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Superoxide Dismutase and Oxidative Stress: Elucidation of Antiaging and Antioxidative Effect on Mammalian Cell Lines

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Abstract: During the course of routine metabolic activities of a cell, it produces different harmful byproducts esp. reactive oxygen species. Superoxide dismutase (SOD; EC 1.15.1.1) is a cellular defense enzyme that neutralizes these free radicals and protects the cell from deleterious effects and increases longevity. In the present work, superoxide dismutase enzyme, purified from a thermophilic bacterium *Bacillus licheniformis* SPB-13, was assessed for its antioxidative effect on HeLa cell lines. An experimental design was constructed in which the animal cells were first of all exposed to an oxidative stress generated using chemical stressors like H_2O_2 and Menadione, a vitamin K analogue. Then the MTT assay was performed to assess the cell viability in the presence of high oxidative stress. In final experiment, the menadione preincubated HeLa cells were treated with purified SOD and kept for an interval for detoxification of ROS. Viability assay was performed and it showed an increase in cell viability by 65 % at a concentration of 30 µg/ml of SOD. This confirmed the antioxidative and antiaging property of thermostable SOD from *Bacillus licheniformis* SPB-13. This potential can be harvested by pharmaceutical industry for topical antiaging formulations.

Key words: Reactive oxygen species, *Bacillus licheniformis* SPB-13, Superoxide dismutase, HeLa cells, menadione.

Introduction

The cells of every aerobic organism liberate certain oxygen species during metabolism that are less reactive but due to some modification within cell, these oxygen species become very reactive and prove very harmful to amino acids, nucleic acids and other entities of the cell. This harm renders these molecules defective and ultimately leads to cell aging and necrosis. Superoxides also wreak havoc by reacting with nitric oxide to form peroxynitrite, another highly reactive molecule that subsequently induces cellular and tissue injury. Peroxynitrite is implicated in several diseases, including stroke, Alzheimer's, and atherosclerosis [2]. This oxidative stress is generated when ROS neutralizing mechanisms of cell go ineffective. Superoxide dismutase, (SOD; EC 1.15.1.1) a metallo enzyme, is one such significant system of cell for free radical detoxification. It catalyzes the dismutation of superoxide free radicals into oxygen and hydrogen peroxide. Three classes of superoxide dismutase have been reported so far on the basis of metal cofactor attached to the enzyme. These include Cu/Zn, Fe/Mn and Ni SOD. Cu/Zn SOD is mostly found in eukaryotes including humans. Fe/Mn SOD is found in prokaryotes and protists and Ni SOD is also found in prokaryotes. Aging is the function of low SOD expression and activity. Researchers have tried to isolate SOD from different sources i.e. plants, animals and fungi [4,5,6]. Prokaryotic sources have also been explored for SOD isolation [7,8,9]. The antioxidative effect of SOD has been reported [1] but no study has been conducted on examination of

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effect of purified microbial SOD on cell lines for quantitiative determination of its antioxidative and antiaging properties. This lacuna provided the base for designing this piece of work.

The focus of present study was to establish the correlation between SOD and cell survival in high oxidative stress. Superoxide dismutase from a thermophilic bacterium *Bacillus licheniformis* SPB-13, isolated from thermal springs of western Himalayas, was purified to homogeneity and its effect on animal cell lines was examined. The simulation of animal model was achieved using Hela cell line. The enzyme was serially purified to homogeneity using ammonium sulphate precipitation and anion exchange (DEAE- Sepharose) chromatography and electrophoretic method was used to confirm single band purification. Animal origin HeLa cell line was used for the experimentation *in vitro*. Oxidative stress was generated in cell lines using chemical stressors like Menadione and H_2O_2. Then, SOD induced cell viability was checked in the presence of high oxidative stress.

Materials and methods

Chemicals

Inorganic salts and media components were purchased from HiMedia Ltd. (Mumbai, India) and the chemicals used for protein purification were purchased from Merck (Germany). Menadione and hydrogen peroxide were purchased from Sigma Aldrich (USA). The chemicals used were molecular biology and electrophoresis grade. RPMI-1640 culture medium was purchased from Sigma Aldrich (USA) and penicillin-streptomycin, amino acids, trypsin, and fetal bovine serum (FBS) were obtained from Gibco, Thermo Fisher, (USA). All other chemicals used in the experiments were of analytical grade.

