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Effect of Multi-berries Drink on Endogenous Antioxidant Activity in Subjects Who Are Regular Smokers or Drinkers

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Abstract The multi-berries drink (MBD) has been demonstrated to possess high total polyphenol content and antioxidant activity measuring by ORAC and CAA assays. The influence of MBD supplement on endogenous antioxidant activity in healthy subjects was evaluated in this study. Twenty adults who smoke or drink alcohol regularly were allocated to MBD (100 mL/d, n = 10) or the control (placebo, 100 mL/d, n = 10) group for a 90-day, randomized, double-blind, placebo-controlled study. The results showed that MBD supplementation significantly increased glutathione (GSH), the activities of glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rd), superoxide dismutase (SOD) and decreased thiobarbituric acid reactive substances (TBARS) compared with the placebo. Additionally, hepatic and renal functions were not adversely altered in MBD-ingested subjects. It is suggested that MBD effectively promotes the endogenous antioxidant activity, and can be consumed as a functional supplement for people who are in danger of suffering oxidative-related chronic diseases.

Keywords: *antioxidant activity, catalase, glutathione, maqui, muiti- berries, superoxide dismutase*

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1. Introduction

Reactive oxygen species (ROS), a group of highly reactive molecules including free radicals and peroxides, generates in the body by several mechanisms involving both endogenous (mitochondrial leak and respiratory burst) and exogenous factors (cigarette smoke, UV lights, ionizing radiation, chemicals or air pollutants) [1]. When the generation of ROS exceeds the antioxidant capacity of a living system, oxidative stress is produced and associated with damage of vital biomolecules including DNA, RNA, lipids, protein and carbohydrates leading to tissue or cellular injury [2,3,4]. A stressful lifestyle, environmental pollutants and declined antioxidant defense capacity as age increased accelerate the pro-oxidant-antioxidation imbalance in human body giving rise to the development of various chronic and degenerative diseases such as cancers, diabetes, inflammation, cardiovascular disease (atherosclerosis), and aging [3,5,6]. In addition, oxidative stress has been demonstrated to be correlated with the pathogenesis of age-related liver diseases, liver fibrogenic response, viral and alcoholic liver diseases [7,8,9]. Oxidative stress also increases in the liver at the beginning stage of many diseases, such as diabetes [10,11]. Under normal physiological conditions, the intrinsic antioxidant defense systems including superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rd), catalase (CAT) and nonenzymatic compound, reduced glutathione (GSH), protect living organisms from the attack of ROS to maintain a dynamic balance and reduce cellular damage [1,12]. The diseased liver can be revealed by direct measurement of the level of oxidative agents. The catalytic mechanism of SOD is known to involve in the dismutation of superoxide anion into oxygen and hydrogen peroxide [13], which is further removed by GSH-Px or CAT. GSH, a non-protein antioxidants, constitutes the glutathione system in the cell together with GSH-Px and GSH-Rd [14]. GSH-Px, widely distributed in almost all tissues, catalyzes the oxidation of GSH to oxidized glutathione (GSSG) accompanying with the reduction of a hydroperoxide such as hydrogen peroxide or lipid hydroperoxide [15]. GSH-Px is also the main scavenger of hydrogen peroxide in the cytosol and mitochondria, and its activity is dependent on the constant availability of GSH [16]. Meanwhile, the oxidation of GSH is reversed by the action of GSH-Rd to keep a high ratio of GSH to GSSG [17], which is important to maintain sulfhydryl-dependent enzymes in the active state [18]. GSH levels appear to decline due to aging and cause an imbalance in the antioxidant enzymes system in a number of tissues, thereby putting cells at increased risk of oxidative stress [19,20]. Treatment with antioxidants can protect against oxidation of the glutathione pool. On the other hand, malondialdehyde (MDA) generated from lipid

hydroperoxides by the hydrolytic conditions of the reaction is considered as a marker of oxidative damage to lipids (lipid peroxidation) [21]. Patients with alcoholic liver disease or chronic viral hepatitis showed a significant decline in GSH level and the ratio between GSH and GSSG, and a rise in MDA level [22,23].

