5μm were cut in the anteroposterior plane on a automate

microtome (HM 355S Automatic Microtome, Thermo Scientific, USA) and mounted in glass slides. The histological slices were deparaffinized through downward alcohol concentrations and stained with Hematoxylin & Eosin (H&E) (Thermo Scientific, USA) using a standardized protocol. The cortical porosity (Ct.Po; %) and cortical thickness (Ct.Th; μm) were assessed in the cortical bone and the BV/TV (%), Tb.Th, Tb.Sp and Tb.N in the trabecular bone were quantified using the BoneJ (DOUBE et al., 2010) plugin of ImageJ software. For that, all micrographs of the histological cuts stained with H&E were split in the RGB channels. A bitwise operation was performed to subtract the green channel, strongly staining the bone marrow area, to the red channel, roughly corresponding to the bone area and the bone marrow, rendering an image of the bone area. These resulting representations of the bone area were treated to remove noise and binarized for histomorphometric evaluation.

Statistical analysis

ANOVA and Wilcoxon / Kruskal-Wallis test were used to determine statistical differences ($P<0.05$) and to compare mean differences between the different times or between study groups. The degree of correlations between the serum BTMs, minerals and E_2 values were assessed by z Spearman correlation test. Statistical analyses were performed with SPSS statistical software (version 23.0, SPSS, Inc., IBM Company, NY, USA).

RESULTS

Serum biochemical evaluation

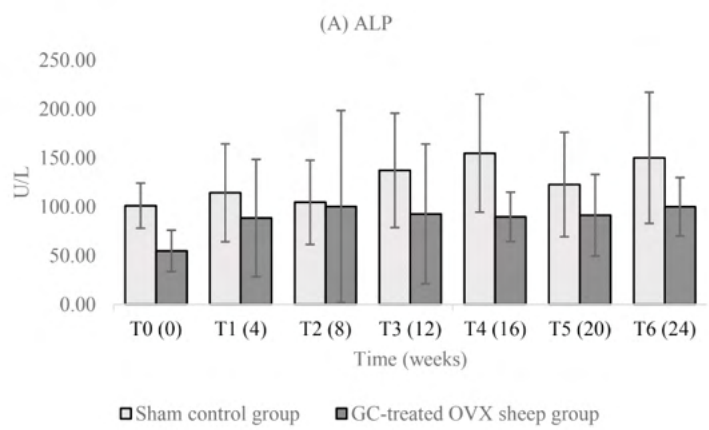
The results of biochemical analyses are shown in Table 1 and Figure 1 and the correlation coefficients between investigate variables in Table 2.

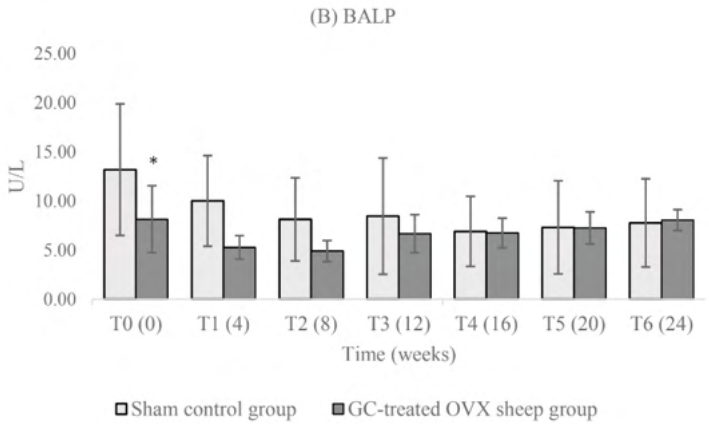
Time (weeks)	Groups	ALP (U/L)	BALP (U/L)	OC (μg/L)	CTX (pg/L)	TRAP (U/L)	Ca (mmol/L)	P (mmol/L)	Mg (mmol/L)	E_2 (pmol/L)
T0 (pre-op.)	Sham control	101.0±23.0	13.2±6.7	18.3±4.7	0.23±0.12	2.17±0.29	2.38±0.11	1.73±0.20	0.95±0.10	199.0±67.9
	GC-treated	54.8±21.2	8.1±3.4*	12.0±9.0	0.39±0.34	2.20±0.29	2.26±0.22	1.99±0.50	0.85±0.09*	253.3±154.2
	OVX sheep									
T1 (4)	Sham control	114.2±50.0	10.0±4.6	16.0±7.1	0.91±1.06	1.99±0.78	2.33±0.09	1.83±0.38	0.94±0.07	177.0±75.6
	GC-treated	88.4±60.1	5.3±1.2	4.6±2.1*†	0.64±0.31	2.36±0.24	2.27±0.13	2.20±0.44	0.88±0.13	171.8±46.8
	OVX sheep									
T2 (8)	Sham control	104.5±43.1	10.0±4.6	16.0±7.1	0.91±1.06	2.20±0.50	2.33±0.09	1.83±0.38	0.94±0.07	177.0±75.6
	GC-treated	100.2±98.3	4.9±1.1	5.0±3.2*†	0.60±0.30	2.46±0.09	2.21±0.17	2.27±0.45*	0.94±0.04	151.3±75.6
	OVX sheep									

