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Artículo original

Extracción del calcio obtenido de residuos agroindustriales y evaluación preliminar de su bioactividad en ratas ovariectomizadas

Extraction and preliminary assessment of calcium obtained from hen eggshells in ovariectomized rats.

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Título abreviado: Revalorización del calcio en ratas ovariectomizadas.

RESUMEN
Este estudio aborda la necesidad de adoptar prácticas sostenibles para el uso de cáscaras de huevo (CH) de gallina resultantes de la producción agroindustrial ante los desafíos de reducir los contaminantes ambientales y sus efectos en la salud pública. Las CH son ricas en calcio y constituyen una materia prima con potencial nutricional o terapéutico, tanto en la salud humana como animal. Por ese motivo, el objetivo de este estudio fue explorar un proceso para reutilizar las CH, extraer el calcio, caracterizar y evaluar su potencial bioactividad en ratas ovariectomizadas (OVX). Dicha materia prima, se analizó y luego se evaluó su bioactividad (3,5-7,0 mg/kg/día) en ratas OVX y en dos ensayos, preventivo y curativo. El contenido de $CaCO_3$ detectado fue de 98,50 ± 0,09%. Las ratas OVX mostraron cambios significativos ($p<0,05$) en el peso corporal (PC), en ciertos parámetros bioquímicos y desarrollaron alteraciones óseas en el fémur. En el ensayo preventivo, las ratas OVX suplementadas con CaCO3 durante 90 días mostraron cambios beneficiosos en el PC y en los parámetros bioquímicos de interés, en comparación con el grupo OVX sin suplementación. Sin embargo, los parámetros óseos no mostraron diferencias estadísticas significativas ($p>0,05$). En conclusión, el $CaCO_3$ obtenido de la CH, mostró propiedades funcionales en el modelo experimental utilizado, en particular en el ensayo preventivo. No obstante, se requieren más estudios para establecer las dosis adecuadas y el tiempo de suplementación.
Palabras clave: Residuos agroindustriales, Calcio; peso corporal, depleción hormonal, Salud pública.

ABSTRACT
This study addresses the need to adopt sustainable practices for the use of chicken eggshells (ES) resulting from agro-industrial production, in the face of the challenges of reducing environmental pollutants and their effects in public health. ES are rich in calcium and constitute a raw material with nutritional potential for use in animal or human food. Thus, the objective was to explore a process for reusing ES as a raw material for therapeutic purpose. To characterize and evaluate their potential bioactivity in ovariectomized (OVX) rats. Once the ES were analyzed, the effect of supplementation (3.5-7.0 mg/kg/day) on OVX rats was studied in two trials, preventive and curative. The CaCO3 content detected was 98.50 ± 0.09%. The OVX rats showed significant changes ($p<0.05$) in body weight (BW) and certain biochemical parameters and developed osteoporosis. In the preventive trial, the OVX rats supplemented with ES for 90 days showed changes in BW and biochemical parameters of interest compared to the OVX group. However, bone parameters were not altered ($p>0.05$). In conclusion, ES contains a high concentration of CaCO3 and, in the *in vivo* trial, showed functional properties in the experimental model used. However further studies are required to recommend its consumption, establish appropriate dosages, and determine the duration of supplementation.
Key words: Waste, calcium; body-weight, hormone depletion, public health.

