

Serum Biomarker Evaluation in an Ovariectomized *Oryctolagus Cuniculus* Model for Osteopenia

Evaluación de Biomarcadores Séricos en un Modelo de Osteopenia en *Oryctolagus Cuniculus* Ovariectomizado

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SUMMARY: Estrogen deficiency accelerates bone turnover and underlies postmenopausal osteoporosis. The ovariectomized rabbit is a model of this condition, but the point at which bone loss becomes established and its temporal biochemical profile remain poorly characterized. This descriptive study assessed whether the serum pattern of estrogen-deficiency bone loss develops and becomes established by week 12 in this model, quantifying four markers of estrogenic status and bone turnover at baseline and at 12 and 20 weeks after ovariectomy. Ten adult female New Zealand White rabbits (*Oryctolagus cuniculus*) underwent bilateral ovariectomy; three additional animals provided baseline reference values. Estradiol, parathyroid hormone, the beta-isomerized C-terminal telopeptide of type I collagen (β -CTx, resorption), and osteocalcin (formation) were measured by chemiluminescence immunoassay. Changes over time were assessed with the Friedman test and Wilcoxon pairwise comparisons with Holm correction. All markers changed significantly. Relative to baseline, by week 12 estradiol had already decreased and β -CTx and osteocalcin had already increased significantly, becoming more pronounced by week 20: estradiol fell by 91 %, β -CTx rose by 145 %, and osteocalcin by 68 %, whereas parathyroid hormone fell by 39 %. The resorption/formation ratio increased steadily, indicating a progressive predominance of resorption. Baseline values in ovariectomized animals did not differ from reference values. These findings describe the establishment, from week 12, of a high-turnover state consistent with osteopenia and its progression to osteoporosis in the ovariectomized rabbit and identify β -CTx as the most sensitive marker of the panel.

KEY WORDS: Osteoporosis; Ovariectomy; Bone remodeling; Estradiol.

INTRODUCTION

Osteoporosis is a systemic skeletal disorder characterized by low bone mass and deterioration of bone microarchitecture, leading to increased bone fragility. In women, the decline in ovarian estrogen production at menopause is a driver of accelerated bone loss, because estrogen deficiency shifts bone remodeling in favor of resorption (NIH Consensus Development Panel, 2001; Riggs, 2000).

Estrogen deficiency disrupts the balance of bone remodeling and is associated with changes in serum markers of bone metabolism. Among these, estradiol is the principal indicator of estrogenic status, whereas parathyroid hormone (PTH) participates in the regulation of calcium homeostasis and bone remodeling (Silva & Bilezikian, 2015). Bone turnover markers, in turn, provide a minimally invasive tool for assessing these changes in *in vivo* models: resorption markers such as the beta-isomerized C-terminal telopeptide

of type I collagen (β -CTx) reflect osteoclastic activity, whereas formation markers such as osteocalcin reflect osteoblastic activity (Vasikaran *et al.*, 2011).

Animal models are essential for studying the sequence and magnitude of these changes under controlled conditions. The ovariectomized (OVX) rodent is the most widely used model, but larger species offer bone dimensions and remodeling dynamics closer to the human condition (Reinwald & Burr, 2008). The rabbit (*Oryctolagus cuniculus*) is of particular interest because, unlike rodents, it attains true skeletal maturity, exhibits intracortical Haversian remodeling, and has a faster rate of bone turnover, features that bring its bone biology closer to that of humans (Permuy *et al.*, 2019).

In rodent and rabbit models, several studies have shown that structural bone loss develops from 12 weeks after OVX (Castañeda *et al.*, 2008; Wanderman *et al.*, 2018; Parra

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et al., 2021a, 2021b). However, evidence on the longitudinal course of serum markers of estrogenic status and bone turnover during this process remains limited. In this context, the present study aims to document the biochemical profile consistent with estrogen-deficiency bone loss, providing evidence to support the use of this model in experimental studies.

The aim of this study was to characterize the temporal course of serum markers of estrogenic status and bone turnover in an ovariectomized rabbit model, in order to identify the time point at which a biochemical profile consistent with a high bone turnover state induced by estrogen deficiency becomes established.

