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Regular consumption of a silicic acid-rich water prevents aluminium-induced alterations of nitrergic neurons in mouse brain: histochemical and immunohistochemical studies

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Summary. Silicon is not generally considered an essential nutrient for mammals and, to date, whether it has a biological role or beneficial effects in humans is not known. The results of a number of studies suggest that dietary silicon supplementation might have a protective effect both for limiting aluminium absorption across the gut and for the removal of systemic aluminium via the urine, hence, preventing potential accumulation of aluminium in the brain. Since our previous studies demonstrated that aluminium exposure reduces the number of nitrergic neurons, the aim of the present study was to compare the distribution and the morphology of NO-containing neurons in brain cortex of mice exposed to aluminium sulphate dissolved in silicic acid-rich or poor drinking water to assess the potential protective role of silicon against aluminium toxicity in the brain. NADPH-d histochemistry and nNOS immunohistochemistry showed that high concentrations of silicon in drinking water were able to minimize the impairment of the function of nitrergic neurons induced by aluminium administration. We found that silicon protected against aluminium-induced damage to the nitrergic system: in particular, we demonstrated that silicon maintains the number of nitrergic neurons and their expression of nitrergic enzymes at physiological levels, even after a 12 and 15 month exposure to aluminium.

Key words: Aluminium, Silicon, Brain, Nitrergic neurons

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Introduction

With a share of approximately 8.1%, aluminium (Al) is the most abundant metal of the Earth's crust and the third most common element overall after oxygen and silicon (Exley, 2003). Next to this high natural abundance, the industrial use of Al has recently increased because of its advantageous material properties (Rossbach et al., 2006): the widespread use of products made from or containing Al is ensuring the presence of this metal in our bodies (Exley, 2009a). Al has been described as omnipresent since it can be found in measurable quantities in food, soil, air and drinking water (Solfrizzi et al., 2003): typical adult dietary Al intake is 5 to 10 mg/day, mostly from food (Pennington and Schoen, 1995), whereas drinking water provides about 1% of normal daily Al intake (Deschamps et al., 2009). Al accumulates in the body with age and particularly so when exposure is high and/or protective gastrointestinal mechanisms are bypassed or renal function is impaired (Kisters et al., 1999).

Al toxicity in humans, even at low levels of exposure (Exley, 2009b), is a well-established fact and the brain is a target organ for Al to exert its deleterious effects (Exley et al., 1996; Exley, 1999; Yokel et al., 1999). The molecular mechanisms of Al neurotoxicity are not completely understood: Al has been reported to alter the blood-brain barrier (Zatta et al., 2003) and is deposited in the human brain (Exley and House, 2011). Even if Al is not a redox metal and, hence, it cannot itself initiate any oxidation/reduction reactions (Yang et al., 1999), it is able to induce oxidative damage by an indirect mechanism because of its ability to potentiate the peroxidative effects of iron (Golub et al., 1999) and also enhance pro-oxidant properties of several transition

metals, like copper or chromium (Bondy et al., 1998). Al exposure has been reported to induce oxidative stress by inflicting damage to membrane lipids, proteins and anti-oxidative enzyme defense system (Exley, 2004; Jyoti et al., 2007).

Moreover, a relationship between high levels of Al and increased risk of some neurodegenerative disorders, including Alzheimer's disease (AD), has been suggested (Exley, 2001; Becaria et al., 2002; Yokel, 2006): the possibility of such relation was proposed first in epidemiological studies reporting that Al in drinking water is associated with geographic prevalence of AD (Martyn et al., 1997). Moreover, some researchers demonstrated the presence of Al in both senile plaques and neurofibrillary degeneration in brain of AD patients (McLachlan et al., 1996) suggesting a role for this metal in the etiopathogenesis of AD.

Some reports showed that Al interferes with glutamatergic neurotransmission (Provan and Yokel, 1992), impairing the neuronal glutamate (Glu)- nitric oxide (NO)- cyclic monophosphate guanosine (cGMP) pathway *in vivo* (Cucarella et al., 1998). The involvement of NO in a number of neurological disorders is a well established fact (Steinert et al., 2010). In recent years, researchers have demonstrated that NO could be involved in many pathological mechanisms related to AD. The ability of NO to exert cellular oxidative damage is probably its primary neurotoxic mechanism. Nevertheless, NO has also been found to function as a neuronal messenger molecule, whose biological function in the brain is of major importance (Förstermann and Sessa, 2012). NO is involved in crucial physiological events, such as neurotransmitters release (Paul and Ekambaram, 2011), long-term potentiation (Taqatqeh et al., 2009) and different mechanisms of synaptic plasticity, including those that play important roles in learning and memory (Paul and Ekambaram, 2011) as well as in some pathological events underlying neurotoxicity (Steinert et al., 2010). The presence of stimuli that lead to NO overproduction will probably cause neuronal damage. Al, for instance, is known to cause neurotoxicity and death of neurons, in particular nitrergic neurons by inducing glutamatergic excitotoxicity and consequently NO overproduction, (Cheng et al., 2008). This kind of alteration in Glu-NO-cGMP pathway can cause a reduction of nitrergic neurons and neuronal nitric oxide in some cerebral cortical areas in animals (Hermenegildo et al., 1999); similar findings have been revealed in brain of AD patients (Tao et al., 1999; Gargiulo et al., 2000).

A decrease of nitrergic transmission has been already observed by our group in rat brains chronically treated with Al sulphate (Rodella et al., 2001, 2006). In particular, we demonstrated that chronic Al oral exposure significantly reduced NADPH-diaphorase (NADPH-d) activity of rat cerebral cortex and cerebellum (Rodella et al., 2001) and that the number of nNOS expressing neurons in the somatosensory cortex (SSC) decreased proportionally to the duration of Al treatment (Rodella et al., 2006). These findings further strengthened the hypothesis that Al in drinking water could be an etiological factor in AD (Exley and Esiri, 2006; Rodella et al., 2008), supporting previous epidemiological studies (Becaria et al., 2002; Yokel, 2006).

