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Artificial Neural Network Modeling to Predict the Non-Linearity in Reaction Conditions of Cholesterol Oxidase from *Streptomyces olivaceus MTCC* 6820

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Abstract

Cholesterol oxidase (COX) is widely used enzyme for total cholesterol estimation in human serum and for the fabrication of electro-chemical biosensors. COX is also used for the bioconversion of cholesterol; for the production of precursors of steroidal drugs and hormones. Enzyme activity depends decisively on defined conditions with respect to pH, temperature, ionic strength of the buffer, substrate concentration, enzyme concentration, reaction time. Standardization of these parameters is desirable to attain optimum activity of the enzyme. The present work aims to build a neural network model using five input parameters (pH, cholesterol concentration, 4-aminoantipyrine concentration, crude COX volume and horseradish peroxidase) and one output *i.e.*, COX activity (U/ml) as a response. A feed forward back propagation neural network with Levenberg-Marquardt training algorithm was used to train the network. The network performance was assessed in terms of regression (R^2), Mean Squared Error (MSE) and Mean Absolute Percentage Error (MAPE). A network topology of 5-10-1 was found to be optimum. The MSE, MAPE and R^2 values of the neural model were 0.0075%, 0.12% and 0.9792% respectively. The maximum predicted activity of COX was 1.073 U/ml, which was close to the experimental value *i.e.*, 1.1 U/ml at simulated optimum assay conditions. MSE and MAPE depicted the precision in the prediction efficiency of the developed ANN model. Higher R^2 value showed a good correlation between the experimental and ANN predicted values. This proved the robustness of the ANN model to predict similar type of system (COX from other *Streptomyces* sp.) within the limits of the trained data set. The COX activity was enhanced by 1.71 folds after optimization of the reaction conditions.

Keywords

Cholesterol Oxidase, Artificial Neural Network, Optimization, *Streptomyces olivaceus*, Prediction

1. Introduction

Cholesterol oxidase (COX) (EC 1.1.3.6) is a bacterial flavoenzyme that catalyzes the oxidation of cholesterol to 4-cholesten-3-one with the simultaneous reduction of molecular oxygen to hydrogen peroxide [1]. Owing to its enormous applications in the field of clinical pathology for the estimation of total serum cholesterol [2], determination of cholesterol concentrations in food samples for the quality control assay, and for the commercial production of precursors of the steroidal hormones from cholesterol and its derivatives [3]; COX has come up as a useful biotechnological tool in present times. An increasing need for specific estimation of this steroid in clinical samples has enhanced the importance and demand of COX in the pharmaceutical industry.

COXs have been isolated and purified from various micro-organisms including *Actinomyces lavendulae* [4], *Streptomyces fradiae* [5], *Streptomyces* sp. [6]. *Streptomyces* are potent producers of valuable antibiotics and are also non-pathogenic in nature. As per the available literature, *Streptomyces* is a promising culture for COX production to meet the above mentioned applications. Various methods have been reported for the estimation of COX activity including colorometric enzymatic method [2], UV method using 4-cholestenone as a standard [7], and fluorometric enzymatic method [8] [9]. Detection of cholesterol by enzymatic method involving COX coupled with H_2O_2 is though extremely simple, specific and highly sensitive. It indicates the relative concentration of cholesterol indirectly by the measurement of H_2O_2. Coupling of H_2O_2 with chromogen like 4-aminoantipyrine and o-dianisidine in the presence of peroxidase yields adduct that exhibits highly absorbing chromophore Quiononeimine allowing more sensitive measurement of cholesterol than any other method. This approach makes it an effective assay method.