MATERIAL AND METHOD

Animals

The animals were managed within a larger project aimed at the morphological characterization of osteoporotic bone and at the application of osteosynthesis systems in the ovariectomized rabbit model, of which the serum data presented here constitute a complementary line and not the sole objective of their experimental management. Thirteen adult female New Zealand White rabbits (*Oryctolagus cuniculus*), approximately 1 year old and weighing around 5 kg, were included and distributed into two groups: an ovariectomized group (OVX; $n = 10$), which underwent bilateral ovariectomy and was followed longitudinally, and a reference group ($n = 3$), from which only baseline values were obtained as a pre-ovariectomy control group. The animal research protocol was approved by the Scientific Ethics Committee of the Universidad de La Frontera (Protocol No. 118/22; Approval Act No. 032-23, 22 March 2023). The sample size corresponded to the animals available for this line within the project and was not derived from a formal power calculation, in accordance with the recommendation of a minimum of five experimental units per group to detect large-magnitude effects in quantitative morphology (Mandarim-de-Lacerda & del Sol, 2017) and with the reduction principle of the 3Rs.

The animals were housed individually in the CEMyQ animal facility under controlled temperature and humidity and a 12-hour light–dark cycle, with pelleted feed and water *ad libitum*, following the management protocol described for rabbit surgery by Olate *et al.* (2019).

Ethical statement

All procedures were performed in accordance with the guidelines for the care and use of laboratory animals, following the ARRIVE 2.0 recommendations and the 3Rs

framework (replacement, reduction, and refinement). Anesthesia was performed with an intramuscular combination of ketamine and xylazine, according to the scheme described by Olate *et al.* (2019) for surgical procedures in rabbits, and animal suffering was minimized. Humanitarian endpoints were defined *a priori* (sustained weight loss, signs of persistent pain or distress, or inability to access food and water). When the planned experimental endpoint was reached, euthanasia was performed under a deep anesthetic plane (ketamine and xylazine, intramuscular), in accordance with the standardized institutional CEMyQ protocol shared across the group's studies (Olate *et al.*, 2019).

Ovariectomy

Estrogen deficiency was induced by bilateral ovariectomy under aseptic conditions. General anesthesia was administered intramuscularly with 10 % ketamine (40 mg/kg) combined with 2 % xylazine (5 mg/kg) and 1 % acepromazine (1 mg/kg); vital signs and clinical parameters were monitored throughout the surgical procedure. Postoperatively, flunixin meglumine (1.1 mg/kg) was administered together with enrofloxacin (5 mg/kg), following the same anesthetic and surgical-access protocol as the group's parallel studies in this model (Olate *et al.*, 2019). In accordance with previous studies (Castañeda *et al.*, 2008; Wanderman *et al.*, 2018), a period of at least 12 weeks after OVX is required for estrogen deficiency to induce bone changes consistent with osteoporosis in the rabbit model. The animals were monitored daily by trained personnel during recovery.

Serum sampling and biochemical analysis

To characterize the biochemical course of the model longitudinally, serum samples were obtained at three time points: baseline, before ovariectomy (initial = T0), at 12 weeks (T1), and at 20 weeks (T2) after surgery. These time points were selected to assess the establishment and progression of the changes associated with estrogen-deficiency bone turnover, given that previous studies have described significant bone loss in this model from 12 weeks onward (Castañeda *et al.*, 2008; Wanderman *et al.*, 2018).

To control for circadian variation in bone turnover markers, all blood draws were performed in the morning at a fixed, predetermined time. The collected blood was centrifuged to separate the serum, and the aliquots were stored at -80°C until analysis. Before processing, the samples were thawed gradually (-20°C and then 4°C) and brought to room temperature ($15 - 20^{\circ}\text{C}$) before being loaded into the analyzer.

