

Antiproliferative ability of a combination regimen of crocodile egg extract, wild radix ginseng and natural *Ganoderma lucidum* on acute myelogenous leukemia

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Abstract. Chinese practitioners have employed the use of traditional Chinese medicine as an anti-cancer agent since the ancient period. Different combinations have been formulated for various purposes. Some have been claimed for post-chemotherapy use but their direct actions on cancer cells may not be significantly reported. In the present study, we have tested the possible anti-leukemia potential of a combination regimen including crocodile egg extract, wild radix ginseng and natural *Ganoderma lucidum* (CGG extract) on acute myelogenous leukemia (AML) *in vitro*. A water soluble CGG extract was prepared and its antiproliferative activity was tested on the KG1a AML cell line and two freshly prepared bone marrow aspirate samples isolated from patients with *de novo* AML during presentation by a MTS/PMS assay. Furthermore, the possible activity of the CGG extract on the regeneration potential of KG1a cells was also investigated using a semi-solid methyl-cellulose colony formation assay. Lastly, the acute toxicity of CGG extract was further examined by a single high-dose oral feeding to rats. We found that the CGG extract could possess significant anti-proliferative activity on AML cells. A strong colony formation inhibition was further demonstrated on KG1a cells. After feeding the rats with an excessive dose of CGG extract, we observed no development of acute toxicity. We concluded that

the CGG extract has growth inhibitory potential on KG1a cells and AML bone marrow samples *in vitro*. An *in vivo* toxicity test revealed that no acute toxicity was observed after feeding the rats a high dosage of the CGG extract. Further animal model tests are necessary to investigate the possible chronic toxicity of the CGG extract.

Introduction

Traditional Chinese medicine (TCM) is believed to have been used for at least five thousand years. Many commonly recognized TCM remedies for cancer treatment including ginseng have been extensively studied (1). Even though most of its active ingredients have been identified and purified, Chinese practitioners continue to use whole ginseng extract for adjunctive cancer therapeutic purposes. *Ganoderma lucidum* is another well-known TCM for cancer treatment. *G. lucidum* extract acts to induce growth inhibition plus apoptosis in breast cancer cell line MCF-7 and prostate cancer cell line PC-3 (2-3). The ratio of the apoptotic protein bax to the two anti-apoptotic proteins bcl-2 and bcl-XL was markedly increased after *G. lucidum* extract incubation. The apoptotic mechanism further involved cell cycle arrest together with change in cyclin level.

The combination of crocodile egg extract (33%), wild radix ginseng (34%) and natural *G. lucidum* (33%) (CGG extract) has been suggested for individuals with poor immunity, deteriorating health, illness due to accumulated hard work, blood deficiency, physical enervation and fatigued patients especially after chemotherapy, radiotherapy and operation. However, the possible direct interaction of this combination towards acute myelogenous leukemia (AML) cells remains uncertain. Here we reported the experimental results of the CGG extract on the KG1a AML cell line plus primary cultured bone marrow cells isolated from *de novo* AML patients. Furthermore, the acute toxicity of the CGG extract was observed by a single-dose oral feeding to rats. Our results

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suggested that the CGG extract has an antiproliferative effect on AML cells and no acute toxicity was found after feeding rats with an excessive dose of the CGG extract.

Materials and methods

Preparation of the CGG extract for the *in vitro* antiproliferative activity test. The CGG extract sample was manufactured by Hong Kong Health and Long Biotechnology Ltd. and provided by Wah Sze Health Products Ltd. in capsule form. The powder was first dissolved in warmed distilled water and solubilization was further facilitated by sonication. Afterwards, the aqueous solution was filtered and warmed with absolute ethanol using an evaporator. Lastly, the remaining powder was weighed and adjusted to a final concentration with complete medium of 5000 $\mu\text{g/ml}$. The clear solution was then filtered using a 0.22- μm syringe filter. The final solution of the CGG extract was then stored at -80°C until use.

Cell line and cell culture. The acute myelogenous leukemia (AML) cell line KG1a was obtained from American Type Culture Collection. It was maintained in RPMI-1640 medium (JRH Biosciences) supplemented with 10% of heat-inactivated fetal bovine serum (Hyclone) together with antibiotics involving penicillin and streptomycin.

