



Consumption of Water-Soluble Egg Yolk Extract on Growth Rate, Changes in Blood Cholesterol Levels, and Immune Modulation in BALB/c Mice

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Abstract

Egg consumption has been limited to avoid cardiovascular diseases such as atherosclerosis and hyperlipidemia, because the yolk contains high levels of cholesterol. This study was conducted to evaluate the effect of the water-soluble component of egg-yolk on the growth efficiency, immune modulation, and changes in serum lipid levels in BALB/c mice. A total 5 wk old 120 BALB/c male mice were divided into 4 groups and were fed 0, 2, 10, and 20 mg/d water-soluble egg yolk extract (WSEYE) for 5 wk. Water-soluble egg yolk extract (WSEYE) uptake resulted in a significant reduction in daily weight gain and feed efficiency rate (FER). The mouse groups treated with 2 and 20 mg/d WSEYE showed a significant increase in populations of monocytes at the third wk and B-lymphocyte activity at the fifth wk. In addition, WSEYE uptake did not influence serum immunoglobulin E levels. In serum lipid-profile studies, treatment of WSEYE did not alter total cholesterol and low-density lipoprotein levels; however, blood triglyceride levels were significantly diminished in mice treated with 2 mg/d at the third wk ($p < 0.05$), and the level of high-density lipoprotein was significantly increased in the mice group treated with 2 and 10 mg/d WSEYE after 5 wk ($p < 0.05$). Taken together, the data demonstrate the beneficial effects of WSEYE in the diet on immune modulation and serum lipid profiles in mouse models; therefore, this study suggests that ingestion of water-soluble fraction of egg yolk might not be related to the increased risk of heart disease, but can be an excellent candidate for maintaining health.

Key words: water-soluble egg yolk extract (WSEYE), cholesterol, immunoglobulin, lymphocyte activation

Introduction

The chicken egg has been known to be the perfect food that contains many nutrients, including essential amino acids, unsaturated fatty acids, folate, vitamins, and minerals (Song *et al.*, 2000). However, for most of the past 40 years, large amount of daily egg consumption by men was limited because egg yolk contains high levels of cholesterol. Elevated low-density lipoprotein cholesterol (LDL-C) levels are a major risk factor for coronary heart disease (CHD). Dietary cholesterol often raises blood

LDL-C levels in human (Stamler *et al.*, 1998). However, data from free-living populations show that egg consumption is not associated with higher cholesterol levels (Kritchevsky and Kritchevsky, 2000). Furthermore, epidemiological literature does not support the idea that egg consumption is a risk factor for CHD. Therefore, the most recent American Heart Association guidelines no longer include a recommendation to limit egg consumption (Kritchevsky, 2004).

Recently, biophysiological functions of the chicken embryo extract that were reported using *in vitro* cultured cell models indicated that the extracts enhanced spleen lymphocyte proliferation and interleukin-2 (IL-2) secretion, while peritoneal macrophage phagocytosis and nitric oxide (NO) production activity were elevated (Li *et al.*,

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2012). In addition, chicken egg yolk has been used as an inexpensive and effective source of immunoglobulin Y (IgY) for the treatment of various bacterial (Sunwoo *et al.*, 2010) and viral (Wallach *et al.*, 2011) infections in animals.

Despite of these beneficial effects of egg extracts, hen eggs are regarded as one of the most prevalent allergens that affect about 1.3% of infants < 3 years of age in the US (Sampson, 2004). Although the allergic reactions are more frequently elicited by egg-white proteins than egg yolk (Anet *et al.*, 1985), some LDLs such as apovitellenins I and VI in egg yolk act as allergens (Anet *et al.*, 1985; Walsh *et al.*, 1988).

