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Immunotherapeutic effects of Ninjin-youei-to on patients with multiple myeloma

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ABSTRACT

The pathology of multiple myeloma (MM) includes the involvement of chemokines, cytokines and growth factors secreted by MM and other cells. Vascular endothelial cells and/or the hemostatic-coagulatory system are thought to play an important role in the development of MM. Ninjin-youei-to (NYT) is a traditional Chinese medicine made from the extracts of 12 plants that affects immunity and inflammation. The effects of NYT on plasma concentrations of various biomarkers were therefore investigated in MM patients. The plasma concentrations of RANTES, sVCAM-1, sE-selectin, Ang-2, VEGF, PAI-1 and PDMP were higher in MM patients than in healthy controls. Melphalan-prednisone (MP) treatment of MM patients for 6 months significantly decreased plasma concentrations of RANTES, sE-selectin, PAI-1 and PDMP, but did not significantly alter plasma concentrations of IL-6, TNF, MCP-1, sVCAM-1, Ang-2 and VEGF. Although treatment with NYT alone did not significantly alter the concentrations of any biomarker, it enhanced the MP-associated reduction of many biomarkers, as well as significantly reduced the concentrations of some markers, such as sVCAM-1, Ang-2 and VEGF, not reduced by MP alone. Furthermore, MP plus NYT significantly improved immunoglobulin concentrations compared with administration of MP alone, and regimens that included NYT significantly improved general fatigue. These results suggest that NYT possess an immunotherapeutic effect or can enhance the effects of MP in patients with MM.

KEYWORDS: multiple myeloma, Ninjin-youei-to, chemokine, endothelial cell, platelet-derived microparticle

INTRODUCTION

Multiple myeloma (MM) is an incurable malignancy of the plasma cells [1]. The majority of MM patients relapse, regardless of their initial treatment [1]. Melphalan-prednisone (MP) has long been the treatment of choice for MM patients over 65 years of age [2]. Although MP plus either thalidomide or bortezomib has been reported to enhance progression-free survival and overall survival, compared with MP alone [3, 4], these combination regimens are accompanied by side effects, including general fatigue, which have a negative effect on patient quality of life (QOL).

Being a tumor of plasma cells at the end stage of stem cell differentiation, MM is characterized by the high production and secretion of cytokines and growth factors [5]. The pathologic characteristics of individual patients therefore differ, depending on the products secreted by MM cells. In addition to secreted cytokines, chemokines and growth factors, vascular endothelial cells and/or the hemostatic-coagulatory system are thought to play

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an important role in the clinical course of MM [5, 6].

Ninjin-youei-to (NYT), a traditional Chinese medicine, is a drug consisting of a spray dried powder of hot water extracts from 12 species of medical plants [7]. Oral NYT has been used to treat fatigue, hypothermia, loss of appetite and anemia, as well as to affect immunity and inflammation [8-10]. However, the effects of NYT on patients with MM and on the plasma concentrations of cytokines, chemokines, soluble factors and coagulatory markers are poorly understood. This study was designed to analyze the effects of NYT treatment, with or without MP, on the plasma levels of various biomarkers in MM patients.

MATERIALS AND METHODS

Patients

The study group consisted of 47 patients newly diagnosed at our hospitals with MM, defined in accordance with the Guidelines for Diagnosis and Treatment of MM in Adults [11], between May 2010 and September 2013 and with symptomatic, measurable disease. As a control group, 30 healthy volunteers were recruited from the hospital staff and other sources. The study protocol was approved by the Institutional Review Board (IRB) of our institutions and written informed consent was obtained from each patient prior to the start of the trial.

Study design

A total of 31 patients were assigned to receive MP (n = 8), MP plus NYT (n = 12), or NYT (n = 11) (Fig. 1). The MP regimen consisted of six 28-day cycles of melphalan (0.18 mg/kg body weight on days 1 through 4) and prednisone (2 mg/kg body weight on days 1 through 4). NYT was administered orally at a dose of 5.0 g/day on days 1 through 28 of each cycle. Treatment was continued until disease progression or the development of unacceptable adverse effects. The primary end point of the study was an improvement in various biomarkers; secondary end points included response rate, response quality and adverse events.

