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BIOMEDICAL SCIENCES

Evaluation of hematology, general serum biochemistry, bone turnover markers and bone marrow cytology in a glucocorticoid treated ovariectomized sheep model for osteoporosis research

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Abstract: Osteoporosis is a metabolic disorder characterized by a loss of bone mass and structure and increasing the risk of fragility fractures, mostly among postmenopausal women. Sheep is a recognized large animal model for osteoporosis research. An experimental group of ewes (3-4 years old) was subjected to ovariectomy (OVX) and weekly glucocorticoid (GC) application for 24 weeks and compared with a sham control group. Blood and bone marrow parameters were analyzed before and 24 weeks after OVX and GC administration. Osteopenia was confirmed through micro-computed tomography and histomorphometric analysis of L4 vertebra in the study end. A statistically significant increase was observed in mean corpuscular volume, mean cell hemoglobin and monocytes and a decrease in red blood count and eosinophils ($p<0.05$). Alkaline phosphatase (ALP), gamma-glutamyl transpeptidase, magnesium and α1-globulin increased, and creatinine, albumin, sodium and estradiol decreased ($p<0.05$). A slight decrease of bone formation markers (bone ALP and osteocalcin) and an increase of bone resorption markers (C-terminal telopeptides of collagen type 1 and tartrate-resistant acid phosphatase) were observed, but without statistical significance. This study aims to contribute to better knowledge of sheep as a model for osteoporosis research and the consequences that a performed induction protocol may impose on organic metabolism.

Key words: animal model, blood analysis, bone marrow cytology, lumbar vertebral micro-structure, osteoporosis, sheep.

INTRODUCTION

Osteoporosis is a common metabolic bone disease resulting from changes in bone remodeling characterized by increased bone resorption and decreased bone formation (Cabrera et al. 2018). These changes result in a loss of bone mass and structure in which bone strength is compromised, increasing the probability of fragility fractures, mainly in the femoral neck, wrist, pelvis and lumbar vertebrae among other skeletal sites (Riggs & Melton 1986, Riggs et al. 2003). Osteoporosis predominantly affects older Caucasian women with low estrogen levels in the postmenopausal period and is considered the most prevalent worldwide metabolic bone disease (Curtis et al. 2017). In order to modify this scenario, more studies are necessary to better

understand all the mechanisms involved in this disease and to improve its early diagnosis.

The sheep is considered an excellent preclinical animal model based on its long life expectancy, physical stature, low cost compared to other animal models, availability and ease of handling, feeding and housing. Moreover, this species allows obtaining a large amount of biological material samples and presents similarities to humans in relation to bone structure, composition and remodeling process (Pearce et al. 2007, Kiełbowicz et al. 2016, Oheim et al. 2016, Cabrera et al. 2018). To date, various studies have used sheep as a large animal model for preclinical and translational studies of postmenopausal osteoporosis, namely for vertebral augmentation, spinal fusion, improvement of the fragile fracture healing process, bone defect repair and development of new anti-osteoporotic pharmacological agents (Oheim et al. 2012, Dias et al. 2018). One of the most common protocols used to mimic the organic and metabolic conditions of osteoporosis in the ovine model is a combination of ovariectomy (OVX) and subsequent administration of glucocorticoids (GCs) aiming to increase bone loss and reduce experiment work time (Cabrera et al. 2018, Dias et al. 2018). Chronic GC administration induces bone loss in a multifaceted process. On one hand it reduces the bone remodeling by directly modulating the bone cells (osteoclast, osteoblast and osteocyte) function (McKay & Cidlowski 2003, Sato et al. 2018). On the other hand, GCs also increase renal calcium (Ca) excretion and decrease its gastrointestinal absorption (McKay & Cidlowski 2003). The resulting decreased serum Ca level enhances the secretion of parathyroid hormone (PTH), over which GCs increase sensitivity, with PTH also increasing the osteoclast activity (Patschan et al. 2001).

For these reasons OVX and the exogenous administration of GCs should cause considerable changes in the organic metabolism of sheep, with a high probability of good evaluation at the hematological and blood serum levels. Also in the bone turnover markers (BTMs), which are indicative of bone metabolism and are used in the diagnosis of osteoporosis, changes are expected occur after the induction of bone loss protocol. Generally, BTMs are divided into (1) bone formation markers; (2) bone resorption markers; and (3) osteoclast regulatory protein markers. The bone formation markers include the total alkaline phosphatase (ALP), its bone-specific isoform (BALP), intact osteocalcin (OC) and procollagen type 1 propeptides. The resorption markers are the products of collagen breakdown, such as the C-terminal (CTX) and N-terminal telopeptides of collagen type 1, CTX-matrix metalloproteinase, hydroxyproline and collagen cross-links (pyridinoline, deoxypyridinoline), and the enzymes secreted by the osteoclasts, namely the tartrate-resistant acid phosphatase (TRAP) 5b isoform. The osteoclast regulatory protein markers are the receptor activator of nuclear factor κB ligand (RANKL), the RANK and the osteoprotegerin (OPG) (Leeming et al. 2006, Cremers et al. 2008, Sousa et al. 2015). The bone remodelling process involves the OPG/RANKL/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 (Liu & Zhang 2015, Ikeda & Takeshita 2016).