Although egg extract has been shown to have either beneficial or detrimental biological effects, the effect of water-soluble components that are removed from lipid cholesterol of egg yolk on diet, blood cholesterol levels, and immune modulation in normal healthy animal models has not yet to be clarified. In the present study, different concentrations of the water-soluble egg yolk extract (WSEYE) were supplied to mice to determine the correlation between WSEYE consumption and health parameters by measuring the changes in body weight, blood cholesterol level, sensitivity to allergy, and immunomodulatory activity, and the advantages and disadvantages of WSEYE consumption have been discussed.

Materials and Methods

Preparation of WSEYE

Commercially available normal fresh eggs were obtained from a local egg farm. The yolk was selected and the white was removed using 3M papers (GE Healthcare Life Science, USA). The egg yolk extraction method was modified from the previous report for extraction of water-soluble yolk components (Kim *et al.*, 1999). Briefly, the selected egg yolk was 10% diluted (*v/v*, 1 volume of egg yolk : 9 volume of water) with distilled water and homogenized for 30 min in ice. The homogenized yolk mixture was centrifuged at 6,000 *g* for 30 min at 4°C to precipitate insoluble yolk components. The water-soluble materials contained supernatant was freeze-dried using a freeze dryer (Ilshintech, Korea) and resuspended in phosphate-buffered saline (PBS) solution.

Animals

A total 5 wk old 120 male BALB/c mice (Nara Biotech Co., Korea) were housed in a conventional temperature- and humidity-controlled room and provided standard lab-

oratory food and water. All animal care protocols were approved by the Konkuk University Institutional Animal Care and Use Committee (IACUC; approval No.: KU13037).

Group design and experiment

Four groups of mice (10 mice/group) were used in the study. Group 1 comprised animals that were treated with an oral dose of 1× PBS as a vehicle for 5 wk. Groups 2, 3, and 4 comprised animals that were treated with an oral dose of 2, 10, and 20 mg/d of WSEYE, respectively, dissolved in 1× PBS for 3 d a week for 5 wk. The changes in body weight (BW) and the average feed intake of the mice were measured every 7 d during WSEYE treatment.

Lymphocyte and serum preparation

Ten mice from each experimental group were sequentially anesthetized using 2.5% (*v/v*) avertinon 0, 7, 21, and 35 d of the experimental period, and total blood samples were collected through direct heart puncture. Approximately 1 mL of blood was collected into tubes containing ethylenediaminetetraacetic acid (EDTA) (Becton Dickinson, USA), and then 0.5 mL of whole blood was mixed with 0.5 mL of Histopaque-1077 (Sigma-Aldrich, USA) and centrifuged at 1,200 *g* for 20 min. The lymphocyte cells were carefully isolated from the middle of the gradient. To separate the serum, 0.5 mL of whole blood was incubated for 1 h at room temperature, the samples were centrifuged at 1,200 *g* for 15 min, and the supernatant was carefully collected and stored at -70°C.

Blood cell analysis

The collected whole-blood samples were inverted several times to prevent coagulation in an EDTA-coated tube. The concentration of mice white blood cells (WBCs) and the percentages of neutrophils, lymphocytes, eosinophils, basophils, and monocytes were compared among the four different treatments. Samples were measured immediately on a Hema Vet 850 (CDC Technologies, UK) analyzer, according to the manufacturer's instructions.

Measurement of lymphocyte activation during WSEYE treatment

Isolated lymphocytes (5×10^5 cells) from each treated group were plated on a 96-well plate and incubated for 1 h at 37°C. Lipopolysaccharide (LPS, 2.5 µg/mL) was added to cultured cells to identify B-lymphocyte activity, and 2.5 µg/mL concavalin A (ConA) was added to assess T-lymphocyte activity. Cells were then incubated for an

additional 48 h. Cell viability and proliferation were analyzed using an EZ-cytotox kit (Daeil Lab Service, Korea), according to the manufacturer's instructions. The cell proliferation rate was then determined on the basis of the absorbance at 550 nm by using a Sunrise microplate reader (Tecan Austria GmbH, Austria).

