FOOD
RESEARCH

Whey protein concentrate mixed beverages and plasma amino acid response in young males

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Abstract

Whey protein isolate (WPI) and whey protein hydrolysate (WPH) contains more protein than whey protein concentrate. However, to achieve a better taste, most commercial products mix WPI and WPH with whey protein concentrate (WPC). This study aimed to compare postprandial blood amino acids following ingestion of three beverages: WPI-mixed with WPC (WPI-mixed), WPH-mixed with WPI (WPH-mixed), and placebo. A total of ten healthy men aged between 18 and 30, who regularly engaged in exercise training, were recruited as participants. They each received 350 mL of each beverage on separate days and 6-day apart. Concentrations of plasma essential amino acids (EAA), branched-chain amino acids (BCAA), and leucine (Leu) were measured in blood samples taken from participants every 15 mins for 2 hrs. Plasma concentrations of EAA, BCAA, and Leu increased after ingestion of the WPI-mixed and WPH-mixed beverages versus the placebo beverage ($P<0.05$). Plasma EAA did not differ between WPI- mixed and WP-mixed trials. In contrast, plasma concentrations of BCAA after WPI-mixed were higher than after WPH-mixed at 30 mins. The same was observed for Leu at 30 and 75 mins after ingestion. The higher plasma BCAA and Leu in the WPI-mixed trial versus the WPH-mixed trial may have been due to the presence of short-chain peptides, as bioactive peptides, in WPH-mixed, which were absorbed. In conclusion, there was no difference in plasma EAA, but plasma concentrations of leucine and BCAA were higher in the WPI-mixed trial than WPH-mixed trial. Therefore, future research should investigate bioactive peptides along with plasma amino acids.

1. Introduction

Whey proteins (WP) are one of the two groups of proteins found in milk, which are removed when the casein proteins are coagulated to form cheese. WP is of interest as a source of high-quality protein with a complete amino acid (AA) profile. It contains the full range of AAs, including all the nine essential amino acids (EAAs) and branched-chain AAs (BCAAs), which are necessary for tissue growth and repair. The EAAs and BCAAs in WP are present in higher concentrations than in other protein sources such as soy, meat, and wheat; they are also efficiently absorbed and utilized (Gangurde *et al*., 2011). The benefits of WP were reported to lessen several risk factors for metabolic diseases (Sousa *et al*., 2012). Additionally, BCAA may

normalize serum amino acids, reduce proteolysis, increase protein synthesis, and improve patients' nutritional status after surgery (Wang *et al*., 2003). However, the primary reason for using WP as a supplement is to help improve muscle protein synthesis, in conjunction with resistance exercise, to gain more muscle.

There are three forms of commercially available WP. Differences in processing techniques from cow milk to WP give rise to each form's different biological efficacy. Those three forms are whey protein concentrate (WPC), whey protein isolate (WPI), and whey protein hydrolysate (WPH) (Patel, 2015). WPC is available in several protein concentrations, such as 34% (WPC34), 50% (WPC50), and 80% (WPC80). Concentrates contain

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a low level of fat and cholesterol but, in general, have higher levels of bioactive compounds and carbohydrates in the form of lactose. WPC can be found in a fluid, concentrate, or dry product form. Most WPC on the market contains about 80% protein (Gangurde *et al*., 2011). The second is WPI, the purest form of whey protein, which contains about 90% protein and 4–6% water. The remaining 4–6% is a combination of fat, lactose, and ash. The main proteins found in WPC and WPI are α-lactoglobulin, α-lactalbumin, glycomacropeptides, bovine serum albumin, immunoglobulins, lactoferrin, and lactoperoxidase (Foegeding *et al*., 2011).

The final form is WPH, which is made by utilizing protease-mediated hydrolysis of intact WP, and which has an amino-acid composition that is identical to intact WP. Hydrolysates are predigested, partially hydrolyzed whey proteins. This process breaks down protein chains into small fractions called peptides, which have structures that are different from those in the intact protein (Foegeding *et al*., 2011). Therefore, peptides may be absorbed slightly better and more quickly than amino acids or whole proteins. However, its cost is usually higher. Highly hydrolyzed whey may be less allergenic than other forms of whey. However, it is very bitter.