Estradiol, PTH, β -CTx, and osteocalcin were measured by chemiluminescence immunoassay on a MAGLUMI 600 analyzer (SNIBE, Shenzhen New Industries Biomedical Engineering Co., Ltd., Shenzhen, China), following the manufacturer's instructions; during measurement, the instrument maintains incubation at approximately 37 °C and the sample and reagent compartment at approximately 12 °C. The analytical limits declared in each assay insert were: estradiol, limit of blank (LoB) 5.0 pg/mL, limit of detection (LoD) 12 pg/mL, and measuring range 5.0 - 6000 pg/mL; PTH, LoB 1.0 pg/mL, LoD 1.5 pg/mL, and range 1.0 - 5000 pg/mL; osteocalcin, LoB 0.13 ng/mL, LoD 0.15 ng/mL, and range 0.13 - 300 ng/mL; and β -CTx, LoD 0.010 ng/mL, limit of quantification 0.030 ng/mL, and linear range 0.030 - 6.00 ng/mL.

All measurements fell within the quantifiable range of the respective assay. Estradiol and PTH are expressed in pg/mL, and β -CTx and osteocalcin in ng/mL, according to the International System of Units. It should be noted that these immunoassays are validated and calibrated against human serum and that cross-reactivity in *Oryctolagus cuniculus* has not been formally established (estradiol being the analyte with the greatest cross-species tolerance); therefore, absolute concentrations should be interpreted within the model and not against human clinical reference ranges.

Statistical analysis

The individual animal was the experimental unit ($n = 10$), and descriptive statistics are presented as mean $\pm$ standard deviation. Normality was assessed with the Shapiro–Wilk test. Given the descriptive nature of the study and the sample size, non-parametric methods were adopted as the primary analysis: the effect of time across the three time points was assessed with the Friedman test, using Kendall's W as the effect size, and pairwise comparisons between time points with the Wilcoxon signed-rank test and Holm correction, applied to the set of the three temporal comparisons for each marker. As a sensitivity analysis, a repeated-measures analysis of variance was performed. Baseline concentrations of the OVX and reference animals were compared with the Mann–Whitney U test. A two-sided p -value < 0.05 was considered significant. Analyses were performed in GraphPad Prism (version 11.0.2).

RESULTS

All four markers changed significantly across the three time points (Friedman test, $p < 0.001$ in all four cases), with a maximal effect size (Kendall's $W = 1.00$ for all four markers), reflecting a consistent direction of change across all animals. The repeated-measures analysis of variance confirmed the effect of time for each marker (all $p < 0.001$). Descriptive values are summarized in Table I.

Table I. Serum bone turnover markers and their temporal change in ovariectomized rabbits ($n = 10$).

Marker (unit)	Time point	Mean $\pm$ SD	Median (min–max)	IQR (Q1–Q3)	Change (%)	p (Friedman)
β -CTx (ng/mL)	Baseline	0.058 $\pm$ 0.008	0.059 (0.045–0.071)	0.055–0.061	+144.5	< 0.001
	Week 12	0.087 $\pm$ 0.009	0.088 (0.068–0.097)	0.083–0.095		
	Week 20	0.141 $\pm$ 0.014	0.141 (0.107–0.161)	0.140–0.147		
Estradiol (pg/mL)	Baseline	135.1 $\pm$ 32.0	132.5 (90.0–200.0)	111.8–153.0	-91.3	< 0.001
	Week 12	72.0 $\pm$ 21.2	68.2 (45.5–112.0)	56.8–82.5		
	Week 20	11.8 $\pm$ 8.3	10.7 (3.2–28.3)	5.3–14.4		
Osteocalcin (ng/mL)	Baseline	5.39 $\pm$ 1.11	5.29 (3.45–7.12)	4.79–6.23	+68.2	< 0.001
	Week 12	6.96 $\pm$ 1.26	6.96 (4.79–8.69)	6.47–7.74		
	Week 20	9.07 $\pm$ 1.72	8.94 (6.04–11.54)	8.41–10.10		
PTH (pg/mL)	Baseline	6.34 $\pm$ 1.59	6.29 (4.21–8.98)	5.05–7.25	-38.5	< 0.001
	Week 12	4.80 $\pm$ 1.27	4.81 (3.20–6.46)	3.67–5.89		
	Week 20	3.90 $\pm$ 1.11	3.72 (2.50–5.42)	3.04–4.88		
β -CTx/osteocalcin	Baseline	0.011 $\pm$ 0.003	0.010 (0.009–0.017)	0.009–0.011	—	< 0.001
	Week 12	0.013 $\pm$ 0.003	0.012 (0.010–0.018)	0.011–0.013		
	Week 20	0.016 $\pm$ 0.004	0.015 (0.013–0.022)	0.013–0.017		

