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Original Article

Prevention potential of *Cordyceps militaris* aqueous extract against cyclophosphamind-induced mutagenicity and sperm abnormality in rats

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ABSTRACT

For decades, many natural products (from plants, animals, fungi and bacteria) have been popularly used for nutrapharmaceutical purposes such as treating and preventing a variety of symptoms and diseases. *Cordyceps militaris* is becoming one of the most popular medicinal mushrooms, especially in Asian countries because of the promising abilities of its extracts in promoting health (antihypertensive, hypoglycemic, sexual potentiative, cancer/tumor prevention, cancer treatment). Nevertheless, research on antimutagenic activity which can protect against DNA damage from chemicals and free radicals is still insufficient. Thus, the present study investigated the antimutagenic effect of aqueous extract from *C. militaris* in male albino rats using micronucleus and sperm morphology assays. Male Wistar rats ($n = 15$) were orally administered with crude *C. militaris* extract at doses of 40 mg/kg bodyweight (bw) and 60 mg/kg bw for 3 wk before mutagenic induction using cyclophosphamind (CP). The normal control group received only distilled water (1 mL/d). The results of antimutagenic assay at 1 wk after CP injection showed that the frequency of micronuclei found in the control group after CP injection was higher than for the standard criterion of rat, while, the frequency in the treated group was significantly lower. However, the ratio of polychromatic erythrocytes to normochromatic erythrocytes in all treated groups was lower than that of the standard and the total sperm abnormalities in all treated groups were not significantly different from the control group. These results revealed the potential preventive benefit of a mutagenic effect of *C. militaris* aqueous extract on male rats.

Introduction

Cordyceps mushroom is a variety of ascomycetic growth that incorporates around 400 species, with all *Cordyceps* species being insect endoparasitoids, for the most part on bugs and different arthropods at different developmental stages (Mitzuno, 1999). These mushrooms (including the species *C. sinensis* which is now classified as *Ophiocordyceps sinensis*) have a long history as uncommon and often outlandish therapeutic use but also have an exceedingly respected foundation in customary Chinese solutions with clearly various wide-ranging restorative impact (Mitzuno, 1999).

In more recent years, *C. militaris* (another species of *Cordyceps* fungi) has become well known among Asian people as a miracle drug as it has been used to prevent and cure a variety of diseases including digestive disorders, peptic ulcers, diabetes and cancer (Shang et al., 2013; Tuli et al., 2014). Several biological activities of *C. militaris* have been studied and evaluated including anti-urolithiatic, anticancer, antioxidant, hypocholesterolemic and hypoglycemic capacities (Gu et al., 2007; Das et al., 2010; Reis et al., 2013; Tuli et al., 2014; Sun et al., 2014).

C. militaris is a species of fungus in the family Cordycipitaceae, and the type species of the genus *Cordyceps* which was originally described by Carl Linnaeus in 1753 as *Clavaria militaris* (Shrestha et al., 2014). Since *C. militaris* has been recognized as a new miracle drug which can cure and prevent many types of diseases, several research groups from different countries have performed some experiments and analyses to prove this claim (Zhu et al., 1998). The fungus has been recommended for several diseases in patients as a

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functional food and nutrapharmaceutical supplements since *C. militaris* and its extracts could exhibit so many biological activities including being antioxidant, anti-inflammatory, anticancer, antitumor, antimicrobial (bacteria, virus, fungi, protozoa), hypoglycemic and hypolipidemic (Ahn et al., 2000; Zhou et al., 2002; Won and Park, 2005). Several bioactive compounds of *Cordyceps* spp. have been discovered such as ergosterol, 3′-amono-3′-deoxyadenosine ($C_{10}H_{14}N_6O_3$), homocitrullyl amino adenosine ($C_{11}H_{27}N_9O_5$), adenine ($C_5H_5N_5$), cordycepic acid ($C_6H_{14}O_6$) and D-mannitol ($C_6H_{14}O_6$), polysaccharides and cordycepin ($C_{10}H_{13}N_5O_3$), with the latter was likely to be the key bioactive compound or the signature metabolite of *C. militaris* (Gu et al., 2007; Khan et al., 2010; Kumar et al., 2010).

