Volume : 3 | Issue : 10 | October 2014 • ISSN No 2277 - 8179

Treatment of Chronic Radiation-Inducedinjury of Lymphocyte With Exogenously Added Superoxide Dismutase Purified From a Sewage Isolated E Coli

Biotechnology

KEYWORDS : SOD,Cell viability, irradiation

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ABSTRACT

Accidental exposure toradiation forms free radicals which causes cell death. Superoxide dismutase (SOD) enzyme produced for dismutation of superoxide into oxygen and hydrogen peroxide to prevent damage caused by superoxide radical. SOD have been implicated in many disease states including inflammatory diseases, ischemia and reperfusion, neurodegenerative diseases, cancer, as well as more subtle roles in cell signaling and perhaps in immune function, SOD is not yet in widespread usage in human clinical medicine. One obstacle has been that none of the three human SODs possesses attractive pharmacological properties to make it a clinically useful therapeutic agent.In this experiment a sewage isolate wasused for production of SOD. Enzyme was purified by mechanical and enzymatic cell disruption then ammonium sulphate precipitation followed by column chromatography method. Then it's effect was studied on irradiated lymphocytes. For comparison1mg of purereference SOD powder (sigma) was used. The cell monolayer was irradiated in 25-cm2 culture flasks in the exponential growth phase. Culture flask was irradiated with dose of 6 Gy.SOD was added after 30 min in the culture and incubated in a humidified atmosphere of 5% CO2 at 37C in CO2 incubator for 72 hours. To check percentage viability, cell culture was stained by Trypan blue. The percentage viability clearly shows that the irradiations were lethalfor cells (17.48%). The addition of superoxide dismutase to cells after irradiation attenuated the death of cells by irradiation and was effective for increased survival. Viability with our SOD increases up to 77.96% which was significantly nearer to viability by pure reference SOD values 83.53%. So it can be concluded that, the SOD treatmentcan be used for recovery of irradiated cells and it increases cell viability.

Introduction

Free radicals are normally produces in the body in metabolic processes. Also can be produced by different factors such as exposure to radiation (sun rays or medical x-rays), exposure to environmental pollutants such as vehicle exhaust fumes and tobacco smoke, exposure to medicines, toxins, chemicals and foods high in fat and unhealthy oils (Wiendow, 2009). Antioxidant enzymes are capable of stabilizing, or deactivating free radicals before they attack cellular components. They act by reducing the energy of the free radicals or by giving up some of their electrons for its use, thereby causing it to become stable. In addition, they may also interrupt with the oxidizing chain reaction to minimize the damage caused by free radicals (Halliwell and Gutteridge 1999). It has been found that a substantial link exists between free radicals and more than sixty different health conditions, including the aging process, cancer, diabetes, Alzheimer's disease, strokes, heart attacks and atherosclerosis (Krishnamurthy &Wadhwani, 2012). By reducing exposure to free radicals and increasing the intake of antioxidant enzyme rich foods or antioxidant enzyme supplements, our body's potential to reducing the risk of free radical related health problems is made more palpable (Slater, 1984). Antioxidant enzymes are, therefore, absolutely critical for maintaining optimal cellular and systemic health and well being. (Percival, 1998).Hydrogen peroxide, which is generated during oxidative stress, including radiations is known to damage proteins, nucleic acids and cell membranes and also has been implicated in cancer, ageing and several chronic neurogenerative diseases (Daroui et al. 2004, Halliwell et al. 1990,Gallin, 1987)It has also been reported to cause DNA damage in the form of chromosomal aberrations (Birnboim, 1986)), ultimately results in cell death (Sofni&Ishidate 1984). SOD is the first enzyme involved in the antioxidant defense system found in both prokaryotic and eukaryotic cells (McCord &Edeas, 2005).SODs are essential for aerobic survival and are ubiquitous among aerobic and aerotolerant organisms (McCord &Fridovich, 1988) and even some anaerobic organisms (Hassan &Fridovich 1977).The present study was aimed at studying the protective effect of SOD against radiation

induced damage on human lymphocyte culture.

