

Effects of Flaxseed Lignan on In Vitro Mitogen-Stimulated T cell Proliferation

Yeong Rhee, Ph.D., Ardith Brunt, Ph.D.

Department of Health, Nutrition and Exercise Sciences, North Dakota State University, Fargo, North Dakota

Department of Health, Nutrition and Exercise Sciences, North Dakota State University, 351 EML, Fargo, ND, 58105.

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ABSTRACT

This study determined the effects of flaxseed lignan, *secoisolariciresinol diglucoside* (SDG), on in vitro lymphocyte reactivity to mitogens. Twelve-hour fasting blood samples were collected four times from a healthy donor.

Heparinized whole blood was diluted with complete RPMI, and then incubated with 0, 10, 50, or 100 μM SDG for 4 hrs at 37° C. Upon completion of the incubation, whole blood cells were incubated with 0, 10, 20, 40, or 80 $\mu\text{g/ml}$ phytohemagglutinin-L (PHA-L) or 0, 12.5, 25, 50, or 100 $\mu\text{g/ml}$ concanavalin A (ConA) for 96 hours at 37° C. Eighteen hours before the end of the incubation, blood cells were pulsed with 1 μCi ^3H -thymidine. The ^3H -thymidine uptake by lymphocytes was counted and reported as disintegrations per minute (DPM). Both PHA-L and ConA significantly increased lymphocyte proliferation ($p < 0.0001$). A higher lymphocyte proliferation was observed with a higher concentration of mitogens; however, the lymphocyte proliferation was higher with PHA-L when compared to ConA. No significant effects of SDG on lymphocyte proliferation were observed.

The results of this study indicate that flaxseed lignan has no effects on the immune response.

INTRODUCTION

Antioxidants have been at the center of focus in chronic disease prevention research recently. Many studies have shown that antioxidants play roles in the prevention of coronary vascular diseases or diabetes¹⁻⁵ and in the immunoregulatory function in humans and animals.⁶⁻⁸

Alpha-tocopherol is known as an antioxidant, and in vitro supplementation of alpha-tocopherol significantly increased mice splenocyte proliferation.^{8,9} Five weeks of feeding antioxidants supplemented biscuits significantly improved immune responses in young prematurely aging mice.¹⁰ Moreover, 15 weeks of antioxidant supplementation (vitamins C and E, zinc, selenium, and β -carotene, 5% and 20% (w/w)) in the diet significantly increased lymphocyte proliferation, natural killer activity, interleukin-2 release while significantly decreasing DNA and lipid oxidative damage in prematurely aging mice.¹¹ Administration of butylated hydroxyanisole, an antioxidant, significantly decreased production of interleukin-1, and tumor necrosis factor- α by pancreatic islets and peritoneal macrophages in streptozotocin induced diabetic rats.¹²

