CANCER
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□ ORIGINAL ARTICLE □

Ethanol Extract of *Inonotus obliquus* Shows Antigenotoxic Effect on Hydrogen Peroxide Induced DNA Damage in Human Lymphocytes

Eunju Park[1], Kyung-Im Jeon[1] and Boo-Hyung Byun[2]

[1]Department of Food and Nutrition, Kyungnam University, Masan 631-701,
[2]Department of Oriental Medicine, Daegu Haany University, Gyeongsan 712-715, Korea

The aim of this study was to evaluate the effect of ethanol extract of *Inonotus obliquus* (Chaga mushroom) on oxidative DNA damage induced by H_2O_2 in human lymphocytes. Cells incubated in medium with 1 % DMSO (negative control) or with various concentration of *Inonotus obliquus* extracts (6.25, 12.5, 25, 50, 100㎍/ml) for 30 min at 37°C were further treated with H_2O_2 (200㎛M) as an oxidative stimulus for 5 min on ice. Cell viability measured by trypan blue exclusion test was above 95% for all treatments. Oxidative damage was evaluated by Comet assay and quantified as % tail DNA. An increased oxidative DNA damage by H_2O_2 was significantly inhibited by pre-incubating with 6.25, 12.5, 25, 50, 100㎍/ml of *Inonotus obliquus* extracts by 46, 52, 69, 70 and 80%, respectively. When human lymphocytes were post-incubated with *Inonotus obliquus* for 30 min after exposure to H_2O_2 or incubated with *Inonotus obliquus* and H_2O_2 simultaneously, the antigenotoxic effect of *Inonotus obliquus* was not changed. These results indicate that *Inonotus obliquus* supplementation to human lymphocytes could inhibite or repair H_2O_2 induced damage to cellular DNA, supporting a protective effect of *Inonotus obliquus* against oxidative damage. **(*Cancer Prev Res* 10, 54-59, 2005)**

Key Words: *Inonotus obliquus* extract, Comet assay, Human lymphocytes, Oxidative DNA damage

INTRODUCTION

In Asia, a variety of dietary products have been used for centuries as popular remedies to prevent or treat different diseases. A large number of herbs and extracts from medicinal mushrooms are used for the treatment of diseases. Mushrooms such as *Ganoderma lucidum* (Reishi), *Lentinus edodes* (Shiitake), *Grifola frondosa* (Maitake), *Hericium erinaceum* (Yamabushitake), and *Inonotus obliquus* (Chaga) have been collected and consumed in China, Korea, and Japan for centuries. Most mushrooms are rich in vitamins, fiber, and amino acids and low in fat, cholesterol, and calories. These mushrooms contain a large variety of biologically active polysaccharides with immunostimulatory properties, which contribute to their anticancer effects. Furthermore, other bioactive substances, including triterpenes, proteins, lipids, cerebrosides, and phenols, have been identified and characterized in medicinal mushrooms.[1)]

Inonotus obliquus is a fungal parasite of living trees, growing primarily on branch stubs on birches, but sometimes on elm, alder, or beech trees in northern latitudes and has been widely used as a folk medicine in Russia, Poland and most of the Baltic countries.[2)] Studies have shown anti-tumor effects of *Inonotus obliquus* water extracts *in vitro*[3,4)] as well as antimicrobial and antiviral activities.[5,6)] A recent report suggested that a hot water extract of *Inonotus obliquus* might suppress cellular

책임저자 : 박은주, ㉾ 631-701, 경남 마산시 월영동 449번지
경남대학교 자연과학대학 식품영양학과
Tel: 055-249-2218, Fax: 055-244-6504
E-mail: pej@kyungnam.ac.kr
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Correspondence to : Eunju Park
Department of Food and Nutrition, Kyungnam University, 449 Woryeong-dong, Masan 631-701, Korea
Tel: +82-55-249-2218, Fax: +82-55-244-6504
E-mail: pej@kyungnam.ac.kr

proliferation in a time-dependent manner in human stomach cancer cell lines.[7] Ham *et al*., using the Ames test, also showed strong anti-mutagenic and anti-cytotoxic effects of ethanol extract, ethyl acetate fraction, water fraction and other sub-fractions from *Inonotus obliquus*.[8,9]

