UPEI-400, a conjugate of lipoic acid and scopoletin, mediates neuroprotection in a rat model of ischemia/reperfusion

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**ABSTRACT**

Previously, our laboratory provided evidence that lipoic acid (LA) covalently bonded to various antioxidants, resulted in enhanced neuroprotection compared to LA on its own. The naturally occurring compound scopoletin, a coumarin derivative, has been shown in various in vitro studies to have both antioxidant and anti-inflammatory mechanism of actions. The present investigation was designed to determine if scopoletin on its own, or a co-drug consisting of LA and scopoletin covalently bonded together, named UPEI-400, would be capable of demonstrating a similar neuroprotective efficacy. Using a rat stroke model, male rats were anesthetized (Inactin®, 100 mg/kg, iv), the middle cerebral artery was permanently occluded for 6 h (pMCAO), or in separate animals, occluded for 30 min followed by 5.5 h of reperfusion (ischemia/reperfusion; I/R). Pre-administration of either scopoletin or UPEI-400 significantly decreased infarct volume in the I/R model (p < 0.05), but not in the pMCAO model of stroke. UPEI-400 was ~1000 times more potent compared to scopoletin alone. Since UPEI-400 was only effective in a model of I/R, it is possible that it may act to enhance neuronal antioxidant capacity and/or upregulate anti-inflammatory pathways to prevent the neuronal cell death.

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1. Introduction

According to the most recent published data in 2016, death and disability due to stroke remains a global medical issue and therapeuetic options remain limited (Mozaffarian et al., 2016). Ischemic stroke, resulting from the occlusion of a cerebral artery, accounts for up to 80% of all strokes (Ginsberg, 2008). A transient or permanent reduction of cerebral blood flow results in hypoxia, hypoglycemia, the failure of ATP-dependent pumps and a disruption of ionic equilibrium, leading to the generation of reactive oxygen species (ROS) and free radicals, and the upregulation of cytokine inflammatory pathways, ultimately ending in neuronal damage and death (Ginsberg, 2008; Margaill et al., 2005). Research into antioxidant and anti-inflammatory therapy to prevent and/or scavange ROS and free radical generation has provided hope that the damage due to reperfusion injury may be mitigated, and that the window of opportunity for therapeutic treatment, such as tissue-plasminogen-activator (TPA), may be extended beyond the currently accepted time frame of 3—4 h following the onset of stroke.

Scopoletin (6-methoxy-7-hydroxycoumarin) is a coumarin derivative found in various plants and has a long history of use for its medicinal properties in traditional Chinese medicine (Kang et al., 1999). Scopoletin is isolated from Canarium patentinervium Miq (Burseraceae Kunth), a rare plant from the family of Burseraceae and genus Canarium found in the Asia-Pacific region and has been used in wound healing by the indigenous people of Malaysia (Mogana et al., 2011). In particular, evidence has been provided to suggest that scopoletin has both antioxidant and anti-inflammatory properties and has been used to ameliorate the symptoms of various inflammatory, rheumatoid and digestive disorders (Kang et al., 1999). In vitro studies have demonstrated that scopoletin has antioxidant properties, is an effective inhibitor of the activation of 5-lipoxygenase and has anti-acetylcholinesterase activity (Mogana et al., 2013). To date, scopoletin has not been tested in any in vivo model of stroke (ischemia or ischemia/reperfusion), but other coumarin derivatives have been tested with some success (Moa et al., 2011; Sun et al., 2003).