The enzyme activity depends on manifold factors and a general understanding of the particular features of enzymes produced by any new microbial source is required. Enzyme activity depends decisively on defined conditions with respect to pH, temperature, ionic strength of the buffer, substrate concentration, enzyme concentration, reaction time, etc. Standardization of these parameters is desirable to attain optimum activity of the enzyme [10]. Enzymes display their highest activity at their respective optimum conditions, and deviations from the optimum cause a reduction in the activity, depending on the degree of deviation. Optimization of reaction conditions for an enzyme provides direct information about the optimal values of the parameter under study and combined effects of various enzyme assay parameters for the en-

hancement of activity [11]. In the past decade, the enzyme assay conditions for cellulase and inulinase have been optimized by classical method [12] and factorial design with surface response analysis [13] respectively. As reported by Singh and Banik, 2014, the reaction conditions for L-Glutaminase was optimized using RSM and ANN models (Singh and Banik, 2014) [11]. Parameters optimization for an enzyme assay is a complex process due to interactions of the factors on which they depend. This complexity can be minimized up to a certain extent with the advent of new tools and methodologies in mathematics and statistics. Therefore, a combination of mathematical and computational methods capable of predicting enzyme activity would be helpful to enhance the COX activity thus optimizing the factors affecting COX activity. ANN mimics the neural functioning of human brain and is a data-driven approach primarily based on input-output data [14]. ANN is a model-independent technique capable of predicting highly variable and non-linear bio-catalytic reaction like enzymatic assays with high accuracy.

In the present paper, we have worked for the optimization of assay conditions for the estimation of COX produced by a new species of *Streptomyces i.e. Streptomyces olivaceus MTCC* 6820 using ANN. In order to achieve enhanced COX activity (U/ml), the assay conditions for new microbial source needs to be standardized as the effective operating conditions for every new system are different. The incorporation of substrate concentration (cholesterol and 4-aminoantipyrine), pH of reaction mixture, enzyme concentrations (COX and peroxidase) as input parameters enable the development of robust ANN network for prediction of the response. To the best of our knowledge, this would be the first report on cholesterol oxidase activity by *Streptomyces olivaceus MTCC* 6820.

2. Materials and Methods

2.1. Chemicals Used

All the chemicals used were of analytical grade. Cholesterol was purchased from Sigma Aldrich Pvt. Ltd and Horseradish peroxidase was purchased from Sisco Research Laboratories, Mumbai, India.

2.2. Microorganisms and Growth Media

Streptomyces sp. including *S. niger MTCC* 4010, *S. fradiae MTCC* 4002, *S. olivaceus MTCC* 6820, *S. hygroscopicus MTCC* 4003, *S. annulatus MTCC* 6818, and *S. clavifer MTCC* 4150 were procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. All the *Streptomyces* cultures were maintained in the *Streptomyces* growth medium containing (g/L): glucose—4, yeast extract—4, malt extract—1, $CaCO_3$—2 and agar—12; pH was adjusted to 7.2 with KOH. The slants were incubated at 30˚C ± 2˚C for 48 - 72 h for the growth of organism, preserved at 4˚C in the refrigerator. All the cultures were routinely sub-cultured in every 30 days.

2.3. Medium Components and Culture Conditions

The production medium for COX contained (g/L): cholesterol—2, glucose—12, starch—9, yeast extract—6, peptone—4, $(NH_4)_2SO_4$—7.5, cholesterol—2, K_2HPO_4—1, $MgSO_4$—0.5, NaCl—1, $MnSO_4$—0.008, $CaSO_4$—0.002, $ZnSO_4$—0.002, $FeSO_4$—0.02, $CaCl_2$—0.0002 and Tween 80—10 ml [5]. COX production was carried out in 250 ml Erlenmeyer flask, incubated at 30˚C ± 2˚C for 72 h in an orbital shaker (Orbitek, Scigenics Biotech Pvt. Ltd., Chennai, India) at 180 rev/min.

2.4. Cholesterol Oxidase Assay and Protein Estimation

COX activity assay was done by modified method of Allain *et al.* [1] [2]. 50 µL of 6 g/L cholesterol (dissolved in dimethyl formamide containing 5% (v/v) Triton X-100) was added to 1 ml of reaction mixture containing 1mM 4-aminoantipyrine, 5 mM phenol, 5 U/ml horseradish peroxidase and sodium phosphate buffer (20 mM, pH 7.0); pre-incubated for 5 min at 30˚C. 100 µL of crude enzyme extract was added to the pre-incubated reaction mixture to start the reaction and the incubation continued for 10 min at 30˚C. The reaction was terminated by placing the samples in a boiling water bath for 2 min and then immediately placed in an ice-bath for 2 min for color development. Absorbance was recorded at 500 nm (UV 1800 Spectrophotometer, Shimadzu, Japan). Blank was prepared by adding inactivated enzyme to the reaction mixture. One unit of cholesterol oxidase activity was defined as the amount of enzyme that converts 1µmol of cholesterol into 4-cholesten-3-one per minute at 30˚C.