Descriptive statistics of the serum bone turnover markers in the ovariectomized group. Serum concentrations of estradiol, parathyroid hormone (PTH), β -CTx, and osteocalcin, and the resorption/formation ratio (β -CTx/osteocalcin), at baseline and at 12 and 20 weeks after ovariectomy; values are presented as mean $\pm$ standard deviation, median (minimum–maximum), interquartile range (IQR, first–third quartile), the percentage change between baseline and week 20, and the overall significance of the effect of time ($n = 10$). All markers changed significantly (Friedman test, $p < 0.001$; Kendall's $W = 1.00$ for all four markers); all pairwise comparisons between time points were significant (Wilcoxon signed-rank test with Holm correction, $p = 0.006$, the minimum attainable value with $n = 10$). Estradiol and PTH are expressed in pg/mL, and β -CTx and osteocalcin in ng/mL. β -CTx, beta-isomerized C-terminal telopeptide of type I collagen; SD, standard deviation; IQR, interquartile range.

Estradiol decreased progressively from a mean baseline of 135.1 ± 32.0 pg/mL to 72.0 ± 21.2 pg/mL at week 12 and 11.8 ± 8.3 pg/mL at week 20, a reduction of 91.3 % relative to baseline; the decrease was monotonic in all ten animals and was already significant at week 12 ($p = 0.006$ for each comparison). β -CTx increased from 0.058 ± 0.008 ng/mL at baseline to 0.087 ± 0.009 ng/mL at week 12 and 0.141 ± 0.014 ng/mL at week 20, an

increase of 144.5 %, monotonic in all ten animals ($p = 0.006$). Osteocalcin increased from 5.39 ± 1.11 ng/mL to 6.96 ± 1.26 ng/mL and 9.07 ± 1.72 ng/mL, an increase of 68.2 %, also monotonic ($p = 0.006$). Thus, the decrease in estradiol and the rise in both bone markers were already significant at week 12 and became more pronounced toward week 20. The distribution at each time point is shown in Fig. 1.

