Original Research

Effects of Dietary Zinc and Polyphenol Intake on Hair Mineral Content in Rats with DMBA-Induced Mammary Cancer

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Abstract

Our study investigated the effects of dietary supplementation with zinc and polyphenol compounds, i.e. resveratrol and genistein, on the effectiveness of changes in the content of select elements (Zn, Mg, Fe, Ca, and P) in hair of rats with chemically (DMBA (7,12-dimethyl-1,2- benz[a]anthracene) induced mammary cancer.

Regardless of the diet (standard; Zn; Zn+genistein) there occurred an increase in Fe and Zn content as well as a decrease in Ca, Mg, and P content in the hair of rats with mammary cancer in comparison with the content of those elements in healthy animals. Only in the group of rats supplemented with Zn and resveratrol were no changes in hair observed as compared with the control group, fed the same diet but without DMBA supplementation.

The process of neoplasia in mammary tissue caused a number of changes in the concentrations of elements in hair. Certain dietary factors seem to have a significant effect on the distribution of elements in hair, but the reason for this phenomenon remains unknown.

Keywords: hair mineral analysis, zinc, polyphenols, breast cancer

Introduction

Among the most frequently applied analytical methods used for preliminary health assessment are blood and urine tests. The homeostatic mechanisms in serum result in rapid alignment of the level of elements, at a cost of tissue reserve. In many investigations it was attempted to use the analysis of micro- and macroelements in hair to assess mineral transformations [1-10]. The level of mineral elements in hair allows one to obtain information about their content over a prolonged period of time such as two or three months [11]. Besides, elemental analysis of hair makes it possible to perform quantitative determinations of some components that are present in the organism in very small amounts [1, 2, 11]. Another advantage of hair mineral analysis is that while the concentrations of micro- and macroelements in the blood may vary for instance as a result of emotions, such fluctuations are not observed in the analysis of hair samples. Particularly valuable is toxic elements hair analysis, thanks to which one can determine the harmful effect of heavy metals on the processes of homeostasis and the levels of toxicity in the body [3]. An additional advantage of using hair mineral analysis is the fact that hair samples can be taken from the donor in an

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easy, painless, and noninvasive way, and sent directly to the lab in an ordinary mail envelope. Yet hair analysis is not commonly used to diagnose diseases. This is due first of all to the difficulties in standardization of the range of reference values. The factors that have a significant but not an unequivocal effect on the content of elements in hair include: age, sex, hair fragment taken for analysis, place of hair intake, hair color, place of residence, diet, or stress [11-13]. It is not known for certain if chronic diseases affect the mineral content of hair. In the case of laboratory animals most of the above problems do not exist, so researchers can try to find the relationship between the obtained results and the disease processes. However, in the literature practically no information is available regarding the differences in the levels of mineral elements in the hair of animals suffering from various diseases, including neoplastic processes [7-10].

Scientific investigations prove that zinc deficiency may be related to the increased risk of cancer [14, 15]. The role of zinc in the onset and progress of a neoplastic process is not yet fully known, although negative effects of zinc deficiency on the immune system are explicit [7]. On the other hand, zinc supplementation is able to reduce oxidative stress and thus may boost the immune system function, which in turn improves immunity and reduces the risk of neoplasia [16]. There are high expectations as concerns the use of some polyphenols in prevention and treatment of cancer. In epidemiologic studies a reverse correlation was observed between the content of flavonoids in the diet and the risk of the onset of some types of cancer [17, 18]. The investigations on animal models confirm those results. It was found that flavonoids can inhibit tumor formation as well as the hyperplasia of the already existing tumors, and additionally it may reduce the frequency of metastases.

The aim of this work was to determine the effect of a zinc-supplemented diet (in amounts exceeding twofold the Zn content in the fodder) and polyphenolic compounds: resveratrol and genistein, on the effectiveness for inducing carcinogenesis and on the changes in concentration of mineral components (Zn, Mg, Ca, Fe, and P) in the hair of rats with DMBA-induced mammary cancer.

