Comparison Between Locally Produced *Chlorella vulgaris* and *Chlorella vulgaris* from Japan on Proliferation and Apoptosis of Liver Cancer Cell Line, HepG2

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**Abstract**

*Chlorella vulgaris* (CV), a unicellular green algae, has been shown to have many positive effects in human and animal studies in Japan and in the United States. In this study, we cultured CV in the laboratory to study its anti-carcinogenic properties in liver cancer cell line, HepG2. At the same time we compared the anti-carcinogenic properties of locally produced CV with that from Japan. Liver cancer cell line, HepG2 and normal liver cell line, WRL68, were treated with hot water extract of CV cultured in the laboratory (CVL) and with hot water extract of CV from Japan (CVJ). Results from the BrdU assay showed that CVJ increased proliferation of HepG2 cells initially and reduced later at higher concentration of CVJ extract. However, HepG2 cell line treated with hot water extract of CVL showed significant decrease in proliferation rate; with a 50% reduction at 1600 µg/ml of CV extract (IC 50 of CVL for HepG2). Proliferation of WRL68 decreased with a 50% reduction at 1700 µg/ml of CV extract (IC 50 of CV for WRL68). The TUNEL assay showed that CVL induced apoptosis in HepG2 cell line at the concentration of 2000 µg/ml while CV from Japan needed a higher concentration (3000 µg/ml) for apoptosis to occur. The extent of DNA damage by comet assay induced by CVL at 2000 µg/ml was found to be more severe (79% damage) compared to the same concentration of CV from Japan (58% damage). From this study it can be concluded that locally grown CV reduced proliferation and induced apoptosis of liver cancer cell line, HepG2 better than CV from Japan, suggesting that locally produced CV has better anticancer effect on hepatoma cell line.

**Keywords:** *Chlorella vulgaris*, apoptosis, antioxidant, oxidative damage.

**Introduction**

Hepatocellular carcinoma (HCC) is the fifth common malignancy worldwide and its incidence is increasing and will likely to continue for decades [1]. The aetiology of liver cancer is multi factorial. Some causes of liver cancer have been attributed to exposure to chemicals such as aromatic hydrocarbon, radiation, infectious agents such as aflatoxin and genetic factors [1]. The process of carcinogenesis involves an initiating event which induces genetic damage, followed by survival and progression of selected clones of the transformed cells to form tumors. The focus is now directed to the progressive dysregulation of apoptosis as a possible causal factor in aging and its associated diseases, including cancer [2]. Tissue homeostasis is a dynamic process that requires a balance between cell proliferation and cell loss. Apoptosis refers to the morphologic features of programmed cell death, which is characterized by cell shrinkage, nuclear condensation, membrane blebbing, fragmentation into membrane bound apoptotic bodies, and membrane changes that eventually lead to phagocytosis of the affected cell [3]. It can be triggered by numerous stimuli including hormones, antigens, carcinogens, chemotherapeutic agents, ionizing radiation, cell-cell interaction, growth factor withdrawal and physical trauma [4]. Much of cancer research over the last two decades has focused on genes that, when mutated, act in either a dominant or recessive manner to enhance proliferation. Imbalance between cell proliferation and apoptotic cell death will result in serious cell death. DNA damage can be assessed by the single cell gel electrophoresis or comet assay [5].

Until now, there is no effective remedy in the treatment of liver cancer besides surgery. Numerous studies have been done in the past to find alternative medications in the treatment of liver cancer. Herbs have been widely used in traditional medicine for the treatment of various cancers including liver cancer. Microalgae has been known to be a useful source of health foods rich in antioxidants [6]. *Chlorella vulgaris*, a unicellular green algae, has been widely used as a food additive and credited with therapeutic abilities [7]. *In vivo* studies have revealed the significant antitumor and antigenotoxic efficacy of CV [8]. *Chlorella vulgaris* is categorized as a green algae with high nutritive value (such as protein, nucleic acid, carbohydrate, chlorophylls, vitamins and minerals) content and potential in pharmaceutical use. The effects of *Chlorella vulgaris* (CV) extract on certain diseases have been reported. A study by Hasegawa et al [9] showed that hot water extract of CV inhibited metastases of...
fibrosarcoma tumor and immunosupression effect induced by 5-fluorouracil. Glycoprotein designated ARS-2 found in CV extract has been shown to have anticancer effect on mice induced fibrosarcoma [10].

