Anti-platelet Effects of Dimethyl Sulfoxide *via* Down-regulation of COX-1 and TXA$_2$ Synthase Activity in Rat Platelets

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In this study, we investigated the effect of DMSO, a highly dipolar organic liquid, in collagen (5 μg/ml)-stimulated platelet aggregation. DMSO inhibited platelet aggregation at 0.5% by inhibiting production of thromboxane A$_2$ (TXA$_2$) which was associated with blocking cyclooxygenase (COX)-1 activity and TXA$_2$ synthase. In addition, DMSO significantly increased the formation of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP) and cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). On the other hand, DMSO (0.1~0.5% concentration) did not affect the LDH release which indicates the cytotoxicity. Based on these results, DMSO has anti-platelet effect by regulation of several platelet signaling pathways, therefore we suggest that DMSO could be a novel strategy on many thrombotic disorders.

Key Words: DMSO, Platelet aggregation, cAMP, cGMP, COX-1 activity, TXA$_2$ synthase, TXA$_2$

INTRODUCTION

When blood vessels are injured, platelet aggregation and coagulation cascades lead to blood clot and thrombosis (Nossel et al., 1969). Abnormal platelet activation is causative of a large number of cardiovascular diseases such as atherosclerosis, stroke, and peripheral vascular diseases (Fitzgerald et al., 1986). Platelets are activated by a number of stimuli, such as collagen, arachidonic acid lead to TXA$_2$ formation through COX-1 and TXA$_2$ synthase pathway

(TXAS) (Baumgartner and Haudenschild, 1972; Hamberg et al., 1975). TXA$_2$, one of the powerful inducer of platelet aggregation, interacts with other platelets, which is increased in thrombotic disorders (Miller et al., 1977; FitzGerald et al., 1987). Therefore, inhibition of platelet aggregation would protect against cardiovascular diseases and offer an opportunity to develop a promising anti-platelet agent.

DMSO, a highly dipolar organic liquid, is used as a powerful solvent in organic synthesis for chemical reactions to increase the solubility and as a cryoprotective agent for cell freezing frequently (Kligman, 1965; Egorin et al., 1998; Wusteman et al., 2008). However, as a chemotherapeutic drug, many studies have established that DMSO has anti-inflammatory effect (Wood and Wood, 1975), lowers blood pressure due to vasodilation (Kligman, 1965), exerts a inhibitory effect on microorganism (Basch and Gadebusch, 1968) and protects against ischemic injury (Shimizu et al., 1997). In addition, platelets have assumed a role in the

development of thrombosis or focal cerebral ischemia, but DMSO impaired platelet aggregation and function *via* P selectin expression (Rosenblum and El-Sabban, 1982; Dujovny et al., 1983). Also, it has been reported to have protective effect of occlusion of middle cerebral artery in animal models, and inhibitory effect of vascular smooth muscle cell (VSMC) proliferation and tissue factor expression (Bardutzky et al., 2005; Camici et al., 2006). Despite all this information, the identities of mechanism for the anti-platelet effect of DMSO are well unknown. In the present report, we investigated whether DMSO has an inhibitory effect on TXA_2-mediated platelet aggregation.

MATERIALS AND METHODS

Materials

All experimental animals were obtained from Daehan Bio Link (Chungbuk, Korea), and collagen was purchased from Chrono-Log Corporation (Havertown, PA, USA). LDH cytotoxicity assay, COX activity assay, and TXB_2 EIA kits were bought from Cayman Chemical (Ann Arbor, MI, USA). cAMP and cGMP EIA kits were purchased from Biovision (Milpitas, CA, USA). PT, aPTT reagent and 0.02 M $CaCl_2$ were bought from Thermo Scientific (Waltham, MA, USA). DMSO and all other chemicals and reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of washed rat platelets

8 weeks-old male Sprague-Dawley rats were housed in an animal facility equipped with a 12~12 hours dark cycle. Rats were allowed free access to a standard rat chow and drinking water and acclimatized for 1 week before the experiments. After starvation for 12 hours, rats were anesthetized with ethyl ether and blood was collected from the abdominal vein with 3.2% sodium citrate (1:9, v/v) for anti-coagulation. Platelet-rich plasma was centrifuged at $125 \times g$ 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 6.9). 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, pH 7.4) to a final concentration of 5×10^8/mL. All of the above procedures were carried out at 25℃ to avoid platelet aggregation on cooling. The Ethical Committees for Animal Experiments of Konyang University (Daejeon, Korea) approved this study.