Among imaging techniques, the micro-computed tomography (μCT) is currently considered to be one of the most advanced non-destructive and minimally-invasive options, providing a 3D reconstruction of the internal architecture and allowing the observation of cross sections (internal sections) of objects (Faot et al. 2015). In regard to bone tissue it is possible

to evaluate its composition and microstructure, and measures the bone mineral density (BMD), which allows assessment for osteopenia or osteoporosis (Campbell et al. 2008), making CT in an important tool for skeletal tissue evaluation. The μCT can obtain results using very small sized samples, which is an advantage as samples can be collected and evaluated at non-terminal time points.

The aim of this study is to contribute to the characterization of the GC-treated OVX sheep through evaluation of the effects of this osteoporosis induction protocol on the haematological and biochemical blood parameters levels, including estradiol (E_2) and a set of formation and resorption BTMs (ALP and BALP, intact OC, CTX and TRAP) and bone marrow composition. Additionally, an evaluation of micro-architectural characteristics and BMD of L4 vertebra that were acquired by μCT and bone histomorphometry was performed.

MATERIALS AND METHODS

Animals, housing, anesthetic and surgical procedure

The study was carried out in Vila Real (latitude 41°19' N, longitude 7°44' W and altitude 479 m), Portugal. Twelve healthy female sheep of the Portuguese Serra-da-Estrela breed with approximately 3 to 4 years old (mean weight of 55.9±4.5 kg) were acclimatized for 4 weeks before the first blood was drawn, and the surgical protocol procedure was performed. The animals were housed indoors under the natural influence of seasonal variations and photoperiod. The barn was spacious, dry, well-drained, ventilated with bedding of regularly changed hay and straw. The animals were fed with grass hay and food pellets (0.250 kg/animal/day) and water provided *ad libitum*. The diet offered had an estimated 1.20 x energy maintenance requirements according to the NCR (1985) recommendations for sheep nutrition.

The sheep were divided into a sham control group and an osteoporosis induction group (n=6/each group) which was subjected to bilateral OVX and a subsequent protocol of weekly injections of 1 mg/kg dexamethasone (combination of 0.6 mg/kg IM, Dexafort; MSD Animal Health, Portugal and 0.4 mg/kg IM, Oradexon, N.V. Organon, The Netherlands), as described by Zarrinkalam et al. (2009). For the OVX procedure, the anesthetic protocol included premedication with acepromazine maleate (0.1 mg/kg EV, Calmivet; Univete, Lisbon, Portugal). The anesthetic induction was carried out with butorphanol tartrate (0.06 mg/kg EV, Torbugesic; Fort Dodge Veterinaria, S.A., Vall de Vianya, Girona, Spain) and propofol 2% (3 mg/kg EV, Propofol-Lipuro; B.Braun, Melsungen, Germany) and anesthesia was maintained with 1.5% isoflurane in oxygen. Analgesia was obtained using flunixin meglumine (1 mg/kg, IM, q24h, Finadyne; Vetlima, Lisbon, Portugal) for 72 hours, and the animals were given antibiotherapy with amoxicillin (15 mg/kg, IM, q48h, Clamoxyl LA; Laboratórios Pfizer, Lda, Barreiro, Portugal) during the first week. During the last four weeks, steroids tapering was performed (3/4, 1/2, 1/4 and 0 of the initial steroids dose), since the complete removal of GCs was necessary for the subsequent use of this animal model in further experiments not related to the scope of this work, namely to study anti-osteoporotic drugs and to evaluate orthopedic implants in the osteopenic and osteoporotic skeleton. All animals were euthanized at the 24th postoperative week, with a lethal EV injection of pentobarbital sodium (Eutasil; Sanofi Veterinária, Miraflores, Algés, Portugal). All procedures, treatments and animal care were in compliance the Directive 2010/63/EU of the European Parliament and of the Portuguese Council on the protection of animals

used for scientific purposes (Authorization DGAV Of. n° 0420/000/000/09).

Collection of blood samples, hematological and serum biochemical analysis

Blood samples were collected from the experimental sheep via jugular venipuncture and collected in EDTA blood tubes (S-Monovette, Sarstedt, Nümbrecht, Germany) for the determination of hematological parameters and for serum tubes without anticoagulant (S-Monovette - Serum Gel S, Sarstedt, Nümbrecht, Germany) for the general biochemical and electrolytic parameters, estradiol and BTMs evaluation. Samples were taken between 9:00 a.m. and 10:00 a.m. at the beginning of the study, repeated 24 weeks later and stored in a thermal box at 4°C during transportation to laboratory facilities.

