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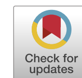
A novel protocol for bacterial ghosts' preparation using tween 80



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ABSTRACT

Bacterial ghosts (BGs) can be prepared by both genetic and chemical means. Genetic method include using lysis gene *E*. Chemical method include incubation with numerous agents for a short time at their minimum inhibitory or minimum growth concentrations (MIC or MGC). The aim of this study is to prepare the BGs with a new protocol via exposing the bacterial cells to tween 80 for an extended period of time followed by sudden reduction of the surrounding pH. *Salmonella enterica serovar typhimurium* ATCC 13311 was used for this purpose. The cells were incubated in 7% $^{v}/_{v}$ tween 80 solution in Muller-Hinton broth for 24 h at 37 °C then pH was decreased to 3.6 by adding lactic acid for one hour. The bacterial pellets were separated by high speed centrifugation, and then washed three times by half normal saline solution. High quality BGs were visualized by scanning electron microscopy (SEM) revealing punctured cells with intact outer shells and at least one intramembranous tunnel. The absence of vital cells was confirmed by subculturing. The release of respective amounts of proteins and DNA is another evidence of ghost's production. In addition, the integrity of cells was proved by visualization of Gram-stained cells using light microscopy. In conclusion, this new protocol is simple, economic and feasible for BGs preparation.
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1. Introduction

The need for safe vaccines, biological carriers and effective drug delivery systems has increased tremendously in recent times. The Bacterial Ghosts (BGs) are one of the biotechnology platforms that can satisfy this need. BG, by definition, is an empty cellular envelop of Gram-negative bacteria conserving their surface and antigenic characters and lacking the internal components (Langemann et al., 2010). This definition was confined to Gram-negative bacteria because of their unique ability to translate highly specific protein E of Phage ϕX174, which was the only means of creating BGs till recently. This protein can create transmembrane tunnel which leads to cell death without full lysis (Lubitz et al., 2009). Gene E of the Phage ϕX174 encode this 91-aa lysis protein (Kwon et al., 2005). Under controlled translation of this gene into the lysis protein has been used for production of Gram-negative bacterial (*Escherichia coli*, *Salmonella typhimurium*, *Salmonella enteritidis*, *Klebsiella pneumoniae*, *Bordetella bronchiseptica, Heliobacter pylori, Vibrio cholerae, Haemophilus influenza, Pasteurella multocida, Pseudomonas aeruginosa*) ghosts (Haidinger et al., 2003; Langemann et al., 2010). The definition of BG has been extended to include Gram-positive bacteria by virtue of using specific chemical agents in critical concentrations and critical times (Vinod et al., 2015).

Bacterial ghosts are useful in different biotechnology applications. Vaccine delivery system using ghost preparation offers effective humoral and cellular immune responses (Peng et al., 2011). Veterinary vaccines also can be prepared using the respective ghosts (Kwon et al., 2006). Targeting chemotherapeutic agents as doxorubicin to human colon cancer cell line was very effective utilizing genetically obtained *E. coli* ghosts leading to less side effects of the anticancer (Paukner et al., 2004). The effective internalization of *Escherichia coli* and *Mannheimia haemolytica* ghosts by human conjunctiva epithelial cells was used as beneficial drug

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carriers (Kudela et al., 2011). Additionally, BGs carrying DNA can be used in gene therapy (Tabrizi et al., 2004; Kudela et al., 2008). Although genetically obtained ghosts' vaccines are relatively safe, they still have a sort of pathogenicity due to the very little chance of viable cells existence. The full population killing can be achieved by extra processing and manipulation (Haidinger et al., 2003). In this study, we introduce a novel chemical induced BG preparation protocol based on using a critical concentration of a chemical agent for long time.

2. Materials and methods

2.1. Target strain

The bacterial strain targeted to be ghosts was *Salmonella enterica serovar typhimurium* ATCC 13311 obtained from American Type Culture Collection, (VA, USA). The lyophilized cells were reconstituted, then cultured in Muller-Hinton broth (Fluka, Milwaukee, WI, USA) and incubated at 37 °C for 24 h.