The effect of CV on hepatoma cells has not been explored. In this study we investigated the anti cancer effect of CV extract on hepatoma cell line, HepG2 by measuring the rate of proliferation, apoptosis and the extent of DNA damage in hepatoma cells. The aim of this study is to find the probable antitumor properties of CV against hepatoma cell line, HepG2.

Materials and methods

Culturing of Chlorella vulgaris

Stock of Chlorella vulgaris Beijerinck (strain 072) was obtained from University of Malaya Algae Culture Collection (UMACC, Malaysia) and grown in Bold Basal Media (BBM) with a 12 hours dark : 12 hours light cycle.

Hot water extract

Cells were harvested by centrifugation at 1000 rpm, followed by a few washings in water. The algae was dried using freeze dryer and then mixed in distilled water at a concentration of 10% (w/v); algae suspension was then boiled at 100°C for 20 minutes using reflux method followed by centrifugation at 10,000 rpm for 20 min. The supernatant was lyophilized with freeze dryer to obtain powdered form of Chlorella vulgaris.

Cell culture and treatment

Liver cancer cell line HepG2 and normal liver cell line WRL68 were maintained in EMEM (Flow Laboratories, Sydney Australia) media supplemented with 1mM sodium pyruvate (SIGMA, St Louis, USA), 2mM glutamine, 10% fetal calf serum and 100 U/ml penicillin and streptomycin at 37°C in humidified 5% CO₂. Cell’s proliferation, apoptosis and Comet assay were performed when cells reached 70% confluence density. Chlorella vulgaris extract was added to cell lines after 24 hours incubation.

Cell Proliferation Assay

96 well plates were seeded with HepG2 and WRL 68 cells at a uniform density, 2 x 10⁴ cells per well. After 24 hours of incubation, cells were treated with hot water extract of CV and incubated further for another 24 hours. Cells were labeled with BrdU during the last 3 hours of CV extract treatment. The cells were fixed with denat solution and incorporation of BrdU was detected by immunoreaction. After substrate solution was added to each well, the amount of BrdU incorporated was determined by measuring absorbance at dual wavelengths 450/690 nm using scanning multi-well spectrophotometer (ELISA reader).

Apoptosis by TUNEL assay

Apoptotic cell death was determined by Dead End TM Colorimetric TUNEL System (Promega, USA). Floating cells and adherent cells in culture were collected (after 24 hours treatment with CV extract) in a tube, trypsinized, centrifuged and washed in PBS buffer. Cells were resuspended and applied onto poly-L-lysine-coated slides and air-dried. Cells were fixed by immersing slides in 4% formalin in PBS for 25 minutes at room temperature. After washing with PBS buffer, cells were permeabilized by immersing the slides in 0.2% TritonR X-100 solution in PBS for 5 minutes at room temperature. Cells were then equilibrated with 100 µl of Equilibration Buffer for 7 minutes. The equilibrated areas were blotted by tissue paper before adding biotinylated nucleotide and TdT reaction mix (100 µl) on the slides. Slides were covered with coverslips to ensure even distribution of the reagent before incubating at 37°C for 60 minutes inside a humidified chamber. Coverslips were removed and the slides were immersed in 2X SSC (sodium chloride 0.15M, trisodium citrate 0.015M) buffer for 15 minutes at room temperature, and washed twice with PBS. Endogenous peroxidases were blocked by immersing the slides in 0.3% hydrogen peroxide for 4 minutes at room temperature and washed again in PBS. Streptavidin Horis Radish Peroxidase (HRP) solution (1:500 in PBS) was added to each slide and incubated for 30 minutes at room temperature. After final washing with PBS, diaminobenzidine (DAB) solution was added on the slides for 20 minutes until light brown staining was observed. Finally, after each slides were mounted with DPX to be examined under light microscope.