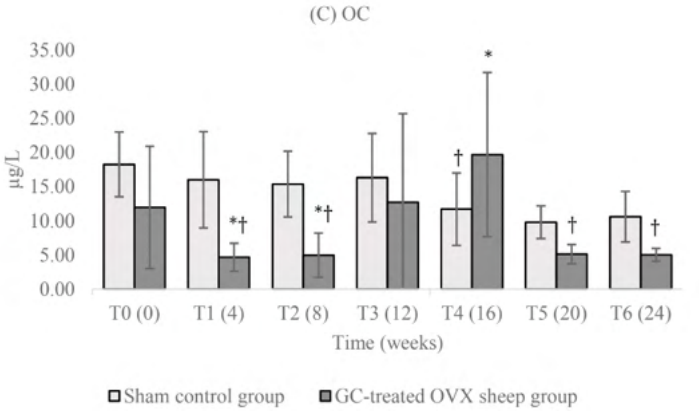
T3 (12)	Sham control	137.2±58.5	8.4±5.9	16.3±6.5	0.18±0.12	2.53±0.53	2.36±0.12	1.85±0.40	0.96±0.09	207.4±83.0
	GC-treated OVX sheep	92.6±71.5	6.6±1.9	12.7±13.0	0.37±0.12	2.22±0.47	2.11±0.23*	2.04±0.22	0.86±0.04*	152.0±92.2
T4 (16)	Sham control	154.7±60.4	6.9±3.6	11.7±5.3†	0.27±0.14	2.22±0.81	2.32±0.03	2.03±0.48	0.94±0.07	143.2±76.0
	GC-treated OVX sheep	89.6±25.3	6.7±1.5	19.7±12.0*	0.56±0.29	2.10±0.32	2.28±0.25	2.58±0.54*†	0.83±0.04*	148.3±97.7
T5 (20)	Sham control	122.7±53.5	7.3±4.7	9.8±2.4	0.27±0.14	2.33±0.31	2.30±0.12	1.70±0.45	0.92±0.03	166.3±89.2
	GC-treated OVX sheep	91.2±41.8	7.2±1.6	5.1±1.4†	0.81±0.13*†	2.90±0.91*	2.14±0.17	2.21±0.37	0.95±0.07	116.0±78.9
T6 (24)	Sham control	150.0±67.2				2.27±0.34				
	GC-treated OVX sheep	100.0±29.9	7.8±4.5 8.0±1.1	10.6±3.7 5.0±0.9†	0.25±0.12 0.72±0.40*	2.54±0.38	2.27±0.15 2.16±0.21	1.89±0.56 2.17±0.60	1.00±0.05 1.01±0.03	152.4±80.8 119.7±57.3