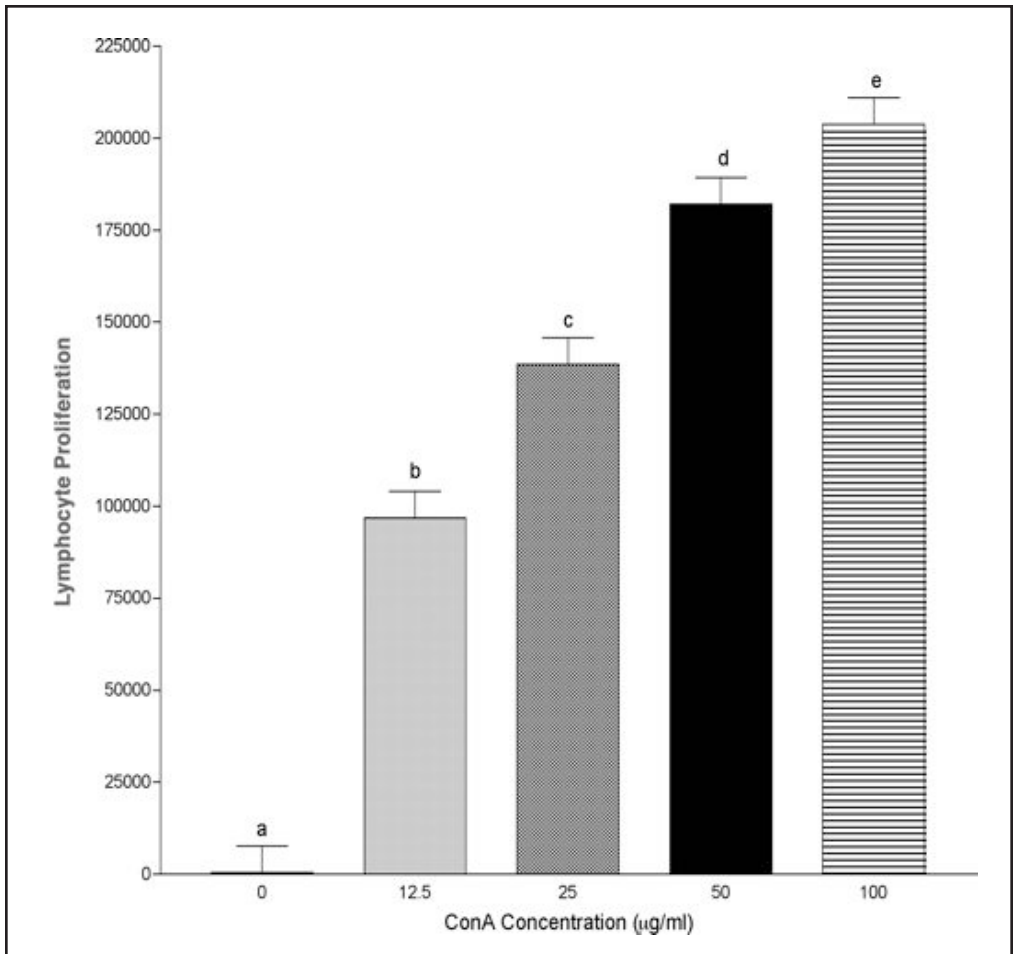


Figure 1. Lymphocyte proliferation with ConA stimulation from whole blood cell cultures; The data shown are representative of four independent experiments, with the mean and SD of the mean shown for each treatment group; When comparing mitogen concentrations, values with different lower case letters are significantly different ($P < 0.05$)

N-acetylcysteine, an antioxidant, supplementation in mice increased chemotaxis and decreased peritoneal lymphocyte adherence.⁶ Vitamin E supplementation as alpha-tocopherol 233 mg/d for 28 days significantly increased phytohemagglutinin (PHA) or lipopolysaccharide (LPS) stimulated lymphocyte proliferation and decreased oxidative stress, plasma malondialdehyde, and urinary DNA adduct 8-hydroxy-2'-deoxyguanosine in healthy individuals.¹³ Vitamin E supplementation in humans also increased lymphocyte proliferation and decreased lymphocyte adherence.⁶

Supplementation with β -carotene, another antioxidant, did not have effects on PHA stimulated lymphocyte proliferation in lactating women.¹⁴ A combined supplementation of vitamin E and selenium significantly increased T lymphocyte proliferation, T helper cells, and interleukin-2 receptor production followed by PHA or LPS stimulation, and decreased hydrogen peroxide production in healthy individuals.⁷ These studies indicate immunoregulatory function of antioxidants in animals and humans.