Plasma levels of platelet-derived microparticle (PDMP), cytokines/chemokines, soluble factors, and PAI-1

Fasting blood samples were obtained from the peripheral veins of patients and controls into vacutainers containing ethylene-diamine tetraacetic acid and acid-citrate-dextrose (EDTA-ACD) (NIPRO Co. Ltd., Osaka, Japan) using 21-gauge needles to minimize platelet activation. The samples were gently mixed by inverting the tubes once or twice and kept at room temperature for a maximum of 2-3 hours.

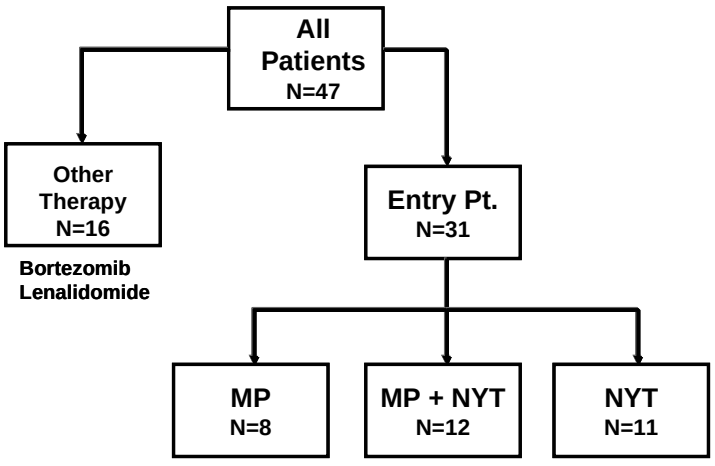

Fig. 1. Randomization and follow-up of the patients included in the trial.
A total of 47 patients underwent randomization; 16 patients were assigned to treatment with bortezomib or lenalidomide, and 31 were treated with MP and/or NYT in the present trial.
MP: melphalan and predonisone; NYT: ninjin-youei-to.

Samples were centrifuged at 8,000 g for 5 minutes, and 200 μl from the top of each 2 ml upper layer were withdrawn to avoid contamination by platelets. These plasma samples were stored at -40 °C until analysis. PDMP was measured by enzyme-linked immunosorbent assay (ELISA; JIMRO Co. Ltd., Tokyo, Japan) [12, 13]. Plasma concentrations of sE-selectin, sVCAM-1, IL-6, TNF, RANTES, MCP-1, Ang-2, VEGF and PAI-1 were measured using monoclonal antibody-based ELISA kits (Invitrogen International Inc., Camarillo, CA, USA). The recombinant products and standard solutions provided with the commercial kits were used as positive controls in each assay. All the kits were used in accordance with the manufacturer's instructions.

Statistical analysis

Data were expressed as the mean ± SD and analyzed using multiple regression analysis (stepwise method), as appropriate. Between-group comparisons were made using the Newman–Keuls test and Scheffe's test. The correlation between uric acid and after continuous-response variables was assessed using multivariate linear regression analysis. All statistical analyses were performed using StatFlex (ver. 6) software, with P-values less than 0.05 considered statistically significant.

RESULTS

The plasma concentrations of biomarkers in patients newly diagnosed with MM and in healthy controls are shown in Table 1. RANTES, sVCAM-1, sE-selectin, Ang-2, VEGF, PAI-1 and PDMP concentrations were higher in patients than in controls. Multivariate analysis showed that IgG, IgA, creatinine, sex, age, and hemoglobin were significantly associated with total protein concentrations in patients with MM (Table 2). In addition, several biomarkers related to coagulation or endothelial activation, including sE-selectin and PAI-1 were significant factors in the multivariate model (Table 2).

The demographic and baseline characteristics of the treated patients were similar among 3 groups (data not shown). Treatment with MP for 6 months significantly reduced the plasma concentrations of RANTES, sE-selectin, PAI-1 and PDMP, relative to baseline, but did not significantly alter the plasma concentrations of IL-6, TNF, MCP-1, sVCAM-1, Ang-2 and VEGF (Table 3). In contrast, treatment with NYT alone had no effect on any of the biomarkers tested (Table 4). The combination of MP and NYT resulted in further reductions in many biomarkers reduced by MP alone, as well as significantly reducing the plasma concentrations of several biomarkers

