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Chronic neuroprotective effects of low concentration lithium on SH-SY5Y cells: possible involvement of stress proteins and gene expression

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Abstract

To investigate the molecular mechanism underlying the neuroprotective effect of lithium on cells, in this study, we exposed SH-SY5Y cells to 0.5 mmol/L lithium carbonate (Li_2CO_2) for 25–50 weeks and then detected the expression levels of some neurobiology related genes and post-translational modifications of stress proteins in SH-SY5Y cells. cDNA arrays showed that pyruvate kinase 2 (PKM2) and calmodulin 3 (CaM 3) expression levels were significantly down-regulated, phosphatase protein PP2A expression was lightly down-regulated, and casein kinase II (CK2), threonine/tyrosine phosphatase 7 (PYST2), and dopamine beta-hydroxylase (DBH) expression levels were significantly up-regulated. Besides, western blot analysis of stress proteins (HSP27, HSP70, GRP78 and GRP94) showed an over-expression of two proteins: a 105 kDa protein which is a hyper-phosphorylated isoform of GRP94, and a 108 kDa protein which is a phosphorylated tetramer of HSP27. These results suggest that the neuroprotective effects of lithium are likely related to gene expressions and post-translational modifications of proteins cited above.

Key Words: *lithium; neuroprotection; kinase; phosphatase; stress proteins; SH-SY5Y cells; gene expression; mechanism of action*

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Introduction

Lithium neuroprotection is provided through multiple, intersecting mechanisms, but the precise mechanism is still under investigation (Rowe and Chuang, 2004). Besides, mood stabilizers require a long-term treatment period to exhibit their beneficial effects. It is hypothesized that alterations of signaling pathways and gene expression may be involved and a great interest has been raised in the investigation of the effects of lithium and other mood stabilizers on gene expression and cellular signaling in both basic and preclinical laboratories (Chuang, 2005). The present work was designed as a contribution to understand some of the cellular mechanisms underlying the neuroprotective effects of lithium on SH-SY5Y cells. The modification of stress proteins and the expression of genes implicated in cell metabolism and signaling pathways under chronic lithium treatment at therapeutically-relevant concentration were investigated. Thus, we studied the genes coding for the pyruvate kinase (PK), casein kinase II (CK2), threonine/tyrosine phosphatase7 (PYST2), calmodulin 3 (CaM 3), phosphatase protein 2A (PP2A) and dopamine beta-hydroxylase (DBH).

Pyruvate kinase M2 (PKM2) is an enzyme involved in glycolysis. It enhances the use of glycolytic intermediates for macromolecular biosynthesis and tumor growth (Warburg effect) (Ferguson and Rathmell, 2008). Casein kinase-2 (CK2) is a serine/threonine protein kinase with prominent prosurvival functions (Song et al., 2003; Chakraborty et al., 2011). Pyst-2 is a Thr/Tyr mitogen-activated protein (MAP) kinase phosphatase protein. MAP kinase isoforms coordinate a wide variety of cellular functions. These include proliferation, differentiation, development, inflammatory responses and apoptosis (Dowd et al., 1998). CaM protein is the ubiquitous intracellular receptor of free calcium (Ca^{2+}) regulating diverse cellular functions by acting as an intracellular second messenger (Singht et al., 2004). The protein serine/threonine phosphatase 2A (PP2A) can interact with a substantial number of proteins and contribute to the regulation of numerous signaling pathways. Active PP2A can inhibit the cell cycle, induce apoptosis, and act as a tumor suppressor (Ivaska et al., 2002). Human DBH, a constituent of catecholamine biosynthetic pathway, catalyzes the conversion of dopamine to noradrenaline or norepinephrine (Kapoor et al., 2011).

We have also studied the stress protein expression in order to verify whether the neuroprotective effects of lithium, under our condition, could be, in part, attributed to a molecular protection conferred by stress protein/chaperone accumulation. Indeed, it was demonstrated that lithium inhibition of GSK-3 is associated with activation of heat shock

factor-1 (Bijur and Jope, 2000), which suggests that lithium treatment may up-regulate heat shock response as part of the neuroprotective mechanisms (Chuang, 2005). The heat shock proteins (HSPs) are molecular chaperones that bind to unfolded or misfolded proteins to ensure proper folding and prevent intracellular protein aggregation (Hendrick and Hartl, 1993; Ma and Hendershot, 2001). Certain HSPs also exert their neuroprotective effects by antagonizing apoptosis-inducing factors (Ravagnan et al., 2001). The endoplasmic reticulum (RE) is known to exert several actions in response to accumulated misfolded and/or unfolded proteins within this organelle, including the transcriptional up-regulation of ER chaperones named glucose regulated proteins (GRPs) (De Gracia et al., 2002).