RESUMO
Este estudo aborda a necessidade de adotar práticas sustentáveis para o aproveitamento de cascas de ovos de galinha (COG) provenientes da produção agroindustrial, considerando os desafios da redução de poluentes ambientais e seus efeitos na saúde pública. As COG são ricas em cálcio e constituem uma matéria-prima com potencial nutricional para uso na alimentação animal e humana. Portanto, o objetivo foi explorar um processo de reutilização das COG como matéria-prima para fins terapêuticos. O estudo visou caracterizar e avaliar sua potencial bioatividade em ratas ovariectomizadas (OVX). Após a análise das COG, o efeito da suplementação (3,5–7,0 mg/kg/dia) em ratas OVX foi estudado em dois ensaios: preventivo e curativo. O teor de $CaCO_3$ detectado foi de 98,50 ± 0,09%. As ratas OVX apresentaram alterações significativas ($p<0,05$) no peso corporal (PC) e em certos parâmetros bioquímicos, além de desenvolverem osteoporose. No ensaio preventivo, ratas ovariectomizadas (OVX) suplementadas com extrato de COG por 90 dias apresentaram alterações no peso corporal e nos parâmetros bioquímicos de interesse em comparação ao grupo OVX. No entanto, os parâmetros ósseos não foram alterados ($p>0,05$). Em conclusão, o CE contém alta concentração de $CaCO_3$ e, no ensaio *in vivo*, demonstrou propriedades funcionais no modelo experimental utilizado. Contudo são necessários mais estudos para recomendar seu consumo, estabelecer dosagens adequadas e determinar a duração da suplementação.
Palavras-chave: Resíduos, calcio; peso corporal, depleção hormonal, saúde pública.

INTRODUCTION

Eggshells are among the most abundant types of agricultural waste, typically originating from domestic and industrial food processing, this results in environmental pollution and wasted resources. The entire shell accounts for approximately 9% to 12% of the total weight of the egg. It has two components (CES): the eggshell (ES) and the eggshell membrane (ESM) [1]. This shell mainly contains calcium in the form of calcite in a range of 94 to 97 %; however, 37 and 39 % of this calcium can be isolated from chicken eggshells [2]. The other components are $Ca_3 (PO4)_2$ (1%), $MgCO_3$ (1%) and organic matter (4%). The ESM, on the other hand, is a protein structure mainly composed of glycosaminoglycans, elastin, collagen and hyaluronic acid, and other biocomponents [3]. Therefore, both ES and ESM are

suitable for medical applications and for the development of dietary supplements [4]. ES can be used as an inexpensive source of Ca^{2+} for treating or preventing bone diseases, and in the development of biomaterials for bone tissue replacement [5]. Human studies have shown that supplementation with dairy products containing eggshell powder significantly increased bone mineral density of the lumbar spine, proximal femur and trochanter [6-8]. Studies on rats fed a high-fat diet showed that supplementation with a low dose of Ca^{2+} in the form of ES reversed obesity-related disorders [9]. It produced a significant improvement in lipid profile, liver enzymes, renal functions, and parathyroid hormone (PTH) levels, as well as antioxidant response [6-9]. Although studies on the bioavailability of calcium from ES are scarce, it has been reported to be similar to that of commercial calcium supplements in salt form, such as calcium carbonate [7]. However, there is controversy about its administration and arguments against it include the occurrence of adverse cardiovascular and gastrointestinal events, as well as the occurrence of kidney stones [10]. The discrepancies in the conclusions of the aforementioned studies could be explained by the differences in experimental design, study subjects, calcium source dosage and administration time. Therefore, this research study focuses on obtaining and using ES as a renewable source of Ca^{2+} to reduce osteoporosis-related complications, using an animal model of osteoporosis (ovariectomised rats), specially for controlling weight and lipid profile after menopause.

MATERIALS AND METHODS

White eggshells, i.e. the raw material (*Gallus gallus* domesticus, L.), were obtained from an agricultural producer of Santa Fe city, Santa Fe province, Argentina. To separate the different parts of the husk, i.e. ESM and ES from CES, the following methodology was applied. First, the ES was sanitised and crushed, and then a flotation process was done to separate the ESM from the solid (ES) by injecting an air-water mixture [3]. Both fractions were then dried in an oven. Effective separation requires optimising the particle size after grinding and the design conditions of the flotation system. The separated material was then washed with distilled water and then subjected to treatment with a 0.45 M sodium chloride solution at 4 °C in a shaking vessel to remove unwanted compounds. Subsequently, the sample was washed once more with distilled water, and oven-dried for 24h at 180 °C to minimise microbiological contamination. Finally, was ground in a micronizing mill. The moisture content of the obtained sample was determined using the AOAC standard method [11]. Microbiological characterisation was performed using a bacteriological kit to determine the presence of total coliforms and Salmonella [3]. The content of metals such as Ca, Mg, Fe and Si, was determined using inductively coupled plasma optical emission spectroscopy (ICP-OES, in a Perkin Elmer, Optima 2100 DV) instrument, after digestion in a 1:5 (V/V) nitric: perchloric acid solution.