A cell blood count was immediately performed on a Sysmex XT2000iV hematology analyser (Sysmex Europe GmbH, Norderstedt, Germany) device using the flow cytometry and impedance methodologies. For serum biochemical analysis, the blood samples were centrifuged at 3000 rpm for 10 minutes and the serum stored in Eppendorf tubes at -20°C for general biochemical parameters, TRAP and estradiol, and at -80°C for analysis of the other BTMs analyses.

The general biochemical parameters were measured with commercially available immunoassay kits ordered from Beckman Coulter (CA, USA): blood urea nitrogen (BUN) (Ref. 6134), creatinine (Crea) (Ref. 6178), total cholesterol (TC) (Ref. 6116), calcium (Ca) (Ref. 60117), phosphorus (P) (Ref. 6122), magnesium (Mg) (Ref. 6189), glucose (Glu) (Ref. 6221), ALP activity (Ref. 6004), aspartate aminotransferase (AST) (Ref. 6109), alanine aminotransferase (ALT) (Ref. 6107), gamma-glutamyl transferase (GGT) (Ref. 6020) and total proteins (TP) (Ref. 6132) using a colorimetric method by molecular absorbance spectrophotometry by an automated biochemistry analyzer (Olympus AU400; Olympus America Inc., PA, USA). Protein electrophoresis was performed on Interlab G26 equipment (Interlab Srl, Rome, Italy). Also the electrolytes – sodium (Na^+), potassium (K^+) and chloride (Cl^-) were determined by Beckman Coulter System ISE modules.

The serum BALP activity was determined by an immunocapture method in a microtiter strip format using a monoclonal anti-BALP antibody adsorbed onto strips that captured the BALP in the sample. Para-nitrophenylphosphate (p-NPP) substrate was used for determining the BALP enzymatic activity (Ref. 4660, EIA kit, Quidel Corporation, CA, USA). The serum levels of intact OC were determined by a competitive method that uses OC coated onto strips, a mouse anti-OC antibody, an anti-mouse IgG-ALP conjugate and a p-NPP substrate (Ref. 8002, EIA kit, QUIDEL Corporation, Santa Clara, CA, USA). Two highly specific monoclonal antibodies determine the CTX (ACP, Ref. O2F1, ELISA kit, IDS, Boldons, UK) against the amino acid sequence of EKAHD-ß-GGR, where the aspartic acid residue (D) is ß-isomerized two chains of EKAHD-ß-GGR must be cross-linked to obtain a specific signal in the ELISA. The TRAP was performed via an enzymatic method and molecular absorption spectrophotometry using commercially available kits (ACP, Ref. 17304, Sentinel Diagnostics, Milan, Italy).

Serum estradiol (E_2) levels (eE2 Ref. 10490889, ADVIA Centaur-Siemens Healthcare Diagnostics, Frimley, UK) were determined by automated direct competitive chemiluminescent immunoassay where monoclonal anti-estradiol antibody was labeled by acridinium ester. The manufacturer's protocol was followed as described and samples were assayed in duplicates. The sensitivity of this assay was 19 pg/mL, and intra- and inter-assay coefficients of variation were 2.3-11.1% and 0.9-2.6%, respectively.

All blood analyses measurements were performed in duplicate.

Bone marrow samples collection and cytology

Bone marrow (BM) samples were obtained from the iliac crest of each sheep before and after osteoporosis induction (OVX and weekly GC administration), while they were restrained in lateral recumbency after sedation and topic anesthesia with 2% lidocaine hydrochloride spray. After aseptic preparation of the sample collection site, a BM aspirate needle (DMNI1x0x, Argon Medical Devices, Frisco, TX, USA) was inserted into the coxal tuberosity at a depth of 3-4 cm. A 10 mL heparinized syringe (1 mL heparin) was attached to the needle to obtain 10 mL BM. A mean of six smears were obtained from the BM samples of each animal and rapidly dried. About 300 cells per slide were evaluated under the Nikon Eclipse 600 fluorescence microscope using 10x, 20x, 50x and 100x immersion oil lenses. One BM smear of each animal was stained with Giemsa stain solution. Aspirated cells were identified on the basis of their morphological characteristics as described by Byers & Kramer (2010).

