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The effect of zinc and phytoestrogen supplementation on the changes in mineral content of the femur of rats with chemically induced mammary carcinogenesis

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ABSTRACT

The aim of this study was to assess skeletal effects of zinc or zinc with phytoestrogen (resveratrol or genistein) supplementation in an animal model of rats with DMBA-induced mammary carcinogenesis. The changes in bone parameters such as the length and mass were examined, as well as the changes in concentrations of selected minerals: calcium, magnesium, zinc, iron and phosphorus. Moreover, the investigations focused on finding the differences between the levels of iron and zinc in other tissues: the liver, spleen and serum of the examined rats.

Fifty-six female Sprague–Dawley rats, 40 days old, were divided into four groups, regardless of the diets: standard (77 mg Zn kg/food), zinc (4.6 mg/mL via gavage), zinc (4.6 mg/mL) plus resveratrol (0.2 mg/kg bw), and zinc (4.6 mg/mL) plus genistein (0.2 mg/kg bw) for a period from 40 days until 20 weeks of age. The study rats were also treated with 7,12-dimethyl-1,2-benz[a]anthracene (DMBA) to induce mammary carcinogenesis.

The applied diet and the advanced mammary cancer did not affect macrometric parameters of the rats' bones, but they strongly affected their mineral content. It was found that mammary cancer, irrespectively of the applied diet, significantly modified the iron level in the femur, liver, spleen and serum of the examined rats. In addition, zinc supplementation significantly lowered the levels of calcium, magnesium and phosphorus in the femur of rats with mammary cancer as compared with respective levels in the control group. So, it was found that additional supplementation with zinc, which is generally considered to be an antioxidant, with the co-existing mammary carcinoma, increased the unfavorable changes as concerns the stability of bone tissue. The appropriate combination of zinc and phytoestrogens (resveratrol or genistein) could help prevent or slow bone loss associated with a range of skeletal disorders in breast cancer.

1. Introduction

In the literature numerous studies can be found dealing with the differences in distribution of trace elements in neoplastic tissues as compared with healthy tissues [1–3]. It was found that breast cancer cells accumulate different mineral components. This, in view of their key importance for biochemical processes can intensify the development of the neoplastic process [2]. The bones, which store a large amount of minerals, can be a significant source of minerals for the growing tumor. Mammary cancer exhibits a particularly strong tendency for malignant neoplasm metastasis to the bones [4,5]. This process results in destruction of the osseous tissue. There are also reports saying that the presence of breast tumors can negatively affect the bone structure even in the case when no metastasis to the bones occurred [6]. The anabolic effect of zinc on bone tissue is confirmed by numerous investigations performed with the participation of both humans and rats [7–9]. It has been known for many years that zinc is an essential trace mineral needed for normal growth. This mineral, which takes part in gene expression,

Abbreviations: Ca, calcium; DMBA, 7,12-dimethylbenz[a]anthracene; CK, protein kinase CK2 (formerly casein kinase 2); FAAS, flame atomic absorption spectrometry; Fe, iron; LOD, limits of detection; Mg, magnesium; MNU, *N*-methyl-*N*-nitrosourea; P, phosphorus; PTHrP, parahormone-related protein; RSD, relative standard deviation; ZIP7, transporter ZIP7; Zn, zinc.

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cell proliferation and regulation of apoptosis, can have a direct influence on the development of different forms of cancer. However, the exact mechanism, and in particular the question how it affects the growth of neoplastic cells, is not yet known [10]. Phytoestrogens suggested to have marked chemopreventic properties and anabolic effect on bone metabolism [11,12]. The combination of nutritional factors may have a potent anabolic effect *in vivo*. In this work we examined the effect of zinc, zinc with resveratrol or genistein supplementation and the coexisting neoplastic process on macrometric parameters (length and mass) and the mineral content (Ca, Mg, Fe, Zn and P) in the femur of rats with the chemically induced (DMBA) mammary carcinogenesis. In order to explain the observed changes, the levels of iron was also analyzed in other tissues, namely in the liver, spleen and serum, in comparison with the control rats.

2. Materials and methods

2.1. Animals

Fifty-six 30-days-old Spraque–Dawley female rats of 100 ± 20 g body weight, were subjected to a 10-day adaptation period. The experiment was conducted for 14 weeks (from 40 days until 20 weeks of age). The animals were kept in constant, controlled conditions (temperature 22 ± 1 °C, with a 12-h day/night cycle) with free access to water and laboratory food Labofeed H (produced by Morawski, Kcynia, Poland). The rats were obtained from the Laboratory of Experimental Animals, Department of General and Experimental Pathology, Medical University of Warsaw (Warsaw, Poland). The animal experiments were approved by the Ethics Committee, Medical University of Warsaw.

