Research Article

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Effect of calcium lactate in standard diet on selected markers of oxidative stress and inflammation in ovariectomized rats

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Abstract: The effectiveness of calcium depends on its source, i.e., salt it is supplied with. This study aimed to determine the effects of calcium lactate in diet on inflammation and oxidative stress markers in ovariectomized rats. A total of 40 female Wistar rats were included in this study, which were divided into four groups. The control group was fed a standard diet, whereas the remaining three groups were ovariectomized and provided a standard diet containing calcium carbonate (OVX), a calcium-deficient diet (OVX_DEF), or a diet containing calcium lactate (OVX_CaL). The nutritional intervention lasted for 12 weeks, and then, the rats were sacrificed. Tissue and blood samples were taken and evaluated for cyclooxygenase 1 (COX-1), cyclooxygenase 2, and thiobarbituric acid reactive substance contents in the liver and serum, and total antioxidant status and lipoxygenase 1 contents only in the serum using enzyme-linked immunosorbent assay. Differences were observed in the effects of calcium carbonate and calcium lactate on the COX-1 content in the serum of ovariectomized rats: a lower COX-1 concentration was observed in the case of the calcium lactate diet. No significant differences were observed for the other parameters.

Keywords: calcium carbonate, calcium lactate, inflammation, oxidative stress, ovariectomy

1 Introduction

Menopause in women is characterized by decreased bone mineral density and increased fragility of the skeleton, due to estrogen deficiency and negative calcium balance [1]. Calcium supplementation during menopause may improve bone mineral density. Calcium can be supplemented via sources such as hydroxyapatite, bone meal, and calcium salts. The most commonly used calcium source is calcium carbonate—inorganic salt, which is commonly found in the earth’s crust, shellfish eggs, oysters, and dark green leafy vegetables. The bioavailability of calcium carbonate is comparable to that of calcium citrate, orange juice fortified with calcium citrate malate, or skim milk. Furthermore, calcium carbonate chelates oxalate and thus prevents the formation of kidney stones [2,3]. It is the most commonly used calcium salt in nutritional intervention research [4–6] and is the chief ingredient of preparations prescribed by orthopedic surgeons and pediatricians (followed by calcium citrate) [7,8]. However, in supplements for postmenopausal women, calcium lactate is commonly used due to its ability to reduce the bone degradation rate [9].

Osteoporosis not only results in the loss of bone mineral mass but also accompanies numerous processes such as oxidative stress and inflammation. It is an inflammatory disease, in which bone resorption is associated with the formation of proinflammatory cytokines and macrophages. Activated leukocytes induce the formation of osteoclasts, and some of the known proinflammatory...
cytokines involved in this process are TNF-α, IL-6, and IL-1 [10]. Cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) – enzymes that lead to the formation of prostaglandins – are also involved in the regulation of inflammation. COX-1 regulates normal body responses and is expressed in most of the cells, whereas COX-2 is only expressed pathologically and is induced by TNF-α, IL-1, and lipopolysaccharide [11]. Therefore, COX-2 plays a key role in response to inflammation. COX-2-stimulated prostaglandin E2 causes inflammation in bone tissue, thus increasing the number of osteoclasts [12]. Although estrogen deficiency is considered a major factor for postmenopausal osteoporosis, oxidative stress also leads to a significant loss of bone mineral mass. An increase in reactive oxygen species (ROS) is associated with aging and regulates bone cell survival [13]. Throughout life, oxidative defense is essential for maintaining bone health, and a reduction in the level of sex hormones weakens oxidative defense and thus increases bone resorption [14]. The imbalance between the antioxidant system and the ROS level is a serious threat to the proper balance between bone structure and bone resorption. Furthermore, monitoring oxidative stress biomarkers is helpful in the diagnosis of postmenopausal osteoporosis [15]. The degree of severity of oxidative stress can be determined by monitoring the concentration of the fat peroxidation products (lipooxygenase 1 [LOX-1] and thiobarbituric acid reactive substances [TBARS]) and total antioxidant status (TAS) [15–17].

Previous studies have reported that an appropriate supplementation of calcium leads to a reduction in inflammation and oxidative stress by lowering the expression of IL-6 and TNF-α and reducing the activity of lipogenesis, as well as restoring the physiological balance between the levels of ROS and antioxidants [18]. Calcium supplementation reduces oxidative stress by increasing the concentration of antioxidant enzymes in the serum [19] and the plasma total antioxidant capacity [20]. In addition, calcium supplementation with other minerals and vitamins lowers the level of malondialdehyde – an indicator of oxidative stress [21,22].