Materials and Methods

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 animals were housed in stainless steel cages under controlled conditions (22±1ºC, a 12 h light-dark cycle), with free access to a standard laboratory diet (Labofeed H Poland) and drinking water. 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.

Experimental Design

The experiment was performed for 14 weeks (from 40 days until 20 weeks of age). After the adaptation period the animals were divided into two experimental groups. In group 1 (DMBA+), study group n = 39) the rats were treated with a dose of 80 mg/body weight of DMBA (7,12-dimethyl-1,2-benz[a]anthracene; Sigma-Aldrich, St. Louis, MO, USA) given in rapeseed oil at 50 and 80 days of age to induce breast cancer (adenocarcinoma), and in group 2 (DMBA-, control group n = 25) the rats were accommodated under the same conditions as those in Group 1, fed the same diet but without DMBA treatment. Tumors were histopathologically evaluated to confirm their malignancy and to prove that they were adenocarcinomas (II and III degree). Spontaneous cancers were not found in the non-DMBA groups.

Animals from both groups were also fed diets different in bioelements. The diets were supplemented with:

- Zn – the exposed group received daily via gavage 0.4 mL of Zn (28.9 mg Zn/kg bw (as $ZnSO_4 \cdot 7H_2O$ in aqueous suspension)
- Zn+resveratrol – the group received daily via gavage 0.4 mL of Zn (28.9 mg Zn/kg bw) and resveratrol (0.2 mg/kg bw) in aqueous suspension
- Zn+genistein – the group received daily via gavage 0.4 mL of Zn (28.9 mg Zn/kg bw) and genistein (0.2 mg/kg.bw) in aqueous suspension.

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, i.e. 9.6 mg Zn/kg bw). The polyphenol dose level was selected based on human average daily consumption (extrapolating on the rats' body weight).

Samples and Measurements

The animals were sacrificed by decapitation at 20 weeks of age, and hair samples were collected. The mineral content was determined after wet microwave mineralization of the samples with atomic absorption spectrometry (FAAS). Approximately 250-300 mg hair samples were also wet digested with 5 ml of nitric acid. The vessels were placed in a microwave system (Plazmatronica, Poland) and their content was mineralized. Prior to mineralization, the hair samples were cleaned. The dirt and grease was removed by thorough hair rinsing first with water, next with acetone (12 h). The hair was finally washed several times in double distilled water, and the oven was dried at 60ºC for 3 h.

After decomposition, the samples were transferred into a 10 ml volumetric flask (class A, Brand®) and filled to volume with double-distilled water. The FAAS technique with air-acetylene flame (PU 9100) was used to analyze the following elements: magnesium (Mg), iron (Fe), zinc (Zn), and calcium (Ca). The phosphorus content was determined using the spectrophotometric method, based on the formation of phosphomolybdate [19].

Table 1. Cancer induction in 7,12-dimethyl-1,2-benz[a]anthracene-treated groups in relation to the diet applied.

Diet	Tumors in group	Number of tumors in one rat	First week at onset
1. standard diet (9.6 mg Zn/kg bw)	9/9 (100%)	1-5	15
2. zinc (28.9 mg Zn/kg bw)	9/9 (100%)	1-5	15
3. zinc (28.9 mg Zn/kg bw) and resveratrol (0.2 mg/kg bw)	10/10 (100%)	2-10	13
4. zinc (28.9 mg Zn/kg bw) and genistein (0.2 mg/kg bw)	10/11 (91%)	1-6	15

The date refers to tumors evaluated at 20 weeks of age (decapitation time)

Recovery

The intralaboratory quality control of determination was based on the following certified Reference Material (percent of recovery for a given element and relative standard deviation (RSD)).

- DC73347 (GSH-1) Hair: Fe – 92%, Ca – 91%, Zn – 93%, Mg – 98%, P – 113%
- RSDs: Fe – 2.6%, Ca – 3.1%, Zn – 3.4%, Mg – 2.4%, P – 5.8%
- Limits of detection (LOD) for: Zn – 0.07 mg/L, Ca – 0.76 mg/L, Fe – 0.42 mg/L, Mg – 0.14 mg/L, and P – 0.22 mg/L

Statistical Analysis

Data are given as means±SD. The results were compared with those in the control animals, in order to elucidate the possible effect of Zn or polyphenols on the mineral composition of hair. The data were analyzed using Student's t-test; differences at $p \leq 0.05$ were considered statistically significant.