2.2. Determination of minimum inhibitory concentration (MIC)

MIC was determined for specific reagents according to the American Society for Microbiology guidelines (Coyle et al., 2005). Stock solutions of Muller-Hinton broth (Fluka, Milwaukee, WI, USA) were prepared containing the following reagents: Tween 80 (5% v/v), SDS (5% w/v), KOH (3% w/v), NaOH (3% w/v), benzoic acid (0.3% w/v) (Lobachemie, Mumbai, India), EDTA (0.2% w/v) (Scharlau, Barcelona, Spain), and lactic acid (0.15% v/v) (WINLAB, East Midlands, England). Tenfold serial dilutions were prepared for each reagent. Aseptically, 100 μl of standard inoculum of S. Typhimurium (matched 0.5 McFarland standard) were added to each diluton. All the tubes were incubated for 24 h before tested for turbidity.

2.3. Preparation of bacterial ghosts

A total number of 9 sterile tubes of 2 mls of Muller-Hinton broth solution supplied with 7% v/v tween 80 were inoculated by 100 μl of standard inoculum of S. Typhimurium. The tubes have divided into 3 groups. Each group involved three tubes which designated for 3 different periods of times: 24, 36 and 48 h. The first group was not treated more. The second group was frozen for one hour at the end of each incubation period. Finally, at the end of each incubation period, the third group was treated by addition of lactic acid (pH = 3.6) and lasting for 20 mins. The third group was examined more by extending the contact time for lactic acid to 30 min, 1, 2 and 3 h. Each experiment was done in triplicate and the results were expressed as average.

Centrifugation was done using (Hettich EBA20S Tuttlingen, Germany) portable centrifuge at 4000×g for 10 min. The supernatant was utilized for quantification of proteins and DNA. The obtained pellets were washed by sterile half normal saline solution three times.

2.4. Quantification of released proteins

Bradford method of protein quantification (Bradford, 1976) was used/applied for determination of protein quantities (μg/ml) released by ghost cells using a NanoDrop™ 2000/2000c (Thermo Scientific, MA, USA) spectrophotometer. The standard bovine serum albumin (BSA) provided by the manufacturer was used for generation of standard curve. All readings were taken at 595 nm. The protein contents of the culture media were considered and calculated by measuring the protein contents in the un-inoculated culture media.

2.5. Quantification of released DNA

The NanoDrop™ 2000/2000c -Thermo Scientific spectrophotometer was used for quantification of released DNA in (μg/ml) in the supernatant at 260 nm. Standard concentrations of DNA were used for generation of standard curve. The ratio of absorbance at 260 nm/280 nm was measured to assess DNA purity.

2.6. Scanning electron microscopy (SEM)

The centrifuged pellets of bacteial cells were investegated by SEM (JEOL-JSM-5500 LV): The samples were fixed by glutaraldehyde (2.5%) and dehydrated by serial dilutions of ethanol using automatic tissue processor (Leica EM TP). The samples were dried using CO_2 critical point drier (Tousimis Audosamdri-815). The samples were coated by gold sputter coater (SPI-Module). Finally, samples were examined by SEM with amplification power of x9500 and 20 kV and using high vacum mode at the Regional Center Mycology and Biotechnology, Cairo, Egypt.

2.7. DNA extraction from pellets and supernatant

The DNA was extracted from both pellets and supernatant using AxyPrep™ multisource genomic DNA miniprep kit (Tewksbury, MA, USA). The pellets were separated through high speed centrifugation. The harvested pellets and the supernatant were suspended in TBE buffer, pH = 8. Both pellets and supernatant were processed according to the supplier's specifications.

2.8. Agarose gel electrophoresis

The extracted DNA was analyzed using agarose 0.8% gel electrophoresis separation. Fifteen microliters of DNA extract were mixed with 5 μl DNA gel loading dye (6X) (Thermo Scientific, Waltham, USA). The separated DNA bands were compared with a standard 1 Kb DNA extension ladder marker (Thermo Scientific, MA, USA).

2.9. Gram staining and light microscopy

The centrifuged pellets of bacteial cells were stained by Gram stain then visualized by light microscope using amplification power of 1000 x in order to invistigate cellular external surface integrity.

2.10. Lyophilization of ghost cells

The obtained ghost cells pellets produced from treatments were lyophilized using Christ lyophilizer (Osterode am Harz, Germany)

2.11. Statistical analysis

The significant differnce between the means at a confidence interval of 95% were done using ANOVA and post hoc analysis utilizing IBM based SPSS program version.23.