However, whether calcium supplementation improves inflammation and antioxidant status in women under menopausal conditions and whether various calcium salts have a similar effect on the body are not known. Therefore, this study aimed to compare the effects of calcium lactate and calcium carbonate (which was used in the standard diet) on selected markers of inflammation and oxidative stress in ovariectomized rats.

2 Methods

2.1 Materials and reagents

Ingredients of the animal diets and their manufacturers were as follows: choline, cysteine, and water-soluble vitamins were purchased from Sigma-Aldrich (Darmstadt, Germany); minerals were purchased from Alfa Chem (Poznań, Poland); fat-soluble vitamins were purchased from a local pharmacy; corn starch, dextrin, casein, and cellulose were purchased from Hortimex (Konin, Poland); and sucrose and oil rapeseed were purchased from a local grocery store. Enzyme-linked immunosorbent assay (ELISA) kits were purchased from Sun Red (Shanghai, China).

2.2 Animals

Forty 12-week-old female Wistar rats were purchased from the Wielkopolska Center for Advanced Technologies (Adam Mickiewicz University, Poznań, Poland). There were no significant differences in the body weight of the rats. The experiment was carried out in accordance with the guidelines for the care and use of laboratory animals. The animals were kept under appropriate conditions: 12 h light/dark cycle, in individual cages. Before the experiment, the animals were acclimatized for 1 week and were provided ad libitum access to deionized water and feed.

2.3 Experimental protocols

Before the introduction of the modified diets, the animals were divided into four groups of ten rats each. Ovariectomy was performed in three groups (30 rats). Throughout the experiment as well as during the adaptation and weekly recovery after ovariectomy, the rats were fed the standard diet AIN-93M. Then, a 12-week nutritional intervention was introduced. The control group (C) was fed the standard diet (standard diet contains calcium carbonate as a source of calcium). The ovariectomized groups were fed as follows: group OVX was fed the standard diet (standard diet contains calcium carbonate as a source of calcium), OVX_DEF group was fed calcium-deficient diet (obtained by not adding
calcium in the standard diet), OVX_CaL was fed the diet with calcium lactate. Calcium salt was added to ensure a constant level of calcium in all diets (except in the deficient one), i.e., about 5 g/kg, which is the same as in the standard AIN-93M diet. The schematic of the experiment is illustrated in Figure 1. Body weight of the rats was measured weekly, and their food consumption was recorded daily. In addition, the rats were subjected to a body composition analysis (Bruker LF90II) as a part of the experiment. After blood sampling, serum was obtained by centrifugation for 10 min at 4°C at 1,200×g. Then, the liver was isolated, weighed, and washed with saline. The tissues were then stored at −80°C until further analyses.

2.4 Diet analysis

Dietary components were determined as follows: lipids – Soxhlet method (PN-EN ISO 3947: 2001; Soxtec System, Foss Tecator); proteins – Kjeldahl method (AOAC, 1995; Foss Tecator); ash – after complete burning in a furnace muffle (AOAC, 2000); carbohydrates – based on the fat, protein, ash, and water contents; and fiber – using the enzymatic gravimetric method.

2.5 Ca analysis in diets

Before evaluating the calcium content in the diet samples, 1 g of each sample was ashed in a muffle furnace at 450°C, and the samples were dissolved in 1 mol/l nitric acid (Merck, Kenilworth, NJ, USA). The samples were then diluted with deionized water and LaCl₃ (0.5%), and the calcium content was determined using flame atomic absorption spectrometry (AAS-3, Carl Zeiss, Jena, Germany). The apparatus was validated with an accuracy of 92% on a reference material (Brown Bread BCR191, Sigma-Aldrich, St. Louis, MO, USA). Each of the samples was analyzed in triplicate.

2.6 Serum parameters

Concentrations of COX-1, COX-2, LOX-1, TAS, TBARS, and estradiol in the serum and concentrations of COX-1, COX-2, and TBARS in the liver were determined using ELISA kits (SunRed, Shanghai, China). Liver tissues were previously homogenized in phosphate-buffered saline by centrifugation at 7,000×g for 20 min. An infinite F50 spectrometer was used for the analysis (Tecan Group Ltd, Mannedorf, Switzerland).

