J. Exp. Biomed. Sci. 2010, 16(4): 377~380

Brief Communication

Anti-Thrombotic Effects of Egg Yolk Lipids *In Vivo*

Hyun-Jeong Cho[1,3], Young-Cheol Ju[2,3] and Hwa-Jin Park[2,†]

[1]*Department of Biomedical Laboratory Science, College of Medical Science, Konyang University, 685, Gasuwon-dong, Seo-gu, Daejeon 302-718, Korea*

[2]*Department of Biomedical Laboratory Science, College of Biomedical Science and Engineering and Regional Research Center, Inje University, 607, Obang-dong, Gimhae, Gyungnam 621-749, Korea*

In this study, we investigated the effect of egg yolk lipids (EYL) on collagen (10 μg/ml)-stimulated platelet aggregation *in vivo*. Dietary EYL significantly inhibited collagen-induced platelet aggregation, in addition, increased the formation of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), intracellular Ca^{2+}-antagonist as aggregation-inhibiting molecules, in collagen-stimulated platelets. These results suggest that EYL inhibits the collagen-induced platelet aggregation by up-regulating the cAMP and cGMP production. On the other hands, prothrombin time (PT) on extrinsic pathway of blood coagulation was potently prolonged by dietary EYL *in vivo*. These findings suggest that EYL prolongs the internal time between the conversion of fibrinogen to fibrin. Accordingly, our data demonstrate that EYL may be a crucial tool for a negative regulator during platelet activation and blood coagulation on thrombotic diseases.

Key Words: Egg yolk lipids (EYL), Platelet aggregation, Cyclic adenosine monophosphate, Cyclic guanosine monophosphate, Blood coagulation, Prothrombin time

Platelet aggregation is absolutely essential to the formation of a hemostatic plug when normal blood vessels are injured. However, the interactions between platelets and collagen can also cause circulatory disorders, such as thrombosis, atherosclerosis, and myocardial infarction (Schwartz et al., 1990). Inhibition of the platelet-collagen interaction provides a promising approach to the prevention of thrombosis. On the other hand, the process of platelet aggregation is regulated, in part, by the second messengers, the cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), which decrease the $[Ca^{2+}]_i$, an essential factor for platelet aggregation. Increase of intracellular cAMP or cGMP productions lead to inhibition of agonist-induced platelet activation: adhesion, release of granule substances, and aggregation (el-Daher et al., 1996; Jang et al., 2002; Rodomski et al., 1990). It is known that platelet aggregation and blood coagulation are common risk factors for pathologic phenomena in thrombotic disease. Blood coagulation systems (secondary hemostasis) have intrinsic blood coagulation system (activated partial thromboplastin time, APTT) from the injured blood vessel, and extrinsic blood coagulation system (prothrombin time, PT) derived from tissue. The prolongation of blood coagulation means the prolonged time of forming fibrin clot and is the mark of inhibited blood coagulation. Extended APTT and PT hint that thrombin is produced with delay (Sano et al., 2003; Davie and Ratnoff, 1964; Furie and Furie, 1988; Akoum et al., 1990). In the present study, we therefore investigated the effect of dietary egg yolk lipids (EYL) on platelet aggregation and blood coagulation.

Hen eggs have been known to be beneficial to health for a long time. However, there are adverse reports that egg yolk proteins are composed of allergens such as lipovitellenin and livetin (Langeland T, 1983; Anet et al., 1985), and that lipids of egg yolk contain cholesterol, which affects plasma

*Received: 5 November, 2010 / Revised: 13 December, 2010
Accepted: 15 December, 2010

[3]Hyun-Jeong Cho and Young-Cheol Ju contributed equally to this work.

[†]Corresponding author: Hwa-Jin Park. Department of Biomedical Laboratory Science, College of Biomedical Science and Engineering and Regional Research Center, Inje University, 607, Obang-dong, Gimhae, Gyungnam 621-749, Korea.
Tel: +82-55-320-3538, Fax: +82-55-334-3426
e-mail: mlsjpark@inje.ac.kr

cholesterol levels (Howell et al., 1997). In our study, we did not analyze the cholesterol content from EYL, however, dietary EYL diminished triglyceride and total cholesterol levels of plasma and liver (data not shown). Also, EYL inhibited the collagen-induced platelet aggregation *in vivo*. Accordingly, we studied whether EYL has anti-platelet and anti-coagulation effects compared with those of control.

We used fresh mature hen eggs from a local market. Hen eggs were broken and egg yolk was collected. A total 20 g of the egg yolk was homogenized with extraction solvents (chloroform/methanol 2:1, v/v) using the method of Folch et al. (1957), and then extracted three times with extraction solvents using an extraction mixer (Lab-Line Instruments, Inc). After centrifugation, the lower layer was collected, frozen at -80℃, and then lyophilized for 24 h. This dehydrated substance was dissolved and extracted in solvent (chloroform/distilled water 1:1, v/v). After centrifugation, the chloroform layer and aqueous layer were separated. The chloroform layer (EYL) was evaporated, and dried. EYL was dissolved in dimethysulfoxide (DMSO), and the effect of DMSO was subtracted from the results.