Almost all organisms possess antioxidant defense and repair systems, but these systems are insufficient to prevent the damage entirely. For this reason, dietary supplementation is necessary to strengthen the intrinsic protection systems. Many researches reveal that food with high antioxidant capacity may help to prevent cellular damaged by oxidative stress [11,12,24,25]. The antioxidant effects exerted by vegetables and fruits have attracted substantial attention [26,27,28]. Fruits contain higher quantity and quality of phenolic antioxidants than vegetables, and present higher antioxidant activity than many isolated pure phenolic compounds [27,28,29]. It may come from the synergistic effect of the active compounds in the fruit extract. Especially, berries which are rich in phenolic compounds possess antioxidant activity, and can inhibit cell damage causing by oxidation, which led to several pathological conditions [27,30,31]. Maqui (*Aristotelia chilensis*), a high-anthocyanin-content berry, possesses antioxidation, cardioprotection, anti-diabetes effects and *in vitro* inhibition of adipogenesis and inflammation [24,32,33,34,35]. Consumption of the Amazonian fruit açaí (*Euterpe oleracea* Mart.) rich in phytochemicals especially polyphenols and flavonoids increases *in vitro* and *in vivo* antioxidation, anti-inflammation, anti-aging, anti-nociceptive capacity and decreases lipid oxidation [36,37,38,39,40,41]. Comparatively, the maqui-based drink displayed higher total phenolic content and *in vitro* antioxidant capacity ($ABTS^{+}$, $DPPH^{\bullet}$ and superoxide scavenging assays) than açaí-based one [42]. Other berries with long-term consumption history such as cranberry, raspberry, blackberry, blueberry and strawberry have demonstrated to perform several bio-functions such as antioxidation, anti-cancer effects and protection against LDL oxidation [43,44,45,46]. Blended juice, juice concentrates and smoothies are considered as health-supporting foods preventing from radical-related chronic diseases [47]. What's more, several studies have interpreted the modulation effects of berries on intrinsic antioxidant systems. Addition of açaí pulp to the rats' diet significantly reduces hepatic MDA levels and increased total hepatic GSH content and GSH-Px gene expression [11]. Goji berry is effective in preventing oxidative stress after exhaustive exercise by elevating SOD and GSH-Px levels and decreasing MDA levels in rats' muscle [48]. Raspberry supplementation is found to increase GSH-Px activities in humans [49].

Berries such as maqui, açaí, camu camu, blackthorn, wolfberry, elderberry, bilberry and chokeberry have been utilized in making blended juices, however, studies for these berries or their blends on antioxidant activity are mostly *in vitro* or by animal experimentation [11,31,37,48,50,51,52]. Thus, the aim of this study was to investigate the effect of multi-berries drink (MBD) ingestion to subjects who are regular smokers or alcohol drinkers on endogenous antioxidant activity after 90 days consumption.

2. Materials and Methods

2.1. Subjects

There were 24 subjects recruited. A total of 20 healthy subjects were included in the study through inclusion/exclusion criteria (Table 1). They were randomly allocated into MBD (n = 10) and control (placebo, n = 10) group. The anthropometric characteristics of the subjects were summarized in Table 2.

Table 1. Inclusion and Exclusion Criteria

Criteria	Details
Inclusion	Adults ages 20–40 years Body mass index (BMI) ≤ 30 Systolic blood pressure (SBP) < 140 mmHg Diastolic blood pressure (DBP) < 90 mmHg Fasting blood glucose (GLU) < 100 mg/dL Blood urea nitrogen (BUN) 7–20 mg/dL Aspartate transaminase (AST) 5–40 IU/L Alanine transaminase (ALT) 5–40 IU/L Smoke regularly (≥ 10 cigarettes / day) or ingest alcohol drink contained more than 30 g alcohol (about 600 mL of beer, 250 mL red wine or 75 mL liquor) per day at least six months Write informed consent to participate in the trial Willingness to complete standard health history questionnaire before recruitment into the study Willingness to change food supplement habits to follow the full treatment procedure Willingness to try new food supplements Available for the entire study duration (90 days),
Exclusion	On a long term medication History of chronic diseases associated with heart, liver, kidney or blood systems Consumption other food supplements or any treatments (medicinal, hormonal, dermatological) with biological effects in the past 2 months Unwilling to change food supplement habits during the study Negative feeling about food supplements, functional food or drinks Pregnancy or breast-feeding a child