Animals. Twenty-eight six-month-old female Wistar rats (262-275 g) were housed in a temperature-controlled room (22±1 °C) under a 12:12 light/dark cycle with free access to food (Ganave Argentina), and water for a one-week adaptation period prior to surgery. At the end of this period, twenty rats underwent bilateral ovariectomy (OVX) and eight underwent a sham-operation (SHAM), with the ovaries left intact [12]. The rats were anaesthetised with ketamine and xylazine for surgery. Ten days after surgery, they were randomly divided into two study groups. A) Study group 1 (treatment of established osteopenia), 90 days after surgery, the rats were divided into three groups of four animals each, a SHAM group and two OVX groups supplemented with ES (3.5-7.0 mg/kg/BW per day). B) Study group 2 (prevention of hormone depletion-induced osteopenia), groups of four rats each, as follows: SHAM, OVX not supplemented, OVX + lower dose of ES, and OVX + higher dose of ES. ES supplementation was administered orally for 90 days, either 10 days after the start of the study

(study 2), or 90 days after castration (Study 1). Calcium dose, was 3.5-7.0 mg/kg/BW per day. All experimental procedures were approved by the Animal Experimentation Ethics Committee (Cicual-Med-UNNE Res 0011/21).

Blood collection and organ weight. On day 90 of both studies, the rats were weighed and bled by cardiac puncture. The blood was collected in heparinised tubes for serum clinical chemistry analysis. The blood was then centrifuged at 2500 × g to separate the serum and was stored in aliquots at −20◦C until the assays were done. The spleen, heart, liver and kidneys were then removed and their weights recorded in grams. In addition, both femurs were dissected for histological analysis.

Serum biochemistry determination. Total cholesterol (TC), high density lipoprotein (cHDL), triglycerides (TG), transaminase (GOT), alkaline phosphatase (ALP), creatinine, and calcium plasma concentrations obtained were assayed using commercial enzymatic kits (Weiner®, Rosario, Santa Fe, Argentina), according to the manufacturer's specifications [3].

Specimen processing and staining. The liver and kidney samples were fixed in 10% neutral buffered formalin and dehydrated in a graded alcohol series, cleared in xylene, and finally embedded in paraffin. The sections were stained with haematoxylin and eosin (H&E, Sigma-Aldrich) according to the standard method [3]. The bones were decalcified in an acid solution and coloured using the previously described methodology. Tissue sections were examined using an optical microscope, with a magnification between 4x and 40x, for histological evaluation.

Statistical analyses. Results were evaluated using Duncan's multiple range test using INFOSTAT software (UNC-Argentina) with a *P* value of ≤0.05 being considered significant.

RESULTS

The composition of the obtained raw material is shown in **Table 1.** ES is mainly composed of calcite ($CaCO_3$ 98.50%), with small percentages of magnesium carbonate, iron carbonate (0.20%) and Si (0.20%). Microbiological analysis of the final product (ES) yielded microbial counts, in colony-forming units, below the standard specified detection limits (<10 CFU/g), except for salmonella, which was not detected. These results are consistent with those reported in the literature and suggest that eggshell waste processed in a similar method developed here is safe for animal health [4, 5].

Food intake and body weight gain. As shown in **Table 2**, the OVX group gained more weight ($P < 0.05$) than the SHAM group, and more than the ES-supplemented group (with both higher and lower doses). However, in the curative trial, ES supplementation had no significant effect on this parameter (data not shown). Still, food intake did not vary between groups throughout the experiment, suggesting that ES could attenuate OVX-induced body weight gain [12-15]. The animals appeared generally healthy, with no signs of disease or lethargy, none of them died during the study. As can be seen in **Table 3**, liver and kidneys weights of OVX animals supplemented with ES were greater than those of the SHAM control group ($P<0.05$); this effect was only observed in animals exposed to the curative trial. Yet no morphological or histopathological changes were observed in these tissues.