Sprague-Dawley rats (male, 180~220 g) were purchased from Hyo-Chang Science Co. (Cheonan, Korea) and acclimatized for 1 week at a temperature of 22±2℃, humidity of 60±5%, and 12-h light/dark cycle with free access to a commercial pellet diet (Samyang Co., Cheonan, Korea) and drinking fresh tap water before the experiments. The experimental groups were fed EYL 1% with commercial pellet diet for 30 days. The Ethical Commitees for Animal Experiments of Inje University approved this study.

Blood was collected, and anticoagulated with ACD solution (0.8% citric acid, 2.2% sodium citrate, 2.45% glucose). Platelet-rich plasma was centrifuged at 125 × *g* for 10 min to remove the red blood cells, and the platelets were washed twice with washing buffer (138 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 5.5 mM glucose, and 1 mM EDTA, pH 7.4). The washed platelets were then resuspended in suspension buffer (138 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 0.49 mM $MgCl_2$, 5.5 mM glucose, 0.25% gelatin, pH 7.4) to a final concentration of 5 × 10^8/ml. To measure the platelet aggregation by dietary EYL, washed platelets (10^8/ml) were preincubated for 3 min at 37℃ in the presence of 2 mM exogenous $CaCl_2$, and then stimulated with collagen (10 μg/ml) for 5 min. Aggregation was monitored using an aggregometer (Chrono-Log, Corp., PA, USA) at a constant stirring speed of 1,000 rpm. Each aggregation rate was evaluated as an increase in light transmission. The suspension buffer was used as the reference. As shown in Fig. 1A, dietary EYL significantly inhibited the collagen stimulated platelet aggregation by 14% as compared with that of control.

Consequently, we focused on the effect of EYL on cAMP and cGMP production in collagen-stimulated platelets. Washed platelets (10^8/ml) of experimental groups were preincubated for 3 min at 37℃ in the presence of 2 mM $CaCl_2$, and then stimulated with collagen (10 μg/ml) for 5 min for platelet aggregation. The aggregation was terminated by the addition of 80% ice-cold ethanol. cAMP and cGMP were measured with Synergy HT Multi-Model Microplate Reader (Bio Teck Instrument, Inc., Winoosku, USA) using cAMP and cGMP EIA kits. As shown in Fig. 1B and C, the basal levels of cAMP and cGMP in washed platelets prepared from control rats were 1.047±0.011 pmol/10^8 platelets and 0.273±0.009 pmol/10^8 platelets, respectively. Collagen itself decreased intracellular cAMP level from 1.047 pmol/10^8 platelets to 0.753 pmol/10^8 platelets, and cGMP level from 0.273 pmol/10^8 platelets to 0.235 pmol/10^8 platelets. However, the dietary EYL up-regulated the intracellular cAMP up to 1.253 pmol/10^8 platelets, and cGMP level up to 0.462 pmol/10^8 platelets in collagen-stimulated platelets. cAMP and cGMP production by dietary EYL were significantly increased by 66.4% and 96.6% as compared with those of control (collagen-stimulated EYL non-treated group), respectively. These results indicate that dietary EYL inhibits the collagen-induced platelet aggregation by increasing the cAMP and cGMP production as negative regulators on platelet aggregation. On the other hand, in the present study, because we did not determine the $[Ca^{2+}]_i$ and TXA_2 level, platelet aggregating molecules, we have not an obvious evidence that EYL decrease the $[Ca^{2+}]_i$ and TXA_2 level. However, it is known that cGMP and cAMP inhibit the collagen-induced platelet aggregation by decreasing the $[Ca^{2+}]_i$ and TXA_2 level (Cho et al., 2007).