Human bone marrow cell collection and isolation. Bone marrow cells were collected from consenting *de novo* AML patients during presentation by bone marrow aspirate methods according to the regulations of the institute. A total of two adult subjects were recruited. Immediately after collection, mononuclear cells were enriched by Ficoll plaque (General Electricity, Amersham) gradient centrifugation. Cells were washed twice with phosphate-buffered saline and re-suspended in complete medium. Viable cell percentage was then estimated by a trypan blue exclusion assay and counted using a hemacytometer.

[3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) assay. Changes in the cellular viability of CGG-treated cells were monitored using the MTS activity assay as reported previously. Briefly, leukemia cells (KG1a and AML bone marrow aspirate samples) were seeded at a concentration of $1 \times 10^5/\text{ml}$. After 6 h, CGG was diluted in complete medium and they were added at the serial diluted concentrations. After 72 h of incubation, medium was removed and the MTS/PMS solution dissolved in PBS was added, and they were further incubated for exactly 30 min. Afterwards, the optical absorbance was determined at 490 nm according to the user's manual with minor modifications (Promega).

Morphological monitoring of the CGG extract-treated colonies. KG1a leukemia cells were mixed with methyl-cellulose and incubated ($1 \times 10^5/\text{ml}$). The 500 $\mu\text{g/ml}$ CGG extract disc contained 10% of 5 mg/ml CGG extract dissolved in complete medium. The vehicle control consisted of 10% complete medium as placebo. Colony formation for both the CGG- and vehicle-incubated leukemia cells was recorded after 7 days.

Proteasome activity assay. KG1a cancer cells were treated with the CGG extract (500 $\mu\text{g/ml}$) for 24 h. Total cellular protein was extracted and quantitated by the Bradford method. Then 30 μg protein extract was mixed with the proteasome specific substrate (Suc-Leu-Leu-Val-Tyr-AMC, 20 μM) and reaction buffer and further incubated at 37°C for 1 h. The resulting relative fluorescence units were then compared to the untreated control. Triplicate tests were performed for each experiment. A total of three experiments were conducted.

***In vivo* acute toxicity test.** This part of the experiment was a consultancy study performed by the State Key Laboratory of Safety Evaluation on New Drugs in Guangzhou, P.R. China according to their regulations on animal testing. Briefly, a total of 40 rats were divided into two groups. Each group had 10 males and 10 females. For the CGG group, all of them were fed 8 g/kg body weight of the CGG while the placebo group was fed only distilled water. Change in physical parameters including body weight were recorded for one week.

Results

CGG extract induced growth inhibition and reduced the colony formation potential of AML cells. After incubating the

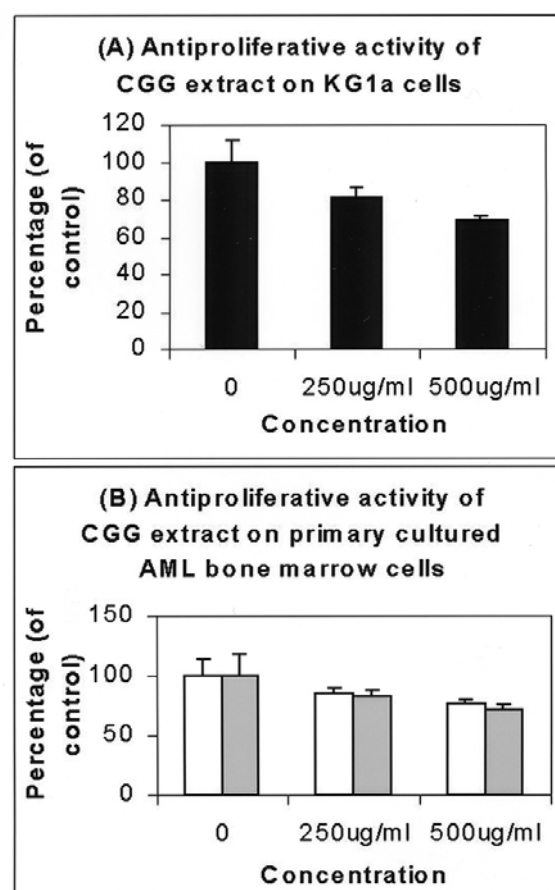


Figure 1. Antiproliferative effect of the CGG extract on (A) KG1a leukemia cell line and (B) two primary cultured AML bone marrow samples after 72 h of incubation and assay by MTS. Result are the mean \pm SD of one representative experiment. Triplicate tests were performed for each experiment. A total of three experiments were performed and similar results obtained.

