

Original Article

Effect of ethanol extract of *alga Laurencia* supplementation on DNA oxidation and alkylation damage in mice

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Laurencia terpenoid extract (LET) had been extracted from the red alga *Laurencia tristicha*. The study is to investigate the effects of LET supplementation on DNA oxidation and alkylation damages in mice. Forty healthy Kunming mice weighing between 18g and 25g were randomly assigned into 4 groups, each consisting of ten animals. The mice were orally intubated respectively for 60 days with the designed concentrations of LET (25, 50, 100mg/kg b.w.) for three exposed groups and salad oil (0.2 ml) for the blank group. Food and water were free for the animals. Mice in the blank and exposed groups were sacrificed after the last treatment and the blood of each animal was quickly taken for further experiments. The spontaneous and oxidized DNA damages of peripheral lymphocytes induced by H₂O₂ were analysed by SCGE. O⁶-Methy-guanine (O⁶-MeG) was measured by high performance capillary zone electrophoresis. There was no significant difference in DNA spontaneous damage on peripheral lymphocytes of all the mice. The oxidative DNA damage in the 50 mg/Kg body weight supplement group are 286AU with the oxidation of 10μmol/L H₂O₂, significantly lower than the blank group 332AU ($p < 0.05$). The contents of O⁶-MeG in plasma in the 50mg/kg b.w. and 100mg/kg b.w. supplement group were 1.50μmol/L and 1.88μmol/L, significantly lower than that of the blank group, which was 2.89μmol/L ($p < 0.05$). The results from the present study indicated that the LET were rich in terpenoids and safety to be taken orally and it could improve antioxidative and decrease DNA damage effectively.

Key Words: *Laurencia*, terpenoid, SCGE, O⁶-methylguanine, DNA damage

Introduction

Seaweeds have attracted much attention as a source of antioxidative preparations, which could find applications in the cosmetic industry, food technology and biomedicine. In previous reports, the antioxidant activity of organic seaweed extracts has been suggested to be related to different compounds such as carotenoids, phlorotannins, amino acids and glucitols.^{1,2} Scholars have isolated a series of active ingredients especially the terpenoids from the alga *Laurencia*. The terpenoids possessed 26 kinds of carbon skeletons and exhibited significant cytotoxicity *in vitro*,³ In our laboratory, an ethanol extract of *Laurencia tristicha* Tseng named LET was prepared and we had detected the content of terpenoids in LET was 63.3% by HPLC. It has received special attention due to their potent pharmacological activities, including immuno-stimulation, antitumor and antioxidant activities.^{4,5} In this investigation, the effect of LET supplement on DNA oxidation and alkylation damage was detected *in vivo*.

Materials and methods

Collection of algal materials and preparation of seaweed extracts

Seaweed species *Laurencia tristicha* Tseng, Chang, E. Z. et. B. M. Xia was hand picked in 2002, from Naozhou beach

(coastal area near Guangdong Province, China) at low tide. Voucher specimens were sent to Dr. Shao Kuishuang as the Institute of Oceanology, Chinese Academy of Sciences, where they were authenticated, and specimens were kept for reference. Fresh algal material was washed, air dried under shade and ground to a fine powder in an electrical miller. For the preparation of extract, 15kg seaweed powder was soaked in ethanol for 1 week, filtered and the extract was evaporated on a rotary vacuum evaporator to dryness. The concentrates yielded brown oil, which was dissolved in salad oil for *in vivo* studies.

Animal and treatment

Based on LD₅₀ studies⁴ and optimum dosage studies, the effective dosage for LET was 25, 50 and 100 mg/kg b.w. respectively. Four-week-old Kunming mice weighing 18–25g were procured from Qingdao Laboratory Animal Center (Qingdao, China) and used for experiments after one week of acclimatization. The animals were maintained on a standard feed.

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They were housed in an air conditioned room at temperature of 35 ± 2 °C and 50–70% humidity with a 12 h light/dark cycle and allowed free access to standard laboratory diet (purchased from Qingdao Laboratory Animal Center). The mice were divided into four groups of ten animals each. Three groups were treated by oral intubation with 25, 50 and 100mg/ kg b.w. LET respectively for a period of 60 days. The blank group received the vehicle (salad oil, 0.2 ml) alone. No mortality was observed throughout the experiments.

