Introduction

Various abnormalities in the content and homeostasis of mineral elements frequently induce specific biochemical alterations characteristic of many conditions, including neoplasia [1]. To exemplify, Se, Zn, and Mg deficiencies may lead to an increased risk for developing cancer [2-5]. Conversely, Fe and Cu supplementation may contribute to neoplastic disease [6-9]. Therefore, attention should be focused on assessing the correlation between concentrations of certain mineral elements (particularly Zn, Cu, and Fe) in cancerous tissues (tumors), and easily obtainable...
The role of the mineral elements is also associated with normal functioning of numerous enzymes and the immune system. Occasionally, their contents in cancerous tissues significantly differ from those in normal tissues. The aim of the study was to evaluate the mineral (Zn, Cu, Ca, Fe, Mg, P) composition of the breast tumor tissue and to investigate the role of Zn and Cu diet contents on tumor development, DMBA-induced rat breast cancer being the experimental model. Apart from the assessment of the elemental content in the formed mammary tumors, evaluation was also performed on rat hair. In many studies, attempts were made to use the analysis of hair micro- and macroelements in an immediate assessment of pathological processes in the body (e.g. As and Cd analysis shows the body’s degree of elemental intoxication) [11-14]. Hair is an easily obtainable tissue, the levels of bioelements are considerably higher than those in blood, and they do not undergo abrupt homeostatic alterations. An attempt was also made to establish whether altered elemental concentrations in rat hair are consistent with the altered elemental composition in tumors, and if the material might be useful in diagnosing breast cancer.

**Material and Methods**

**Animals**

Forty-five 30-day-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, Poland. The animal experiments were approved by the ethics committee at the Medical University of Warsaw.

**Experimental Design**

The experiment was conducted 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 (study group, n=24), 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 (control group, n=20) 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 degrees). 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 a daily via gavage, 0.4 ml of Zn (6.9 mg/mL) (i.e. 231 mg Zn/kg diet) (as ZnSO₄·7H₂O in aqueous suspension)
- Cu – the exposed group received a daily via gavage, 0.4 ml of Cu (1.3 mg/mL) (i.e. 42.6 mg Cu/kg diet) (as CuSO₄·5H₂O in aqueous suspension)
- with the standard laboratory diet (Labofeed H, Poland) the group received a daily 0.4 ml of water in an identical manner

The doses of bioelements were established by redoubling the basal value standard Labofeed H diet (i.e. 77 mg Zn/kg diet; 21.3 mg Cu/kg diet) (Table 1).

**Samples and Measurements**

The animals were sacrificed by decapitation at 20 weeks of age, and tumor, normal tissues of the mammary gland and hair samples were collected. The samples were stored at a temperature of -70ºC until the test time. The min-
eral content was determined after wet microwave mineralization of the samples with atomic absorption spectrometry (AAS) (for hair) and ICP-OES technique (for tumor, normal mammary tissue and fodder).

The samples were mineralized according to the procedure developed at the Central Forensic Laboratory of the Police in Warsaw. Approximately 500-800 mg samples of tumors and normal mammary tissues or fodder were placed in a teflon vessel with 3.5 ml of 65% nitric acid added (Suprapur®, Merck, Germany). The vessel was placed in a microwave system (MULTIWA VE, Anton Paar, Perkin Elmer) and mineralized.

Subsequently, approximately 250-300 mg hair samples were also wet digested with 5 ml of nitric acid. The vessel was placed in a microwave system (Plazmatronica, Poland) and mineralized. Prior to mineralization, the hair samples were cleaned. The dirt and grease had been 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 oven-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 ICP-OES technique (Optima 3100XL, Perkin Elmer) was used to analyze the following elements: magnesium (Mg), iron (Fe), zinc (Zn), and calcium (Ca). Copper (Cu) content in samples was estimated in an atomic absorption spectrophotometer (PU 9100) using air-acetylene flame. Phosphorus content was determined using the spectrophotometric method based on the formation of phosphomolybdate [15].