It is well known that DNA damage is a crucial mechanism in cancer development[10] and individuals who body cells show high levels of spontaneous or induced DNA damage (e.g. smokers) may be prone to cancers.[11~13] Therefore, it appears reasonable in the study of anticarcinogenic mechanisms to search for substances that prevent DNA damage. The presence of DNA damage has become one of the most sensitive biological markers for evaluating the oxidative stresses resulting from the imbalance between free radical generation and control through antioxidant systems. DNA damage is detectable in single mammalian cells by the single cell gel electrophoresis, also known as the comet assay, which is a very simple, rapid and sensitive technique to analyze DNA damage and specifically for detecting DNA strand breaks.[14]

The objective of this work was to determine the antigenetoxic activity of ethanol extract of *Inonotus obliquus* on oxidative DNA damage induced by H_2O_2 in human lymphocytes in order to evaluate its potential as a natural antioxidant source for medicinal and food industry.

MATERIALS AND METHODS

1. Sample preparation

Inonotus obliquus, obtained from Russia, were dried in sunlight and crushed into a powder. The extraction and separation of *Inonotus obliquus* was performed according to a method described by Mizuno *et al*.[3]

2. Preparation of human lymphocytes

Blood samples were obtained from two healthy male volunteers (non-smokers, 24 and 25 years old, respectively). Five ml of fresh whole blood was added to 5 ml of phosphorous buffered saline (PBS) and layered onto 5 ml of Histopaque 1077. After centrifugation for 30 min at 400× *g* at room temperature, the lymphocytes were collected from the just above the boundary with the Histopaque 1077, washed with 5 ml PBS. Finally, they were freshly used for comet assay or resuspended in freezing medium (90% fetal calf serum, 10% demethyl sulfoxide) at 6×10^6 cells/ml. The cells were frozen to -80°C using a Nalgene Cryo 1°C freezing container (Nalgene, Rochester, NY) and stored in liquid nitrogen. The cell were thawed rapidly prior to each experiment in a water bath at 37°C.

3. Treatment of *Inonotus obliquus* extracts on human lymphocytes

Cells were incubated with *Inonotus obliquus* extract in three different treatments. (1) Lymphocytes were incubated with various concentrations of *Inonotus obliquus* extracts (0, 6.25, 12.5, 25, 50, 100㎍/ml) for 30 min at 37°C in a dark incubator and then were resuspended in PBS with 200㎛M H_2O_2 for 5 min on ice. (2) Lymphocytes were damaged oxidatively with 200㎛M H_2O_2 for 5 min on ice and then incubated with *Inonotus obliquus* extracts (0, 6.25, 12.5, 25, 50, 100㎍/ ml) for 30 min at 37°C. (3) Lymphocytes were incubated simultaneously with 200㎛M H_2O_2 and *Inonotus obliquus* extract. After each treatment, samples were centrifuged at 1,450 rpm for 5 min and washed with PBS. All the experiments were repeated twice with lymphocytes from each of two donors on the separate day.

4. Determination of DNA damage (comet assay)

The alkaline comet assay was conducted according to Singh *et al*.[15] with little modification (Fig. 1). The cell suspension was mixed with 75㎕ of 0.5% low melting agarose (LMA), and added to the slides precoated with 1.0% normal melting agarose. After solidifaction of the agarose, slides were covered with another 75㎕ of 0.5% LMA, and then immersed in lysis solution (2.5 mM NaCl, 100 mM EDTA, 10 mM Tris, and 1% sodium laurylasarcosine; 1% Triton X-100 and 10% DMSO) for 1 h at 4°C. The slides were next placed into an electrophoresis tank containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for 40 min for DNA unwinding. For electrophoresis of the DNA, an electric current of 25 V/300 mA was applied for 20 min at 4°C. The slides were washed three times with a neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min at 4°C, and then treated with ethanol for another 5 minutes before staining with 50 L of ethidium bromide (20 ㎍/ml). Measurements were made by image analysis (Kinetic Imaging, Komet 5.0, U.K) and fluorescence microscope (LEICA DMLB, Germany), determining the percentage of fluorescence in the tail (tail intensity, TI; 50 cells from each