Clinically, single drug therapies following stroke have not yielded the positive clinical trial results expected when compared to the laboratory testing of these compounds. Due to the multifactorial nature of ischemia and reperfusion injury, it is likely that a particular drug will be unable to affect several pathways simultaneously. Thus,
there has been a recent interest in the use of co-drugs as a therapeutic approach in various pathologies (Bansal and Sliakari, 2014; Minnerup and Schabitz, 2009). The development and administration of a co-drug, containing lipoic acid (LA) covalently linked to another compound, have been demonstrated to have a greater efficacy/potency compared to the administration of a mixture of the two drugs (Connell et al., 2012a, 2012b, 2014; Das et al., 2010; Saleh et al., 2014; Sozio et al., 2010). The antioxidant LA, is a known free radical scavenger (Bilska and Wlodek, 2005; Biewengo et al., 1997), and many researchers have shown that in several different stroke models administration of lipoic acid on its own can be neuroprotective but only at relatively high doses (Connell et al., 2011; Richard et al., 2011; Clarke et al., 2001; Panigrahi et al., 1996; Wolz and Krieglstein, 1996; Cao and Phillis, 1995). Further, combining lipoic acid with other drugs has been shown to produce an additive or synergistic protective effect in several different animal models of pathology (Mufherjee et al., 2011; Bano and Bhatt, 2010; Babu et al., 2011; Garcia-Estrada et al., 2009; Shotton et al., 2004; Gonzalez-Perez et al., 2002), when compared to the effect of either drug alone. Our laboratory has demonstrated that LA covalently bonded to various naturally occurring antioxidant compounds has provided superior neuroprotection following I/R injury compared to either drug alone or a mixture of 2 compounds administered simultaneously (Connell et al., 2012a, 2012b, 2014; Saleh et al., 2014, 2015). Based on the above observations demonstrating the beneficial effects of developing a co-drug, our laboratory has developed a new synthetic co-drug that is a covalent conjugate between LA and scopoletin, named UPEI-400.

In the present study, using a model of acute stroke and reperfusion injury (I/R) in male rats developed in our laboratory (Connell and Saleh, 2010), we determined whether the administration of scopoletin alone is neuroprotective, and if the administration of UPEI-400 would provide a further improvement of the neuroprotection observed with scopoletin alone. Further, a neuroprotective dose of UPEI-400 was then tested at various time points following reperfusion to examine the neuroprotective capability of UPEI-400 in a more clinically relevant paradigm.

2. Materials and methods

2.1. Ethics statement

All experiments were carried out in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the University of Prince Edward Island Animal Care Committee (protocol #11-045 and 13-036).

2.2. General surgical procedures for in vivo stroke surgery

All experiments were conducted on male Sprague-Dawley rats (300–350 g; Charles Rivers; Montreal, PQ, CAN). For all animals, food and tap water were available ad libitum. Rats were anesthetized with the long-lasting barbiturate anesthetic, sodium thiobutabarbital (Inactin®; Sigma-Aldridge; St. Louis, MO, USA; 100 mg/kg; ip). Supplements were given if a withdrawal reflex was elicited following corneal or toe pinch every 10 min. All animals were tracheotomized to facilitate breathing and then placed on a heating blanket to maintain body temperature at 37 ± 1 °C (Physitemp Instruments; Clifton, NJ, USA).

2.3. Transient and permanent middle cerebral artery occlusion (tMCAO and pMCAO)

We have previously published the detailed methodology for occlusion of the middle cerebral artery (Connell et al., 2012a; Connell and Saleh, 2010; Saleh et al., 2014, 2015) in both permanent (pMCAO) and transient (tMCAO) models. Briefly, the middle cerebral artery (MCA) was exposed via a craniotomy, and suture thread placed under 3 separate branches of the MCA. The sutures were either occluded for 6 h (pMCAO) or occluded for 30 min, followed by removal of the sutures and the MCA allowed to reperfuse for an additional 5.5 h (tMCAO).

2.4. UPEI-400 synthesis

Scopoletin and lipoic acid were chemically linked via an ester bond using a simple synthetic route. Accordingly, scopoletin (0.012 g) was placed in a dry 100 ml flask followed by lipoic acid (0.025M in 0.02 ml of dimethylaminopyridine; DMAP) in 100 ml anhydrous dichloromethane (CH₂Cl₂). Dicyclo-hexylcarbodiimide (DCC) (0.025M) was added in small quantities over a period of 1 h under nitrogen atmosphere. The reaction was stirred overnight and the compound was purified using silica column chromatography after an aqueous work up. Proton (1H) nuclear magnetic resonance spectroscopy and mass spectrometry was used to characterized the pure compound, UPEI-400.