$$\text{CHOLESTEROL} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{4-CHOLESTEN-3-ONE} + H_2O_2$$

$$H_2O_2 + \text{PHENOL} + \text{4-AMINOANTIPYRINE} \xrightarrow[\text{Peroxidase}]{\text{Horseradish}} \text{QUINONEIMINE DYE } (A_{500\text{nm}})$$

Protein concentration was determined by Bradford's method using Coommassie Brilliant Blue G-250 dye. The standard curve of bovine serum albumin (BSA) was prepared in the concentration range of 0.01 to 0.2 mg/ml. Absorbance was recorded at 595 nm [15].

2.5. Screening of COX Producing Microorganism

Six different *Streptomyces* sp. as listed in **Table 1** were procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India.

Table 1. Estimation of cholesterol oxidase activity among different *Streptomyces* sp.

Streptomyces sp.	Cholesterol oxidase activity (U/ml)
S. niger MTCC 4010	0.27
S. fradiae MTCC 4002	0.32
S. olivaceus MTCC 6820	0.625
S. hygroscopicus MTCC 4003	0.472
S. annulatus MTCC 6818	0.355
S. clavifer MTCC 4150	0.254

All the six strains were examined for their extracellular COX producing ability under un-optimized enzyme assay conditions.

2.6. Input Parameters and Design Matrix

Enzyme activity is significantly influenced by substrate concentration, enzyme concentration, pH, and incubation temperature. The enzyme assay parameters were optimized by classical method in our preliminary studies, out of which five most effective parameters were considered for ANN modeling. Five assay parameters *viz.* pH of the reaction mixture, cholesterol concentration, 4-aminoantipyrine concentration, crude COX volume and horseradish peroxidase were the determinants of COX activity assay and served as network inputs, whereas COX activity (U/ml) was the output. The range and levels of the independent factors studied are given in Table 2. The input range provided in the Minitab was based on the results of the preliminary studies; which resulted in a five-level-five factor CCD. The experimental design was obtained by applying central composite design (CCD) to five independent variables using Minitab version 17.0. The experimental design matrix consisting of the central values used for modeling are presented in Table 3.

2.7. Artificial Neural Network Modeling

The neural model was created in MATLAB 2017a (Mathworks, USA) using neural network toolbox (command *nftool*) with five input variables (X_1, X_2, X_3, X_4 and X_5) and one output variable (COX activity). A multilayer feed forward back propagation neural network was employed for the modeling and prediction of COX activity. The network architecture consisted of one input layer, one hidden layer and one output layer (shown in Figure 1). The network was trained using Levenberg-Marquardt learning algorithm till the preferred network accuracy was achieved by multiple trainings of the network concurrently adjusting the number of neurons in the hidden layer. The input and output dataset were divided into three sets: Training (22), Validation (5) and Testing (5). Network performance was monitored through performance plot and regression analysis at each step consisting of training, validation and testing.

Table 2. Independent variables used in the experimental design for ANN modeling along with their levels.

Factors	Name of factors	Levels				
		$-\alpha$	−1	0	+1	$+\alpha$
X_1	pH of the reaction mixture	6	7	8	9	10
X_2	Cholesterol concentration	0.2	0.4	0.6	0.8	1.0
X_3	4-Aminoantipyrine	−0.5	0.5	1.0	1.5	2.5
X_4	Crude COX volume	0	50	100	150	200
X_5	Horseradish Peroxidase	0	5	10	15	20

Table 3. CCD of five independent variables displaying experimental and ANN predicted COX activity.