Culture of *Bacillus licheniformis* SPB-13

The culture of *Bacillus licheniformis* SPB-13 used in present work was isolated previously at Department of Biotechnology, Himachal Pradesh University, Shimla, India.

Superoxide dismutase (SOD)

The superoxide dismutase was isolated from bacterial strain *Bacillus licheniformis* SPB-13 and purified to homogeneity using ammonium sulpahte and DEAE sepharose ion exchange chromatography. The enzyme activity was assayed using NBT-NADH and PMS dye reduction method [10]. The purified SOD with an enzyme activity of 3966 U /mg, was used for antioxidative experiment on cell lines.

Cell culture and treatment

Exponentially growing HeLa cells (human cervical cancer) were procured from National Centre for Cell Science (NCCS) Pune, India. HeLa cells were allowed to adhere for 48 h before putting in experimental conditions. The cells were cultured under a humidified 5 % carbon dioxide, 95 % air atmosphere at 37°C. The cell density was maintained at lesser than 3x10^5 cells/ ml in 25 cm^2 plastic tissue culture flasks with 10 ml of RPMI-1640 culture medium supplemented with 10 % fetal calf serum.

Cell viability/cytotoxicity assay

Cytotoxicity/cell viability was determined using the MTT assay [11]. This assay is based on the capability of mitochondrial enzyme succinate dehydrogenase of live cells to reduce the yellow water soluble substrate 3-, 5-diphenyl tetrazolium bromide (MTT) into an insoluble, purple coloured product, formazon. This formazon is then measured spectrophotometrically. The formazon production is directly proportional to viable cell number and inversely proportional to degree of cytotoxicity.

Evaluation of cytotoxicity of SOD

The cytotoxic effect of SOD was studied on HeLa cells by the MTT method. The cells were cultured in 96-well plates at a density of 1×10^4 cells per well at different concentration of SOD (10, 20, 30 40, 50, 60, 70 µg/ml). After incubation for 24 h, MTT dissolved in PBS was added to each well at a final concentration of 5 mg/ml and then incubated at 37°C and 5 % CO_2 for 2 h. The water insoluble formazan was dissolved in DMSO. The optical density was measured by a micro plate reader (Bio-Rad) at a wavelength of 570 nm and IC_{50} value was obtained.

Experimental setup for evaluation of antioxidative property of SOD

To generate oxidative stress in mammalian cells, two chemical stressors i.e. menadione and hydrogen peroxide were used. Individual cytotoxicity (IC_{50}) value was analyzed for each stressor. The cells were cultured in 96-well plates at a density of 1×10^4 cells per well at different concentration of menadione and H_2O_2 (10, 20, 30, 40, 50, 60, 70 µM). Cell density was kept low in order to avoid the effect of media starvation. After incubation for 24 h, MTT dissolved in PBS was added to each well at a final concentration of 5 mg/ml and then incubated at 37°C and 5 % CO_2 for 2 h. The formazan was dissolved in DMSO and the absorbance was measured by a micro plate reader (Bio-Rad) at a wavelength of 570 nm. For final experiment, stressor exhibiting good cytotoxicity value was chosen. Then we hypothesized that free radicals generated by menadione will be scavenged by SOD and it will lead to increase in cell viability. So for that, cells were cultured in 96-well plates at a density of 1×10^4 cells per well and pretreated with menadione (20 µM) for 4 hours. After incubation for 4 h, SOD was added at concentration less then IC_{50} value i.e. 25 µg/ml and incubated for 24 hours. MTT dissolved in PBS was added to each well at a final concentration of 5 mg/ml and then incubated at 37°C and 5 % CO_2 for 2 h. The water insoluble dark blue formazan was dissolved in DMSO. The optical density was measured by a micro plate reader (Bio-Rad) at a wavelength of 570 nm. The increase or decrease in cell density before and after the SOD treatment was seen and cell viability was observed.

Results

Assessment of cytotoxic effect of SOD

The cytotoxic effect of SOD was studied on HeLa cells by the MTT method. Briefly, the cells were cultured in 96-well plate at a density of 1×10^4 cells per well at different concentration of SOD (10, 20, 30 40, 50, 60, 70 µg/ml).). After incubation for 24 h, MTT dissolved in PBS was added to each well at a final concentration of 5 mg/ml and then incubated at 37°C and 5 % CO_2 for 2 h. After two hours, optical density was checked at 570 nm and it showed the IC_{50} value of SOD as 30 µg/ml (Fig. 1).