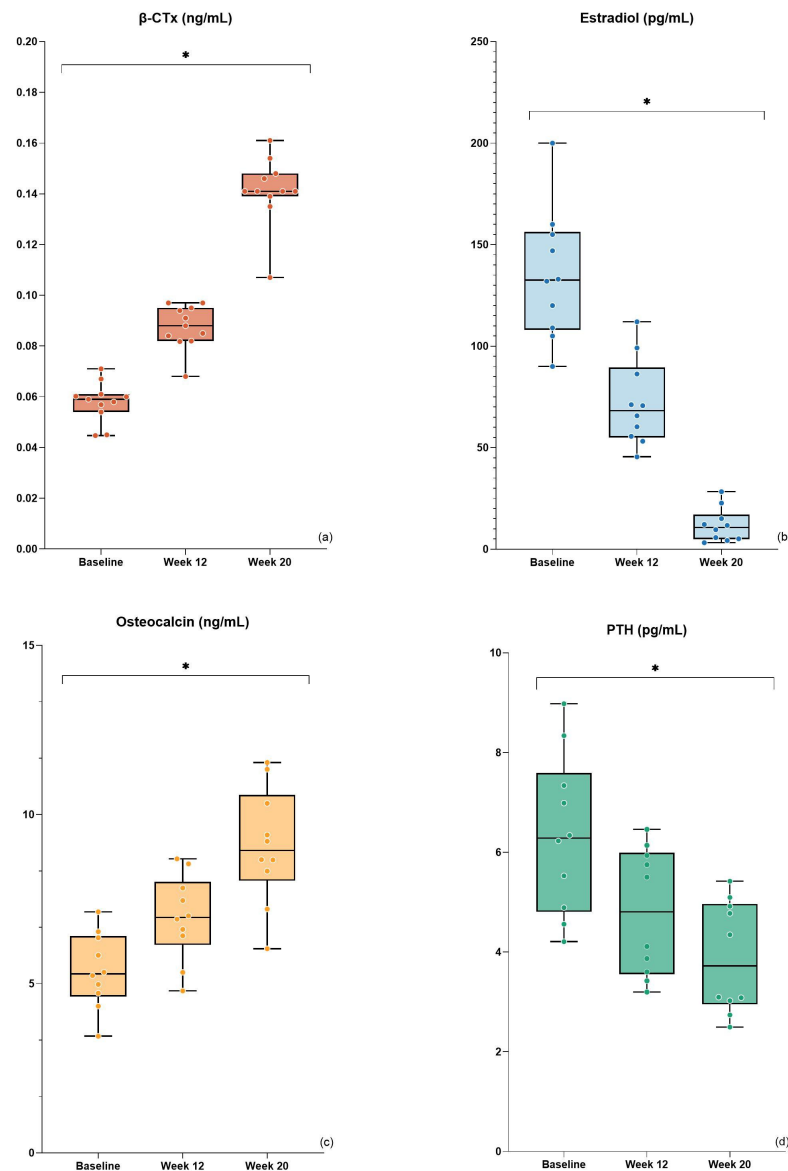


Fig. 1. Serum profile of the bone turnover markers in ovariectomized rabbits at baseline and at 12 and 20 weeks. Box plots of (a) β -CTx, (b) estradiol, (c) osteocalcin, and (d) parathyroid hormone (PTH), measured at baseline (before ovariectomy) and at 12 and 20 weeks after ovariectomy in the ten rabbits of the ovariectomized group. In each box, the central line represents the median, the box limits the first and third quartiles (interquartile range), and the whiskers the minimum and maximum values; each point corresponds to an individual animal. Estradiol and PTH are expressed in pg/mL; β -CTx and osteocalcin in ng/mL. All markers changed significantly over time (Friedman test, $p < 0.001$); the asterisk (*) indicates that all pairwise comparisons between time points were significant (Wilcoxon signed-rank test with Holm correction, $p = 0.006$). β -CTx, beta-isomerized C-terminal telopeptide of type I collagen; PTH, parathyroid hormone.

PTH decreased progressively from 6.34 ± 1.59 pg/mL at baseline to 4.80 ± 1.27 pg/mL at week 12 and 3.90 ± 1.11 pg/mL at week 20, a reduction of 38.5 %, decreasing monotonically in all ten animals; all pairwise comparisons were significant ($p = 0.006$).

At baseline, serum concentrations of the ovariectomized animals did not differ significantly from those of the reference animals for any marker (Mann–Whitney U test, all $p > 0.39$), indicating comparable pre-intervention values.

Concurrently, the decrease in estradiol was accompanied by the rise in β -CTx and osteocalcin in the same animals and at the same time points. The individual trajectories of each of the four markers, with one line per animal, illustrate the consistency of this coordinated temporal behavior: the decreases in estradiol and PTH and the increases in β -CTx and osteocalcin were monotonic in all ten animals (Fig. 2). The resorption/formation ratio (β -CTx/osteocalcin) increased progressively from 0.011 ± 0.003 at baseline to 0.013 ± 0.003 at week 12 and 0.016 ± 0.004 at week 20, indicating a relative and progressive predominance of resorption throughout the study period (Fig. 3).