Biochemical analyses. As shown in Table 4, OVX resulted in a significant increase in serum TC and ALP levels, as well as a reduction in creatinine levels, compared to the SHAM group ($p < 0.05$). In contrast, ES supplementation decreased TG levels in both, preventive and curative trials, compared to

the non-supplemented OVX group ($p < 0.05$). ES supplementation also reduced TC and alkaline phosphatase levels in rats exposed to the preventive trial compared to the non-supplemented OVX group. An increase in creatinine levels was detected in animals supplemented with ES, compared to the OVX group, but remained within the reference range in the SHAM group (**Table 4**). In contrast, in the curative trial, ES was found not to reverse the levels of ALP or creatinine in OVX supplemented animals compared to the SHAM group.

Histological studies. No significant differences were observed between heart, liver and kidney samples. These samples came from each group (data not shown). Microscopic evaluation of liver tissue from control rats, revealed normal liver parenchyma, including hepatic cords, central veins and portal areas. Similarly, ES-supplemented rat livers showed a preserved structure, with normal hepatic lobules. Macroscopically, the kidneys presented a clear boundary between the cortex and medulla, indicating an intact structure in all experimental groups. Likewise, the renal capsule was not firmly attached to the parenchyma. Microscopic examination of renal tissue also revealed normal cortical and medullary structures, including renal tubules and glomeruli, with no histopathological changes observed.

Femur histology. Light microscopy images of the SHAM (surgery) group showed a cortical zone formed by compact bone of adequate thickness and the osteon complexes, with central and lateral canals and corresponding irrigation. In addition, each central canal was surrounded by a bone matrix in which osteocytes were arranged in lamellae, which is characteristic of lamellar bone. Moving from the cortex towards the centre of the bone, there is a preserved cancellous bone, periosteum and endosteum. These consist of dense connective tissue containing osteoblasts and bone lining cells, constituting the haematopoietic bone marrow. In contrast, in unsupplemented OVX rats, a significant decrease of the cortical bone thickness can be observed (data not shown). The bone begins to be replaced by cancellous bone and the osteone complex presents structural alterations. The osteocytes lose their concentric organisation around the central canals of the osteons. The continuity of both the periosteum and endosteum around the bone matrix is reduced, and cancellous bone with a disorganised bone trabeculae is detected in the central region of the bone. In the bone marrow, less haematopoietic tissue and more unicellular or white adipose tissue can be seen. This evidence of cellular disorganisation can be related to an osteoporotic phenotype, in comparison to the SHAM group (without ovariectomy). However, supplementation with ES for 90 days did not attenuate the effects caused by OVX in either the preventive or curative trials). Macroscopic and microscopic analysis of the femur showed no significant differences in the studied variables (cortical and trabecular bone structure) between the experimental groups in both trials (data not shown).

Table 1. Composition of ES obtained from discarded eggshells.

Sensory properties	**Data**
Appearance/Colour	Powder (>100 mesh)
Chemical composition	
Water, %	1.06
CO_3Ca, %	98.50
CO_3Mg, %	0.20
CO_3Fe, %	0.04
Si, %	0.20
Microbiological analysis	
Total plate count, UFC.g-1	<1000
Salmonella	Negative
Total coliforms, UFC.g-1	<10

Values are means ± standard deviations (n=3).
Some results were expressed as % dry matter.

Table 2. Evolution of body weight (g) during the preventive trial.

Preventive assay	**BWI**	**BWF**
SHAM	160.05±9.00	213.19±7.70
OVX	168.01±6.60	229.22±9.36*
OVX ESLD	170.88±7.01	203.38±8.03#
OVX ESHD	173.41±5.36	205.08±7.42#

Data are given as mean ± SD (n=4). LD= Low dose HD= High dose.
* indicates a significant difference compared to the SHAM group ($p<0.05$).
# indicates a significant difference compared to the OVX group by Tuckey test ($p<0.05$).