In contrast, while the health benefits of Silicon (Si), with particular regard to skeletal status and function, are widely recognised, the mechanism of Si essentiality remains to be confirmed (Exley, 2009a). Si is the second most abundant element in the Earth's crust and, after carbon, shows the most diverse chemistry (Dobbie, 1982). In man, Si is present in blood at levels similar to other physiologically important elements (i.e. iron, copper and zinc) (Dobbie and Smith, 1982), but whether Si has a biological role is not known (Exley, 1998). The main route of entry of Si to the body is the gastrointestinal tract. Silicic acid is the most available source of Si to man; it is ubiquitous in the diet and natural waters (Bellia et al., 1994) and, unlike crystalline silica, it has no associated toxicity (Jugdaohsingh et al., 2000). Silicic acid is able to limit Al neurotoxicity and it has been proposed as a geochemical control of the biological availability of the metal (Exley and Birchall, 1992; Exley, 1998; Parry et al., 1998). It has been suggested that silicic acid can reduce Al oral absorption, enhance Al excretion and protect against Al adverse effects, facilitating the urinary elimination of Al, perhaps by interacting with filterable Al in renal tubules, forming hydroxyaluminosilicates and impeding reabsorption of the metal (Reffitt et al., 1999; Exley et al., 2006). However, there is currently very little research regarding the possible beneficial effects of Si on neural toxicity, on the antioxidant system and on stress markers of certain neurological disorders (Domingo et al., 2011).

Since Al exposure was demonstrated to reduce nitrergic neurons, the aim of this study was to compare the distribution and the morphology of NO-containing neurons in brain cortex of mice exposed to Al sulphate dissolved in silicic acid-rich or silicic acid-poor drinking water to assess the potential protective role of Si against Al toxicity in the brain.

Materials and methods

Animal parameters

Forty male C57BL/6 mice, 5 weeks old at the beginning of the experimental protocol (0 months) (21.05±1.65 g b.w.) were used in this study. They were housed in a controlled environment, including a regular 12-h light/dark cycle and constant temperature (20±1°C). Food and water were provided *ad libitum*. For each group, body weights and daily water consumption of the mice were monitored three times a week. Averages of body weight and water consumption measurements for each group at the beginning (0 months) and end of the experiments (12 months and 15 months) were calculated.

Animal treatments

Two different kinds of mineral water were used as they are commercially available, or, in the treated groups, as a solvent for aluminium and given to the mice as their source of drinking water: 1) Silicic acid-poor drinking water, which contains low Si levels (SiO_2 10 mg/L, Si<5 mg/L); 2) Silicic acid-rich drinking water, which contains high Si levels (SiO_2 86 mg/L, Si<43 mg/L).

The chemical composition of the waters is described in Table 1.

Neither water contained a significant concentration of Al. Aluminium treatment was prepared by dissolving 25 g/L aluminium sulphate hexadecahydrate ($Al_2(SO_4)_3 \bullet 16H_2O$) (Fluka Analytical, Sigma-Aldrich Chemie GmbH, GERMANY), to give a final concentration of 0.04M aluminium sulphate. Once prepared, mineral waters with Al supplementation were left for at least two days to equilibrate, before being given to the mice. Each treatment was replaced three times a week to reduce loss of aluminium through precipitation. The pH of the different drinking water treatments are given in table (Table 2).

Animal groups

The animals were divided in 8 groups (5 animals per group): GROUP 1: mice treated with the silicic acid-poor mineral water for 12 months; GROUP 2: mice treated with silicic acid-rich mineral water for 12 months; GROUP 3: mice treated with the silicic acid-poor mineral water plus 0.04M aluminium sulphate for 12 months; GROUP 4: mice treated with silicic acid-rich mineral water plus 0.04M aluminium sulphate for 12 months; GROUP 5: mice treated with the silicic acid-poor mineral water for 15 months; GROUP 6: mice treated with silicic acid-rich mineral water for 15 months; GROUP 7: mice treated with the silicic acid-poor mineral water plus 0.04M aluminium sulphate for 15 months; GROUP 8: mice treated with silicic acid-rich mineral water plus 0.04M aluminium sulphate for 15 months.

At the end of treatments, all the animals were anaesthetized with sodium pentobarbital (40 mg/kg i.p.) and transcardically perfused with saline followed by 4% paraformaldehyde in phosphate buffer 0.1M, pH 7.4. Every effort was made to minimize animal suffering and the studies were performed according to Italian Law on the protection of laboratory animals. All the experimental procedures were approved by the Italian Ministry of Health.

Tissue processing

After fixation, brains were removed, post-fixed in 4% paraformaldehyde in phosphate buffer for 2h, washed in phosphate buffer 0.1M and cryoprotected in 30% sucrose at 4°C for 24h. Frozen serial cortical sections (30 μm thick) from each animal were cut using a cryostat between the bregma -3.28 and bregma +4.84 according to Franklin and Paxinos atlas of mouse brain (Franklin and Paxinos, 1997) for localizing the SSC and then collected in phosphate-buffered saline.

Alternate sections were processed either for the histochemical analysis of the nitrergic system or the immunohistochemistry reaction against nNOS, a specific marker commonly used to visualize nitrergic neurons, or incubated with Toluidine blue for morphological control.

NADPH-diaphorase histochemistry

Five randomly selected sections per animal were stained. Briefly, freely-floating cryostat sections were pre-treated with 0.1% Triton X-100 in phosphate buffer for 20 min and then incubated for 1h in the dark with NADPH-d reaction mixture containing 0.1 mg/mL nitroblue of tetrazolium (NBT) (Sigma, St. Louis, MO, USA) previously dissolved in 0.5 mL dimethyl-formamide (Sigma, St. Louis, MO, USA), 1 mg/mL ß-NADPH (Sigma, St. Louis, MO, USA) and 0.01% Triton X-100 in phosphate buffer pH 7.4. The sections were then rinsed in a phosphate buffer, mounted on slides and dehydrated. Control sections were stained identically except for the omission of the substrate: in this way, the specific NADPH-d reaction was eliminated in the

Table 1. Chemical composition of the mineral waters used in the experimental protocol of this study, conducted respectively by the University of Pavia, Department of General Chemistry (30th June 2008) for the silicic acid-poor drinking mineral water and by the University "Federico II" of Napoli, Department of Preventive Medicine, Section of Hygiene (22nd June 2008) for the silicic acid-rich drinking mineral water.