Analysis of immunoglobulin

The immunoglobulin G (IgG) and immunoglobulin E (IgE) concentrations in the total blood samples were measured using a mouse IgG and IgE enzyme-linked immunosorbent assay (ELISA) Quantitation Kit (Bethyl Laboratory Inc., USA), according to the manufacturer's instructions. Briefly, anti-mouse IgG and IgE (1 µg each) antibodies were diluted using 100 µL of the coating buffer and incubated for 1 h on 96-well plates. The non-binding excess antibodies were then aspirated, and the plates were washed 3 times with the washing solution. The blocking solution was added, and the samples were incubated for 30 min; then, 100 µL of the reaction mixture was added to the samples, which were then incubated for an additional 1 h. The samples were then washed 5 times with the washing solution. Horseradish peroxidase (HRP)-conjugated secondary antibodies were added, and the samples were incubated for 1 h at room temperature. The HRP solution was then removed, and the samples were washed 5 times with the washing solution. 3,3',5,5'-Tetramethylbenzidine (TMB) solution (100 µL/well) was added, and the samples were incubated for 20 min at room temperature. Finally, 100 µL of H₂SO₄ was added, and the concentration of each Ig was determined on the basis of the absorbance at 450 nm by using the microplate reader. Determination of the concentration of IgG and IgE in serum was evaluated by the comparison with concentrations of serial dilutions of each IgG and IgE standard proteins provided from the kit.

Analyses of cholesterol and lipids

Total cholesterol (TC) and triglyceride (TG) concentrations were determined enzymatically using the cholesterol oxidase-phenol aminophenazone (CHOD-PAP) and lipase/glycerol-3-phosphate oxidase (GPO)/PAP meth-

ods, respectively, on a modular analytics (Roche, Basel, Switzerland). High-density lipoprotein cholesterol (HDL-C) was subsequently measured by precipitation with phosphotungstic acid and MgCl₂ (Roche, Basel, Switzerland). The LDL cholesterol (LDL-C) assay was performed according to the manufacturer's instructions on a modular analytics by using an LDL-C Plus 2nd Generation kit (Roche, Switzerland).

Statistical analysis

One-way ANOVA was performed on calculations of the results by using GraphPad Prism 4[®] (GraphPad Software, Inc., USA) for Windows XP. Tukey's multiple comparison test was used for comparison between groups. All data from figures are expressed as mean (±SD). The null hypothesis was rejected when the probability was $p < 0.05$.

Results and Discussion

Body weight gain, feed intake, and feed efficiency rate

The BWs of mice were measured after WSEYE treatment with 0, 2, 10, 20 mg/d for 5 wk to ensure biosafety and changes of body weight. The dosages of WSEYE used in this study were determined by comparing the average BW of humans and 5-week-old mice (60 kg and 20 g, respectively). If humans ate one egg/d, 15 g of egg yolk would be consumed, and 15 g of egg-yolk consumption is approximately 5 mg for a 20-g mouse; therefore, treatment of 2, 10, and 20 mg/d WSEYE is equal to 1/2.5, 2, and 4 egg yolks consumed/d in mice. The daily BW gains in the control, 2, 10, and 20 mg/d WSEYE treatments were 0.150±0.034, 0.105±0.021, 0.140±0.036, and 0.119±0.023 g, respectively (Table 1). The daily BW gain of the 2 and 20 mg/d WSEYE-treated groups showed a significant decrease ($p < 0.05$) compared to that in the control; however, the 10 mg/d WSEYE-treated group did not show any significant change (Table 1). In the group treated with 10 mg/d WSEYE, there was a significant increase in average feed intake for 5 wk (Table 1); therefore, this significant increase appeared to have a correlation with an increase in feed intake. Analysis of the feed efficiency ratio (FER)

Table 1. Changes in daily body weight gain and daily feed intake in mice treated with water-soluble egg yolk extract