Some reports suggest promising antioxidant activity which could possibly protect the somatic and germ cells from DNA damage which would be beneficial for antimutagenic properties (Cho et al., 2003). Even though there have been a few reports on the antimutagenic property of *C. militaris* extracts using several complicated extraction solvent systems (Kim et al., 2001; Cho et al., 2003), the antimutagenic activity of its aqueous extract using *in vivo* assay (animal model) has never been reported before. According to these reports, only *in vitro* assays were performed to evaluate its antimutagenic activity using the Ames test on a human cell line induced by several chemical agents such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, 4-nitroquinoline-1-oxide, benzo(alpha)pyrene, and 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indol. Therefore, *in vivo* assay is required to evaluate the antimutagenic effect of *C. militaris* aqueous extract.

Thus, the present study aimed: 1) to investigate the antimutagenic effect of the aqueous extract from *C. militaris* on cyclophosphamind-induced male rats using micronucleus and sperm morphology assays; and 2) to observe genotoxic evidence to ensure the safety of using *Cordyceps* as a “nonmutagenic side effect” nutrapharmaceutical agent.

Materials and methods

Crude extract preparation and extraction

Mushrooms of *C. militaris* were grown under static conditions in peptone yeast extract glucose broth medium for 30 d at 25 °C. All mycelial hyphae were collected and washed with distilled water. The aqueous extraction process was performed using 2 h boiling of the mycelia o the washed mushrooms, percolating with filtrate paper and freeze drying to obtain the *Cordyceps militaris* (CM) extract powder. The powder of the aqueous extract was prepared after freeze drying.

Animals and treatments

Healthy young male Wistar rats (*Rattus norvegicus*), aged approximately 4–5 wk and weighing 120–150 g were purchased from the National Laboratory Animal Center, Salaya, Nakhon Pathom, Thailand. They were allowed to acclimatize in the departmental animal facility for 1 wk prior to the day of the experiment. They had access to water and standard diet (C.P. 082). Three groups of five replicates were maintained in air-conditioned rooms at 25 ± 2 °C and 50 ± 10% relative humidity, with a 12hr light:dark natural cycle. All procedures involving animals were conducted with strict adherence to guidelines and procedures reviewed and approved by the Institutional Animal Care and Use Committee of Research Institutes, Kasetsart University (*Approval no. ACKU 04359*).

To evaluate the antimutagenicity of *Cordyceps militaris* (CM) aqueous extract, five animal groups were orally treated with three different doses (0 mg/kg bodyweight (bw), 40 mg/kg bw, 60 mg/kg bw of CM aqueous extract powder for 3 wk. The experimental design is illustrated in Fig. 1. All 15 rats were divided into three groups: 1) control group (treated with distilled water); 2) the first treatment group (treated with 40 mg/kg bw); and 3) the second treatment group (treated with 60 mg/kg bw). Mutagenicity of young erythrocytes and sperm abnormalities were induced using a single intraperitoneal injection of 80 mg/kg bw of cyclophosphamind (CP) on day 22. At 1 wk after exposure, the rats were sacrificed. Both micronucleus and sperm morphology abnormality assays were performed to confirm the antimutagenic effects of CM extract.

Micronucleus assay

The formation of the micronucleus in young erythrocyte in bone was observed and micronucleated polychromatic erythrocytes (MNPCE) scoring was carried out according to the procedure of Schmid (1975). Briefly, the rats were euthanized using 200 mg/kg of Nembutal® (sodium pentobarbital) that was injected intraperitoneally, femurs were dissected, opened, and the young erythrocytes from bone marrow were gently flushed out using fetal bovine serum. The product of homogenization of the bone marrow in serum was centrifuged at 1000 revolutions per minute for 5 min. The bone marrow cells were smeared on glass slides, air-dried and then fixed in absolute methanol for 5 min and stained with Giemsa to detect MNPCEs. For each animal, three slides were prepared and a minimum of 2000 polychromatic erythrocytes (PCE) were counted to determine the MNPCE frequency. For each animal, 1000 normochromatic erythrocytes (NCE) were counted under high power using a light microscope, as well as the PCE frequency within the same slide area, and the PCE:NCE ratio was then calculated to evaluate the cytotoxicity.