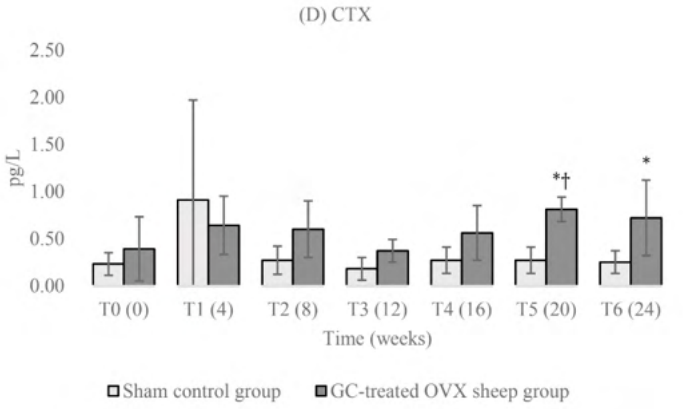
Total alkaline phosphatase (ALP); bone isoform of alkaline phosphatase (BALP); intact osteocalcin (OC); C-terminal telopeptides of collagen type I (CTX); tartrate-resistant acid phosphatase (TRAP); calcium (Ca); phosphorus (P); magnesium (Mg); 17β-estradiol (E_2); glucocorticoids (GC); ovariectomy (OVX); *significant differences compared with sham control group for the same time (*P<0.05); †significant differences compared to their preoperative (baseline) values (†P<0.05)

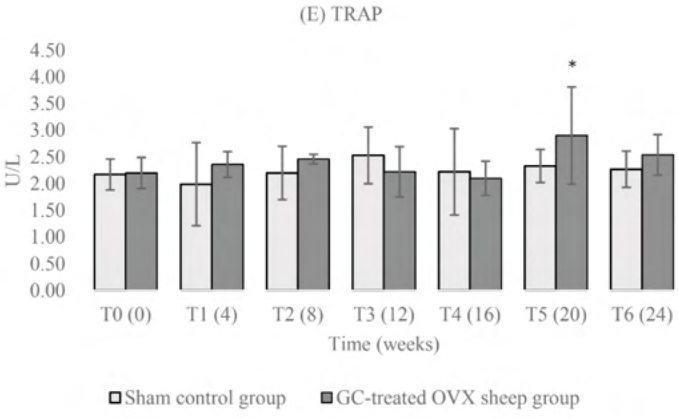
Table 1: Means±SD of serum bone turnover markers, minerals and 17β-estradiol levels in the sham control and glucocorticoid-treated ovariectomized sheep groups.

