Suppressive effects on immune cells and oxidative cytotoxicity of ozonated olive oil

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Abstract

To investigate antiinflammatory effects of ozonated olive oil, its effects on lymphocyte mitogenesis, production of nitric oxide (NO) from macrophage, and oxidative cytotoxicity were determined. Ozonated olive oil of 0.025% suppressed mitogenesis of mouse splenic lymphocytes. It suggested that immune reactions involving lymphocytes are suppressed by ozonated olive oil. Ozonated olive oil of 0.05% eliminated the production of NO from mouse macrophage-like cell line, ozonated olive oil is supposed to suppress inflammation derived from NO from macrophage. Results from determination using cell lines overprodecing superoxide dismutase (SOD) showed that ozonated olive oil of 0.5% has oxidative cytotoxicity.

Introduction

Application and efficacy of ozonated olive oil on decubitus and intractable inveterate fistula have been reported[1-3]. Topical application of ozonated olive oil disinfects their affected area, and enhances formation of granulation tissue and proliferation of epithelial cells. One of effective components of ozonated olive oil was reported to be trioleintriozonide which has oxidizing action[4]. However, detail mechanism of the action has not been elucidated.

Recently, it was reported that ozonated olive oil inhibited phosphorylation of IκB, which inhibits NF-κB as intracellular signal transduction factors, and ozonated olive oil inhibited expression of cyclooxygenase-2, resulting in suppression of generation of proinflammatory prostaglandins[5]. To investigate the possibility for ozonated olive oil to suppress other proinflammatory factors beside prostaglandins, its effects on lymphocyte mitogenesis and production of nitric oxide (NO) from macrophage. Additionally, oxidative cytotoxicity of ozonated olive oil was also evaluated.

Material and methods

1) Lymphocyte mitogenesis test

Cells were prepared from a spleen of 5-week-old male BALB/c mouse, washed with RPMI-1640 medium and 2×10^5 cells were dispensed into each well of a 96-well microplate. Olive oil or ozonated olive oil was added with or without 2 mg/mL concanavalin A (Con A). The plate was incubated at 37°C in 5% CO_2 for 4 days.

Cells were washed with phosphate buffered saline (PBS), lysed with 0.05% sodium dodecyl sulfate, and 50 mg/mL ethidium bromide was added. Fluorescence of complexes of DNA and ethidium bromide was measured at emission wavelength 515 nm and excitation wavelength 620 nm as indication of cell number[6].

2) Evaluation of activity of inducible NO synthase

Mouse macrophage cell line J774.1 was cultured, and 1×10^5 cells were put into each well of a 48-well microplate. Ozonated olive oil was added and cultured at 37°C in 5% CO_2 for 24 hours. After incubation, cells were washed with Krebs-Linger buffer and cultured further 24 hours in new medium with or without 10 mg/mL of lipopolysaccharide. The cells were washed with Krebs-Linger buffer, and incubated in new buffer containing 100 μmol/L of L-arginine and 1 μmol/L of diaminofluorescein-2 for 2 hours at 37°C, while NO was produced by inducible NO synthase from substrate L-arginine and was diffused from the cell, and reacted with diaminofluorescein-2 in the buffer to produce fluorescent substance diaminofluorescein-2T. The fluorescence of diaminofluorescein-2T was measured at excitation wavelength 515 nm and emission wavelength 620 nm, and the concentration was calculated by a calibration curve using authentic diaminofluorescein-2T.

3) Oxidative cytotoxicity to SOD-highly-expressing cell line

HeLa cells transfected with an SOD expression vector or a control vector[8] were kindly gifted by Dr. Imura. Cell lines were maintained by passage culture, 1×10^4 cells were seeded in each well of a 96-well microplate. After cultivation at 37°C in 5% CO_2 for 24 hours, olive oil or ozonated olive oil was added and incubated at 37°C for 48 hours . After cells were washed with PBS, PBS containing calcein-AM [9] was added and incubated at 37°C for 30 minutes. Fluorescent substance produced by intracellular esterases was estimated with excitation wavelength 495 nm, and elimination wavelength 515 nm as indication of cell number.

Results and discussion

1) Suppressive effect of ozonated olive oil on lymphocyte mitogenesis

Replication of lymphocytes is usually arrested and when lymphocytes encounter xenobiotics, they are activated to proliferate, namely, they undergo mitogenesis. Antigen-nonspecific lymphocyte mitogenesis can be induced by concanavalin A (Con A), a lectin from Jack bean (*Canavalia ensiformis*). The effect of ozonated olive oil on mouse splenic lymphocytes was estimated with nonspecific mitogenesis induced by Con A. (Fig. 1).

Mitogenesis of the cells without Con A was not observed, and neither olive oil nor ozonated olive oil induced mitogenesis. It suggests that olive oil and ozonated olive oil have little allergenisity.

Mitogenesis of the cells with Con A was suppressed by 0.01% olive oil, and olive oil more than 0.025% did not suppress it further. These results may suggest that 0.01% olive oil floated and covered surface of culture medium and impaired culture conditions.

Lymphocyte mitogenesis induced by Con A was almost completely inhibited by 0.025% ozonated olive oil. It was suggested that ozonated olive oil suppresses immune reactions related to lymphocytes.