Run Order	X_1	X_2	X_3	X_4	X_5	COX Activity (U/ml)	
						Experimental	ANN Predicted
1	7	0.8	0.5	50	5	0.047	0.058
2	8	0.6	1.5	100	20	0.803	0.796
3	8	0.6	1.5	100	10	1.100	1.073
4	9	0.4	2.5	150	5	0.580	0.596
5	8	0.6	1.5	100	10	1.070	1.073
6	8	0.6	1.5	200	10	0.671	0.664
7	7	0.8	2.5	50	15	0.595	0.392
8	8	0.6	1.5	100	10	1.080	1.073
9	6	0.6	1.5	100	10	0.656	0.615
10	7	0.4	0.5	50	15	0.545	0.471
11	8	0.6	1.5	100	10	1.100	1.073
12	9	0.4	0.5	50	5	0.005	0.007
13	7	0.4	2.5	50	5	0.509	0.355
14	9	0.4	2.5	50	15	0.504	0.500
15	8	0.6	1.5	100	10	1.090	1.073
16	8	0.6	-0.5	100	10	0.322	0.413
17	8	0.6	1.5	100	0	0.268	0.256
18	8	1.0	1.5	100	10	0.919	0.891
19	9	0.8	2.5	150	15	0.882	0.873
20	7	0.8	2.5	150	5	0.705	0.769
21	9	0.8	2.5	50	5	0.444	0.501
22	7	0.4	2.5	150	15	0.532	0.764
23	8	0.6	3.5	100	10	0.788	0.809
24	8	0.6	1.5	0	10	0.105	0.097
25	7	0.4	0.5	150	5	0.387	0.364
26	8	0.2	1.5	100	10	0.667	0.643
27	9	0.8	0.5	150	5	0.473	0.265
28	9	0.4	0.5	150	15	0.657	0.730
29	8	0.6	1.5	100	10	1.080	1.073
30	7	0.8	0.5	150	15	0.809	0.757
31	10	0.6	1.5	100	10	0.588	0.601
32	9	0.8	0.5	50	15	0.455	0.247

The performance of the ANN was determined in terms of regression coefficient (R^2) and the prediction efficiency was evaluated statistically by Mean Absolute

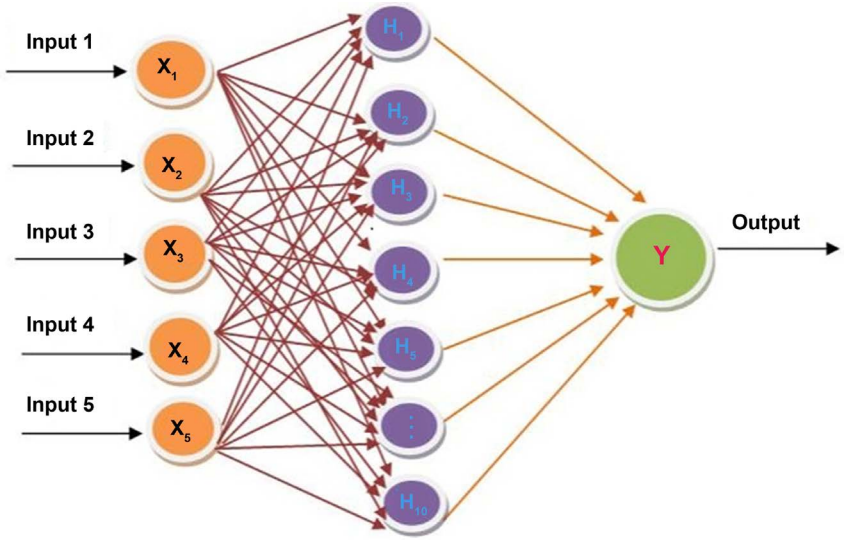

Figure 1. ANN architecture showing five input parameters at Input Layer (X_1, X_2, X_3, X_4 and X_5), neurons in the Hidden Layer (H_1, H_2, H_3, H_4, …, H_{10}) and Output Layer (Y).

Percentage Error (MAPE) and Mean Squared Error (MSE). R^2, MAPE and MSE were calculated according to Equations (1)-(3) respectively [11] [14] [16].

$$R^2 = 1 - \frac{\sum_{i=1}^{n}\left(y_i - y_{di}\right)^2}{\sum_{i=1}^{n}\left(y_{di} - y_m\right)^2} \tag{1}$$

$$\text{MAPE} = \frac{100}{n}\sum_{i=1}^{n}\left|y_{di} - y_i / y_{di}\right| \tag{2}$$

$$\text{MSE} = \frac{100}{n}\sum_{i=1}^{n}\left(\theta_i, p - \theta_i, e\right)^2 \tag{3}$$

where, y_{di} is the experimental value; y_i is the predicted response; y_m is the average of actual values and n is the number of experiments. θ_i, p is predicted value obtained from ANN model, θ_i, e is experimental value.