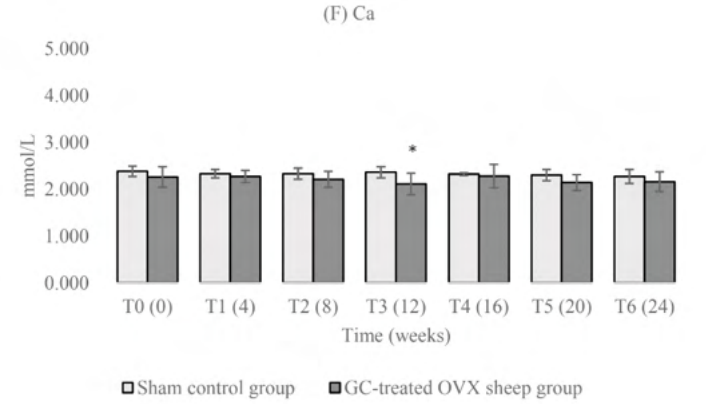


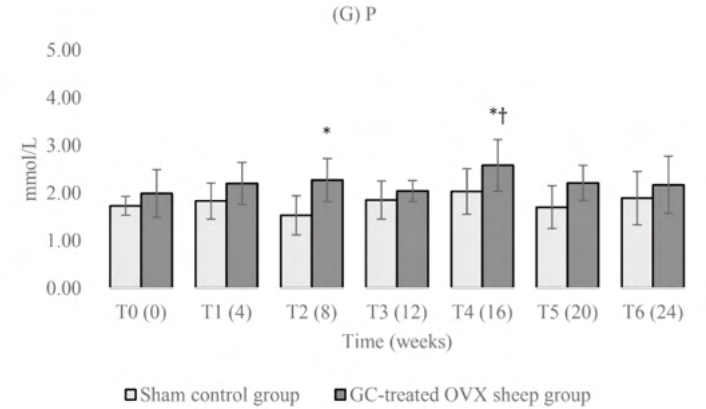


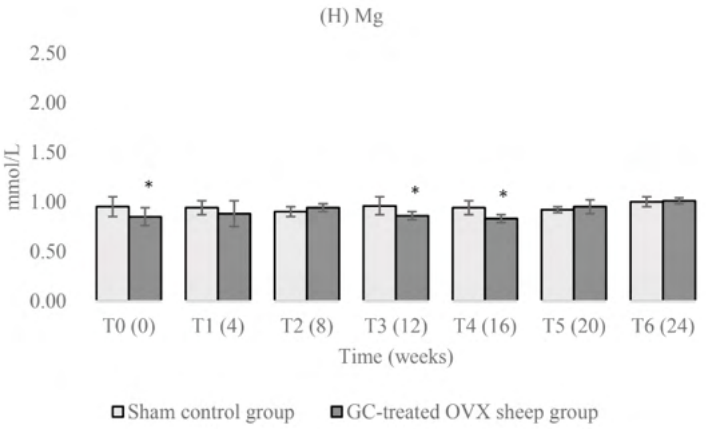

(C) OC
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
μg/L
*†
*†
†
*
†
†
T0 (0)
T1 (4)
T2 (8)
T3 (12)
T4 (16)
T5 (20)
T6 (24)
Time (weeks)
Sham control group
GC-treated OVX sheep group


(D) CTX
2.50
2.00
1.50
1.00
0.50
0.00
pg/L
*†
*
T0 (0)
T1 (4)
T2 (8)
T3 (12)
T4 (16)
T5 (20)
T6 (24)
Time (weeks)
Sham control group
GC-treated OVX sheep group


(E) TRAP
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
U/L
*
T0 (0)
T1 (4)
T2 (8)
T3 (12)
T4 (16)
T5 (20)
T6 (24)
Time (weeks)
Sham control group
GC-treated OVX sheep group


(F) Ca
5.000
4.000
3.000
2.000
1.000
0.000
mmol/L
*
T0 (0)
T1 (4)
T2 (8)
T3 (12)
T4 (16)
T5 (20)
T6 (24)
Time (weeks)
Sham control group
GC-treated OVX sheep group


(G) P
5.00
4.00
3.00
2.00
1.00
0.00
mmol/L
*
*†
T0 (0)
T1 (4)
T2 (8)
T3 (12)
T4 (16)
T5 (20)
T6 (24)
Time (weeks)
Sham control group
GC-treated OVX sheep group


(H) Mg
2.50
2.00
1.50
1.00
0.50
0.00
mmol/L
*
*
*
T0 (0)
T1 (4)
T2 (8)
T3 (12)
T4 (16)
T5 (20)
T6 (24)
Time (weeks)
Sham control group
GC-treated OVX sheep group

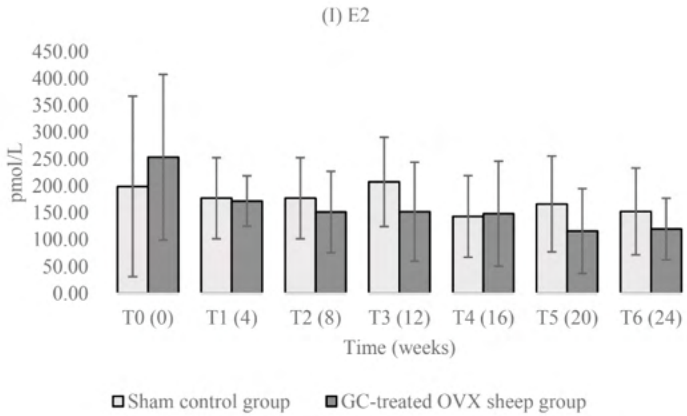


Figure 1: Time-related (preoperatively and every 4 weeks until the 24th postoperative weeks – T0 to T6) changes of serum (A) total alkaline phosphatase (ALP) and (B) bone isoform of alkaline phosphatase (BALP) activities, (C) intact osteocalcin (OC) level, (D) C-terminal telopeptides of collagen type I (CTX) level, (E) tartrate-resistant acid phosphatase (TRAP) activity, (F, G and H) serum minerals – calcium (Ca), phosphorus (P) and magnesium (Mg) concentrations and (I) 17β-estradiol (E_2) level at the preoperative time and during the postoperative period (mean±SD); *significant differences compared with sham control group for the same time (*P<0.05); †significant differences compared to their preoperative (baseline) values (†P<0.05).