Results

Carcinogenesis

It is a relevant issue to assess the severity of carcinogenesis expressed by tumor weight and number in particular rat groups as well as the onset time of initial tumors. Irrespective of the diet applied, the effectiveness of DMBA-induced carcinogenesis was 100% (Table 1). Only in the group of rats supplemented with Zn+genistein did one animal develop no tumors. There were both single and multiple tumors – on average up to 6 tumors per rat, the group supplemented with Zn+resveratrol was characterized by a maximum number of 10 tumors per rat. In this group (receiving Zn+resveratrol) the first tumors appeared at 13 weeks of age, i.e. two weeks earlier than in the groups receiving Zn or Zn+genistein or only the standard diet.

Minerals in Hair of Rats on Standard Diet

The levels of zinc, magnesium, calcium, iron, and phosphorus belonging to hair of cancerous rats fed the standard diet with DMBA and control groups receiving the standard diet are summarized in Table 2. On the basis of the obtained results it can be said that in hair of rats fed the standard diet and receiving DMBA as compared with hair of the control group of rats there are statistically significant changes regarding the content of the analyzed elements. In rat hair of the examined animals there was a clear increase of Fe and Zn concentration, as well as a decrease of Ca, Mg, and P levels as compared with controls.

Table 2. Comparison of mean concentrations of select elements in hair of study group (DMBA+) to control group (DMBA-) obtaining standard diet.

Elements (µg/g)	Study group (DMBA+) $\bar{X}$±*SD*		Control group (DMBA-) $\bar{X}$±*SD*	
Iron	12.77±2.91*	(8)	9.92±1.08	(6)
Calcium	307.9±34.8*	(8)	434.2±54.1	(6)
Zinc	195.7±7.14*	(8)	163.8±10.9	(6)
Magnesium	124.0±2.99*	(7)	168.5±7.60	(5)
Phosphorus	305.5±10.1*	(8)	358.2±41.3	(6)

*$p \leq 0.05$ significantly different from controls
(n) – test number, $\bar{X}$ – mean, *SD* – standard deviation

Minerals in Hair of Rats on Diet: + Zn, or Zn+Genistein

The levels of zinc, magnesium, calcium, iron, and phosphorus in hair of cancerous rats receiving different diets and control groups receiving the same diets are summarized in Table 3. The results of the performed investigations show that in hair of rats fed the standard diet supplemented with zinc and treated with DMBA there was a statistically significant increase of Fe, with a simultaneous decrease of Ca, Mg, and P levels. Similar results were observed in hair of rats receiving the standard diet supplemented with zinc and genistein. In addition, in this case there was also an increase of Zn concentration.

Minerals in Hair of Rats on Diet: Zn+Resveratrol

It is worth emphasizing that in the case of rats receiving the standard diet, additionally supplemented with zinc and resveratrol no statistically significant changes were observed in the content of the analyzed minerals (Table 3).

Table 3. Comparison of mean concentrations of select elements in hair of study group (DMBA+) to control group (DMBA-) of each type of diet.

Elements (μg/g)	Standard diet + Zn		Standard diet + Zn + resveratrol		Standard diet + Zn + genistein	
	Study group $\bar{X}$±*SD*	Control group $\bar{X}$±*SD*	Study group $\bar{X}$±*SD*	Control group $\bar{X}$±*SD*	Study group $\bar{X}$±*SD*	Control group $\bar{X}$±*SD*
Iron	12.36±2.27* (7)	9.86±0.99 (6)	13.78±3.16 (7)	9.96±2.64 (5)	16.24±4.34* (10)	10.82±0.44 (5)
Calcium	316.2±44.9* (8)	417.6±22.8 (6)	322.2±176.0 (8)	335.6±134.5 (6)	369.6±32.5* (10)	474.1±83.65 (6)
Zinc	232.5±34.3 (8)	239.2±13.9 (6)	207.8±55.1 (7)	213.8±63.9 (6)	254.3±14.8*(11)	215.7±13.67 (5)
Magnesium	134.2±13.2* (8)	192.2±9.10 (6)	119.0±176.0 (7)	147.6±47.1 (6)	110.6 ±14.9* (11)	169.4±9.34 (5)
Phosphorus	316.1±37.7* (7)	395.8±70.0 (7)	203.0±68.4 (8)	293.7±103.4 (6)	255.6±43.3 (11)	290.4±55.7 (6)