3. Results

MICs of the following reagents: KOH, NaOH, lactic acid, benzoic acid and EDTA were: 0.0234% w/v, 0.0469% w/v, 0.075%v/v, 0.15% w/v and 0.025%w/v respectively. It was found that the tested serial dilutions of tween 80 and SDS could not inhibit bacterial growth.

Table 1
The effect of different treatments on bacterial ghost production.

Released proteins (average) µg/ml*			Released DNA (average) µg/ml*			Electron microscope revealing pores			Light microscope revealing perfect cells			Subculture Growth		
Tween 80	Tween 80 +Freezing	Tween 80+ Lactic acid	Tween 80	Tween 80 +Freezing	Tween 80 +Lactic acid	Tween 80	Tween 80 +Freezing	Tween 80 +Lactic acid	Tween 80	Tween 80 +Freezing	Tween 80 +Lactic acid	Tween 80	Tween 80 +Freezing	Tween 80 +Lactic acid
Incubation with tween 80 (7%) for 24 h														
507.5	593.8	2975	0.85	1.5	785.9	+	++	+++[a]	+	+	+	+	+	–

* *P* value < .05.
[a] +: The observed number of pores are relatively low; ++: medium; +++: high.

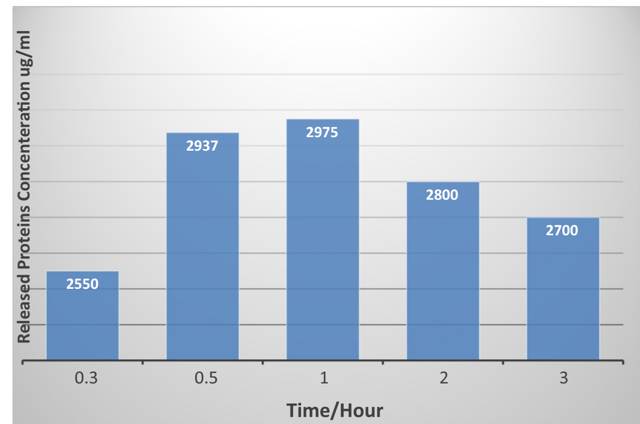

Fig. 1. Time course of released proteins from *Salmonella enterica* serovar typhimurium ATCC 13311, after incubation with lactic acid (pH = 3.6).

None of the used reagents produced high quality ghost cells when added to the broth culture after incubation for 24 h at 37 °C except tween 80. Incubation of S. Typhimurium in Muller-Hinton broth containing 5% w/w tween 80 resulted in cells' perforation. All used reagents other than tween 80 has produced cells lacking the integrity of their outer surface. The produced pores were proved by (SEM) and the release of proteins and DNA quantities. Incubating the cells with tween 80 at 5%, 6% and 7% concentrations led to a variation in the proteins and DNA released. It was found that using 7% of tween 80 induces the release of the largest quantities of proteins but not the largest quantities of DNA. The largest amount of DNA was released after using 6% tween 80.

The subculture of isolated cells was positive which indicates incomplete ghosts' preparation. In order to optimize the product quality, extra process was needed. After incubation of S. Typhimurium with tween 80 at concentrations of 5%, 6% and 7% for 24, 36 and 48 h, all the cells were frozen for one hour then thawed. The largest amount of proteins and DNA were released after using 7% tween for 24 h. In addition, SEM images showed surface pores but cells were grown after subculture. The partial killing of the culture indicates incomplete BG production, also it will be unsafe product. The effect of low pH was examined after incubation of S. Typhimurium with tween 80. The significant increase of released proteins and DNA occurred when using tween 80, 7% for 24 h followed by addition of lactic acid at pH 3.6 for one hour. No growth has occurred after subculture indicating full killed population and safe product.

By comparing the three trials; incubation with tween 80 only, post tween 80 freezing and post tween 80 reduction of pH, a significant increase in the amount of released proteins and DNA, (*P*-value < .05 at 95% confidence interval) was found when using lactic acid after tween 80(7%). These conditions induced the release of the largest quantity of proteins and DNA in addition to puncturing the cells and killing the entire population (Table 1).