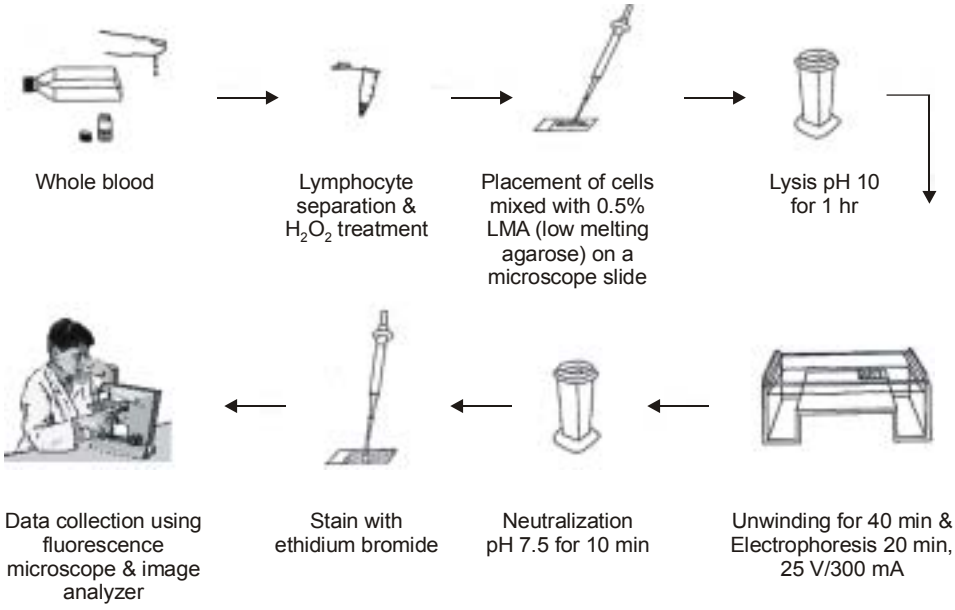

Fig. 1. Alkaline comet assay scheme.

