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Antineoplastic effects of *Chlorella pyrenoidosa* in the breast cancer model

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ABSTRACT

Objectives: There has been considerable interest in both clinical and preclinical research about the role of phytochemicals in the reduction of risk for cancer in humans. The aim of this study was to determine the antineoplastic effects of *Chlorella pyrenoidosa* in experimental breast cancer in vivo and in vitro.
Methods: In this experiment, the antineoplastic effects of *C. pyrenoidosa* in the chemoprevention of *N*-methyl-*N*-nitrosourea–induced mammary carcinogenesis in female rats were evaluated. Chlorella powder was administered through diet at concentrations of 0.3% and 3%. The experiment was terminated 14 wk after carcinogen administration. At autopsy, mammary tumors were removed and prepared for histopathological and immunohistochemical analysis. In vitro cytotoxicity assay, parameters of apoptosis, and proliferation after chlorella treatment in human breast adenocarcinoma (MCF-7) cells were carried out.
Results: Basic parameters of experimental carcinogenesis, mechanism of action (biomarkers of apoptosis, proliferation, and angiogenesis), chosen metabolic variables, and side effects after long-term chlorella treatment in animals were assessed. Chlorella at higher concentration suppressed tumor frequency by 61% ($P < 0.02$) and lengthened tumor latency by 12.5 d ($P < 0.02$) in comparison with the controls. Immunohistochemical analysis of rat tumor cells showed caspase-7 expression increase by 73.5% ($P < 0.001$) and vascular endothelial growth factor receptor-2 expression decrease by 19% ($P = 0.07$) after chlorella treatment. In a parallel in vitro study, chlorella significantly decreased survival of MCF-7 cells in a dose-dependent manner. In chlorella-treated MCF-7 cells, a significant increase in cells having sub-G_0/G_1 DNA content and significant increase of early apoptotic and late apoptotic/necrotic cells after annexin V/PI staining assay were found. Decreases in mitochondrial membrane potential and increasing reactive oxygen species generation were observed in the chlorella-treated MCF-7 cells.
Conclusions: This study is the first report on the antineoplastic effects of *C. pyrenoidosa* in experimental breast cancer in vivo and in vitro.

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Introduction

The aim of chemopreventive trials is to find an efficient substance that can be administered for a long period with minimum adverse effects [1]. Epidemiologic studies have consistently shown the protective effects of increased consumption of fruits, vegetables, whole grains, and other plant ingredients against the risk for developing chronic diseases such as cancer or cardiovascular disease. It is estimated that there are >5000 individual phytochemicals in plant-based foods [2]. Their identification and mechanism of action evaluation need to be resolved before we can fully understand the health benefits in humans. The phytochemicals demonstrated antiproliferative, anti-inflammatory, antiangiogenic, and pro-apoptotic effects, or the ability to reduce oxidative stress [3], and thus they are of high interest to scientists around the world and the general public. In vitro studies on different cancer cell lines proved the role of polyphenols as growth inhibitors, either by induction of G1-cell cycle arrest [4], G2/M arrest [5], or cell death [6]. Similarly, different carotenoids demonstrated G-1 arrest [7] and apoptosis [8] in various cancer cells. Some polyphenolic compounds demonstrably decreased the levels of one of the most important molecules in angiogenesis, vascular endothelial growth factor (VEGF), thereby inhibiting capillary formation [9].

Chlorella pyrenoidosa—freshwater algae—is an important source of different carotenoids [10]. Polyphenols in *C. pyrenoidosa* are present in lower levels. The positive effects of chlorella in terms of treatment and prevention of cancer have been reported in several in vitro experiments [11–13] and also in clinical study [14]. In one animal study, *C. pyrenoidosa* demonstrated significant chemopreventive effects in chemically induced rat hepatocarcinogenesis [15].

C. pyrenoidosa has not been tested in experimental mammary carcinogenesis in vivo and in vitro so far. The main aim of this study was to evaluate the preventive effects of long-term chlorella administration in a well-established model of *N*-methyl-*N*-nitrosourea (NMU)–induced mammary carcinogenesis in female rats. In rat mammary tumors, the immunohistochemical analysis of caspase-3, caspase-7, Bax, and bcl-2 proteins as the apoptotic parameters, VEGF and VEGFR-2 as parameters of angiogenesis, and finally Ki67 as a proliferation parameter after chlorella treatment were determined. Another aim of this study—a histomorphologic analysis of carcinomas—may have implications for assessment of chlorella effects on the differentiation and prognosis of the tumors. Some side effects of chlorella after long-term administration in animals were observed. To obtain more complex results, the parallel in vitro study with cytotoxicity data, parameters of apoptosis, and proliferation in MCF-7 cells after chlorella treatment was carried out.

Materials and methods

Animals and induction of mammary carcinogenesis, design of experiment

Female rats of the Sprague-Dawley strain (Charles River Laboratories, Sulzfeld, Germany) aged 30 to 34 d were used in the experiment. The animals were adapted to standard vivarium conditions with temperature 23°C ± 2°C, relative humidity 40% to 60%, and artificial regimen (light/dark 12:12 h). During the experiment, the animals were fed the Sniff diet (Soest, Germany) and drank tap water ad libitum. Mammary carcinogenesis was induced by NMU (Sigma, Deisenhofen, Germany) administered intraperitoneally in a single dose of 50 mg/kg body weight on average on the postnatal day 42.