	ALP	BALP	OC	CTX	TRAP	Ca	P	Mg	E_2
Sham control group									
ALP	R=1.00 P=1.00	-	-	-	-	-	-	-	-
BALP	R=-0.3558 P=0.0262	R=1.00 P=1.00	-	-	-	-	-	-	-
OC	R=-0.2606 NS	R=0.4184 P=0.0077	R=1.00 P=1.00	-	-	-	-	-	-
CTX	R=-0.4741 P=0.0023	R=0.2452 NS	R=0.2463 NS	R=1.00 P=1.00	-	-	-	-	-
TRAP	R=0.5072 P=0.0010	R=-0.5083 P=0.0010	R=-0.3237 P=0.0444	R=-0.6182 P<0.0001	R=1.00 P=1.00	-	-	-	-
Ca	R=-0.0566 NS	R=0.2500 P=0.0490	R=-0.0336 NS	R=-0.0322 NS	R=-0.0056 NS	R=1.00 P=1.00	-	-	-
P	R=0.3796 P=0.0132	R=-0.5196 P=0.0007	R=-0.1238 NS	R=-0.2592 P=0.0129	R=0.3947 NS	R=-0.2031 NS	R=1.00 P=1.00	-	-
Mg	R=-0.1516 NS	R=0.1237 NS	R=0.1852 NS	R=0.1281 NS	R=-0.0429 NS	R=-0.4135 P=0.0065	R=-0.1665 NS	R=1.00 P=1.00	-
E_2	R=-0.5299 P=0.0003	R=0.6864 P<0.0001	R=0.3942 P=0.0118	R=0.3116 NS	R=-0.4489 P=0.0042	R=0.4489 P=0.0029	R=-0.6057 P<0.0001	R=0.0698 NS	R=1.00 P=1.00
Glucocorticoid-treated ovariectomized sheep group									
ALP	R=1.00 P=1.00	-	-	-	-	-	-	-	-
BALP	R=0.1231 NS	R=1.00 P=1.00	-	-	-	-	-	-	-
OC	R=-0.3453 NS	R=0.3312 NS	R=1.00 P=1.00	-	-	-	-	-	-

CTX	R=0.3131 NS	R=0.0594 NS	R=-0.1652 NS	R=1.00 P=1.00	-	-	-	-	-
TRAP	R=0.1120 NS	R=0.2243 NS	R=-0.2366 P=0.0490	R=0.0912 NS	R=1.00 P=1.00	-	-	-	-
Ca	R=-0.3688 P=0.0347	R=0.1518 NS	R=0.2844 NS	R=0.0611 NS	R=-0.0943 NS	R=1.00 P=1.00	-	-	-
P	R=0.5079 P=0.0025	R=-0.2100 NS	R=0.0561 NS	R=0.0102 NS	R=0.0657 NS	R=-0.4934 P=0.0026	R=1.00 P=1.00	-	-
Mg	R=0.1415 NS	R=0.0979 NS	R=-0.2811 NS	R=0.1917 NS	R=0.4687 P=0.0052	R=-0.0373 NS	R=0.1546 NS	R=1.00 P=1.00	-
E_2	R=-0.0961 NS	R=0.0289 NS	R=-0.0272 NS	R=-0.0078 NS	R=-0.0055 NS	R=-0.0847 NS	R=0.0403 NS	R=-0.0456 NS	R=1.00 P=1.00

Total alkaline phosphatase (ALP); bone isoform of alkaline phosphatase (BALP); intact osteocalcin (OC); C-terminal telopeptides of collagen type I (CTX); tartrate-resistant acid phosphatase (TRAP); calcium (Ca); phosphorus (P); magnesium (Mg); 17β-estradiol (E_2)

Table 2: Correlation between serum bone turnover markers, minerals and 17β-estradiol levels in the sham control and glucocorticoid-treated ovariectomized sheep groups.