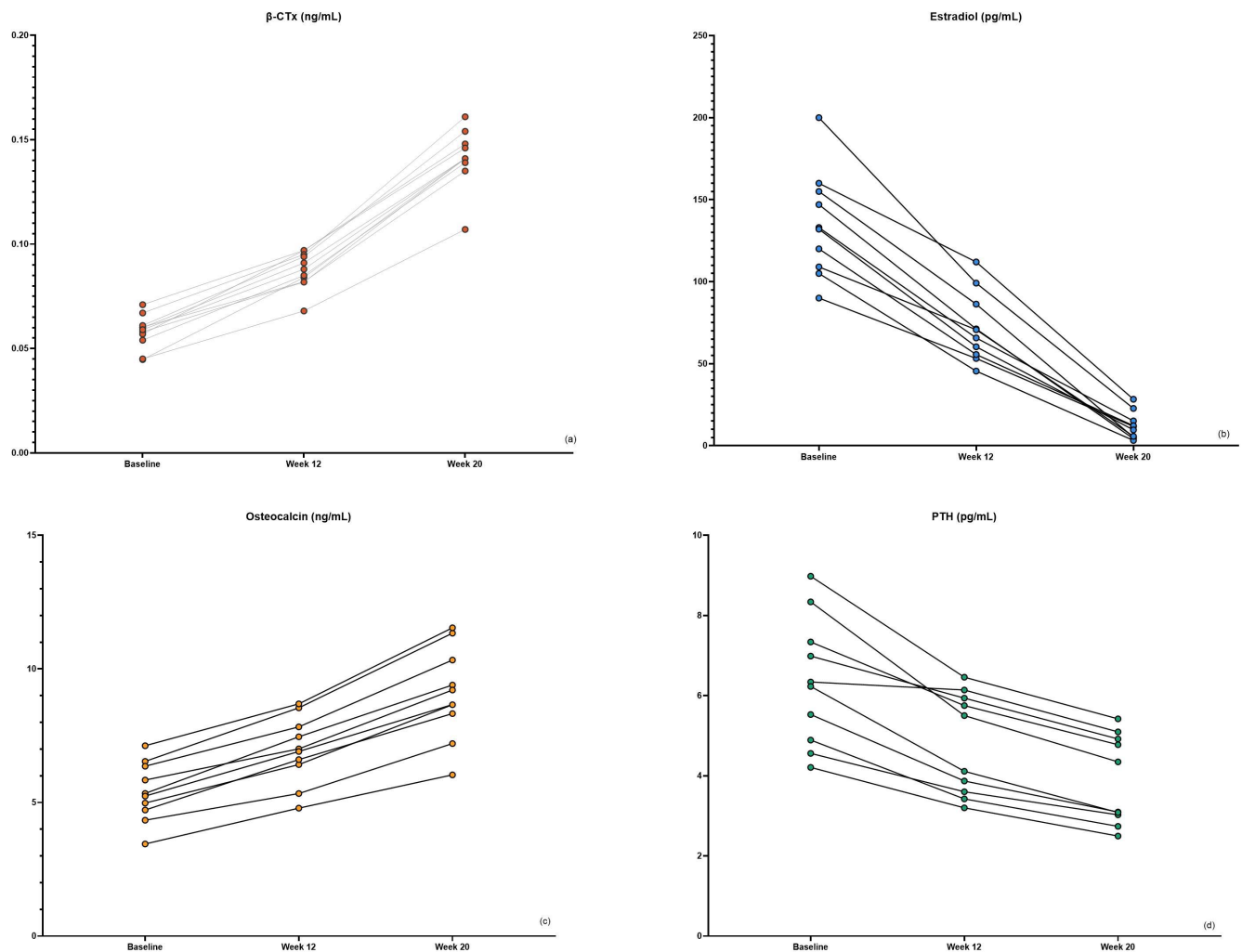


Fig. 2. Individual trajectories of the bone turnover markers over time. Individual course of (a) β -CTx, (b) estradiol, (c) osteocalcin, and (d) PTH in each of the ten ovariectomized rabbits, at baseline and at 12 and 20 weeks after ovariectomy; each line joins the three serial measurements of the same animal. Estradiol and PTH are expressed in pg/mL; β -CTx and osteocalcin in ng/mL. The decrease in estradiol and PTH and the increase in β -CTx and osteocalcin were monotonic in all ten animals (complete agreement across individuals; Kendall's $W = 1.00$), illustrating the consistency of the change over the follow-up.

