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Effects of Epoxidised Soya Bean Oil on Humoral Immune Response in Mice

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ABSTRACT *: Epoxidised soya bean oil (ESBO, 1000, 2000 or 4000 mg/kg) was orally administered to BALB/c mice daily for 28 consecutive days, and the control mice were exposed to vehicle (corn oil). Mice were immunized and challenged with sheep red blood cells (SRBC) or bovine serum albumin (BSA). In groups exposed to ESBO, the body weight gains and the relative lymphoid organ weights were not significantly changed as compared with control group. Secondary IgG antibody response to BSA was not significantly changed by ESBO, but plaque-forming cell (PFC) response to SRBC was significantly suppressed in mice treated with 4000 mg ESBO/kg/day. The mitogenic response of splenic B cells induced by LPS was not affected by ESBO in any of the groups. These results indicate that ESBO did not induce significant humoral immune response at a dose less than 2000 mg/kg/day in mice.*

Key Words *: Epoxidised soya bean oil, Immunotoxicity, Plaque-forming cell, Splenocytes proliferation*

I. INTRODUCTION

Epoxidised soya bean oil (ESBO) is used as a plasticizer in PVC gaskets in lids for glass jars used for packaging of ready-cooked baby food, used in the manufacture of plastic wraps, and widely used in marketing both raw and cooked poultries. Plasticizers are a group of additives used in plastic materials to improve the properties of the plastics. Examples of plasticizers are adipates, phthalates, citrates, phosphates, and ESBO. ESBO is listed as a prior sanctioned food ingredient by the FDA (1977). Castle *et al.* (1990) reported that the levels of ESBO in food wrapped in PVC films ranged from 1 to 85 mg/kg and in baby food packed in glass jars and metal cans from <0.1 to 7.6 mg/kg. The migration of ESBO from the lids has been determined in 81 samples of different dishes of baby food, including purees of beef, pork, fish, poultry, berries, and vegetables. Residues of ESBO were found in all dishes except in blueberries. The level of ESBO ranged from <1.5 to 50.8 mg/kg, with a mean of 11.9 mg/kg and a median of 7.8 mg/kg in baby foods with detectable levels (Hammarling *et al.*, 1998). Epoxidised fatty acids may also occur naturally in food.

Epoxidation at some or all of the points of unsaturation in the fatty acid chains gives a variety of products known as ESBO. The toxicity of ESBO varies with its grade and specifications. In rabbits a commercial grade ESBO was a mild skin irritant and another grade irritated the eyes. The vapour has caused asthmatic reactions in a worker but attempts to sensitize guinea pig skin using dilute solutions were unsuccessful (BIBRA, 1997). ESBO has a low acute toxicity when given orally to rats and applied dermally to rabbits (BIBRA, 1997; Krauze *et al.*, 1961). In rats, repeated oral administration of some grades caused liver, and kidney enlargement. Gross and microscopic examination of a comprehensive range of organs revealed slight changes in the testis and uterus. No convincing effects on reproduction or on offspring development were seen in rats given ESBO orally by stomach tubing during mating and pregnancy. There was no evidence of carcinogenicity in

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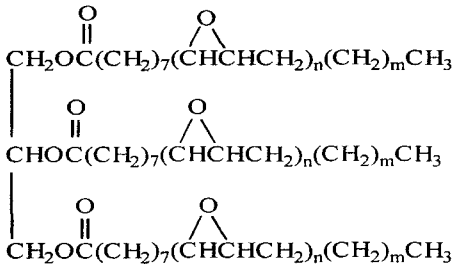
rats given ESBO in the diet or in mice treated by the oral and the dermal route (BIBRA, 1997; Larson *et al*, 1960; Weisburger *et al.*, 1965). ESBO neither damage the chromosomes nor induce mutations in mammalian cells in culture. In Ames tests there was no indication of mutagenicity (BIBRA, 1997; Heath *et al.*, 1982). However, there has been no report on the immunotoxicity of ESBO. Therefore, the purpose of this study was to investigate the immunotoxicity of ESBO.