X-ray micro-computed tomography (µCT)

After euthanasia, samples obtained from the body of the L4 vertebra body (6 mm diameter biopsies) of both groups in the study were scanned using an X-ray scanner (µ-CT; SkyScan 1272; Bruecker, Kontich, Belgium). The samples were maintained in wet conditions by wrapping them with filter paper soaked in saline. A series of two-dimensional projections with a resolution of 7 µm were acquired over a rotation range of 180° with a rotation step of 0.45°, by cone-beam acquisition and using a 0.35 mm copper + 0.15 mm aluminium filter.

The cross-section slices were reconstructed using the NRecon software (version 1.6.6.0, Skyscan) and analyzed in a CT analyzer (version 1.17.0.0, Skyscan). The region of interest (ROI) was defined as a 4.5 mm diameter circle centered over the specimen. Auto-interpolation of manually defined ROI with the inner and outer limits of trabecular bone yielded a volume of interest (VOI) representative of the sample, which was the basis for the quantitative analyses. The BMD (g/cm^3) of each sample was determined using 8 mm phantom calibrators of 0.25 and 0.75 g/cm^3. The ratio between bone volume/total volume (BV/TV; %), specific bone surface (BS/BV; %), trabecular thickness (Tb.Th; µm), trabecular number (Tb.N; 1/mm) and trabecular spacing (Tb.Sp; mm), closed porosity (Po(cl); %), open porosity (Po(op); %), and total porosity (Po(tot); %) were calculated using the BatMan tool of CT analyzer software. For the 3D analysis, the bone region of each section was automatically defined (Ridler-Calvard method) and the resulting binarised image was despeckled to remove the background (for bright speckles < 40 voxels). The 3D reconstructions were produced using the CTVOX software.

Bone histomorphometry

Biopsies harvested from the L4 vertebra (6 mm diameter cylinders) were fixed in 10% formalin (NBF-neutral buffered formalin, Thermo Scientific, USA) and stored at 4°C. For histological preparations, the bone samples were decalcified by incubation in a solution of TBD-2 (Thermo Scientific, USA) with mechanical stirring for 7 days. The decalcification endpoint was defined as two consecutive days with negative tests for the presence of Ca in the decalcification solution supernatant. In brief, to 0.5 mL of supernatant were added 1.0 mL of citrate-phosphate buffer (0.20 M citric acid and 0.16 M dibasic potassium phosphate, pH 3.2-3.6) and 2.5 mL of saturated ammonium oxalate. After 20 minutes a Ca 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 5 µm were cut in the anteroposterior plane on a automate microtome (HM 355S Automatic Microtome, Thermo Scientific, USA) and mounted on glass slides. Lastly, the histological slices were deparaffinized using decreasing alcohol concentrations and stained with Hematoxylin & Eosin (H&E) (Thermo Scientific, USA) using standardized protocols.

The cortical porosity (Ct.Po; %) and cortical thickness (Ct.Th; µm) were assessed in the cortical bone and the BV/TV (%), Tb.Th (µm), Tb.Sp (µm) and Tb.N (#/µm) in the trabecular bone were quantified using the BoneJ (Doube et al. 2010) plugin of ImageJ software. For this, all micrographs of the H&E histological cuts 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. The resulting representations of the bone area were treated to remove noise and binarized for the histomorphometric evaluation.

Statistical analysis

The values are presented as medians. To determine statistical differences, Steel-Dwass Method were performed to compare median of the distributions differences between the different times and/or each study groups. All statistical analyses were performed with SPSS statistical software (version 23.0, SPSS, Inc., IBM Company, NY, USA). The p-values were considered significant at $p<0.05$.

RESULTS

Hematological and serum biochemical analysis and bone marrow cytology analysis

Mean corpuscular volume (MCV), mean cell hemoglobin (MCH), monocytes levels presented a statistically significant increase, and red blood cell count (RBC) and eosinophil levels decreased after the bone loss induction protocol. In particular, the MCV showed values above their reference interval after the bone loss induction protocol. The other blood elements also showed increased levels with an exception for eosinophils, which decreased (Table I). For the serum biochemical parameters statistical differences were observed for Crea, ALP, GGT, Mg, albumin, α1-globulin, Na^+ and E_2 (Table II). From those, ALT, GGT and α1-globulin increased their serum levels, while Crea, albumin, Na^+ and E_2 decreased. The BUN, GGT and CTX presented serum levels slightly above the superior level of their reference intervals after the bone loss induction protocol. Regarding the BM cytology, there were no statistically significant differences after the bone loss induction protocol for hematopoietic cell lines (Table III). The various hematopoietic cell lines were within the published values for the sheep species (Al Izzi et al. 2007, Byers & Kramer 2010).