Materials and Methods

Chemicals and reagents

Lithium carbonate (Li_2CO_3) was purchased from Prolabo/Rhone-Poulenc (France); HSP27, phosphorylated HSP27, HSP72/73, GRP78, GRP94, KDEL and phospho-serine epitopes monoclonal antibodies from StressGen Biotechnologies (France); Super-Signal® West Pico Chemiluminescent substrate from Pierce Biotechnology, Inc (Rockford, IL, USA); Atlas™ Nylon cDNA arrays (Human neurobiology Arrays, 7736-1) from Clontech (Saint-Germain-en-Laye, France); fetal calf serum (FCS) and other cell culture reagents from Institut Jacques Boy, Reims, France; proteases inhibitors, *i.e.*, phenylmethanesulfonylfluoride (PMSF), N-ethylmaleimide (NEM), and aprotinin from Sigma-Aldrich (St. Louis, MO, USA).

Cell cultures

Human neuroblastoma SH-SY5Y cell line, a subline of bone marrow biopsy-derived line SK-N-SH (ECACC#86012802) (a generous gift from Cécile Monnet, Centre de Physiopathologie de Toulouse-Purpan, Inserm U563, Toulouse, France) were cultured in Dulbecco's modified Eagle medium (DMEM; 25 mmol/L glucose) supplemented with 10% fetal calf serum (FCS) and 20 μg/mL gentamycin and grown at 37°C in humidified 5% CO_2 atmosphere.

The experiments of chronic lithium treatment were initiated by seeding cells in T25 flasks containing 8 mL culture medium at a density of 20,000/cm^2 (*i.e.*, 500,000/flask) and allowed to attach overnight. Then, Li_2CO_3 (150 mmol/L stock solution, pH 7) was added in half of the cultures in order to obtain a final concentration of 0.5 mmol/L. For 1 year, once a week, cells from control (cultured without lithium) and lithium treated cultures were dissociated by trypsin-EDTA treatment, counted and then re-seeded at low density, as above (20,000/cm^2), lithium being added 12 hours later in lithium treated cultures. Control and lithium-treated cells were periodically (every week) collected for analysis.

Gene expression analysis using human neurobiology arrays

After cultured by the culture medium with and without 0.5 mmol/L Li_2CO_3 for 42 weeks, the culture medium was rapidly sucked out and cell layers were frozen at −80°C until analysis. Total RNAs were extracted using the RNAble® procedure (Eurobio, Paris, France). cDNA probe synthesis and hybridization were carried out as specified by the manufacturer. Briefly, the $polyA^+$ RNA fraction was isolated with biotinylatedoligo-dT bound to streptavidin-coated magnetic beads. ^{32}P-labeled cDNA probes were synthesized on captured $polyA^+$ RNA with a Moloney Murine Leukemia Virus (MMLV) reverse transcriptase in the presence of [α-^{32}P] ATP and a gene specific primer mix. This mix only contained primers for genes represented on the array. This procedure provides the lowest background signal by concentrating the entire radioactive label into the newly synthesized DNA. ^{32}P-labeled cDNA probes were purified on a chromatography column and labeling was checked by scintillation counting. Probes were then hybridized onto the cDNA arrays which contain duplicate spots of cDNA fragments from 588 human genes involved in the neurobiology. Hybridized membranes were imaged with a phosphorimager (PharosFX System from Bio-Rad Laboratories, Inc., Hercules, CA, USA). Comparison of expression profiles and quantitative analysis were carried out using the Atlas image™ program (Clontech). The global normalization mode was used to normalize the signal intensity between the lithium treated cells and control cells.