Table 1. Plasma levels of cytokines, chemokines and soluble factors in the controls and patients. Abbreviations: IL-6, interleukin-6; TNFα, tumor necrotizing factorα; MCP-1, monocyte chemotactant protein-1; RANTES, regulated on activation normally T-cell expressed and secreted; sVCAM-1, soluble vascular cell adhesion molecule-1; sE-selectin, soluble endothelial-selectin; Ang-2, angiopoietin-2; VEGF, vascular endothelial growth factor; PAI-1, plasminogen activator inhibitor; PDMP, platelet-derived microparticles. Data are shown as mean ± SD. P value: patients vs. controls. N.S.: not significant.

	controls	patients
N	**30**	**47**
IL-6 (pg/ml)	2.92 ± 1.12	3.86 ± 4.14 $^{N.S.}$
TNFα (pg/ml)	13.3 ± 10.1	23.1 ± 16.3 $^{N.S.}$
MCP-1 (pg/ml)	340 ± 59	411 ± 67 $^{N.S.}$
RANTES (ng/ml)	40.7 ± 10.3	98.0 ± 22.1 $^{P < 0.01}$
sVCAM-1 (ng/ml)	624 ± 214	1,836 ± 1,192 $^{P < 0.01}$
sE-selectin (ng/ml)	43.9 ± 7.3	97.8 ± 17.4 $^{P < 0.01}$
Ang-2 (pg/ml)	982 ± 213	2,065 ± 1,054 $^{P < 0.01}$
VEGF (pg/ml)	314 ± 75	650 ± 257 $^{P < 0.01}$
PAI-1 (ng/ml)	9.4 ± 2.3	36.4 ± 7.4 $^{P < 0.01}$
PDMP (U/ml)	8.1 ± 1.4	27.4 ± 4.2 $^{P < 0.01}$

Table 2. Multiregression analysis of TP, sE-selectin and PAI-1 in myeloma patients. This analysis was done by stepwise regression. Abbreviations: CRTN, creatinine; HB, hemoglobin. Other abbreviations: see Table 1. β: standardized regression coefficient.

Variable	β	SE(β)	std β	t value	P value
Objective Variable: TP (N = 47)					
IgG	0.00081	0.00005	0.9038	15.7933	0.0000
IgA	0.00072	0.00008	0.5923	9.55294	0.0000
CRTN	0.50831	0.04921	0.6396	10.3286	0.0000
Sex	-0.4965	0.11241	-0.3000	4.41731	0.0001
Age	-0.0205	0.00452	-0.2416	4.52989	0.0001
Hb	0.12571	0.02461	0.3062	5.10807	0.0000
Objective Variable: sE-selectin (N = 47)					
PDMP	3.09288	0.44954	0.7422	6.88004	0.0000
sVCAM-1	0.00521	0.00157	0.3578	3.31009	0.0020
PAI-1	-0.5728	0.24276	-0.2440	2.35953	0.0233
IgG	0.00390	0.00176	0.2084	2.21558	0.0325
Hb	-1.8164	0.82788	-0.2154	2.19408	0.0341
Objective Variable: PAI-1 (N = 47)					
RANTES	0.11693	0.03888	0.3493	3.00727	0.0044
sVCAM-1	0.00304	0.00080	0.4892	3.80221	0.0005
MCP-1	-0.0286	0.01389	-0.2583	2.05629	0.0460

Table 3. Changes in the plasma levels of PDMP, soluble factors, and cytokine/chemokines before and after MP treatment in myeloma patients. Abbreviations: see Table 1. 3M, 3 months after therapy; 6M, 6 months after therapy. Data are shown as mean ± SD. P value: before vs. 3 or 6 months; N.S.: not significant.