µCT analysis

Figure 1 illustrates 3D reconstructions of consecutive µCT images harvest from L4 sheep vertebra from the sham control and GC-treated OVX sheep groups. The comparison of the micro-architectural parameters and trabecular BMD of L4 vertebra body between the sham control and the experimental groups did not show any statistical differences using Steel-Dwass Method ($p>0.05$). However, a slight decrease of BV/TV (-4.6%), Tb.N (-10%) and BMD (-10.5%) and an

Table I. Hematological parameters before and after OVX and exogenous GC administration in sheep (median values) and reported reference range for ovine species [reference intervals from Byers & Kramer (2010) and Meyer & Harvey (1998) (between parentheses)].

Hematological parameters	GC-treated OVX sheep group		Ovine species reference range
	Before	After	
Red blood cell parameters			
Erythrocytes ($x10^6/\mu L$)	9.12	8.65*	9-15
Hemoglobin (g/dL)	10.9	10.9	9-15
Hematocrit (%)	40.4	42.3	27-45
Mean corpuscular volume (fL)	39.7	47.9*	28-40
Mean cell hemoglobin (pg)	11.1	12.5*	8-12
Mean corpuscular hemoglobin concentration (%)	28.0	26.3	31-34
White blood cell parameters			
Leucocytes ($x10^3/\mu L$)	7.32	11.11	4.0-12.0
Neutrophils (band cells) ($x10^3/\mu L$)	-	-	rare
Neutrophils (polymorphonuclear cells) ($x10^3/\mu L$)	2.53	7.92	0.7-6.0
Lymphocytes ($x10^3/\mu L$)	4.16	2.92	2.0-9.0
Monocytes ($x10^3/\mu L$)	0.21	0.55*	0.0-0.75
Eosinophils ($x10^3/\mu L$)	0.31	0.02*	0.0-1.0
Basophils ($x10^3/\mu L$)	0.02	0.02	0.0-0.3
Other parameters			
Total platelet count ($x10^3/\mu L$)	352	410	800-1100 (300-800)

Medians after osteoporosis induction followed by asterisk differ from the medians before induction by nonparametric comparisons for all pairs using Steel-Dwass Method (*p<0.05).

increase in BS/BV (+14.3%) and Po(tot) (+13.5%) were observed (Table IV).

Bone histomorphometry

The Steel-Dwass Method demonstrated statistical differences after OVX and GC administration at the 24th postoperative week at trabecular bone level of L4 vertebra for Tb.Sp, which significantly increased ($p<0.05$) (Table IV). Although without a statisticaly significance result, an apparent increase in was visible Ct.Po and Ct.Th values and a decrease in the BV/TV, Tb.Th and Tb.N values.

Table II. Serum biochemical parameters before and after OVX and exogenous GC administration in sheep (median values) and reported reference range for ovine species [reference intervals from Radostits et al. (2000); reference intervals of bone turnover markers from Dias et al. (2008), Kiełbowicz et al. (2015) and Camassa et al. (2017); reference interval of estradiol from Sigrist et al. (2007) and Kiełbowicz et al. (2015) (between parentheses)].

	GC-treated OVX sheep group		Ovine species reference range
Serum biochemical parameters	**Before**	**After**	
Metabolites			
Glucose (mmol/L)	3.28	2.89	2.78-4.45
Total cholesterol (mmol/L)	1.62	1.39	1.11-2.66
Renal function			
Blood nitrogen urea (mmol/L)	5.0	7.5	1.67-5.83
Creatinine (µmol/L)	73.4	59.2*	106-168
Enzymes			
Total alkaline phosphatase (U/L)	52	98*	70-390
Aspartate aminotransferase (U/L)	132	133	60-280
Alanine aminotransferase (U/L)	31	24	22-38
Gamma-glutamyl transferase (U/L)	56	81*	20-52
Minerals			
Total calcium (mmol/L)	2.33	2.1	2.88-3.25
Phosphorus (mmol/L)	1.84	2.16	1.6-2.4
Magnesium (mmol/L)	0.84	1.0*	0.90-1.15
Proteins			
Total protein (g/dL)	7.5	6.4	6-7.9
Albumin (g/dL)	3.64	3.15*	2.4-3
Alpha 1-globulin (g/dL)	0.34	0.37*	-
Alpha 2-globulin (g/dL)	0.81	0.81	-
Beta-globulin (g/dL)	0.40	0.53	-
Gamma-globulin (g/dL)	2.44	1.97	-
Electrolytes			
Sodium (mmol/L)	151	149*	145-152
Potassium (mmol/L)	4.6	4.9	3.9-5.4
Chloride (mmol/L)	110	102	95-103

Table II. Continuation

	GC-treated OVX sheep group		Ovine species reference range
Serum biochemical parameters	**Before**	**After**	
Bone turnover markers			
Bone-specific alkaline phosphatase (U/L)	7.06	8.5	5.8-25.8
Intact osteocalcin (µg/L)	9.54	4.89	9.3-16
C-terminal telopeptides of collagen type 1 (pg/L)	0.31	0.52	0.2-0.45
Tartrate-resistant acid phosphatase (U/L)	2.1	2.5	0.14-5.9
Hormones			
Estradiol (pmol/L)	183.5	139.5*	76.3 (mean) (29-117)

Medians after osteoporosis induction followed by asterisk differ from the medians before induction by nonparametric comparisons for all pairs using Steel-Dwass Method (*p<0.05).