The comet assay

Chemical reagents

Low melting agarose (LMA), normal melting agarose (NMA), Triton X-100, sodium N-lauroyl sarcosinate, ethylene di-aminetetraacetic acid disodium salt (Na_2 EDTA), Tris-(hydroxy methyl)-aminomethane (Tris-HCl), 1,6-Diphenyl-1,3,5-hexatriene (DPH) and all other common chemicals were obtained from Sigma (St. Louis, MO). Cyclophosphamide (CP), purchased from Endoxan Asta, (Asta Medica A.G., Germany) was dissolved in distilled water just before use. LMA and NMA were prepared in phosphate buffered salt solution (136mM NaCl, 2.68mM KCl, 8.10mM Na_2HPO_4 , 1.47mM KH_2PO_4 , pH 7.4). 4,6-Diamidine-2-Phenindole dihydrochloride (DAPI) purchased from Boehringer Mannheim.

Blood sample collection and preparation of slides

A volume of 60–80 μL of blood was collected from the orbital vessels of each animal at the end of the treatment. The comet assay was performed as described by Singh.⁶ Slides were prepared in five plicate per sample. Fully frosted microscopic slides were covered with 140 μL of 0.75% regular melting agarose. After application of a cover slip the slides were allowed to gel at 4°C for 10 min. Mean while, 20 μL of whole blood was then added to 0.5% of 110 μL of LMA (37°C). After carefully removing the coverslips, a second layer of 110 μL of sample mixture was pipetted out on the precoated slides and allowed to solidify at 4 °C for 10 min. The coverslips were removed and a third layer of 110 μL of LMA was pipetted out on the slides and allowed to gel at 4 °C for 10 min. The slides (without cover slips) were immersed in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris-HCl pH 10, 1% sodium N-lauroyl sarcosinate with 1% Triton X-100& 10% DMSO), DMSO added just before use and refrigerated overnight. Slides were then placed in alkaline buffer for 20 min to allow unwinding of the DNA to occur. Electrophoresis was conducted for 25 min at 25V (0.66V/cm) adjusted to 300 mA by raising or lowering the buffer level in the tank. Slides were then drained, placed on a tray and washed slowly with three changes of 5 min each of neutralization buffer. DNA was precipitated and slides were dehydrated in absolute methanol for 10 min and were left at room temperature to dry. The whole procedure was carried out in dim light to minimize artifactual DNA damage.

Microscopic examination

Slides were stained with DAPI and viewed under a fluorescence microscope. A total of 500 individual cells were screened per sample (100 cells from each of five replicate

slides). Undamaged cells look like an intact nucleus without a tail and damaged cells have the appearance of a comet. In addition, cells were graded by eye into five predefined categories (A–E) according to the amount of DNA in the Comet tail, as previously described by Anderson: A, no damage; B, low level damage; C, medium level damage; D, high level damage; and E, total damage (Fig 1).⁷ To quantify the damage in this scoring, a rank number ranging from A to E was assigned to each of the categories, in order to calculate a mean of DNA damage grade for all the samples analyzed.

Plasma O^6 -MeG

Instruments

Autokinetic capillary electrophoresis (P/ACE5000), to match ultraviolet detector (Beckman Instrument Company), capillary tube (75 μm id \times 57cm) O^6 -MeG standard preparation purchased from Sigma, borax buffer solution were obtained from Beckman. O^6 -MeG in plasma was detected by high efficiency performance capillary zone electrophoresis. It was directly introduced into the capillary employing sodium borate at pH9.0 as buffer. The condition of electrophoresis: voltage: 30KV, experimental temperature: 23°C, detection wavelength: 214nm, pressure of injection: 20Kpa.s⁻¹, column cleaning : 0.1mol/LNaOH flush for 2min, distilled water flush for 2min, borax buffer solution flush for 1min.

Analysis of O^6 -MeG

A volume of 0.3ml of blood was collected from the orbital vessels of each animal at the end of the treatment and mixed with 0.3ml of sulfosalicylic acid. After centrifuged twice at 6000r min for 15 min, the supernatant fluid was directly injected into the CE system. The Plasma O^6 -MeG contents were detected as described by Du.⁸The