Total ALP activity did not present statistically significant differences between both groups in study along time, but with the values of the GC-treated OVX sheep group showing lower levels relatively to sham control group after induction protocol. No statistically significant differences were observed along time within each study group. Relatively to serum minerals concentration, Ca did not show statistically significant differences between both groups in study along time, except for T3. Nevertheless, the values of the GC-treated OVX sheep group were always below the values of the sham control group, similarly to ALP for each time point. For P and Mg, also statistical differences were obtained between groups in study along time ($P<0.05$ and $P<0.01$, respectively). The P presented statistical differences between groups at the T2 and T4 with the values of GC-treated OVX sheep group superior to those of the sham control group and for Mg statistical differences were observed at the T3 and T4 with the values of GC-treated OVX sheep group inferior to those of the sham control group. For P, just within the GC-treated OVX sheep group, it was observed a statistically significant increase in values between the preoperative time and T4. The Mg showed statistical differences in sham control group between the T3 and T6 and in the GC-treated OVX sheep group between the preoperative time and the T5 and T6. For E_2, after the preoperative period, it was possible to observe in the GC-treated OVX sheep group a decrease in its values until T6, while sham control group just presented a slight fluctuation during the study.

For the BTMs, the BALP and TRAP activities did not present statistically significant differences between both groups in study along time, but intact OC and CTX concentrations presented statistical difference between both groups in study ($P<0.001$ and $P<0.01$, respectively). However, serum BALP activity showed in the GC-treated OVX group lower values than those obtained in the sham control group until the T4. The intact OC levels

were markedly higher in sham control group in the T1 and T2 compared to GC-treated OVX sheep group, but at T4 the value was significantly higher in the GC-treated OVX sheep. The CTX levels also presented significant differences between study group at the T5 and T6 with the GC-treated OVX sheep group presenting the highest values. For the intact OC values it was possible to verify that within the sham control group there were significant differences between the preoperative time and from the T4 in which a decrease of the intact OC values could be observed. In the GC-treated OVX sheep group the intact OC showed statistical differences between the preoperative time and the T1, T2, T5 and T6, which presented lower values. For the CTX levels no statistical differences were observed in the sham control group along time but in the GC-treated OVX sheep group there was a statistical difference between the preoperative time and the T5, with this last one value presenting a marked increase.

μ-CT analysis

No statistical differences were observed during the comparison of the micro-architectural L4 vertebra sample parameters and trabecular BMD between the sham control and the GC-treated OVX sheep groups ($P>0.05$). However, a slight decrease of BV/TV – 44.1% to 42.5% (-4.6%), Tb.N – 3.39(1/mm) to 3.05(1/mm) (-10%) and BMD – 0.69g/cm^3 to 6.62g/cm^3 (-10.5%) and an increase in BS/BV – 18.1(1/mm) to 18.6(1/mm) (+14.3%) and Po(tot) – 52.9% to 59.5% (+13.5%), were observed between the sham control group and the GC-treated OVX sheep group, respectively.

Bone histomorphometric analysis

Statistical differences were observed after OVX and GC administration at the 24th postoperative week at trabecular bone level of L4 vertebra for Tb.Sp – 366.7*μ*m in the sham control group and 430.8*μ*m in the GC-treated OVX group, which significantly increased ($P<0.05$). Although there was no statistically significant result, it was also possible to observe an increase in Ct.Th – 693.8*μ*m to 721.4*μ*m (+3.8%) and Ct.Po – 5.0% to 8.8% (+60%) and a decrease in the values of BV/TV – 43.2% to 34.7% (-19.7%), Tb.Th – 218.2*μ*m to 174.8*μ*m (-19.9%) and Tb.N – 3.7#/mm to 3.2#/mm (-13.5%), were observed between the sham control group and the GC-treated OVX sheep group, respectively.