II. MATERIALS AND METHODS

1. Materials

ESBO was purchased from Shin Dong Bang Co., (Seoul, Korea) and its molecular structure and specification are shown in Fig. 1. Phosphate buffered-saline (PBS), bovine serum albumin (BSA), penicillin, streptomycin, peroxidase-conjugated goat anti-mouse IgG, lipopolysaccharide (LPS), o-phenylenediamine (OPD), and phenazine methosulfate (PMS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Alsever's solution, fetal bovine serum (FBS), RPMI-1640, and guinea pig complement were from Gibco BRL (Grand Island, NY, USA). 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetra-zolium (MTS) were purchased from Promega Co. (Medison, WI, USA) and DEAE Dextran



n = 0, 1, 2, or 3; m = 9-3n or n=0 and m=7

Appearance	Light yellow and transparent liquid
Molecular Weight	About 1,000
Oxirane Oxygen Content (%)	6.6 Min.
Iodine Value (%)	3.0 Max.
Acid Value (%)	0.5 Max.
Viscosity (25°C, cp)	350 Max.
Refractive Index (25°C)	1,468~1,472
Specific Gravity (25°C)	0.982~1.002

Fig. 1. Molecular structure and specification of ESBO.

were from Pharmacia LKB Biotechnology (Piscataway, NJ, USA). Isotonic detergent and hemoglobin lysing reagent were purchased from Biochemical Systems (Arezzo, Italy) and other chemicals were from Sigma Chemical Co.

2. Experimental animals and administration

BALB/c inbred female mice, 7 weeks old, were provided from National Institute of Toxicological Research (NITR, Seoul, Korea). Animals were acclimated in polycarbonate cages of animal facilities which were regulated at 23±1°C of temperature, 55±5% of humidity, 10~18 circulations/hour of ventilation, 12 hour cycle of light/dark and 300~500 lux of illumination before use. All animals were maintained on standard rodent chows (Cheil Chedang, Seoul, Korea) and tap water ad libitum with the acclimation period of was 7 days. ESBO (dissolved in corn oil) 1000, 2000, and 4000 mg/kg, was orally administered to mice daily for 28 consecutive days.

3. Immunization

Sheep red blood cell (SRBC) was kept at 4°C in sterile Alsever's solution and was washed three times in PBS (pH 7.2) after centrifugation at 400×g for 10 min and diluted to provide a desired concentration for hemacytometer count. BSA was diluted to 2% solution in PBS. All mice were administered by intraperitoneal (i.p.) injection of 1 ml SRBC suspension (1×10^8 cells/m*l*) 4 days prior to each assay as described by Holsapple (1995). To assess the secondary IgG antibody response to BSA, mice were immunized by i.p. injection of 0.1 m*l* BSA 2% on the day 0, 14th, and 21th of treatment.

4. Preparation of spleen cell suspensions

BALB/c mice were killed by cervical dislocation and their spleens were removed aseptically. Cell suspensions from the spleens were prepared in complete medium (RPMI-1640 medium supplemented with 10% FBS, 100 unit/m*l* penicillin, 100 μg/m*l* streptomycin, and 2 mM L-glutamine). In brief, the suspensions were then minced, and gently squeezed into fragments between the frosted ends of two sterile microscope

slides in cold complete RPMI 1640 medium. The cell suspensions were washed three times by centrifugation and finally suspended in cold complete medium. Cell viability was determined by trypan blue exclusion test (Mishell *et al.*, 1980).

5. Primary IgM antibody response to SRBC

The primary IgM response to the T-dependent antigen SRBC was assessed as described by Holsapple *et al.* (1986). DEAE Dextran solution (30 mg/m*l*) was added into agar solution, in which 250 mg of agar crystals was dissolved completely in 50 m*l* PBS. The agar will turn cloudy. A 100 μ*l* of spleen cell suspension, a 25 μ*l* guinea pig complement and a 100 μ*l* agar solution containing 25 μ*l* SRBC were used in this test. The petri dishes containing above mixture solution under the cover slips were incubated at 37°C for 3 hr. The number of PFC was counted with a dissecting microscope.

6. Secondary IgG antibody response to bovine serum albumin (BSA)

The influence of ESBO on the splenic IgG antibody forming cell response to BSA was assessed as described by Holsapple *et al.* (1986). Blood taken by heart puncture was stood at room temperature for more than 30 min and centrifuged at 1,500×g, for 10 min at 4°C, and serum was stored at -20°C until using. 96-well plates were coated with 100 μ*l* 2% BSA in coating buffer containing 1.592 g sodium carbonate and 2.932 g sodium hydrogen carbonate dissolved in 1000 m*l* distilled water. These plate were rinsed 3 times with 0.05% Tween 20-PBS (PBST), and then each well was blocked with adding 200 μ*l* fresh 0.25% BSA/PBST. Thereafter, the plates were incubated for 1 hr at 37°C, and blocking solution was removed by tapping in the sink and the plates were washed 3 times with PBST. After the diluted serum solution was added in the ELISA plates coated by antigen, those plates were incubated for 90 min at 37°C. The supernatant was siphoned off and all wells were washed three times with 150 μ*l* PBST. Washed plates were incubated with peroxidase-conjugated goat anti-mouse IgG diluted 1:1000 for 90 min at 37°C. For peroxidase, OPD (dissolved 20 mg OPD and 20 μ*l* 30% H_2O_2 in 50 m*l* citric-phosphate buffer) was used as the substrate and optical density (OD) read at 490 nm wavelength with an ELISA microplate reader (Molecular Device, Sunnyvale, CA, USA).