Measurement of platelet aggregation

Washed platelets (10^8/mL) were pre-incubated for 3 min at 37℃ in the presence of 2 mM exogenous $CaCl_2$ with or without DMSO and then stimulated with collagen (5 μg/mL) for 5 min. Aggregation was monitored using an aggregometer (Chrono-Log, Havertown, PA) at a constant stirring speed of 1,200 rpm. Each aggregation rate was evaluated as an increase in light transmission. The suspension buffer was used as the reference.

Measurement of TXB_2

Washed platelets (10^8/mL) were pre-incubated with various concentrations of DMSO for 3 min in the presence of 2 mM $CaCl_2$ at 37℃, and stimulated with collagen (5 μg/mL) for 5 min. The reactions were terminated by the addition of ice-cold EDTA (5 mM) and indomethacin (0.2 mM). The amount of TXB_2, a stable metabolite of TXA_2, was determined using a TXB_2 EIA kit according to the procedure described by the manufacturer.

COX-1 activity assay

Platelets in suspending buffer containing 1% protease inhibitor were sonicated with a VCX 130 sonicator (Vibra-Cell, Newtown, CT, USA). The platelet lysates were prepared to assay the COX-1 activity in the cytosol. The platelet lysates (10 μg-protein) were incubated with or without DMSO (0.5%), and the reactions were initiated by the addition of arachidonic acid. After the incubation for 1 min at 25℃, COX-1 activity was measured by a COX-1 activity assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's recommendations. SC-560 (330 nM) was used as a positive control.

TXA₂ synthase activity assay

Platelets in suspending buffer containing 1% protease inhibitor were sonicated with a VCX 130 sonicator (Vibra-Cell, Newtown, CT, USA). The platelet lysates were prepared to assay the TXA_2 synthase activity in the cytosol. Platelet lysates (10 μg-protein) were pre-incubated with or without DMSO (0.5%) at 37℃ for 5 min, and the reactions were initiated by the addition of PGH_2. After the incubation for 1 min at 37℃, the reactions were terminated by the addition of 1 M citric acid, and neutralized with 1 N NaOH. The amount of TXB_2, a stable metabolite of TXA_2, was measured by a TXB_2 EIA kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's recommendations. Ozagrel (11 nM) was used as a positive control.
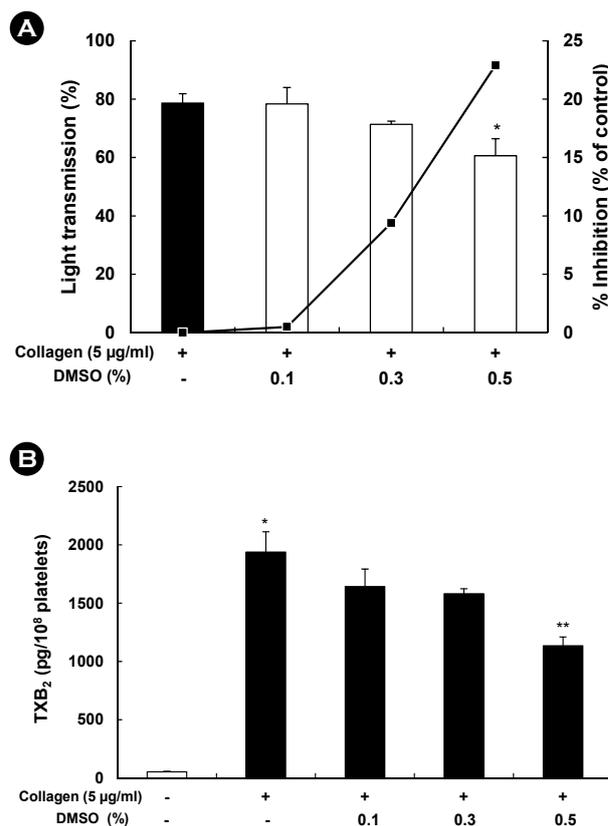
Measurement of cAMP and cGMP

Washed platelets (10^8/mL) were pre-incubated for 3 min at 37℃ with various concentrations of DMSO in the presence of 2 mM $CaCl_2$, and then were stimulated with collagen (5 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 using cAMP and cGMP EIA kits (Biovision, Milpitas, CA, USA).