2.7 Statistical analysis

The Statistica program (StatSoft, Tulsa, OK, USA) was used for statistical analysis. Normality of the distribution of variables was determined using the Shapiro–Wilk test. Tukey’s post hoc test was used to determine the differences between the groups. A p-value of <0.05 was considered statistically significant.
3 Results

The composition of diets is presented in Table 1. The highest calorific value, as well as the highest carbohydrate content, was observed in the calcium-deficient group. This is probably due to adding corn starch as an alternative to calcium carbonate. As expected, the lowest calcium content was observed in the calcium-deficient group. Higher body weight and fat content were observed in the ovariectomized rats, with no differences between the three groups (Table 2). In addition, a significantly lower relative weight of the liver was observed in the OVX_DEF and OVX_CaL groups compared to the control group.

In this experiment, the concentrations of selected parameters related to oxidative stress and inflammation in the serum and liver of the ovariectomized rats were measured (Table 3). The calcium-deficient diet was not found to affect the serum concentration of COX-1 and COX-2, but some changes were observed in the rats fed with different types of calcium salts. In the OVX group, the concentration of COX-1 in the serum was similar to that in the control group, whereas the addition of calcium lactate to the diet decreased the concentration of COX-1 in the serum. Calcium lactate significantly decreased the concentration of COX-1 in the serum compared to the calcium carbonate in the diet: OVX and C groups. Calcium lactate also increased the concentration of COX-2 in the serum compared to the control group.

4 Discussion

The results showed that there are differences in the effects of calcium lactate and standard diet with calcium carbonate on cyclooxygenases in ovariectomized rats, which is a novel finding of this study.

Calcium lactate significantly decreased the serum COX-1 concentration in ovariectomized rats, which may indicate less inflammation in this group. Moreover, the COX-2 concentration in the liver was the lowest (but not significantly) in the calcium lactate group, which may confirm the beneficial effect of calcium lactate. Similarly, in the study by Cha et al. in rats on a high-fat diet, supplementation with organic and inorganic calcium resulted in

Table 1: Composition of diets (mean ± standard deviation)

<table>
<thead>
<tr>
<th>Components</th>
<th>Diets</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Calcium carbonate (standard diet) (C/OVX)</td>
</tr>
<tr>
<td>Caloric value (kcal/100 g)</td>
<td>326.37 ± 4.48</td>
</tr>
<tr>
<td>Carbohydrates (g/100 g)</td>
<td>47.92 ± 0.60</td>
</tr>
<tr>
<td>Fiber (g/100 g)</td>
<td>23.04 ± 0.60</td>
</tr>
<tr>
<td>Fat (g/100 g)</td>
<td>3.76 ± 0.41</td>
</tr>
<tr>
<td>Protein (g/100 g)</td>
<td>13.70 ± 0.21</td>
</tr>
<tr>
<td>Ca (mg/g)</td>
<td>5.63 ± 0.37</td>
</tr>
</tbody>
</table>

C, control group; OVX, ovariectomized group with standard diet; OVX_DEF, calcium-deficient ovariectomized group; OVX_CaL, ovariectomized group with calcium lactate.

Table 2: Daily intake and body composition in rats (mean ± standard deviation)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>C</th>
<th>OVX</th>
<th>OVX_DEF</th>
<th>OVX_CaL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily intake of diet (g)</td>
<td>25.08 ± 0.63</td>
<td>25.11 ± 1.70</td>
<td>26.14 ± 1.87</td>
<td>25.90 ± 0.55</td>
</tr>
<tr>
<td>Daily intake of calcium (mg)</td>
<td>141.12 ± 3.56b</td>
<td>141.30 ± 9.57b</td>
<td>16.77 ± 1.20a</td>
<td>147.03 ± 3.11b</td>
</tr>
<tr>
<td>Body mass (g)</td>
<td>325.86 ± 25.97a</td>
<td>421.90 ± 55.10b</td>
<td>441.00 ± 70.97b</td>
<td>428.40 ± 51.10b</td>
</tr>
<tr>
<td>Fat tissue (%)</td>
<td>36.95 ± 8.63a</td>
<td>54.43 ± 10.26b</td>
<td>55.73 ± 11.11b</td>
<td>58.59 ± 5.89b</td>
</tr>
<tr>
<td>Relative weight of liver (%)</td>
<td>2.82 ± 0.24b</td>
<td>2.49 ± 0.68ab</td>
<td>2.21 ± 0.17a</td>
<td>2.22 ± 0.14a</td>
</tr>
</tbody>
</table>

