Research Article

Ethanol Extract of Lepidium apetalum Seed Elicits Contractile Response and Attenuates Atrial Natriuretic Peptide Secretion in Beating Rabbit Atria

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The seeds of Lepidium apetalum Willdenow (called “Tinglizi” in China and “Jungryukza” in Korea) have been used to discharge phlegm and improve dropsy in Oriental medicine. The present study investigated the effects of ethanol extract of the seeds of Lepidium apetalum (ELA) on atrial dynamics and atrial natriuretic peptide (ANP) secretion in beating rabbit atria. ELA increased atrial stroke volume, pulse pressure, and cAMP efflux, concomitantly attenuating ANP secretion in a dose-dependent manner. ELA-induced increases in atrial stroke volume, pulse pressure, and cAMP levels and decrease in ANP secretion were not inhibited by pretreatment with staurosporine, a nonspecific protein kinase inhibitor, or diltiazem and verapamil, the L-type Ca \(^{2+}\) channel blockers, respectively. Helveticoside, a well-known digitalis-like cardiac glycosidic constituent of ELA, also increased atrial dynamics, including stroke volume and pulse pressure, without changing cAMP efflux and ANP secretion, and the effects of helveticoside were not inhibited by pretreatment with staurosporine, diltiazem, and verapamil. These results suggest that the ELA-induced positive inotropic activity in beating rabbit atria might, at least partly, be due to the digitalis-like activity of helveticoside rather than an increase in cAMP efflux.

1. Introduction

Cardiac glycosides are a diverse family of naturally derived compounds that bind to and inhibit Na\(^{+}\)/K\(^{+}\)-ATPase. Members of this family have been used for many years for the treatment of heart failure and atrial arrhythmia, and the mechanism of their positive inotropic effect is well characterized. There are many different well-described clinical trials of drugs for the treatment of chronic heart failure, including cardiac glycosides, sympathomimetics, phosphodiesterase (PDE) III inhibitors, diuretics, and angiotensin-converting enzyme inhibitors [1]. Stimulation of β-adrenergic receptor with a sympathomimetic agent induces positive inotropic effects, which are dependent on protein kinases (PKs) and L-type Ca\(^{2+}\) channels [2, 3]. The increase in cyclic adenosine monophosphate (cAMP) levels induced by PDE III inhibitors also accentuates cardiac contractility via activation of protein kinases and L-type Ca\(^{2+}\) channels [4]. Treatment of heart failure patients with cardiac glycosides like digitalis, which augment pump function by increasing the contractility of cardiac myocytes, is known to improve hemodynamics and exercise tolerance [5, 6]. In the regulation of cardiac contractility, Ca\(^{2+}\) plays a pivotal role and has been implicated in the functional mechanism of various agents involved in the modulation of cardiac action [7, 8]. In addition, several signal transduction factors like cAMP, inositol triphosphate (IP\(_{3}\),...
diacylglycerol (DAG), PK, and adenyl cyclase (AC) influence the generation of cardiac contractile forces and regulate intracellular Ca\(^{2+}\) concentrations [9].

Atrial natriuretic peptide (ANP) is synthesized and stored in atrial cardiomyocytes and secreted into the bloodstream by atrial stimulation [10]. The secretion of ANP from cardiomyocytes under mechanical stimulation has been known to regulate body fluid levels through relaxation of vascular smooth muscle and inhibition of water and renal electrolyte reabsorption [11].

The seeds of *Lepidium apetalum* Willdenow (Cruciferae, called “Tinglizi” in China and “Jungryukza” in Korea) have been used to discharge phlegm and improve dropsy in Oriental medicine. From the seeds of *Lepidium apetalum*, compounds such as helveticoside, linoleic acid, and olein have been isolated [12]. Recently, it was reported that an extract of the seeds of *Lepidium apetalum* inhibits skin pigmentation mediated by IL-6-driven signaling. However, to the best of our knowledge, the inotropic effect of *Lepidium apetalum* in perfused beating atria has not been defined. Therefore, we performed this study to elucidate the mechanism of ELA-induced positive inotropic activity in perfused beating rabbit atria.