After luminal and brush-border peptidase digestion of proteins, only di- and tri-peptides are absorbed intact. In contrast, tetrapeptides and higher peptides require prior brush-border hydrolysis before their hydrolysis products can be absorbed. Protein hydrolysates that provide mainly di- and tri-peptides are superior to intact (whole) proteins and free amino acids in skeletal muscle protein anabolism (Manninen, 2009). Morifuji *et al.* (Morifuji *et al*., 2010) compared the effects of different sources and degrees of hydrolysis of dietary protein on plasma amino acid and dipeptide levels and insulin responses in humans. The results showed that WPH was absorbed more rapidly as plasma amino acids than was nonhydrolyzed protein. WP also produced more rapid increases in EAA and BCAA concentrations than soy protein. Besides, protein hydrolysates caused significant increases in Val-Leu and Ile-Leu concentrations compared to nonhydrolyzed protein. WPH also induced significantly greater stimulation of insulin release than the other proteins and also stimulated muscle protein synthesis (MPS) to a greater degree than soy after resistance exercise (Tang *et al*., 2009). These differences may be related to the rate of protein digestion (i.e., fast vs. slow) or possibly due to the small differences in each protein's leucine content. The results demonstrate that whey protein hydrolysates significantly increase plasma concentrations of amino acids, dipeptides, and insulin. Studies have shown that protein ingestion increases amino acids in the blood, stimulating muscle protein synthesis rates in healthy adults (Burd *et al*., 2015; Van Vliet *et al*., 2015; Gorissen and Witard, 2018). The postprandial increase in muscle protein synthesis rate is in response to the rise in circulating amino acids (Devries and Phillips, 2015).

WPC contains less protein than WPI, and WPH is the semi-digested form of the protein, which is absorbed slightly better and more quickly (Patel, 2015). Due to the cost and bitter taste, the most common forms of WP used in high protein bars, beverages, and supplements are WPC or WPI. In the market, WP products mostly contain mixed WPC or either WPI or WPH. There are studies of the effect of WPC, WPI, and WPH supplementation and postprandial blood amino acids. To our knowledge, no prior studies have examined mixed WP products. Therefore, this study aimed to compare postprandial blood amino acids following ingestion of three beverages: WPI-mixed with WPC (WPI-mixed) and WPH-mixed with WPI (WPH-mixed), versus a placebo beverage.

2. Materials and methods

A double-blind, repeated measures design was applied for this study. Each participant consumed the three drinks on separate days in a double-blinded manner, with 6 days in between. The researcher informed all participants to consume a regular diet during project participation. They came to the laboratory in the early morning, having fasted for at least 10 hrs. The order of the drinks consumed was randomized, and participants were blinded to the drink's identification. Following consumption of the test drinks, participants were not allowed to eat or drink anything until they had completed the 2-hr test period. A flexible 18-gauge catheter was inserted into a dorsal hand vein. After resting, a blood sample was drawn at 0 min for baseline. The subject then consumed a 350 mL serving of a test drink. Blood samples were drawn at 15, 30, 45, 60, 75, 90, 105, and 120 mins after consumption of the test drink.

During this time, the subjects remained seated or engaged in minimal movement. Participants were in a suitable room with a temperature of 25°C. Only the participants, researchers, and technicians were inside the room. The laboratory atmosphere was conventional for relaxation; they could talk, play games, and partake in other minimal-movement activities.

On each occasion, the researchers observed and recorded participants using a tolerance questionnaire to measure the intensity of gastrointestinal disturbances (heartburn, belching, bloating, nausea, vomiting,

flatulence, diarrhoea, and constipation), light-headedness, headache, dizziness, thirst, and dry mouth. In between the visits, participants were followed up via the LINE application and a telephone call. These follow-ups were scheduled approximately 5 days after each study visit in order to identify adverse events and assess their tolerance of the interventions. Reasons for the premature discontinuation of participation in the study included the occurrence of any conditions that presented a health risk for subjects (at the supervising clinicians' discretion) and in which a subject had decided to withdraw from further participation in the study voluntarily.