C, control group; OVX, ovariectomized group with standard diet; OVX_DEF, calcium-deficient ovariectomized group; OVX_CaL, ovariectomized group with calcium lactate.

a,b Significant differences between groups (p < 0.05).
a reduction in inflammation, which was attributable to a change in the composition of the intestinal microbiota [23]. Increases in the serum COX-2 level in rats with calcium lactate observed in this study may be a result of ovariectomy and the diet because changes were observed between the control group (without ovariectomy) and the ovariectomized group with calcium lactate. These findings need to be further explained in future experimental and clinical studies. Several authors did not observe any influence of calcium on inflammation biomarkers and did not find any difference between the calcium salts used. Amini et al. reported no significant changes in the levels of inflammatory markers (IL-6, TNF-α) after nutritional intervention with calcium carbonate [24]. In patients with colorectal cancer, the possible anti-inflammatory effects of organic and inorganic calcium salts (carbonate and citrate) were compared, but no significant changes in the levels of inflammatory markers (CRP, proinflammatory interleukins) were observed [25]. However, in rats with nonalcoholic fatty liver disease (NAFLD), calcium carbonate inhibited the synthesis of fatty acids in the liver, which reduces inflammation [26]. As reported in previous studies, ovariectomy is related to inflammation [27–29], and COX-2 is activated by factors related to inflammation [30–32]. In this study, we did not observe significant influence of ovariectomy on inflammation markers in rats (results in C and OVX were comparable). It was observed that calcium-deprived diet slightly decreased COX-1 and COX-2 levels in serum and liver in ovariectomized rats. Disorders in extracellular calcium ion levels may be involved in these results because calcium ions are implicated in the pathophysiology of immune stress and regulate COX-2 expression [33].

In this study, the calcium salts did not affect the biomarkers of oxidative stress. However, minor associations were found. Lower serum TAS values were recorded in the groups with high levels of COX-2 in the serum. However, Rabbani et al. reported that the reduction in oxidative stress is positively correlated with the concentration markers of inflammation such as COX-2, IL-8, and CCL2 proteins [34]. The lowest concentration of TAS was observed in the ovariectomized group with calcium carbonate or calcium lactate, which may indicate an increase in oxidative stress in these groups, but it may be a consequence of ovariectomy [35,36]. Yang et al. reported that calcium supplementation does not reduce oxidative stress, which was reflected in the concentration of F2 isoprostanes in the plasma [25]. Another marker of oxidative stress is the concentration of TBARS—a fat degradation product whose concentration increases with the growth of ROS [37]. In the present study, the highest serum TBARS concentration was observed in the group fed with calcium lactate, which corresponds to a high concentration of COX-2 in the serum. MacDowell et al. reported a similar increase in the COX-2 concentration with a simultaneous decrease in antioxidant properties in patients with borderline personality disorder [38].

The results obtained in this study may be related to changes caused by ovariectomy in rats. Ovariectomy increased body mass as was expected but did not impact inflammation and oxidative stress parameters. The reduction in estrogen levels is the primary factor for the increase in body weight and the adipose tissue (AT) content in postmenopausal women. During the reproductive period, estrogens interact with AT genes, reduce the central mass, and increase the subcutaneous AT content in the femoral

**Table 3: Levels of parameters of oxidative stress and inflammation in rats (mean ± standard deviation)**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>C</th>
<th>OVX</th>
<th>OVX_DEF</th>
<th>OVX_CaL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>COX-1 (ng/mL)</td>
<td>5.16 ± 1.28b</td>
<td>4.86 ± 1.11b</td>
<td>4.22 ± 0.58ab</td>
<td>3.74 ± 0.35a</td>
</tr>
<tr>
<td></td>
<td>COX-2 (ng/mL)</td>
<td>17.04 ± 3.16c</td>
<td>20.41 ± 0.79ab</td>
<td>19.19 ± 3.26ab</td>
<td>21.64 ± 2.22b</td>
</tr>
<tr>
<td></td>
<td>LOX-1 (ng/mL)</td>
<td>8.57 ± 1.19</td>
<td>9.25 ± 1.3</td>
<td>8.32 ± 0.94</td>
<td>8.95 ± 1.45</td>
</tr>
<tr>
<td></td>
<td>TBARS (nmol/mL)</td>
<td>11.91 ± 2.51</td>
<td>10.69 ± 2.36</td>
<td>10.93 ± 2.59</td>
<td>13.14 ± 2.13</td>
</tr>
<tr>
<td></td>
<td>TAS (U/mL)</td>
<td>10.25 ± 2.83</td>
<td>8.7 ± 1.77</td>
<td>9.03 ± 1.69</td>
<td>8.1 ± 1.5</td>
</tr>
<tr>
<td>Liver</td>
<td>COX-1 (ng/g)</td>
<td>25.14 ± 6.03</td>
<td>22.5 ± 7.59</td>
<td>19.48 ± 4.39</td>
<td>24.27 ± 6.77</td>
</tr>
<tr>
<td></td>
<td>COX-2 (ng/g)</td>
<td>53.54 ± 11.23</td>
<td>55.27 ± 17.54</td>
<td>48.56 ± 13.06</td>
<td>44.46 ± 5.18</td>
</tr>
<tr>
<td></td>
<td>TBARS (nmol/g)</td>
<td>39.39 ± 10.01</td>
<td>33.37 ± 4.05</td>
<td>46.4 ± 12.79</td>
<td>44.46 ± 6.64</td>
</tr>
</tbody>
</table>