In order to optimize BG quality, different periods of exposure to lactic acid were tested. The optimum times of exposure that led to release of the highest quantity of proteins and DNA were one hour and half an hour respectively as indicated in Figs. 1 and 2. Also, trans-membrane tunnels were revealed by SEM (Fig. 3A & B). SEM image for *Salmonella enterica* serovar *typhimurium* ATCC 13311 cells. (A): Untreated cells showing unaffected surfaces; (B): Treated cells by tween then lactic acid, showing some surface pores.

Gel electrophoresis analysis of DNA that was extracted from the pellets of untreated and treated cells showed differences in band intensities. The control pellets (untreated) showed more intense

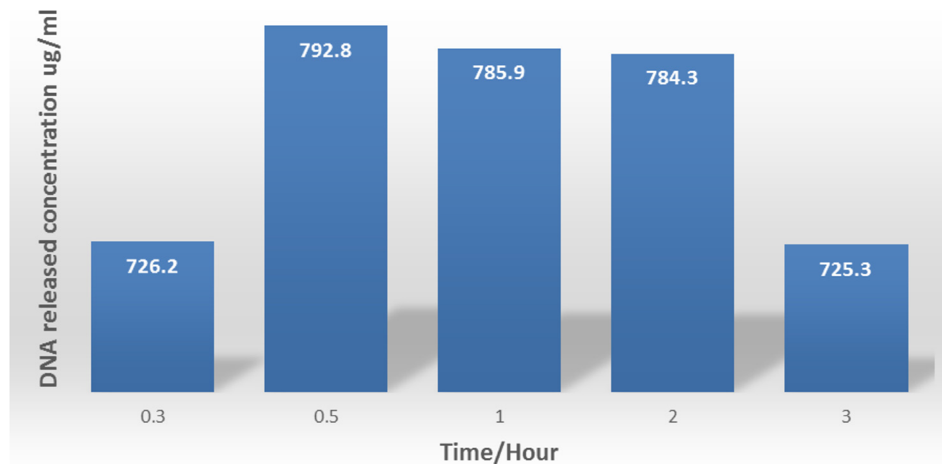

Fig. 2. Time course of released DNA from *Salmonella enterica* serovar typhimurium ATCC 13311, after incubation with lactic acid (pH = 3.6).

