Inhibition of hTERT Gene Expression by Silibinin-Loaded PLGA-PEG-Fe₃O₄ in T47D Breast Cancer Cell Line

Zohreh Ebrahimnezhad¹,², Nosratollah Zarghami¹,², Abolfazl Akbarzadeh³, Mohammad Rahmati², Zohreh Mohammad Taheri⁴, Kazem Nejati-Koshki⁵

¹Tuberculosis and Lung Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
²Department of Clinical Biochemistry and Laboratory, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
³Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran
⁴Faculty of Advanced Medical Sciences, Department of Medical Nanotechnology, Tabriz University of Medical Sciences, Tabriz, Iran
⁵National Research Institute of Tuberculosis and Lung Diseases, Tehran, Iran

Introduction: Nowadays, using drug delivery is an essential method to improve cancer therapy through decreasing drug toxicity and increasing efficiency of treatment. Silibinin (C₂₅H₂₂O₁₀), a polyphenolic flavonoid which is isolated from the milk thistle plant, has various applications in cancer therapy but it has hydrophobic structure with low water solubility and bioavailability. To increase the effect of silibinin, silibinin-loaded PLGA-PEG-Fe₃O₄ was prepared to determine the inhibitory effect of this nanodrug on Telomerase gene expression.

Methods: The rate of silibinin loaded into PLGA-PEG-Fe₃O₄ was measured. Then, the cytotoxic effect of silibinin-loaded PLGA-PEG-Fe₃O₄ was determined through Methyl Thiazol Tetrazolium (MTT) assay. After that, inhibition of Telomerase gene expression was indicated through Real-time PCR.

Results: Data analysis from MTT assay showed that silibinin-loaded PLGA-PEG-Fe₃O₄ had dose dependent cytotoxic effect on T47D cell line. Real Time PCR analysis showed that the level of telomerase gene expression more efficiently decreased with silibinin-loaded PLGA-PEG-Fe₃O₄ than with free silibinin alone.

Conclusion: The present study indicates that this nanodrug causes down-regulation of Telomerase gene expression in cancer cells. Therefore, PLGA-PEG-Fe₃O₄ could be an appropriate carrier for hydrophobic agents such as silibinin to improve their action in cancer therapy.

© 2013 by Tabriz University of Medical Sciences
The most commonly used materials for preparing nanoparticle carriers are polymer, liposome, dandrimer and micelles. PLGA-PEG (poly (DL-lactic-co-glycolic acid)-polyethylene glycol)-Fe₃O₄ is a kind of nanoparticle that can increase the therapeutic effect of silibinin. The silibinin and the pharmaceutical drugs can be physically attached to PLGA-PEG-Fe₃O₄ surface and could be released into the target site due to its external localized magnetic-field gradient. The high possibility of nonspecific toxicities and more accurate target-based dose of PLGA-PEG-Fe₃O₄ make better bioavailability and solubility of silibinin.

In this study, we investigated the inhibitory effect of silibinin-loaded PLGA-PEG-Fe₃O₄ on Telomerase expression in T47D human breast cancer cell line.

Materials and methods

Cell Culture

Breast cancer epithelial-like cell line (T47D) was provided from Pasteur Institute cell bank of Iran (code: C203). This cell line was cultured in RPMI 1640 cell culture medium (Gibco, Invitrogen, UK). One liter of RPMI 1640 was supplemented with 2 mg sodium bicarbonate (Merck co, Germany), 0.08 mg penicillin G (Serva co, Germany), 50 mg streptomycin (Merck co, Germany), 0.2 mg amphotericin B and 10% heat-inactivated fetal bovine serum (FBS (Fetal Bovine Serum) (Gibco, Invitrogen, UK). One liter of RPMI was used and then 5 ml culture medium was added to each of them. Finally, they were incubated in 5% CO₂ and 37°C).

Materials and experiment for preparation of silibinin-loaded PLGA-PEG-Fe₃O₄

Ferric chloride hexahydrate (FeCl₂·6H₂O), ferrous chloride tetrahydrate (FeCl₂·4H₂O) and ammonium hydroxide (25wt%) were purchased from Fluka (Buchs, Switzerland). D, L-lactide and glycolide were purchased from Sigma and recrystallized with ethyl acetate. Stannous octoate (Sn(Oct)₂) was purchased from Sigma (St Louis, USA). PEG 4000 was prepared by ring opening polymerization of D, L-lactide and glycolide (molecular weight, 4000) and DMSO were dehydrated under vacuum at 70°C for 12 h and were used without further purification. Silibinin was purchased from Sigma (St Louis, USA).