Table 2. Anthropometric Characteristics of the Subjects

Parameter	Age (yr)	Height (cm)	Weight (kg)	BMI (kg/m^2)	Gender (F/M)
MBD	27.7 ± 5.2	169.4 ± 3.8	75.3 ± 11.2	26.2 ± 3.9	2 / 8
Control	22.9 ± 1.9	175.6 ± 3.7	71.2 ± 11.9	23.1 ± 3.17	2/ 8

2.2. Sample Preparation

MBD (Maqui Plus-Multi fruits & berries concentrate, Beyonde™) contained 12 fruit concentrates including maqui berry, açaí, artichoke (*Cynara scolymus*), *Lycium barbarum* (goji berry), acerola (*Malpighia glabra*), raspberry (*Rubus idaeus*), red grape (*Vitis vinifera* L.) and grape seed extract, chokeberry (aronia, *Aronia melanocarpa*), cranberry (*Vaccinium macrocarpon*), apple, strawberry and cherry was provided by Unilever Thai Trading Ltd., Bangkok, Thailand. Total polyphenol (TP) [53], total anthocyanin (TA) [54] content, *in vitro* antioxidant activity by using ORAC (Oxygen Radical Absorbance Capacity) assay [55,56,57,58,59] and the CAA (Cellular Antioxidant Activity) without a PBS wash [60] of MBD have been measured. The placebo, which is purple-colored de-ionized water with 0.1% ColorFruit® Magenta 109 WS (Chr. Hansen, Hørsholm, Denmark), has similar appearance with MBD.

2.3. Study Design

This study took place in National Pingtung University of Science and Technology and has received the certificate of approval from Antai Medical Care Cooperation Antai-Tian-Sheng Memorial Hospital Institutional Review Board (IRB) with the TSMH IRB No. 13-044-A2, and has been undertaken according to the Helsinki Declaration. A randomized, double-blind, placebo-controlled trial was conducted. The subjects ingested 50 mL of MBD or placebo twice a day (before lunch and dinner) for a period of 90 days. All of the subjects in both groups completed the trial. The fasting blood of each subject was drawn and measured the serum biochemical and antioxidant activity-related parameters on day 0, 30 and 90 of the trial. The body weight and blood pressure (systolic blood pressure [SBP] and diastolic blood pressure [DBP]) were also monitored throughout the experiment.

2.4. Serum Biochemical and Oxidative Stress-Related Parameters

The collected blood samples were centrifuged at 3000 rpm for 10 min at 4°C to obtain the serum. The serum samples were stored at –70°C for the following analysis. Levels of serum biochemical parameters including fasting blood glucose (GLU), blood urine nitrogen (BUN), creatinine (Cr), aspartate transaminase (AST), alanine transaminase (ALT), total cholesterol (TC), and triglyceride (TG) were determined. The concentration of GSH, thiobarbituric acid reactive substances (TBARS) and the activities of antioxidant enzymes (SOD, GSH-Px and GSH-Rd) were measured by using commercially available assays, which were the GSH assay kit (#703002, Cayman Chemical Company, Ann Arbor, MI), the NWLSS™ MDA assay kit (#NWK-MDA01, Northwest Life Science Specialties, LLC., Vancouver, WA), the SOD assay kit (#706002, Cayman Chemical Company, Ann Arbor, MI), GSH-Px assay kit (#703102, Cayman Chemical Company, Ann Arbor, MI) and GSH-Rd assay kit (#703202, Cayman Chemical Company, Ann Arbor, MI), respectively. The assays were performed according to the manufacturer's instructions.

2.5. Statistical Analysis

Descriptive data were generated for all variables and expressed as mean ± standard deviation (SD). 2 (groups) × 3 (times) analysis of variance (ANOVA) was performed using the Statistical Product and Service Solutions (SPSS® 14.0; SPSS Inc., Chicago, IL) to determine the significance of treatment, followed by a LSD post hoc test. Statistical significance in the present study was set at p value < 0.05.