2. Materials and Methods

2.1. Plant Materials and Extraction. The seeds of *Lepidium apetalum* Willdenow were commercially available and purchased from the herbal market in Iksan, Jeonbuk Province, and authenticated by professor Tae-Oh Kwon, College of Life Sciences and Natural Resources, Wonkwang University. A herbarium voucher specimen (HBI-048) was deposited in the herbarium of the Professional Graduate School of Oriental Medicine, Wonkwang University, Iksan, Jeonbuk, South Korea. The dried seeds of *Lepidium apetalum* (600 g) were subjected to extraction procedures with 1L of 95% ethanol thrice, with each extraction being performed for 24h. The ethanol extract was filtered through a Whatman No. 3 filter paper, concentrated using a rotary evaporator (ELA, 1.5 g), and then used in experiments.

2.2. Preparation of Perfused Beating Rabbit Atria and Determination of Atrial Stroke Volume and Pulse Pressure. New Zealand white male rabbits weighing 2 kg were used as the source of rabbit atria. Each rabbit was anesthetized by injecting ketamine-HCl, and its chest was opened. An isolated perfused atrial preparation was prepared by a slightly modified version of Cho’s method [13], allowing atrial pacing and perfused atrial preparation was prepared by a slightly modified ketamine-HCl, and its chest was opened. An isolated perfused atrium was then transferred to an organ chamber containing 3 mL of buffer at 34°C. The pericardial space of the organ chamber was opened to air so as not to restrict atrial dynamics. The atrium was immediately perfused with N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffer solution by means of a peristaltic pump (1mL/min). The buffer was prepared using the following constituents: 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl\(_2\), 1.2 mM MgCl\(_2\), 25 mM NaHCO\(_3\), 10.0 mM glucose, and 10.0 mM HEPES (adjusted to pH 7.4 with 1M NaOH) and 0.1% bovin serum albumin (BSA). Soon after setup of the perfused atrium, transmural electrical field stimulation at 1.3 Hz (duration, 0.3–0.5 ms; voltage, twice the threshold intensity, 20–30 V; distention, 6.1 cm H\(_2\)O) was started with a luminal electrode. The organ chamber was fixed so as to allow axial rotation to change the height of the atrial cannula and intra-atrial pressure. The perfusate was prewarmed to 34°C by passage through silicone tubing in a mixed gas chamber. The buffer in the organ chamber was oxygenated.

2.3. Measurement of ANP Levels in Perfusates. The levels of immunoreactive ANP in the perfusate were measured by radioimmunoassay, as previously described [13]. The radioimmunoassay was performed in tris (hydroxymethyl) aminomethane (Tris)-acetate buffer (0.1 mM EDTA, 0.005% soybean trypsin inhibitor, 0.02% sodium azide, 0.0004% phenylmethylsulfonyl fluoride, and 1% BSA, at pH 7.4). The sample volume used for radioimmunoassay was 50 μL, and the total assay volume was 300 μL. Standard or perfusate samples were incubated with 100 μL of anti-ANP antibody and 100 μL of \(^{125}\)I-labeled ANP for 24 h at 4°C. The separation of free tracer from antibody-bound tracer was achieved by adding 1.0 mL of dextran-charcoal suspension (charcoal, 6.0 g; Dextran T-70, 0.625 g; phenylmercuric acetate, 34 mg; and neomycin, 2 g in 1L of Tris-acetate buffer, 0.1 M, pH 7.4). Radioimmunoassay for ANP was performed on the day of the experiments, and all samples in an experiment were analyzed in a single assay. The secreted amount of ANP was expressed as nanograms of ANP per minute per gram of atrial wet weight.

2.4. Preparation of Samples for cAMP Assay. To prepare the perfusates for cAMP assay, 100 μL of the perfusate was treated with trichloroacetic acid (100 μL) to a final concentration of 6% for 15 min at room temperature and centrifuged at 4°C. The supernatant (100 μL) was transferred to a polypropylene tube, extracted 3 times with water-saturated ether (300 μL), and dried using a speedVac concentrator (Savant, Farmingdale, NY, USA). The dried samples were resuspended in 50 mM sodium acetate buffer (pH 4.85).