	Silicic acid-poor drinking water	Silicic acid-rich drinking water
Carbon dioxide	31 mg/L	2310 mg/L
Bicarbonate	305 mg/L	1403 mg/L
Calcium	85 mg/L	365 mg/L
Silica	10 mg/L	86 mg/L
Potassium	1 mg/L	52 mg/L
Magnesium	28 mg/L	18 mg/L
Nitrate	3 mg/L	5 mg/L
Sodium	3 mg/L	50 mg/L
Chloride	2 mg/L	20 mg/L
Sulphate	87 mg/L	3 mg/L
Fluoride	0.05 mg/L	1.1 mg/L
Salinity (at 180°C)	384 mg/L	1300 mg/L
Conductivity (at 20°C)	547 uS/cm	1810 uS/cm

Table 2. pH values of each mineral water used in the study.

WATER	pH
Silicic acid-poor drinking water	7.4
Silicic acid-rich drinking water	6.1
Silicic acid-poor drinking water + Aluminum sulphate 0.04M	3.2
Silicic acid-rich drinking water + Aluminum sulphate 0.04M	3.5

control sections.

Neuronal NOS immunohistochemistry

Five randomly selected sections per animal were stained. Briefly, the sections were incubated in normal goat serum (10% in phosphate-buffered saline (PBS) containing 0.1% Triton X-100) for 30 min and then incubated in rabbit polyclonal primary antibody directed against nNOS (Chemicon, USA) diluted 1:1000 in PBS containing 3% normal goat serum and 0.1% Triton X-100, for 24h at 37°C. After incubation in the primary antibody, the sections were sequentially incubated in biotinylated goat anti-rabbit immunoglobulins (IgG) and avidin-biotin peroxidase complex (Vector Labs., Burlingame, CA, USA). The reaction product was visualized using hydrogen peroxide (3%) and diaminobenzidine (DAB 5mg/10ml PBS) (Sigma, St. Louis, MO, USA) as chromogen. The immunohistochemistry control was performed by omitting the primary antibody and in the presence of isotype matched IgGs: in this way, the specific nNOS reaction product was eliminated in the control sections. In the reaction, nNOS staining appeared brown, due to the diaminobenzidine (DAB) reaction product.

Neuronal NOS immunofluorescence

Five randomly selected sections per animal were stained. Briefly, the sections were incubated in 1% bovine serum albumin (Sigma Aldrich, St. Louis, MO, US) for 2h at room temperature. Successively, the sections were incubated with polyclonal rabbit primary antibody against nNOS (Chemicon, US) diluted 1:1000 in BSA 1% for 1h at room temperature and then overnight at 4°C. Then, sections were labeled using 1:200 diluted anti-rabbit Alexa Fluor 555 conjugated secondary antibodies (Invitrogen, UK). Finally, the samples were counterstained with DAPI, mounted and observed with a confocal microscope (LSM 510 Zeiss, Germany) using a 40x magnification objective. The immunofluorescent control was performed by omitting the primary antibody and in the presence of isotype matched IgGs.

Semi-quantitative and quantitative analyses

Size of nNOS positive neurons

The cross-sectional areas of nNOS positive cell bodies were measured in the SSC of each mouse section using an Olympus light microscope (Olympus, Germany) (x1000), connected to an image analyser (Image Pro Plus, Milan, Italy); 100 randomly selected neurons were measured in each experimental group.

NADPH-d and nNOS positive neuron counting

NADPH-d and nNOS positive neurons in the SSC were counted using an Olympus light microscope (Olympus, Germany) (x100), connected to an image analyser (Image Pro Plus, Milan, Italy). The nitrergic neurons in the SSC were counted in randomly selected cortical areas (5 selected fields for each section, and 5 sections per animal). NADPH-d and nNOS positive neurons were scored if they showed cytoplasmic staining and a clear unstained nucleus. To compare the data, the number of nNOS positive neurons in the SSC were referred to a standardized area (0.5 mm^2). All the counting was done by two different investigators unaware of animal group assignment.

Semi-quantitative and quantitative integrated optical density evaluation of nNOS staining

For the evaluation of the intensity of nNOS staining, all samples were analyzed and semi-quantitatively scored blindly. Cytoplasmic staining intensity was graded as non detected (N.D.) when staining was completely absent; (+/-) when staining was very weak; (+) when staining was weak; (++) when staining was moderate; (+++) when staining was strong.

Successively, the intensity of nNOS staining was also computed as integrated optical density (IOD) and measured in 100 nNOS positive neurons for each experimental group. The neurons were randomly selected by two different investigators unaware of the animal group assignement and their boundaries were manually traced before being measured. Digitally fixed images of the neurons at high magnification (x400) were analysed under optical microscope (Olympus, Germany) equipped with an image analyser (Image Pro Plus, Milan, Italy) that automatically calculated the IOD referred to a standardized area and the area.

Statistical analyses

The results were presented as means ± standard error (S.E.) of values. Statistical analyses were performed using one-way ANOVA for parametric results and Bonferroni's test for multiple comparisons. Differences among groups were considered significant at $P<0.05$.

Results

Animal parameters

Data about animal body weights and daily amounts of water consumption were measured for each experimental group and summarized in Figure 1A and 1B respectively. Neither body weights nor daily amount of water consumption showed significant differences among the experimental groups (Fig. 1A,B).

No animals died in any of the treatments, showing that oral 0.04M aluminium sulphate administrated in this study is under the lethally toxic dose for this metal in mice. Moreover, we did not observe particular behavioural alterations among the animal groups (in

particular no differences in food consumption, no cage behavioural disorders and no aggressiveness due to aluminium treatment were observed).

Morphology and size of nitrergic neurons

NADPH-d staining revealed a number of scattered, medium-size, heavily-stained neurons in the SSC (Fig. 2) of all animals, showing very clear cellular processes and clearly unstained nuclei. The immunohistochemical demonstration of nNOS revealed a pattern of staining that quantitatively and qualitatively overlapped NADPH-d histochemistry, showing clearly brown-stained neurons scattered within the SSC, with brown major dendritic processes and cell nuclei free of staining. From a morphological point of view, we found that the population of nitrergic neurons in SSC was composed of neurons having various shapes. In particular, we observed multipolar, as well as bipolar and triangular neurons with no differences among the experimental groups.