Treat (mg/d)	0	2	10	20
Weight gain (g/d)	0.150±0.034	0.105±0.021*	0.140±0.036	0.119±0.023*
Feed intake (g/d)	3.251±0.124	3.034±0.212	4.040±0.553*	3.749±0.475
FER	0.046	0.035	0.035	0.032

p -value was detected in between 0 mg/d (control) and each WSEYE treatment for 5 wk ($*p < 0.05$, $n = 10$). FER; feed efficiency rate. WSEYE; water-soluble egg yolk extract.

showed a decrease in FER in all WSEYE-treated groups, compared to that in the control (Table 1). These data are also similar to the results of previous reports that an egg breakfast induced greater satiety and significantly reduced body weight in women (Vander Wal *et al.*, 2005). Taken together, our data showed that WSEYE consumption did not reduce feed intake but did decrease daily weight gain and FER, and regular consumption of WSEYE might result in growth inhibition in normal healthy mice. This observation suggests that WSEYE could be a promising regulator for BW.

Changes in blood immune-cell populations and lymphocyte activation

To investigate whether WSEYE can influence the population of blood immune cells, changes in the number of WBCs, granulocytes, and monocytes were analyzed. No significant differences in the populations of WBCs, lymphocytes, neutrophils, eosinophils, and basophils were identified among the treatment groups following the time courses (Figs. 1A-E). The percentage of monocytes was significantly increased in the 2 mg/d WSEYE-treated group at the third week (Fig. 1F). Interestingly, the populations of other immune cells were within the normal

ranges; however, the average population of monocytes in the 10 and 20 mg/d WSEYE-treated groups at the first week, 2 and 10 mg/d WSEYE-treated groups at the third week, and 2 and 20 mg/d WSEYE-treated groups at the fifth week were over the normal range (Fig. 1F). Although the populations of lymphocytes were not changed among the different WSEYE-treated groups following the time courses, activation of B and T lymphocytes was compared among the control and WSEYE-treated groups. The effect of WSEYE (20 mg/d) on B-cell activation was significantly higher than that in the control groups; however, T-cell activity was neither increased nor decreased compared to that in the control group (Fig. 2). The increased number of monocytes and B-lymphocyte activation suggest a putative role for WSEYE in the proliferation of monocytes and activation of B lymphocytes. Blood monocytes are an important mediator of innate immunity and are likely to be differentiated into antigen-presenting cells (APCs) such as macrophages and dendritic cells in tissues (Cheong *et al.*, 2010). In addition, lymphocytes are activated and controlled by APCs that differentiated from monocytes in lymph nodes (Ahrens *et al.*, 2009; Cheong *et al.*, 2010; Moussion and Girard, 2011); therefore, WSEYE can be involved in the activation of APC as an immune