Table III. Bone marrow composition before and after OVX and exogenous GC administration in sheep (median values).

Bone marrow composition	**GC-treated OVX sheep group**	
Cell type	**Before**	**After**
Hematopoietic cells – Myeloid series		
Myeloblasts and promyelocytes (%)	3.0	3.1
Myelocytes, metamyelocytes and segmented granulocytes (%)	32.0	31.7
Eosinophils (%)	6.2	7.5
Basophils (%)	0.0	0.0
Total myeloid series (%)	41.2	42.3
Hematopoietic cells – Erythroid series		
Rubriblasts and prorubricytes (%)	2.0	3.55
Rubricytes and metarubricytes (%)	54.3	52.9
Total erythroid series (%)	56.3	56.4
Myeloid/erythroid ratio (%)	0.76	0.75
Lymphocytes (Lymph) (%)	1.7	0.65
Plasma cells (%)	0.5	0.18
Monocytes (%)	0.0	0.35
Megakaryocytes (%)	0.5	0.55

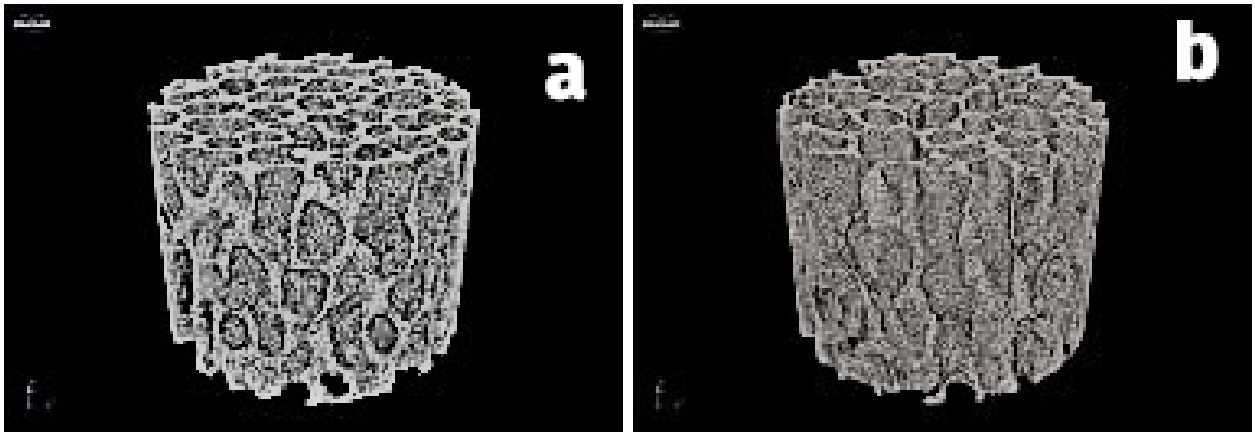

Figure 1. Representative micro-computed tomography 3D reconstructions of L4 vertebral bodies from (a) the sham control and (b) GC-treated OVX sheep groups at the 24th postoperative week.

Table IV. Micro-architectural parameters and trabecular BMD obtained by µCT and by histomorphometric analysis from the L4 vertebra of both groups in study (median values).

	L4 vertebral body	
	Sham control group	**GC-treated OVX sheep group**
µCT parameters		
BV/TV (%)	44.1	42.5
BS/BV (1/mm)	18.1	18.6
Tb.Th (mm)	0.13	0.14
Tb.Sp (mm)	0.35	0.40
Tb.N (1/mm)	3.39	3.05
Po(cl) (%)	0.20	0.15
Po(op) (%)	52.7	59.4
Po(tot) (%)	52.9	59.5
BMD (g/cm^3)	0.69	0.62
Histomorphometric parameters		
Ct.Th (µm)	693.8	721.4
Ct.Po (%)	5.0	8.8
BV/TV (%)	43.2	34.7
Tb.Th (µm)	218.2	174.8
Tb.Sp (µm)	366.7	430.8*
Tb.N (#/mm)	3.7	3.2

Medians after osteoporosis induction followed by asterisk differ from the medians before induction by nonparametric comparisons for all pairs using Steel-Dwass Method (*p<0.05).