Preparation of PLGA-PEG tri-block co-polymer

Poly(lactide–co–glycolide)–poly(polyethylene glycol), PLGA–PEG co-polymers with molecular weight of polyethylene glycol (PEG₄₀₀₀) as an initiator was prepared by a melt polymerization process under vacuum using stannous octoate (Sn(Oct)₂) as catalyst. D, L-lactide (14.4 g), glycolide (3.86 g) and PEG₄₀₀₀ 8g (45% w/w) in a bottle-neck flask were heated to 140°C under nitrogen atmosphere for complete melting. The molar ratio of D, L-lactide and glycolide was 3:1. Then, 0.05% (w/w) stannous octoate was added and the temperature of the reaction mixture was raised to 180°C. The temperature was maintained for 4 h. The polymerization was carried out under vacuum. The co-polymer was recovered by dissolution in methylene chloride following by precipitation in ice-cold diethyl ether.

Preparation of silibinin-loaded PLGA-PEG-Fe₃O₄

First, Fe₃O₄ and the polymer were mixed through the double emulsion method and then the mixture encapsulated the drug physically. To prepare silibinin-loaded PLGA-PEG-Fe₃O₄, it was physically conjugated to the PLGA-PEG-Fe₃O₄ in compliance with Dilnawaz et al. protocol. For conjugation, 240 mg of nanoparticle was dissolved in 20 ml chloroform and then 8 mg of Fe₃O₄ was added. In the next step, 20 mg of silibinin was added to mixture, and then these two solutions were homogenized by homogenizer. 20 ml 1% PVA (poly vinyl alcohol) was added as a stabilizer and was mixed for 8 min. It was then centrifuged for 30 min at 11000 rpm to become sediment. After that the mixture was leached, put in room temperature for 5 hours to dry, and pulverized into powder by mortar. Successful loading of silibinin was confirmed by FTIR measurement.
Nanodelivery in breast cancer therapy

**Drug Release Experiment**

3 mg silibinin-loaded PLGA-PEG-Fe₃O₄ was dissolved in 30 ml PBS (pH=7); it was incubated at 37°C while being shaken. At specific times, with an interval of 3 h, 3 ml was picked up from the solution and 3 ml fresh PBS was added. Supernatant was collected for estimations. The amount of released silibinin was measured through UV-vis spectrophotometry at 325 nm.

**Cytotoxicity Assay and Cell Treatment**

Cytotoxicity of silibinin-loaded PLGA-PEG-Fe₃O₄ was measured at 24, 48 and 72 h using the MTT (3-[4, 5-dimethylthiazol-2yl]-2, 5-diphenyl tetrazolium bromide)(MTT; Sigma-Aldrich) assay. First, 2 × 10⁴ cells per well were seeded and kept for 24 h in the incubator to promote cell attachment. Then, cells were treated with different concentrations of free silibinin and silibinin-loaded PLGA-PEG-Fe₃O₄ (20-120 µM) in replicates of four. T47D cells were exposed to free silibinin and the silibinin-loaded PLGA-PEG-Fe₃O₄ in the logarithmic phase of growth. Three controls were used; the first was 1% DMSO; the second was PLGA-PEG-Fe₃O₄ control for assessment of nanoparticle effect; and the third one was cells alone. After 24, 48 and 72 h exposure times, the cell culture medium was replaced with 200 µl fresh medium for 24 h, after that the cells were incubated with MTT solution for 4 h. Then, contents of all wells were removed and 200 µl of pure DMSO were added to the wells followed by adding 25 µl Sorensen’s glycine buffer to each well. The absorbance was read at 570 nm ELISA-reader and IC₅₀ was calculated at most within 1 h.

A concentration of 70 µM of free Silibinin and 3 concentrations of 20, 40 and 60 µM of silibinin-loaded PLGA-PEG-Fe₃O₄ were used for T47D cells treatment. The control and treated cells were incubated at 37°C and 5% CO₂ for 24 h.

