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Original Article

Optimal conditions for cordycepin production in surface liquid-cultured *Cordyceps militaris* treated with porcine liver extracts for suppression of oral cancer

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ABSTRACT

Cordycepin is one of the most crucial bioactive compounds produced by *Cordyceps militaris* and has exhibited antitumor activity in various cancers. However, industrial production of large amounts of cordycepin is difficult. The porcine liver is abundant in proteins, vitamins, and adenosine, and these ingredients may increase cordycepin production and bioconversion during *C. militaris* fermentation. We observed that porcine liver extracts increased cordycepin production. In addition, air supply (2 h/d) significantly increased the cordycepin level in surface liquid-cultured *C. militaris* after 14 days. Moreover, blue light light-emitting diode irradiation (16 h/d) increased cordycepin production. These findings indicated that these conditions are suitable for increasing cordycepin production. We used these conditions to obtain water extract from the mycelia of surface liquid-cultured *C. militaris* (WECM) and evaluated the anti-oral cancer activity of this extract *in vitro* and *in vivo*. The results revealed that WECM inhibited the cell viability of SCC-4 oral cancer cells and arrested the cell cycle in the G2/M phase. Oxidative stress and mitochondrial dysfunction (mitochondrial fission) were observed in SCC-4 cells treated with WECM for 12 hours. Furthermore,

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porcine liver extracts
water extract

WECM reduced tumor formation in 7,12-dimethylbenz[a]anthracene-induced hamster buccal pouch carcinogenesis through the downregulation of proliferating cell nuclear antigen, vascular endothelial growth factor, and c-fos expression. The results indicated that porcine liver extracts irradiated with blue light light-emitting diode and supplied with air can be used as a suitable medium for the growth of mycelia and production of cordycepin, which can be used in the treatment of oral cancer.

1. Introduction

Oral cancer is one of the most common cancers worldwide and causes 135,000 deaths annually [1]. Oral squamous cell carcinoma (OSCC) accounts for 90% of oral malignant tumors. Therapeutic targets for cancer, such as gain-of-function mutations in oncogenes and loss-of-function mutations in tumor suppressor genes, are key factors for head and neck carcinogenesis. Tobacco and alcohol consumption have been reported to be major factors contributing to oral cancer development [2]. However, betel quid chewing is one of the major causes of oral cancer in Taiwan, which has high mortality and poor prognosis. Therefore, new therapeutic approaches focusing on molecular targets and mechanisms mediating tumor cell growth or cell death have gained increasing attention for improving patients' survival and quality of life.

The hamster buccal pouch (HBP) carcinogenesis model is the most well-characterized animal model for investigating the development of oral cancer and the effect of intervention with chemopreventive agents. The development of both OSCC and HBP carcinoma is associated with sustained genetic mutations leading to excessive cell proliferation, prolonged cell survival, and apoptosis evasion [3]. In addition, various morphological and histological similarities are present between human OSCC and HBP carcinoma [4,5], providing a rationale for analyzing the effect of putative chemopreventive agents on the HBP model. Moreover, the HBP model has been used in several studies on oral cancer [6–10].

Chemoprevention through dietary agents has evolved as an effective strategy for controlling the incidence of oral cancer. In recent years, natural food products have received increasing attention because of their potential role in the prevention of and intervention in carcinogenesis and neoplastic progression [11,12]. Therefore, chemotherapy by using functional foods may aid in cancer prevention.

The fungus *Cordyceps militaris* has been used as a herbal tonic in traditional Chinese medicine for > 300 years. Numerous studies have recently reported that the anticancer activity of *Cordyceps sinensis*, including the inhibition of B16 melanoma [13], leukemia [14], thyroid carcinoma [15], hepatocellular carcinoma [16], and renal cancer [17] cells, is due to cordycepin, a crucial bioactive compound produced by *C. militaris*. However, tumor inhibition by cordycepin is attenuated by adenosine deaminase catalysis *in vivo* [18]. In addition, the inhibition of oral cancer cells by cordycepin and *C. militaris* remains unclear.

A study reported the use of a biochemical medium as a fermentation medium in the surface liquid cultivation of *C. militaris* [19]. In addition, another study indicated that natural materials can be used as the components of a fungal medium [20]. These studies suggested that natural materials can be used to cultivate *C. militaris*. In addition, recent studies have investigated optimal carbon and nitrogen resources and other nutrients required for *C. militaris* cultivation and cordycepin production [21]. Extracts from the porcine liver contain a high amount of vitamins, proteins, adenosine, and Mg^{2+}; however, the effect of porcine liver water extracts on the surface liquid cultivation of *C. militaris* for cordycepin production remains unknown. Therefore, in this study, we investigated the effect of porcine liver extracts on the surface liquid cultivation of *C. militaris* for cordycepin production. In addition, we evaluated the anti-oral-cancer activity of water extract isolated from the mycelia of surface liquid-cultured *C. militaris* (WECM). Furthermore, we investigated whether WECM inhibits tumor growth in 7,12-dimethylbenz[a]anthracene (DMBA)-induced HBP carcinogenesis.

2. Materials and methods

2.1. Materials

Crystal violet, propidium iodide, DMBA, sodium dodecyl sulfate (SDS), cordycepin, Triton X-100, trypsin, and trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum was purchased from Life Technologies (Auckland, New Zealand). MitoSOX Red and Mitotracker Deep-Red FM were purchased from Invitrogen (Carlsbad, CA, USA). Porcine liver (500 g) extract was prepared through treatment in water (5 L) at 4°C for 24 hours. The liquid extract was filtered and freeze dried, and the porcine liver powder extract was stored at −80°C.

2.2. Sample preparation

C. militaris (BCRC34380) was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). The liquid seed culture was inoculated with a mycelial mat from a stock slant and cultured in a 500-mL conical flask containing 200 mL medium (10 g/L peptone, 30 g/L glucose, 0.5 g/L $MgSO_4$, 0.5 g/L K_2HPO_4, and 0.5 g/L KH_2PO_4) at 25°C on a rotary shaker at 150 rpm for 7 days for maintenance. To evaluate the effects of porcine liver powder extracts on cordycepin production in the

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