DISCUSSION

By far the most common osteoporotic small ruminant model is the sheep. Two main induction protocols have been used in this model: 1) OVX sheep 12 months or more postoperatively as a validated large animal model of postmenopausal osteoporosis; or 2) the combined treatment of OVX sheep associated with a calcium/vitamin-D deficient diet and exogenous GC administration for 6 months, thereby reducing the time necessary to obtain bone mass loss (Dias et al. 2018). The need for inducing the disease in ovine species stems from the natural resilience to loss of bone mass in the adult (3 to 8 years old) or in the mature/geriatric (over 8 years old) sheep (Zarrinkalam et al. 2009). Although this animal model does not accurately reproduce the natural osteopenia/osteoporosis pathogenesis that occurs in humans, it has been widely used as an animal model capable of representing a significant bone loss for preclinical trials of pharmacological or surgical treatments in humans after submission to the published protocols for that purpose (Oheim et al. 2012). Andreasen et al. (2015) concluded that GC-treated OVX aged sheep induced a significant bone loss, promoted by an arrest of the reversal phase, resulting in an uncoupling of bone formation and resorption, as demonstrated in postmenopausal women with GC-induced osteoporosis (Jensen et al. 2011, Andersen et al. 2013). A more recent study elucidates the osteocyte regulation of OPG/RANKL 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).

Based on that finding, this study aimed to contribute to the characterization of sheep for osteoporosis research through OVX and a postoperative protocol of GC administration over a 6 months period as described by Zarrinkalam et al. (2009). For that purpose, variations in a panel of analytical blood and bone marrow parameters in sheep were assessed before and after OVX and exogenous GC administration to add to the knowledge of its metabolic and organic effects. Moreover, the microstructural bone tissue parameters of L4 lumbar vertebrae were studied by µCT analysis, bone histology and histomorphometry.

Corticosteroids are key regulators of whole-body homeostasis and provide capacity to resist environmental changes and invasion of foreign substances (McKay & Cidlowski 2003). They affect all the major body systems, especially the cardiovascular, musculoskeletal, nervous, endocrine and immune systems. Their action targets the intermediate metabolism – carbohydrate, protein and lipid metabolism, and the modulation of electrolyte and water balance (McKay & Cidlowski 2003). Glucocorticoid administration also induces hematological and immunosuppressive effects, among other adverse effects (McKay & Cidlowski 2003).

Among the hematologic effects of GCs is increasing hemoglobin (Hg) and RBC levels, not observed in the present study, most likely as consequence of retarded erythrophagocytosis (McKay & Cidlowski 2003). In addition, GCs are correlated with an increase in circulating white blood cell numbers as observed in the present study. This increase is justified by a transfer of polymorphonuclear cells to the circulating compartment from the marginal, once an increased BM release from mature neutrophils occurs and also a reduced neutrophil output from the vascular compartment to the inflammatory focus (McKay & Cidlowski 2003). On the other hand GCs promote a decrease of the lymphocytes, eosinophils and monocytes number due to redistribution of these cells.

However, this cell number could rise 24 to 72 hours after exogenous GCs treatment (Pountain et al. 1993), which could justify the elevation of the monocytes in this study after the GCs withdrawal. Lymphopenia is caused by the redistribution of circulating lymphocytes, which remain temporarily sequestered in lymphoid tissues or BM instead of circulating into the lymphatic system or into the blood. Monocytosis is caused by a similar effect to that of neutrophils, that is, the mobilization of cells from the marginal compartment to the blood circulation (Poetker & Reh 2010). The basophil number decrease has unknown mechanism (McKay & Cidlowski 2003). On the other hand, the GCs have a negative effect on neutrophils, reducing their adhesion to the vascular endothelium and reducing its bactericidal activity and they also inhibited the function of macrophages by limiting chemotaxis, phagocytosis and cytokine release (tumor necrosis factor and interleukin-1) (Poetker & Reh 2010).

Regarding serum biochemical parameters, the Crea level, before and after the OVX and GC administration, presented values below the reference range for the ovine species. Bearing in mind that high GC levels cause muscle wasting associated with their catabolic effects on protein metabolism (McKay & Cidlowski 2003, Klein 2015), elevated serum Crea levels should be expected. Nevertheless, this decrease could also be explained by the progressive discontinuation of the GCs during the last 4 weeks treatment of the induction protocol and since creatinine values were already diminished before OVX and GC administration. The significant increase of ALP and GGT in treated animals, could be an indicator of liver disease (namely cholestasis) as a consequence of long-term CG doses administered at higher than physiologic levels (LiverTox 2012). Glucocorticoid use can result in hepatic enlargement and steatosis or glycogenosis (LiverTox 2012). The ALP activity increase is attributable mostly to isoenzymes of hepatic origin: liver ALP and the corticosteroid-induced enzyme of ALP (Solter et al. 1993). Furthermore, GCs have also been demonstrated to cause oxidative stress in other tissues, namely bone, nervous tissue and possibly muscle (Klein 2015). Gamma-glutamyl transferase has been considered as one of the most reliable biomarkers of whole-body oxidative stress (Lee et al. 2004, Koenig & Seneff 2015).