*p ≤ 0.05 significantly different from controls
(n) – test number, $\bar{X}$ – mean, *SD* – standard deviation

Discussion

Human hair is composed mainly (ca. 80%) of proteins. The amount and type of particular amino acids that compose hair proteins varies and depends on many factors, such as genetic factors, diet, state of health, atmospheric and environmental conditions. Water is another important component of human hair (ca. 15%). Its amount depends on the relative humidity of the surroundings. Lipids form 1% to 9% of hair composition. This component is obtained from the sebum and is composed of a mixture of free fatty acids, mono-, di-, and triglycerides, waxes, hydrocarbons, and alcohols. Chemical composition of hair also includes trace elements, which are the smallest group of compounds and their content ranges from 0.25% to 0.95% [11].

There are significant differences between growth cycles in hair of various mammals. In humans, guinea pigs, dogs, and cats the hair growth cycle proceeds in a mosaic pattern. Each hair follicle has its own growth cycle, which is not affected by the activity of the vicinal follicles. In some other mammals, e.g. rats, mice, or rabbits, the growth of one follicle affects the neighboring follicles and results in phases of growth and rest, beginning from the head and moving toward the tail, and from the abdominal part to the dorsal part of the animal body. This process repeats every few weeks. Besides, in different species of mammals the pattern of hair growth, as well as the time of the follicle phase, are different [20].

The performed investigations show that the Fe level in hair of the examined rats which received DMBA is significantly higher than the content of this mineral in hair of control rats. This is true of almost all groups, except the group supplemented with Zn+resveratrol (Tables 2 and 3). The stimulating effect of Fe for the formation of free radicals and other reactive forms of oxygen causing cell damage is well known [21]. It was also found that iron takes part in the process of angiogenesis and thus may induce the formation of neoplastic cells [22]. The works of other researchers agree with the obtained results. In many investigations an increased Fe level was found in the hair of patients with different types of cancer [9, 24, 25]. The Fe concentration was determined not only in hair, but also in neoplastic tissues or the blood serum. Thus, Reddy et al. [21] analyzed the mineral content in the tissue of colorectal cancer and of control tissues. They found an increased Fe content in cancerous patients (287 μg/g dry mass) in comparison with the tissues of healthy patients (123 μg/g dry mass). On the other hand, data concerning the Fe content in blood serum vary and there is no unequivocal confirmation of the correlation between the increased Fe content and risk of cancer. The reason why there is increased Fe migration to rat hair remains unknown. A tendency to increased Fe concentration that was found both in human hair studies and rats' hair investigations indicates that iron could be used as a mineral useful in early diagnosis of neoplastic processes.

Zinc is another element whose increased level was found in hair of rats fed the standard diet and supplemented with Zn and genistein (Tables 2 and 3). What is interesting in the rats supplemented with Zn and Zn+resveratrol is that no differences were observed in the amount of this mineral in hair in comparison with control groups that received analogical diets. Zinc plays an important role in many cellular processes, such as cell proliferation or reproduction, and also plays a protective role against free radical formation [15]. The direction of the obtained changes adequately matches the investigations of other researchers concerning human hair [21, 23]. However, contrary to those results, some authors report that in cancerous patients there is a decreased Zn concentration. For instance, Memon et al. [7] showed that in the hair of women suffering from breast cancer the Zn level was 114.5 μg/g, whereas in the control group of women it was equal to 246.9 μg/g. Those results were confirmed by Kolmogorov et al. [24], who found that in the hair of women with breast cancer a statistically significant decrease of zinc content was observed as compared with healthy women (129 μg/g against 163 μg/g). These substantial differences between the results of zinc analysis in human hair are difficult to explain. They may depend on many factors concerning the same diagnostic material (hair color and length, age of patient, sex, diet, or geographical location). Undoubtedly, in our investigations concerned rat hair there were no big differences observed. The direction of changes of zinc concentration in rat hair showed an increase of this element in the animals with chemically induced mammary cancer. However, the direction of those changes was not as equivocal as in the case of iron. For unclear reasons in the groups supplemented with Zn and Zn+resveratrol, no differences were observed in the Zn level in hair of cancerous rats as compared with the control group.