2.2. Groups and diets

The rats were divided into study group ($n = 32$) and control group ($n = 24$). To induce mammary tumorigenesis, the animals from the study group were administered DMBA (7,12-dimethyl-1,2-benz[a]anthracene; Sigma–Aldrich, St. Louis, MO, USA) *via* gavage in rapeseed oil at a dose of 80 mg/kg body weight at 50 and 80 days of age. Tumors were histopathologically evaluated to confirm their malignancy and to prove that they were adenocarcinomas (II and III degree). The control groups included the rats kept in the same conditions and fed the same diet as the animals from the study groups, but not DMBA-treated. Spontaneous cancers were not found in the non-DMBA groups.

The following groups were used:

1: STANDARD group (standard diet Labofeed H – containing 77 mg Zn/kg food) and 0.4 mL water daily *via* gavage (study group DMBA+; and control group DMBA-)

2: ZINC group – received a daily *via* gavage, 0.4 mL of Zn (6.9 mg/mL; *i.e.* 231 mg Zn/kg diet) (as $ZnSO_4 \cdot 7H_2O$ in aqueous suspension) (study group DMBA+; and control group DMBA-).

3: ZINC + RESVERATROL group – received a daily *via* gavage, 0.4 mL of Zn (6.9 mg/mL) and resveratrol (0.1 mg/mL; *i.e.* 0.2 mg/kg bw) in aqueous suspension (study group DMBA+; and control group DMBA-).

4: ZINC + GENISTEIN group – received a daily *via* gavage, 0.4 mL of Zn (6.9 mg/mL) and genistein (0.1 mg/mL; *i.e.* 0.2 mg/kg bw) in aqueous suspension (study group DMBA+; and control group DMBA-).

The animals without supplementation recived daily *via* gavage 0.4 mL of water.

The dose of Zn was established based on the value used in the Labofeed H diet (standard diet) (77 mg Zn/kg diet). The applied Zn dose – exceeding threefold the amount of that element in the fodder. The polyphenols dose level was selected based on human average daily consumption (extrapolating on the rats' body weight).

2.3. Samples collection and preparation

The animals were sacrificed by decapitation at 20 weeks of age, and right femur, liver, spleen were collected. The samples were stored at a temperature of −80 °C until the test time. The mineral content was determined after wet microwave mineralization of the samples. Femur and samples of tissues or fodder were placed in a teflon vessel and with added 3.5 mL of 65% nitric acid (Suprapur®, Merck, Germany). The vessels were placed in a microwave system (Plazmatronica, Poland) and their content was mineralized. The FAAS technique (PUnicam, 9100) was used to analyze the following elements: magnesium (Mg), iron (Fe), zinc (Zn) and calcium (Ca). Phosphorus (P) content was determined using the spectrophotometric method based on the formation of phosphomolybdate [13]. The analysis of fodder was performed in order to check if the Zn content was in agreement with the amount declared by the producer. The obtained results were satisfactory, *i.e.* in agreement with the content declared by the producer (date not shown).

2.4. Blood collection and preparation

In the investigations we used the serum of female Spraque–Dawley rats of body mass ±210 g. The serum samples were stored in a deep freezer at −80°C until performing the investigations. Before the determinations the serum was thawed and then centrifuged at 2500 rpm during 15 min. The determination of zinc and iron was carried out in serum samples by air-acetylene flame atomic absorption spectrometer (PU-9100). Before the mineral determinations the serum was thawed and then centrifuged at 2500 rpm during 15 min. For the determination of minerals, serum samples of about 0.5 mL each were diluted with deionized water in the proportion 1:10. Standard solutions of Fe and Zn were prepared from their standard solutions (1000 mg/L in water) from Central Office of Measures, Warsaw, (Poland).

3. Recovery and detection limit

The intralaboratory quality control of determination was done using the following certified Reference Materials (percent of recovery for a given element and relative standard deviation (RSD)):

NCS ZC 71001 Beef Liver recoveries: Fe – 101%; Ca – 106%; Zn – 104%; Mg – 97%; P – 110%; RSDs – Fe – 1.7%; Ca – 9%; Zn – 1.8%; Mg – 1.8%; P – 7.9%

Limits of detection (LOD) for: Zn – 0.03 mg/L; Ca – 1.47 mg/L; Fe – 0.17 mg/L; Mg – 0.04 mg/L; P – 0.22 mg/L

Seronorm Trace Elements Serum -1 (SERO201405) recoveries: Fe – 100%; Ca – 90%; Zn – 101%, Mg – 96%; RSDs: Fe – 4%; Ca – 3.8%; Zn – 2.3 %, Mg – 2.3%

Limits of detection (LOD) for: Fe – 0.38 mg/L; Ca – 0.25 mg/L; Zn – 0.01 mg/L; Mg – 0.12 mg/L

4. Statistical analysis

Results were expressed as mean and standard deviation. The results were compared to those in the control animals on the same diet, in order to elucidate the possible effect of Zn or polyphenols compounds supply on the mineral composition of femur, liver, spleen and serum. The data were analyzed using Student's *t*-test for two-groups comparisons, differences at $p \leq 0.05$ were considered statistically significant. Multi-factor analysis of variance (ANOVA using Statistica 12 package) was calculated in order to