Chemoprevention with *C. pyrenoidosa* (Green Ways, Prague, Czech Republic; country of the origin: Taiwan) began 1 wk before carcinogen administration and lasted until the end of the experiment—14 wk after NMU administration. Chlorella powder was administered in the diet at concentrations of 3 (0.3%) and 30 g/kg (3%). Animals were randomly assigned to one of three experimental groups: control group without chemoprevention; chemoprevention with chlorella at a concentration of 0.3% (CHLO 0.3); or chemoprevention with chlorella at a concentration of 3% (CHLO 3). Each group consisted of 25 animals. The animals were weighed and palpated weekly in order to register the presence, number, location, and size of each palpable tumor. Food intake per cage during 24 h was monitored during weeks 7 and 13 of the experiment (the value obtained was divided by the number of animals in the cage and thus we determined the average food intake per animal in the relevant cage). The measurements were taken four times (twice in weeks 7 and 13). The chlorella doses per animal and day were calculated in accordance with the amount of chow consumed. In the last week of the experiment (week 14), the animals were quickly decapitated, the blood from each animal was collected, mammary tumors were excised, and tumor size was recorded. Macroscopic changes in selected organs (liver, spleen, kidney, stomach, intestine, and lung) were evaluated at autopsy.

Histopathological and immunohistochemical analysis of rat tumors

A tissue sample of each mammary tumor was routinely formalin-fixed and paraffin-embedded. The tumors were classified according to the criteria for the classification of rat mammary tumors [16]. The additional parameter—grade of invasive carcinomas—was used. Tumor samples were divided into low-grade (LG) and high-grade (HG) carcinomas. The criteria for categorization (solidization, cell atypia, mitotic activity index, and necrosis) were chosen according to the standard diagnostic method of classification. HG carcinomas were considered to be tumors with ≥2 positive criteria; LG carcinomas were tumors with ≤1 positive criterion. Serum lipid parameters were evaluated using an Olympus AU640 (Olympus Optical, Tokyo, Japan) automatic biochemical analyser.

The paraffin block with the most representative tumor area of each mammary tumor was chosen for immunohistochemical analysis. The detection of selected proteins was carried out by indirect immunohistochemical method on whole paraffin sections, utilizing commercially available rat-specific antibodies (Santa Cruz Biotechnology Inc., Paso Robles, CA, USA; Dako, Glostrup, Denmark; Abcam, Cambridge, UK). After deparaffinization, endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in methanol for 30 min. Sections were pretreated in a microwave generator for 15 min in 10 mM citrate buffer (pH 6.0) and incubated with the primary antibody in phosphate-buffered saline (PBS) containing 1% bovine serum albumin, for 60 min at room temperature. The primary antibodies were visualized by a secondary staining system (EnVision, Dua Link System-HRP, cat. No. K4061, Dako North America Inc., Carpinteria, CA, USA) using diaminobenzidine tetrahydrochloride as a substrate. The sections were counterstained with hematoxylin, dehydrated, and mounted in Canadian balsam. Negative controls included sections where the primary antibody was omitted in the staining process. Immunohistochemically detected antigen expression was evaluated by precise morphometric method. Sections were screened and digital images of microscopic views at magnifications of ×200 were taken with an Olympus Evolt E-420 installed in an Olympus BX41N microscope. Expression of VEGF, caspase-3, and caspase-7 was analyzed in the cytoplasm of tumor cells. Ki67 was detected within the nucleus. Receptors for VEGF were observed in the cell membrane. Bcl-2 and Bax were detected as a membrane-associated oncoproteins. Expression of proteins was quantified as the average percentage of antigen-positive area in standard fields (0.5655 mm^2) of tumor hotspots. Morphometric analysis of the digital images was done using QuickPhoto Micro software, version 2.3 (Promicra, Prague, Czech Republic). The antigen-positive area was evaluated by phase analysis with standard thresholds for weak, mild, and strong intensities of immunoreactivity. The values of protein expression were compared only between effectively treated (CHLO 3 group) and nontreated (control group) carcinoma cells of female rats (the changes in the CHLO 0.3 group were not expected due to the main in vivo results). At least 40 images for one protein were analyzed (280 images for seven proteins).

Antiproliferative activity: Tumor cell lines and cell proliferation assay

MCF-7 (human breast adenocarcinoma, estrogen receptor-positive) cell line was provided by Dr. M. Hajdúch (Olomouc, Czech Republic). The cells were routinely maintained in Dulbecco's modified Eagle's medium with Glutamax-I supplemented with 10% fetal calf serum, penicillin (100 IU × mL^{-1}), and streptomycin (100 μg × mL^{-1}) (all from Invitrogen, Carlsbad, CA, USA) in humidified air with 5% carbon dioxide at 37°C. Before each proliferation assay, cell viability was determined using the trypan blue exclusion method and found to be >95%.

The effects of compounds on cell proliferation were determined using colorimetric microculture assay with the 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide (MTT) end point [17]. Briefly, 3 × 10^3 cells were plated per well in 96-well polystyrene microplates (Sarstedt, Germany) in the culture medium containing tested chemicals at final concentrations of 0.0012 to 1.25 mg/mL or Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Fluka, Buchs, Schwitzerland) at final concentrations 100 and 300 μM, or at mutual combinations. After 72 h of incubation, 10 μL of MTT (5 mg × mL^{-1}) (Sigma, Germany) were added to each well. After an additional 4 h, during which