LDH release assay

To assess whether DMSO has cytotoxicity on platelet aggregation reaction, we examined the effect of DMSO on LDH release. LDH is a stable enzyme normally found in the cytosol of cells, but it rapidly releases into the supernatant upon damage of cell membrane. Washed platelets (10^8/ml) were incubated for 5 min at 37℃ with varying concentrations of DMSO, and then the supernatant was measured by an LDH assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's recommendations.

Statistical analysis

The experiment results are expressed as the means ± S.D. Statistical analysis was performed by two tailed unpaired Student's t-test or ANOVA as appropriate. Each group was compared by the Scheffe's method for post hoc tests.

Fig. 1. Anti-platelet effect of DMSO. (A) Effect of DMSO on collagen-induced platelet aggregation. Washed platelets (10^8/mL) were pre-incubated with various concentrations of DMSO (0.1, 0.3 and 0.5%) in the presence of 2 mM $CaCl_2$ for 3 min at 37℃, and then stimulated with collagen (5 μg/mL) for 5 min. Platelet aggregation (%) was recorded as an increase in light transmission. Inhibition by DMSO was recorded as percentage of the collagen-induced aggregation rate. *$P<0.05$ was compared with that of collagen-induced control. **(B)** Effects of DMSO on collagen-induced TXA_2 production. Washed platelets (10^8/mL) were pre-incubated with various concentrations of DMSO (0.1 0.3 and 0.5%) in the presence of 2 mM $CaCl_2$ for 3 min at 37℃, and then stimulated with collagen (5 μg/mL) for 5 min. The reactions were terminated by the addition of ice-cold EDTA (5 mM) and indomethacin (0.2 mM). The amount of TXB_2 was determined using a TXB_2 EIA kit. Data are expressed as means ± S.D. (n=3). *$P<0.05$ was compared with that of intact cell. **$P<0.05$ was compared with that of collagen-induced control.

RESULTS

DMSO inhibits platelet aggregation

Washed platelets (10^8/mL) were activated with collagen (5 μg/mL) in the presence of 2 mM $CaCl_2$ with or without various concentrations of DMSO (0.1, 0.3 and 0.5%). As a result, DMSO significantly reduced the collagen-stimulated

platelet aggregation from 78.7 ± 3.2 to $60.7 \pm 5.8\%$ (22.9% Inhibition at 0.5% DMSO) (Fig. 1A).

DMSO decreases TXA$_2$ production

TXA$_2$ is a positive feedback mediator produced following platelet aggregation (Paul et al., 1999). A signaling pathway of TXA$_2$ is activated by collagen treatment. Therefore, we next examined whether DMSO inhibits the production of TXA$_2$ in collagen-treated platelets. After stimulating with collagen (5 μg/ml), the amount of TXA$_2$ was increased from 54.2 ± 3.6 to 1937.8 ± 174.6 pg/10^8 platelets. However, DMSO reduced the production of TXA$_2$ to 1133.5 pg/10^8 platelets on collagen-induced platelet aggregation (41.5% inhibition at 0.5% DMSO). These results mean that DMSO has the anti-platelet effect by inhibiting TXA$_2$ production (Fig. 1B). These results suggest DMSO could be directly related to inhibition of its metabolic enzyme, COX-1 activity or TXA$_2$ synthase.