3. Results

MBD contains TP 5.682 mg/mL and TA 0.3 mg/mL. The ORAC values of MBD against free radicals which are singlet oxygen, hydroxyl radicals, superoxide anion, peroxyl radicals and peroxynitrite were 126.46, 80.40, 70.48, 51.47 and 3.49 μmole Trolox/mL, respectively (totally 332.30 μmole Trolox/mL). In addition, MBD possessed a CAA value of 19.37 μmole quercetin equivalents (QE)/mL.

On the other hand, mean body weight on day 90 of MBD or the control group was not significantly different compared with the baseline (on day 0). Blood pressure of both groups remained in the normal range throughout the trial (data not shown). The serum biochemical parameters were presented in Table 3. The baseline of GLU, BUN, Cr, AST, ALT, TC and TG showed no significant difference between MBD and the control groups (p > 0.05). After administrating for 30 and 90 days, except Cr, other parameters showed no significant difference between two groups. Though Cr levels of MBD group on day 30 and 90 were significantly lower than those of the control group, there was not significantly changed when comparing baseline, day 30 and day 90 in MBD group.

Table 3. Serum Biochemical Profiles of the Control and Multi-berries Drink (MBD) Group on Day 0, 30 and 90 of the Study

	Control			MBD		
	0	30	90	0	30	90
GLU (mg/dL)	97.20 ± 9.32	102.60 ± 4.12	106.20 ± 7.04	98.80 ± 8.42	103.70 ± 6.62	105.60 ± 6.67
BUN (mg/dL)	14.71 ± 2.63	13.51 ± 3.41	12.53 ± 2.75	13.45 ± 1.76	13.17 ± 2.31	10.80 ± 2.63
Cr (mg/dL)	1.26 ± 0.12	1.21 ± 0.07	1.23 ± 0.07	1.16 ± 0.15	1.08 ± 0.10*	1.08 ± 0.10*
AST (U/L)	26.30 ± 4.24	25.00 ± 2.67	15.70 ± 6.09	30.20 ± 7.21	21.60 ± 6.77	16.30 ± 5.52
ALT (U/L)	31.00 ± 6.70	27.70 ± 10.01	25.80 ± 19.58	31.50 ± 11.86	29.20 ± 10.91	26.00 ± 11.49
TC (mg/dL)	182.30 ± 14.56	172.70 ± 12.94	172.50 ± 19.60	186.90 ± 14.19	162.10 ± 24.76	174.80 ± 16.53
TG (mg/dL)	81.20 ± 13.92	83.40 ± 10.85	72.30 ± 11.48	99.20 ± 35.40	84.90 ± 24.52	81.20 ± 32.04

All values represent mean ± SD (n = 10).
*The asterisk symbol indicated the significant difference between the control and multi-berries drink (MBD) group at the same time (p < 0.05).

Furthermore, the results of oxidative stress-related parameters in Figure 1 showed that GSH ($p < 0.001$), GSH-Px ($p = 0.004$), GSH-Rd ($p < 0.001$) and SOD ($p < 0.001$) significantly increased 34.86%, 15.50%, 108.41%, and 70.78%, respectively, whilst TBARS ($p < 0.001$) significantly decreased 30.60% for MBD group when compared with those of the control on day 90. In addition, all these parameters of MBD group significantly improved with increasing treatment period, however, those of the control did not show the same results. Also, GSH and GSH-Rd had presented significant 20.33% and 25.14% elevation after 30-day MBD ingestion.