 Comet assay

Microscope slides were each covered with a thin layer of 1% normal melting agarose (NMA) and allowed to dry overnight. After treatment, cell suspensions were prepared by washing the cells with PBS and treating them with trypsin/EDTA for 5 min. The cells treated with the same concentration were pooled, centrifuged (100 x g, 5 min) and resuspended in 500 µl PBS. 20 µl of cells (5 x 10⁴ cells) prepared for analysis were then mixed with 75 µl of 0.5% low melting agar (LMA) agarose at 37°C. The cell suspension was rapidly spread onto a precoated slide, covered with 25mm x 25mm coverslip and placed at 4°C for 5 min. The coverslip was gently removed and 75 µl of 0.5% LMA agarose was layered and covered as previously described. Coverslips were removed and the slides was exposed to lysis solution for at least 1 hr. After lysis, slides were exposed to alkaline electrophoresis buffer (pH 13) for 40 min and subjected to electrophoresis for 20 min (300 mA, 25 mV). Then, the alkali was neutralized with Tris buffer and DNA stained with 80 µl of a 5 mg/l ethidium bromide solution.
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Statistical analysis

Results refer to mean ± SEM with the experiment repeated at least 3 times. Statistical evaluation was done using the Student’s t-test. A p value of <0.05 was considered significant.

Results

Results of the proliferation assay showed that initially there was an increase in proliferation of HepG2 cell line when treated with hot water extract of CV from Japan (CVJ) and started to decrease at 1600 µg/ml of the CVJ extract (Figure 1). Proliferation of WRL68 cells followed the same pattern with an initial increase of proliferation and finally a decrease in proliferation beyond 1400 µg/ml. HepG2 cell line when treated with hot water extract of locally grown CV (CVL) showed significant decrease in proliferation with a 50% reduction at 1600 µg/ml of CV extract (IC50 of CVL for HepG2). Proliferation of WRL68 cells was decreased when treated with CVL extract resulting in a 50% reduction at 1700 µg/ml (IC50 of CVL for WRL68) and 3500 µg/ml respectively (IC50 of CVJ for WRL68). A 100% inhibition of proliferation of HepG2 cells was achieved at approximately 3000 µg/ml of CVL but at the same concentration, CV from Japan did not inhibit completely the growth of HepG2 cells.

Figure 1: The effect of CV extract on proliferation of HepG2 and WRL68 cell lines treated with different concentration of CV extract. Data are represented as mean ± SD. * showed significant changes (p<0.05) for n=3 triplicate after 24 hours treatment with different concentration of CV extract using BrdU assay. CVJ : Chlorella vulgaris from Japan. CVL : Hot water extract of locally grown Chlorella vulgaris.

The extent of DNA damage by comet assay at 2000 µg/ml induced by CVL was found to be mild to severe (79% damage) compared to the same concentration of CVJ 58% damage). Comet assay showed that at 3000 µg/ml, CVL induced 42% of severe DNA damage compared to 20% by CVJ (Figure 4).

Discussion

Previous studies by Hasegawa et al [10] and Noda et al [11] found an antitumor substance in the culture supernatant of Chlorella vulgaris (CV), a galactose-rich glycoprotein, consisting of a 6-linked β1-6 galactopyranose TUNEL assay showed that CVL induced apoptosis in HepG2 cell line at the concentration of 2000 µg/ml with distinct nuclear condensation and blebbing of the plasma membrane (Figure 2). Percent apoptosis of HepG2 cells induced by CVL is significantly higher (7%) compared to normal cells, WRL 68 (15%) (Figure 3). CVJ induced low rate of apoptosis (10%) in HepG2 cells at 2000 µg/ml, however at higher concentration (3000 µg/ml) HepG2 cells underwent apoptosis which was almost at the same rate for CVL (= 7%).

Figure 2: Comparison of morphology of WRL68 and HepG2 cells before and after treatment with 2000 µg/ml CVL extract. Apoptotic cells are absent in untreated WRL 68 (a) and in treated (2000 µg/ml CVL) WRL68 cells (b). Apoptotic cells are absent in untreated HepG2 cells (c), but present in treated (2000 µg/ml CVL) HepG2 cells (d). Arrow shows condensation of chromatin and cell blebbing on HepG2 cells.

