Full Length Research Paper

Impact of the application of humic acid and sodium nitroprusside on nickel toxicity: Analysis of relative gene expression

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Nickel (Ni) is an essential micronutrient for plants but in high concentrations may turn toxic. This paper discusses the potential role of humic acid (HA) and sodium nitroprusside in modulating or preventing oxidative stress in rice plants. Three genes [superoxide dismutase (SOD) glutathione reductase (GR) and ascorbate peroxidase (APx) were selected for an expression study using a real time PCR technique. Three different treatments (T1 = nickel [nickel chloride (NiCl$_2$·6H$_2$O)] 300 mg L^{-1}, T2 = nickel-humic acid, T3 = nickel-sodium nitroprusside) were used to determine the effect of humic acid and sodium nitroprusside on nickel toxicity in rice plants. Rice plants grown in T2 appeared green and well developed. In leaves and roots, the expression of superoxide dismutase and ascorbate peroxidase was higher in T3 (nickel-sodium nitroprusside); glutathione reductase expression in roots was lower in T1 (sand with Ni solution) compared to T2 (nickel 300 mg L^{-1} and HA) where the expression was higher; significant differences were found between both treatments. In leaves, the behavior of this gene was similar in all treatments. This research suggests that nickel toxicity cannot be diminished when HA or SNP are used, and they induce oxidative stress in rice plants.

Key words: Nickel toxicity, heavy metals, gene expression, oxidative stress.

INTRODUCTION

Soil contamination with heavy metals like lead (Pb), Cadmium (Cd) and nickel (Ni) is an environmental problem worldwide because these metals may bio-accumulate and they do not have specific metabolic functions for living beings. This pollution is mainly due to

the intense industrialization and urbanization (Wei and Yang, 2010; Yaylali-Abanuz, 2011; Mireles et al., 2012). Nickel is a ubiquitous trace metal and compounds such as nickel acetate, nickel carbonate, nickel hydroxide and nickel oxide are used in a wide range of industrial processes.

These compounds ultimately accumulate in soil and environment, and can be easily absorbed by plants. Thus, they can enter the food chain and cause deleterious effects to animals and humans (Cempel and Nikel, 2006). Human exposure to nickel and its compounds has the potential to produce a variety of pathological effects, which may include cutaneous inflammations such as swelling, reddening, eczema and itching on skins, and may also induce allergy reactions and teratogenicity in the human body. The most concerning adverse health effects due to nickel exposure are lung fibrosis and lung cancer (Zhao et al., 2009). Higher plants have developed a series of protective mechanisms to counteract Ni-toxicity and to control the generation of excessive reactive oxygen species (ROS). These mechanisms include anti-oxidative enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and catalase (CAT) (Xu et al., 2010).

In rice plants, an increase in Ni levels produces diverse toxicity symptoms such as chlorosis and necrosis (Samantaray et al., 1997); also, the number of lateral roots considerably decreases (Seregin and Kozhevnikova, 2006). With the development of the global economy, both type and content of heavy metals in the soil caused by human activities have gradually increased in recent years, which have resulted in serious environmental deterioration (Su et al., 2014). One way to diminish heavy metals toxicity on plants is the use of natural products, such as humic acid (HA) and nitric oxide donors like sodium nitroprusside (SNP). Application of humic acid not only effectively improves soil physical and chemical properties, to provide a more suitable environment for plant growth, but also significantly reduces the use of chemical fertilizers and pesticides in soils. As an important way for increasing yield in agricultural production, the use of humic acid can also accelerate remediation of contaminated soil by heavy metals (Xu et al., 2010).

In the case of the SNP the number of studies that have examined the exogenous NO effects on reducing heavy metal toxicity in plants has increased. Application of SNP under different heavy metals toxic conditions may protect rice seedlings from Cd and As (arsenic) (Panda et al., 2011; Singh et al., 2009). These studies strongly suggest that exogenous NO can protect plants from the harmful impacts of toxic heavy metals concentrations. The aim of this research was to evaluate the effect of Humic Acid and Sodium Nitroprusside on diminishment of toxicity in rice plants exposed to nickel through a gene expression

Table 1. Composition of the Hoagland`s nutrient solution.