2.1 Participants

The sample size was calculated from the following formula, which totally of ten participants should be included.

$$n = \frac{\left(Z_\beta + Z_\frac{\alpha}{2}\right)^2 (\sigma_1^2 + \sigma_2^2)}{(\mu_1 - \mu_2)^2}$$

A total of 10 healthy men who regularly engaged in exercise training, aged between 18 and 30 years, were recruited as eligible volunteers for the study. Subjects were screened by using a questionnaire to confirm that they met the following criteria and would adhere to it before entering into the study: (1) maintain a diet consisting of 15-20% protein, 45-65% carbohydrate, and 25-30% fat according to a 3-day dietary food recall, (2) had not taken performance-enhancing supplements in the previous 6 weeks, (3) was a non-smoker, (4) were not taking amino acid supplements, (5) were not using anabolic or catabolic hormones, (6) were not taking medication or supplements known to influence any of the variables measured in the study, (7) were free of metabolic diseases and gastrointestinal tract disorders; and (8) did not have lactose intolerance and hypersensitivity of the constituents of the products. After the questionnaire screening, all participants had a physical examination, which was performed by a registered physician.

All participants obtained information about the process and the possible risks associated with the experimental procedures before providing written informed consent to participate in the study. This study was approved by the Ethical Review Committee for Human Research, Faculty of Public Health, Mahidol University (COA. No. MUPH 2019-132).

2.2 Drinks

This study used 3 test beverages: (1) mixed formula with WPH and WPC (WPH-mixed), containing 7.2% WPC, 2.0% WPH, and 29 g total protein, (2) mixed formula with WPI and WPC (WPI-mixed), containing 7.14% WPC, 1.57% WPI, 0.9% additional BCAA, and 29 grams total protein, (3) placebo which contained no added protein, only carbohydrate to provide an equal amount of energy as the two test beverages. Each beverage contained 200-220 kcal per 350 mL serving.

2.3 Blood collection and analysis

Blood samples were immediately put into EDTA tubes and put on ice. A total of 3 mL of blood was taken at each time point. Protein precipitation was performed using a methanol precipitation technique described by McGaw *et al.* (2010) with some modifications. The blood samples were centrifuged at 2,500 rpm for 15 mins at 4°C, 200 µL of blood plasma was then removed and mixed with 1,600 µL of methanol. After mixing for 30 s, plasma samples were stored at -80°C until analysis.

Free plasma amino acids were analyzed according to a method described by Thanatsang *et al.* (2020), with some modifications. Plasma samples (200 µL) were transferred to a 2 mL GC glass vial, to which 50 µL of norleucine (200 nmol/mL) was added as the internal standard. The mixture was then dried at 60°C for 1-2 hrs, combined with 50 µL dichloromethane, and dried again for 30 mins to remove the residual water. The dried samples were mixed with 50 µL of derivatizing agent, N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-Butyldimethylchlorosilane (MTBSTFA + 1% TBDMSCl) and 50 µL of acetonitrile. The sample vials were sealed with an aluminium cap with PTFE/Red Rubber Septa and incubated at 100°C for 4 hrs in a hot-air oven. After the sample had reached room temperature, 2 µL was analyzed by GC-TQ.

The chromatographic analysis was performed using gas chromatography (Agilent; 7890B) equipped with a mass spectrometer (Agilent, 7000D) and PAL autosampler system (CTC Analytics AG, Switzerland). Aliquots of the derivatized amino acids (2 µL) were injected by using pulsed split mode at a 1:5 split ratio at 280°C into an HP-5MS column (30 m, 0.25 mm i.d., Agilent J&W GC column). Helium was used as a carrier gas with a constant flow rate of 1.4 mL/min. The GC oven was programmed as follows: ramped from 130°C to 190°C (6°C/min) and 230°C (30°C/min), held at 230°C for 5 mins, then ramped to 325°C, and held at 325°C for 6 mins. The transfer line, ion source (EI), and quadrupole were set as 325°C, 240°C, and 180°C, respectively. The mass spectrometer was operated in selected ion monitoring (SIM) mode.

2.4 Data and statistical analysis

Statistical analysis was performed by using PASW Statistics for Windows, Version 18.0. (SPSS Inc.,

Chicago, USA). All values are presented as mean ± standard deviation (SD). The primary interaction between time and drink was determined using a two-way analysis of variance (repeated measures). When there was a significant effect, the Bonferroni posthoc test was applied to determine the differences between the three drinks at each timepoint (time 0 mins). The area under the curve was calculated as the incremental area under the plasma amino acid curve (IAUC). A one-way analysis of variance (repeated measures) and Tukey's post hoc analysis were used to compare the area under the curve. Differences with $P<0.05$ were considered significant.