C, control group; OVX, ovariectomized group with standard diet; OVX_DEF, calcium-deficient ovariectomized group; OVX_CaL, ovariectomized group with calcium lactate; COX-1, cyclooxygenase 1; COX-2, cyclooxygenase 2; LOX-1, lipoxygenase 1; TBARS, thiobarbituric acid reactive substances; TAS, total antioxidant status.

a,bStatistical differences between groups (p < 0.05).
area [39]. Normally, menopause is associated with the development of metabolic diseases, cardiovascular diseases, and type 2 diabetes. In addition, an increase in the AT content and redistribution are observed; lipids from subcutaneous AT penetrate visceral AT and are stored there. Higher body fat is associated with increased adipocyte size and insulin resistance and a higher expression of inflammatory cytokines [40]. Therefore, it can be supposed that ovariectomized rats would have increased levels of COX-2 in both serum and liver, indicating an increase in inflammation, which was not confirmed in the present study. In this study, we observed that ovariectomy and a calcium-deprived diet did not significantly affect markers of inflammation and oxidative stress. However, the source of calcium in the diet may impact cyclooxygenases level and calcium lactate may be more beneficial for COX-1 level than calcium carbonate in menopause.

Due to several limitations of this study, some of the obtained results could not be highlighted here. In this study only selected parameters of oxidative stress and inflammation were analyzed. Some indicators of oxidative stress such as superoxide dismutase and malondialdehyde and other inflammation cytokines were not analyzed because only a limited volume of serum was obtained from rats. Moreover, only two calcium salts: calcium carbonate and calcium lactate were compared in this study.

5 Conclusions

In ovariectomized rats, calcium carbonate and calcium lactate affect the serum COX-1 concentration in different ways. Calcium lactate exhibits a serum-COX-1-lowering effect in ovariectomized rats.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>COX-1</td>
<td>cyclooxygenase 1</td>
</tr>
<tr>
<td>COX-2</td>
<td>cyclooxygenase 2</td>
</tr>
<tr>
<td>CRP</td>
<td>C reactive protein</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>E2</td>
<td>estradiol</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin-1</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>LOX-1</td>
<td>lipoxygenase 1</td>
</tr>
<tr>
<td>NAFLD</td>
<td>nonalcoholic fatty liver disease</td>
</tr>
<tr>
<td>TAS</td>
<td>total antioxidant status</td>
</tr>
<tr>
<td>TBARS</td>
<td>thiobarbituric acid reactive substances</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor</td>
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</table>

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Author contributions: Conceptualization: N.W., J.S.; methodology: J.S., N.W.; software: J.S., N.W.; validation: N.W., J.S.; formal analysis: J.S.; investigation: N.W., P.K., J.S.; resources: A.G.M.; data curation: J.S., N.W.; writing – original draft preparation: N.W., J.S.; writing – review and editing: all authors; visualization: N.W., J.S.; supervision: J.S.; project administration: A.G.M.; funding acquisition: A.G.M. All authors have read and agreed to the published version of the manuscript.

Conflict of interest: The authors have declared that they have no conflict of interest.

Ethical approval: All experimental procedures were performed in accordance with the EU Directive 2010/63/EU for animal experiments. Approval for the study was obtained from the Local Ethics Committee in Poznań (no. 34/2019). The reporting in the manuscript follows the recommendations in the ARRIVE guidelines.

Data availability statement: The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

References