Hydrogen peroxide produced during phagocytosis functions as a second mes-

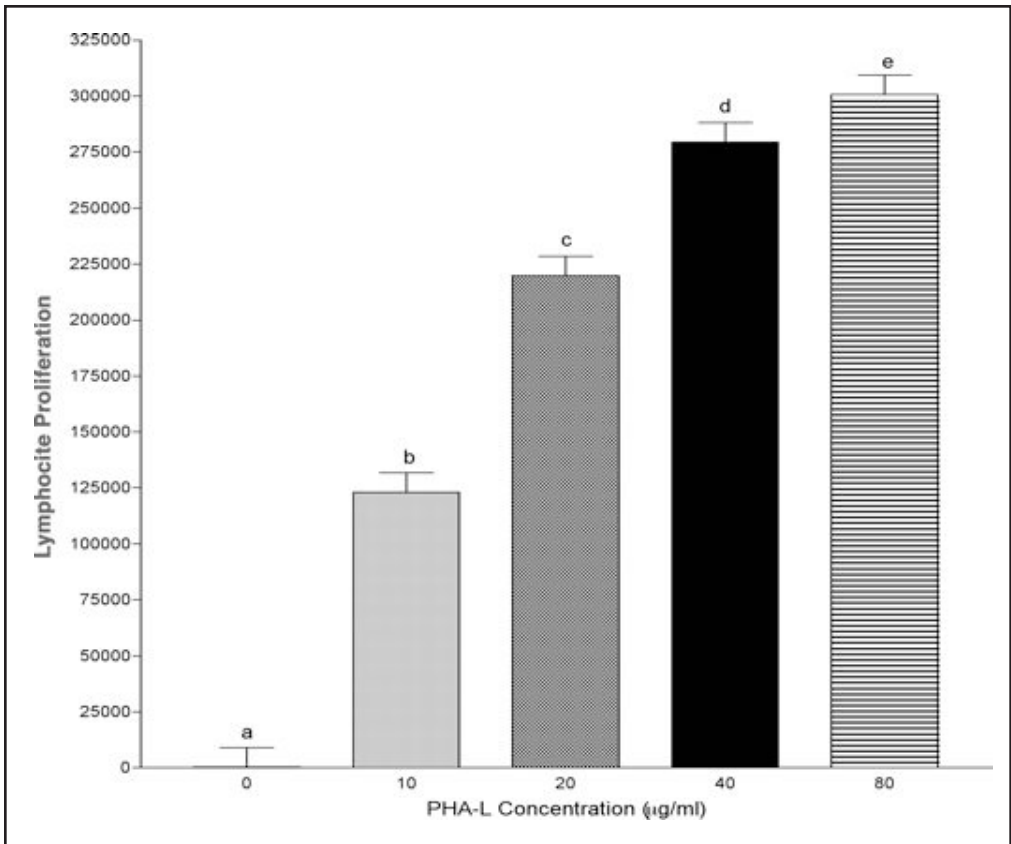


Figure 2. Lymphocyte proliferation with PHA-L stimulation from whole blood cell cultures; The data shown are representative of four independent experiments, with the mean and SD of the mean shown for each treatment group; When comparing mitogen concentrations, values with different lower case letters are significantly different ($P < 0.05$)

senger to signal T cell activation. A short period exposure (2 min) of lymphocyte to hydrogen peroxide followed by catalase addition to the cell culture significantly increased T cell proliferation. However, cell proliferation was significantly decreased or blocked as hydrogen peroxide increases.¹⁵ This study showed that an antioxidant is required for optimal cell growth. Hydrogen peroxide produced by phagocytes decreased PHA stimulated T cell proliferation and interleukin-2 production.¹⁶

Secoisolariciresinol diglucoside (SDG), a flaxseed lignan, and its metabolites secoisolariciresinol (SECO), enterodiol (ED), and enterolactone (EL), which are all formed in the body have antioxidant activity.^{2,17,18} In vitro,

linoleic acid peroxidation and DNA scissions, indicators of the antioxidant activity of lignan, were decreased by SDG, ED, and EL.¹⁹ In addition, SDG supplementation decreased reactive oxygen species (ROS) producing activity of white blood cells and was associated with decreased serum or pancreatic malondialdehyde, which is a lipid peroxidation product.^{2,20} Moreover, the antioxidant activity of SDG, SECO, ED, and EL was found to be 1.27, 4.86, 5.02, and 4.35 times more potent than that of vitamin E, respectively.¹⁸

A diet high in flaxseed (40% of the diet, w/w) significantly decreased mitogen stimulated splenocyte proliferation in pregnant and F1 generation rats; however, defatted flaxseed meal or a lower concen-