**UPEI-400**

![Chemical Formula: C₁₈H₂₀O₅S₂](attachment)

**Exact Mass:** 380.07522

**Molecular Weight:** 380.47840

### Preparation of UPEI-400 (synthetic scopoletin-lipoic acid combination)

![Preparation Diagram]
2.5. Drug injection protocol in tMCAO model

Scopoletin, UPEI-400 or vehicle were administered (1 ml/kg; i.v.) 30 min prior to the onset of MCAO (−60 on schematic). The sutures were left in place for 30 min (duration of ischemia; MCAO on schematic), and then removed to allow the return of blood flow and reperfusion which lasted for 5.5 h (0−330 min on the schematic). Post-MCAO administration of UPEI-400 was investigated by administering the lowest dose of UPEI-400 which was previously shown to produce significant neuroprotection at the following time points during the ischemia/reperfusion protocol: 15 min prior to the onset of reperfusion (−15), and 30, 90, 150 or 210 min following the start of reperfusion.

2.6. Effect of scopoletin on ischemia-reperfusion (tMCAO model)

In the first set of animals, scopoletin (1.0 (n = 6), 0.1 (n = 6) or 0.01 (n = 6) mg/kg) was administered in a volume of 1 ml/kg (i.v.) or vehicle (30% DMSO; 1 ml/kg; i.v.; n = 8) 30 min prior to the onset of MCAO (−30 min). Following this 30 min occlusion, sutures were removed and the region allowed to reperfuse for an additional 5.5 h.

2.7. Effect of UPEI-400 on ischemia-reperfusion (tMCAO model)

In the second set of experiments, UPEI-400 (0.1 (n = 6), 0.01 (n = 6), 0.001 (n = 6) or 0.0001 (n = 6) mg/kg) was administered in a volume of 1 ml/kg (i.v.) or vehicle (0.05% DMSO; 1 ml/kg; i.v.; n = 6) 30 min prior to the onset of MCAO (−30 min). The sutures were left in place for 30 min, followed by 5.5 h of reperfusion.

2.8. Effect of UPEI-400 on infarct volume when administered either during the occlusion or reperfusion

To determine if UPEI-400 was neuroprotective when administered during the 30 min period of occlusion, the optimal dose of UPEI-400, which based on the dose-response-curve described above was 0.001 mg/kg, was administered 15 min into the 30 min occlusion period. The sutures were then removed for an additional 5.5 h of reperfusion.

To examine the effect of UPEI-400 on reperfusion injury alone, injections of the optimal dose of UPEI-400 (0.001 mg/kg; 1 ml/kg; i.v; n = 5–6 per group) were made 30, 90, 150 or 210 min following suture removal (start of reperfusion). Experiments were then terminated at the end of the 5.5 h of reperfusion. In all cases, the comparison group far statistical analysis was UPEI-400 vehicle (0.05% DMSO; n = 6).

2.9. Effect of UPEI-400 on ischemia alone (permanent occlusion; pMCAO)

To determine if administration of UPEI-400 (0.001 mg/kg; iv; 1 ml/kg; n = 5) was neuroprotective on ischemia-induced cell death only, injections of UPEI-400 were made 30 min prior to pMCAO. Following 6 h of occlusion, the experiment was terminated. Similar to the studies on tMCAO described above, the vehicle for UPEI-400 tested in the pMCAO model was also 0.05% DMSO (1 ml/kg; iv; n = 5).

2.10. Effect of UPEI-400 on baseline blood pressure and heart rate

To determine the hemodynamic effect of UPEI-400 on baseline (resting) blood pressure and heart rate, continuous recordings of blood pressure and heart rate were taken prior to and for 2 h following UPEI-400 (0.001 mg/kg; 1 ml/kg; iv; n = 4) or vehicle (0.05% DMSO, 1 ml/kg; iv) administration. Cardiovascular data acquisition and analysis was done as described in our previous studies (Connell and Saleh, 2010). Measurements of the blood pressure and heart rate values were made 5 min immediately prior to drug administration and at 5, 10, 15, 30, 45, 60, 90 and 120 min following drug administration.

2.11. Histological procedures

At the end of each experiment where infarct volume was measured, animals were overdosed with Inactin (100 mg/kg), their diaphragm transected and left ventricle of the heart perfused with phosphate buffered saline (PBS 0.1 M; 200 ml) via an 18-gauge needle. The brains were removed and infarct volumes measured as previously described (Connell et al., 2012a; Connell and Saleh, 2010; Saleh et al., 2014, 2015). Digital images were coded prior to being sent for analysis of infarct volume to allow the analyst to be blinded to treatment groups.