Figure 3: The effect of CV extract on apoptosis of HepG2 and WRL68 cell lines. Data are represented as mean ± SD. * showed significant changes (p<0.05) for n=3 triplicate after 24 hours treatment with different concentrations of CVJ and CVL extract using TUNEL assay. Percent apoptotic cells are scored by taking an average of 1000 cells in 10 random fields. Apoptotic cells are absent in untreated and at 1000 µg/ml of CV but present quantitatively in HepG2 cells at higher concentrations of CV. The extent of DNA damage by comet assay at 2000 µg/ml induced by CVL was found to be mild to severe (79% damage) compared to the same concentration of CVJ 58% damage). Comet assay showed that at 3000 µg/ml, CVL induced 42% of severe DNA damage compared to 20% by CVJ (Figure 4).
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Figure 4: Detection of DNA damage in WRL 68 and Hep G2 cells as measured by comet assay after 24 hours treatment with CVL and CVJ. Data are represented as mean ± SD for n=3 triplicate after 24 hours treated with CV extract. (a) WRL68 cells treated with hot water extract of local CV. (b) WRL68 cells treated with hot water extract of Japan CV. (c) HepG2 cells treated with hot water extract of local CV. (d) HepG2 cells treated with hot water extract of Japan CV.

- rich containing carbohydrate (70%) and protein (35%). The glycoprotein is a component of the 55-67% of protein found in chlorella cells besides other nutrients: 1-4% chlorophylls, dietary fiber 9-18% and large amounts of minerals and vitamins. From this study, it is clear that the anticancer mechanism of CV in hepatoma cell line, HepG2 is by inhibiting DNA synthesis, causing DNA damage and inducing apoptosis. This is shown by the reduced incorporation of BrdU into replicating DNA of HepG2 cells treated with the CVL (*Chlorella vulgaris* grown locally), extensive DNA damage of HepG2 cells as shown by COMET assay and an increased number of apoptotic bodies in HepG2 cells as evidenced by TUNEL assay.

The induction of apoptosis is known to be an efficient strategy for cancer therapy [12]. Many Chinese herbal remedies such as *Rubus coreanum* [13] and *Paeoniae Radix*, [14] have been demonstrated to possess the ability in triggering the apoptotic pathway [15-16] in HepG2 cells. We also demonstrated that locally grown CV (CVL) inhibit proliferation and induced apoptosis of human liver cancer cell line (HepG2) better than CV obtained from Japan (CVJ). CVL inhibited the proliferation of liver cancer cells in a concentration dependent manner, ranging from 0-4000 µg/ml as shown by BrdU proliferation assay. Since high concentration of CVL and CVJ was required to induce significant cell death in our study, the antiproliferation effect of CV in human liver cancer cells
seems to be contributed by the induction of apoptosis pathway. CVL seemed to have performed better than CVJ in inducing apoptosis in liver cancer cell line. One probable reason could be the difference of nutrient contents including the glycoprotein rich substance between CVL and CVJ, which is determined by several factors, for example the preparation methods, plant species and location [9]. Since CV from Japan was cultured in a large scale, therefore some nutrient loss might be inevitable in comparison with CVL which was cultured in the lab at a much smaller scale, hence higher nutrient contents. Although CVL also inhibited the growth of normal WRL 68 cells, this however did not induce significant apoptosis in normal cells as shown in figure 3. The reduction in the growth of WRL 68 cells could be due to temporary arrest in growth at the G0 stage of its cell cycle. Most anticancer drugs exert their anticancer effects at the G1 or G2 stage to inhibit cell cycle progression [17].

Our dose response experiments showed that CVL generated larger comets in HepG2 cells than CVJ at 2000 µg/ml providing evidence that HepG2 cells treated with CVL were more prone to DNA damage at low doses of CVL compared to CVJ. However, it is clear that all samples treated with 3000 µg/ml of both CVL and CVJ generated comets larger than the control. The comets could have resulted in the event of apoptosis.

Antiproliferative and apoptotic effects of CV on liver cancer cell lines may be due to the glycoprotein rich substance derived from CV extract and its antioxidant properties such as vitamin C and E which would be the subject of interest in our future study.

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