Moreover, comparing NADPH-d/nNOS staining with parallel sections processed for a standard morphological staining (Toluidine Blue staining), we found that approximately 1% of the total number of neurons of the SSC were clearly NADPH-d/nNOS positive.

Finally, we evaluated the size of the cell body of nNOS positive neurons (measured as cross sectional area) to assess whether different treatments administered to the mice could induce alterations in nitrergic body size (Fig. 3). We showed a significant increase ($P<0.05$) in body size of SSC nitrergic neurons of mice treated with silicic acid-rich mineral water (Fig. 3A (b) and (d)), both with or without aluminium sulphate (respectively GROUP 4,8 and GROUP 2,6), if compared with silicic acid-poor mineral water treated groups (GROUP 1,3,5,7) (Fig. 3A (a) and (c)), independently of the duration of the treatment (12 or 15 months). Quantitative data about cross sectional areas of nNOS positive neurons from the different groups are shown in Fig. 3B.

Nitrergic neuron counting

The count of SSC positive neurons to NADPH-d histochemical analysis revealed a clear and significant ($P<0.05$) decrease of the number of nitrergic neurons in silicic acid-poor mineral water + aluminium sulphate 0.04 M (GROUP 3, 7) (Fig. 4A (c)) and silicic acid-rich mineral water + aluminium sulphate 0.04 M (GROUP 4, 8) (Fig. 4A (d)), if compared with silicic acid-poor mineral water (GROUP 1, 5) (Fig. 4A (a)) and silicic acid-rich mineral water (GROUP 2, 6) (Fig. 4A (b)) treated mice.

In particular, this reduction was more consistent between groups treated with silicic acid-poor mineral water with and without the aluminium sulphate 0.04M addition. In groups 3, 7 the reduction of NADPH-d positive neurons was about 30% with respect to those counted in groups 1, 5. In silicic acid-rich mineral water + aluminium sulphate 0.04M groups (GROUP 4, 8), instead, it was not possible to see such a considerable

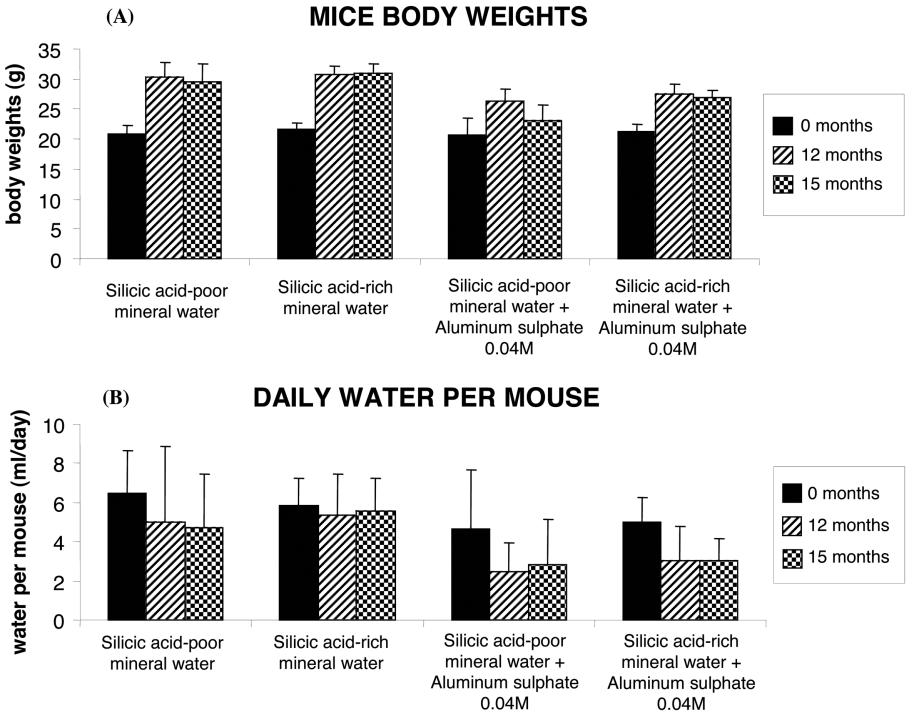

Fig. 1. Data about animals body weights **(A)** and daily amount of water consumption per mouse **(B)** for each experimental group.

reduction of nitrergic neurons with respect to GROUPS 2, 6, even if the diminution was significant as well, around 13-14% (Fig. 4B).

Moreover, comparing mice which received the same treatment for different experimental times, we observed no statistical differences between the number of neurons counted at 12 and 15 months of treatment (GROUP 1 vs 5; GROUP 2 vs 6; GROUP 3 vs 7; GROUP 4 vs 8) (Fig. 4B).

Finally, no significant differences were seen between mice which received silicic acid-poor mineral water and mice administered with silicic acid-rich mineral water (GROUP 1 vs 2; GROUP 5 vs 6), suggesting that Si addition to water could not cause alterations in the number of nitrergic neurons itself.

nNOS immunohistochemistry confirmed the results about the number of nitrergic neurons obtained with NADPH-d analysis either after 12 months or 15 months of treatment.

Semi-quantitative and quantitative integrated optical density evaluation of nNOS staining

First of all, we analyzed the expression of nNOS in the SSC nitrergic neurons of all experimental groups giving a semi-quantitative evaluation of the staining (Table 3). From first observation, we underlined that in all the experimental groups nNOS immunostaining within the neurons was diffuse but exclusively restricted to the cytoplasm, while nuclei showed no immunohistochemical signals (N.D.).

In 12 months silicic acid-poor mineral water + aluminium sulphate 0.04 M treated mice (GROUP 3), a weak intensity of nNOS staining could be observed, especially if compared with 12 months silicic acid-poor mineral water treated mice (GROUP 1) in which a strong immunohistochemical signal was detected. A very weak staining could be also described for 15 month silicic acid-poor mineral water + aluminium sulphate 0.04 M treated mice (GROUP 7), when it was compared to the strong intensity of the staining in 15 month silicic acid-poor mineral water treated mice (GROUP 5).

Moreover, a slight difference between the immunohistochemical signals could also be found in 15 month silicic acid-rich mineral water + aluminium sulphate 0.04 M treated mice (GROUP 8), in which the intensity was moderate with respect to 15 month silicic acid-rich mineral water treated mice (GROUP 6), in which we found a strong staining.