Component	Stock Solution g L^{-1}
Macronutrients	
2 M KNO_3	202
1 M $Ca(NO_3)_2 \cdot 4H_2O$	236 g $0.5L^{-1}$
Iron (Sprint 138 iron chelate)	15
2 M $MgSO_4 \cdot 7H_2O$	493
1 M NH_4NO_3	80
Micronutrients	
H_3BO_3	2.86
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.22
$CuSO_4 \cdot 5H_2O$	0.051
$H_3MoO_4 \cdot H_2O$ or	0.09
$Na_2MoO_4 \cdot 2H_2O$	0.12
Phosphate	
1 M KH_2PO_4 (pH to 6.0)	136

analysis of some gene related to antioxidant activity in plants.

MATERIALS AND METHODS

Plant material

Rice (*Oryza sativa*) var. Tetep was kindly provided by National Institute of Agricultural Science (INCA, Cuba). Seeds were sterilized with 5% sodium hypochlorite for 15 min, and then rinsed with distilled water for three times. Seeds were sown in pots filled with distilled water and covered with a thin cloth to avoid water evaporation. Pots were placed in a phytotron with a temperature of 35°C and a relative humidity of 32 to 35%. After one week seedlings were transplanted to 1 L pots filled with sand and Hoagland's nutrient solution (600 mL) (Table 1) (four seedlings by pots) with Ni [nickel chloride ($NiCl_2 \cdot 6H_2O$)] 300 mg L^{-1} (T1 = control), ($NiCl_2 \cdot 6H_2O$) 300 mg L^{-1} + HA (T2), or ($NiCl_2 \cdot 6H_2O$) 300 mg L^{-1} + SNP (T3). HA were used at 46 mg L^{-1} (Garcia et al., 2012) and SNP at 7.2 mg L^{-1} (Zhao et al., 2013). The experiments were carried out in a glasshouse under natural daylight (September to December 2014) with temperatures in the range of 20 to 30°C. The Hoagland solution with Ni was changed weekly, and the total volume was completed with water once a week. Roots and leaves were collected after 30 days and stored at -80°C for further analysis.

Total RNA isolation

Total RNA was extracted according to Gao et al. (2001) method

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Table 2. Sequences of the primers used for real-time PCR.

Oligo name	Length (pb)	Tm (°C)	Sequence (5`-3`)
APx			
OsAPx1 mRNA F	20	64.8	5'GCGACTACAAAGGGAGGGTC3'
OsAPx2 mRNA R	20	64.3	5'TTAGATCCAGGCTCCTGTGC3'
GR			
OsGr2 mRNA F	24	64.3	5'GGTGATGAACCTACCAAACCAGAT 3'
OsGr3 mRNA R	19	66.9	5' GGGTGGTTGGGAGAAAACG 3'
SOD			
OsSOD3 mRNA F	20	66.8	5' CTCCAGAGCGCCATCAAGTT 3'
OsSOD5 mRNA R	23	63.3	5'TCCAGAAGATCGAATGATTGACA 3'

($LiCl_2$) using an NTES buffer (0.2 M Tris-HCl pH 8.0; 25 mM EDTA, 0.3 M NaCl; 2 % SDS). Leaves and roots samples were ground in N_2 and homogenized in a mixture containing 4.5 mL NTES buffer and 3 mL of phenol:chloroform (1:1). Homogenized samples were centrifuged at 12,000 × *g* for 10 min at 4°C and supernatant was transferred to a new tube. Total RNA was precipitated by adding 1/10 volume of 2 M sodium acetate pH 4.8 ($NaOAc_{DEPC}$) and one volume of cold isopropanol. Samples were placed at -20°C for 2 h and then centrifuged at 12,000 × *g* for 10 min. Pellets were re-suspended in 2.5 mL of H_2O-DEPC and precipitated again by the addition of 2.5 mL of 4 M lithium chloride pH 4.8 ($LiCl_2$-DEPC). After centrifugation at 12,000 × *g* for 10 min, pellets were washed with 70% ethanol and dissolved in 0.1 mL H_2O-DEPC. RNA was then stored at -80°C until use. The RNA concentration was determined using a NanoDrop Spectrophotometer (NanoDrop 1000 Thermo Fisher Scientific) prior a complementary DNA (cDNA) experiment.

cDNA first strand synthesis

The first strand of cDNA was synthesized using a Taq Man Reverse Transcription kit (Applied Biosystems, Inc.) A polymerase chain reaction (PCR) of three sequential steps of one cycle was performed at 42°C for 5 min, 50°C for 50 min and another at 70°C for 15 min.