7. LPS-induced splenocyte proliferation

This test was performed using the modified method described by Buttke *et al.* (1993). Splenocytes (2×10^5 cells/well) were incubated in 96 well flat-bottomed tissue culture plates in 0.1 m*l* culture media with LPS. Mitogens were added to the cultures to give final concentrations of 20 μg/m*l* for LPS. The cultures were incubated at 37°C under 5% CO_2-air for 72 hrs, 20 μ*l* of combined MTS/PMS solution was added into each well of the 96 well assay plate containing the samples. After 1 hr incubation at 37°C in a humidified 5% CO_2 atmosphere, the absorbance at 490 nm was recorded using an ELISA microplate reader.

8. The number of peripheral blood cells

Blood samples for measuring blood cells in mice were collected into tubes containing EDTA by puncture of retro-orbital venous plexus of mice on the first day after last ESBO treatment. Those samples were diluted with isotonic detergent and hemoglobin lysing reagent was dropped into this solution until clear, and blood cells were counted by auto-blood analyzer (H1 system, Technicon Co., Tarrytown, NY, USA).

9. Statistical analysis

Statistical significance of the differences among groups was examined at a 5% level of significance by ANOVA analysis. When a significant effect was found, subtesting using Dunnett's test for multiple comparisons was performed. All data are expressed in terms of a mean ± standard deviation (S.D.).

III. RESULTS

1. Body and lymphoid organ weights

The body weight gains were not changed in mice treated with ESBO as compared with those of control mice (Table 1). The relative thymus and spleen

Table 1. Effects of ESBO on the body weight gain and organ weights in female BALB/c mice

Group	Dose (mg/kg/day)	Body weight gain[a] (%)	Relative organ weight[b] (%)		
			Liver	Spleen	Thymus
Control	0	109.2±4.16	5.62±0.115	0.60±0.0480	0.36±0.0592
1	1000	110.7±5.40	5.94±0.410	0.71±0.232	0.33±0.0457
2	2000	113.1±3.86	5.87±0.124	0.58±0.0756	0.28±0.0164
3	4000	116.6±5.84	6.67±0.344*	0.48±0.0550	0.29±0.0432

Mice received ESBO orally for consecutive 28 days at the dose of 1000, 2000, or 4000 mg/kg. Each value represents the mean±S.D. of results obtained from 10 mice.
*$p<0.05$, significantly different from control group.
[a]Body weight gains (%) = (final body weight/initial body weight)×100.
[b]Relative organ weight (%) = (organ weight/final body weight)×100.

weights were slightly decreased in the mice treated with ESBO as compared with control group except relative spleen weight at 1000 mg ESBO/kg/day, but not significant (Table 1). The relative liver weights were not changed in mice treated with 1000 and 2000 mg ESBO/kg/day, but they were increased significantly in the treatment group of 4000 mg ESBO/kg/day (Table 1).