Table 3. Evolution of relative organ weight (g) during the curative trial.

Curative assay	**SHAM**	**OVX ES LD**	**OVX ES HD**
Heart	0.31±0.02	0.33±0.04	0.35±0.03*
Kidney	0.37±0.03	0.42±0.06*	0.41±0.04*
Spleen	0.26±0.05	0.28±0.06	0.26±0.03
Liver	3.08±0.06	3.55±0.09*	3.45±0.05*

Data are given as mean ± SD (n=4). LD= Low dose HD= High dose.
* indicates a significant difference compared to the SHAM group by Tuckey test ($p< 0.05$).

Table 4. Effects of ES on the concentrations of some blood biochemical parameters in rats.

Preventive assay	SHAM	OVX	OVX ESLD	OVX ESHD
GLU mg/dL	119.33±19.89	122.0±21.09	109.25±22.56	114.75±24.48
TG mg/dL	95.37±19.14	96.03±14.24	80.25±12.07#	76.25±11.65#
TCmg/dL	54.66±4.83	76.34±5.09*	55.00±3.81#	54.02±2.87#
HDLmg/dL	30.07±2.13	33.51±1.05	33.25±3.06	32.75±2.75
AST UI/L	68.51±16.01	88.55±21.78	73.83±18.94	91.20±24.93
Creat mg /dL	0.77±0.22	0.42±0.05*	0.55±0.14#	0.66±0.16#
ALP UI/L	195.03±58.01	480.78±154.27*	315.25±92.11#	310.75±85.51#
Ca mg/dL	11.06±1.01	12.56±1.01	10.23±1.29#	9.78±1.07#

Data are given as mean ± SD (n=4). LD= Low dose HD= High dose.
* indicates a significant difference compared to the SHAM group ($p< 0.05$).
# indicates a significant difference compared to the OVX group by Tuckey test ($p< 0.05$).

DISCUSSION

The raw material obtained from eggshells contains a high percentage of calcium carbonate (CO_3Ca) and trace elements (CO_3Mg, CO_3Fe, Si) that are essential for the body. As it is harmless, it could be used as an ingredient to address calcium deficiency in other foods or for other health-related purposes. Table 2 shows the results obtained in the preventive trial. The table reveals significant differences in body weight between the SHAM, OVX, and OVX + ES groups. Supplementation reduces the increase in body weight caused by OVX. However, food intake remained unchanged throughout the trial. These findings are consistent with those reported by other researchers, who have observed a negative correlation between calcium intake and body mass index, waist circumference, and hip circumference. This prevents the development of overweight and obesity in rodents and humans [12-15]. Similarly, other researchers found that calcium intake reduces postprandial lipaemia, linking this effect to lower fat absorption and higher faecal lipid excretion. They also found a decrease in adipogenesis gene expression and an increase in the expression in lipolytic gene expression. [16]. These findings could explain, at least in part, the reduction in body weight observed in this study. An increase in the weight of the liver, heart, and kidneys was also observed in OVX + ES animals exposed to the curative trial ($P<0.05$) (Table 3). These organs were macroscopically and microscopically examined, and no morphological or histopathological changes were detected. This could be due to an indirect effect of ES on these organs´ tissues, which could manifest as symptoms or signs not analysed in this study. In fact, high calcium intake has been associated with some adverse effects in susceptible individuals, including kidney stones, myocardial infarction, and acute gastrointestinal discomfort, among others [10]. Regarding biochemical parameters, a significant increase ($p<0.05$) in serum TC and TG levels was observed in OVX rats, compared to the SHAM group. These data are partially consistent with studies showing an unfavourable lipid profile but are characteristic of the most advance stage of hormonal depletion in female rats [6]. Meanwhile, ES supplementation decreased triglyceride levels in both the preventive and curative trials compared with the non-supplemented OVX group ($p < 0.05$). It has been reported that a calcium-rich diet reduces fatty acid synthase enzyme activity and triglyceride levels, while increasing lipolytic activity in rat adipose tissue, resulting in a lower adiposity index [13-15]. Therefore, the observed results could be due to reduced fat absorption in the small intestine (Table 4). ES supplementation also reduced TC in rats exposed to the preventive trial ($P < 0.05$). These findings are consistent with previous studies which found that calcium administration reduces total