In confirmation of the semi-quantitative analysis, nNOS expression was also evaluated by measuring the values of integrated optical density (IOD). IOD measurements of nNOS confirmed the previous semi-quantitative analysis, showing a decrease of staining intensity exclusively in the cytoplasm of SSC nitrergic neurons of mice treated with silicic acid-poor mineral water + aluminium sulphate 0.04 M administered for 12 (IOD= 22.62±0.47) and 15 months (IOD= 17.86±1.37) compared to groups treated with silicic acid-poor mineral water without aluminium for 12 months (IOD= 35.07±1.68) and 15 months (IOD= 28.76±0.82). The

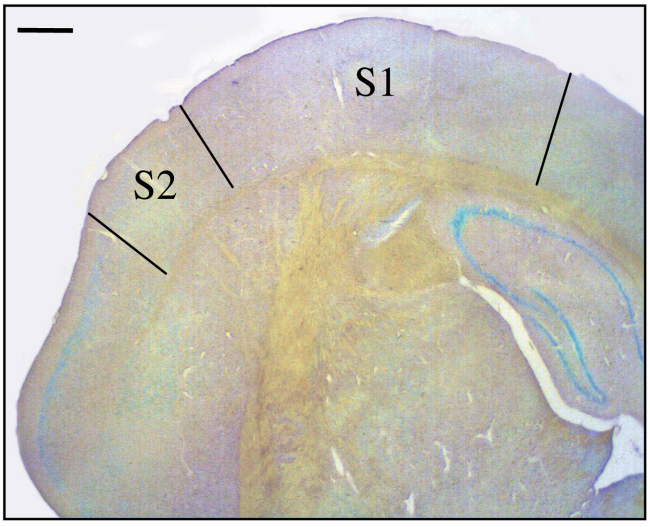

Fig. 2. Low magnification of the SSC region of mouse brain stained with Toluidine Blue. (S1: Primary somatosensory cortex; S2: Secondary somatosensory cortex). Scale bar: 1000 μm.

Table 3. Semiquantitative analysis of nNOS staining intensity observed in the different experimental groups.

GROUP	CYTOPLASMIC STAINING	NUCLEAR STAINING
Silicic acid-poor drinking water 12 months (GROUP 1)	+++	N.D.
Silicic acid-rich drinking water 12 months (GROUP 2)	+++	N.D.
Silicic acid-poor drinking water + Aluminum sulphate 0.04M 12 months (GROUP 3)	+	N.D.
Silicic acid-rich drinking water + Aluminum sulphate 0.04M 12 months (GROUP 4)	+++	N.D.
Silicic acid-poor drinking water 15 months (GROUP 5)	+++	N.D.
Silicic acid-rich drinking water 15 months (GROUP 6)	+++	N.D.
Silicic acid-poor drinking water + Aluminum sulphate 0.04M 15 months (GROUP 7)	+ \-	N.D.
Silicic acid-rich drinking water + Aluminum sulphate 0.04M 15 months (GROUP 8)	++	N.D.

The data are expressed as not detected (N.D.), very weak (+/-), weak (+), moderate (++) and strong (+++).

weak reduction in staining intensity that was found with the semiquantitative analysis in mice which received 15 month silicic acid-rich mineral water + aluminium sulphate 0.04 M (GROUP 8) compared to mice which received 15 month silicic acid-rich mineral water (GROUP 6), was, instead, not confirmed by IOD measurements, because no statistically significant differences could be detected between the two groups (IOD= 32.23±1.21 in GROUP 6 vs IOD= 30.25±1.28 in GROUP 8). Quantitative data about nNOS expression are displayed in Fig. 5C.

Discussion

In this study, the morphological features obtained by Toluidine Blue staining and the quantitative data from NADPH-d histochemistry, a simple and useful method to investigate nitrergic neurons in cerebral cortex (Hope et al., 1991), showed scattered nitrergic neurons in SSC that represent about 1% of total SSC neurons. We found a distribution of nitrergic neurons that is in agreement with the data previously obtained by other authors (Jope and Johnson, 1992; Rodrigo et al., 1994) and by our group (Rodella et al., 2006) from the cerebral cortex of control animals.

There is evidence that the brain is a target organ for Al toxicity, raising the suspicion that chronic exposure to this metal could play a role in AD and other neurodegenerative diseases (Bonkale et al., 1995). Impairment of the Glu-NO-cGMP pathway in the brain may be responsible for some Al-induced neurological alterations. In particular, Al accumulation in the brain is able to alter neuronal signal transduction pathways associated with glutamate receptors (Llansola et al., 1999). Many authors showed that chronic Al exposure could affect the proteins of the Glu-NO-cGMP pathway, inducing a decrease in neuronal NOS and nitrergic neurotrasmission in SSC (Bonkale et al., 1995; Llansola et al., 1999; Rodella et al., 2006). For these reasons, in the present study, we firstly wanted to identify