Recovery

The intralaboratory quality control of determination was based on the following certified Reference Material:
- DC73347 (GSH-1) Hair (Cu – 91%; Fe 92%; Ca – 90%; Zn – 93%; Mg – 98%, P – 113%) – NCS ZC 71001 Beef Liver (Cu – 118; Fe – 109%; Ca – 115%; Zn – 105%; Mg – 96%; P – 103%)
- NIST 1577b Bovine liver (Cu – 116%; Fe – 103%; Ca – 103%; Zn – 107%; Mg – 105%)

Statistics

Data are given as means±SD. The results were compared to those in the control animals, in order to elucidate the possible effect of Zn or Cu supply on tumorigenesis and the mineral composition of tumor and hair. The data were analyzed using Student’s t-test; differences at p≤0.05 were considered statistically significant.

Results

Weight Gain

Dietary composition of the basal diet is presented in Table 1. The concentrate used in this study contained 77 mg Zn and 21.3 mg Cu per kg diet. The Zn and Cu content of drinking water was negligible. The Zn-exposed group received zinc via gavage in an amount exceeding three times its fodder level (i.e. 231 mg Zn/kg diet).

The Cu-exposed group received copper via gavage, in the amount exceeding twice its fodder level (i.e. 42.6 mg Cu/kg diet).

The results of our experiments show that, in comparison to the controls, the body weight gain in the standard laboratory diet group decreased significantly (by approximately 20%), while Zn and Cu supplementation produced no changes (Table 2).

Carcinogenesis

It is relevant 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 (Table 3). In the group receiving Cu, the first tumors appeared at 14 weeks of age, i.e. one week earlier than in the group receiving Zn or the standard diet.

The results of our experiments show that, in comparison to the controls, the body weight gain in the standard laboratory diet group decreased significantly (by approximately 20%), while Zn and Cu supplementation produced no changes (Table 2).

Magnesium and Iron Content in Tumor and Hair

Mg content in cancerous tissue and hair from the study rats receiving carcinogens was decreased in relation to the control groups, regardless of the diet (Table 4). Reversely, the tumor Fe content was significantly increased as compared with the normal mammary tissue in the control group. This was particularly evident in the study group fed only a standard diet (over 180%) in contrast to the groups supplemented with Zn or Cu (over 50%) (Table 4). Altered Fe content in rat hair is diet-related. Thus, the standard diet and

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**Table 2. Effects of differentiated diet on rat body weight.**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Study group</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body weight (g)</td>
<td>Body weight (g)</td>
</tr>
<tr>
<td></td>
<td>X±SD (n)</td>
<td>X±SD (n)</td>
</tr>
<tr>
<td>standard</td>
<td>206±8* (8)</td>
<td>253±14 (6)</td>
</tr>
<tr>
<td>Zn</td>
<td>230±19 (8)</td>
<td>220±21 (7)</td>
</tr>
<tr>
<td>Cu</td>
<td>231±22 (8)</td>
<td>210±15 (7)</td>
</tr>
</tbody>
</table>

(n) – test number, X – mean, SD – standard deviation *p<0.05 significantly different from controls

**Table 3. Cancer induction in 7,12-dimethyl-1,2-benz[a]anthracene-treated groups in relation to diet.**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Tumors in group</th>
<th>Number of tumors in one rat</th>
<th>Tumor weight (g)</th>
<th>First week at onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>standard</td>
<td>9/9 (100%)</td>
<td>1-5</td>
<td>0.3-11.9</td>
<td>15</td>
</tr>
<tr>
<td>Zn</td>
<td>9/9 (100%)</td>
<td>1-5</td>
<td>0.38-20.4</td>
<td>15</td>
</tr>
<tr>
<td>Cu</td>
<td>9/9 (100%)</td>
<td>1-5</td>
<td>0.34-7.24</td>
<td>14</td>
</tr>
</tbody>
</table>

The date refer to tumors evaluated at 20 weeks of the animals' age (decapitation time)
Zn supplementation yielded an approximately 25% Fe growth in the hair of the study rats in relation to the control groups; however, Cu supplementation produced an opposite effect, i.e. a significant Fe depletion in the hair of DMBA-treated rats (by 30%) (Table 4).