With regard to serum minerals, Ca suffered a slight decrease and P a consequent slight increase, but without statistical significance. Magnesium presented a significant increase in this study which could be related to the fact that this mineral is involved in many of the biochemical reactions that take place in the cells and particularly in processes involving the formation and utilization of adenosine triphosphate (Paunier 1992). At the cellular level, Mg^{+} has a key role in ionic transport processes (Paunier 1992). Contrary to a described increase in Na^{+} retention and K^{+} excretion associated with GCs (McKay & Cidlowski 2003), in the present study a slight but statistically significant decrease in Na^{+} was observed. Again, this observation may be associated with the fact that serum measurements at the 24^{th} week were made after the gradual and total removal of the GCs.

Albumin, the most abundant plasma protein and the main determinant of coloidosmotic pressure, presented a statistically significant decrease at the 24^{th} week, which could be related with a slight change in liver function. Corticosteroids are also shown to inhibit immunoglobulin (Ig) synthesis, to kill B cells and decrease production of components of the complement system (McKay & Cidlowski 2003, Poetker & Reh 2010). Although of no statistically significant value, in the 24^{th} week it was still possible to observe a decrease in

gamma-globulin, mostly composed of IgG but also the IgA, IgM, IgD and IgE (Poetker & Reh 2010).

In regard to BTMs levels, no statistically significant differences were observed after OVX and GC administration. Nevertheless, a slight decrease of bone formation markers levels – BALP and total OC, and an increase of bone resorption markers – CTX and TRAP, support the tendency for an imbalance in the bone remodeling process towards bone resorption. Finally, a significant decrease in E_2 at the 24th week was observed, which should be related to the OVX procedure.

In the present study, after OVX and exogenous GC administration, the microstructural measurements obtained by μCT reveal a decrease of bone mass at trabecular L4 vertebra level, but without statistically significant differences. A slight reduction in BV/TV, Tb.N and trabecular BMD, conjugated with an increase in BS/BV and Po(tot), was observed. Concerning the histomorphometric evaluation of trabecular bone tissue, this method already revealed a statistically significant decrease of bone mass at trabecular level of L4 vertebra, especially based on the increase of Tb.Sp, confirming the osteopenia induction. Similar changes in microstructural measurements at vertebral level in sheep are reported in other studies that developed this animal model with this protocol (Lill et al. 2002a, Schorlemmer et al. 2003). More profound changes were acquired in the studies of Lill et al. (2002b), Zarrinkalam et al. (2009) and Eschler et al. (2015) with OVX, GC treatment and an associated of a diet with reduced calcium/phosphorus/vitamin D, validating this combined protocol to induce osteopenia in sheep. A reason for this discreet bone loss may be due to the fact that a balanced diet was maintained with no introduction of a diet deficient in minerals and vitamin D. This study maintained a conventional diet from the start so that deficient levels of Ca and P in the diet did not impose any changes through of exogenous causes to the values of these serum minerals and interrelated parameters.

Another aspect to be mentioned in the justification of these results is the fact that microstructural parameter measurements were focused on the L4 vertebral body (axial skeleton), and this location is less subjected to the process of bone remodeling relative to the appendicular skeleton (Schorlemmer et al. 2003, Osterhoff et al. 2016). It should also be noted that in the present study there was no comparison with preoperative values within the same group, but only between the GC-treated OVX sheep group and the sham control group (normal physiological condition) at the 24th postoperative week. So, the possibility of a more pronounced decrease in these parameters within the GC-treated OVX sheep group relative to the preoperative values should not be totally excluded.

In conclusion, this study contributed to the evaluation of this animal model and the consequences that ovariectomy (OVX) and weekly glucocorticoid (GC) application may impose at organic and metabolic levels. From this study we can better understand the general clinical status of these animals when they are subsequently included in pharmacological or surgical trials for biomedical research.

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All authors of this paper have read and approved the final version of the manuscript submitted, they also agree to be accountable for all aspects of the work in ensuring that questions relating to the accuracy or integrity of any part of the work are appropriately investigated and resolved and all of them have made substantive contributions to the work, namely: Substantially contributed to conception or design (CC, JB, PF, PC, CV, ID). Contributed to acquisition and analysis – blood and bone marrow analysis (CC, JB, ID), µ-CT (PB) and histomorphometry (VB, PB), or interpretation of data (CC, JB, JC, VB, PB, MG, RR, JA, JR, PF, PC, CV, ID). Drafted the manuscript (CC, JB, ID). Critically revised the manuscript for important intellectual content (CC, JB, JC, VB, PB, MG, RR, JA, JR, PF, PC, CV, ID).