Effect of SOD on cell viability

The cytotoxic effect of chemical stressors i.e. H_2O_2 and menadione was studied on HeLa cells by the MTT method. The IC_{50} value of H_2O_2 and menadione was found to be 70 µM and 30 µM respectively (Fig. 2a & b). Menadione proved to be best oxidative stress generator among two. For analysis of effect of SOD on mammalian cell lines, the cells were cultured in 96-well plates at a density of 1×10^4 cells per well and pretreated with menadione (20 µM). After incubation for 4 h, SOD was added at concentration less than IC_{50} value i.e. 25 µg/ ml and incubated for 24 hours. MTT dissolved in PBS was added to each well at a

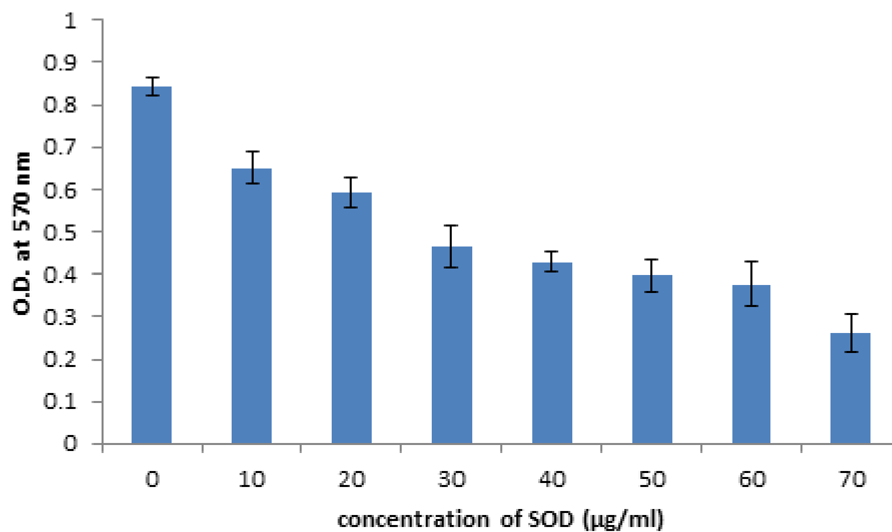

Fig. 1. Assessment of IC_{50} value of SOD

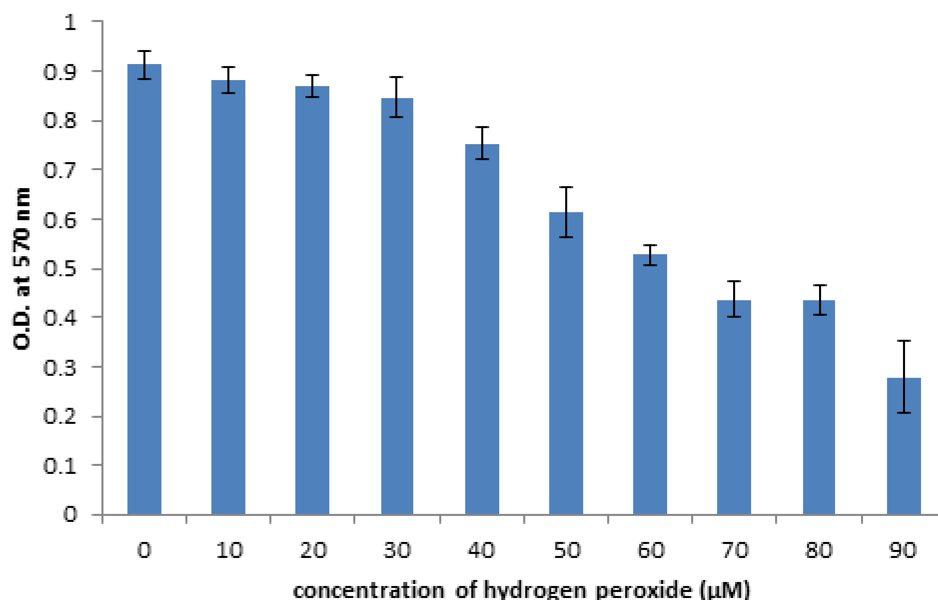

Fig. 2a. Assessment of IC_{50} value of H_2O_2

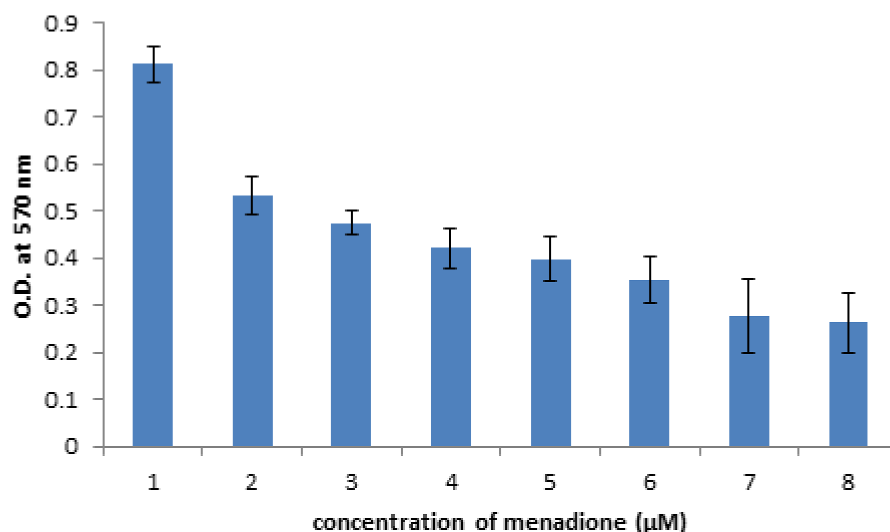

Fig. 2b. Assessment of IC_{50} value of menadione

final concentration of 5 mg/ml and then incubated at 37°C and 5 % CO_2 for 2 h. The water insoluble dark blue formazan was dissolved in DMSO. The optical density was measured at 570 nm and it showed that SOD prevented the cell death till a concentration of 40 µg/ml but at higher concentration, it showed saturation of antioxidant effect of SOD. Maximum free radical scavenging activity was shown at 30 µg/ml (Fig. 3).

Discussion

Superoxide dismutase causes the neutralization of reactive oxygen species and prevents the cells of the organisms from oxidative damage. In the present piece of research work, we tried to evaluate the antioxidative effect of thermostable Fe/Mn SOD isolated from thermophilic bacterium *Bacillus licheniformis* SPB-13. The mammalian origin HeLa cell line was used for experimental purpose. Chemical stressors like menadione and H_2O_2 were used to induce oxidative stress and cell survival in oxidative stress was observed. Then the cell viability of HeLa cells was checked after SOD treatment was given to the cells. We