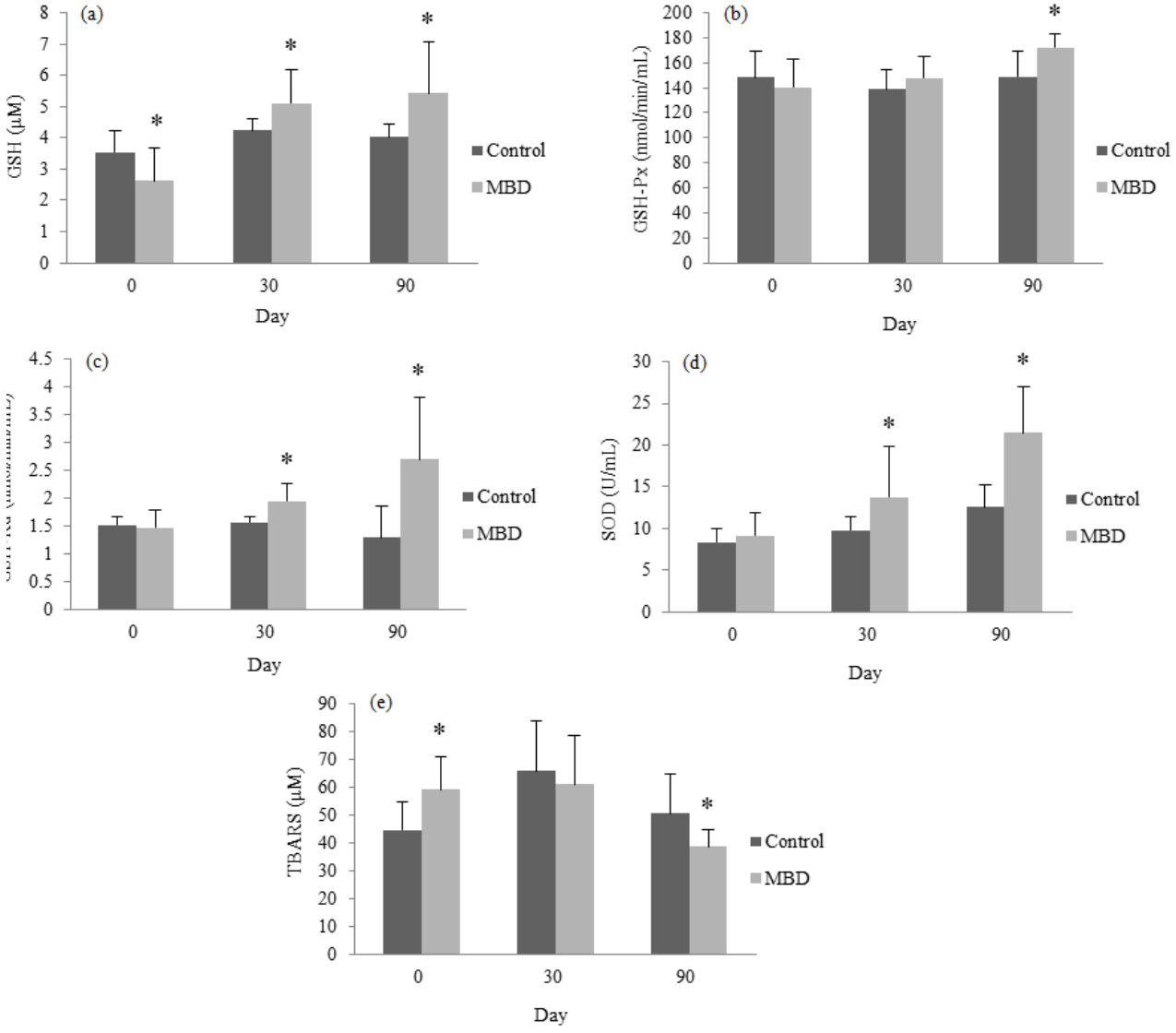

Figure 1. Oxidative stress-related parameters (a) GSH, (b) GSH-Px, (c) GSH-Rd, (d) SOD, (e) TBARS of the multi-berries drink (MBD) and the control group on day 0, 30 and 90 of the study. *The asterisk symbol indicated the significant difference between the control and MBD group at the same time ($p < 0.05$)

4. Discussion

Because ROS is relatively low in healthy nonsmokers, changes in biomarkers of antioxidant capacity and oxidative stress might be insufficient to measure [47,49]. However, functional effects would be achieved by prolonged consumption of the drink. The changes may be much likely to occur under acute or chronic oxidative stress conditions, for example, people who smoke or drink alcohol frequently. As a result, in present study, the influence of 90-day MBD ingestion on endogenous antioxidant activity was investigated for the subjects who are smoking or drinking alcohol regularly.