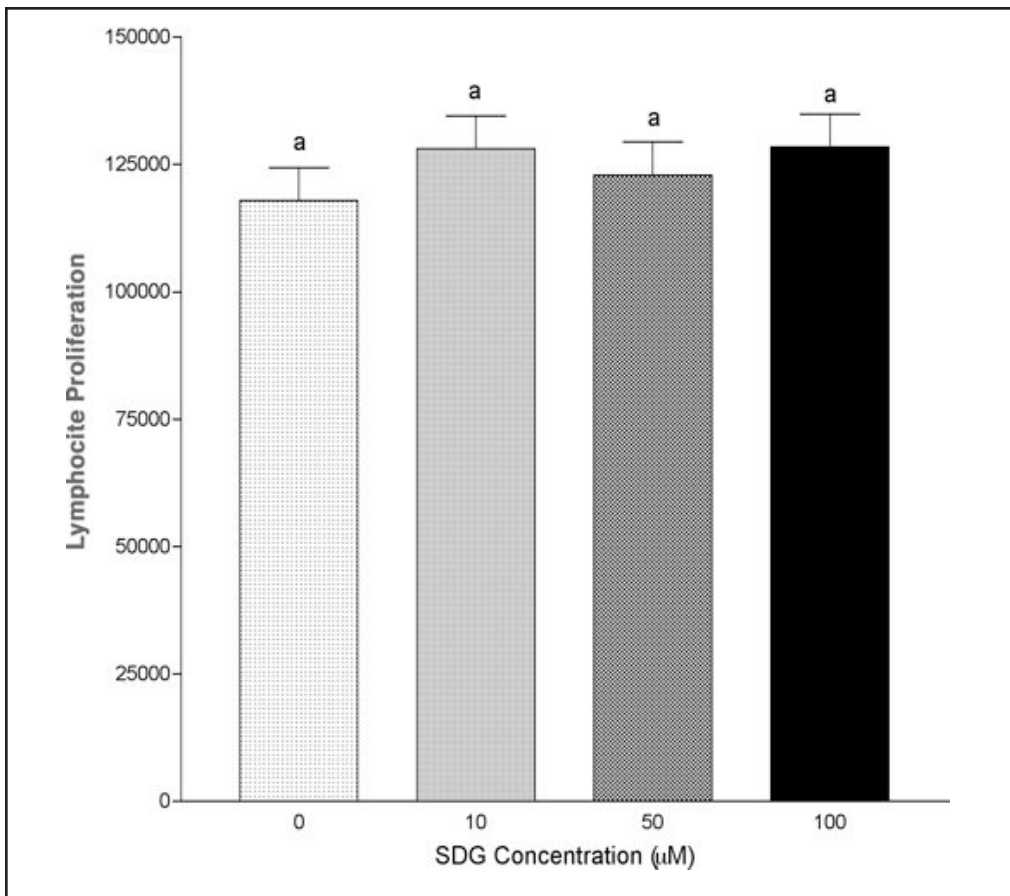


Figure 3. Effects of flaxseed lignan SDG on T cell proliferation (ConA stimulated) from whole blood cell cultures; The data shown are representative of four independent experiments, with the mean and SD of the mean shown for each treatment group; When comparing SDG concentrations, values with different lower case letters are significantly different ($P < 0.05$)

tration of flaxseed (20% of the diet, w/w) did not change the lymphocyte proliferation.²¹ Dietary flaxseed (10% of diet, w/w) significantly inhibited superoxide generation by rat peritoneal exudates cells.²²

Studies have shown antioxidant activity of flaxseed lignan, SDG; however, few studies have investigated the antioxidant function of flaxseed lignan in immune responses. Therefore, the current study was conducted to determine the effects of SDG on mitogen stimulated human lymphocyte proliferation in vitro.

MATERIALS AND METHODS

The research protocol was reviewed and approved by the Institutional Review

Board, Institutional Biosafety Committee, and Institutional Radiation Safety Committee at North Dakota State University.

Secoisolariciresinol diglucoside (SDG) preparation

Purified flaxseed SDG (Chromadex Co., Santa Ana, CA) was dissolved in a 0.1% ethanol solution (v/v) and then diluted into 10 μ M, 50 μ M, and 100 μ M. For a control (0 μ M SDG), 0.1% ethanol solution was used.