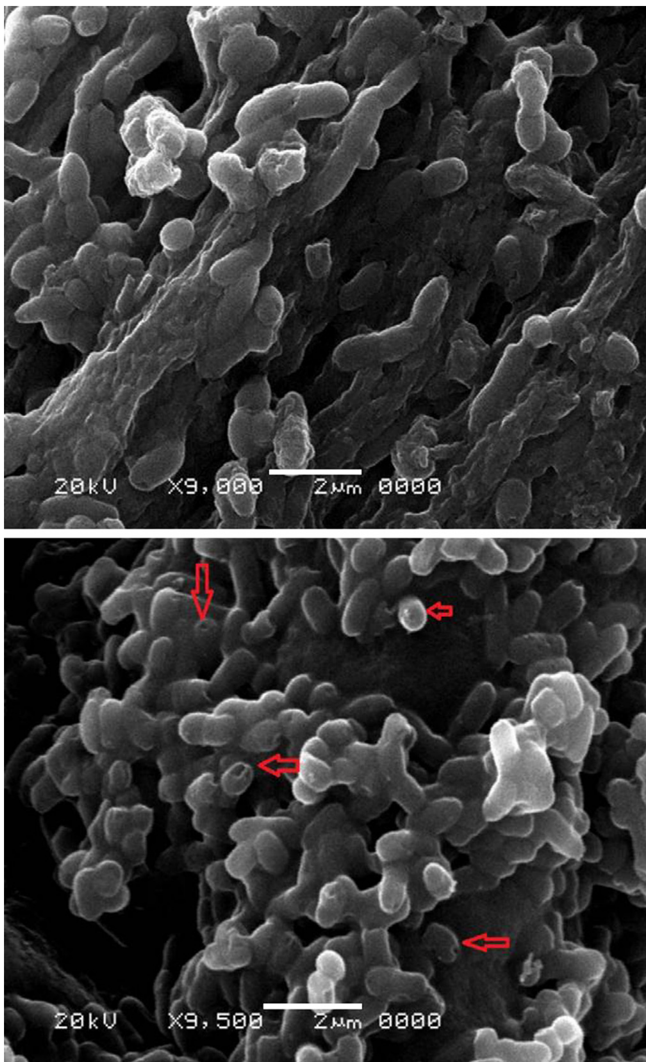

Fig. 3. Scanning electron microscope (SEM) image for *Salmonella enterica* serovar typhimurium ATCC 13311 cells. (A): Untreated cells showing unaffected surfaces (B): Treated cells by tween then lactic acid, showing some surface pores.

bands than those treated by tween 80 and subjected to different incubation periods with lactic acid, which showed less intense bands. The faintness in intensity of DNA bands was proportionally related to the treatment conditions and indicated successful evacuation of cells from large quantities of DNA. Consequently, the optimum treatment for producing the desired evacuated, killed and perfect cellular shells was achieved by incubating the bacterial cells with 7% tween 80 for 24 h then reducing the pH to 3.6 by adding lactic acid for one hour as shown in the flow chart (Fig. 4).

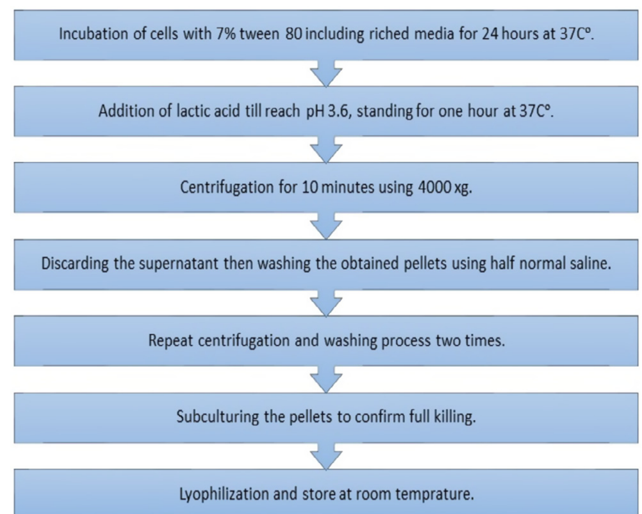

Fig. 4. Flow chart showing bacterial ghost preparation protocol steps.

4. Discussion

The controlled activation of *E*-lysis gene that is used for preparation of bacterial ghosts is limited to Gram negative bacteria (Kudela et al., 2010). Multiple steps should be applied to obtain hundred percent of non-reproductive lysed cells (Kwon et al., 2006). The high cost and sophistication are limiting factors of using the genetic methods for preparation of bacterial ghosts. Alternatively, Chemical agents can be used in critical concentrations and periods of times for preparation of Gram positive BGs (Vinod et al., 2015; Wu et al., 2016; Amara, 2017), Gram negative BGs (Amara et al., 2013; Vinod et al., 2014; Park et al., 2016; Menisy et al., 2017), yeasts' ghosts (Amara, 2015) and even viral ghosts (Abd El-Baky and Amara, 2014). Gram-positive bacterial ghosts can be chemically obtained by the same concepts that are used for preparation of Gram-negative bacterial ghosts (Vinod et al.,

2015). High quality ghosts have been obtained by using the suitable chemicals, optimizing their concentrations, temperature and shacking speed (Amro et al., 2014).