Detection of stress protein expression by western blot analysis

Cell layers were rinsed with ice-cold phosphate-buffered saline (PBS; pH 7.4), collected into a lysis buffer (PBS containing 0.5% NP40 and protease inhibitors: 10 mmol/L EDTA, 2 mmol/L PMSF, 5 mmol/L NEM, 1 μg/mL aprotinin, pH 7.4) and stored at −20°C until use. 100 μL of lysis buffer was used for about 10^6 cells. For analysis, cell lysates were sonicated for 10 seconds and protein concentration was determined. Equal amounts of proteins (20 μg) from each homogenate were separated by sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE; 10% acrylamide) and electro-transferred onto a 0.45 μm nitrocellulose membrane (70 V, 90 minutes). To saturate the unspecific sites, the membranes were incubated for 1 hour at 37°C into a Tris-buffered saline (TBS: 10 mmol/L Tris; 140 mmol/L NaCl, pH 7.4) containing 0.5% Tween 20 (TBST). The membranes were then incubated for 1 hour at room temperature under continuous shaking in the presence of primary antibodies: mouse anti-HSP27 (dilution 1:10,000), rabbit anti-phospho-HSP27 (dilution 1:50,000), mouse anti-HSP72/73 (dilution 1:40,000), mouse anti-GRP78 (dilution 1:40,000), rat anti-GRP94 (dilution 1:30,000) and mouse anti-KDEL (dilution 1:1,000), diluted respectively in PBST to 1:10,000, 1:50,000, 1:40,000, 1:40,000, 1:30,000, and 1:1,000 then allowed to stand overnight at 4°C. Monoclonal anti-phosphoserine antibody was diluted to 1:80,000. After washed with TBS twice for 5 minutes each, with TBS containing 0.1% Nonidet-P40 for 15 minutes, and then with TBS twice again for 5 minutes each, blots were incubated at room temperature, in the dark, for 2 hours under continuous shaking in the presence of peroxidase-conjugated secondary antibodies (anti-rat IgG (dilution 1:30,000); anti-rabbit IgG (dilution

Figure 1 Immunoblots showing stress protein expression (GRP94, GRP78, HSP72/73) in lithium (Li)-treated (0.5 mmol/L for 47 weeks) SH-SY5Y cells and control cells (C).
Equal amounts of proteins (20 μg) were loaded in each lane.

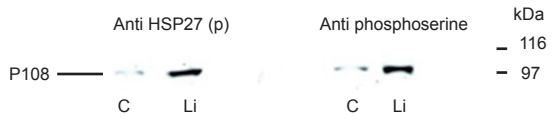

Figure 3 Immunoblots showing the expression of P108 protein recognized by the anti-phospho HSP27 (Ser 78) and the anti-phosphoserine in lithium (Li)-treated (0.5 mmol/L for 47 weeks) SH-SY5Y cells and control cells (C).
Equal amounts of proteins (20 μg) were loaded in each lane.

1:40,000); anti-mouse IgG (dilution 1:80,000); Stressgen Biotechnologies Corporation, Victoria, Canada) diluted in TBS containing 3% dry skimmed milk. After washing, stress protein expression was detected using SuperSignal® West Pico chemiluminescent substrate and Bio Max light-1 film, Kodak® (Kodak, New York, NY, USA).

Results

Differential expression of genes involved in the neurobiology of SH-SY5Y cells exposed to 0.5 mmol/L Li_2CO_3 using cDNA macroarrays

Expression of genes was analyzed in cells cultured with Li_2CO_3 at the therapeutical concentration 0.5 mmol/L for 42 weeks. Ratio of gene expression in transcripts from lithium treated cells to controls is shown in Table 1. As recommended by the manufacturer, changes were only considered as significant when the ratios were < 0.5 or > 2.

Results showed that the transcriptional levels of genes coding for casein kinase protein CK2 (ratio 2.7), threonine/tyrosine phosphatase7 (PYST2) (ratio 2.3), dopamine beta-hydroxylase (ratio 2.5), and thymosin beta-10 (ratio 2) were significantly increased in lithium treated cells compared to in control cells. However, the transcriptional levels of the genes coding for PKM2 (ratio 0.27) and CaM 3 (ratio 0.37) were significantly down-regulated. Also, the transcriptional level of genes coding for phosphatase-2A (PP2A) protein (ratio 0.54) was decreased.