Materials and method

Chemicals

Nutrient broth, DEAE cellulose andsephadex 75 column, Monobasic sodium phosphate, Dibasic sodium phosphate, Riboflavin, Methionine, Nitrobluetetrazolium, EDTA, Phosphate buffer saline (PBS), Blood sample, Ficoll-Hypaque, Heparin,RPMI 1640 media, Fetal Bovine serum, Antibiotic Pen-Strep, Phytohaemagglutinin (PHA) Pure SOD powder (sigma) activity 1000U/mgTyphan blue. All other chemical reagents used were of analytical grade purchased either from MERCK or sigma. Centrifuge (Remi), micropipettes, sterile tips, haemocytometer. Gammator (model M Gammator, Radiation International) Optical measurements were achieved with a spectrophotometer (UV-1800,Shimadzu, Japan) at 560 nm. and 650nm. Microscopic study carried out using NikonYS100 Microscope.

Isolation and Purification of Superoxide Dismutase

As more free radicals are present in sewage, chances of getting higher SOD producer from sewage are more. So from 6 different sites around Kolhapur sewage samples were collected. 10ml of sample was inoculated in 200 ml Nutrient broth. It was incubated for 24 Hrs. at 37C (Kirby et al. 1980) 12 samples of sewage isolate were selected for SOD assay. After SOD assay, higher sewage isolate was selected for production and partial purification of enzyme. Cells were collected by centrifugation (10000 g, 10 min). They were washed twice with0.1M phosphate buffer (pH 7.5).The suspension was ground in mortar with glass beads and vortexed for 5 min. in presence of lysozyme. The disrupted product was centrifuged (10000g, 10min). The supernatant was recentrifuged (25000g, 30min).The resulting supernatant was 30 % saturated with solid ammonium sulfate and the precipitate was discarded. More ammonium sulfate was added to the supernatant to reach 80 % saturation and the precipitate was dissolved in the same 0.1M phosphate buffer (pH 7.5) and dialyzed against the same buffer overnight then centrifuged (25000 g, 20 min)("**Methods in Enzymology," 1990**). Supernatant containing SOD activity was applied to a DEAE-cellulose column (40×1.6 cm), which was equilibrated with 0.05 M phosphate buffer (pH 7.4) at 40^0C. Elution of the enzyme was achieved by establishing a linear gradient with a 0.06-0.140 M phosphate buffer (pH 7.4) atflow rate of0.5 ml/min (Etovi et al. 2004). Fractions containing SOD activity were loaded onto a Sephadex G-75 (90×1.6 cm) column equilibrated with 0.05 M phosphate buffer pH 7.4 and eluted with the same buffer at a flow rate of 0.21 ml/min. Thus this purified enzyme was used for further analysis(Aydem'ir&Tarhan ,2001).

Media preparation:

RPMI 1640 media prepared by dissolving 1.64gm in 100ml autoclaved distilled water.Fetal Bovine serum 11 ml used for 100ml Lymphocyte media.Antibiotic Pen-Strep used 3 ml per 100 ml lymphocyte media.Phytohaemagglutinin (PHA) used 2 µl in 100 ml lymphocyte media. All ingredients were added in autoclaved distilled water in proper proportion. The media was taken in 5ml sterile syringe. The sterile filter was attached to syringe and the syringe was pressed slowly so that the media get filtered and collected in the culture bottle.

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Lymphocytes culture

Peripheral blood (10ml) collected by venipuncture. Blood sample was diluted1:2 with PBS. 1ml of Ficoll-Hypaque solution added such that it should form a layer on it. It was centrifuged at 3000 rpm for 10 - 15 minutes at RT. Lymphocytes and monocytes were recovered at the Ficoll plasma interface where they form a white band. The RBC and PMN cells were sedimented through Ficoll-Hypaque and form a pellet. The cells at the interface aspirated without removing Ficoll as it leads to granulocyte contamination. The cells were washed in large volume of PBS at 2000 rpm for 10 minutes. Then supernatant was removed and the pellet was taken into media bottle containing medium and gently mixed(24)

Treatment:

The cell monolayer was irradiated by Cesium-137 (^{137}Cs) gammator (model M Gammator, Radiation International) at a dose rate of 6.0 Gy/min for 1 min, on a rotating platform in 25-cm^2 culture flasks in the exponential growth phase.

a.Pure/ reference SOD stock preparation(pure):1mg of pure SOD powder (sigma) was dissolved in 10ml distilled water (100U/ml solution).The 50 µl/ml solution from stock was added to the culture.

b.Isolated SOD: The 50 µl/mlisolated SOD solution (100U/ml) used to the culture.