The chemical agents were chosen because of their impact on outer surfaces of cells. The permeabilization effect on the outer membranes or on the cell walls is the common effective factor in these agents (Vaara, 1992). In the previous protocols of chemically induced ghost preparation, bacterial cells in their stationary growth phase were exposed to either one chemical agent (Park et al., 2016) or number of chemicals with or without physical factors for short time (Amara et al., 2013; Amro et al., 2014). Using of sodium hydroxide was highly effective in producing high quality bacterial ghosts of some Gram-negative bacteria such as: *Salmonella enteritidis* (Vinod et al., 2014) and *Vibrio parahaemolyticus* (Park et al., 2016). In addition, it was used for preparation of the Gram-positive bacteria *Staphylococcus aureus* (Vinod et al., 2015). In a chemically induced BG preparation procedure called sponge-like protocol, sodium hydroxide was combined with other compounds in specific concentrations and added for short times to produce the desired ghosts (Amara et al., 2013). Alkalies have a damaging effect on the outer membrane of Gram-negative bacteria (Hinto and Ingram, 2006). Sodium dodecyl sulfate (SDS) is an amphoteric compound which has hydrophilic head and hydrophobic tail so it affects the outer membrane permeability and hydrophobicity and keeps bacteria more liable to disinfectants and antimicrobials (Walton et al., 2008). Hydrogen peroxide oxidizes essential external cellular components by producing hydroxyl free radicals. The lack of catalase enzyme in Gram-positive bacteria limits the effect of hydrogen peroxide on their walls. In contrast, Gram-negative bacteria are more liable to hydrogen peroxide effect due to the existence of catalase enzyme. (McDonnell and Russell, 1999).

Accordingly, tween 80 is the sole key-player in this protocol. High quality- perfect three dimensional bacterial shells- was achieved using different concentrations of tween 80 alone without inhibiting growth or killing the whole population.

The proposed mechanism of penetrating effect of ethylenediaminetetraacetic acid (EDTA) was the chelation with divalent cations (for example: calcium and magnesium) which maintain the outer membrane integrity. It can also liberate a high quantity of LPS and decrease the permeability and hydrophobicity of the outer shell of *Salmonella enterica serovar typhimurium* (Alakomi, 2007; Alakomi et al., 2003). Organic acids also act as permeabilizers. Benzoic acid and lactic acid can disturb outer membrane permeability by penetration and acidifying cytoplasm resulting in deactivation of acid sensitive enzymes (Alkomi et al., 2000). Disturbing the outer membrane of *Salmonella infantis, Salmonella typhimurium, Escherichia coli and Pseudomonas aeruginosa* could be indicated by Lipopolysaccharides (LPS) release and N-phenyl-1-naphthylamine (NPN) uptake. Lactic acid was the strongest organic acid that can liberate LPS in comparison with other acids (Alakomi, 2007).

Our protocol involves exposure of bacterial cells to tween 80 starting from lag growth phase till stationary growth phase for relatively a long time. Tween 80 (polysorbate 80) is a nonionic surfactant used in food and drug industry. It is used in solubilization of hydrophobic components. In case of *Pseudomonas aeruginosa*, the longer the exposure of the cells to tween 80, the higher the degree of leakage of outer membrane components (Brown and Winsely, 1969). It was obvious that tween 80 in- concentrations of 5% and 6%- was very effective in potentiation of bactericidal activity of number of antibiotics against *Pseudomonas aeruginosa.* However, the *Pseudomonas aeruginosa* resistance to theses antibiotics without addition of tween 80 suggests that tween 80 affect outer membrane permeability by disturbing porin proteins, efflux pumps and finally uptake mechanisms (Al-Thamir et al., 2010).

The release of reasonable quantities of proteins and DNA is a strong indicator for effective cellular evacuation**.** In our protocol, the maximum liberated quantities of proteins and DNA were 2975 μg/ml and 793 μg/ml respectively. In another protocol of preparing BG of *E. coli*, the released proteins' amounts were 3425 μg/ml, while DNA released amounts were 179 μg/ml (Amara et al., 2013). It was obvious that the quantified liberated DNA were consistent with gel electrophoresis results. DNA bands' intensity proportionally increased with the increased DNA quantity. The proposed mechanism of BG preparation is due to the long exposure time of bacterial cells to tween 80 which leads to the dissolution of the hydrophobic components of Salmonella's outer membrane forming weak points. These points facilitate punctures formation that caused by the sudden exposure to lactic acid. Indeed, the full antigenic characters of obtained ghosts need further investigation by observing their ability to elicit valuable immune response. The applicability of this protocol in preparation of BG of Gram positive bacteria should be examined.

5. Conclusion

The current protocol of chemically induced ghost preparation can be used for obtaining high quality and surface integrated ghosts of S. Typhimurium. It is cheap, simple, and feasible, avoids the complicated genetic approaches and after all produces reproducible results. Further investigation is needed to study their utilization as a drug delivery systems, biological carriers or vaccines.

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Conflict of interest

We declare no conflicts of interest.

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