Original Article

Expression of *Leishmania major LmSTI1* in Yeast *Pichia Pastoris*

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Abstract

Background: *Leishmania major LmSTI1* is a conserved protein among different species of leishmania, and expressed in both amastigote and promastigote forms of L. major life cycle. It has previously been expressed in bacterial systems.

Materials and Methods: To express *LmSTI1* in the methylotrophic yeast *Pichia pastoris* (*P. pastoris*), the shuttle vector pPICZA containing gene lmsti1 was constructed under the control of the AOX1 promoter. The recombinant vector was electro-transformed into *P. pastoris*, and induced by 0.5% methanol in the buffered medium. The expression of the LmSTI1 protein was visualized in the total soluble protein of *P. pastoris* by 12% SDS-PAGE, and further confirmed by Western blotting with *L.major*-infected mouse sera and HRP-conjugated goat anti-mouse IgG as the first and secondary antibodies, respectively.

Results: The expression level was 0.2% of total soluble proteins.

Conclusion: It might be possible to use this formulation as a whole yeast candidate vaccine against cutaneous leishmanization.

Keywords: *Pichia pastoris*, LmSTI1, *Leishmania major*

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Introduction

Leishmaniasis is an endemic disease, ranging from localized cutaneous to visceral forms. Cutaneous leishmaniasis is caused by different *leishmania* parasites including *leishmania major* (*L.major*) , *L. tropica, L. aethiopica, L. mexicana, L. guyanensis, L. peruviana, and L. braziliensi*[1,2]. According to WHO estimation, the annual incidence of leishmaniasis in new cases is about 2 million people worldwide, most of which (1.5 million) suffer from cutaneous leishmaniasis (http://www.who.org).

Leishmaniasis is primarily mediated by T lymphocytes. To neutralize intracellular parasitic infections such as *leishmania*, cellular immunity was demonstrated to play a critical role in inducing protective immunity[3]. In mouse models of *leishmania* infection, the Th1 response phenotype is observed in protected animals such as C57BL/6 mice by increasing IFN-*gamma* and TNF-*alpha* while the Th2 response phenotype producing IL-4 and IL-5 is associated with susceptibility or disease progression[4-6]. In the

susceptible BALB/c mice, immunization elicited Th1 responses and established resistance to *L. major* (7, 8), so that each protective vaccine should be able to induce broad and intense Th1 Tcell responses.

Antigen presenting cells (APCs) are one of the most important cells in Th1 responses. APCs, mostly dendritic cells (DCs), by producing IL-12 increase the activity of different effectors including T cells, natural killer cells and macrophages in early stages of leishmaniasis[9]. In addition, DCs are able to differentiate responses into producing Th1 cells. In contrast, Th2 lymphocytes lead T cells to producing anti-leishmania cytokines such as IFN-γ[10,11]. Furthermore, these cells have a key role in stimulating cytotoxic T lymphocytes in a process, known as cross-presentation (12). On this subject, DCs can both endocytosis and process exogenous antigens, present molecules to CD8+ T cells (cytotoxic T cells) through the major histocompatibility complex (MHC) class I pathway, and activate naive antigen-specific CD8+ T cells[13].

L. major stress-inducible protein 1 (*LmSTI1*) is a heat shock protein expressed in both amastigote and promastigote forms of *L. major*[14]. In a study, CAMPOS-NETO et al. found that LmSTI1 and TSA plus IL-12 were able to induce protection against cutaneous leishmaniasis in both murine and nonhuman primate models with the strong response of Th1 and higher amounts of interferon gamma; however, the amount of cytokine produced by LmSTI1 was much more than that of TSA[15]. In the other study, vaccination by using protein LmSTI1 and liposome with sequences containing CpG, as an adjuvant, high proportion of IgG2a/ IgG1 and efficient protection in mice were observed up to 14 weeks after challenge[16]. On the other hand, studies on vaccines that has reached the stage of trial (Leish111-f) represent the strongest antibody response and highest IF-γ in vaccinated mice with LmSTI1 compared to other vaccine components (TSA, and LeIF)[17]. Above all, lmSTI1 alone can elicit robust protective immunity by as much as compositions of protein antigens with each other, making this protein a good candidate for the design of vaccines against cutaneous leishmaniasis.