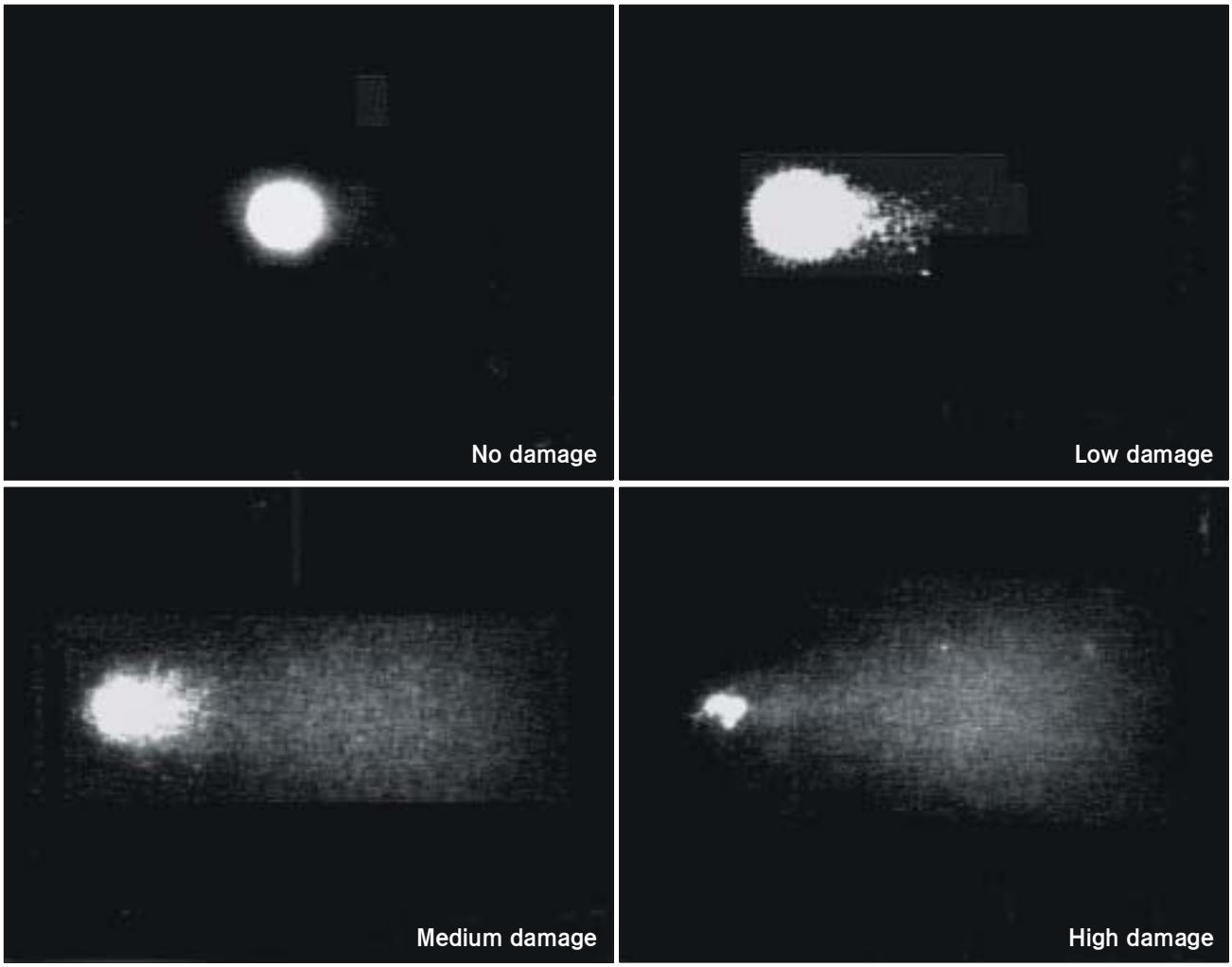

Fig. 2. Images of comets obtained by single-cell gel electrophoresis representing different degrees of DNA damage.

of two replicate slides)(Fig. 2). Cell viability measured by trypan blue exclusion test was above 95% for all treatments.

5. Statistical analysis

The mean values of the DNA damage (tail intensity) from each treatment were compared using one-way analysis of variance (ANOVA) followed by Duncan's test. p-value of less than 0.05 was considered significant.

RESULTS

Pretreatment of the cells for 30 min with *Inonotus obliquus* extract significantly reduced the genotoxicity of hydrogen peroxide measured as DNA strand breaks (Fig. 3). The DNA damage inhibitory effect of *Inonotus obliquus* extract increased as its concentration increased from 6.25 to 100㎍/ml by 46 to 80% in *Inonotus obliquus* extracts of H_2O_2 treated positive control. Especially, the highest concentration (100㎍/ml) of the *Inonotus obliquus* was shown that there was no statistical difference compared to DMSO-treated negative control.

When human lymphocytes were post-incubated with rice hull extract for 30 min after exposure to hydrogen peroxide, the protective ability of the *Inonotus obliquus* was not changed (Fig. 4). Post-treatment with *Inonotus obliquus* extracts to H_2O_2 induced DNA damaged lymphocytes at 6.25, 12.5, 25, 50, 100㎍/ml reduced the degree of DNA damage by 32%, 49%, 53%, 72% and 77%, respectively, compared with the positive controls.

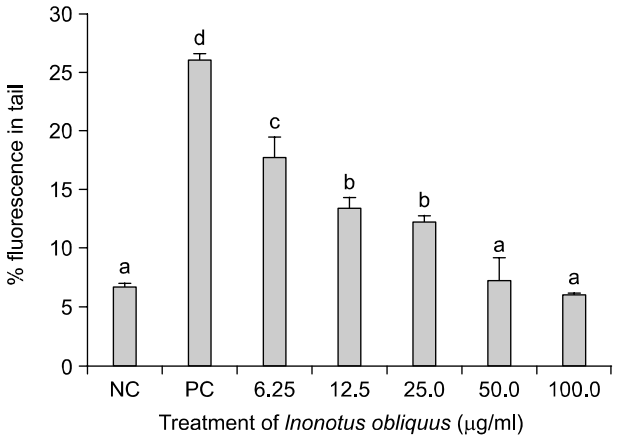

Fig. 4. The protective effect of supplementation *in vitro* with different concentration of EtOH extract of *Inonotus obliquus* on 200㎛M H_2O_2-induced human lymphocytes DNA damage. Values are mean with standard error of duplicate experiments with lymphocytes from each of two different donors.
[a~d]Values not sharing same letters are significanlty different from one another ($p < 0.05$).
NC: negative control (DMSO 1%), PC: positive control (200㎛M H_2O_2).