3. Results and Discussion

COX from various microbial sources including *Streptomyces* sp., *Nocardia* sp., *Pseudomonas* sp., are commercially available but the COX obtained from *Streptomyces* has been found to be superior. *Streptomyces* being the producers of extracellular COXs are preferred for their potential in-vitro applications such as clinical pathology [2] [17], pharmaceutical and food industry [3] [18], biosensors [19] [20], etc. A number of *Streptomyces* sp. have been reported to produce extracellular COX, on the basis of which six *Streptomyces* strains were used in this study to get a potent strain with high COX yielding capacity, listed in **Table 1**. All of the six *Streptomyces* strains showed positive results for extracellular COX production. Amongst the six *Streptomyces* sp. used, *Streptomyces olivaceus MTCC* 6820 was found to produce significantly high level of COX under unoptimized assay conditions, therefore, it was further selected for the optimization study of COX enzyme assay conditions.

ANN model was developed with pH, cholesterol concentration, 4-aminoantipyrine, crude COX volume and horseradish peroxidase as five model inputs, Table 3. An experimental design matrix was prepared using CCD consisting of 32 experiments, as shown in Table 3. COX activity (U/ml) served as an output of the ANN, gives the quantitative prediction of the increase in enzyme activity when all the five independent variables are optimized. ANN captures the non-linear behavior of the input parameters thus, affecting the output of the system. The enzyme activity is largely affected by the physico-chemical parameters of the reaction conditions provided. The above mentioned reaction conditions needed to be optimized for a new source of COX from *Streptomyces olivaceus* to predict the optimum activity of COX. The optimum values of assay parameters were found to be: pH of the reaction mixture (8.0), cholesterol concentration (0.6% (w/v)), 4-aminoantipyrine (1.5 mM), crude COX volume (100 µl) and horseradish peroxidase (10.0 U/ml), which are different from those reported for cholesterol oxidase activity from other sources of cholesterol oxidase [21] [22]. Maximum cholesterol oxidase activity obtained was 1.1 U/ml (predicted-1.073 U/ml) at optimum levels of parameters, which was very close to the predicted response and is 1.71 times higher than the control. The optimized enzyme activity is the result of a combined effect of these assay parameters as a whole, which shows non-linearity. Hence, ANN modeling was the preferable method.

The hit and trial method was employed to determine the optimal number of neurons in the hidden layer to train the network. The network topology of 5-10-1 was found to be optimum and is presented in Figure 2. The ANN predicted values of the output (COX activity U/ml) for different range of 5 input parameters (pH, cholesterol concentration, 4-aminoantipyrine concentration, crude COX volume and horseradish peroxidase concentration) were presented in Table 3. The performance of the neural network and prediction efficiency of ANN were measured in terms of regression coefficient (R^2), MSE and MAPE, which were 0.9792%, 0.0075% and 0.12% respectively. The regression analysis of the training, validation and testing phase of the ANN is displayed in Figure 3. The obtained R^2 value is close to unity which confirms the excellent network performance and indicates that 97.92% variability of the data can be explained by the ANN model while only 2.08% of the total variation is not explained by the

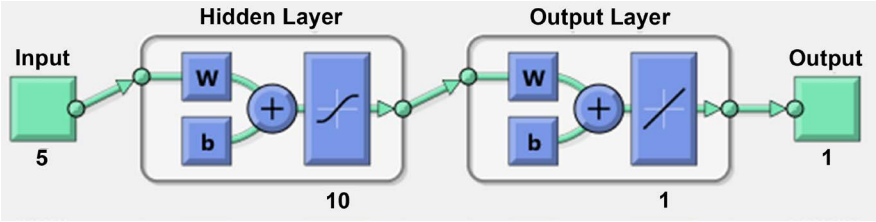

Figure 2. An optimized ANN topology with five input variables, 10 neurons at hidden layer with tansigmoid activation function and one output layer with Purelin transfer function. "*w*" and "*b*" in the hidden and output layer represents the weight and bias respectively; their values were adjusted automatically by the ANN function.