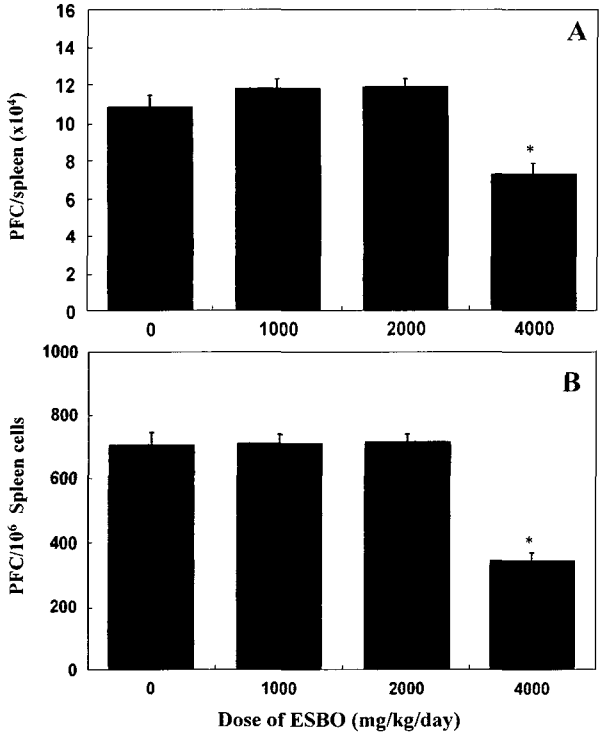


Fig. 2. Effects of ESBO on the primary antibody response to SRBC in female BALB/c mice. Mice received ESBO orally for consecutive 28 days at a dose of 1000, 2000, or 4000 mg/kg. Mice were immunized SRBC intraperitoneally 4 days before the enumeration of plaque forming cells. (A) Antibody responses to SRBC expressed as PFC/spleen; (B) Antibody responses to SRBC expressed as PFC/10^6 spleen cells. Each value represents the mean±S.D. of results obtained from 10 mice. *$p<0.05$, significantly different from control group.

2. Plaque forming cell (PFC) response to SRBC

The IgM antibody response of the spleen to the T-dependent antigen SRBC was assessed (Fig. 2). The significant change of splenic PFC responses were not observed in 1000 and 2000 mg ESBO/kg/day treatment group as compared with those in control group. However, PFC response was significantly decreased in mice treated with 4000 mg ESBO/kg/day.

3. Secondary IgG antibody response to BSA

The IgG antibody response of the spleen to BSA is shown in Fig. 3. Secondary antibody (IgG) production was not significantly decreased in all mice treated with ESBO as compared with those in control mice.

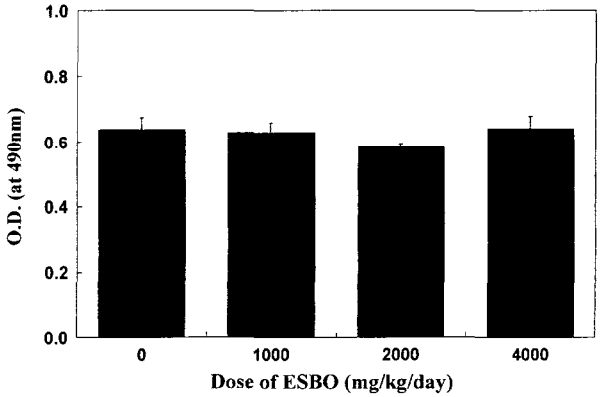


Fig. 3. Effects of ESBO on the secondary antibody response to BSA in female BALB/c mice. Mice received ESBO orally for consecutive 28 days at a dose of 1000, 2000, or 4000 mg/kg. Animals administered BSA intraperitoneally on the day 0, 14, and 21th of treatment and the levels of serum IgG were measured by ELISA. Each value represents the mean± S.D. of results obtained from 10 mice.

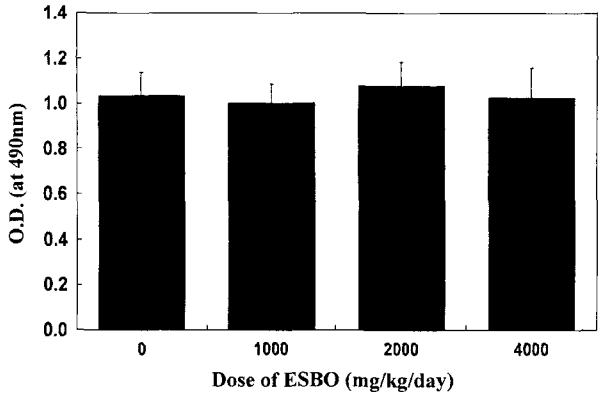


Fig. 4. Effects of ESBO on the LPS-induced mitogenic response in female BALB/c mice. Mice received ESBO orally for consecutive 28 days at a dose of 1000, 2000, or 4000 mg/kg. Spleen cells were cultured with LPS for 72 hours, respectively. MTS/PMS solution was added into each well and $O.D._{490}$ was measured by ELISA reader 1 hour after incubation. Each value represents the mean±S.D. of results obtained from 10 mice.

4. LPS-induced splenocyte proliferation

B cell proliferation induced by LPS were observed in ESBO-treated mice (Fig. 4). B cell proliferation to LPS were not decreased in mice treated with ESBO as compared with those in control mice.

5. The number of peripheral blood cells

Table 2 shows the effects of ESBO on the number of peripheral blood cells in ESBO-treated mice. Generally, the number of peripheral blood cells was not significantly affected in the mice treated with ESBO.