Analysis of the expression levels by real time-PCR

The differential expression of some genes involved in oxidative stress response [Ascorbate peroxidase (OsAPx1, OsAPx2), glutathione reductase (OsGR2, OsGR3) and superoxide dismutase (OsSOD3, OsSOD5) was confirmed by real-time PCR. The primers were designed using Primer Express 2.00 (Applied Biosystems software), based on sequences retrieved from the National Center of Biotechnology Information (NCBI) database. Primers sequences specificity and melting curve of the final PCR reaction were analyzed through TIGR (http://rice.plantbiology.msu.edu) and with NCBI (http://www.ncbi.nlm.nih.gov). All primers used are listed in Table 2. RT-PCR was performed using a Platinum®SYBR® Green qPCR Super MIX-UDG (Invitrogen) in a reaction mixture of 20 µL containing: 0.5 µL of each primer (10 pmol·L^{-1}) (Table 2), 10 µL of SYBR Green qPCR Super MIX-UDG, 2 µL of cDNA and 7 µL of RNase-free water, with 48-wells plates and the standard cycling program. PCR reactions were as follows: 10 min at 95°C, 40 amplification cycles at 95°C for 15 s and 60°C for 1 min (annealing, extension and fluorescence detection), followed by the "melting curve" accomplished by the increase in temperature at intervals of

0.3°C, from 60 to 95°C in order to verify the specificity of the reaction. Reaction conditions (10 mL volumes) were optimized to increase PCR efficiency by changing the primer concentration and annealing temperature to minimize primer-dimer formation. The absence of primer-dimers or accumulation of non-specific products was checked by melting-curve analysis. PCR efficiency was determined through a standard curve with serial dilutions of cDNA using Actin primer. The housekeeping gene Actin was used as an internal reference for normalization of gene as the "driver". Each sample was analyzed in triplicated. The rate of gene expression was calculated using the delta-delta CT (ΔΔCT) method (Livak and Schmittgen, 2001). At first, the threshold cycles (CT) of the duplicate PCR results of each gene were averaged and used for quantification of the transcripts. Then, the average of the CT value of the Ubiquitin (*UBQ5*) gene was subtracted from the average of the CT value of the target gene to obtain the ΔCT value. The 2ΔΔCT value was given to estimate the relative expression rate of each gene. Each value was obtained from two independent experiments. A standard deviation was given to each value and the results were analyzed by the Student's t-test. A P-value of ≤0.05 was considered significant.

RESULTS

Phenotypical differences between treatments

Reductions in plants growth and chlorosis of leaves was observed in T1 (Figure 1A) while in T2 (Ni 300 mg L^{-1}+ HA) phenotypical characteristics of rice seedling like plant growth and area of leaves were better (Figure 1B) compared to T1 and T3 (Figure 1C). In T2 (nickel 300 mg L^{-1}+ HA) plants grew well, leaves showed normal size and green color, maybe because HA improves plant development in environments polluted with heavy metals and also reduces availability and mobility of heavy metals in the soils.

Analysis of the expression levels of ascorbate peroxidase (OsAPx1, OsAPx2), glutathione reductase (OsGR2, OsGR3) and superoxide dismutase (OsSOD3, OsSOD5) by real time PCR