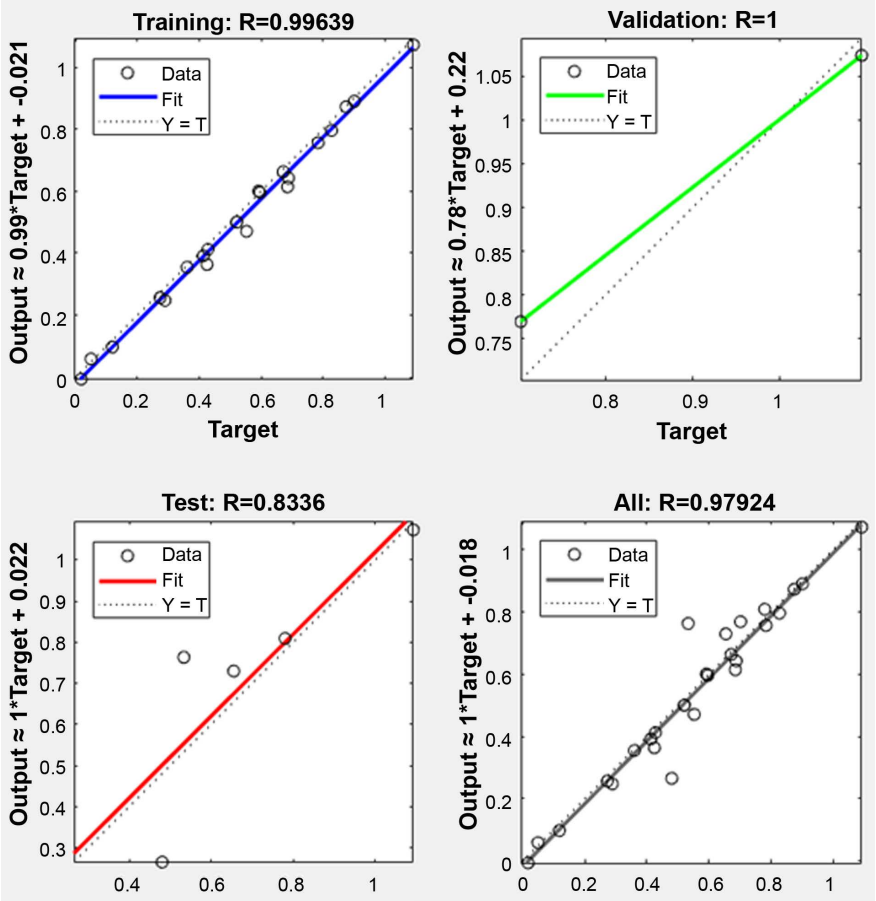

Figure 3. Regression analysis of ANN model generated by the ANN toolbox during training, validation and testing phase.

model [23]. The MSE and MAPE values determine the scale for prediction accuracy. MAPE ≤ 10% indicates high prediction efficiency, 10% ≤ MAPE ≤ 20% depicts good prediction, 20% ≤ MAPE ≤ 50% suggests reasonable prediction while MAPE ≥ 50% indicates inaccurate prediction [5]. The small MSE and MAPE values so obtained prove excellent prediction efficiency of the model. The values of R^2, MSE and MAPE altogether substantiated an efficient neural model for the prediction of responses.

4. Conclusion

The developed ANN model was successful in predicting the COX activity of *Streptomyces olivaceus MTCC* 6820. In addition, the excellent prediction accuracy of the ANN model illustrates its robustness in predicting the COX activity in similar type of systems within the range of dataset. ANN technique was based on the machine-learning approach, considering input-output data. This method was helpful in minimizing the labor, cost and enhancing the COX activity to a greater extent. The activity of COX was enhanced by 1.71 folds after optimization of reaction conditions *viz.* pH of reaction mixture (8.0), cholesterol concentration (0.6% w/v), 4-aminoantipyrine (1.5 mM), crude COX volume (100 µl)

and horseradish peroxidase (10.0 U/ml). The generated ANN model will work as a template for the prediction, modeling and estimation of COX from other *Streptomyces* sp. or microorganisms using similar reaction conditions studied in this work.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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