**Real-time PCR Assay**

After 24 h, cells were washed with PBS and their total RNA was extracted from each sample using TRIZOL reagent (Invitrogen, USA). Through absorbance measurement, RNA concentrations were determined by UV-vis Spectrophotometer (Eppendorf BioPhotometer) and at 260-280 nm purity of RNAs were estimated. The integrity of RNA was confirmed by electrophoresis of the individual samples on a 2% agarose gel. Complementary DNA (cDNA) was reverse-transcribed using the First Strand cDNA Synthesis Kit (Fermentase). To synthesize cDNA, the reaction of mixture was prepared on ice as for each reaction, 2 µl of 5 X PrimeScript Buffer, 0.5 µl of Prime-Script RT Enzyme Mix1, 0.5 µl of Oligo dT Primer and 0.5 µl of Random 6 mers accompanied by 500 ng of total RNA were used that reached to 10 µl by adding RNase Free dH2O. The reaction mixture was incubated under the following conditions: 37°C, 15 minutes (Reverse Transcription); 85°C, 5 sec (inactivation of reverse transcriptase with heat treatment); 4°C. Levels of hTERT expression were determined by real-time PCR (RT-PCR). We used hTERT primers (Genbank accession: NM_198255, bp 2165-2362) and beta-actin primers (Genbank accession: NM_001101, bp 787-917) for real time PCR. For hTERT, a 198 bp amplicon and for beta actin, a 131 bp amplicon were generated in a mixture that contained 10 picomolar of forward and reverse primers of hTERT (5′CCGCGCTAGCTGTCACTTTGT3′, 5′CAGGTGAGGCACGAAACTGT3′) or beta-actin (5′TCCCTGGAGAGACGCTACG3′, 5′GTAGTTTCGTGGATGCCACA3′), 2 µl template cDNA and 2x PCR master mix of Syber Ex Taq II. Each RNA sample was divided into equal amounts and then, hTERT and beta-actin in parallel with each other were amplified by real-time PCR in triplicate. 20 µl of PCR reaction mixture contained 10 µl of SYBER Ex Taq II (2x) PCR master mix, 2 µl template cDNA, 6.4 µl dH₂O water per reaction. Negative controls were prepared each time with 2 µl ddH₂O instead of the cDNA template. Real time PCR amplification was performed using a Corbett (Rotor Gena 6000) system with the following setting as manufacture protocol. The reaction mixture was incubated under the following conditions: 95°C, 2 minutes, 1 cycle (Holding step); 65°C, 20 seconds, 45 cycles (Annealing); 72°C, 20 seconds, 45 cycles (Extension); 75-99°C, 1 cycle (Melting).

**Statistical analysis**

SPSS 16 was used for Statistical analysis. The differences...
in mRNA levels of hTERT between control and treated cells were assessed by ANOVA and Tukey’s test. A p-value < 0.05 was considered as significant.

**Results**

**1H NMR spectrum of PEG–PLGA co-polymer**
The basic chemical structure of PEG–PLGA co-polymer was confirmed by 1H NMR spectra that were recorded at RT with a Bruker DRX 300 spectrometer operating at 400 MHz. Chemical shift (δ) was measured in ppm using tetramethylsilane (TMS) as an internal reference (Fig. 2). One of the striking features was a large peak at 3.65 ppm, corresponding to the methylene groups of the PEG. Overlapping doublets at 1.55 ppm were attributed to the methyl groups of the D-lactic and L-lactic acid repeat units. The multiples at 5.2 and 4.8 ppm corresponded to the lactic acid CH and the glycolic acid CH, respectively, with the high complexity of the 2 peaks resulting from different D-lactic, L-lactic and glycolic acid sequences in the polymer backbone.

**Size and size distribution (SEM)**
The surface morphology of the nanospheres during the incubation period was observed by SEM. The nanographs of pure Fe₃O₄ nanoparticles (and silibinin-loaded Fe₃O₄ magnetic nanoparticles modified with PLGA-PEG copolymers are shown in Fig. 3(a) and Fig. 3(b), respectively. Observing the photograph, it can be seen that the nanoparticles were well aggregated due to the nanosize of the Fe₃O₄ of about 10 nm. After encapsulation and modification of the silibinin-loaded Fe₃O₄ magnetic nanoparticles with PLGA-PEG copolymers, the size of particles changed to 25–75 nm and dispersion of the particles was greatly improved (Fig. 3(b)). This can be explained by the electrostatic repulsion force and steric hindrance between the copolymer chains on the encapsulated Fe₃O₄ nanoparticles. The samples were coated with gold particles.