Sperm morphology assay

Both epididymides were removed for sperm morphology evaluation. The sperm smears were stained with eosin and methylene blue and examined under light microscopy. Two hundred sperm were scored for each animal. Abnormal morphologies were classified into normal, having head defects, tail defects, and both head and tail defects.

Statistical analysis

All parameters were expressed as means ± S.D. The data were subjected to a one-way analysis of variance (ANOVA) to determine the level of significance between the control and treatment. Further comparison between groups was performed using the least significant difference and Tukey's test. All differences were considered significant at the 95% confident level, ($p < 0.05$).

Results

Preventive effect on micronucleus induction bone marrow cells

The results of micronucleus formation in male rats receiving different doses of crude extracts from *C. militaris* for 3 wk are summarized in Fig. 2B. The frequency of micronuclei found in rats treated with high doses of *C. militaris* (40 mg/kg bw and 60 mg/kg bw) was significantly lower than for the control group. The ratio of polychromatic erythrocytes to normochromatic erythrocytes (PCE:NCE) was similar in both test groups. The characterization on MNPCE, PCE and NCE was undertaken according to Fig. 2A. Only rats in the control group that had received distilled water had a significantly lower PCE:NCE value than either of the treatment groups.

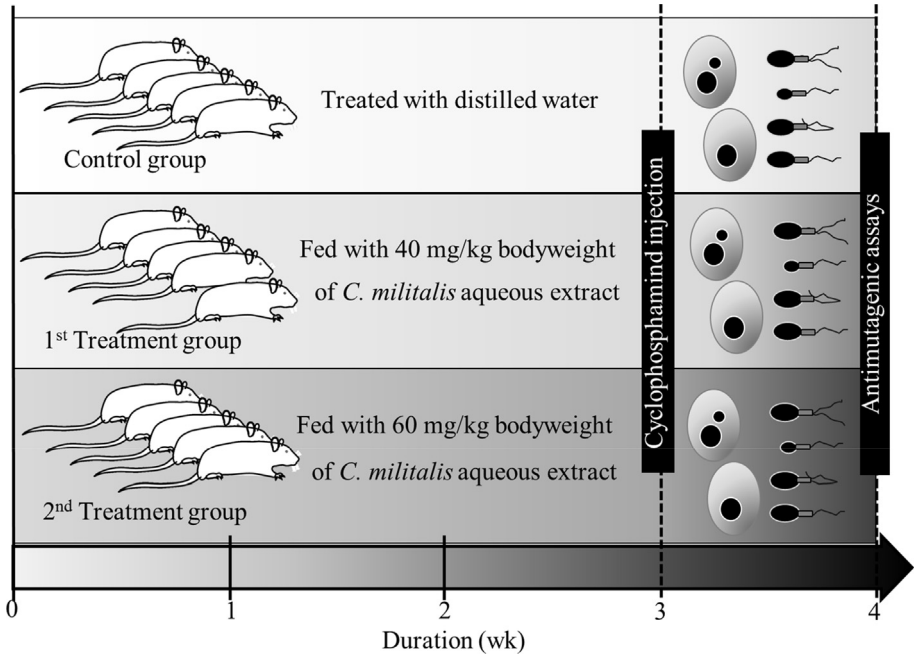

Fig. 1. Summary of experimental design and workflow to evaluate the antimutagenic effect of cyclophosphamind aqueous extract on cyclophosphamind-induced mutagenic rats using both micronucleus and sperm morphological abnormality assays.