	Before	3M	6M
N = 8			
IL-6 (pg/ml)	4.63 ± 7.30	6.09 ± 12.0 $^{N.S.}$	6.93 ± 12.1 $^{N.S.}$
TNFα (pg/ml)	26.1 ± 19.7	29.5 ± 18.6 $^{N.S.}$	27.4 ± 17.1 $^{N.S.}$
MCP-1 (pg/ml)	391 ± 60	377 ± 59 $^{N.S.}$	385 ± 38 $^{N.S.}$
RANTES (ng/ml)	83.6 ± 11.8	81.3 ± 15.9 $^{N.S.}$	75.3 ± 8.3 $^{P < 0.05}$
sVCAM-1 (ng/ml)	2,069 ± 1,605	1,940 ± 1,201 $^{N.S.}$	1,871 ± 846 $^{N.S.}$
sE-selectin (ng/ml)	100.5 ± 23.5	92.8 ± 16.2 $^{N.S.}$	86.1 ± 16.8 $^{P < 0.05}$
Ang-2 (pg/ml)	2,022 ± 989	1,879 ± 735 $^{N.S.}$	1,831 ± 784 $^{N.S.}$
VEGF (pg/ml)	557 ± 336	570 ± 324 $^{N.S.}$	581 ± 245 $^{N.S.}$
PAI-1 (ng/ml)	38.1 ± 5.1	33.6 ± 5.1 $^{N.S.}$	28.5 ± 6.6 $^{P < 0.05}$
PDMP (U/ml)	27.6 ± 6.2	24.6 ± 7.3 $^{N.S.}$	22.3 ± 5.2 $^{P < 0.05}$

not significantly altered by MP alone (Table 5). Testing of immunoglobulin concentrations before and after treatment showed no significant differences between patients treated with MP alone and NYT alone (Fig. 2). However, the combination of MP plus NYT significantly enhanced the decrease of immunoglobulin concentrations compared with MP alone (Fig. 2).

Table 4. Changes in the plasma levels of PDMP, soluble factors, and cytokine/chemokines before and after ninjin-youei-to treatment in myeloma patients. Abbreviations: see Table 1. Data are shown as mean ± SD. P value: before vs. 3 or 6 months; N.S.: not significant.

	Before	3M	6M
N = 11			
IL-6 (pg/ml)	2.83 ± 1.7	2.50 ± 1.09 N.S.	2.75 ± 1.8 N.S.
TNFα (pg/ml)	23.8 ± 14.9	24.1 ± 12.9 N.S.	23.4 ± 17.5 N.S.
MCP-1 (pg/ml)	410 ± 52	416 ± 17 N.S.	400 ± 28 N.S.
RANTES (ng/ml)	85.3 ± 14.7	83.8 ± 8.8 N.S.	84.2 ± 9.1 N.S.
sVCAM-1 (ng/ml)	1,402 ± 542	1,370 ± 597 N.S.	1,329 ± 536 N.S.
sE-selectin (ng/ml)	97.7 ± 14.0	96.3 ± 11.6 N.S.	94.4 ± 12.8 N.S.
Ang-2 (pg/ml)	1,842 ± 776	1,861 ± 683 N.S.	1,817 ± 626 N.S.
VEGF (pg/ml)	605 ± 317	639 ± 242 N.S.	641 ± 234 N.S.
PAI-1 (ng/ml)	30.6 ± 9.8	31.8 ± 5.7 N.S.	31.5 ± 6.4 N.S.
PDMP (U/ml)	27.1 ± 3.6	24.8 ± 3.7 N.S.	24.4 ± 3.9 N.S.

Table 5. Changes in the plasma levels of PDMP, soluble factors, and cytokine/chemokines before and after MP plus ninjin-yoiei-to treatment in myeloma patients. Abbreviations: see Table 1. Data are shown as mean ± SD. P value: before vs. 3 or 6 months; N.S.: not significant.

	Before	3M	6M
N = 12			
IL-6 (pg/ml)	3.79 ± 4.30	3.08 ± 2.25 N.S.	2.54 ± 0.64 N.S.
TNF α (pg/ml)	26.5 ± 19.3	24.3 ± 16.2 N.S.	23.9 ± 17.4 N.S.
MCP-1 (pg/ml)	433 ± 99	398 ± 88 N.S.	390 ± 81 N.S.
RANTES (ng/ml)	110.1 ± 20.1	94.3 ± 17.6 P < 0.05	83.5 ± 12.8 P < 0.01
sVCAM-1 (ng/ml)	2,557 ± 1,220	1,780 ± 654 P < 0.05	1,593 ± 686 P < 0.01
sE-selectin (ng/ml)	104.5 ± 19.2	93.1 ± 15.0 P < 0.01	86.8 ± 14.4 P < 0.01
Ang-2 (pg/ml)	2,246 ± 800	2,031 ± 712 P < 0.05	1,906 ± 660 P < 0.05
VEGF (pg/ml)	703 ± 187	622 ± 157 P < 0.01	576 ± 146 P < 0.01
PAI-1 (ng/ml)	41.3 ± 2.0	29.6 ± 6.0 P < 0.01	23.3 ± 4.1 P < 0.01
PDMP (U/ml)	28.5 ± 3.6	21.0 ± 3.5 P < 0.01	18.1 ± 5.1 P < 0.01