### Zinc and Copper Content in Tumor and Hair

Neither the Labofeed H standard diet nor that supplemented with Zn or Cu exerted any effect on the Zn content in tumors when compared with the normal tissue (Table 5). Interestingly, no alterations were found in the Zn content in the hair of study rats fed the Zn supplemented diet in relation to the corresponding control group. However, in the remaining groups fed the standard diet, the Zn level in rat hair was significantly increased, and in the Cu supplemented groups, the hair content was lower than that in the non-DMBA control groups.

Copper content in tumors increased significantly (by over 90%) in both the Zn and Cu supplemented groups (with no change for the standard diet) (Table 5). This had no effect on the Cu level in the rat hair, except for those in the Cu-supplemented group that showed a 35% reduction in relation to the rat hair in the control group (Table 5).

### Calcium and Phosphorus Content in Tumor and Hair

Regardless of the diet, the Ca and P content in tumor tissue showed no differences in relation to the normal mammary tissue in the control animal groups (Table 6). Only the Cu supplemented study group showed increased P levels. However, the direction in elemental alterations in the rat hair is highly evident and stable. Namely, regardless of the diet type, the Ca and P levels in the hair of DMBA-treated rats were depleted, which was particularly significant in the case of calcium.

### Discussion

Altered concentrations of different bioelements in different tissues (also in tumors) and body fluids during carcinogenesis in the body are indeed a subject of numerous studies and reports [1, 7, 10, 14]. However, it is not clear if the alterations in the levels of elements are a cause or a consequence of disease. Does the tumor, in order to increase its size, have to potently accumulate certain elements and effectively eliminate the other ones? The novelty in the paper is an attempt to assess if the dietary components (i.e. zinc and copper ions provided in excess and over a long period of time) will alter the concentration of selected elements in tumor tissue as compared to normal tissue or, more precisely, if Zn and Cu taken in the diet may significantly modify the process of mammary oncogenesis in the rat. In order to assess the process, we assumed the speed of the onset and number of the first palpable tumors. The choice of Zn ions was associated with its potent documented antioxidant and antitumor effect and naturally inhibiting copper ions (as a prooxidant and an angiogenic factor) [4, 9, 16, 17]. Since the Zn dose used in multiple studies as a supportive therapy in antiangiogenic treatment is threefold of the daily Zn requirement in humans [8, 9], we decided, in order to effectively diminish the Cu dose in long-term therapy, to supplement rats with a threefold Zn dose of that contained in their fodder. Supplementation with Cu ions was used to achieve a reverse effect, i.e. to find out if an additional supply of Cu ions intensifies the process of carcinogenesis [8, 9, 18].

Zinc and copper in the doses administered in our study rats did not cause symptoms of intoxication, and there was no body weight loss in rats in relation to the control groups.

### Table 4. Altered magnesium and iron content in cancerous tissue and hair in the study rats vs. controls.

<table>
<thead>
<tr>
<th>Group →</th>
<th>Controls X±SD (n)</th>
<th>Study group X±SD (n)</th>
<th>Controls X±SD (n)</th>
<th>Study group X±SD (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>diet↓</td>
<td>magnesium (μg/g wet tissue)</td>
<td>iron (μg/g)</td>
<td>magnesium (μg/g dry tissue)</td>
<td>iron (μg/g)</td>
</tr>
<tr>
<td></td>
<td>normal tissue</td>
<td>cancer tissue</td>
<td>hair</td>
<td>hair</td>
</tr>
<tr>
<td>standard</td>
<td>224.4±33.8 (5)</td>
<td>153.6±10.5* (7)</td>
<td>168.5±7.6 (6)</td>
<td>124.0±2.99* (7)</td>
</tr>
<tr>
<td>Zn</td>
<td>263.5±7.66 (6)</td>
<td>166.5±11.3* (8)</td>
<td>192.2±9.1 (6)</td>
<td>134.2±13.2* (8)</td>
</tr>
<tr>
<td>Cu</td>
<td>245.9±11.9 (7)</td>
<td>171.9±12.6* (8)</td>
<td>133.6±17.5 (6)</td>
<td>85.2±22.2* (7)</td>
</tr>
<tr>
<td></td>
<td>17.7±3.07 (5)</td>
<td>50.7±22.6* (7)</td>
<td>9.92±1.08 (6)</td>
<td>12.7±2.91* (8)</td>
</tr>
<tr>
<td>Zn</td>
<td>18.6±2.2 (5)</td>
<td>28.9±6.3* (8)</td>
<td>9.86±0.99 (6)</td>
<td>12.4±2.27* (7)</td>
</tr>
<tr>
<td>Cu</td>
<td>17.4±2.9 (7)</td>
<td>39.7±15.5* (8)</td>
<td>9.22±0.50 (7)</td>
<td>6.73±1.72* (8)</td>
</tr>
</tbody>
</table>