Real Time PCR analysis showed different expression
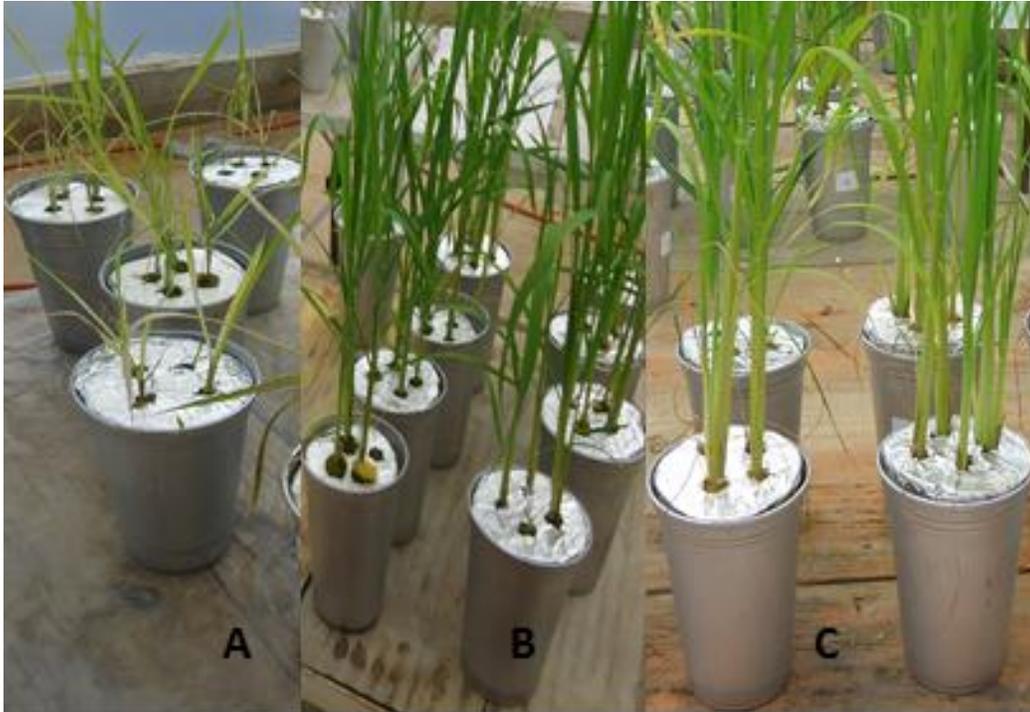
Figure 1. Phenotypical characteristics of rice (*O. sativa*) seedlings. **A,** Hoagland's nutrient solution + Ni 300 mg·L^{-1} (T1). **B,** Ni 300 mg·L^{-1} + HA (T2). **C.** Ni 300 mg·L^{-1} + SNP (T3).

profiles of APx, GR and SOD in roots and leaves of rice variety Tetep (Figure 2A, B, C). According to Figure 2A, there was a significant expression of ascorbate peroxidase (APx) in roots in T3 (nickel 300 mg L^{-1}+ SNP), indicating the possible toxicity of SNP, while in T1 (control only with Ni) and T2 (nickel 300 mg L^{-1}+ HA) expression in this organs was lower. Significant differences in leaves for this variable were not found. The expression of GR is shown in Figure 2B. Significant differences were found, being the expression of this gene in T2 higher in roots compared to T1 and T3. In leaves, the expression of this gene was similar in all treatments. The induction of SOD was similar in root and leaves being significantly higher in T3 (nickel 300 mg L^{-1} + SNP) and lower in T2 (nickel 300 mg L^{-1} + HA) in both organs analyzed.

DISCUSSION

In this research, the effect of HA and SNP in rice plants with Ni heavy metal was investigated according to the expression of some important gene related with oxidative stress in plants. Several studies showed that the toxic effect of Ni causes various physiological alterations and diverse toxicity symptoms such as chlorosis and necrosis in a variety of plant species (Zornoza et al., 1999; Pandey and Sharma, 2002), and specifically in rice (Samantaray et al., 1997). The toxic effects of Ni and some other

heavy metals are manifested first by the inhibition of plant growth (Seregin et al., 2003; Nagajyoti et al., 2010) as was shown in this research where rice plants showed growth affectation with Ni at 300 mg·L^{-1} (Figure 1A). Ni has strong influence on metabolic reactions in plants, and it can induce reactive oxygen species (ROS) that can lead to oxidative stress (Sreekanth et al., 2013). The application of humic substances to plants has been proved to stimulate their biochemical-physiological mechanisms, growth and development. Humified materials exhibit structural characteristics that allow interactions with heavy metal cations dissolved in aqueous environments (Garcia et al., 2012). APx and SOD expression was lower in root when HA was used, possibly because HA is considered as organic matter and contributes to change a particular form of toxic elements, in this case Ni.