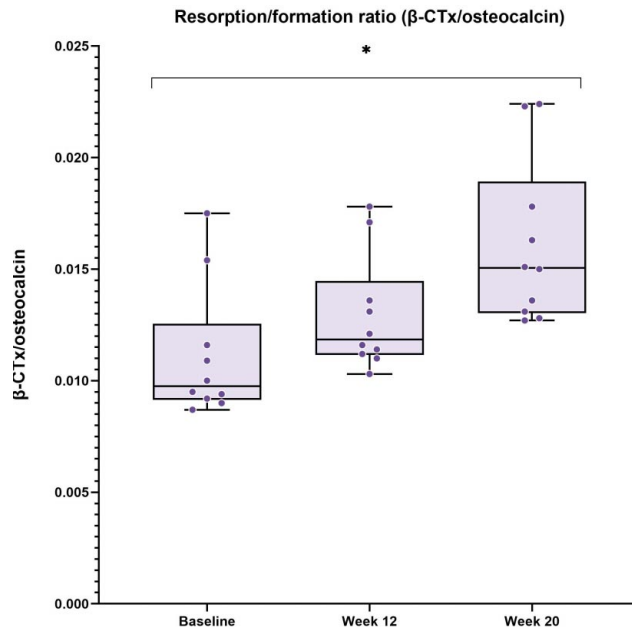


Fig. 3. Resorption/formation ratio (β -CTx/osteocalcin) over time. Box plots of the ratio between the resorption marker and the formation marker in each animal, at baseline and at 12 and 20 weeks after ovariectomy ($n = 10$). In each box, the central line represents the median, the box limits the first and third quartiles, and the whiskers the minimum and maximum values; each point corresponds to one animal. The ratio increased progressively over the follow-up (Friedman test, $p < 0.001$); the asterisk indicates that all pairwise comparisons between time points were significant (Wilcoxon signed-rank test with Holm correction, $p = 0.006$). β -CTx, beta-isomerized C-terminal telopeptide of type I collagen.

DISCUSSION

The present study described the temporal serum profile of bone turnover in the ovariectomized rabbit and showed that estrogen withdrawal induces, as early as week 12, a coordinated pattern of high-turnover bone loss: a marked and progressive decrease in estradiol accompanied by a rise in the resorption marker β -CTx and the formation marker osteocalcin, together with a decrease in parathyroid hormone. This pattern, which becomes more pronounced toward week 20, is that expected for the development of osteopenia and its progression to osteoporosis due to estrogen deficiency, and places week 12 as the time point at which the altered bone state is already biochemically detectable in this model.

The direction of these changes is consistent with the established role of estrogen in restraining osteoclastic resorption; its withdrawal accelerates resorption and increases the release of type I collagen degradation products such as β -CTx (Riggs, 2000). At the molecular level, estrogen deficiency increases the expression of

RANK ligand (RANKL) in bone marrow cells, a direct determinant of the increase in resorption (Eghbali-Fatourehchi *et al.*, 2003). The concurrent rise in osteocalcin indicates that formation was also elevated rather than suppressed, reflecting the coupled high-turnover state characteristic of early estrogen-deficiency bone loss rather than a purely resorptive process; this coupling between resorption and formation within the basic multicellular unit is a well-characterized physiological phenomenon (Sims & Martin, 2014). The sustained increase in the β -CTx/osteocalcin ratio shows, however, that resorption gained relative predominance over the interval, consistent with the negative bone balance that underlies bone loss in this context (Riggs, 2000).

Among the four analytes, β -CTx showed the greatest relative change and a strictly monotonic increase in each animal, which identifies it as the most sensitive marker of the panel and an appropriate primary outcome for future studies in this model. This is consistent with the use of β -CTx as the reference resorption marker in clinical and experimental osteoporosis research (Vasikaran *et al.*, 2011); indeed, β -CTx (CTX-I) and PINP are the resorption and formation markers recommended for clinical use because of their performance and reproducibility (Eastell & Szulc, 2017). The coordinate rise in resorption and formation markers is congruent with findings in the ovariectomized rat, in which serum osteocalcin and β CTx increase after ovariectomy and rise progressively over time (Yoon *et al.*, 2012; Guo *et al.*, 2022). The same pattern of decreasing estradiol and increasing bone markers has been documented in ovariectomized sheep followed longitudinally (Newton *et al.*, 2004; Cabrera *et al.*, 2018) and in the ovariectomized rabbit followed over comparable intervals (Qiu *et al.*, 2019), reinforcing the consistency of bone loss development. The greater relative increase in β -CTx observed here, compared with some reports in rodents, should be interpreted in light of the longer follow-up (20 weeks), the faster intracortical remodeling of the rabbit, and the longitudinal within-subject design, which references each animal to its own baseline value.