IV. DISCUSSION

In this study, the doses of ESBO were selected based on preliminary experiments including the initial dose ranging studies such as PFC, mitogenic response and bone marrow assay. All mice treated with 8000 mg ESBO/kg/day died within 3 days after administration. The above parameters were not changed in mice treated with 300 mg ESBO/kg/day, and even at 1000 mg ESBO/kg/day, PFC and mitogenic response were not decreased. Based above experiments, we decided 1000 mg/kg/day as dose that do not be shown immunotoxicity, 4000 mg/kg/day as maximum tolerated dose (MTD), therefore 1000, 2000, and 4000 mg ESBO/kg/day were selected.

The body weight gains were not affected in all group treated with 1000, 2000, and 4000 mg ESBO/kg/day (Table 1). This result was supported in previous studies reporting that growth appeared to be essentially normal at the 2.5% dietary level (about 1.25 g/kg/day) for up to 2 yr (Larson, 1960). However this result is different to the report that ingestion of 5% ESBO (approximately 2.5 g/kg/day) in the diet of six rats for 12 days resulted in decreased growth compared with controls treated with soya bean oil (Mounie *et al*, 1988). There was a dissimilarity in ESBO specification used in above experiments. These results suggest that difference in specification of ESBO make different effects in experimental animals. Those of relative lymphoid organ weights indirectly indicate that immune tissues are not influenced by ESBO. The

Table 2. Hematological parameters in female BALB/c mice orally exposed to ESBO for consecutive 28 days

Parameters	Dose (mg/kg/day)			
	0	1000	2000	4000
WBC ($\times 10^3/\mu l$)	4.96±1.31	4.63±0.853	5.11±0.418	4.74±1.63
RBC ($\times 10^6/\mu l$)	9.76±0.690	9.90±0.687	9.68±0.562	9.73±0.255
HGB (g/dl)	15.5±1.10	15.3±0.115	15.6±0.619	15.5±0.625
HCT (%)	50.8±3.93	49.4±1.74	48.7±2.25	48.8±1.83
MCV (fl)	52.0±1.51	50.0±1.76	50.3±1.24	50.1±0.701
MCH (pg)	15.9±0.436	15.8±0.608	16.1±0.680	16.0±0.251
MCHC (g/dl)	30.6±0.444	31.6±0.115	32.0±0.804	31.8±0.597
Platelet ($\times 10^3/\mu l$)	893±142	990±210	1164±185	959±142
Neutrophil ($\times 10^3/\mu l$)	0.908±0.446	1.05±0.477	0.896±0.236	0.998±0.289
Lymphocyte ($\times 10^3/\mu l$)	3.14±0.804	2.71±1.20	3.61±0.419	3.07±0.978
Monocyte ($\times 10^3/\mu l$)	0.372±0.087	0.367±0.263	0.280±0.118	0.248±0.144
Eosinophil ($\times 10^3/\mu l$)	0.0780±0.124	0.147±0.107	0.0880±00.0709	0.0800±0.0158
Basophil ($\times 10^3/\mu l$)	0.0880±0.0606	0.0800±0.0624	0.0460±0.0207	0.0460±0.0167

WBC : white blood cell, RBC : red blood cell, HGB : hemoglobin, HCT : hematocrit, MCV : mean corpuscular volume, MCH : mean corpuscular hemoglobin, MCHC : mean corpuscular hemoglobin concentration. Values are mean±S.D. from 10 mice.

increase of relative liver weight in group treated with 4000 mg ESBO/kg/day is supported by BIBRA's report (1997) that liver enlargement and fatty acid deposition were observed in rats given diets containing 2.5% ESBO (about 1.3 g/kg/day) for 15 weeks.

The production of antigen-specific antibodies by plasma cells of B-lymphocyte lineage represents a major defense mechanism of humoral immune responses. Optimal antibody production to antigens requires a complex interaction between antigen presenting cells T lymphocyte, B lymphocyte, cytokines, functional major histocompatibility antigens and cell surface receptors. PFC is an ideal model to monitor the primary effector function of the B cell. In this study, PFC and IgG were determined in order to evaluate humoral immunotoxicity. Secondary IgG antibody production did not change (Fig. 3), however PFC response was significantly suppressed in mice treated with 4000 mg ESBO/kg/day (Fig. 2).

The proliferation of specially sensitized lymphocytes by antigen has also been used to assess the immunotoxic potential of drugs and chemicals in human and experimental animals. Nowell (1960) first described *in vitro* lymphocyte stimulation or transformation. In this study, mitogenic response to LPS was not altered by ESBO (Fig. 4).

Consequently, it is predicted that ESBO does not affect humoral immune response of mice at dose less than 2000 mg/kg/day. However, other immunotoxicity studies including cellular immune function have been performing to evaluate overall immunotoxicity of ESBO.

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