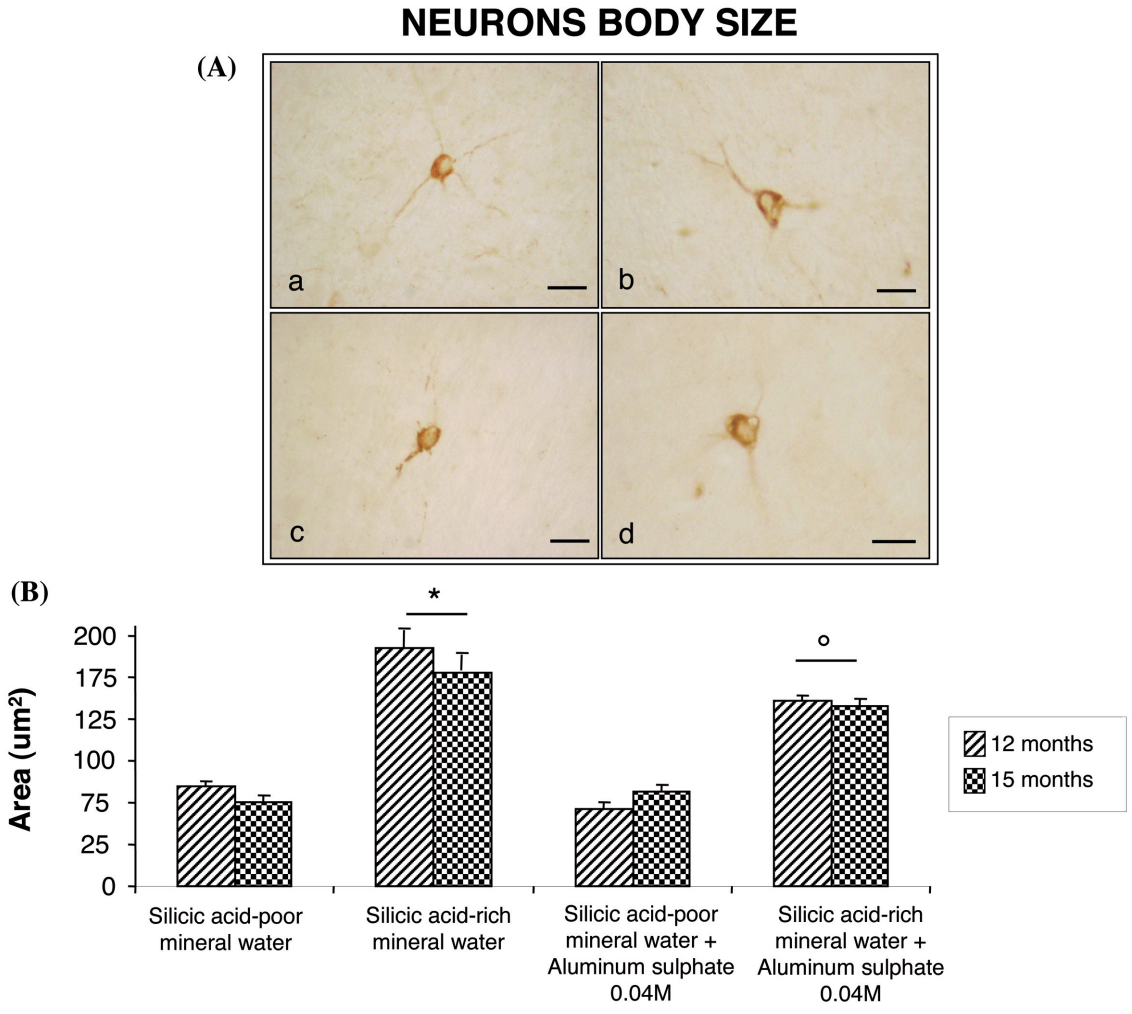

Fig. 3. A. nNOS positive neurons in the SSC of mice treated for 15 months with the silicic acid-poor drinking mineral water (a), with the silicic acid-rich drinking mineral water (b), with the silicic acid-poor drinking mineral water + aluminium sulphate 0,04M (c) and with the silicic acid-rich drinking mineral water + aluminium sulphate 0,04M (d). Scale bar: 20 μm.
B. Quantitative data about cross sectional area measurements of nNOS positive neurons from the different experimental groups. Data are expressed as mean ± S.E. (*P<0.05 vs silicic acid-poor drinking mineral water; °P<0.05 vs silicic acid-poor drinking mineral water + aluminium sulphate 0.04M).

qualitative and quantitative alterations induced by chronic (12 and 15 months) oral aluminium administration in the nitrergic neuronal pattern. In this regard, here we demonstrated a 30% reduction of the number of NADPH-d positive neurons in SSC of mice treated with Al sulphate dissolved in silicic acid-poor mineral water (GROUPS 3 and 7), respect to control groups (GROUPS 1 and 5, as reported above).

These findings firstly confirmed our previous studies in which we had already showed that exposure to Al administered in drinking water could decrease the number of nitrergic neurons scattered in the SSC of mice in a time dependent manner, presenting greater diminutions with chronic administrations (6 to 12 months) with respect to sub-chronic ones (15 days-1 month) (Rodella et al., 2006). In particular, here we found that such a reduction seems to be a little more consistent in the 15 months Al-treated group (GROUP 7), than in the 12 months treated one (GROUP 3), even if the difference between the two groups is not statistically significant. Moreover, these findings confirmed our previous results in which we showed that 6 and 12 months of Al exposure caused effects of similar extent on nitrergic neurons (Rodella et al., 2006), suggesting that aluminium needs a long time to accumulate in the nervous system and to impair certain defence mechanisms able to protect neurons from excessive Al toxicity.

In addition, because the main aim of our study was to assess the potential protective role of Si against Al toxicity in NO-containing neurons in SSC of mice brain, the successive step of our study was to countercheck if the decrease in the number of nitrergic neurons, previously detected in silicic acid-poor mineral water + aluminium sulphate treated animals, could also be noticed when Al sulphate was dissolved in silicic acid-rich water. In this respect, it is interesting to note that Al dissolved in high Si water did not induce a similar reduction in the number of NADPH-d positive neurons either after 12 or 15 months of treatment (we found only a little 13-14 % reduction), suggesting a protective role of Si against aluminium toxicity in nitrergic neurons.