Berries have been interpreted their high content and wild diversity of phenolic compounds exerting high antioxidant activity [31]. MBD contains considerable phenolics and radical absorbance capacity against singlet oxygen, hydroxyl radicals, superoxide anion, peroxyl radicals and peroxynitrite. Additionally, the CAA values of berries and fruits, which indicate the antioxidative capacity within liver cells, were found to be within 0.09 and 1.71 μmole QE/g fruit [60], while MBD possessed a high CAA value of 19.37 μmole QE/mL indicating its potent cellular antioxidant capacity. These results demonstrate that MBD has a great potential to be consumed as an antioxidant drink.

After food consumption, the body naturally strictly regulates blood glucose levels as part of metabolic homeostasis. GLU is the important index of healthy metabolic function. In our study, though GLU of both groups was slightly increased at the end of the experiment, statistical analysis did not indicate any significant

difference between the two groups that meant the intake of MBD did not cause a serious change on the glucose metabolism. Similarly, Guerra *et al.* [11] reported that administration with 2% açaí pulp in rats did not affect the levels of glucose, insulin and body weight comparing with the controls. The elevated serum level of either BUN or Cr can be monitored to evaluate the renal dysfunction due to foods or drug administration [61]. From our results, there was no significant different in BUN between MBD and the control groups. MBD group had a slightly lower Cr relative to the control group due to the different baseline between those two groups. Among each group, BUN and Cr did not significantly changed throughout the trial implying that MBD has no adverse effect on kidneys. On the other hand, liver is a crucial organ participated in a variety of metabolic activities such as detoxification and oxidation. Its metabolites (e.g., AST and ALT), TC and TG were used as indicators for both hepatic and cardiovascular health [62,63]. AST, ALT, TC, and TG of the subjects consumed MBD for 90 days had no significant difference when comparing with the control group. These biomarkers of both groups before and after the trial were all within reference ranges for health adults. It is suggested MBD neither detrimentally affects the liver function nor induces the possibility of cardiovascular diseases.

In present study, though the baseline of GSH was significantly lower in MBD group than the control group, it was significantly elevated after MBD intake for 30 days, and the effect was lasting to the end of the experiment. GSH-Px, GSH-Rd and SOD also had significantly increment after ingesting MBD for 90, 30 and 90 days, respectively. The increased GSH-Rd after 30-day MBD intake might elucidate the increase of GSH, and subsequently inducing the latter elevation of GSH-Px and SOD. These results indicate the antioxidant enzymes boosting effect by MBD ingestion. On the other hand, assay of TBARS measures MDA present in the sample. TBARS of MBD group was significantly higher on day 0 and became no difference on day 30 relative to that of the control group. After consecutively 90-day consumption, MBD group presented significantly decreased TBARS compared with the control suggesting the suppression effect of MBD on lipid peroxidation. According to the previous studies, the antioxidant activity of berries might result from the high contents of phenolic compounds such as anthocyanin, flavonols and flavanols [27,64]. High positive correlations between the antioxidant capacity and the total phenolic content were observed for various berries and grapes [65,66].

The functional drink market has been increased for decades. Novel fruit/vegetable-based products are hugely launched on the market [47]. However, relevant clinical researches are relatively limited. Hence, the present study provides an available clinical evidence for the improvement of endogenous antioxidant activity in human by MBD ingestion.

5. Conclusions

After consuming MBD for 90 days, though minor changes were observed in the biochemical parameters, they were all within the normal ranges. It was revealed no adverse effect of MBD to the subjects. MBD consumption demonstrated the beneficial effects on promotion of the intrinsic antioxidant activity (GSH, GSH-Px, GSH-Rd and SOD) and suppression of lipid peroxidation as proven by TBARS reduction result. Therefore, MBD can be considered as an effective antioxidant supplement.

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Statement of Competing Interests

The authors have no competing interests.

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