The use of whole recombinant yeast vaccines is considered as a novel approach to induce protective immunity in cancer and infectious diseases[18,19]. This system offers several advantages over conventional methods for vaccine development. Because of the intrinsic adjuvant properties of yeast cell walls, efficient presentation of both MHC class I and II, the ability to express multiple antigens together, safety, ease of handling and low cost of mass production, yeast is considered as the best strategy to construct a vector for vaccine application[20-22]. Importantly, several previous studies showed that internalized whole yeast by macrophages and dendritic cells (DCs) induces DC maturation and IL-12 expression[20,23,24]. In the present study, we constructed whole recombinant yeast capable of expressing LmSTI1 in the yeast *Pichia pastoris* (*P. pastoris*) strain x33 as a model system for potential application in vaccine studies against leishmaniasis.

Methods

Parasite culture: The *L. major* strain MRHO/IR/75/ER (Iran vaccine strain, kindly provided by Dr. Javadian, School of Public Health, Tehran University of Medical Sciences) was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum with 100 U/ml penicillin and 100 µg/ml streptomycin, and grown to the mid-logarithmic phase (approximately 10 million parasites) at 24°C. Parasites were then harvested by centrifugation (4000 RPM for 10min at 4°C), and stored at -20°C.

DNA extraction: Genomic DNA was extracted as described by Kelly (25). Briefly, the frozen parasites were thawed and resuspended in 1ml of sterile lysis buffer (125 mMNaCl, 125 mM EDTA, 2.5% w/v (SDS), 125 mMTris, pH 8.0) containing 100 µg/ml proteinase K (Sigma-Aldrich). Subsequently, the DNA was extracted by phenol/chloroform and ethanol precipitation. The extracted DNA was dissolved in 100 µl of distilled water and stored at -20°C.

Construction of expression vector: The full-length *lmsti1* gene (1641base pairs; a 62.1 KDa ORF) was amplified from the extracted genomic DNA using following sets of PCR primers (LM and LR in Table 1). The 5'oligonucleotide primers contain a *BamH*I restriction site (boldface) preceding the ATG initiation codon (underlined) followed by sequences derived from the first seven amino-acid residues of *LmSTI1*.

The 3'oligonucleotides contain an *Eco*RI restriction site (boldface) immediately followed by the termination codon (underlined) and sequences comprising the last seven amino acid residues of *LmSTI1*. For long-term storage of the *lmsti1* gene, the resulting PCR amplicons were digested with *BamH*I and *Eco*RI enzymes, ligated into the similarly-digested pUC18 vector and then transformed into competent *E. coli* cells. Ampicilin-resistant transformants were identified by restriction analysis, and the sequence fidelity was confirmed by sequencing. The second PCR primers were designed (PLM-F and PLM-R in table 1) to introduce a yeast kozak sequence (underlined) and *Eco*RI (boldface) in the forward primer at the N-terminal of the *lmsti1* gene and *Kpn*I restriction site (boldface), without termination codon in the reverse primer at the C-terminal of the gene sequence. The latter PCR product and pPICZA vectors were digested with *Eco*RI and *Kpn*I, ligated and then transformed into DH5α competent cells. Ampicilin-resistant transformants were identified by restriction analysis, and the sequence fidelity was confirmed by sequencing. In this strategy of cloning, the recombinant plasmid (pPICZA_LmSTI1) was constructed (figure 1) by inserting gene *lmsti1* in frame with 6×His-tag and under the control of the strong promoter AOX1, allowing the high expression level of the protein by methanol induction.