3. Results

Plasma EAA, BCAA, and Leu response up to 120 mins after ingestion of the two kinds of WP mixed beverage versus placebo were tested in 10 healthy young men who exercised regularly. Both WPI-mixed and WPH-mixed produced significantly more plasma EAA, BCAA, and leucine concentrations at every time point from baseline when compared with the placebo. The placebo did not produce any significant increase in plasma EAA, BCAA, and Leu concentrations after ingestion; indeed, there was a steady decrease in plasma EAA, BCAA, and Leu concentrations at every timepoint.

For plasma EAA, there were no differences between WPI-mixed and WPH-mixed at any time point. The appearance of EAA in the plasma sharply increased by 30 mins post-ingestion of each protein drink and peaked at 30 and 45 mins post-ingestion of WPI-mixed and WPH-mixed, respectively. At that point, plasma EAA concentrations gradually decreased (Figure 1).
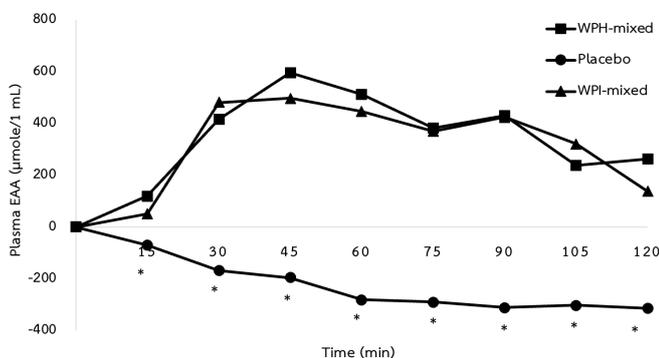

Figure 1. Plasma EAA concentration (μmol/1 mL) at 0 min (baseline) and 15, 30, 45, 60, 75, 90, 105 and 120 mins after ingestion of 350 mL of WPH-mixed, placebo, and WPI-mixed drinks.
*Significant difference between placebo and both WPH-mixed and WPI-mixed, $P<0.05$.

For plasma BCAA, WPI-mixed resulted in an earlier response than that observed when ingesting WPH-mixed. The appearance of plasma BCAA was significantly greater 30 mins after ingesting WPI-mixed compared with WPH-mixed. However, a gradual reduction in plasma BCAA concentrations was observed 45 mins after the ingestion of both protein drinks (Figure 2).
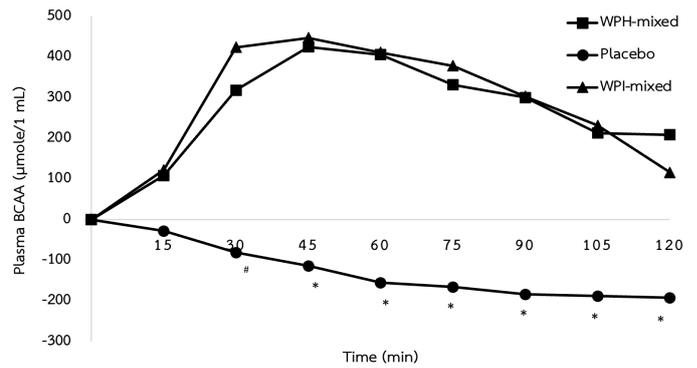

Figure 2. Plasma BCAA concentration (μmol/1 mL) at 0 min (baseline) and 15, 30, 45, 60, 75, 90, 105 and 120 mins after ingestion of 350 mL of WPH-mixed, placebo, and WPI-mixed beverages.
*Significant difference between placebo and both WPH-mixed and WPI-mixed, $P<0.05$.
#Significant difference between all of the products (WPH-mixed, placebo, and WPI-mixed), $P<0.05$.