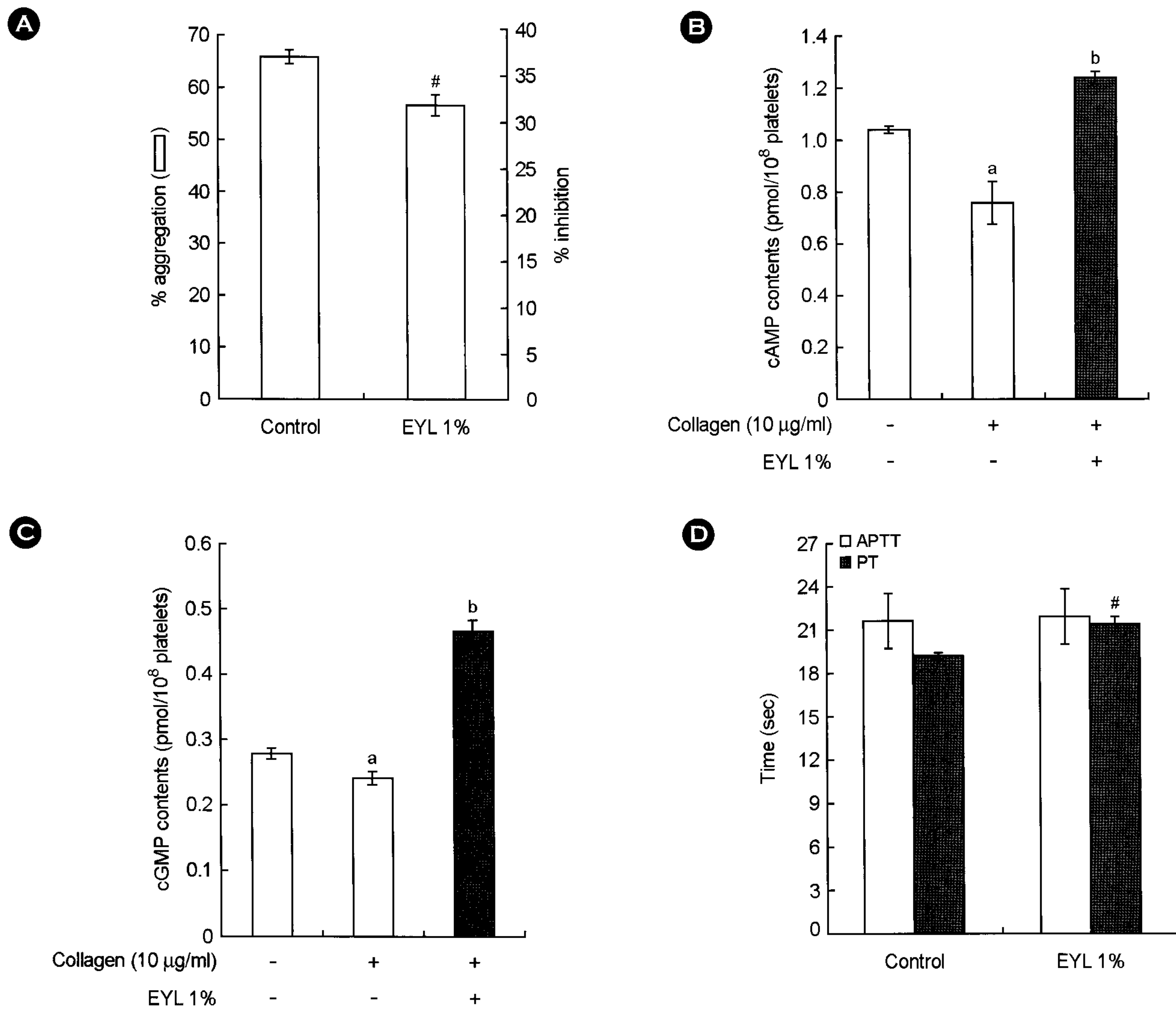

Fig. 1. The Anti-thrombotic effects of dietary egg yolk lipids (EYL) in collagen-stimulated rat platelets *in vivo*. (A) Effects of dietary EYL on collagen-induced platelet aggregation. Washed platelets (10^8/ml) were preincubated in the presence of 2 mM $CaCl_2$ for 3 min at 37℃, and then stimulated with collagen for 5 min. Platelet aggregation (%) was recorded as an increase in light transmission. Inhibition by EYL was recorded as percentage of that of control. $^{\#}P<0.05$ vs control. (B) Effects of dietary EYL on cAMP production on collagen-induced platelet aggregation. (C) Effects of dietary EYL on cGMP production on collagen-induced platelet aggregation. Washed platelets (10^8/ml) were preincubated in the presence of 2 mM $CaCl_2$ and then stimulated with collagen (10 μg/ml) for 5 min at 37℃. The reactions were terminated by adding 80% ice-cold ethanol. cAMP and cGMP contents were measured using EIA kits. $^{a}P<0.05$ *vs* intact platelets. $^{b}P<0.001$ *vs* collagen-stimulated EYL non-treated platelets. (D) Effects of dietary EYL on PT and APTT. Data are expressed as mean ± SEM (n=6). $^{\#}P<0.05$ *vs* control. Control, EYL non-treated group; EYL 1%, EYL 1%-treated group.