2.12. Statistical analysis

All data was analyzed using SigmaStat and SigmaPlot (Jandel Scientific, Tujunga, CA). Data are presented as a mean ± standard error of the mean (S.E.M). Using analysis of variance (ANOVA) followed by a Bonferroni posthoc analysis, differences were considered significant if p ≤ 0.05. If only two groups were being compared, Student’s t-test was used.

3. Results

3.1. The effect of pre-administration of scopoletin on ischemia-reperfusion (tMCAO)

Pre-administration of scopoletin resulted in a dose-dependent neuroprotection with 1.0 mg/kg resulting in a significant decrease in infarct volume compared to the administration of vehicle (p ≤ 0.05; Fig. 1A and B). (A) Representative photomicrographs of TTC-stained, 1 mm thick coronal slices illustrating the extent of the infarct within the
3.2. The effect of pre-administration of UPEI-400 on ischemia-reperfusion (tMCAO)

Pre-administration of UPEI-400 resulted in a dose-dependent neuroprotection with 0.001, 0.01 and 0.1 mg/kg resulting in a significant decrease in infarct volume compared to the administration of vehicle (p ≤ 0.05; Fig. 2A1 and 2B1).

(A) Representative photomicrographs of TTC-stained, 1 mm thick coronal slices illustrating the extent of the infarct within the prefrontal cortex following pretreatment (30 min prior to MCAO; i.v.) with either vehicle (0.05% DMSO) or UPEI-400 (0.001 mg/kg) following ischemia-reperfusion (tMCAO). (B) Bar graph summarizing the dose-response relationship between increasing doses of UPEI-400 and infarct volume (mm³) calculated from TTC-stained, 1 mm thick coronal sections following tMCAO. Each bar represents the mean S.E.M. (n = 6 or 8/group) and * indicates significance (p ≤ 0.05) from the vehicle-treated control group.

3.3. The effect of pre-administration of UPEI-400 on permanent ischemia (pMCAO)

To examine the effect of UPEI-400 on ischemia-induced cell death only, administration of the optimal dose of UPEI-400 identified in the dose-response curve in Fig. 2A2 (0.001 mg/kg) or vehicle were made 30 min prior to 6 h of pMCAO (sutures left in place for 6 h). UPEI-400 pre-administration did not produce significant neuroprotection when infarct volume was measured 6 h following the start of pMCAO (p ≥ 0.05; Fig. 2B2).
3.4. The effect of UPEI-400 administration on infarct volume when administered either during ischemia or after the start of reperfusion

Administration of UPEI-400 (0.001 mg/kg) 15 min into the 30 min period of MCAO (15 min prior to the start of reperfusion; –15 min on schematic) produced significant neuroprotection compared to vehicle when infarct volume was measured following 5.5 h of reperfusion (p ≤ 0.05; Fig. 3A).

(A) Representative photomicrographs of TTC-stained, 1 mm thick coronal slices illustrating the extent of the infarct within the prefrontal cortex following treatment with either vehicle (0.05% DMSO) or UPEI-400 (0.001 mg/kg) 15 min prior to the beginning of reperfusion (–15), immediately prior to suture removal (0), or 30, 90, 150, or 210 min (n = 5 or 6/group). (B) Effect of UPEI-400 (0.001 mg/kg) on infarct volume (mm$^3$) following injection 15 min prior to the beginning of reperfusion (–15), immediately prior to suture removal (0), or 30, 90, 150, or 210 min (n = 5 or 6/group) following the start of reperfusion. Each bar represents the mean ± S.E.M. and * indicates significance (p ≤ 0.05) from the vehicle control group (0.05% DMSO; n = 5) injected 30 min prior to tMCAO.

We determined the effect of UPEI-400 on reperfusion injury only by measuring the infarct volume when the drug was only administered at 30, 90, 150 or 210 min after the start of reperfusion. Administration of UPEI-400 at all time points up to 150 min following the start of reperfusion resulted in significantly smaller infarct volumes compared to vehicle administration (p ≤ 0.05 at each time point; Fig. 3B).