2.5. Measurement of cAMP Levels in Perfusates. Production of cAMP was measured in an equilibrated radioimmunoassay, as described previously [14]. Briefly, standards or samples were made up to a final volume of 100 μL in 50 mM sodium acetate buffer (pH 4.8) containing theophylline (8 mM). Then, 100 μL of diluted cAMP antiserum and iodinated \(^{3}\)-O-monomosuccinyl-adenosine \(^{3}\)-cyclic monophosphate tyrosyl methyl ester (\(^{125}\)I-ScAMP-TME, 10,000 counts/min [cpm])
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per 100 μL) were added, and the mixture was incubated for
24 h at 4°C. For the acetylation reaction, 5 μL of a mixture of
acetic anhydride and triethylamine (1:2 dilution) was added
to the assay tube before adding antiserum and tracer as well.
The bound form was separated from the free form by charcoal
displacement at 4°C. Trichloroacetic acid, NaI (0.2 mg/mL)
and sodium metabisulfite (0.2 mg/mL) were added, and the mixture was incubated for
24 h at 4°C. For the acetylation reaction, 5 μL of a mixture of
acetic anhydride and triethylamine (1:2 dilution) was added
to the assay tube before adding antiserum and tracer as well.
The bound form was separated from the free form by charcoal
displacement at 4°C. Trichloroacetic acid, NaI (0.2 mg/mL)
and sodium metabisulfite (0.2 mg/mL) were added, and the reaction was terminated
with sodium metabisulfite (0.2 mg/mL) and NaI (5 mM).

2.6. Measurement of K⁺ Concentration in Perfusates. Before
and after the perfusion of beating rabbit atria with HEPES
buffer, the K⁺ concentration in the perfusates was measured
by using an ion selective electrode analyzer (NOVA 5, Biochemical, Walt-
mann, MA, USA) and expressed as mmol/L.

2.7. Reagents. HEPES, sodium chloride, potassium chloride,
calcium chloride, magnesium chloride, sodium bicarbonate,
glucose, BSA, sodium acetate, aprotinin, glycine, lysosome,
thephrine, sodium azide, potassium phosphate monobasic,
inorganic phosphate dibasic, charcoal, diltiazem, ver-
apamil, ouabain, and helveticoside were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The following
reference materials were obtained from the sources specified:
anti-cAMP (Merck Bioscience Calbiochem, USA), anti-ANP
(Homemade, Korea), staurosporine (Biological Research Labor-
atories Inc, USA), and NaII (Amersham Biosciences, Swe-
den). Stock solutions of diltiazem, verapamil, staurosporine,
and helveticoside were prepared in DMSO. Control experi-
ments demonstrated that the highest DMSO level (0.2%) had
no effect on beating rabbit atria.

2.8. Statistical Analysis. The results are shown as means ± SE.
Data was analyzed by repeated measures ANOVA followed
by Bonferroni’s multiple-comparison test. Student’s t-test for
unpaired data was also applied. Statistical significance was
defined as P < 0.05.

3. Results

3.1. Effect of ELA on the Atrial Dynamics, cAMP Efflux,
and ANP Secretion. In beating rabbit atria, treatment with
ELA increased stroke volume and pulse pressure in a dose-
dependent manner (Figures 1(a)(A) and 1(a)(B)). Treatment
with ELA also increased cAMP efflux in beating rabbit atria
(Figure 1(a)(C)). On the other hand, treatment with ELA
markedly decreased ANP secretion in beating rabbit atria
(Figure 1(a)(D)). Ouabain, which was used as a positive con-
trol, significantly increased stroke volume (Figure 1(b)(A))
and pulse pressure (Figure 1(b)(B)), with no change in cAMP
efflux (Figure 1(b)(C)) and ANP secretion (Figure 1(b)(D)).