**FTIR spectroscopy**
The FTIR spectrum is consistent with the structure of expected copolymer. FTIR spectroscopy was used to show the structure of Fe₃O₄ and PLGA-PEG copolymer nanoparticles. From the infrared spectra shown in Fig. 4, the absorption peaks at 720 cm⁻¹ belonged to the stretching vibration mode of Fe–O bonds in Fe₂O₃. In addition, the absorption band at 3509.9 cm⁻¹ is assigned to terminal hydroxyl groups in the copolymer which PEG homopolymer has been removed from. The bands at 3010 cm⁻¹ and 2955 cm⁻¹ are due to C–H stretch of CH, and 2885 cm⁻¹ due to C–H stretch of CH. A strong band at 1630 cm⁻¹ is assigned to C=O stretch. Absorption at 1186–1089.6 cm⁻¹ is due to C–O stretch. FTIR spectroscopy was done by Shimadzu spectrophotometer (Fig. 4).

**In Vitro silibinin-loaded PLGA-PEG-Fe₃O₄ Releasing**
UV-vis spectrophotometer of 325 nm wavelength was used for drawing the curve indicating samples 1 to 15 of the nanoparticle release with nearly the same proportion of silibinin during the time (Table 1). 16th sample was a solution which remained from the aqua phase of loading procedure and showed percentage of silibinin in the environment. It is measured about 24%. 1 mg of silibinin-loaded PLGA-PEG-Fe₃O₄ included 760 µg silibinin. The curve indicates that this nanodrug has an appropriate amount of magnetic nanoparticle and silibinin in total.

**MTT Assay**
Cell viability was assessed by MTT assay through exposing T47D cell line to different concentrations of free silibinin and silibinin-loaded PLGA-PEG-Fe₃O₄ (20-120

---

**Fig. 2. 1H NMR spectrum of PEG-PLGA co-polymer.**
µM) during 24, 48, 72 h. The results in all cases show that the toxicity effect increased by increasing the drug dose, leading to the fact that these drugs are dose-dependent. This is quite opposite for the viability factor.

The free silibinin had cytotoxic effect on T47D cell line with inhibitory concentration at 50% (IC50), 107 µM for 24 h, 73 µM for 48 h and 47 µM for 72 h (Fig. 5).

The nanodrug, on the other hand, had cytotoxic effect of 73 µM for 24 h, 77 µM for 48 h and 74 µM for 72 h at IC50 (Fig. 6). The sameness of results for different periods shows time-independency of this drug. The IC50 of nanodrug was dose-dependent and did not show significant time dependency. The graph was drawn by SPSS 16 (Figs. 5 and 6). Sub-optimums cytotoxic dose of free silibinin (70 µM) and the silibinin-loaded PLGA-PEG-Fe₃O₄ (20, 40 and 60 µM) were taken.