Transformation of the recombinant pPICZA into P. pastoris: To intergrate the recombinant pPICZA-LmSTI1 plasmid into the *P. pastoris* genome, 10 µg of recombinant plasmids were linearized by *sac*I, cutting the 5' AOX (alcohol oxidase) region of the plasmid, followed by electroporation into *P. pastoris* according to supplier's instructions (Easy Select *Pichia* Expression manual, Invitrogen). Briefly, a single colony of *P. pastoris* strain x33 (INVITROGEN, CA, USA) grown on YPD (1% yeast extract, 2% peptone and 2% dextrose) agar plates was transferred into 5 ml of YPD medium in a 50-ml Erlenmeyer flask overnight at 28°c with shaking at 250 rpm. Subsequently, 500 µl of the overnight culture was inoculated into 50 ml of fresh YPD medium in a 1-liter flask and grown overnight again under the same conditions until reaching an OD_{600} of 1.3-1.5. The cells were then centrifuged at 1500g for 5 min at 4°C, and washed two times with 50 ml ice-cold sterile water and once with 20 ml ice-cold 1M sorbitol. The 5–10 µg of linearized recombinant plasmids was mixed with 80 µl of competent X33 cells. The mixture was immediately transferred into a pre-chilled 0.2 cm electroporation cuvette and incubated on ice for 5 min prior to electroporation on a gene pulser (Biorad, USA). Electroporation was performed at 1.5 kV, 25 µF and 200 Ω. Approximately 1 ml ice-cold 1M sorbitol was immediately added to the cuvette after electroporation. Subsequently, the electroporated cells were plated on the YPD agar plates containing Zeocin (500 µg/ml) and incubated for 3-7 days at 28°c. Individual transformant colonies were then picked from the plates and screened on MD (Minimal Dextrose Medium; 1.34% YNB, 4×10^{-5}% biotin, 2% dextrose) agar plates during 2-3 days to confirm the mut^+ phenotype of the X-33 (INVITROGEN, EasySelect *Pichia* Expression Kit). To screen the integration of the expression cassette into the *P. pastoris* genome, DNA from 10 Zeocin-resistant colonies were extracted by the method described by Hanna et al (26), and then PCR using 5'AOX1 and 3'AOX1 primers was performed following the standard protocol.

Expression and extraction of LmSTI1 protein: The expression of the *LmSTI1* protein was performed according to the standard protocols. In this regard, a single colony from ten transformants was picked and grown overnight at 30°C in 5 ml of YPD medium + 100 µg/ml of Zeocin in a shake flask until the OD_{600}: 2-3. The cells were then inoculated in 50 ml BMGY media (Buffered Glycerol-complex Medium; 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol), and incubated for one more night until OD_{600}: 2-10. The cell pellets were then harvested by centrifugation (3500 g, 5 minutes, at RT) and resuspended in the same volume of BMMY induction medium (Buffered Methanol-complex Medium; 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol), for 3-5 days under the same conditions. During the induction period, sterile 100% methanol to a final concentration of 0.5% was added daily to the cultures. Afterward, 5 ml of cultures were harvested by centrifugation at 6000 rpm for 5min at 4°C, and the cell pellets were

Table 1: Primers used for cloning of *LmSTI1* into pUC18 (LM-F and LM-R) and pPICZA (PLM-F and PLM-R).

primers	Sequences
LM-F	5'-A-**GGA-TCC**-ATG-GAC-GCA-ACT-GAG-CTG-AAG-3'
LM-R	5'-T-**GAA-TTC**-CTA-CTG-ACC-AAA-ACG-AAT-GAT-GCC-3'
PLM-F	5'-AGA-**GAA-TTC**-GAC-ATG-GAC-GCA-ACT-GAG-CTG-3'
PLM-R	5'- GAA-**GGT-ACC**-CTG-ACC-AAA-ACG-AAT-GAT-GCC-3'

stored at -80°C for the expression analysis.

SDS PAGE and Western Blotting: Cell pellets were thawed quickly, placed on ice and then resuspended by adding an equal volume of the breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM PMSF [phenylmethylsulfonyl fluoride or other protease inhibitors], 1 mM EDTA, 5% glycerol) and glass beads (equal amounts of 150-212 µm and 710-1,180 µm). The cells were disrupted by vortexing 8 times for 30 seconds, with 30-second intervals of incubation on ice. The disrupted cells were then centrifuged for 5–10 minutes at 12,000 × g at 4°C, and the clear supernatants were then transferred into fresh containers. To analyze the protein expression, 10µl of each supernatant was resolved by the standard Laemmli SDS-PAGE (12%) method [27], and then visualized by Coomassie Brilliant Blue staining. Expression of the *LmSTI1* protein was confirmed by Western Blotting according to standard protocols; in brief, the protein bands were transferred into a polyvinylidene difluoride membrane (Sigma-Aldrich) by electroblotting at 350 mA for 90 min. The membrane was then probed with mouse sera (at 1:100 dilution) containing a high titter of antibodies against the *LmSTI1* protein (data not shown), as a primary antibody, and further incubated with HRP-conjugated mouse anti IgG (Sigma-Aldrich) for 1h as a secondary antibody, followed by developing with diaminobenzidine (DAB) (Roche, Switzerland) as a substrate. Immediately after visualization, the membrane was rinsed with PBS to stop reaction.