WPI-mixed significantly enhanced plasma Leu concentrations, which were significantly higher than WPH-mixed. Leu, after both WPI and WPH beverages, seemed to peak around 45 mins. The ingestion of protein drinks produced a sharp increase in plasma Leu at 30 mins and a peak at 45 mins post-ingestion, followed by a plateau until 60 mins. Interestingly, at 105 mins post-ingestion, plasma Leu reduced after WPI-mixed ingestion more than WPH-mixed (Figure 3).
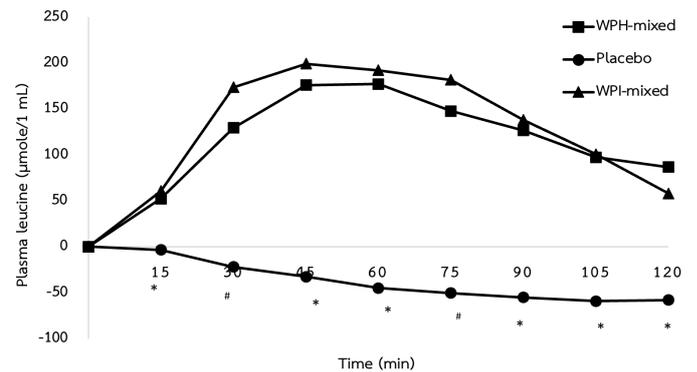

Figure 3. Plasma leucine concentration (μmol/1 mL) at 0 minutes (baseline) and 15, 30, 45, 60, 75, 90, 105, and 120 minutes after ingestion of 350 mL of WPH-mixed, placebo, and WPI-mixed beverages.
*Significant difference between placebo, and both WPH-mixed and WPI-mixed, $P<0.05$.
#Significant difference between all products (WPH-mixed, placebo, and WPI-mixed), $P<0.05$.

4. Discussion

Most previous research on WP studied the appearance of plasma amino acids following ingestion of pure WPC, WPI, or WPH. However, for price and taste

reasons, the commercially available forms of WP are mostly mixtures of WPC and either WPI or WPH. Therefore, the primary purpose of this research was to compare postprandial plasma amino acids (EAA, BCAA, and Leu) following ingestion of three beverages: WPI-mixed with WPC (WPI-mixed), and WPH-mixed with WPC (WPH-mixed), and placebo. The results showed that postprandial EAA, BCAA, and Leu significantly increased after ingestion of WPH-mixed and WPI-mixed beverages and peaked at 45 mins, while the concentrations of these amino acids decreased following the consumption of the placebo beverage. Peak plasma EAA after ingestion of the WPH-mixed drink was not different from the WPI-mixed drink. Additionally, for iAUC analysis, WPH-mixed resulted in lower concentrations of plasma BCAA and Leu than WPI-mixed (P<0.05).

The previous research showed that the higher the caloric density of nutrition consumes, the more gastric emptying delays. Luiking *et al.* (2016) studied protein type and the modulation of postprandial amino acid profile by the caloric density of protein supplements. They found that a low caloric leucine-enriched whey protein nutritional supplement provided a higher rise in serum levels of TAA, EAA, and leucine than high caloric products. Therefore, in our study, each drink contained a similar caloric density, so caloric content did not affect the postprandial blood amino acids. The differences in postprandial plasma amino acids should have occurred due to the test beverages' protein content.

Previous research has shown that plasma amino acids increase sharply as early as 30 mins after ingesting a high-quality protein source when consumed in a fasting state (Park *et al.*, 2020). The peak plasma amino acids, BCAA, and Leu were still about 30 mins and gradually decreased (Sharp *et al.*, 2019). Amino acids in blood similarly responded to the test drinks as in the study of Farnfield *et al.* (2009). They found that plasma Leu was significantly higher after ingesting WPI compared with WPH.

Leucine has been reported to play a crucial role in activating mTORC1 and, consequently, stimulating muscle protein synthesis (Devries *et al.*, 2018). The addition of free leucine to low dietary protein activates mTORC1, thereby amplifying the anabolic response and enhancing muscle anabolism, as reported in previous studies (Churchward-Venne *et al.*, 2014). In contrast, there is no evidence of a beneficial effect in healthy young subjects when free leucine is added to intact protein (Van Loon, 2012). In our study, plasma leucine concentrations were higher following ingestion of WPI-mixed than WPH-mixed. Only increasing plasma leucine

will not enhance muscle anabolism because the availability of the other EAA will become rate-limiting.