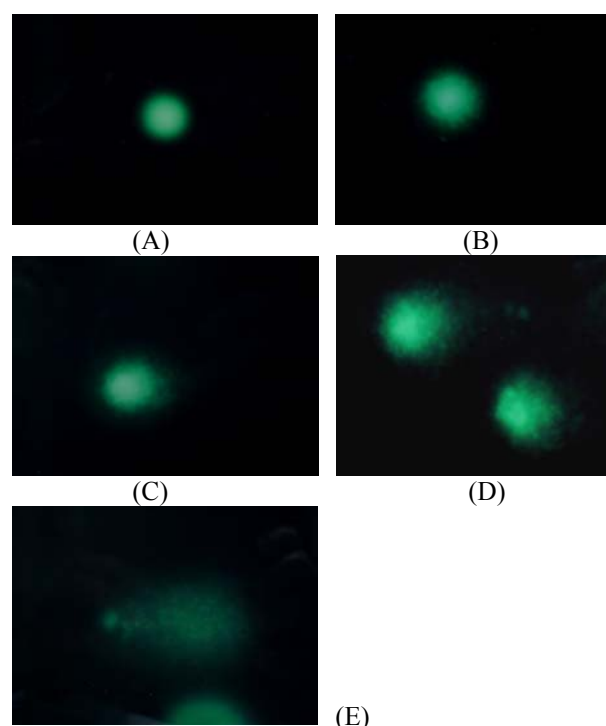


Figure 1. The grade of cells detected in the comet assay. A, no damage; B, low level damage; C, medium level damage; D, high level damage; and E, total damage

Table 1. Effect of LET on DNA oxidative damage of lymphocyte in mice ($\bar{x} \pm s$)

Group	dosage/ (mg.kg ⁻¹)	0μmol/L	5μmol/L	10μmol/L	20μmol/L
blank		161±23.8	305±28.0	332±10.1	380±3.8
Low-dose	25	121±38.3	256±34.8	324±16.1	380±7.8
mid-dose	50	118±35.0	234±39.8	286±30.3 ^a	378±8.7
High-dose	100	130±24.6	287±28.2	346±14.6	387±3.8

a: $p < 0.05$ vs. blank group

Table 2. Effect of LET on plasma O⁶-Methy-guanine in mice ($\bar{x} \pm s$)

Group	N	dosage/ (mg.kg ⁻¹)	O ⁶ -MeG (μmol/L)
blank group	10	-	2.89±0.35
Low-dose	10	25	2.34±0.34
mid-dose	10	50	1.50±0.25 ^a
High-dose	10	100	1.88±0.34 ^a

a: $p < 0.05$ vs. blank group

calibration curve showed good linearity, $A=1.8675C-0.00758$ ($r=0.9987$), C is the contents of the O⁶-MeG standard, A is the peak absorbance, CVs were 1.51% for intra-day and 1.93% for inter-day. O⁶-MeG contents of the samples were automatically calculated in relation to the peak absorbance.

Statistical analysis

All the grouped data were statistically evaluated with SPSS/11.0 software. Data were analyzed using ANOVA followed by Dunnett's test. $p < 0.05$ was considered to indicate statistical significance. All the results were expressed as mean±SD.

Results

The comet assay

Table 1 showed the results when the peripheral blood leukocytes had been classified into five categories from A–E according to the amount of DNA in the tail, as detailed above. From our results it was observed that there was no significantly difference in DNA spontaneous damage in peripheral lymphocytes of all the mice. Results showed significant decreasing tendency of the DNA damage induced by 10μmol/L H₂O₂ in peripheral lymphocytes of the 50 mg/kg supplement group as compare to the blank ($p < 0.05$). However, when comparing the average values of the DNA damage from 100 mg/kg supplement and the blank, the difference is not relevant.

Analysis of O⁶-MeG

As can be seen in table 2, the levels of O⁶-MeG in plasma in the 50 mg/kg and the 100mg/kg supplement group were significantly lower than the blank ($p < 0.05$).

Discussion

The use of the Comet assay is increasing because its advantages for genotoxicity studies are many and well-documented.^{9,10} And from the available data, it appears to

be sensitive enough for the detection of DNA damage induced by low-dose exposures.¹¹ It is applicable to any cell culture or tissue from which a single-cell suspension can be obtained. In this technique, a suspension of cells is embedded in agarose, subjected to electrophoresis and stained with a fluorescent DNA-binding dye. Single stranded DNA fragments can migrate out of the nucleus during alkaline gel electrophoresis whereas full length DNA strands cannot. The resulting images named for their appearance as comets, are measured to determine the extent of DNA damage.

Free radicals are continually produced in the body as a result of normal metabolic processes and interaction with environmental stimuli. They are considered to be of great importance as the cause of many disorders, including inflammation, cancer, arteriosclerosis, hypertension, aging and diabetes.¹²⁻¹⁶ Mammalian cells are equipped with both enzymic and non-enzymic antioxidant defenses to minimize the cellular damage caused by interaction between cellular constituents and oxygen free radicals.¹⁷ Impairment of the oxidant-antioxidant equilibrium in favour of the former provokes a situation of oxidative stress and generally results from hyperproduction of reactive oxygen species.¹⁸ Hydroxyl radical is the most reactive radical known in chemistry. It can abstract hydrogen atoms from biological molecules, including thiols, leading to the formation of sulfur radicals capable to combine with oxygen to generate oxysulfur radicals, a number of which damage biological molecules.¹⁹ Hydroxyl radicals can also lead to lipid peroxidation, which make structure abnormal and dysfunction in the biofilms, such as cell membrane, mitochondrial membrane. In our experiment, the comet assay was used to determine DNA damage measured as strand breaks and alkali-labile sites on blood peripheral lymphocytes of Kunming mice. No single strand breaks or alkali-labile sites were induced in cells after LET treatments compared with the blank. Bearing in mind that the increases of reactive oxygen species (ROS) concentrations