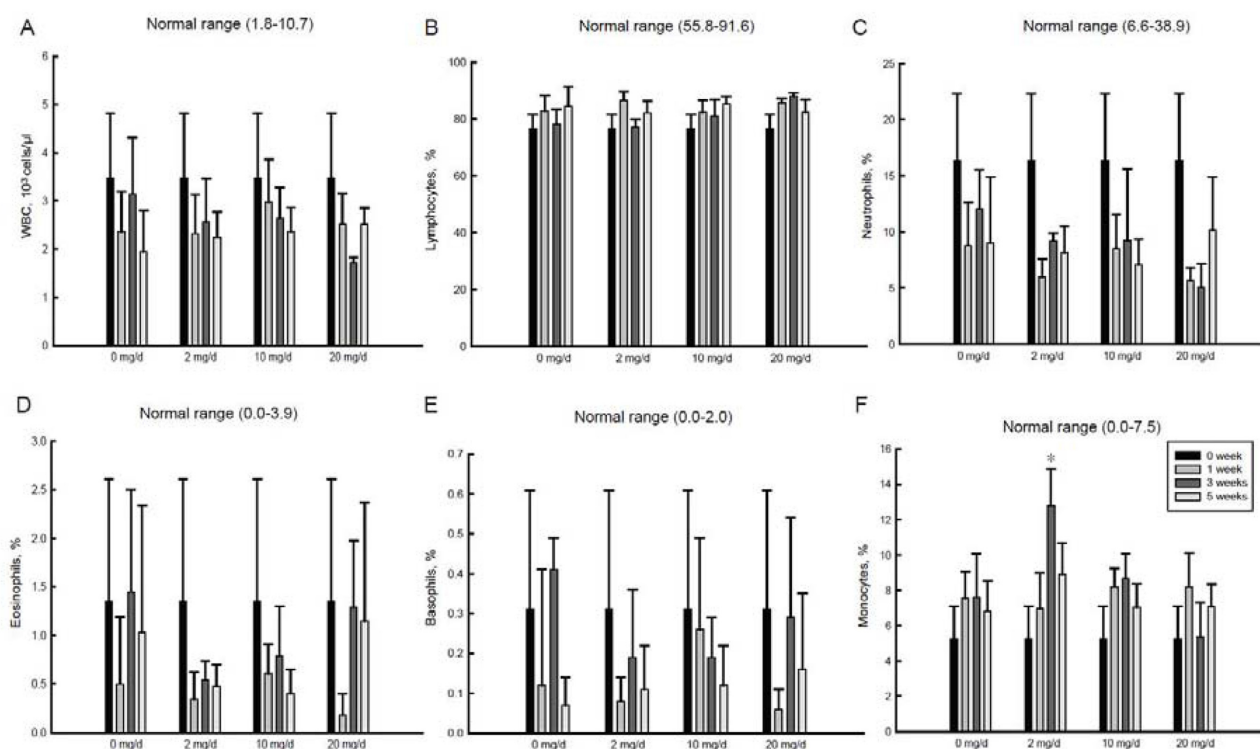


Fig. 1. Number of white blood cells (WBC), lymphocytes, granulocytes, and monocytes. (A) WBC and (B) lymphocytes are represented by the number of blood cells. (C) Neutrophils, (D) eosinophils, (E) basophils, and (F) monocytes are presented as percentages of blood cells. Statistical analysis was conducted between 0 mg/d (control) and each WSEYE treatment in the same week ($*p < 0.05$, $n = 10$). WSEYE: water-soluble egg yolk extract