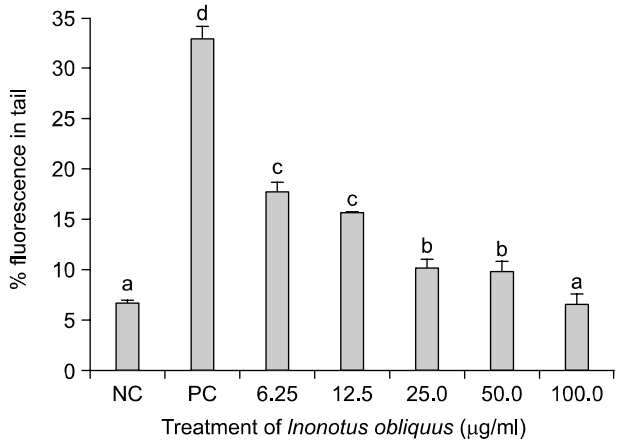

Fig. 3. The preventive effect of supplementation *in vitro* with different concentration of EtOH extract of *Inonotus obliquus* on 200㎛M H_2O_2-induced human lymphocytes DNA damage. Values are mean with standard error of duplicate experiments with lymphocytes from each of two different donors.
[a~d]Values not sharing same letters are significanlty different from one another ($p < 0.05$).
NC: negative control (DMSO 1%), PC: positive control (200㎛M H_2O_2).

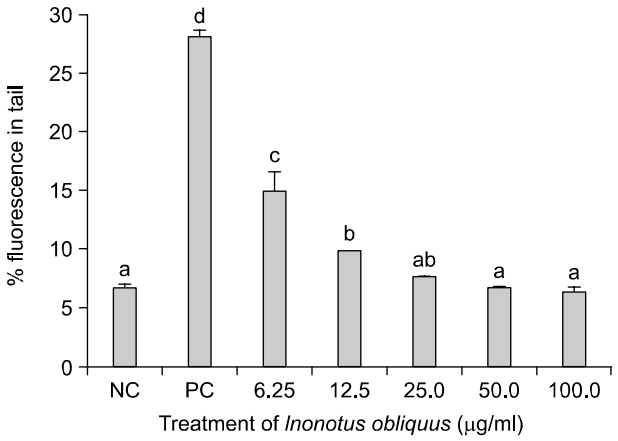

Fig. 5. The effect of supplementation *in vitro* with different concentration of EtOH extract of *Inonotus obliquus* and 200㎛M H_2O_2 simultaneously in human lymphocytes. Values are mean with standard error of duplicate experiments with lymphocytes from each of two different donors.
[a~d]Values not sharing same letters are significanlty different from one another ($p < 0.05$).
NC: negative control (DMSO 1%), PC: positive control (200㎛M H_2O_2).

This antigenotoxic effect of *Inonotus obliquus* extract was the case when the *Inonotus obliquus* and H_2O_2 treated simultaneously on ice for 5 min (Fig. 5).

DISCUSSION

The genotoxic effects of H_2O_2 and the protective effect of *Inonotus obliquus* extract were assessed in normal human lymphocytes by comet assay.

Hydrogen peroxide is believed to cause DNA strand breakage by generation of the hydroxyl radical (OH) close to the DNA molecule, via the Fenton reaction.[16] The possible mechanism by which *Inonotus obliquus* extract inhibited oxidative DNA damage in human lymphocytes can be ascribed to the antioxidant properties of the compound. Recently, Cui Y *et al*.[2] have found that the polyphenolic, triterpenoids and steroid extract from the *Inonotus obliquus* had a strong antioxidant activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide, and peroxyl radicals. They found also that the polyphenolic extract protected these cells against hydrogen peroxide-induced oxidative stress. The polyphenolic, triterpenoids and steroid compound in *Inonotus obliquus* may work by providing hydrogen atoms from their phenolic hydroxyl groups to scavenge hydroxyl radical generated from hydrogen peroxide.[17,18]

The damaged DNA by ROS can be repaired by DNA repair pathway and it is clear that individual variations in repair capability would have a bearing on cancer risk.[19,20] Collins *et al*. reported that when fresh isolated human lymphocytes were incubated after hydrogen peroxide treatment, repair of strand breaks appears to be unusually slow.[21] In the present study, however, hydrogen peroxide induced DNA damage in human lymphocytes was effectively repaired to almost similar level as DMSO treated negative control by post-treatment of *Inonotus obliquus* extract for 30 min. Although the exact mechanism for DNA repair activity needs to be elucidated, the *Inonotus obliquus* extract may contribute to stimulation of DNA repair.

CONCLUSION

These results indicate that *Inonotus obliquus* supplementation to human lymphocytes could inhibit H_2O_2 induced damage to cellular DNA, supporting a protective effect of *Inonotus obliquus* against oxidative damage.

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