Results

The whole genomic DNA of *L.major* was extracted from about ten million parasites using the salting-out procedure. The full-length *lmsti1* gene (1641bp) was successfully amplified by the first PCR with LM-F and LM-R primers (Figure 2). The first PCR amplicon was confirmed by sequencing, and inserted into the PUC18 vector to maintain the *lmsti1* gene as a template (data not shown).

Since the kozak sequence is required for high-throughput protein expression in the yeast expression

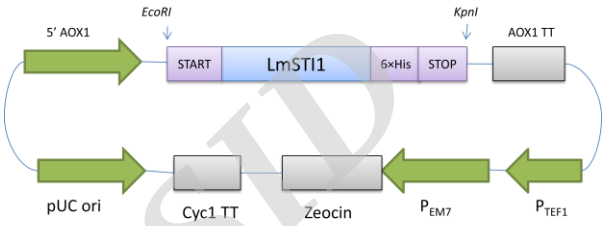

Figure 1. Schematic diagram of the pPICZA vector harboring *lmsti1* gene. *lmsti1* gene was cloned into the pPICZA vector via *Eco*RI and *Kpn*I sites in frame with 6×His, downstream of 5'AOX1 promoter. AOX1 TT, 6xHis and Stop indicate to the transcription termination sequence, His tag and stop codon, respectively.

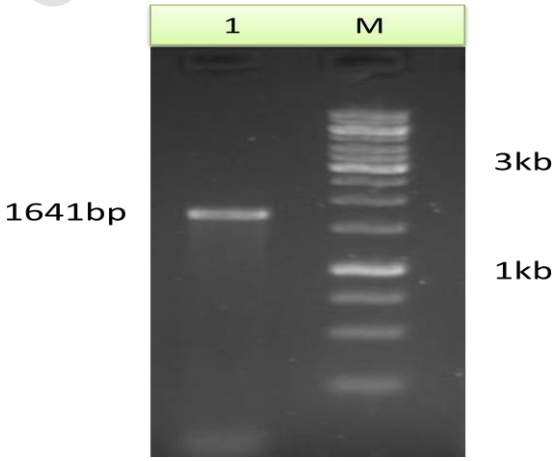

Figure 2. Agarose gel electrophoresis of the full length *lmsti1* amplicon. Genomic DNA of *L.major* was extracted and then amplified by the first PCR reaction. Lane 1: full length of *lmsti1* gene; Lane M: DNA 1 Kb ladder.

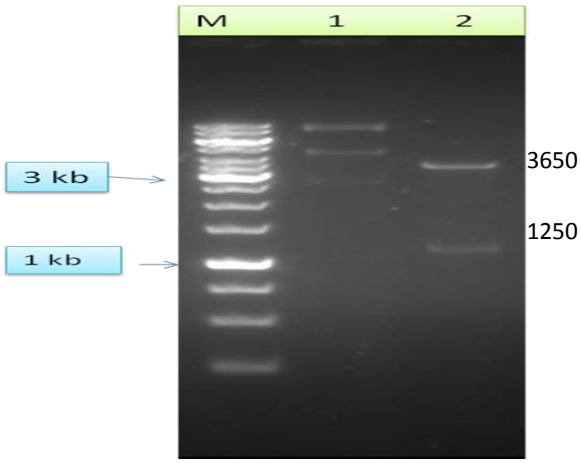

Figure 3. Restriction analysis of PICZ-*LmSTI1*. M, marker; 1, recombinant plasmid; 2, vector digested by *Hind*III enzyme that produced 2 fragments (~1250, 3650).