3.2. Effect of Staurosporine on ELA-Induced Changes. To
define the role of protein kinases in the ELA-induced positive
inotropic effect, the effects of staurosporine, a nonspecific
PK inhibitor, on beating rabbit atria were tested. Treatment
with ELA (5 × 10⁻⁴ g/mL) induced an increase in stroke
volume, pulse pressure, and cAMP efflux and a decrease
in ANP secretion in beating rabbit atria (Figures 2(a)(A),
2(a)(B), 2(a)(C), and 2(a)(D)). Treatment of beating atria
with staurosporine (1 × 10⁻⁶ M) significantly decreased stroke
volume and pulse pressure, in comparison with the corre-
sponding levels in controls (Figures 2(b)(A) and 2(b)(B)).
However, subsequent treatment with ELA (5 × 10⁻⁴ g/mL)
reverted the changes in atrial stroke volume and pulse
pressure and increased the values to levels much higher
than basal levels (Figures 2(b)(A) and 2(b)(B)). Staurosporine
did not affect cAMP efflux in beating atria. However, ELA
substantially increased cAMP efflux in the staurosporine-
pretreated atrium (Figure 2(b)(C)).

In addition, staurosporine had no effect on ANP
secretion in beating atria. However, ELA markedly decreased
ANP secretion in the staurosporine-pretreated atrium
(Figure 2(b)(D)).

3.3. Effect of Diltiazem on ELA-Induced Changes. To inves-
tigate whether Ca²⁺ channels are involved in the ELA-
induced positive inotropic activity, diltiazem, an L-type Ca²⁺
channel blocker, was used to pretreat beating atria. Treatment
of beating atrium with diltiazem (5 × 10⁻⁶ M) markedly
decreased stroke volume and pulse pressure (Figures 3(a)(A)
and 3(a)(B)). However, the diltiazem-induced reductions in
atrial stroke volume and pulse pressure were reverted to
levels greater than the basal levels by subsequent treatment
with ELA (5 × 10⁻⁴ g/mL) (Figures 3(a)(A) and 3(a)(B)). As
shown in Figure 3(g)(A), cAMP efflux level was not altered
by treatment with diltiazem but increased by perfusion with
ELA after the pretreatment with diltiazem (Figure 3(a)(C)).
Diltiazem had no effect on ANP secretion in beating atria.
However, ELA markedly decreased ANP secretion in the
diltiazem-pretreated atrium (Figure 3(a)(D)).

3.4. Effect of Verapamil on ELA-Induced Changes. To con-
firm that L-type Ca²⁺ channels are involved in the ELA-
induced positive inotropic effect, verapamil, another L-
type Ca²⁺ channel blocker, was also tested. Treatment with
### Figure 1: Dose-response curves of ELA (a) and ouabain (b) for stroke volume (A), pulse pressure (B), cAMP efflux (C), and ANP secretion (D) in beating rabbit atria. Values shown are mean ± SE (n = 4); *P < 0.05 versus control; **P < 0.01 versus ELA (5 × 10^{-5} g/mL) or ouabain (3 × 10^{-7} M); ***P < 0.001 versus ELA (1 × 10^{-4} g/mL) or ouabain (1 × 10^{-6} M) (compared with values for the last 3 fractions of control).
Figure 2: Effects of ELA (a) and staurosporine (b) on ELA-induced changes in stroke volume (A), pulse pressure (B), cAMP efflux (C), and ANP secretion (D) in beating rabbit atria (1.3 Hz). Values shown are mean ± SE (n = 4); **P < 0.001 versus control; ###P < 0.001 versus staurosporine (compared with values for the last 3 fractions of control or staurosporine).
Figure 3: Effects of diltiazem (a) and verapamil (b) on ELA-induced changes in stroke volume (A), pulse pressure (B), cAMP efflux (C), and ANP secretion (D) in beating rabbit atria. Values shown are mean ± SE (n = 4); ***P < 0.001 versus control; **P < 0.001 versus diltiazem or verapamil (compared with values for the last 3 fractions of control, diltiazem, or verapamil).
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3.7. Effect of Staurosporine on Helveticoside-Induced Changes. To define the roles of protein kinases in the helveticoside-induced positive inotropic activity, beating atria were treated with staurosporine. The atrial stroke volume and pulse pressure after treatment of beating atrium with staurosporine ($1 \times 10^{-6}$ M) were significantly lower than those in the controls (Figures 5(b)(A) and 5(b)(B)). When staurosporine-pretreated beating atria were treated with helveticoside ($2 \times 10^{-5}$ M), atrial stroke volume and pulse pressure reverted to levels higher than those observed after the staurosporine treatment alone (Figures 5(b)(A) and 5(b)(B)). Staurosporine had no effect on cAMP efflux and ANP secretion in beating atria. Treatment of helveticoside with staurosporine also caused no changes in cAMP efflux and ANP secretion in beating atria (Figures 5(b)(C) and 5(b)(D)). The data were expressed $\Delta %$ changes of the mean values of fraction number 29/30 over the values of fraction number 17/18 (Figure 7).