**Quantitative mRNA analysis**

The levels of hTERT gene expression were measured by Real-Time PCR. Changes in hTERT expression levels between the Control and treated T47D cells were normalized to beta-actin mRNA levels and then calculated by the 2-ΔΔct method. As amount of nanodrug increased, levels of hTERT gene expression decreased accordingly (Table 2). Real-time PCR data analysis indicated that by increasing amount of silibinin-loaded PLGA-PEG-Fe₃O₄, hTERT mRNA level expression would be decreased (Fig. 7). Each experiment was repeated three times.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Conc</th>
<th>WL 325.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.041</td>
<td>0.029</td>
</tr>
<tr>
<td>2</td>
<td>0.036</td>
<td>0.012</td>
</tr>
<tr>
<td>3</td>
<td>0.037</td>
<td>0.015</td>
</tr>
<tr>
<td>4</td>
<td>0.035</td>
<td>0.008</td>
</tr>
<tr>
<td>5</td>
<td>0.035</td>
<td>0.008</td>
</tr>
<tr>
<td>6</td>
<td>0.036</td>
<td>0.009</td>
</tr>
<tr>
<td>7</td>
<td>0.036</td>
<td>0.011</td>
</tr>
<tr>
<td>8</td>
<td>0.041</td>
<td>0.028</td>
</tr>
<tr>
<td>9</td>
<td>0.037</td>
<td>0.014</td>
</tr>
<tr>
<td>10</td>
<td>0.038</td>
<td>0.016</td>
</tr>
<tr>
<td>11</td>
<td>0.041</td>
<td>0.029</td>
</tr>
<tr>
<td>12</td>
<td>0.038</td>
<td>0.016</td>
</tr>
<tr>
<td>13</td>
<td>0.037</td>
<td>0.014</td>
</tr>
<tr>
<td>14</td>
<td>0.040</td>
<td>0.023</td>
</tr>
<tr>
<td>15</td>
<td>0.039</td>
<td>0.022</td>
</tr>
<tr>
<td>16</td>
<td>0.247</td>
<td>0.747</td>
</tr>
</tbody>
</table>

Fig. 3. Scanning electron microscopy of (a) Fe₃O₄ magnetic nanoparticles, (b) Silibinin-loaded Fe₃O₄ magnetic nanoparticles modified with PLGA-PEG copolymers. Abbreviations: PEG, poly (ethylene glycol), PLGA, poly (D, L-lactic-co-glycolic acid).
Gene expression can be reduced to 70% in dose of 70 µM of this free drug. In addition, at doses of 20, 40 and 60 µM, nanodrug injection can inhibit gene expression up to 71, 90 and 98%, respectively.

**Discussion**

In this work, we tested anti-growth factor of silibinin-loaded PLGA-PEG-Fe$_3$O$_4$ on breast cancer cell line T47D and MTT assay showed that silibinin-loaded PLGA-PEG-Fe$_3$O$_4$ inhibited Telomerase in a dose–dependent manner. The results show that Silobilin can significantly inhibit hTERT gene expression (70%) and also silibinin-loaded PLGA-PEG-Fe$_3$O$_4$ has more inhibitory effect than free drug (up to 98%) in this cell line.

Silibinin-loaded PLGA-PEG-Fe$_3$O$_4$ was used instead of free silibinin, demonstrating that PLGA-PEG-Fe$_3$O$_4$ enhances silibinin delivery through higher uptake by cells; Telomerase gene expression in comparison with control cell was significantly inhibited via using silibinin-PLGA-PEG-Fe$_3$O$_4$ in T47D breast cancer cell line because it has poor bio-availability and solubility in water; thus, PLGA-PEG-Fe$_3$O$_4$ was used to enhance drug delivery. Then based on these results, the nanodrug could be an appropriate inhibitor for telomerase expression and could inhibit efficiently hTERT expression in T47D breast cancer cell line. Silibinin has anti-carcinogenic effect on human prostate, breast and cervical carcinoma cells. Evidence shows that anti-cancer properties of silibinins make it a great agent for cancer therapy and these activities of silibinin have been published in the scientific literature.

One reason to select PLGA-PEG is that the PEG is located on the surface of the nanoparticles and easily exposed to the water surrounding the nanoparticles, whereas the...
Nanodelivery in breast cancer therapy

PLGA part of the copolymer is mainly located in the relatively hydrophobic core of nanoparticles. The silibinin is loaded to the surface of the PLGA-PEG with Fe₃O₄ released at the target site due to its external localized magnetic field gradient. It is a suitable object with nonspecific toxicities producing more accurate effect on targeted tissue even when used in lower amount of silibinin. The surface and size of the PLGA-PEG are important for monitoring systemic tumor response to drug treatment. PLGA-PEG-Fe₃O₄ which was used in this study captured maximum percentage of polymer compared to 2000 and 3000. As the rate of PEG drug loading will have better outcome, irrespective of PLGA-PEG composition, the average molecular weight and the molecular weight distribution of the PLGA-PEG remain stable over an incubation period of 7 days and can improve bioavailability of the silibinin. PLGA-PEG-Fe₃O₄ remains for few days and its rapid nanoparticle degradation in vivo would be advantageous because it would result in rapid polymer removal from the body. More interestingly, silibinin and nanoparticle with silibinin are suitable inhibitors of transcription factors like NFKB and AP-1. In this study, different concentrations of silibinin-loaded PLGA-PEG-Fe₃O₄ were used for three different times 24, 48 and 72 h by MTT assay to demonstrate cytotoxic effect of silibinin-PLGA-PEG-Fe₃O₄ on T47D breast cancer cell line. For this aim, preparation of silibinin-PLGA-PEG-Fe₃O₄ was made. The proportion of silibinin loaded into PLGA: PEG₄₀₀₀ was about 76%, which means about 76% of silibinin was loaded into PLGA-PEG-Fe₃O₄. This proportion is appropriate for silibinin uptake by cells. As can be seen, the result is much better than those modified with PLGA: PEG₃₀₀₀ PLGA: PEG₄₀₀₀ copolymers which were 69.5% and 73%, respectively.