Assessment of subjective symptoms in these patients showed that general fatigue was significantly reduced in patients receiving NYT and MP+NYT than in those receiving MP alone (Fig. 3). Pain symptoms, however, did differ significantly among the three patient groups (Fig. 3).

DISCUSSION

Bleeding and thrombosis are complications in patients with hematologic malignancies, with epidemiological, clinical and pathophysiologic significance [14, 15]. Several disease- and treatment-related factors have been found to affect the coagulation system, as well as

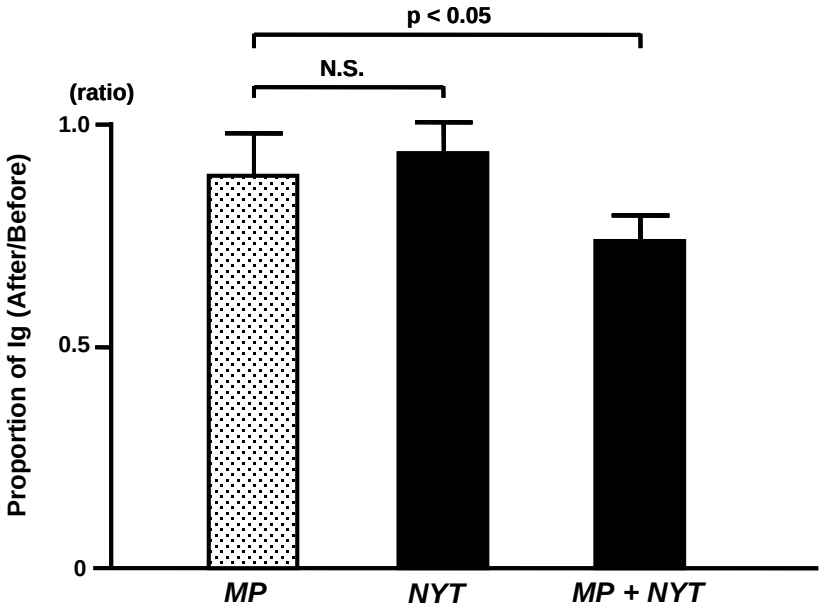

Fig. 2. Immunoglobulin concentrations before and after treatment. MP: melphalan and predonisone; NYT: ninjin-youei-to. P value: MP vs. NYT or MP+NYT; N.S.: not significant.

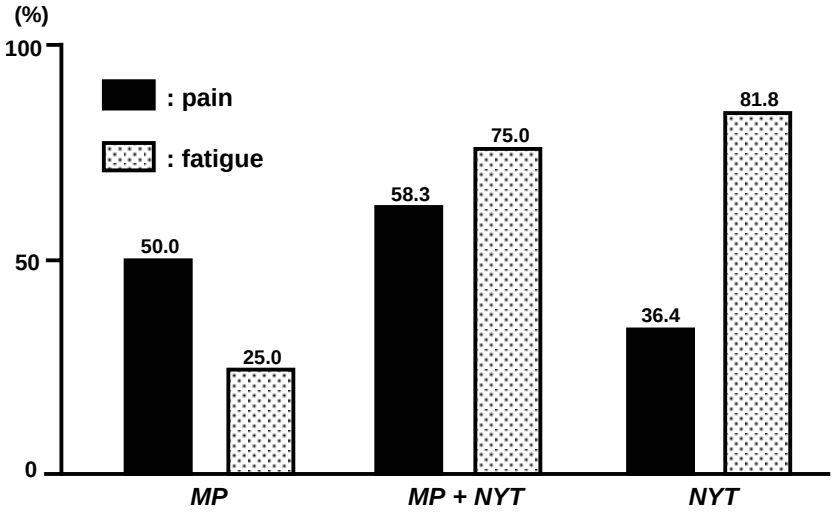

Fig. 3. Improved frequencies of subjective symptoms in these patients. MP: melphalan and predonisone; NYT: ninjin-youei-to.