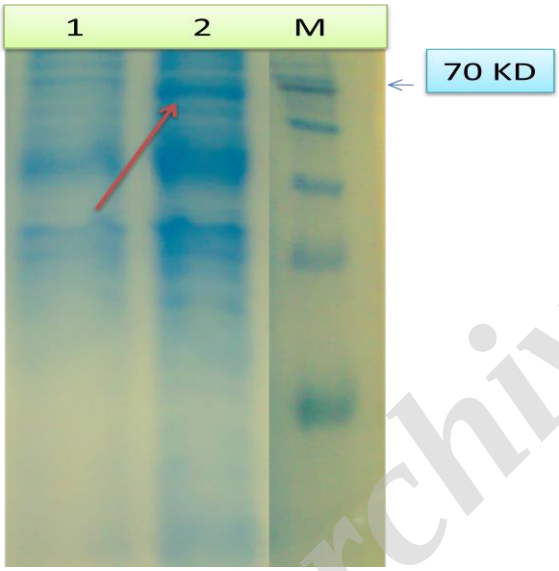

Figure 4. SDS-PAGE of intracellular protein extracted from X-33. Lane 1, uninduced sample; lane 2, induced sample; lane M, prestained molecular-weight marker, arrow indicates *LmSTI* protein expressed in X-33.

system, an additional PCR reaction was performed to add this sequence to the upstream of the gene *lmsti1*, and the PCR amplicon was then inserted into the pPICZA vector. The new recombinant vector, pPICZA-LmSTI1, was confirmed by restriction enzyme analyses and sequencing with 5' AOX and 3' AOX universal primers. The *Hind*III enzyme was used for restriction enzyme analysis, which cut the recombinant vector at two specific sites, resulting in two fragments with a size of approximately 1250 bp and 3650 bp (Figure 3).

Screening of recombinant P. pastoris strains: In order to obtain highest possible multiple copies of the expression cassette in *P. pastoris* strain x33, the concentration of Zeocin increased up to 2000 µg/ml according to supplier's instructions (Easy Select *Pichia* Expression manual, Invitrogen). In these criteria, transformants containing multiple copies of vectors integrated into the host genome were only able to grow on. Approximately six transformants of X33 strains survived in high concentration of the antibiotic and they were screened for the integrative plasmid by PCR using 5'AOX1 and 3'AOX1 primers. The positive PCR colonies were then transferred into the expression medium for analyzing protein expression profiles.

Intracellular expression analysis: Following the induction of the *LmSTI1* protein with methanol in the BMMY medium, the X33 cells were harvested and the total intracellular proteins were extracted using the breaking buffer and glass beads. Protein electrophoresis (SDS-PAGE) was performed and the expected ~67 KD band (the 62.1 KD protein of interest plus 5 KD peptide sequence for detection and purification of the protein) was visualized on 12% SDS-PAGE gel (Figure 4). Results from this study indicated that there is no similar band in the X33 negative control. The position of the protein on the gel was also confirmed by western blotting analysis using antibodies against *LmSTI1* protein and HRP-conjugated mouse anti IgG as primary and secondary antibodies, respectively (Figure 5). The level of

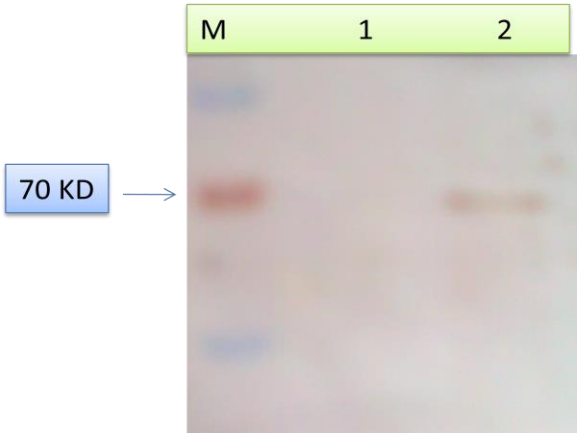

Figure 5. Western-blot analysis of the *LmSTI1* protein expressed in *P. pastoris*. Lane M, prestained molecular-weight marker; Lane 1, here is X33 transformed by only pPICZA vactor (without *lmsti1* gene); Lane 2, induced sample.

expression was 0.2% of total proteins when estimated by a densitometric scan (ImageJ software) of the SDS-PAGE and Western blotting.