(n) – test number, X – mean, SD – standard deviation

*p≤0.05 significantly different from controls
body weight loss in mice. However, reports show that excess Zn in the animal body may exacerbate tumorigenesis [16]. In our study, only a rat group receiving the standard diet showed considerable weight loss, probably due to a progressive malignancy. The achieved results may lead to the conclusion that the Zn or Cu supplementation administered in selected doses protected the animals against body weight loss. Unfortunately, this was not equivalent to inhibition of carcinogenesis.

Regardless of diet, the efficacy of DMBA-induced carcinogenesis amounted to 100%. The tumors were both single and multiple, i.e. a maximum of up to five lesions per animal. Additionally, the first palpable tumors found in the Cu supplemented rat group were detected a week earlier than in the remaining groups. It is generally assumed that one week of a rat’s age corresponds to six months of human age [19]. Guoqing Hou et al. [17] treated their rats with 119 mg Zn/kg body weight injected into the side, and they also did not report reduction in the tumor number and size, although there was a depletion in ceruloplasmin (Cp) activity. However, treatment with tetrathiomolybdenum (TM) resulted in significantly reduced tumor growth. It is suggested that TM exerts its effect on circulating copper and chelates Cu secreted by tumor cells. However, Zn induces intestinal metallothionein and inhibits only dietary Cu absorption and endogenic Cu resorption [17]. Brem et al. [18] have already proved that the administration of copper-reducing and chelating agents in anticancer treatment reduces the mass of transplanted tumors of rabbit brain, although it does not increase the animals’ survival.
Our studies showed that regardless of the diet type (standard; Zn; Cu), the induced tumors demonstrated both an increased Fe level and a reduced Mg level in comparison with the normal breast tissue in the control rats. An increased Fe concentration may be due to tumorigenesis, since cancer cells differ from normal cells both biochemically and histologically. In high concentrations, Fe initiates lipid peroxidation, damaging cells by catalyzing the reaction of free radical formation and causing DNA mutation. It may also initiate an inflammatory process and stimulate tumor growth by intensifying angiogenesis [6, 20-23]. Our study results are compatible with those reported by others [21, 22, 24], who also showed significantly higher Fe concentrations in the mammary tumor tissues in women versus that in the control tissues.

Our study demonstrated that the rat diet supplemented with zinc or copper increased the Cu content in a cancerous tissue, as compared to normal tissue, but not leading to changes in the Zn level. As shown in our paper, an increased Cu concentration in cancer tissues probably stimulates mammary tumorigenesis by oxidative damage to DNA and angiogenesis [8, 9, 21, 25]. A significant increase in Cu concentration in cancer tissues of various types compared with normal rat tissues also has been reported by other authors [21, 26]; moreover, a statistically significant rise in Cu concentration was described in the serum of women with breast cancer [26]. Koksoy C. et al. [27] explain that the higher Cu concentration is most probably due to Cu released from cells undergoing necrosis caused by ongoing inflammatory processes. The researchers also focus on a decreased Cu erythrocyte concentration in women with breast cancer. Copper ions are cofactors of superoxide dismutase and DNA and RNA polymerase. In the course of carcinogenesis, multiple cell divisions and an increased production of oxygen radicals, require enzyme activation, which results in an excessive use of intracellular Cu storage, and this may cause a reduced Cu level in erythrocytes.