3.5. The effect of UPEI-400 administration on blood pressure and heart rate

The following experiment was designed to determine the hemodynamic effect of administration of UPEI-400 (0.001 mg/kg) on mean arterial blood pressure and heart rate for a period of 2 h following administration. Baseline mean arterial pressure and mean heart rate prior to drug administration was 110 ± 9 mm/Hg and 385 ± 16 bpm for the vehicle (0.05% DMSO) treated group, and, 112 ± 5 mm/Hg and 390 ± 15 bpm for the UPEI-400 (0.001 mg/kg) treated group. Administration of either UPEI-400 or vehicle did not
significantly alter either arterial blood pressure or heart rate (p ≥ 0.05) at any time point compared to respective pre-administration (baseline) values. Further, drug administration values for blood pressure and heart rate were not significantly different from those measured in vehicle-treated animals at any time point (p ≥ 0.05 at each time point).

4. Discussion

In this study, we determined that UPEI-400, a chemical combination of two naturally occurring compounds, LA, and scopoletin, produced dose-dependent neuroprotection against neuronal cell death as observed in a previously validated, novel model of ischemia-reperfusion (I/R) (Connell and Saleh, 2010). The results demonstrated that UPEI-400 produced neuroprotection following 5.5 h of reperfusion in a model of focal ischemia, which is restricted to the prefrontal cerebral cortex. Further, the dose of UPEI-400 required to produce significant neuroprotection (0.001 mg/kg) was 1000 fold less compared to the dose required for scopoletin alone (1.0 mg/kg), and 5000 fold less compared to the optimal neuroprotective dose of LA alone, observed previously in our lab (Connell et al., 2011). Also, the optimal dose of UPEI-400 produced significant neuroprotection when administered 15 min prior to the start of reperfusion, and when administered 30, 90, and 150 min following the onset of reperfusion. Clinically, the paradigm of administering UPEI-400 during, and/or following the occlusion was to mimic the clinical situation in which therapy would be administered at the time a patient presents to a hospital following the onset of a stroke, or following administration of thrombolytic therapy (to prevent reperfusion injury). Interestingly, UPEI-400 did not produce neuroprotection when administered prior to a 6-h permanent occlusive stroke (no reperfusion; pMCAO). This suggests that UPEI-400 may act to attenuate the oxidative stress observed following transient occlusion (I/R), whereas UPEI-400 is ineffective against the necrotic cell death primarily observed in permanent ischemia. These results also suggest that UPEI-400 provides neuroprotection against reperfusion injury alone, likely due to the scavenging of free radicals, by the attenuation of ROS production, or the inhibition of upregulated inflammation pathways.

Many studies have demonstrated anti-inflammatory and anti-oxidant properties of scopoletin, but to the best of our knowledge, the neuroprotective capacity of scopoletin has not been tested in an in vivo stroke model. The utility of drug therapy on reactive oxygen species and inflammation-induced growth of ischemic injury using models of reperfusion injury have been established by many labs (Ginsberg, 2008; Margalil et al., 2005; Wang et al., 2007). In vitro studies have demonstrated that scopoletin suppressed the production of pro-inflammatory cytokines in RAW 264.7 and HMC-1 cell lines (Moon et al., 2007; Kim et al., 2004). Scopoletin has been shown to inhibit the enzymatic activity of 5-lipoxygenase and acetylcarnelinate (Mogan et al., 2013; Khunawatunantham et al., 2016) and selectively inhibit MAO-b and increase dopamine levels in rodent brain tissue in vitro (Basu et al., 2016). Scopoletin has also been shown to have antioxidant properties when tested in various antioxidant assays in vitro (Shaw et al., 2003; Malik et al., 2011) and is protective against glutamate-induced neurotoxicity as well as anti-cholinesterase activity in an HT22 cell line (Lee et al., 2015). To date, most of the investigations on the therapeutic efficacy of scopoletin in vivo have focused on systemic disorders. In vivo studies have demonstrated that scopoletin is able to ameliorate the symptoms of various inflammatory, rheumatoid, and digestive disorders (TN1 Deng et al., 2012; XIDing et al., 2012).