Changes in the expression level of stress proteins induced by 0.5 mmol/L Li_2CO_3 treatments

After 47-week exposure to 0.5 mmol/L Li_2CO_3, the expression levels of GRP78 and HSP73/72 were not significantly altered, as determined by immunoblotting (Figure 1). The anti-GRP94 and the anti-KDEL recognized a protein band of 98 kDa, corresponding to the apparent molecular mass of GRP94. This protein was not modified by the treatment.

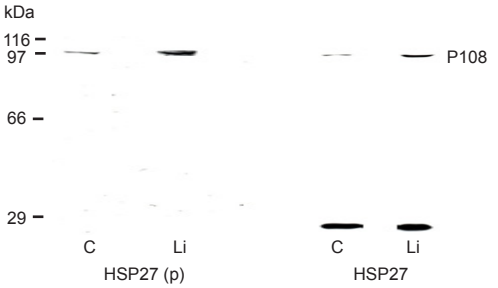

Figure 2 Immunoblots showing expression changes of HSP27 and phospho-HSP27 (Ser 78) in lithium (Li)-treated (0.5 mmol/L for 47 weeks) SH-SY5Y cells and control cells (C).
Equal amounts of proteins (20 μg) were loaded in each lane.

A protein band of about 105 kDa (P105), overexpressed in lithium treated cells compared to control cells, was also immunodetected by the same antibodies. This suggests that this protein could correspond to a post-translational modified form of GRP94. The use of anti-phosphoserine antibody recognized also the same protein P105 which confirms that P105 was a serine-phosphorylated form of GRP94. In addition to P105, the anti-phosphoserine antibody recognized another protein band of about 108 kDa (P108).

Figure 2 shows that the expression level of monomeric HSP27 was similar in lithium treated cells and control cells. However, the phosphorylated (ser78) monomeric form of HSP27 was not detected. At the same time, a P108 band was recognized by both the anti-HSP27 and anti-phospho HSP27 antibodies. This protein was overexpressed in lithium treated cells. It could correspond to a phosphorylated tetrameric form of HSP27.

The simultaneous use of anti-phospho HSP27 and anti-phosphoserine antibodies recognized a protein band of about 108 kDa confirming that P108 was a serine-phosphorylated tetrameric form of HSP27 (Figure 3).

Discussion

Evidence obtained from a diverse array of investigations performed during the last few years indicates that the therapeutic actions of lithium may arise from its effects on multiple targets. One component of this is the evidence that lithium is a neuroprotectant, supporting neuronal survival in the environment of potentially lethal insults (Li et al., 2002).

In this context, we have recently shown that SH-SY5Y cells cultured in the presence of 0.5 mmol/L Li_2CO_3 for 25–50 weeks had the ability to increase glucose consumption and enhance glycolytic activity. This causes an important release of pyruvate, suggesting that chronic exposure to lithium induces adaptive changes in metabolism of SH-SY5Y cells including a higher cell growth rate and a better resistance to oxidative stress (Nciri et al., 2013). The present study was undertaken to investigate the effects of this treatment on the expression level of some genes related to cell neurobiology and on the post-translational modifications of stress proteins. Besides, we tried to evaluate the involvement of these genes and proteins in neuroprotective effect of lithium.

Using cDNA macroarrays, differential expression of genes was analyzed in cells cultured for 42 weeks in the presence of 0.5 mmol/L Li_2CO_3. Thus, we founded that lithium strongly inhibited the transcriptional level of PKM2 gene. Surprisingly, the inhibition of PKM2 gene expression was accompanied by the stimulation of glucose uptake and glycolysis, as evidenced by rapid pH increase, increased glucose consumption, enhanced production of pyruvate and, to a lesser extent enhanced production of lactate and alanine (Nciri et al., 2013). Moreover, lactate dehydrogenase activity was enhanced in lithium treated cells (data not shown). An increase of glucokinase, 6-phosphofructokinase and pyruvate kinase activities was observed in lithium treated rats (Rodriguez et al., 2000).