With this system, the nanoparticle could be used as drug-loading vehicle for hydrophobic drugs with the advantage of being directed into the target tissue by its magnetic section.

Based on our knowledge, the effect of silibinin-loaded PLGA-PEG-Fe₃O₄ on hTERT gene expression in T47D cell line has never been studied up to now. Although there is no study in the literature to be compared with this study, this nanoparticle was used and confirmed for drug delivery system in other research and articles.

**Conclusion**

The results of this work demonstrate that silibinin-loaded PLGA-PEG-Fe₃O₄ in T47D had inhibitory effect on T47D breast cancer cell line more than free silibinin. This inhibition was dose-dependent, but it was not time-dependent. Silibinin-loaded PLGA-PEG₄₀₀₀-Fe₃O₄ cytotoxic effect increased by increasing the concentration of silibinin-loaded PLGA-PEG-Fe₃O₄. Analysis of data distinguished that decreasing hTERT expression level is directly associated with increasing silibinin-loaded PLGA-PEG-Fe₃O₄ concentration. In summary, our data showed that silibinin-loaded PLGA-PEG-Fe₃O₄ inhibited hTERT expression and this complex could be used as an anti-cancer drug in the breast cancer.

**Acknowledgement**

This work was supported by Tuberculosis and Lung Research Center of Tabriz University of Medical Sciences and we thank this centre for funding the study.

---

**Table 2. hTER mRNA level in Real-time PCR measurement**

<table>
<thead>
<tr>
<th>Silibinin-loaded PLGA-PEG-Fe₃O₄</th>
<th>Sample Ct</th>
<th>Internal control Ct</th>
<th>ΔCt</th>
<th>2-ΔΔCt</th>
</tr>
</thead>
<tbody>
<tr>
<td>60µM</td>
<td>24.38</td>
<td>18.86</td>
<td>5.42</td>
<td>0.029</td>
</tr>
<tr>
<td>40µM</td>
<td>22.55</td>
<td>18.89</td>
<td>3.66</td>
<td>0.106</td>
</tr>
<tr>
<td>20µM</td>
<td>21.17</td>
<td>18.94</td>
<td>2.23</td>
<td>0.294</td>
</tr>
<tr>
<td>70µM</td>
<td>20.85</td>
<td>18.87</td>
<td>1.99</td>
<td>0.342</td>
</tr>
<tr>
<td>DMSO control</td>
<td>19.56</td>
<td>18.93</td>
<td>0.63</td>
<td>0.876</td>
</tr>
<tr>
<td>PLGA-PEG-Fe₃O₄ control</td>
<td>19.51</td>
<td>18.95</td>
<td>0.56</td>
<td>0.920</td>
</tr>
<tr>
<td>Untreated cell control</td>
<td>19.41</td>
<td>18.97</td>
<td>0.44</td>
<td>1.000</td>
</tr>
</tbody>
</table>

---

*Fig. 7. Decreasing trend in the hTERT mRNA levels with increasing silibinin-loaded PLGA-PEG₄₀₀₀-Fe₃O₄ concentration in T47-D breast cancer cell line (concentration: 70 µM is concentration of free silibinin. 20, 40 and 60 µM show silibinin-loaded PLGA-PEG₄₀₀₀-Fe₃O₄ concentrations.*
Ethical issues
Not applicable in this study.

Competing interests
The authors report no competing interests.

References