The progressive decrease in parathyroid hormone should be interpreted with caution. It is compatible with intact calcium homeostasis in the presence of increased skeletal resorption; indeed, in early estrogen deficiency the increase in bone turnover occurs without a concurrent rise in parathyroid hormone, which increases only later and indirectly (Khosla *et al.*, 1997). Calcium and phosphate were not measured, which limits the interpretation of the calcium-regulating axis and constitutes a limitation to be addressed in future work, together with the absence of a concurrent non-ovariectomized group followed over the same interval,

since the reference animals provided only baseline values. The sample size, although sufficient to reveal large and consistent changes, was small and corresponded to the animals available within the project; the study is therefore descriptive and hypothesis-generating. Because the repeated measurements of each animal are not independent, the study describes the temporal trajectory of each marker separately rather than computing correlations between markers. The assays were calibrated against human serum, so absolute concentrations should be interpreted within the model. In addition, β -CTx shows marked circadian variation and decreases with food intake, which was controlled by collecting all samples early in the morning at a fixed time (Qvist *et al.*, 2002).

Within these limitations, the coordinated pattern of the markers and its temporal consolidation provide a biochemical characterization of the development of osteopenia and osteoporosis in the ovariectomized rabbit from week 12, complementing morphological and histomorphometric approaches and situating the model within the broader study of osteoporotic bone.

CONCLUSION

It can be concluded that bilateral ovariectomy in the rabbit produces a coordinated, progressive, and consistent change in serum markers, with a decrease in estradiol, an increase in β -CTx and osteocalcin, and a decrease in parathyroid hormone, detectable from week 12 and accentuated toward week 20. This biochemical profile describes the early establishment of a high-turnover state consistent with osteopenia and its potential progression to osteoporosis due to estrogen deficiency in this model, with β -CTx as the most sensitive marker. Further studies should characterize the calcium-PTH axis and incorporate a non-ovariectomized group followed in parallel to confirm these findings.

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BRITO, L. L. & OLATE, S. Evaluación de biomarcadores séricos en un modelo de osteopenia en *Oryctolagus cuniculus* ovariectomizado. *Int. J. Morphol.*, 44(3):1047-1054, 2026.

RESUMEN: La deficiencia de estrógenos acelera el recambio óseo y subyace a la osteoporosis posmenopáusica. El conejo ovariectomizado es un modelo de esta condición, pero el momento en que se instaura, la pérdida ósea y su perfil bioquímico

temporal han sido poco caracterizados. Este estudio descriptivo evaluó si el patrón sérico de la pérdida ósea por deficiencia de estrógenos se desarrolla y se establece desde la semana 12 en este modelo, cuantificando cuatro marcadores del estado estrogénico y del recambio óseo en el estado basal y a las 12 y 20 semanas tras la ovariectomía. Diez conejas adultas New Zealand White (*Oryctolagus cuniculus*) fueron sometidas a ovariectomía bilateral; tres animales adicionales aportaron valores de referencia basales. El estradiol, la hormona paratiroidea, el telopéptido C-terminal beta-isomerizado del colágeno tipo I (β -CTx, resorción) y la osteocalcina (formación) se cuantificaron por inmunoensayo de quimioluminiscencia. Los cambios en el tiempo se evaluaron con la prueba de Friedman y comparaciones pareadas de Wilcoxon con corrección de Holm. Todos los marcadores cambiaron significativamente. Respecto del estado basal, en la semana 12 el estradiol ya había descendido y el β -CTx y la osteocalcina ya habían aumentado de forma significativa, acentuándose en la semana 20: el estradiol disminuyó un 91 %, el β -CTx aumentó un 145 % y la osteocalcina un 68 %; la hormona paratiroidea disminuyó un 39 %. La razón resorción/formación aumentó de forma sostenida, indicando un predominio progresivo de la resorción. Los valores basales de los animales ovariectomizados no difirieron de los de referencia. Estos hallazgos describen el establecimiento, desde la semana 12, de un estado de alto recambio compatible con osteopenia y su progresión a osteoporosis en el conejo ovariectomizado e identifican al β -CTx como el marcador más sensible del panel.

PALABRAS CLAVE: Osteoporosis; Ovariectomía; Remodelación ósea; Estradiol.

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