In the case of the remaining analyzed elements, i.e. Mg, Ca, and P a statistically significant decrease of their content was observed in all investigated groups except the group additionally supplemented with Zn and resveratrol (Tables 2 and 3). The role of magnesium in the development of neoplasia is not yet fully known. On the one hand, Mg deficiency may lead to the development of neoplasia, while Mg supplementation in some cases has an antineoplastic effect [24]. In one of their studies, Ren et al. [26] found a decreased Mg content in the hair of patients with lung cancer. The investigations of Wang et al. [27] carried out on patients with liver cancer or stomach cancer also confirmed the decreased Mg level in their hair. Similar results were obtained by Guo et al. [9], who found decreased content of this mineral in men with prostate cancer as compared with the control group of patients. The lower Mg content was observed not only in hair but also in cancerous tissues. In the literature there are also reports which say that in patients with malignant neoplastic diseases the Mg level is sometimes increased in comparison with the normal level. Such results were obtained in the investigations of Pasha et al. [23], who found an increased Mg content in the hair of cancer patients as compared with healthy patients. The available papers do not confirm unequivocally that magnesium deficiency in various tissues, including hair, may be connected with the development of cancer, but the majority of papers suggest that both magnesium distribution and bioavailability are changed in the course of a neoplastic disease, which may lead to tumor activation and development [28].

Some researchers, e.g. Ren et al. [26], also found a reduced calcium content in the hair of patients with prostate cancer, as compared with the Ca content of healthy persons. These results were confirmed by Wang et al. [27], who analyzed hair samples of patients with different kinds of cancer. They found that the Ca level in hair was equal to 609 μg/g in cancer patients, whereas in healthy persons it was equal to 939 μg/g. In the hair of patients with prostate cancer a decrease of this mineral also was observed [9]. Contrary to those data, increased calcium content was found by Pasha et al. [23]. In the majority of available papers concerned with the analysis of Ca concentrations the determinations were performed in cancerous tissues. Thus the high calcium level observed in the tissues of breast cancer in women may be related to the fact that the pathologically altered tissue collects large quantities of calcium, forming numerous microcalcifications. Such microcalcifications commonly occur in this disease entity, being its characteristic feature which is used for diagnostic purposes [29]. Phosphorus plays a significant role in energy transfer and storage. In order to attain its maximum activity, it needs the presence of vitamin D and calcium. In the course of the performed investigations a statistically significant decrease of phosphorus level was found in hair of the examined rats from the groups receiving the standard diet and Zn-supplemented diet. The obtained results are in agreement with those obtained by Guo et al. [9], who found a reduced phosphorus content equal to 190.5 μg/g in the hair of men with prostate cancer. For comparison, in the tissue of patients from the control group the level of this mineral was almost twofold higher, being equal to 360.4 μg/g. The decrease of phosphorus level was also found in the hair of patients suffering from lung cancer [26].

As in the group of rats receiving the standard diet the direction of changes in the content of minerals was basically the same as the changes of these elements in hair of rats fed the Zn-supplemented diet as well as Zn+genistein supplemented diet. This could indicate that the applied diet has no effect on the process of migration of minerals to animal hair. Hence the diet should also have no effect on the number of tumors formed and generally on carcinogenesis. However, in the group of rats receiving simultaneously Zn and resveratrol, tumor formation was significantly and rapidly accelerated by as much as 2 weeks, which is equivalent to a period of one year in humans. What is interesting is that despite this accelerated growth no differences were found between the concentrations of the examined elements in rat hair. The studies performed over the last few years emphasize the antioxidative as well as antiproliferative activity of resveratrol. That is why it is considered one of the factors of anticancer and/or chemopreventive potential [30, 31]. The mechanism of anticancer activity of resveratrol involves the inhibition of ribonucleotide reductase, DNA polymerase, protein kinase C, or cyclooxygenase-2, inhibition of reactive oxygen species which are intermediates in the process of carcinogenesis, and also the inhibition of cell division and activation of apoptosis [31]. However, the potential anticancer effect of resveratrol strongly depends on its bioavailability and the applied dose.