On the other hand, we investigated the effects of dietary EYL on blood coagulation. Citrated platelet-poor plasma (PPP) was prepared by centrifuging the blood remaining after the removal of PRP at 1,300 × *g* for 10 min. The PPP (0.1 ml) was preincubated in a two-channel coagulator (KG Behnk Elektronik GMBH & Co., Norderstedt, Germany) cup (BioMérieux Corp., France) with gentle stirring for 1 min at 37℃. PT was determined as the time interval between the addition of PT reagent (0.1 ml) to the PPP and the formation of a fibrin clot. After preincubation for APTT measurement, 0.1 ml of APTT reagent was added to the PPP and incubated for 3 min at 37℃. Following incubation, 0.1 ml of 25 mM $CaCl_2$ was rapidly added to the PPP solution containing APTT reagent. APTT was determined as the time required to form a fibrin clot. As shown in Fig. 1D, dietary EYL prolonged the PT (21.5±0.5 sec) by 14.4% as compared with that (18.8±0.2 sec) of control without affecting the APTT. It is known that the value of

PT differs between human and rat, and the sensitivity of PT is very different according to the kinds of thromboplastin in rat: recombinant thromboplastin (9.4±0.79 sec), Dade (26.7±0.58 sec), simplastin (18.6±0.93 sec) (García-Mansano et al., 2001). When was used the simplastin as thromboplastin for determination of PT, as the results (Fig. 1D), control PT (18.8±0.2 sec), a reference, is equal to that (18.6±0.93 sec) of simplastin. This means that dietary EYL is a crucial regulator by affecting the extrinsic pathways on blood coagulation system.

With all above results, dietary EYL inhibits collagen-induced platelet aggregation by acting a potent cAMP/cGMP up-regulator, and prolongs the PT on blood coagulation system. In conclusion, these results suggest that dietary EYL may have an inhibitory effect on platelet aggregation- and blood coagulation-mediated thrombotic diseases by reacting as physiologically an effective negative regulator during thrombosis.

Acknowledgements

This study was supported (in part) by a grant from the Ministry of Commerce, Industry and Energy (MOCIE) and Korean Institute of Industrial Technology Evaluation & Planning (ITEP) through the Biohealth Products Research Center (BPRC) of Inje University, Korea.

REFERENCES

Akoum A, Josefonvicz J, Sigot M. Anticoagulant activity of a bacterial glycopeptide. Thromb Res. 1990. 60: 9-18.

Anet J, Anet J, Back JF, Baker RS, Barnett D, Burley RW, Howden ME. Allergens in the white and yolk of hen's egg. A study of IgE binding by egg proteins. Int Arch Allergy Appl Immunol. 1985. 77: 364-371.

Cho HJ, Cho JY, Rhee MH, Park HJ. Cordycepin (3'-deoxyadenosine) inhibits human platelet aggregation in a cyclic AMP- and cyclic GMP-dependent manner. Eur J Pharmacol. 2007. 558: 43-51.

Davie EW, Ratnoff OD. Waterfall sequence for intrinsic blood clotting. Science 1964. 145: 1310-1312.

el-Daher SS, Eigenthaler M, Walter U, Furuichi T, Miyawaki A, Mikoshiba K, Kakkar VV, Authi KS. Distribution and activation of cAMP- and cGMP-dependent protein kinases in highly purified human platelet plasma and intracellular membranes. Thromb Haemost. 1996. 76: 1063-1071.

Folch J, Lees M, Sloane-Starley GH. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem. 1957. 226: 497-509.

Furie B, Furie BC. The molecular basis of blood coagulation. Cell 1988. 53: 505-518.

García-Manzano A, González-Llaven J, Lemini C, Rubio-Póo C. Standardization of rat blood clotting tests with reagents used for humans. Proc West Pharmacol Soc. 2001. 44: 153-155.

Howell WH, McNamara DJ, Tosca MA, Smith BT, Gaines JA. Plasma lipid and lipoprotein responses to dietary fat and cholesterol: a meta-analysis. Am J Clin Nutr. 1997. 65: 1747 -1764.

Jang EK, Azzam JE, Dickinson NT, Davidson MM, Haslam RJ. Roles for both cyclic GMP and cyclic AMP in the inhibition of collagen-induced platelet aggregation by nitroprusside. Br J Haematol. 2002. 117: 664-675.

Langeland T. A clinical and immunological study of allergy to hen's egg white. IV. Specific IGE-antibodies to individual allergens in hen's egg white related to clinical and immunological parameters in egg-allergic patients. Allergy 1983. 38: 493-500.

Rodomski MW, Palmer RMJ, Monacade S. An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. Proc Natl Acad Sci. 1990. 87: 5193-5197.

Sano Y, Sato K, Uchida M, Murata M. Blood coagulation Fibrinolysis of Rats Fed Fish Oil: Reduced Coagulation Factors Especially Involved in Intrinsic Pathway and Increased Activity of Plasminogen Activator Inhibitor. Biosic Biotechnol Biochem. 2003. 67: 2100-2105.

Schwartz SM, Heinmark RL, Majesky MW. Developmental mechanisms underlying pathology of arteries. Physiol Rev. 1990. 70: 1177-1209.