the risks of bleeding and thrombotic complications in patients with MM [6, 16, 17]. Similar to findings in other cancers, malignant clones in patients with MM induce a cytokine environment responsible for a hypercoagulable state [14]. Circulating monoclonal proteins increase blood viscosity and impair platelet and coagulation function, which are considered key mechanisms in the hemostatic abnormalities frequently detected in patients with MM [6]. This study assessed the plasma concentrations of several biomarkers of hemostasis, coagulation and endothelial dysfunction in patients newly diagnosed with MM. We found that the concentrations of RANTES, sVCAM-1, sE-selectin, Ang-2, VEGF, PAI-1 and

PDMP were higher in MM patients than in healthy controls. A multivariate analysis showed that, among the cohort of patients newly diagnosed with MM, IgG, IgA, creatinine, sex, age, and hemoglobin were significantly associated with total protein levels. In addition, sE-selectin, PAI-1 and VEGF, all of which are biomarkers related to coagulation and endothelial activation, were significant factors in our multivariate model. These results suggest that patients with MM are likely to have coagulation- and/or endothelial cell activation-related risk factors for coagulation abnormalities.

Melphalan is one of the drugs most frequently used to treat MM, and is administered with predonisone [18]. MP is also administered for hematopoietic stem cell support and when new drugs, such as bortezomib and lenalidomide, are used in treatment [19-21]. In particular, MP plus bortezomib has been reported to improve progression-free survival and overall survival when compared with MP alone [3, 4, 21, 22].

Most elderly patients are considered unsuitable for stem cell transplantation, because high dose melphalan is strongly toxic in these patients. Therefore, MP is considered as standard therapy in elderly people. This study therefore examined the relevance of various biomarkers in the treatment for MM. Treatment with MP for 6 months significantly reduced the plasma concentrations of RANTES, sE-selectin, PAI-1 and PDMP, but had no effect on the plasma concentrations of IL-6, TNF, MCP-1, sVCAM-1, Ang-2 and VEGF. Although treatment with NYT alone for 6 months did not significantly alter the concentrations of any of the biomarkers tested, the combination of MP and NYT further reduced the concentrations of several biomarkers reduced by MP, as well as significantly reducing the concentrations of several biomarkers unchanged by MP. These results suggest that NYT can enhance the effects of MP on various biomarkers. Furthermore, MP plus NYT significantly improved immunoglobulin concentrations compared with MP alone, and regimens that included NYT significantly reduced general fatigue.

The precise mechanism by which NYT enhances the effects of MP on various biomarkers is as yet undetermined, although natural killer (NK) cells are likely involved. NK cells can induce spontaneous "natural" cytotoxicity in tumor cells deficient in major histocompatibility complex class I [23, 24]. *In vitro*, both autologous and allogeneic NK cells have been found to effectively eliminate MM cells [25, 26]. In addition, immunomodulatory drugs such as thalidomide and lenalidomide can broadly stimulate the functions of NK cells to treat cancer, including MM [27, 28]. NYT was shown to enhance the effects of NK cell activity in elderly patients with lung carcinoma, as well as inhibiting the reduction of NK activity during chemotherapy treatment of patients with malignant glioma [9, 29, 30]. In particular, NYT can maintain and reinforce the immune surveillance system, a mechanism that may protect against carcinogenesis [9]. Although our findings support this hypothesis, we could not confirm a direct connection between NYT enhancement of MP effects and NK cell activity. Additional studies are required to elucidate the mechanism by which NYT enhances the effects of MP therapy in patients with MM.

CONCLUSION

The effects of NYT on plasma concentrations of various biomarkers were investigated in MM patients. NYT alone did not significantly alter the concentrations of all the examined biomarkers. However, it enhanced the MP-associated reduction of many biomarkers. Furthermore, MP plus NYT significantly improved immunoglobulin concentrations compared with administration of MP alone, and regimens that included NYT significantly improved general fatigue. These results suggest that NYT possess an immunotherapeutic effect or can enhance the effects of MP in patients with MM.

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CONFLICT OF INTEREST STATEMENT

The authors do not have any conflicts of interest to report in this work.

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