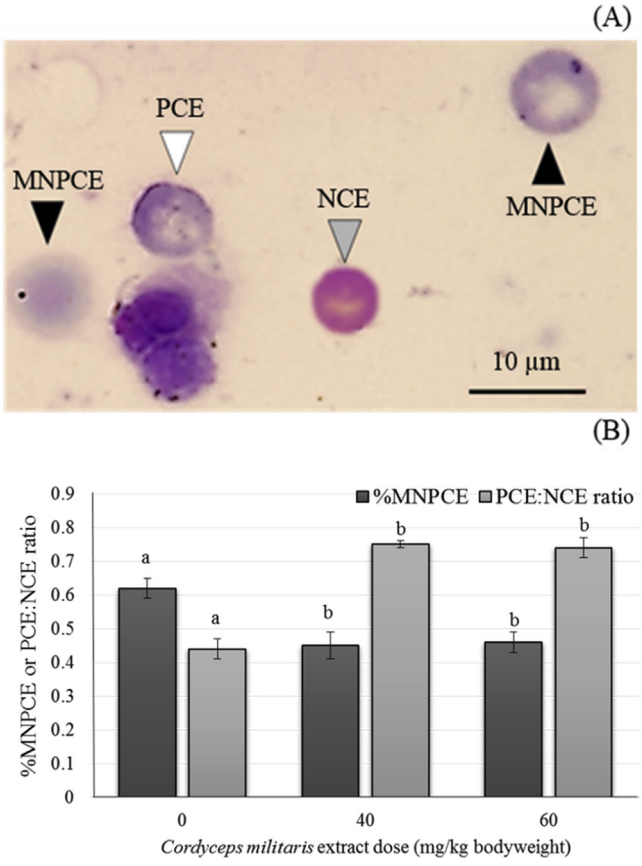

Fig. 2. (A) Bone marrow cells, showing normochromatic erythrocytes (NCE), polychromatic erythrocytes (PCE), and larger cells containing micronucleus polychromatic erythrocytes (MNPCE); (B) Frequencies (%MNPCE and PCE:NCE) in rat bone marrow cells treated with cyclophosphamind (CP) after treatment with different doses of CM aqueous extract for 3 wk, where columns indicated by different letters (a and b) are significantly ($p < 0.05$) different using one-way ANOVA and error bars indicate ± SD.

Preventive effect on sperm abnormalities

The results from the sperm morphology assay are shown in Table 1. Statistical analysis of the data indicated that the frequency of total abnormal sperm in the groups treated with *C. militaris* extracts was not significantly different from that of the control. When considering the sperm abnormality in each category, it was found that only rats treated with *C. militaris* extract at a dose of 60 mg/kg bw had a significantly lower percentage of head defects than the control. However, the percentage of tail-only defects and both head and tail defects in all treated groups was not different from that of the control.

Discussion

The global interest in medicinal plants has increased in recent decades and many natural products have been rushed onto the market to meet the demand for such health care products. The toxicity and side effects of plant-based products requires thousands of research studies to prove the safety of medicinal plants including most natural products and genotoxic effects are one of the most investigated topics (Tansuwanwong et al., 2007). Moreover, antimutagenic effects are of interest for the possible discovery of compounds that can aid in the treatment of cancer and DNA damage (Zhao et al., 2004). A reduction in frequency of MNPCE after micronucleus induction reflects the potential of a substance to prevent chromosome breakage or uneven chromosome distribution (Yener and Dikmenli, 2009). The micronucleus test is one of the most accepted methods for mutagenic evaluation in animals (Ledebur and Schmid, 1973) and the mutagenic effects on male germ cells can be quantified using sperm morphology assay (Anderson et al., 1997). This present study investigated the mutagenicity of aqueous extracts from *C. militaris*, a popular medicinal fungus in Asia. Notably, there have been some scientific reports regarding the positive correlation between the antioxidant and antimutagenic abilities from both natural products and synthetic compounds (Yoshimoto et al., 1999; Paniagua-Perez et al., 2009;

Table 1
Number of abnormal sperm defects by type in male rats treated with *C. militaris*.

Groups	CM extract dose (mg/kg bw)	Number of abnormal sperm (%)		
		Head only	Tail only	Head and tail
Control	0	4[a]	94[a]	2[a]
First treatment	40	3[ab]	96[a]	1[a]
Second treatment	60	2[b]	95[a]	3[a]

CM = *Cordyceps militaris*.
[a,b] letters with different lowercase superscript letters in the same column are significantly different ($p < 0.05$) using Tukey's test.