DMSO down-regulates COX-1 activity and TXA$_2$ synthase

To assess whether DMSO affects COX-1 activity, COX-1 activity assay were examined. DMSO significantly inhibited COX-1 activity from 87.5 ± 3.8 to 76.0 ± 3.4 pmol/min/mg-protein (13.1% inhibition at 0.5% DMSO). 330 nM of SC-560, a selective COX-1 inhibitor, was used as a positive control, it decreased COX-1 activity to 64.7 ± 3.2 pmol/min/mg-protein (Fig. 2A). TXA$_2$ synthase catalyzes the conversion of prostaglandin H$_2$ to TXA$_2$ in platelets. To determine whether DMSO has the effect on TXA$_2$ synthase, TXA$_2$ synthase assay was assessed by measuring TXB$_2$ formation. After the addition of PGH$_2$ (5 μM) for 1 min at 37℃, platelet lysate with DMSO inhibited TXA$_2$ production from 321.1 ± 11.7 to 275.6 ± 12.6 ng/min/mg-protein (14.2% inhibition at 0.5% DMSO). When ozagrel (11 nM), a TXA$_2$ synthase inhibitor, was used as a positive control, it decreased TXA$_2$ level to 211.1 ± 10.7 ng/min/mg-protein (Fig. 2B).
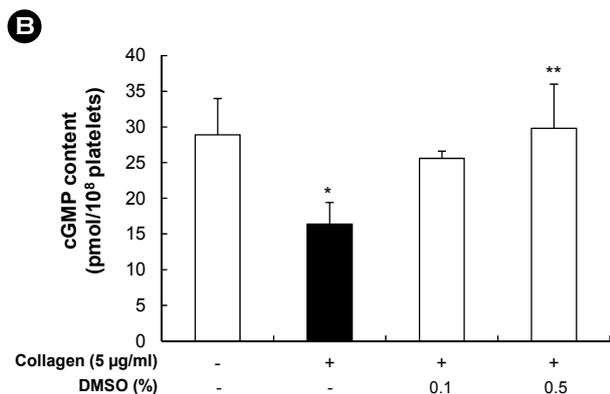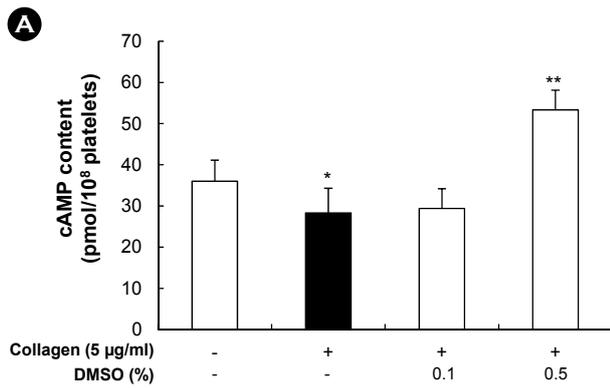
DMSO elevates cAMP and cGMP levels

Increased platelet aggregation by platelet activating-stimulator is known to be lowered by the production of
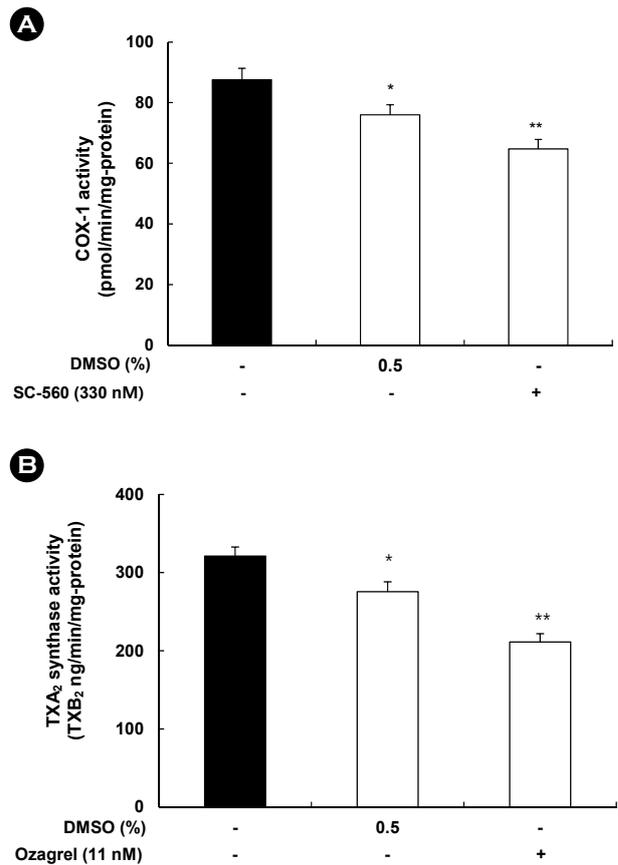
Fig. 2. Effects of DMSO on COX-1 activity and TXAS. (A) Effects of DMSO on COX-1 activity. Platelet lysate was incubated with or without DMSO (0.5%), and SC-560 (330 nM) as known as selective COX-1 inhibitor at 25℃ for 30 min. COX-1 activity was determined by measuring peroxidase activity of COX. Data represent means $\pm$ S.D. (n=3). *$P<0.05$, **$P<0.01$ was compared with that of control. **(B)** Effect of DMSO on TXA$_2$ synthase activity. Washed platelets lysated by sonicator was pre-incubated with or without DMSO (0.5%), and ozagrel (11 nM) as known as TXA$_2$ synthase inhibitor at 37℃ for 30 min. Then PGH$_2$ solution (5 μM) was added as a substrate for TXA$_2$ synthase. TXA$_2$ synthase activity was determined by measuring TXA$_2$ formation. Data represent means $\pm$ S.D. (n=3). * $P<0.01$, **$P<0.001$ were compared with that of control.