In vitro mitogenic proliferative responsiveness

In vitro mitogen stimulated T cell prolifer-

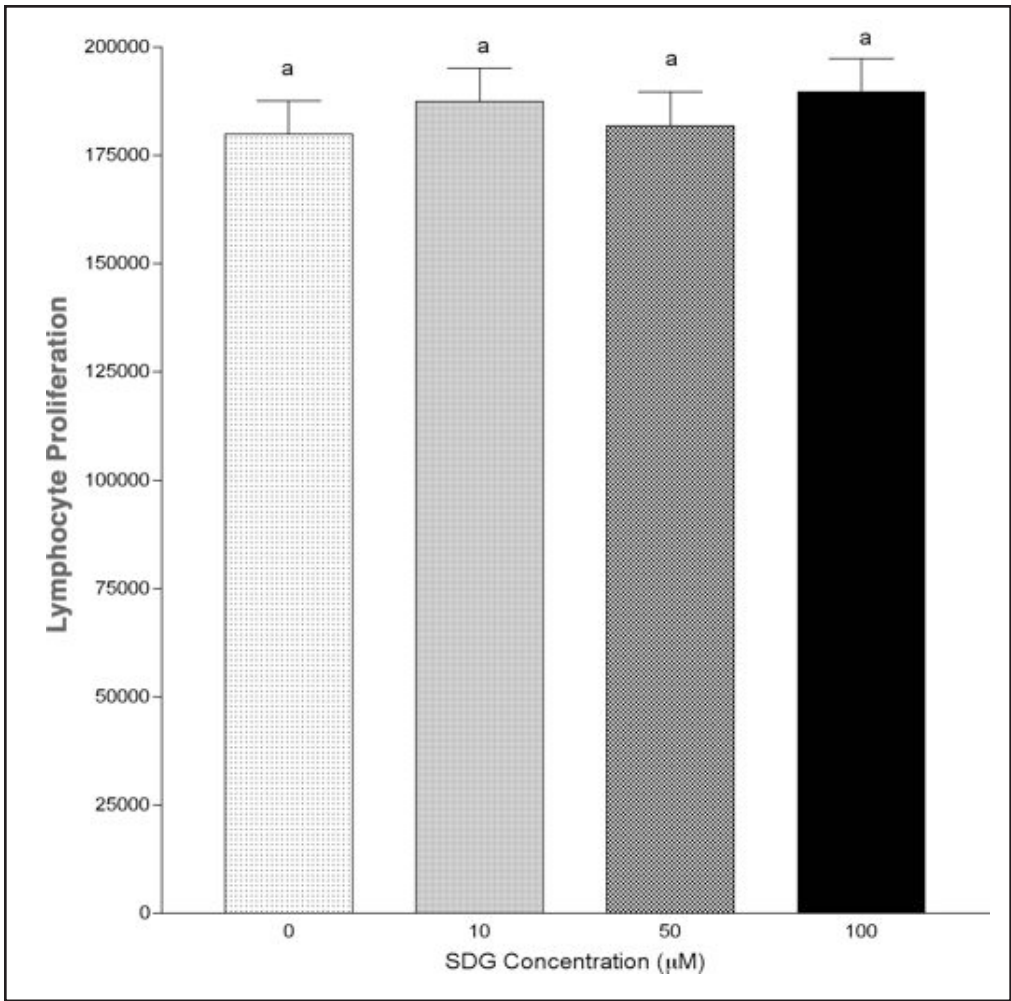


Figure 4. Effects of flaxseed lignan SDG on T cell proliferation (PHA-L stimulated) from whole blood cell cultures; The data shown are representative of four independent experiments, with the mean and SD of the mean shown for each treatment group. When comparing SDG concentrations, values with different lower case letters are significantly different ($P < 0.05$)

eration, using human whole blood cell cultures, was measured following the method of Kramer and Burri.²³ Twelve-hour fasting blood samples were collected in lithium-heparin coated tubes from a healthy donor on the day of each experiment. Blood samples were collected at four different times within a week. The blood donor reported not taking any medication, nor any vitamin, mineral, herbal, or flaxseed supplements. The blood was drawn by a phlebotomist at the MeritCare Health System in Fargo, North Dakota. Collected blood

samples were kept at room temperature (20-22°C), and processed within 3 hours after collection.

Complete RPMI (CRPMI)-1640 culture medium was made by adding 2.0 mM/L of L-glutamine, 100,000 U/L of penicillin, and 100 mg/L of streptomycin to RPMI-1640 culture medium. The cell culture medium, reagents, PHA-L, and concanavalin A (ConA) were purchased from Sigma Chemical company (St. Louis, MO).

Four hundred ml of heparinized blood were diluted with 800 ml CRPMI.

Four hundred μl of 0 μM (0.1% ethanol), 10 μM , 50 μM , or 100 μM SDG were added into the diluted whole blood in 4.0 ml polystyrene tubes and then incubated at 37° C, in 5% CO₂ with 95% humidified air for 4 hours. Upon the completion of the incubation, 50 ml of SDG pretreated-diluted blood and 50 ml of CRPMI (unstimulated), 50 ml of PHA-L at 10-80 $\mu\text{g}/\text{ml}$ (stimulated), or 50 ml of ConA at 12.5-100 $\mu\text{g}/\text{ml}$ (stimulated) were added to each set of triplicate culture wells in 96-well tissue culture plates, followed by addition of 100 ml of CRPMI. The cell cultures were incubated in a 5% CO₂ incubator at 37° C for 96 hours. At 18 hours before termination of incubation, 1.0 mCi methyl-³H-thymidine (Sigma Co., St. Louis, MO) was added to each culture well. After incubation, cells were harvested onto fiberglass filters (Skatron Instruments, Inc., Sterling, VA) using a cell harvester (Skatron Instruments AS, Lier, Norway) and air dried. ³H-thymidine incorporation by blood lymphocytes was counted in a beta liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA) with the activity reported in disintegrations per minute (dpm) per culture.