3.8. Effect of Ca$^{2+}$ Channel Blockers on Helveticoside-Induced Changes. To investigate whether Ca$^{2+}$ channels are involved in the helveticoside-induced positive inotropic activity, beating rabbit atria were pretreated with diltiazem or verapamil. Treatment of beating atrium with diltiazem ($5 \times 10^{-5}$ M) markedly decreased stroke volume and pulse pressure (Figures 6(a)(A) and 6(a)(B)). However, the diltiazem-induced reductions in atrial stroke volume and pulse pressure were significantly reverted by subsequent perfusion with helveticoside (Figures 6(a)(A) and 6(a)(B)). Verapamil ($1 \times 10^{-5}$ M) also markedly decreased stroke volume and pulse pressure (Figures 6(b)(A) and 6(b)(B)), which recovered upon subsequent treatment with helveticoside ($2 \times 10^{-5}$ M) (Figures 6(b)(A) and 6(b)(B)). Diltiazem and verapamil had no effect on cAMP efflux and ANP secretion in beating atria. Treatment of helveticoside with diltiazem or verapamil also caused no changes in cAMP efflux and ANP secretion in beating atria (Figures 6(a)(C) and 6(a)(D), 6(b)(C), and 6(b)(D)). The data were expressed $\Delta %$ changes of the mean values of fraction number 29/30 over the values of fraction number 17/18 (Figure 7).

4. Discussion

This study clearly shows that ELA increases stroke volume, pulse pressure, and cAMP efflux in beating rabbit atria. Because the cAMP-signaling pathway modulates the activation of L-type Ca$^{2+}$ channels and PKs, leading to accentuation of cardiac contractility in beating atria [9, 16–18], it was expected that cAMP would be involved in the ELA-induced increase in atrial dynamics via the L-type Ca$^{2+}$ channels and/or PKs. However, our results showed that blocking of L-type Ca$^{2+}$ channels with diltiazem or verapamil had no effect on the ELA-induced increases in stroke volume, pulse pressure, and cAMP efflux in beating rabbit atria. Similarly, inhibition of PKs with staurosporine did not affect the ELA-induced increases in stroke volume, pulse pressure, and cAMP efflux. It has been reported that Convallaria keiskei, which contains the cardiac glycoside-like molecule convallatoxin, increases stroke volume and pulse pressure without an associated increase in cAMP efflux in the perfusate [19]. These results suggest that ELA-induced positive inotropic activity is
Figure 5: Effects of helveticoside (a) and staurosporine (b) on helveticoside-induced changes in stroke volume (A), pulse pressure (B), cAMP efflux (C), and ANP secretion (D) in beating rabbit atria. Values shown are mean ± SE (n = 4); **P < 0.01, ***P < 0.01 versus control; ###P < 0.001 versus staurosporine (compared with values for the last 3 fractions of control or staurosporine).
Figure 6: Effects of diltiazem (a) and verapamil (b) on helveticoside-induced changes in stroke volume (A), pulse pressure (B), cAMP efflux (C), and ANP secretion (D) in beating rabbit atria. Values are mean ± SE (n = 4); ***P < 0.001 versus control; ****P < 0.001 versus diltiazem, or verapamil (compared with values of the last 3 fractions of control, diltiazem, or verapamil).
not caused by the pathway mediated by L-type Ca\textsuperscript{2+} channels and/or protein kinases.

Stimulation of β-adrenergic receptors with a sympathomimetic agent induces positive inotropic effects that are dependent on PKs and L-type Ca\textsuperscript{2+} channels [2, 3]. Stimulation of β-adrenergic receptors results primarily in an increase in cAMP production and consequent activation of PKs and phosphorylation of L-type Ca\textsuperscript{2+} channels, thereby further increasing the channel open time and/or the probability of opening of functional Ca\textsuperscript{2+} channels [20]. Increase in the cAMP level by PDE III inhibitors also accentuates cardiac contractility via activation of PKs and L-type Ca\textsuperscript{2+} channels [4]. In accordance with our hypothesis, the ELA-induced positive inotropic effect was not altered by pretreatment with L-type Ca\textsuperscript{2+} channel blockers and a protein kinase inhibitor.