DISCUSSION

The present work aimed to fully and thoroughly evaluate the variation of BTMs concentration and their correlations with serum minerals and E_2 levels in the GC-treated OXV sheep as a model for osteoporosis research. The osteoporosis induction in this animal model has proved similarities with the pathophysiological mechanisms that occur in both postmenopausal and GC-induced osteoporosis in humans by deficient bone formation resulting from an uncoupling of the bone formation and resorption during the reversal phase

(JENSEN et al., 2011; ANDERSEN et al., 2013; ANDREASEN et al., 2015). In this way, the evaluation of a comprehensive analytical panel of BTMs, prior to OVX and then in a monthly pattern over 6 months post-OVX sheep, in which GC were administered weekly, was complemented by bone microstructural and composition evaluation through the analysis of bone biopsies obtained at the body level of the L4 vertebra by means of μ-CT and histomorphometry.

During bone loss in osteoporosis, BTMs, namely BALP, OC, CTX and TRAP, are released into the blood stream and/or excreted by kidneys in urine (SEIBEL, 2005). The BALP is a plasma membrane-bound enzyme expressed by osteoblasts used when assessing the phenotype or developmental maturity of mineralized tissue cells (MILLAN, 2006; GOLUB & BOESZE-BATTAGLIA, 2007). It is involved in initiating calcification by increasing the local concentration of inorganic phosphate, a mineralization promoter, and by decreasing the concentration of extracellular pyrophosphate, an inhibitor of mineral formation (WHYTE, 1994; GOLUB & BOESZE-BATTAGLIA, 2007). The OC is a hydroxyapatite-binding protein synthesized by mature osteoblasts odontoblasts, and hypertrophic chondrocytes. At bone tissue level constitutes 15% of the non-collagenous bone matrix, with a small amount released to blood stream during the bone formation process and also during bone resorption process (SEEBECK et al., 2005; CREMERS et al., 2008). It acts as a regulator of bone mineralization, by binding calcium and consequently promoting hydroxyapatite nucleation, also regulating osteoblast and osteoclast activity (NEVE et al., 2013). The CTX are type I collagen molecule breakdown products generated by the activity of the enzyme cathepsin K, composed by short peptide sequences from the non-helical domain of this molecule, which are released during bone resorption process (CREMERS et al., 2008; SHETTY et al., 2016). Otherwise, TRAP 5b isoform is an enzyme, which is resistant to degradation by tartrate, most specifically expressed in the ruffled border of osteoclasts, activated macrophages and dendritic cells and cleaves type 1 collagen into fragments during bone resorption (HALLEEN et al., 2001; LEEMING et al., 2006; SHETTY et al., 2016). The normal values of BTMs in sheep were assessed in previously published studies (KLEIN et al., 2004; SEEBECK et al., 2005; DIAS et al., 2008; SOUSA et al., 2014; COELHO et al., 2020).

In the present study, the bone loss at T6 after the GC-treated OXV implementation protocol in sheep was proven through the microstructural parameter results obtained at L4 vertebra level. It was observed a slight reduction in BV/TV, Tb.N and trabecular BMD, conjugated to an increase in BS/BV and Po(tot) obtained by μ-CT and an increase of Tb.Sp obtained by histomorphometric analysis, confirming the osteopenia induction. LILL et al. (2002) and SCHORLEMMER et al. (2003), with an identical animal model, also describe similar changes in microstructural measurements at vertebral level in sheep. Other studies described more profound changes in bone loss, possibly due to the fact that they also associated to the protocol a Ca/P/vit. D deficient diet to the GC-treated OXV implementation protocol (LILL et al., 2002; ZARRINKALAM et al., 2009; ESCHLER et al., 2015). However,

in this study it was decided to maintain a balanced diet since deficient levels of Ca and P would not alter the possible correlations of these minerals at serum level with the other blood parameters under study.