SNP is a chemical compound used as Nitric oxide (NO) donor (Beligni and Lamattina, 2002). Several studies have shown the protective effect of NO against abiotic stress and also its mediated reduction of ROS in plants (Hsu and Kao, 2004). However, in this study with Ni as a heavy metal, the oxidative stress increased with this substance significantly in roots of rice plants, as evidence of the high expression of APx and SOD. APx gene expression in the leaves of rice plants was higher than in roots in all treatments. In roots, significant values were found in T3 compared to the other two treatments where exogenous SNP significantly induced activities of APX.
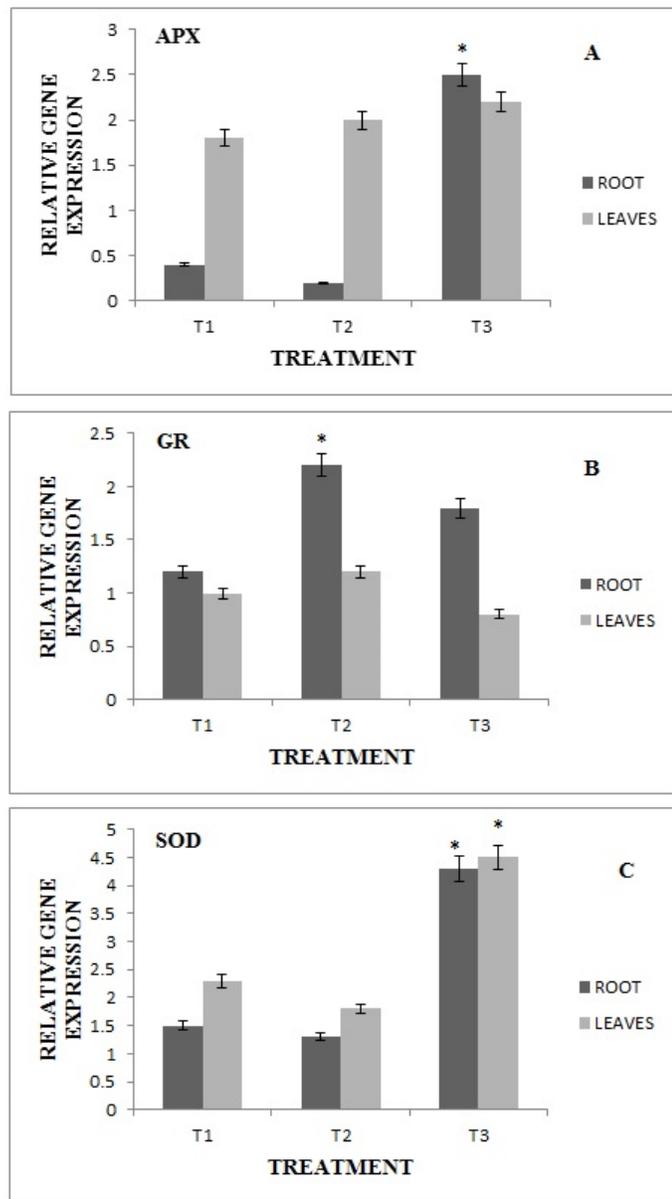
Figure 2. Relative expression of transcriptionally-ascorbate peroxidase (APx). **(A)** glutathione reductase (GR). **(B)** Superoxide dismutase (SOD). **(C)** Rice seedlings grown in different treatments in root and leaves. Each value is the mean ± standard error of three replicates. Data points marked with asterisk (*P ≤ 0.05) indicate statistically significant differences.

This discrepancy in expression for the OsAPx genes might be due to differences in organs (Hong et al., 2007).

In leaves, APx activity increased in all treatments because cytosolic APx (OsAPx1, OsAPx2) is essential for oxidative protection of chloroplasts against stress (Miller et al., 2007; Koussevitzky et al., 2008; Maruta et al., 2010). Also, the H_2O_2 quantity significantly increased in wheat leaves under Ni-stress (Gajewska and Skłodowska, 2007); this behavior can be extrapolated to rice because

both plants belong to the same family (grasses). Ascorbate peroxidase (APX) activities increased in leaves under Ni-stress because this enzyme may play a significant role in the cleaning of H_2O_2 from the leaves of Ni-stressed plants (Gajewska and Skłodowska, 2007); this can explain why the expression of this gene is higher in leaves even with HA and SNP.