Zegura et al., 2011; Kocyigit et al., 2016). According to the reports on the promising antioxidant property of *Cordyceps* extracts (Yamaguchi et al., 2000; Zhan et al., 2006; Yu et al., 2007; Reis et al., 2013), the present study hypothesized that the CM extract could protect or prevent or both the somatic and germ cells against genotoxic chemicals. Although the extracts from entomopathogenic fungal species was able to cause a significantly lower frequency of micronuclei in both treatment groups compared to the control group, the MN frequency in most tested groups was higher (0.12–0.41%) than the spontaneous frequency of that in laboratory rat (Kilbey et al., 1984). In the present study, only those rats in the treatment groups (that received the extract of *C. militaris* at doses of 40 mg/kg bw and 60 mg/kg bw) had lower values of MN than the maximum value of the standard range (Table 1). A significant difference in PCE:NCE from the control group was only found in these two groups. These results indicated the potential antimutagenic effect of CM aqueous extract to prevent DNA damage in CP-induced male rats. The result also showed the safety of aqueous extracts from *C. militaris* in terms of non-mutagenic or non-genotoxic induction, since there was no increase in the percentage of micronucleus (DNA damage) or sperm abnormality. Notably, a Korean research group has performed stepwise solvent extraction using n-hexane, chloroform, ethyl acetate, butanol and water (Kim et al., 2001) and reported that butanol (which seems to be a cordycepin-rich fraction) has the highest antimutagenic effect. However, another research group pointed out that the pure cordycepin isolated using butanol extract did not show any mutagenic activity (Cho et al., 2003). This conflicting information influenced the present study as it suggested that there must be some other compounds playing roles in this antimutagenic effect.

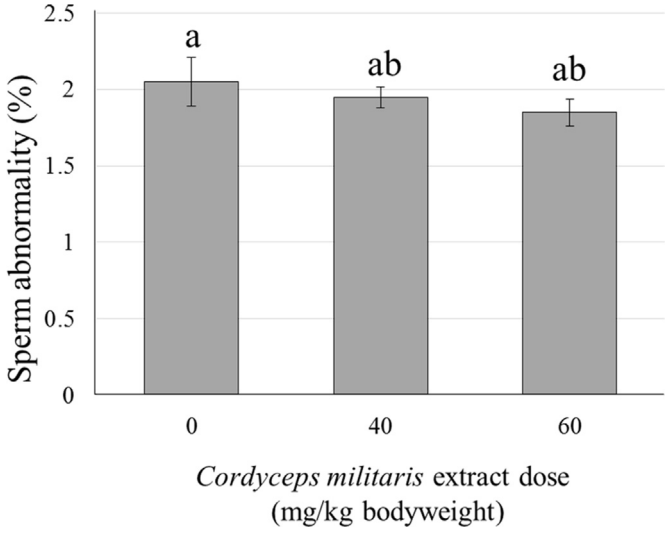

Fig. 3. Percentage of abnormal sperm in male rats treated with *C. militaris*, where different letters (a and ab) indicate significant ($p < 0.05$) differences using Student's t-test and error bars indicate ± SD.

The results from the tests of several chemicals have demonstrated a high correlation between the mutagenicity of an agent and its ability to cause sperm abnormalities (Chauhan et al., 2000). In the present study, the fact that the total count of sperm morphology abnormalities was not different between the control and tested rats (Fig. 3) helped to further confirm the anti -mutagenic effect and preventive effect of the CM aqueous extract on sperm morphological abnormality. Although the number of total sperm morphology abnormalities of rats treated with CM aqueous extract was similar to that of control, the number of head defects in the 60 mg/kg bw group was significantly higher than that of the control (Fig. 3). Since this difference was not dose-dependent, the conclusion of an anti-mutagenic effect of CM aqueous extract by sperm morphology assay was not likely. The results obtained from the present study indicated that aqueous extract from *C. militaris* did not cause somatic or germ cell chromosome damage induction and also showed some preventive effect against CP-mutagenic induction. However, the results of previous studies found increasing spermatogenesis ability of *C. militaris* mycelium extract (Lin et al., 2007; Chang et al., 2008). Deep and extensive research is needed (sub-chronic/chronic administration of CM aqueous extract at high doses) to assess the anti-mutagenic properties of CM on male germ cells.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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