(All these solutions were filter sterilized before addition.)

Bottle A :(control): Lymphocyte
Bottle B :(Toxicity): Irradiatedlymphocyte
Bottle C: Irradiated lymphocyte + Pure/reference SOD
Bottle D: Irradiated lymphocyte + Isolated SOD

Three sets of these bottles were formed and tested. These bottles were incubated in a humidified atmosphere of 5% CO_2 at 37° C in CO_2 incubator for 24 hours. Then viability count was taken.

Viability counting:

Cell suspension prepared using distilled water (approximately 10^6/ml). It was diluted 1:1 using 0.4 % Trypan blue solution. The counting chambers of haemocytometer were loaded with the suspension. The preparation was allowed to settle for few min (1-2). Four corners of haemocytometer made wet and cover slip was fixed on it. Corner squares of haemocytometer were focused using 10X objective. The number of unstained, stained and total number of cellswas counted(Freshney, 1987).

Result and Conclusion:

(Table 1 Should be added here)

The percentage viability (Table 1) clearly shows that the radiations are lethal for cells and the SOD treatment can reduce the effect of free radicals on cells.Percentage viabilityof irradiated lymphocytes with reference SOD was 78.63 % while for our isolated SOD it was 76.33%. that means for recovery of lymphocytes reference SOD is 2.3% more effective than our isolated SOD.

Discussion

Oxidative stress dependent upon superoxide radical, pollution, toxicity, drug and stress effect can account for a number of acute and chronic disease states, which include inflammation and ischemia-reperfusion (Ment et al. 1984, **Burton,** 1985). Cu/ZnSOD added exogenously to cell culture is known to be incorporated into cells and increases total SOD activity (Edeas et al.(1996) Further studies reported that exogenously-added Cu/ZnSOD induced neutrophil apoptosis via accumulation of H_2O_2,(Yasui& Baba 2006) and effectively blocked monocytic differentiation of leukemic cells with platelet-activating factor (PAF)(Nishihira et al. 1994) and stimulation of neonatal rat hepatocyte growth by tumor promoters (Armato et al. 1984)). Tominaga*et.al* (2012) reported, the addition of low concentrations of Cu/ZnSOD stimulated angiogenesis in control as well as in irradiated cells. Superoxide dismutase rescues human primary umbilical vein endothelial cells from endothelial dysfunction caused by irradiation (Epperly et al. 2003). The ECSOD gene is about 60% homologous with Cu/ZnSOD, especially in the region of active sites, but no similarity with MnSOD was observed (Gao et al. 2008). The central core of ECSOD polypeptide is homologous with Cu/ZnSOD but possesses extensions at the N- and C-termini (Culotta et al. 2006). Human ECSOD is related to dimeric Cu/ZnSOD, which is typically produced in

vascular smooth muscle cells, and is secreted into the extracellular environment where it binds to the extracellular matrix and endothelial surface components. Thus, Cu/ZnSOD is structurally similar to ECSOD. In contrast, MnSOD is located in the matrix of mitochondria in eukaryotes, and mitochondrial localization is required for protection of cells by MnSOD from irradiation damage (Petersen et al. 2003). There is no similarity in sequence or structure between Cu/ZnSOD and MnSOD. Exogenously-added Cu/ZnSOD may act in a way similar to ECSOD, whereas an alternative mechanism may involve the suppression of $O_2^{\bullet-}$ generating systems such as NADPH oxidase by extracellular Cu/ZnSOD (Armato et al. 1984).

Table no 1: Cytotoxicity (Cell Viability Count)

Culture bottle	Additions	Live cell count	Dead cell count	Percentage viability (%)
Bottle A	Lymphocytes	13.6×10^6	0.70×10^6	94.8
Bottle B	Irradiated Lymphocyte	2.5×10^6	11.8×10^6	17.48
Bottle C	Irradiated Lymphocyte + Reference SOD	10.3×10^6	2.8×10^6	78.63
Bottle D	Irradiated Lymphocyte + Isolated SOD	10×10^6	3.1×10^6	76.33

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