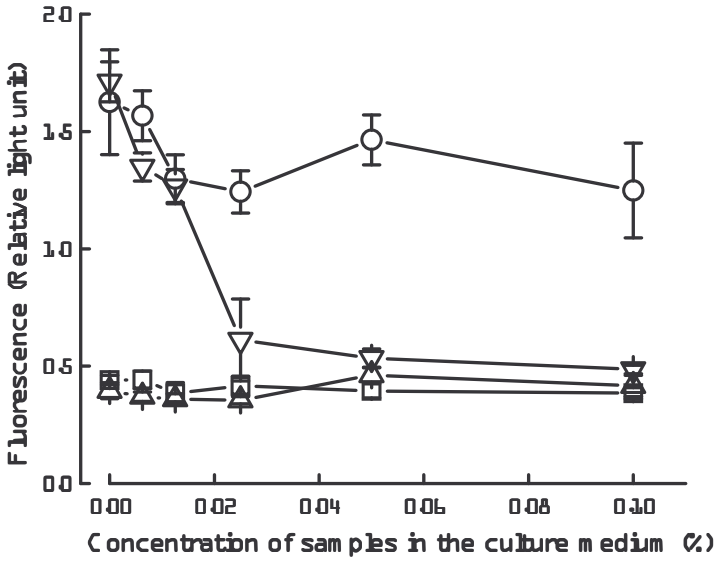

Figure 1. Suppressive effect of ozonated olive oil on lymphocyte mitogenesis

□: Olive oil □: Ozonated olive oil ○: Con A + olive oil □: Con A + ozonated olive oil

2) Suppressive effect of ozonated olive oil on NO synthesis from macrophages.

Macrophages can be activated by lipopolysaccharide as constituent in outer membrane of *Escherichia coli* and produce NO by enzymatic reaction of iNOS due to attack the bacteria. Mouse macrophage cell line J774.1 was stimulated with lipopolysaccharide and an effect of ozonated olive oil on activity of iNOS was determined (Fig. 2).

Less than 0.025% of ozonated olive oil did not affected NO production, and 0.05% of ozonated olive oil eliminated iNOS activity. This result showed the possibility of ozonated olive oil to suppress inflammation derived from NO production in macrophage.

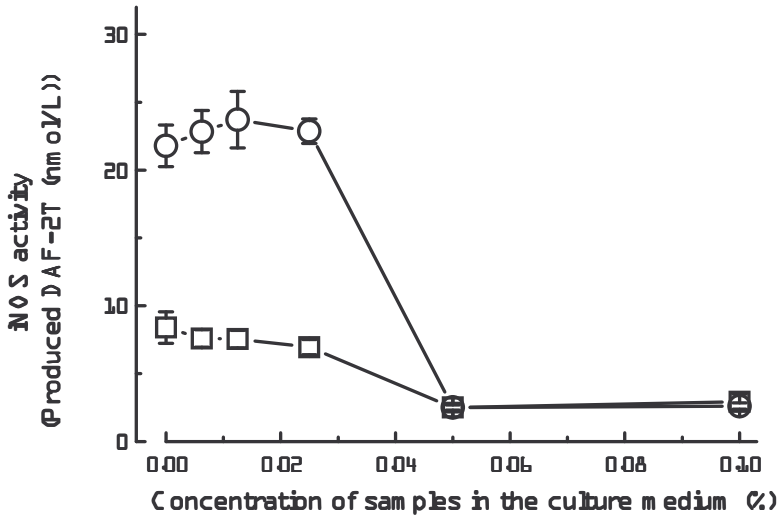

Figure 2. Suppressive effect of ozonated olive oil on NO synthesis from macrophages

□: Not stimulated ○: Lipopolysaccharide-stimulated

3) Oxidative cytotoxicity of ozonated olive oil

It is suspected that ozonated olive oil contained trioleintriozonide gives oxidative stress to cells. A human uterine cancer cell line HeLa was transfected by superoxide dismutase (SOD), one of enzymes eliminating reactive oxygen species, expressing vector or vehicle vector and they were exposed to olive oil or ozonated olive oil. The difference of viability between two cell lines of normal cell and SOD overproduced cell was examined as the indicator of the oxidative cytotoxicity (Fig. 3).

Up to 1% of olive oil was not toxic for neither control cell line nor SOD overproducing cell line. On the other hands, more than 0.2% of ozonated olive oil was toxic for both cell lines and 0.5% ozonated olive oil was toxic more on control cell line than transfected cell line. It shows that ozonated olive oil has oxidative cytotoxicity on HeLa cell and high dose of ozonated olive oil may be toxic for cells.

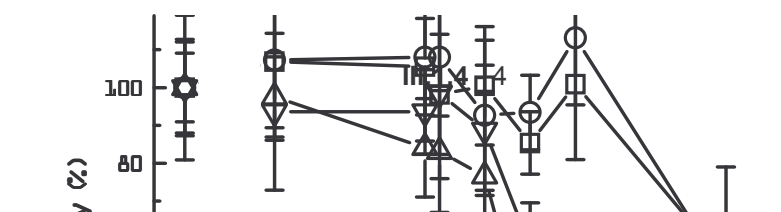

Figure 3. Oxidative cytotoxicity of ozonated olive oil

□ : Control cell line + olive oil, ○: SOD-overproducing cell line + olive oil,

□: Control cell line + ozonated olive oil, □: SOD- overproducing cell line + ozonated olive oil

Conclusion

Ozonated olive oil was added into culture medium and modification of cell functions were determined. 0.025% of ozonated olive oil suppresses lymphocyte mitogenesis, 0.05% ozonated olive oil suppresses NO production from macrophages. These results suggest that ozonated olive oil depresses lymphocyte and macrophage functions during inflammation period. And from the result that 0.5% of ozonated olive oil showed oxidative cytotoxicity, high dose of ozonated olive oil may be toxic for cells.

These results may suggest a possibility that under non-toxic level of oxidative potential, ozonated olive oil affected redox signaling in lymphocyte and macrophage, actions of NF-κB were suppressed, and consequently lymphocyte mitogenesis and NO production from macrophage were depressed. Additionally, the result of oxidative cytotoxicity indicates that physicians who attempt to use ozonated olive oil should administer the appropriate dose owing to its oxidative cytotoxicity.

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