Elevated plasma BCAA is often associated with insulin resistance and type 2 diabetes, resulting from reduced cellular utilization and incomplete BCAA oxidation (Lackey *et al.*, 2013). The toxic catabolic intermediates of BCAA can cause insulin resistance and are involved in different mechanisms in different metabolic tissues. In skeletal muscle, valine produces 3-hydroxy-isobutyrate, which promotes skeletal muscle fatty acid uptake, resulting in the accumulation of incompletely oxidized lipids, causing skeletal muscle insulin resistance. In the liver, branched-chain α-keto acids decompose in large amounts, promote hepatic gluconeogenesis, and lead to the accumulation of multiple acylcarnitines. Acylcarnitines damage the mitochondrial tricarboxylic acid cycle, resulting in the accumulation of incomplete oxidation products, oxidative stress in mitochondria, and hepatic insulin resistance. In adipose tissue, the expression of BCAA catabolic enzymes (BCAA transaminase, branched-chain α-keto acid dehydrogenase) is reduced. This reduction causes an increased plasma BCAA, thereby producing massive decomposition of BCAA in tissues such as skeletal muscle and liver and inducing insulin resistance. However, BCAA, as a well-known nutritional supplement for athletes, does not induce insulin resistance. A possible explanation for this phenomenon is that exercise can enhance the mitochondrial oxidative potential of BCAA, alleviate or even eliminate the accumulation of BCAA catabolic intermediates, and promote BCAA catabolism into beta-aminoisobutyric acid, thereby increasing plasma beta-aminoisobutyric acid concentration and improving insulin resistance (Shou *et al.*, 2019).

A physiologically significant increase in muscle protein synthesis rate requires adequate availability of all amino acid precursors. The source of EAAs for muscle protein synthesis in the post-absorptive state is the free intracellular pool. Intracellular free EAAs that are available for incorporation into protein are derived from muscle protein breakdown. About 70% of EAAs released by muscle protein breakdown are reincorporated into muscle protein under normal conditions. The efficiency of reincorporating EAAs from protein breakdown back into muscle protein can only be increased to a limited extent. For this fundamental reason, a dietary supplement of BCAA alone or leucine cannot support an increased rate of muscle protein synthesis. The availability of the other EAAs will rapidly become rate-limiting for accelerated protein synthesis. Consistent with this perspective, the few studies in human subjects have reported decreases, rather than increases, in muscle

protein synthesis after intake of BCAA. We conclude that dietary BCAA supplements alone do not promote muscle anabolism (Wolfe, 2017).

Another point is that our study did not measure plasma peptides. Luiking *et al*. (2016) have shown that after ingestion of WPH, plasma peptide levels were markedly elevated. The BCAA-containing peptides may act as bioactive peptides in glycogen and muscle protein synthesis in skeletal muscle. In their study, ingestion of WPH caused significantly greater phosphorylation of mTOR levels than a mixture of amino acids, despite the plasma levels of amino acids in AA and WPH being similar. These results indicated that active components, such as bioactive peptides in WPH, significantly affect mTOR phosphorylation more than leucine degraded from WPH. They also demonstrated that phosphorylation of S6K1 and 4E-BP1, which influence translation initiation and elongation, was higher in the WPH group than in the AA group. These results indicate that WPH caused more significant phosphorylation of S6K1 and 4E-BP1 via mTOR activation than AA. Their results suggest that specific bioactive peptides in WPH may activate S6K1 via phosphorylation of Thr421/Ser424 without activating mTOR. They concluded that ingestion of WPH increases muscle protein synthesis more than a mixture of amino acids after endurance exercise due to higher phosphorylation of mTOR levels. Furthermore, it indicated that WPH might contain active components, such as bioactive peptides, that are superior to leucine for stimulating mTOR signalling. These findings provide new insights into the effects of different dietary protein forms for sports nutrition (Kanda *et al*., 2013).

5. Conclusion

This study reports the effect of the ingestion of WPI-mixed and WPH-mixed beverages on plasma amino acids. We found no difference in plasma EAA between WPI-mixed and WPH-mixed beverages. However, plasma concentrations of leucine and BCAA after ingesting the WPI-mixed beverage were higher than after the WPH-mixed beverage, similar to other studies' findings. Therefore, it would be interesting to investigate bioactive peptides together with plasma amino acids in future research.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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