In our case, the inhibition of PKM2 gene expression could be explained by the feedback of the very strong increase of pyruvate release in the extracellular medium of lithium treated cells. Thus, we showed that pyruvate could not enter the Krebs cycle to be converted into acetyl-CoA and would rather be excreted. In addition, Krebs cycle activity in lithium treated cells seems to be reduced since a light decrease of intracellular succinate and glutamate levels was detected, as compared to controls (Dowd et al., 1998; Christofk et al., 2008a, b). Chronic lithium treatment (42 weeks) at therapeutic concentration (0.5 mmol/L) had significantly inhibited (ratio 0.37) the gene coding for calmodulin 3 (CaM 3). CaM is the ubiquitous intracellular calcium receptor. The calcium/CaM complexallosterically activates numerous proteins, including calcium/CaM dependent protein kinase II (CaMKII). CaMKII is a multifunctional enzyme that is highly expressed in brain and muscle. The kinase is believed to serve important roles in synaptic transmission, gene transcription, and cell growth (Singht et al., 2004).

One study showed that, in rat, chronic lithium treatment (serum lithium concentration 0.8 mmol/L) induced an inhibition of CaMKII activity in presynaptic vesicles of frontal/prefrontal cortex (Celano et al., 2003). These authors demonstrated that the down-regulation of vesicular kinase activity induced by lithium was functionally related to a decrease in glutamate release from nerve terminals. This effect could contribute to the recently well-characterized neuroprotective effect of lithium.

In addition to PKM2 and CaM3, the gene coding for PP2A seemed to be down-regulated in the cells exposed to 0.5 mmol/L Li_2CO_3 for 42 weeks than in the control cells. PP2A comprises a family of protein serine/threonine phosphatases. They can interact with a substantial number of proteins and contribute to the regulation of numerous signaling pathways. Active PP2A can inhibit the cell cycle, predominantly at the G2/M checkpoint, induce apoptosis, and act as a tumor suppressor (Ivaska et al., 2002). Moreover, PP2A activation may concomitantly dephosphorylate and activate GSK-3β directly or indirectly by dephosphorylating Akt protein (Ivaska et al., 2002). Besides, lithium has been identified as a selective and direct inhibitor of GSK-3β (Li et al., 2002). Thus, lithium reduces GSK-3β activity, directly, by acting as a competitive inhibitor of Mg^{2+} and, indirectly, by reducing protein phosphatase activity, which leaves GSK-3β phosphorylated and inactive. Lithium GSK-3β phosphorylation is mediated through a PI 3-kinase-Akt pathway (Lin et al., 2007). Indeed, by inhibiting the expression of PP2A, lithium could indirectly inhibit the GSK-3β and therefore block its facilitation of apoptosis. This constitutes a part of the neuroprotectant properties of lithium.

Conversely, our results showed that the transcriptional levels of genes coding for threonine/tyrosine phosphatase 7 (PYST2) (ratio 2.3) were significantly increased in lithium treated cells compared to control cells. It was demonstrated that this 42 kDa isoform of MAP kinases (also known as ERK2 or MAPK2) responds vigorously to growth factors (Cobb et al., 1994). In the same way, Pyst2 transcript in human skin fibroblasts does accumulate rapidly following serum stimulation which could be due to the growth factor effect (Nciri et al., 2013).

We have recently reported that lithium-mediated neuroprotective and neurotrophic effects involve mechanisms highly relevant to the post-stroke population including the increased expression of brain derived neurotrophic factor (BDNF) (Riadh et al., 2011). This suggests that neuroprective effect of lithium may be, in part, mediated by BDNF/PYST2/ERK2 signaling pathway.

The transcriptional level of gene coding for DBH was found up-regulated in lithium treated cells as compared to controls. DBH is an important therapeutic target that has been associated to and implicated in several diseases and pathological conditions including Parkinson's disease and Alzheimer's disease (Kapoor et al., 2011).

It was reported that DBH activity could mediate predisposition to Parkinson's disease through its role in catalyzing the conversion of dopamine to noradrenaline. Thus, lower levels of DBH protein might lead to increased ratios of dopamine to noradrenaline. In Alzheimer's disease, significant reductions in noradrenergic neurones as well as reduced brain noradrenaline levels have often been reported (Haavik and Toska, 1998; Matéo et al., 2006). Besides, plasma DBH activity was shown increased in bipolar disorder patients treated with lithium (Sofuoglu et al., 1995). Therefore, lithium treatment of neurodegenerative diseases is mediated by the increase of DBH activity and then the increase of noradrenaline.