have been associated with the induction of DNA strand breaks,²⁰ our results suggest that LET treatment does not induce increments of ROS or other compounds related with this endpoint at this level.

The present research work indicates significant inhibition of DNA oxidative damage on peripheral lymphocytes exposed to hydrogen peroxide (H₂O₂). The mechanism reported here could have resulted in an increase in the repair efficiency and/or loss of heavily damaged cells leading to the subsequent DNA repair. The probable mechanism is that LET rich in terpenoids, the concentration of which is 63.29%. The skeleton of terpenoid is isoprenes which have ethylenic linkage. So its chemical property is active and the addition reaction is amiable. As a strong reductant, it tends to react with strong oxidizer and protect the unsaturated fatty acid of the biofilms from being oxidized and cellular functions are maintained.

The result shows that the LET decreased DNA damage in 50 mg/kg supplement group induced by 10μmol/L H₂O₂ while the higher dose of LET supplement with the same concentration of H₂O₂ didn't have antioxidative effect. It imply that 50 mg/kg supplement is the most suitable dosage in LET to play the antioxidant ability. And supplement with excessive LET such as 100 mg/kg would damage macromolecule and decrease the cell functions.

It's very important of the appropriate environment for antioxidation in the Comet assay. Treatment should be taken at 4°C to minimize the possibility of cellular processing of damage. Electrophoresis should be conducted at the appropriate voltage and current. The comet tail of the damaged cell would be longer or disappear when the voltage and the current go beyond the limit. The whole procedure should be carried out in dim light to minimize artifactual DNA damage.

DNA methylating agents such as environmental nitrosamines and the anti-cancer drugs procarbazine, dacarbazine, streptozotocine and temozolomide are mutagenic, genotoxic and cytotoxic.²¹ A critical lesion responsible for these endpoints is O⁶-methylguanine (O⁶-MeG), which is induced in minor amounts (maximally 8% of total alkylation products) in the DNA of exposed cells.²²

O⁶-MeG is repaired by the DNA repair protein O⁶-methylguanine-DNA methyltransferase (MGMT).²³ This enzyme prevents G:C to A:T transitions and cells which are unable to repair O⁶-MeG because of lack of expression of MGMT are highly sensitive to O⁶-methylating agents compared to MGMT competent cells.²⁴ This and other data (for review see^{23,25}) provided compelling evidence for a critical role of O⁶-MeG in the geno- and cytotoxic response of cells upon methylation. If O⁶-MeG is not repaired or repair is saturated with a high dose of a methylating agent, N-alkylations may become dominant in inducing genotoxic and killing effects.²⁶ Cytotoxicity induced by O⁶-methylating agents in rodent fibroblasts, human lymphocytes and lymphoblastoid cells deficient for MGMT is due to apoptosis, which indicates that O⁶-MeG is a critical pro-apoptotic DNA lesion.²⁷⁻³⁰ In contrast, over expression of O⁶-methylguanine-DNA methyltransferase (MGMT), also known as alkyltransferase, has been shown to render tissues more resistant to the carcinogenic effects of alkylating agents. MGMT directly

repairs O⁶-methylguanine (O⁶-MeG) lesions in DNA by transferring the alkyl group from a base lesion to a cysteine residue, thereby restoring the integrity of the DNA without creating additional DNA damage. So High MGMT levels protect tissues from alkylation-induced carcinogenesis and O⁶-MeG is thus a procarcinogenic DNA lesion.³¹ In our experiment, we observed that the levels of O⁶-MeG in plasma in the 50 mg/kg b.w. and the 100mg/kg b.w. LET supplement group were significantly lower than the blank. It indicated that LET have the ability of anti alkylation damage. The probable mechanism is that the LET supplement antagonisms the methylation in DNA induced by the alkylating agent or improves the activity and content of MGMT.

Conclusion

Current results demonstrated that the LET could improve antioxidative and decrease DNA damage effectively. Laurencia is the plentiful origin of terpenoids and distributed widely in the nature, so it has the potentials for developing.

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