As expected, among the assessed BTMs level, total ALP and BALP activities and intact OC concentration, almost always, showed consistently higher values over time in the sham control group than in the GC-treated OVX sheep group (except for the intact OC level at T4) (Table 1, Figure 1). Just the bone resorption marker CTX, always presented higher values in the GC-treated OVX sheep group than in sham control group (Table 1, Figure 1). These results are in line with those presented by ANDREASEN et al. (2015), KIELBOWICZ et al. (2015) and CABRERA et al. (2018) who also determined intact OC and CTX levels in a similar animal model, with those of DING et al. (2010) for intact OC and also with the CTX level results obtained in the study of ZARRINKALAM et al. (2009). Thereby, following BTMs level it is possible to consider that sheep model reaches the highest bone remodeling 8 weeks start the bone loss induction protocol. Other authors support the hypothesis that the sheep model undergoes the highest remodeling in short term and might be not suitable for long term studies (SIGRIST et al., 2007; ZARRINKALAM et al., 2009). However, in the present study it was also possible to observe a second significant reduction of intact OC level associated with an increase of CTX level (Table 1, Figure 1) after T5. The TRAP activity did not present statistical differences between the two groups which could be justified by the fact that this enzyme represents the set of two known isoforms – the TRAP 5a and 5b, and not just of the 5b isoform, which is the specific biomarker of osteoclastic resorption activity. Thus, the TRAP activity result might represent the metabolic expression of different organic tissues, and not just of bone tissue.

In the sham control group, serum Ca concentration always showed higher levels over the postoperative period compared to the GC-treated OVX sheep group, and the contrary was observed for P concentration (Table 1, Figure 1). These differences allow to perceive the involvement of these minerals in the bone turnover process and the negative correlation of the variation between these two parameters. Positive correlations in normal physiologic conditions between the formation marker BALP activity with serum Ca concentration and negative with serum P concentration, support the involvement of this BTM with the mineralization process. Also, the significant positive correlation between BALP activity and intact OC level, and negative correlation between these two formation markers and the resorption marker TRAP activity, could help to confirm their opposite function.

It is noteworthy that the serum E_2 level remained in the GC-treated OVX sheep group always with values below those of the sham control group, declining until T6 (Table 1, Figure 1), as expected and as reported by SIGRIST et al. (2007) and KIELBOWICZ et al. (2015). Also, regarding E_2 level, the result with high statistical significance should be highlighted in the study of correlations obtained in the sham control group in which this parameter varies positively with the formation markers – BALP activity and intact OC level,

and total Ca concentration and negatively with the resorption marker TRAP activity, and P concentration, which is extremely elucidative about the role of this hormone in the process of bone turnover. It should also be noted that all correlations obtained between E_2 level and serum BTMs and minerals levels under normal physiological conditions change and disappear in the GC-treated OVX sheep.

CONCLUSION

This study contributes to a better knowledge of the GC-treated OVX sheep model for osteoporosis research and the consequences that the present induction protocol may impose on its bone metabolism. From the physiologic hemodynamic point of view, these results confirm the reliability of these serum BTMs level determination in sheep for orthopedic research, proving the decreased bone formation by decreased of BALP activity and intact OC level, which indicate delay or disruption in the process of bone tissue mineralization, and increased bone resorption through increased CTX level in the GC-treated OVX sheep. Namely, the variations of the intact OC, as stated by VERGNAUD et al. (1997) and CLEMENS & KARSENTY (2001), and of CTX levels, as referred by VASIKARAN et al. (2011), in this animal model confirm these BTMs as reliable and sensitive indicators of diminished quality of bone and for the treatment of osteoporosis monitoring. Also, the obtained correlations between these serum BTMs, minerals and E_2 levels clarify about their intervention and interrelation in the bone turnover process.

FUNDING AND ACKNOWLEDGEMENTS

This work is funded by national funds through Portuguese Foundation for Science and Technology (FCT) under the projects UIDB/AGR/04033/2020 and UIDB/CVT/00772/2020. J. A. CAMASSA acknowledges to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil, for his PhD scholarship 202248/2015-1. Authors thank Dr. JOÃO A. BORDELO and Dr. CAMILA C. DIOGO for their assistance during the animal model osteoporosis induction protocol realization and also to ARIUM enterprise, Portugal, for technical assistance in the bone turnover markers determination.

CONFLICT OF INTERESTS STATEMENT

The authors declare that there is no conflict of interests.

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