Other coumarin derivatives have been shown to have antioxidant properties, which translated to neuroprotection. The coumarin derivative esculetin has been shown to reduce oxidative stress in several in vitro models (Lee et al., 2011; Kim et al., 2008) and demonstrate anti-inflammatory properties in vivo (Kwon et al., 2011; Witaicenis et al., 2010). Esculetin has been shown to be neuroprotective in a mouse model of I/R following transient occlusion of the middle cerebral artery (Wang et al., 2012). These authors also demonstrated that esculetin administration decreased cleaved caspase-3 levels, upregulated levels of Bcl-2 and downregulated levels of Bax. In addition, delayed administration of esculetin up to 4 h following the start of reperfusion resulted in a decreased infarct size after 24 h of reperfusion. The coumarin derivative, osthole, displayed dose-dependent neuroprotection when cultured cortical neurons were exposed to 4 h of oxygen-glucose deprivation followed by 24 h of reperfusion (Chen et al., 2011). These authors demonstrated that osthole prolonged the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and prevented to activation of c-Jun N-terminal kinase (JNK), two members of the family of mitogen-activated protein kinases (MAPK). Osthole has also been tested in an vivo rodent stroke model. Osthole administration at the beginning of reperfusion reduced infarct volume following stroke induced by 2 h of monofilament suture placement at the base of the MCA (Moa et al., 2011). Osthole has also been reported to be protective against myocardial, renal and intestinal ischemia/reperfusion (Dong et al., 2013; XY1Dong et al., 2013; Zheng et al., 2013).

In the present study, UPEI-400 was not able to protect against neuronal death measured following permanent MCAO. Permanent ischemia is characterized by glutamate-induced neuronal toxicity ultimately leading to necrosis (Ginsberg, 2008). Generation of oxidative radicals and the upregulation of inflammatory pathways do not appear to play an important role in ischemic damage and subsequent cell death in an environment where there is a lack of blood flow. Therefore, anti-oxidant and anti-inflammatory agents such as scopoletin or lipoidic acid, would not be effective in combating the permanent ischemia and necrotic cell death as demonstrated in the pMCAO model used in the current study.

Our results indicate that the chemical combination of scopoletin and lipoidic acid produced a 1000-fold increase in I/R-induced neuroprotection compared to scopoletin alone. Similar results have also been presented from our laboratory. For example, using the same stroke model, the synthetic combination of lipoidic acid with resveratrol (UPEI-200; (Saleh et al., 2014)) produced a 200 fold increase in neuroprotection compared to resveratrol alone, a 100 fold increase in neuroprotection was found when lipoidic acid was combined with apocynin (UPEI-100; (Connell et al., 2012a)), and a 5 fold increase in neuroprotection was found when lipoidic acid was combined with edaravone (UPEI-300; (Connell et al., 2014)). Other laboratories have also demonstrated the benefits of using chemical combinations of LA with other compounds to provide neuroprotective effects greater than either of the two compounds administered on their own. Covalent linkage of LA with L-dopa or dopamine decreased neuronal oxidative damage due to the infusion of Aβ (1-40) (Minnerup and Schabitz, 2009). Also, a co-drug produced by chemically linking LA with L-dopa or dopamine decreased neuronal oxidative damage associated with the administration of L-dopa or dopamine on their own (Di Stefano et al., 2006).

Many authors have described the multiplicity of mechanisms involved in ischemia- and reperfusion-induced neuronal damage following an occlusive stroke and the difficulty in providing treatment in a clinical setting (Ginsberg, 2008; Green, 2008). Therefore, drugs targeting multiple pathways might overcome this dilemma. The synthesis and development of co-drugs using biologically relevant molecules provide the ability to simultaneously target
multiple pathways involved in the pathogenesis of neurological diseases, specifically, pathways involved in the initiation of oxidative stress and inflammation following reperfusion. The results presented above support the idea that the combination of antioxidant and/or anti-inflammatory compounds at subthreshold doses can produce equal or enhanced neuroprotection. The advantage of using lower doses to achieve comparable or improved therapeutic effects is to minimize side effects on other physiological systems as is seen in many current therapies. Our findings support the advantage of combination therapy in stroke treatment both prophylactically and even during ischemia-reperfusion. UPEI-400 is a potential therapeutic candidate to protect against the negative outcomes associated with reperfusion injury.

Author contributions

TMS, BJC and MCS conceived and designed the experiments; BC and MCS performed the experiments; TMS and BJC analyzed the data; DR contributed the UPEI-400 material; TMS wrote the paper.

Conflicts of interest

The authors declare no conflict of interest.

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