either cGMP or cAMP (Wang et al., 1998). Therefore, we next investigated whether DMSO increases the cellular levels of cAMP and cGMP. Collagen decreased intracellular cAMP level from 36.0 ± 5.1 pmol/10^8 platelets (basal level), to 28.3 ± 6.0 pmol/10^8 platelets in the washed platelets. In Fig. 3A, when the platelets were incubated in the presence of both DMSO and collagen, DMSO (0.5%) significantly increased cAMP levels to 53.3 ± 7.2 pmol/10^8 platelets. In Fig. 3B, collagen decreased intracellular
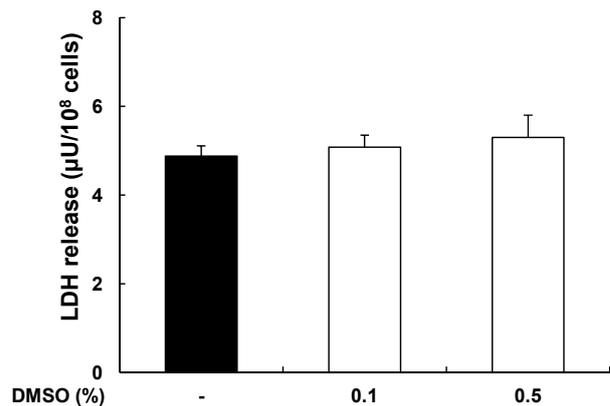
Fig. 4. Effect of DMSO on the LDH release in washed platelets. After 3 min incubation of washed platelets (10^8/mL) with DMSO (0.1 and 0.5%), LDH release was measured using LDH assay kit. Data are expressed as means $\pm$ S.D. (n=3).

Fig. 3. Effects of DMSO on cAMP and cGMP production in collagen-stimulated platelets. Washed platelets (10^8/mL) were pre-incubated with or without DMSO for 3 min in the presence of 2 mM CaCl$_2$ and then stimulated with collagen (5 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)** Effects of DMSO on cAMP production in collagen-stimulated platelets. **(B)** Effects of DMSO on cAMP production in collagen-stimulated platelets. Data are expressed as means $\pm$ S.D. (n=3). *$P<0.05$ was compared with that of basal level. **$P<0.001$ was compared with that of collagen-stimulated platelets.

concentrations of DMSO for 5 min as the same method of platelet aggregation reaction, and then LDH level was assayed. As the results, DMSO (0.1, 0.3 and 0.5 %) did not have toxicity as compared with that of control in rat platelets (Fig. 4).

DISCUSSION

In this study, we investigated that DMSO (0.5%) inhibits collagen (5 μg/ml)-stimulated platelet aggregation (Fig. 1A). Consistent with this observation, DMSO also reduces TXA$_2$ formation *via* suppression of COX-1 and TXA$_2$ synthase activity on collagen (5 μg/ml)-induced platelet aggregation (Fig. 1B, 2A and B). Also, anti-platelet effect of DMSO by 0.5% concentration has does not any toxicity on platelets (Fig. 4). Camici *et al*. (2006) suggested that DMSO inhibits tissue factor expression and activation in HAECs and VSMCs at concentration of 1.0% without any toxic effect. Especially in platelets, not only the concentration of 0.5% DMSO for suppressing platelet activation, also the concentration of DMSO as high as 3% for inhibition on platelet function and P selectin expression has been used without any toxicity (Cetin et al., 2001; Asmis et al., 2010). Therefore, we suggest that DMSO has anti-platelet effects without cytotoxicity on 0.5%.