The concentration of resveratrol in tissues was examined after intravenous and oral administration in rabbits, mice, and rats. It was found that after intravenous administering of 20 mg t-Resveratrol/kg, the maximum concentration (42.8±4.4 μM) that was reached in the blood 5 min after administration, after 60 min decreased down to 0.9±0.2 μM. The same dose of resveratrol administered orally resulted in a maximum increase of the concentration of the compound to merely 2-3 μM during the first 5 min after administration, whereas during 60 min a decrease to less than 0.1 μM was observed [31]. This could be due to the fact that only 1.5% of orally administered resveratrol reached the plasma compartment, or else due to rapid metabolism of resveratrol in the liver and no extravascular accumulation. The role of the liver in the metabolism of resveratrol was examined by treatment of isolated hepatocytes of the rats with 20 μM of t-Resveratrol. During 20 minutes the hepatocytes were able to metabolize about 80% of the administered polyphenol. This indicates that the liver metabolism can remove most of the resveratrol in the bloodstream, and the high rate of this process may be favorable when high doses of resveratrol are administered. One should also take into account the presence of resveratrol metabolites, which may also exhibit biological activity in plasma and other tissues. Over 95% of resveratrol that is present in the blood, plasma, and urea, occurs in the form of metabolites (as glucuronides and sulphates). Also, the rate of intestinal absorption of resveratrol varies depending on the species. In humans, 24.6% of t-Resveratrol was found in urea, whereas in rats it was only 1.5% [31]. It should also be mentioned here that despite the oncoprotective properties of resveratrol, its administration to rats was found to cause numerous adverse effects [32] such as loss of appetite, increased levels of creatinine, alkaline phosphatase, alanine aminotransaminase, and bilirubine, or a decrease of hemoglobin levels and the number of erythrocytes, and also leucocytosis. In addition, histopathological changes were found in kidneys and the gall-bladder that showed the characteristics of hyperplasia (at a dose of 1 and 3 g/kg bw) [32].

In our investigations in the rats receiving only Zn- or Zn+genistein-supplemented diet, significant changes in hair were observed, similar to those observed in the group fed the standard diet, without deviations as concerns the time of appearance of the first palpable tumors.

The combination of zinc supplementation with resveratrol significantly promoted the rate of appearance of tumors and increased their number, which is indicative of an additional induction of oncogenesis. Perhaps a given phytoestrogen can have opposing effects on mammary cancer risk, depending on the timing of exposure. The precise mechanism of this phenomenon is not known, especially as each of these compounds is attributed chemopreventive properties in fighting neoplasms. The presented results show that simultaneous diet supplementation with several antioxidants involves the risk of obtaining, instead of the expected antioxidative effect that would reduce carcinogenesis, a prooxidative effect. This also shows how dangerous it may be to disturb the oxidation-reduction equilibrium and, moreover, how easily such disturbance can be provoked by incompetent activities.

Conclusions

1. The process of neoplasia in the mammary tissue resulted in a number of changes regarding the concentrations of mineral elements in rat hair.
2. In the majority of the examined groups an increased content of iron and zinc was observed as well as a reduced level of calcium, magnesium, and phosphorus in hair of the examined rats, as compared with the control groups.
3. Supplementation of the rats' diet with zinc ions together with resveratrol strongly intensified the rate of oncogenesis of the mammary gland, without causing any changes in the hair mineral content.

Further studies are necessary to investigate the correlations between the physiopathological state of the organism taking into account the applied diet and the content of mineral elements in hair, so that the observed changes would have diagnostic value.

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