According to Shigeoka et al. (2002), APX activity generally increases in response to environmental stress as occurred in this study at a molecular level. Normally, APx2 in *Arabidopsis thaliana* L. is inducible mainly under extreme light or heat stress conditions (Karpinski et al., 1999; Panchuk et al., 2002) but in this research OsAPx gene is inducible by Ni heavy metal. The cDNA of OsGR2 was first isolated in 1998. Subcellular fractionation showed that the OsGR2 protein is localized primarily in cytosol; mRNA and protein of OsGR2 were observed mainly in roots and calli but little in leaf tissues (Kaminaka et al., 1998); that is why the activity is higher in this tissue (roots). Together with OsGS1, OsGR3 is a specific *Poaceae Isoform* targeted to chloroplasts and mitochondria (Tsung-Meng et al., 2013). One GR cytosolic isoform (OsGR2) and two chloroplastic isoforms (OsGR1 and OsGR3) have been identified in rice (Rouhier et al., 2006; Bashir et al., 2007). The lower expression of GR in T1 (sand with Ni solution) may be caused by the possible temporal expression of this gene, mainly transitional. However, this response also could be a consequence of GR-related transcriptional activity gene (Perez et al., 2013).

Recently, it was reported that HA applied to the roots of rice plants stimulated several enzymatic mechanisms associated with the antioxidative defense system (Garcia et al., 2012), as shown in Figure 2B where this substance increases GR expression in rice roots, but this increase indicates oxidative stress. Tsung-Meng et al. (2013) suggested that, through an expression analysis the involvement of OsGR3 is in response to salt stress and salicylic acid (SA), a signal molecule of systemic resistance. They also showed that OsGR3 is a functional GR. From studies using transgenic plants, it has been proved that GR plays a prominent role in conferring resistance to oxidative stress caused by drought, ozone, heavy metals, high light, salinity, cold stress, etc. There has been found an enhanced GR activity in *A. thaliana*, *Vigna mungo* L., *Triticum aestivum* L., *Capsicum annuum* L. and *Brassica juncea* L. after cadmium treatments. Sharma and Dubey (2005) have found an increased GR activity in *O. sativa* seedlings during drought conditions. All these results show the role of this enzyme in plant protection against abiotic stress. The significant increase in GR gene expression found in rice roots with Humic Acid (T2) means an increase in oxidative stress. In this case HA and SNP enhance the expression of this gene mainly in roots, meaning that the aim to diminish Ni toxicity with these products was not accomplished.

In this research, the involvement of OsGR2 and OsGR3

in relation to Ni stress is suggestive. Further functional studies are required to clarify whether OsGR2 and OsGR3 are involved in heavy metal-stress tolerance. The activation of SOD could be useful to reduce O_2^- accumulation, decrease H_2O_2 and alleviate some heavy metals stress (Wang et al., 2008). The expression of SOD (Figure 2C) increases significantly in T3 for both organs (roots and leaves) revealing the presence of oxidative stress. Some researches obtained positive results with the use of SNP, like Yu et al. (2013) who found that in cucumber plants, exogenous application of SNP increases the antioxidant capacity in this crop. Also, Zhao et al. (2013) in rice under cadmium (Cd) toxic conditions demonstrated that applications of SNP may protect rice seedlings from Cd stress (Zhao et al., 2013). The concentration of SNP used in this research, maybe the interaction with Ni, induced increases in gene expression related with antioxidant enzymes. Bai et al. (2015) used SNP in rice seedlings at different concentrations in the presence of lead (Pb) and they found that Pb- induced oxidative damage was reduced with 50, 100 and 200 µM of SNP however, 400 µM of SNP had no obvious alleviating effect in Pb toxicity. These authors demonstrated the effect of SNP concentration on heavy metal toxicity. In this specific case, Ni effects may not be mediated only by oxidative stress, but by some additional mechanisms susceptible to NO.

Conclusion

Exogenous HA and SNP application had no effect on diminishment of Ni toxicity in rice var. Tetep at molecular level. Based on the results, it can be concluded that the effects of SNP did not alleviate Ni stress in rice, may be due to NO from it, and the mechanism and interaction of $NiCl_2$ and SNP should be further investigated.

Conflict of interests

The authors did not declare any conflict of interest.

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