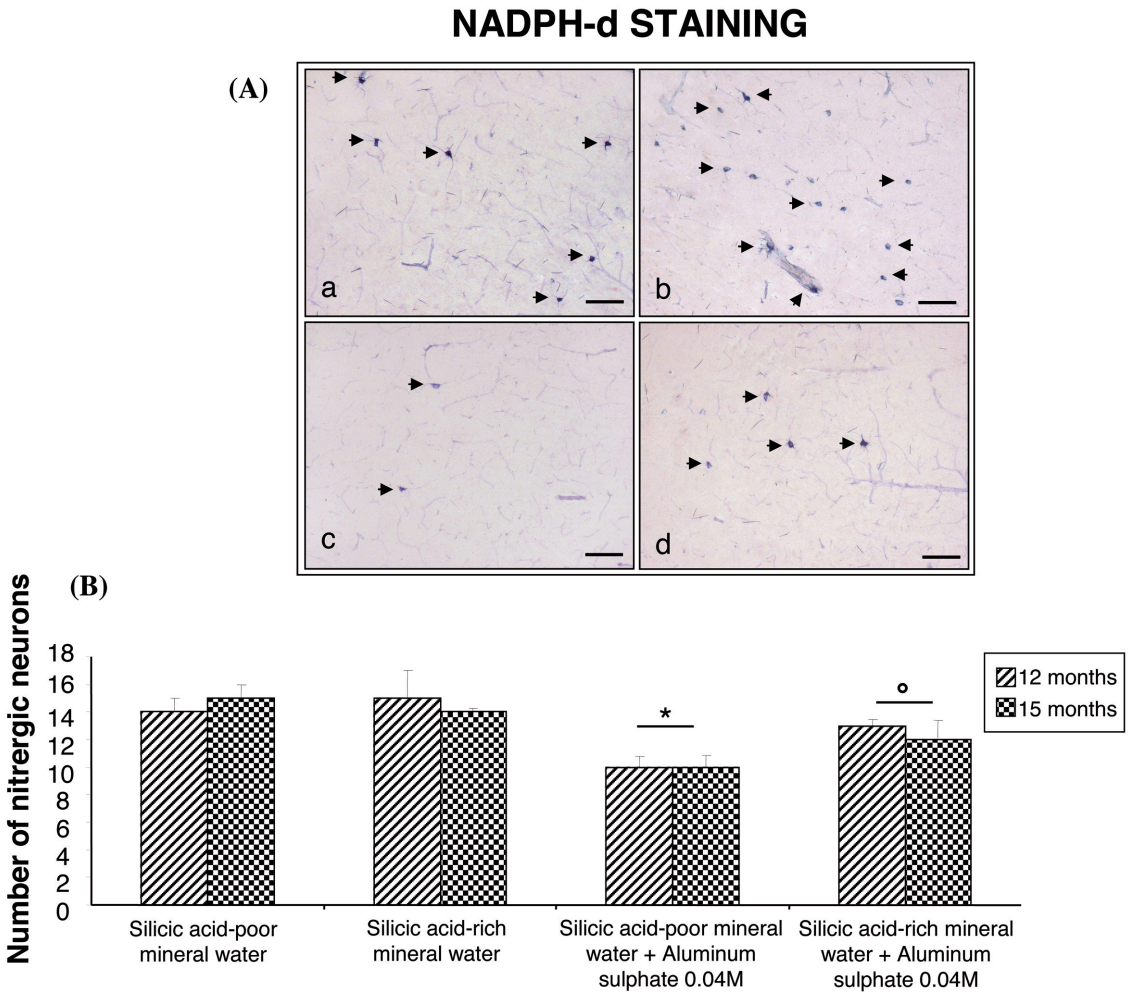

Fig. 4. A. NADPH-d stained neurons in the SSC of mice treated for 15 months with the silicic acid-poor drinking mineral water (a), with the silicic acid-rich drinking mineral water (b), with the silicic acid-poor drinking mineral water + aluminium sulphate 0,04M (c) and with the silicic acid-rich drinking mineral water + aluminium sulphate 0,04M (d). Cytoplasmic NADPH-d staining in blue. Scale bar: 200 μm. **B.** Quantitative evaluation of the number of NADPH-d positive neurons in the SSC of mice from the different experimental groups. The data represent the number of neurons referred to a conventional field. Data are expressed as mean ± S.E. (*P<0.05 vs silicic acid-poor drinking mineral water; °P<0.05 vs silicic acid-poor drinking mineral water + aluminium sulphate 0.04M).

What is more, since NADPH-diaphorase histochemistry could not provide an exact measurement of nNOS activity, because of the presence of some diaphorase-like enzymes in the brain, we preferred to confirm our histochemical results with specific immunohistochemistry for nNOS enzyme. Again, nNOS immunohistochemistry revealed a pattern of staining that, at least within the neurons, quantitatively and qualitatively overlapped NADPH-d results, indicating that the observed diminution of the expression of nitrergic system markers is attributable to the reduction of nNOS/NADPH-d positive neurons more than to changes in the expression of other diaphorasic enzymes.

It is important to note that, along with a decrease in the number of nitrergic neurons, we also demonstrated a decrease in the intensity of nNOS single stained neurons (measured as IOD, integrated optical density of the staining) in animals treated with silicic acid-poor mineral water + aluminium sulphate 0,04M with respect to silicic acid-poor mineral water alone, but not in silicic acid-rich mineral water + aluminium sulphate 0.04M compared to silicic acid-rich mineral water alone.

Finally, our analysis of the morphology and the size of nNOS positive neurons scattered in SSC of mice