Data analysis

Different treatment effects on mitogen stimulated lymphocyte proliferation were analyzed using SAS software (version 9.1; SAS, Cary, NC). A generalized linear model (GLM) was used for analysis. ANOVA used a two-way factorial model with mitogen (either ConA or PHA-L) and SDG as factors. Significance level was set at $p < 0.05$. All data were reported as mean \pm standard deviation (SD).

Results

There were significant differences in lymphocyte proliferation followed by ConA or PHA-L stimulation. Mitogen

stimulated lymphocyte proliferation was significantly increased compared to control without mitogen stimulation ($p < 0.0001$, Figures 1 and 2). Lymphocyte proliferation was significantly higher with higher concentrations of ConA or PHA-L in each cell culture. However, there were no significant effects of SDG on lymphocyte proliferation followed by either ConA or PHA-L stimulation (Figures 3 and 4).

Discussion

Counting the lymphocytes using a Hemocytometer showed >95% of cell viability. In the current study, whole blood cell cultures were used for mitogen stimulated lymphocyte proliferation instead of using lymphocyte cultures which were separated from whole blood by a density gradient. Whole blood cell cultures maintain cell populations without any changes of cell mediators in cell culture which might affect lymphocyte responses to mitogens.^{24,25}

Studies have shown that ROS play an important role in T lymphocyte proliferation.^{6,15,26} Generated ROS, especially hydrogen peroxide, followed by mitogen stimulation of the T lymphocyte, function as a second messenger to signal T cell activation.¹⁵ After T cell activation by hydrogen peroxide, prolonged exposure of T cells to hydrogen peroxide inhibits cell growth.¹⁵ Thus, an antioxidant is required for optimal cell growth by neutralizing hydrogen peroxide and preventing oxidative stress which is caused by ROS.¹⁵ As shown in Los et al.'s study,¹⁵ T-lymphocytes should be activated by hydrogen peroxide to induce T-lymphocyte proliferation. T-lymphocytes reach their maximum activity at about 48 hours of incubation, and ³H-thymidine uptake by lymphocytes significantly increases 16-18 hours post mitogen stimulation.⁹ Mitogen activated protein kinase initiates the oxidative signaling process during T cell cycle entry from G0 to G1

phase transition. Antioxidants either decreased or inhibited mitogen activated protein kinase activity.²⁶

Antioxidant effects on T-lymphocyte proliferations are controversial. Either stimulatory or inhibitory effects of antioxidant on T cell proliferation were observed by other investigators.^{6,14,8,9} The discrepancies in results among studies may be due to the introduction of the antioxidants at different times within the incubation; if the antioxidant was incorporated into the cell culture before T cell activation by ROS, then antioxidant might have prevented T cell activation; thus, T cells might have not proliferated. However, if antioxidant was incorporated into cell culture after T cell activation by ROS, then the antioxidant may have neutralized ROS; thus, it might have promoted optimal cell growth and stimulated T cell proliferation.

SDG is known as an antioxidant which is found in the flaxseed.^{2,17,18,27} SDG did not have any effects on T cell proliferation in the current study. This may be due to the pre-treatment of cells with SDG prior to mitogen stimulation. Pre-existence of SDG in the cell culture might have neutralized ROS including hydrogen peroxide, thus T cell activation by ROS could not occur, which resulted in no significant changes of T cell proliferation.

Babu et al.²¹ reported inhibitory effects of a high flaxseed diet (40% of diet, w/w) on lymphocyte proliferation. These authors concluded that dietary unsaturated fatty acid is known to have inhibitory effects on lymphocyte proliferation, and the decreased lymphocyte proliferation could be due to the high alpha linolenic acids concentration in the flaxseed.²¹ Moreover, defatted flaxseed or low flaxseed diet had no effects on lymphocyte proliferation in the same study by Babu et al.²¹

Although dietary flaxseed did not

affect lymphocyte proliferation, dietary flaxseed (10% of diet, w/w) significantly inhibited superoxide generation by rat peritoneal exudates cells²² which indicates antioxidant activity of flaxseed.

No effects of SDG on T cell proliferation followed by mitogen stimulation in the current study might be explained in the following way: since SDG treatment of the cells occurred before mitogen stimulation, T cells could not be activated by ROS. Therefore, there were no changes in T cell proliferation. However, if cells were treated with SDG after the mitogen stimulation and before the pulse with ³H-thymidine, T cell proliferation might have exerted different responses to SDG treatments.

The timing of the addition of SDG appears to be critical. The different incorporation time of SDG, and production of ROS in the cell culture before and after SDG treatment should be measured to clarify SDG effects on mitogen stimulated T cell proliferation.

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