The results of our study also showed a decreased Mg concentration in breast cancer cells. A depleted Mg level was also confirmed in patients with nasal polyps, papilloma or laryngeal cancer [20, 25, 27]. Its decreased concentration in tissue biopsy specimens in patients with cancerous lesions may contribute to reduced activity of antioxidative enzymes, and an increased severity of cancerous lesions [3, 20, 25, 27, 28]. Mg impact on the DNA repair mechanisms is associated with DNA structural stabilization, and the function of the cofactor of the enzymes regulating the metabolism of nucleic acids, e.g. β-polymerase, ligase, and DNA endonuclease. Therefore, magnesium is indispensable in DNA copying and repair, and its deficiency contributes to DNA mutations leading to the initiation of the first stage of carcinogenesis. However, the protective Mg role in the process of carcinogenesis depends largely on the progression stage of the disease. Many reports have shown that a change from a low-magnesium level to a normal diet results in dramatic tumor growth [29], which may be partly explained by an intensified angiogenesis since the endothelial cells are sensitive to the extracellular level of the this cation. Hence, both Mg deficiency and excess modulate tumor angiogenesis by acting on the endothelial cells (in a different manner) and inducing an inflammatory condition.

Tissue biopsy obtained for biochemical assessment is an invasive procedure and, in order to avoid any disadvantages, attention was focused on altered Fe, Cu, and Mg measured in the serum and reflecting carcinogenesis. There were no results showing an unequivocally existing correlation; therefore, a search is being continued for other easily obtainable tissues to be used as the diagnostic material. Therefore, a search is being conducted for an elemental analysis of hair in cancer patients. However, there has been no unequivocal evidence of a correlation between cancer (the more so of a particular organ) and elemental alteration in hair [1, 12, 30-34]. A question therefore arises as to the degree to which hair may “reflect” alterations in the body’s demands for particular elements, or about the ongoing process of carcinogenesis. Our study has shown that in the standard diet rats, and also in the Zn-supplemented animals, the character of Mg and Fe alterations in the animal hair was identical as in the case of their tumor concentrations. There was also a fall in hair Ca and P levels in cancer-induced rats. Reports from other researchers [12, 13, 35] show a full correlation with our study results. They found an increased Fe level in the hair of cancer subjects as compared to that in the healthy individuals. The remaining elements in our study, i.e. Mg, Ca, and P, showed a significant reduction as compared with the control group. These results were confirmed by other researchers [13, 30, 35], who noted decreased Mg concentrations in patients with lung, liver, prostate, or stomach cancer. The authors of the above reports do not unequivocally confirm that the altered Fe and Mg hair contents positively correlate with carcinogenesis; however, the majority of studies indicate that the distribution and location of the elements alters in the course of the cancer disease. In mammary cancer, the accumulation of large amounts of calcium results in multiple characteristic microcalcifications that are helpful in diagnostic procedures [36]. A frequent metabolic complication found in cancer disease is hypercalcaemia, which is mainly due to a higher resorption of bone tissue [37]. The lower level of calcium in rat hair versus that in the controls (as found in our study), may serve as evidence of calcium distribution also from animal hair. In their reports on human hair analysis, many authors confirm decreased calcium levels in the hair of cancer patients [13, 30, 35].

However, Cu supplementation produced different reactions in the elemental levels in animal hair than in the patient groups discussed above, in whom the profile of changes in the tumor tissue was found, to a high degree, to be corresponding to those in the animal hair. The group demonstrated a fall in Fe, Cu, and Mg levels, and a rise in Ca and P concentrations. Palpable tumors in the same group occurred at the fastest rate. It appears that the Cu supply significantly alters distribution of the assessed elements to and from the hair. Interestingly, the Zn and Cu supplementation did not yield any change in the Zn level in the assessed diagnostic material in spite of known interactions between these elements.
Conclusions

In summary, diet supplementation with zinc, which is a natural copper inhibitor, had no effect on a decreased copper level in tumor tissue and inhibited mammary carcinogenesis in the rat. Presumably, this may be associated with the Zn effect exerted particularly on intestinal absorption and not on binding serum circulating copper. On the contrary, Cu excess in diet had effectively increased the occurrence rate of palpable tumors, most probably due to the faster angiogenesis caused by a higher tumor Cu concentration. An increased Fe content and a simultaneous reduction in Mg levels in the mammary tumor as compared to those in normal tissue, and the same direction of alterations in the rat hair, appear to be of interest; however, it is too early to formulate any conclusions related to the existing correlation.

References

29. ANASTASSOPOULOU J., THEOPHANIDES T. Magnesium-DNA interaction and the possible relation of magnesium-DNA interaction and the possible relation of