CK2 gene was strongly up-regulated in lithium treated cells compared to control cells (ratio 2.5), which suggests that chronic lithium treatment at therapeutically relevant concentration stimulates CK2 activity. CK2 phosphorylates many transcription factors, proto-oncoproteins, and tumor suppressor proteins (Kapoor et al., 2011). *In vitro*, it was demonstrated that the Golgi system serine/threonine kinase and casein kinase were potentially able to phosphorylate the GRP94 (Cala and Jones, 1994; Brunati et al., 2000). This suggests that the increase of CK2 activity should be accompanied with an increase of GRP94 phosphorylation level. Accordingly, we showed that a hyperphophorylated form of GRP94 named P105 was overexpressed in the cells exposed to 0.5 mmol/L Li_2CO_3 for 47 weeks.

Strong evidence suggests that lithium inhibits protein ag-

gregation in Alzheimer's disease through up-regulation of molecular chaperones and/or stress proteins (Kitamura and Nomura, 2003; Smith et al., 2005; Shao et al., 2006). Thus, it was demonstrated that stress proteins such as HSP27, HSP70, GRP78 and GRP94 are involved in the neuroprotective effects of lithium (Ren et al., 2003; Hiroi et al., 2005). In this context, one study reported that lithium (1 mmol/L for 1 week) slightly increased mRNA and protein levels of GRP94 and GRP78 in primary culture of cerebral cortical cells without generating ER stress (Shao et al., 2006).

In our case, lithium treatment had not modified GRP78, HSP73/HSP72, and the 98 kDa band of GRP94. By contrast, it up-regulated P105, a serine-phosphorylated form of GRP94. Same result was reported in A549 and in SH-SY5Y cells (Allagui et al., 2007, 2009). These authors suggested that glycosylation and/or phosphorylation could explain GRP94 apparent mass increase. They explained the appearance of phosphorylated form of GRP94 (P105) by the ability of lithium to inhibit various phosphatases and to increase kinase activity. *In vivo*, we have recently demonstrated that oral administration of lithium carbonate in mice (1–2 g/kg food for 6 months) resulted in serum concentrations in the range of therapeutic doses used in humans. Under these experimental conditions, no significant oxidative stress was observed in liver and GRP94 stress protein was overexpressed, which suggest that lithium has cytoprotective effects against oxidative stress and the overexpression of GRP94 could be part of cellular resilience induced by lithium against the oxidative stress in liver (Nciri et al. 2010).

Although the expression level of monomeric HSP27 was similar in control and lithium treated cells, we showed that anti HSP27, anti-phospho HSP27 (Ser78), and anti-phosphoserine antibodies recognized a serine phosphorylated protein band of about 108 KDa, named P108. This protein was over-expressed in lithium treated cells. It could correspond to a serine-phosphorylated tetrameric form of HSP27. It was also showed that chronic exposure (25 weeks) of SH-SY5Y cells was accompanied by an increase of tetrameric form of phospho-HSP27 (Ser78) (Allagui et al., 2009).

In stress conditions, the chaperone properties of HSP27 seem to correlate with the ability of this protein to form large oligomers, as long as significant amounts of large Hsp27 oligomers could be formed. Conversely, phosphorylation of Hsp27 down-regulates its chaperone properties by dissociation of the large oligomers to tetramers (Rogalla et al., 1999). Moreover, small phosphorylated oligomers of HSP27 have the capacity to bind to actin filaments and to stabilize microfilaments (Lavoie et al., 1995; Mounier and Arrigo, 2002). It was also reported that HSP27 and phosphorylated HSP27 bind directly to pathologically phosphorylated tau protein, facilitating its degradation and thus attenuating its toxicity (Kosik and Shimura, 2005).

Overall this work, we conclude that neuroprotective effects of lithium following chronic treatment at therapeutically relevant concentration could be mediated by its ability to regulate various phosphatases and kinases activities. This acts on post-translational level and then on protein activities.

Author contributions: *Nciri R was responsible for data collection, analysis and interpretation and drafted the manuscript. Bourogaa E provided critical revision of the manuscript for intellectual content. Jbahi S was in charge of statistical expertise. Allagui MS obtained the funds, provided suggestions in procedure performance and material application and supervised the study. Elfeki A, Vincent C and Croute F designed this study. All authors approved the final version of the paper.*

Conflicts of interest: *None declared.*

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