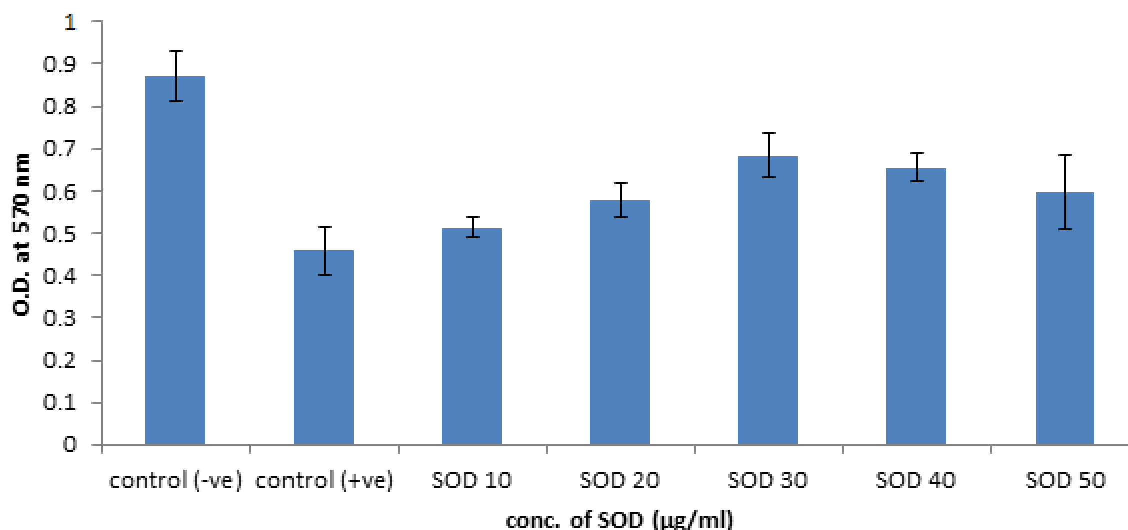

Fig. 3. Assessment of free radical scavenging activity of SOD

found that there was a maximum of 65 % increase in cell survival after the SOD was added to growing HeLa cells. Maximum cell viability was seen at a concentration of 30 µg/ml of SOD added and for 40 µg/ml, there was a slight decrease in cell viability and at higher concentrations of SOD, rate of cell survival decreased. In one study, SOD from garlic was checked for this property and it showed only non significant difference in cell viability before and after SOD treatment [3]. Counted together, the above investigation indicates that the thermostable SOD purified from *Bacillus licheniformis* SPB-13 modulates the levels of harmful superoxide anions and hydroxyl radicals. But the exact mechanism of working of SOD is not clear whether it enters the cell or acts from outside the cell.

Apart from this lacuna, the present piece of work vividly shows the antioxidative role of SOD in body cells and it functions in increasing the longevity of the organism. This property coupled with thermostable nature of SOD from *Bacillus licheniformis* SPB-13 can be used by pharmaceutical industry for antiaging formulations.

Conclusion

The results of *in-vitro* experimentation of antioxidative nature of SOD from *Bacillus licheniformis* SPB-13 performed on mammalian origin HeLa cell line clearly show that SOD increases the cell survival by 65 % at a concentration of 30 µg/ml. This property holds an immense potential for the use of SOD in pharmaceutical industry as an antiaging formulation.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgement

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References

1. **Lingnert, H., Aakesson, G., Eriksson, C.E. (1989).** Antioxidative effect of superoxide dismutase from *Saccharomyces cerevisiae* in model systems. J. Agric. Food Chem. 37(1): 23-28.
2. **Shin, S.G., Kim, J.Y., Chung, H.Y., Jeong, J.C. (2005).** Zingerone as an antioxidant against peroxynitrite. J. Agric. Food Chem. 53(19): 7617-22.
3. **Sfaxi, I.H., Ferraro, D., Fasano, E., Pani, G., Limam, F., Marzouki, M.N. (2009).** Inhibitory effects of a manganese superoxide dismutase isolated from garlic (*Allium sativum* L.) on *in vitro* tumoral cell growth. Biotechnol Prog. 25(1): 257-64.

4. **Shrestha, P., Yun, J., Kim, W.T., Kim, T., Lee, W. (2016).** Cloning, purification and characterization of recombinant human extracellular superoxide dismutase in SF9 insect cells. Mol. Cells. 39(3): 242-249.
5. **Wang, S., Shao, B., Liu, S., Ye, X., Rao, P. (2012).** Purification and characterization of Cu, Zn-superoxide dismutase from black soybean. Food Research International. 47: 374-379.
6. **Namir, I.H., Xia, W.C., Huang, Y.W., Yuan, Q.S. (2003).** Isolation and purification of superoxide dismutase from Radix Lithospermi Seed. Pharm. Biotechnol. 10: 358-362.
7. **Santos, W.G.D., Pacheco, I., Liu, M., Teixeira, M., Xavier, A.V., LeGall, J. (2000).** Purification and characterization of an iron superoxide dismutase and a catalase from the sulfate-reducing *bacterium Desulfovibrio* gigas. J. Bacteriol. 182: 796-804.
8. **Seatovic, S., Gligic, L., Radulovic, L., Jankov, R.M. (2004).** Purification and partial characterization of superoxide dismutase from the thermophilic bacteria *Thermothrix* sp. J. Serb. Chem. Soc. 69(1): 9-16.
9. **Boyadzhieva, I.P., Atanasova, M., Emanuilova, E. (2010).** A novel, thermostable manganese-containing superoxide dismutase from *Bacillus licheniformis*. Biotechnol Lett. 32: 1893-1896.
10. **Kakkar, P., Dos, B. and Viswnathan, P.N. (1984).** A modified spectrophotometric assay of superoxide dismutase. Indian J. Biochem. 21: 130-32
11. **Mosmann, T. (1983).** Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods. 65: 55-63.