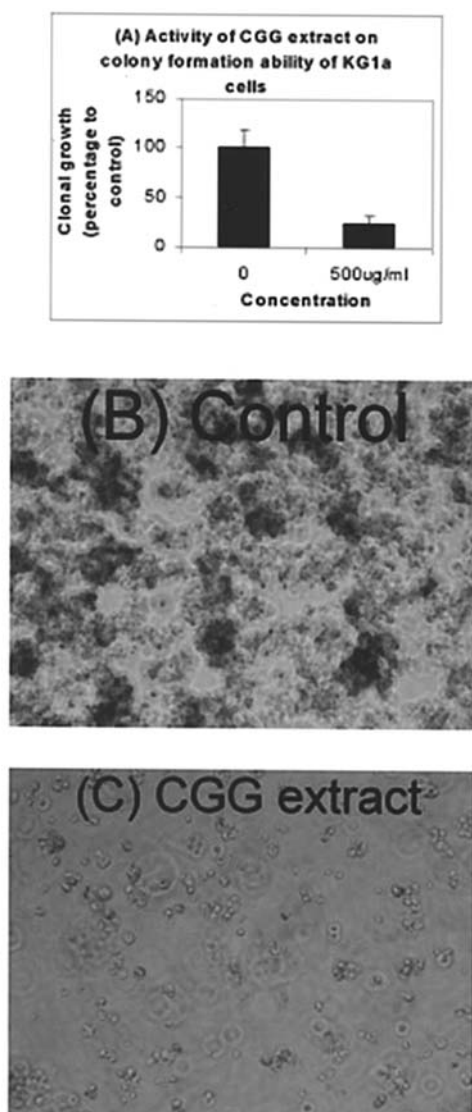


Figure 2. (A) Comparison of the colony formation ability of KG1a cells in the presence of the CGG extract. Results are the mean \pm SD of one representative experiment. Triplicate tests were performed for each experiment. A total of three experiments were performed and similar results obtained. (B) KG1a cells cultured with vehicle (10% complete medium, v:v). (C) KG1a cells cultured with the CGG extract (500 μ g/ml in complete medium, 10% v:v). A viable colony reduced the MTT to intracellular dark formazan.

KG1a cell line with the CGG extract for 72 h, we observed that there was a significant antiproliferative effect exerted by the CGG extract on this leukemia cell line. The influence was dose-dependent (Fig. 1A). Similarly, the CGG extract was also active on the two bone marrow samples isolated from the AML patients (Fig. 1B).

To see whether continuous exposure of the CGG extract to KG1a cells would inhibit their regeneration potential, a colony formation assay was performed. As shown in Fig. 2, a strong inhibitory activity on the colony formation ability of the KG1a cells was observed.

KG1a proteasome activity was reduced by the CGG extract. A significant reduction in relative fluorescence units from the CGG extract-treated cells (500 μ g/ml for 24 h) was observed. The relative mean percentage value dropped to ~77% of the