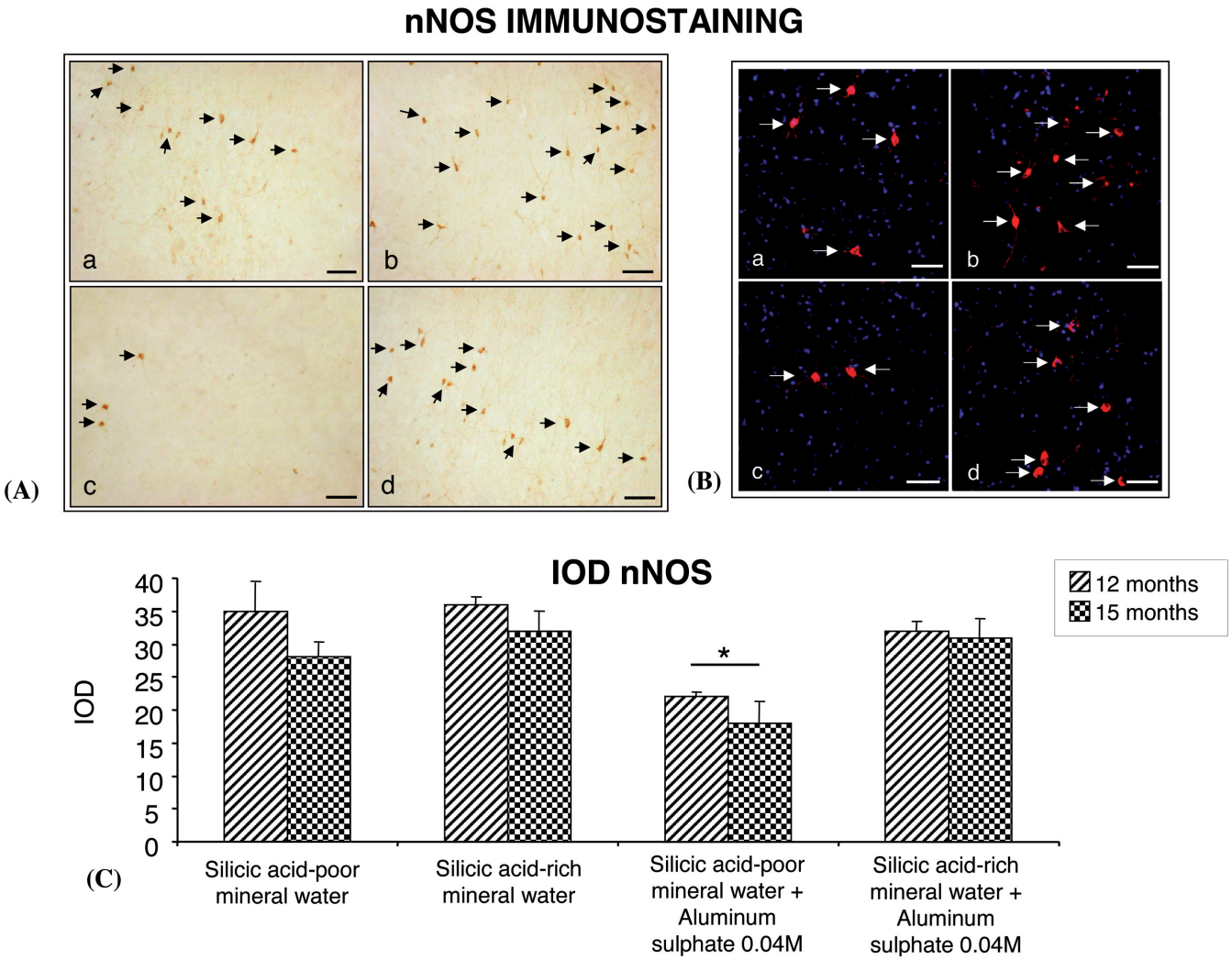

Fig. 5. A. nNOS positive neurons in the SSC of mice treated for 15 months with the silicic acid-poor drinking mineral water (a), with the silicic acid-rich drinking mineral water (b), with the silicic acid-poor drinking mineral water + aluminium sulphate 0,04M (c) and with the silicic acid-rich drinking mineral water + aluminium sulphate 0,04M (d). Cytoplasmic nNOS immunostaining in brown. Scale bar: 200 μm. **B.** Immunofluorescence images of nNOS (red) in the cytoplasm of nitrergic neurons of SSC of mice treated for 15 months with the silicic acid-poor drinking mineral water (a), with the silicic acid-rich drinking mineral water (b), with the silicic acid-poor drinking mineral water + aluminium sulphate 0,04M (c) and with the silicic acid-rich drinking mineral water + aluminium sulphate 0,04M (d). Nuclei are stained with DAPI (blue). Scale bar: 50 μm. **C.** The data represent values of integrated optical density (IOD) of the cytoplasmic nNOS immunostaining from the different experimental groups. Data are expressed as mean ± S.E. (*P<0.05 vs silicic acid-poor drinking mineral water).

allowed us to speculate about a possible neurotrophic action of Si. We showed, in fact, a significant increase in the size of nitrergic neurons in brain of mice treated with silicic acid-rich mineral water either with or without aluminium, compared to silicic acid-poor mineral water. This may suggest that silicic acid is protecting against other sources of biologically available aluminium, for example, in the mouse chow. Our hypothesis is corroborated by the results obtained by Carlisle and Curran, showing that Si may be an essential element for brain of rats and that Si supplementation appeared to be protective in the aging of the nervous system (Carlisle and Curran, 1987).

On the other hand, we did not observe any aluminium-induced alterations in shape or size of nitrergic neurons, suggesting that aluminium at the tested doses was not lethal for nitrergic neurons, since we observed a similar tissue morphology between Al treated or non-treated groups. This could indicate that the toxic action of Al on the nitrergic system prevalently consists of impairing nNOS expression, decreasing NO production and nitrergic neurotransmission. The fact that we also observed a decrease in the number of nitrergic neurons, could be explained by the hypothesis that those neurons that in basal conditions produced lower levels of nNOS, seemed to disappear after Al exposure. In fact they did not die, but merely became undetectable for NADPH-d histochemical staining and nNOS immunohistochemistry. This is confirmed by Toluidine blue staining that did not show a significant amount of degenerating neurons in either Al treated or non treated groups.

Taken together, our results seem to confirm that the benefits of regular consumption of silicic acid-rich water in counteracting Al neurotoxicity may be due to the Si both reducing the uptake of Al form diet and facilitating the removal of systemic Al via the kidney and urine. We currently know very little about the dynamics of urinary Al excretion, whether following the ingestion of silicic acid-rich mineral water or not. It has been suggested that orthosilicic acid is able to increase the urinary output of Al perhaps by interacting with filterable Al in renal tubules, forming hydroxyaluminosilicates, and preventing re-absorption of Al (Reffitt et al., 1999). We theorized, as already proposed by other authors, that in our experiments regular drinking of mineral water containing a high concentration of silicic acid reduced biologically available aluminium in the nervous system, probably both reducing gastrointestinal uptake of dietary Al and facilitating urinary excretion of systemic Al (Exley et al., 2006; Domingo et al., 2011). Our hypothesis is supported by data which showed that renal clearance of Al and Si are similar (Bellia et al., 1996; King et al., 1997) and by a previous study in which other authors demonstrated that regular drinking of mineral water containing a high concentration of silicic acid significantly increased urinary excretion of Al (Exley et al., 2006). Moreover, the knowledge that none of the other constituents of the mineral waters tested in the present study, except silicic acid, could influence Al deposition and the fact that no known aluminium chelators or well-known diuretic elements, that may markedly interfere with Al uptake and excretion, were contained in the tested bottled waters, allowed us to suggest that the reduction of Al damage on nitrergic system was linked to the increase in silicic acid assumption.

In conclusion, based on these results, Si dietary supplementation, in its biologically available form, silicic acid, would appear to be potentially useful in preventing nitrergic system damage caused by chronic Al exposure in the mice brain. Nevertheless, further studies demonstrating that this protection was achieved primarily by limiting the gut absorption of Al and by removal of systemic Al appear to be necessary.

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Conflict of interest statement. The authors declare that there is no conflict of interests in this study.

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