Discussion

E. coli offers several advantages for recombinant protein expression, including low cost, rapid growth rate and high protein yields. However, there are limitations including the lack of post-translational modification, presence of endotoxin and insolubility, as well as aggregation of certain proteins as inclusion bodies[28]. Additionally, mammalian cells (e.g., Chinese hamster ovary cells) suffer from high cost and more complex nutritional requirements. In the last decades, *P. pastoris* has proved to be a potentially powerful expression system to produce recombinant proteins[29,30]. *P. pastoris* has the ability to grow on minimal medium, prepare all criteria required for eukaryotic protein production and lack toxic elements[31].

In this study, *LmSTI1* was intracellularly expressed in *P. pastoris* strain X33. Webb JR et al. previously expressed such protein in two different systems pQE and pET in *E. coli*, showing inclusion bodies and more procedures such as solubilization and refolding[17,32]. In this study, the pPICZA shuttle vector was used to express the protein in the yeast. In this regard, the *lmsti1* gene was inserted downstream of the strong promoter AOX1 immediately after the initiation codon ATG in a KOZAK sequence. The recombinant vector was subsequently transformed into the yeast. It is well-known that integration into the genome limits gene copy numbers. This was resolved by increased zeocin concentrations to 2000 µg/ml, leaving only one to ten colonies when compared to the lower concentration of Zeocin, 100 µg/ml. These recombinant colonies were then chosen for protein expression.

When supernatants and cell pellets were assessed after cell lysis, it turned out that most proteins were intracellulary expressed in yeast[33], resulting in the soluble recombinant protein with no aggregation. Moreover, because there was no signal sequence in the pPICZA vector, the protein was not observed out of the cells (data not shown). As shown in SDS-PAGE and Western blotting (Figures 4 and 5), the protein size increased from 62.1 KD to about 67 KD, due to addition of other sequences such as His-Tag and c-myc.

The optimum criterion for expression was found in the buffered complex medium (e.g, BMMY) three days after induction by purified methanol at a final concentration of 0.5%. Because protein expression was performed in buffered media, the PH of the media seems to exhibit no effects on protein production. Consistent with several researchers[31,34,35], mut^+ phenotype superior, but not mut^s, could successfully express our protein (data not shown); however, the mut^s phenotype was preferred by some researchers[36,37]. Despite the lower expression level (0.2% of TSP), the protein band was detected in SDS-PAGE and Western blotting. Nowadays, codon optimization is one suitable approach to increase gene expression in yeasts[38]. Some studies demonstrated that codon optimization could improve malaria gene expression in *P. pastoris*[39,40]. Anjali Yadava et al. showed that Plasmodium falciparum F2 domain expression in *P. pastoris*, based on the *pichia* codon usage, improved nine-fold compared to that in *E. coli*- based codon usage, which itself expressed that protein four-fold more than native F2 domain in *E. coli*. Furthermore, proteins produced in *Pichia*- based codon usage could be detected by Coomassie blue staining, Western blotting and ELISA, while the native protein failed to be detected when expressed in *P. pastoris*[41]. Therefore, it might be possible to improve protein expression levels by *pichia* codon optimization.

Whole recombinant yeast vaccine is an appropriate method to elicit cellular immune responses against intracellular microorganisms, offering potential advantages including intrinsic adjuvant property, safety, protein purification elimination and increased MHC class I and II expression on antigen processing cells[19]. Based on this system, the protein *LmSTI1* was cloned and expressed in yeast *P. pastoris* to use in vaccination. To our knowledge, this is the first report of the cloning and expression of the *lmsti1* gene in yeasts. Some reports showed that a low amount of protein against E.G7-OVA tumor cells, and even as few as 2 ng human immunodeficiency virus Gag antigens, elicited protective immunity in tumor-transplanted mice[18].

Conclusion

Taken together, whole yeast vaccine might be one of the best strategies, because of safety, ease of production and the lack of downstream processes. In the future, we will evaluate whole yeast vaccine expressing *LmSTI1* against cutaneous leishmaniasis in animal models.

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Conflict of interest

The authors have no financial interest in the products discussed in this article.

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