These findings suggest that the activities of sympathomimetics and PDE III inhibitors could be excluded from the possible mechanism of the ELA-induced positive inotropic effect.

We also determined the effects of helveticoside on atrial dynamics, cAMP efflux, and ANP secretion in beating rabbit atria. Helveticoside, the main constituent of ELA, is a well-known digitalis-like compound. Similar to digitalis, helveticoside markedly increased the pulse pressure and stroke volume, without increasing cAMP efflux, in beating rabbit atria. Helveticoside also increased pulse pressure and stroke volume in the staurosporine-pretreated atria. Furthermore, helveticoside induced positive inotropic activity in diltiazem- and verapamil-pretreated atria. In cAMP and ANP regulation, we cannot rule out other component’s possibility from ELA except for helveticoside. Thus, further study is needed to

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**Figure 7**: Effects of modulators on helveticoside-induced Δ% changes in pulse pressure, stroke volume, cAMP efflux, and ANP secretion. Data were derived from Figures 5 and 6. The values are Δ% changes of mean values of fraction number 29/30 over mean values of fraction number 17/18. Values are means ± SE. *P < 0.05 versus the mean values of two fractions before angles are changed (in modulators: staurosporine, diltiazem, and verapamil).
clarify the effect of linoleic acid or olein on the cAMP efflux and ANP secretion.

A previous report suggested that helveticoside could inhibit Na+/K+-ATPase activity in an in vitro enzyme assay [21]. In this study, we show that treatment with ELA markedly increased K+ concentration in beating atria-derived perfusate. Many lines of evidence have demonstrated that digitalis-like cardiac glycosides increase cardiac contractility by elevating intracellular Ca2+ concentration via Na+/K+-ATPase inhibition-mediated activation of Na+/Ca2+ exchanger [6, 22, 23]. In this case, the K+ efflux would be increased because K+ influx is inhibited due to the inhibition of Na+/K+-ATPase in the myocardium. In our study, ouabain markedly increased the pulse pressure and stroke volume without increasing the cAMP efflux in beating rabbit atria, resulting in a positive inotropic effect via inhibition of Na+/K+-ATPase activity. These findings suggest that the digitalis-like activity of helveticoside might be associated, at least in part, with ELA-induced positive inotropic activity.

The heart is also an endocrine gland, secreting ANP which is involved in the regulation of body fluid and blood pressure [11, 24]. The present study shows that ELA significantly decreased ANP secretion in beating rabbit atria. Diltiazem or verapamil, but not staurosporine, slightly increased ANP secretion in beating rabbit atria. The ELA-induced reduction in ANP secretion was not affected by pretreatment with diltiazem, verapamil, or staurosporine. The potential roles of cyclic nucleotide and Ca2+ in the regulation of ANP release have been the subject of interest of many studies. cGMP and Ca2+ inhibit ANP secretion in perfused atria [25]. On the other hand, cAMP increases the ANP secretion in rat cardiomyocytes [26], isolated atrium [27], and perfused rat atria [28]. However, there are diverse reports on the effects of cAMP in the regulation of ANP secretion. Forskolin, an adenylyl cyclase activator, has been shown to decrease ANP secretion from cultured atrial myocytes [29, 30] and in perfuse beating rat hearts [31]. Likewise, 3-isobutyl-1-methylxanthine (IBMX) and 8-bromoadenosine 3′,5′-cyclic monophosphate (8-BrcAMP), a nonselective PDE inhibitor and a cAMP agonist, respectively, inhibit ANP secretion [30, 31]. Collectively, ELA significantly decreased the ANP secretion associated with increase in cAMP efflux in beating rabbit atria, consistent with other reports [26, 30, 31].

Taken together, the present study suggests that the ELA-induced positive inotropic activity may, at least in part, be due to inhibition of Na+/K+-ATPase activity by helveticoside-like cardiac glycosides.

**Conflict of Interests**

The authors report that they have no conflict of interests.

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**References**