cholesterol (TC) and triglycerides (TG) while increasing high-density lipoprotein levels (HDL) in rats [13, 15]. Although the mechanism by which calcium reduces TC remains unclear, this effect may be due to the influence of calcium ions on the precipitation of fatty acids. This reduces the solubilisation capacity of mixed micelles in the diet, thereby decreasing the levels of solubilised (bioaccessible) cholesterol [13-16]. Another possible mechanism involves a decrease in bioaccessible saturated fatty acids, which could suppress cholesterol synthesis in the liver [13-16]. A slight increase in creatinine levels was observed in animals supplemented with ES compared to the OVX group, though these levels remained below the reference range in the SHAM group (Table 4). This finding is consistent with a previous study which indicated a relationship between calcium supplementation and increased blood creatinine levels in healthy adults. The cause is unknown, but this could be due to an indirect effect on renal function or calcium interaction with metabolism [18]. OVX rats also had elevated serum ALP levels. These results are consistent with other studies indicating that hormone depletion could induce an increase in total serum ALP activity because of the high bone turnover characteristic of this life stage. However, ES supplementation decreased ALP levels in OVX rats (Table 4). Suggesting that ES supplementation contributes to attenuating bone turnover caused by OVX. In addition, a decrease in serum calcium levels was observed in OVX animals supplemented with ES compared to OVX group. Yet, given that the results are similar to those of the SHAM group, it is suggested that the reduction could be due to adaptive changes that slow down its release from bone tissue because of OVX. In contrast, the results of the curative trial indicate that there were no significant differences between the SHAM group and the OVX-supplemented group in the analysed variables (except for TG). These findings suggest that early supplementation can mitigate the negative effects of hormone depletion in rats, or that a critical threshold exists beyond which such effects are difficult to attenuate. Macroscopic and microscopic analysis of the femur showed that OVX causes critical changes to the bone matrix of the rat femur and that ES supplementation did not reverse these effects. In this study, the supplementation period was brief, and the ES was administered in its raw form. In other words, it was not enriched with compounds that could promote calcium absorption and fixation [8,9]. Despite this, the results suggest that the rat organism may benefit from the components present in ES (CO_3Ca, CO_3Mg, CO_3Fe and Si), which contribute to multiple biological processes in which these elements are required. These functions include maintaining bone and dental health, cell signalling, the functioning of certain enzymes, muscle and nervous system function, blood clotting, regulation of hormone secretions and cardiovascular health, among others. Therefore, longer-term studies are needed to understand the systemic effects of ES supplementation including its impact on long-term bone metabolism.

CONCLUSION

The developed extraction method proved to be efficient and cost-effective for producing contaminant-free raw material with multiple potential applications in health science. Supplementing rats with ES for 90 days from an early stage mitigated the adverse effects caused by OVX, but did not significantly influence bone structure. It can therefore be concluded that ES produces systemic improvements when administered preventatively, and that it can be used to minimise environmental contamination in different industries. These finding highlight the strong potential to develop a marketable product based on ES, positioning it as a promising resource for start-ups seeking to launch innovative, sustainable solutions. The efficient and cost-effective extraction method enables scalable production of high-purity raw material, supporting commercial feasibility. In addition to its demonstrated biological and material benefits, ES offers versatility for applications in materials science, biomedical products, and environmentally responsible technologies. This creates an opportunity for start-ups to translate scientific evidence into value-added products with clear differentiation in the market, addressing both industrial needs and environmental sustainability.

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