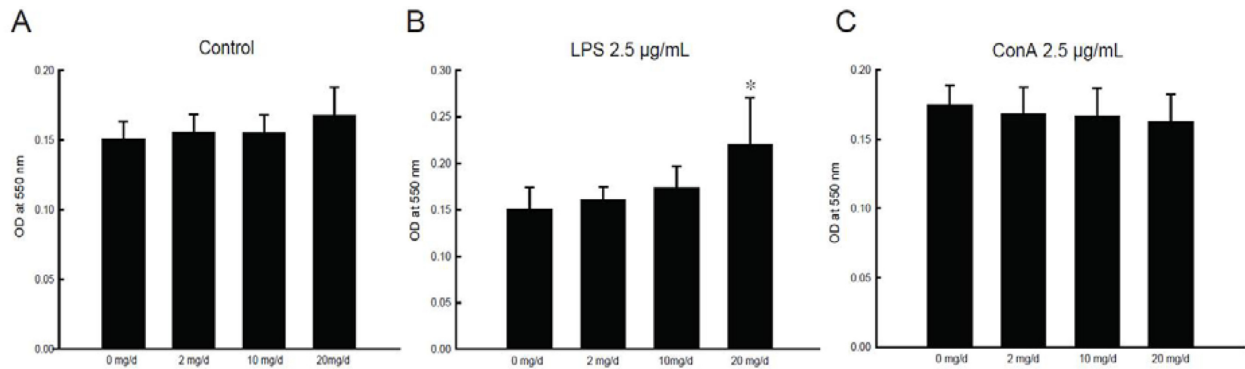


Fig. 2. B- and T-lymphocyte activation by water-soluble egg yolk extract. Lymphocytes were isolated from WSEYE (at 0, 2, 10, and 20 mg/d)-treated mice on week 0, 1, 3, and 5. Isolated lymphocytes (5×10^5 cells) were treated with (A) distilled water, (B) $2.5 \mu\text{g/mL}$ lipopolysaccharides (LPS) for B-cell activation, and (C) $2.5 \mu\text{g/mL}$ concavalin A (Con A) for T-cell activation. *p*-value was detected between 0 mg/d (control) and each WSEYE treatment at the fifth week ($*p < 0.05$, $n = 10$). WSEYE: water-soluble egg yolk extract; OD: optical density

modulator, and these activated APCs might initiate lymphocyte activation in mice.

In addition, chicken-egg yolk has been known to be a source of IgY which is also a water soluble protein, and chicken-egg extracts were used for the treatment of various bacterial and viral infections in animals (Farrelly *et al.*, 1992; Ikemori *et al.*, 1992; Li *et al.*, 2012). It can also be speculated that treatment with WSEYE might stimulate innate immunity by increasing the population of monocytes in the blood; therefore, treatment with WSEYE on animals that are infected with a bacterium or virus might increase both innate immunity by the stimulation of the monocyte population and passive immunity by increased IgY activity.

Effect of WSEYE on immunoglobulin levels

To determine the ability of WSEYE to modulate blood Ig levels in mice, IgG and IgE concentrations in the blood were analyzed at the fifth week of WSEYE treatment. No significant alteration in blood IgG concentration was observed in each WSEYE treatment group compared to that in the control (Fig. 3A). Blood IgE concentration was also not significantly altered among the treatment groups (Fig. 3). Egg allergy has been known to be the most common food allergy in children with atopic dermatitis (Niggemann *et al.*, 1999). The food allergy is frequently the result of an IgE-mediated hypersensitivity reaction, and serum IgE level has a strong association with allergies (Burrows *et al.*, 1989). Egg white contains several allergenic proteins, including ovomucoid, ovalbumin, ovomucoprotein, lysozyme, and ovomucin (Heine *et al.*, 2006).

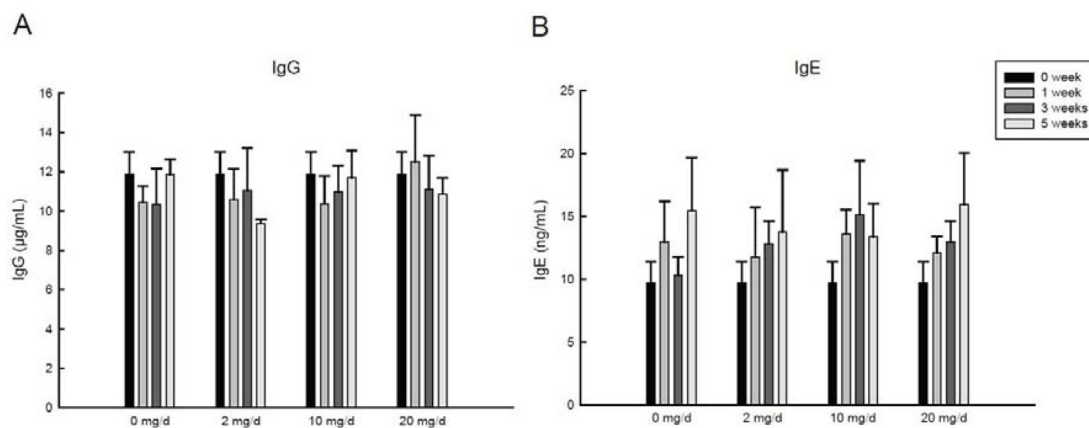


Fig. 3. Effect of water-soluble egg yolk extract treatment on mice immunoglobulin production. Blood samples were collected from WSEYE (at 0, 2, 10, and 20 mg/d)-treated mice on week 0, 1, 3, and 5. Samples were incubated with anti-mouse IgG (A) and IgE (B) antibodies. Horseradish peroxidase (HRP)-conjugated secondary antibody was used to detect the absorbance of each Ig. *p*-value was detected in between 0 mg/d (control) and each WSEYE treatment at the fifth week ($*p < 0.05$, $n = 10$). WSEYE: water-soluble egg yolk extract