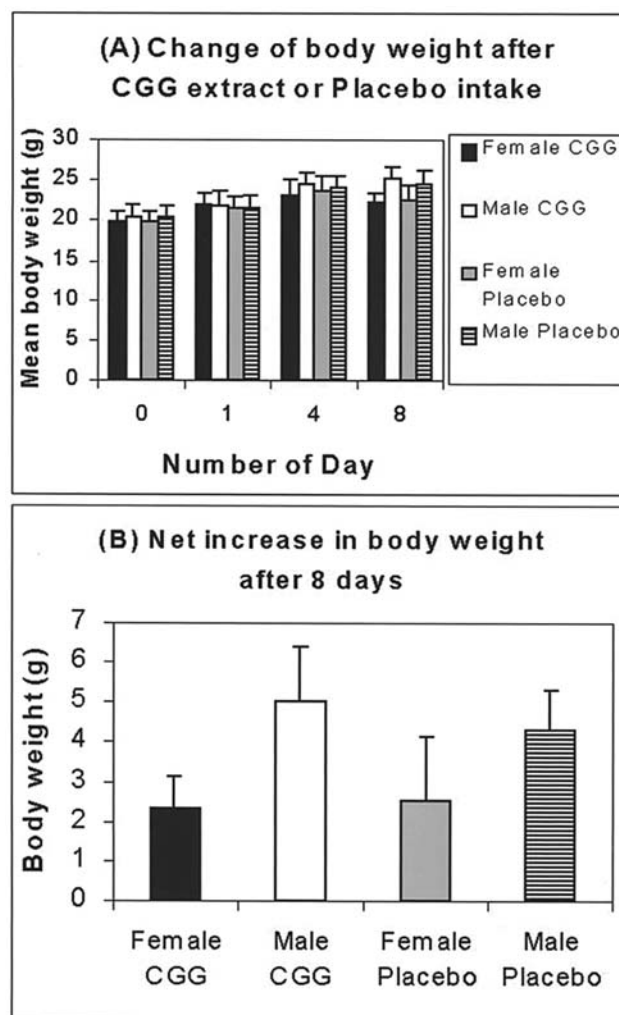


Figure 3. (A) Continuous monitoring of the changes in the animal body weight after a single high oral dose of the CGG extract. Each sex group had 10 rats. (B) Net change in body weight of the individual groups after 8 days. Each sex group contained 10 rats. Results are shown as mean \pm SD.

untreated control. Results were obtained from the mean of three independent experiments with each experiment performed in triplicate.

In vivo acute toxicity test. After feeding the rats with a high dose of the CGG extract (8 g/kg), no mortality was found. No abnormal behavior was detected. Investigation from the autopsy samples including the liver, the lung, and the reproductive and gastrointestinal systems revealed no pathological transformation. Lastly, there was a similar increment in the mean body weight of the rats in both the placebo and test groups (Fig. 3).

Discussion

Ginseng and *G. lucidum* have been used as a home remedy in TCM for a long time in South East Asia. Both are commonly used in alternative or supplemental cancer treatment. Recently, the use of *G. lucidum* on malignant cells of hematologic origin is especially attractive. Müller *et al* has performed an extensive investigation on the activity of the ethanol extract

of *G. lucidum* on a panel of leukemia, lymphoma and myeloma cell lines *in vitro*. Their results strongly confirmed that *G. lucidum* may inhibit a distinct signal transduction pathway in different cancer cells (4). It is believed that the use of both ginseng and *G. lucidum* in cancer therapy is promising. The use of crocodile product as a medicine is also well recognized in China. Traditionally, the use of dried crocodile meat is an important therapeutic regimen in the treatment of asthma. The combination regimen including crocodile egg protein extract, ginseng and *G. lucidum* is commonly used to boost the immune response. Here, we asked whether such a combination could be active on AML cells including the KG1a cell line and bone marrow samples from patients.

We prepared a maximum aqueous extract concentration of 5000 µg/ml. Using the MTS and colony formation assays, we confirmed that the CGG extract was active on both the KG1a cell line and two primary cultured AML samples. The antiproliferative activity was dose-dependent. Furthermore, a strong inhibition of regeneration potential on the KG1a cells was exerted by the CGG extract.

Proteasome is involved in mediating the protein degradation of many regulatory proteins concerned with normal physiological processes including programmed cell death and cell division (5). Therefore any naturally occurring or synthetic proteasome-specific inhibitors are believed to have potential use in cancer therapy. Recently, we demonstrated the proteasome inhibitory potential of *Gleditsia sinensis* fruit extract (6). Here we further demonstrated that the CGG extract could also contain potential natural proteasome inhibitor(s).

The major limitation to *in vitro* models is that cell cultures are only partly representative of *in vivo* conditions. As we know, the complexity of the metabolism of the human body and the interactions among constituents as well as many unknown factors cannot simply be predicted by cell lines and *in vitro* models (7). Here, our data demonstrated the fundamental step that the CGG extract was active on both the cell line and the primary culture of AML, and it is a potential proteasome inhibitor. This should provide us with a preliminary implication to further examine the possible anti-leukemia ability of the CGG extract.

Both acute and chronic toxicity is an important issue for the consumption of TCM remedies. *In vivo* animal experimental results suggested that the oral administration of the CGG extract at a dose of 8g/kg induced no development of acute toxicity on both sexes of rats. Assuming that an adult has a body weight of 50 kg with a total blood volume of 5 liters (8), the calculated concentration of the CGG extract, based on our *in vitro* data, would be 50 mg/kg in the human body. Thus theoretically our therapeutic concentration would still be far from the tested dose obtained from the animal experiment. Further SCID mice with an AML model might possibly explain whether oral administration of the CGG at the dosage predicted from *in vitro* data induces leukemia remission *in vivo*.

We have examined the anti-cancer potential of some TCM extracts including *G. sinensis* fruit (9), *Radix Sophorae Tonkinensis*, *Scutellaria barbata* (10) and *Brucea javanica* (11). All were found to be active in cancer of different origins including leukemia. Our results here further support

the view that TCM could be an alternative choice for leukemia patients as a supplement to traditional chemotherapy (12).

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