TXA$_2$ is a potent inducer on platelet activation and it is

cGMP level from 28.9 $\pm$ 5.1 pmol/10^8 platelets (basal level), to 16.4 $\pm$ 3.0 pmol/10^8 platelets. When the platelets were incubated in the presence of both DMSO and collagen, DMSO significantly increased the cGMP levels to 29.8 $\pm$ 6.2 pmol/10^8 platelets. From these results, DMSO has the anti-platelet effect by up-regulating the intracellular cAMP and cGMP production in collagen-induced platelets.

DMSO does not exert toxicity in platelets

To investigate whether DMSO has potential toxicity, washed platelets (10^8/ml) were incubated with various

synthesized from COX-dependent endoperoxidase (Abe et al., 1995). Furthermore, inhibiting TXA$_2$ production plays important roles in certain coronary syndrome because of its vasoconstrictor activity (Bunting et al., 1983; Bush et al., 1984). Also, the decrease of TXAS reduces TXA$_2$ production and increases the synthesis of PGI$_2$ and PGD$_2$, which have anti-aggregatory effects (Gresele et al., 1991). Thus, TXAS inhibitor can be a promising agent for protection of thrombotic disorder cause (Vilahur et al., 2007). In the present study, DMSO blocked TXA$_2$ production from 1937.8 $\pm$ 174.6 to 1133.5 pg/10^8 platelets (Fig. 1B) by inhibiting the TXAS activity from 321.1 $\pm$ 11.7 to 275.6 $\pm$ 12.6 ng/min/mg-protein at 0.5% concentration (Fig. 2B). Asmis *et al*. (2010) found DMSO also inhibits platelet adherence which is essential step for the physiological formation of stable occlusive thrombus. These results can clearly demonstrate DMSO could represent not only anti-thrombus agent but also a novel agent for drug eluting stents to protect acute stent thrombosis. In addition, nonsteroidal anti-inflammatory drugs (NSAIDs), like aspirin, are known to have anti-platelet effect by blocking COX activity (Smith et al., 1990). In Fig. 2A, DMSO inhibited COX-1 activity from 87.5 $\pm$ 3.8 to 76.0 $\pm$ 3.4 pmol/min/mg-protein. From this result, DMSO may have the beneficial property like aspirin as a COX-1 inhibitor. Therefore, it could be used as a novel agent with aspirin or its alternative drug.

On the other hand, increases of cAMP and cGMP levels affected activating PKA and PKG, which phophorylate substrate protein, vasodilator-stimulated phosphoprotein (VASP) entailed in the inhibitory effect of platelet aggregation (Halbrugge et al., 1990; Li et al., 2003). Thus, cAMP and cGMP are anti-platelet second messenger in platelet aggregation, and a substance which elevate the levels of them may control platelet aggregation. DMSO (0.5%) significantly increased the cAMP and cGMP level on collagen-induced platelet aggregation dose-dependently (28.3 $\pm$ 6.0 to 53.3 $\pm$ 7.2 pmol/10^8 platelets, 16.4 $\pm$ 3.0 to 29.8 $\pm$ 6.2 pmol/10^8 platelets, respectively, Figs. 3A and B). Therefore, we suggest DMSO has therapeutic effects on cardiovascular disease as a regulator of platelet aggregation *via* elevation of cAMP and cGMP levels.

In conclusion, the present study provides that DMSO at 0.5% concentration increases intracellular cAMP and cGMP levels, and inhibits TXA$_2$ formation via down-regulation the COX-1 and TXA$_2$ synthase activity without cytotoxicity. Therefore, DMSO could be a novel agent for anti-platelet and promising strategy for thrombotic disease by having negative feedback regulator during platelet aggregation.

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