In addition, egg yolk alpha livetin is a major allergen in egg yolk; other allergens have been identified in egg yolk, including vitellinin and apoprotein B, although their role remains unclear (Szepefalusi *et al.*, 1994). In this study, serum IgE levels were neither decreased nor increased in WSEYE-treated groups compared to those in the control group. Allergic reactions vary with individuals; some individuals do not have allergies to specific allergens that can induce an allergic reaction in sensitive individuals. Generally, healthy people show less sensitivity to allergens than immunologically unstable individuals. In this study, treatment of mice with WSEYE in different concentrations did not prompt allergic reactions in mice at ages 5 to 10 wk. At 5 wk of age, male mice were similar to the post-pubertal stage of a male human, suggesting that consumption of egg yolk does not induce an allergic reaction in post-pubertal humans. It is necessary to develop efficient technology for reducing food allergies, such as heat processing (Coombs and McLaughlan, 1984), enzymatic hydrolysis (Van der Plancken *et al.*, 2004), and gamma irradiation (Seo *et al.*, 2004).

Effect of WSEYE on serum lipid profile

Serum lipid levels were identified following the ingestion of different concentrations of WSEYE (Fig. 4). The level of TC was not significantly changed among the treatment groups over the experimental periods (Fig. 4A), but the level of serum TG was significantly decreased in the 2 mg/d WSEYE-treated group at the fifth week (Fig. 4B). Although the intake of WSEYE did not affect LDL-C levels, it is remarkable that the level of HDL-C was significantly increased in the 2 and 10 mg/d WSEYE treated groups after 5 wk (Figs. 4C and D). Because egg contains approximately 210 mg of TC, the recommended egg consumption was limited to 3 eggs/wk, which is about 300 mg of TC/d, to prevent heart disorders (AHA, 1973). Previous report suggests dietary cholesterol raises levels of total and LDL-cholesterol that promote coronary heart disease (Hu *et al.*, 1999). However, many researchers refuted that the intake of dietary cholesterol does not increase blood cholesterol levels (Clark *et al.*, 1997; Howell *et al.*, 1997; Krumholz *et al.*, 1994), because intake of dietary cholesterol induced a decrease in internal cho-

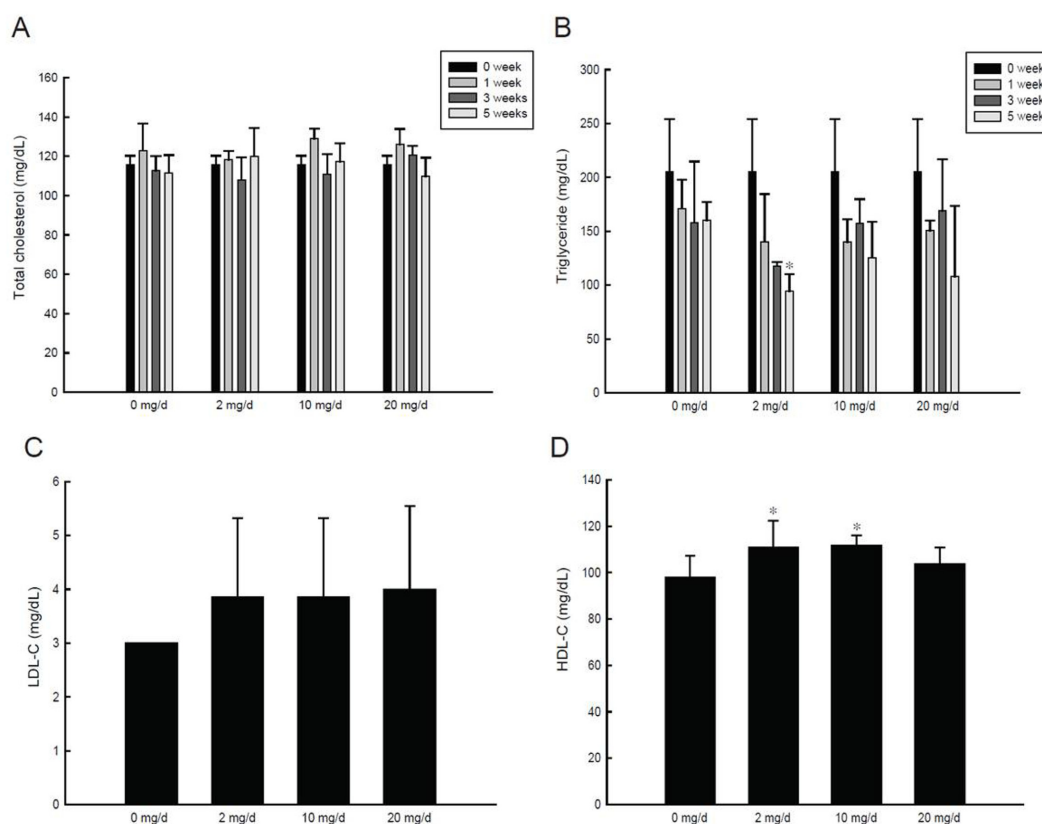


Fig. 4. Changes in serum lipid profiles by water-soluble egg yolk extract treatment. *P*-value for (A) total cholesterol (TC) and (B) triglycerides were detected between 0 mg/d (control) and each WSEYE treatment in the same week ($*p < 0.05$, $n = 10$). *P*-value for (C) low-density lipoprotein cholesterol (LDL-C) and (D) high-density lipoprotein cholesterol (HDL-C) were detected between 0 mg/d (control) and each WSEYE treatment at the fifth week ($*p < 0.05$, $n = 10$). WSEYE: water-soluble egg yolk extract.

lesterol synthesis in the liver and the conversion of the internal cholesterol to bile acid, which is excreted (McNamara, 2000). Supporting these previous reports, the 2 mg/mL WSEYE-treated mice did not show an increase in TC but a decrease in TG. These data are in accordance with previous reports that a high cholesterol diet decreased hepatic TG levels but increased the level of fecal TG (Jang *et al.*, 2011). Chicken egg yolk also contains lecithin and sphingomyelin that controls the blood cholesterol levels by inhibiting the reuptake of bile acid (Eckhardt *et al.*, 2002; Jiang *et al.*, 2001; Nagaoka *et al.*, 2002; Noh and Koo, 2003). In this aspect, we assume that WSEYE contained mostly water-soluble components that are livetin, IgY, unknown peptides, amino acids, carbohydrates, and vitamin, etc. Therefore, it is possible that WSEYE contains cholesterol-regulating materials that might inhibit the increase of TC levels and decrease the TG levels in mice. In addition, a significant increase HDL-C in the 2 and 10 mg/d WSEYE-treated groups were also remarkable, because HDL-C is known to eliminate blood cholesterol to prevent atherosclerosis and hyperlipidemia. Taken together, the intake of WSEYE does not show an increase in serum cholesterol levels, whereas it decreases TG and increases HDL-C; this suggests that WSEYE has a positive effect on regulating serum lipid levels.

Conclusion

This study was designed to investigate the physiological effect of water-soluble components of egg yolk extract on mice. The present results suggested that consumption of WSEYE decreased FER, daily weight gain and level of TG, but increase of monocyte population in WBC, activity of B lymphocytes and level of HDL-C. Therefore, WSEYE may not only have an important role in weight control, but also have a role in regulating physiological process in immune and cardiovascular system.

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