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Effect of Hyperthermia on Antioxidative Status of a Primary Hepatocyte Culture

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Abstract

The present study was conducted to investigate the effect of heat stress on antioxidative status of chicken embryo hepatocyte culture. Primary cell culture was prepared by using 14 to 16 day-old chicken embryos. Cell monolayer was incubated for 45 minutes in CO_2 incubator separately at 43±0.5°C, 40±0.5°C and 37±0.50C temperature with 95% humidity and 5% CO_2. After incubation, the cells were returned to 37°C temperature and allowed to recover for two hours before harvesting for preparation of cell lysate. Activity of different antioxidative enzymes and protein concentration at different incubation temperatures was estimated. Significantly ($P \leq 0.01$) lower glutathione reductase (GSH-R) and catalase (CAT) activity was recorded at 40°C and 43°C as compared to 37°C incubation temperature whereas no significant ($P \geq 0.05$) difference occurred between GSH-R and CAT activity at 40°C and 43°C. Significantly ($P \leq 0.05$) higher superoxide dismutase (SOD) activity and protein concentration was recorded at 40°C and 43°C as compared to 37°C incubation temperature whereas no significant ($P \geq 0.05$) difference occurred between SOD activity and protein concentration at 40°C and 43°C.

Keywords: Antioxidative Enzymes; Heat Stress; Hepatocyte Culture; Cell Lysate

Introduction

Cell culture is almost-universally used in laboratories worldwide to examine metabolic pathways and to elucidate the mechanisms involved in signal transduction, regulation of gene expression, cell proliferation and cell death. Many cell culture studies are done with malignant cell lines, because such cells are robust and grow and divide easily in culture. An alternative is primary culture, where cells are harvested from a tissue and plated. The cells which survive culture shock appear to be those that have adapted rapidly, with multiple changes in gene expression, metabolic activity and the levels of enzymes (Bishop *et al*., 1985). Rise in incubation temperature/ heat stress leads to overproduction of reactive oxygen species (ROS) such as superoxide radicals ($O2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$) in different cell types (Zhao *et al*., 2006). An imbalance of the equilibrium between ROS generation and elimination by antioxidant defense systems can lead to oxidative stress. Cells are at high risk of damage by heat-induced oxidative stress due to their high oxygen turnover and low levels of antioxidant defense enzymes (Huber *et al*., 2006). Restoration of cellular homeostasis often lead to secretion of structural proteins and enzymatic (glutathione peroxidase, glutathione reductase, superoxide dismutase, catalase) and non-enzymatic antioxidants (glutathione, pyridine nucleotides, ascorbate, retinoic acid, tocopherols). Supply of necessary oxidation reduction potential is necessary to maintain the cells in a state of redox balance (Dalton *et al*., 1999). The present investigation aims to study the effect of heat stress on antioxidative status in chicken embryo hepatocyte culture.

Materials and methods

Primary Chicken Embryo Hepatocyte Culture

Primary cell culture was prepared by using 14 to 16 day-old chicken embryos. The embryos (8-10 nos.) were collected aseptically and liver were harvested and washed in Hanks Buffered Salt Solution (HBSS). The liver were then cut into small pieces

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with scissors and minced properly in HBSS. The minced tissue was washed again with HBSS and trypsinized with 0.125% trypsin solution for 15 minutes with constant stirring. The cells were filtered through sterilized muslin cloth and filtrate was centrifuged at 3000 rpm for 10 minutes at 40°C. The cells were washed in HBSS and finally in RPMI 1640 media. The packed liver cells were diluted at the rate of 1:150 (v/v) in RPMI 1640 medium containing 10% fetal calf serum (FCS). The suspension was dispensed in 25 cm^2 size tissue culture bottles at 8 ml/bottle. After 24 and 48 hours, media was changed. The seeded bottles were incubated at 370°C in CO_2 incubator at 5% CO_2 level until a confluent monolayer was formed.

Heat shock procedure

Monolayer cells were incubated for 45 minutes in CO_2 incubator separately at 43±0.5°C, 40±0.5°C and 37±0.50C temperature with 95% humidity and 5% CO_2. After incubation, the cells were returned to 37°C temperature and allowed to recover for two hours before the cells were harvested for preparation of cell lysate (Douglas *et al*., 1996).

Harvesting of Cells

The growth media from tissue culture bottles showing confluent monolayer was decanted and monolayer was washed thrice with sterile calcium-magnesium free phosphate buffer saline (pH 7.2). Then 1ml of 0.125% trypsin solution was added in each bottle, and it was shaken gently for 1-2 minutes. The trypsin solution was then removed and culture bottle was incubated at 37°C for 5-10 minutes until cells get detached. To each culture bottle, 2 ml of PBS was added and the cells were collected in centrifuge tubes along with PBS for the further processing of cells.

Preparation of Cell Lysate

Harvested cells were centrifuged at 1700 rpm for 10 minutes and washed with cold PBS. The pellet was suspended with 0.5 ml of lysis buffer (Tris-Cl-0.10M, Titron X 100-0.5% and Distilled Water) per 1-5 million cells. Thorough mixing was done by repeated pipetting. The suspension was centrifuged at 13000 rpm for 5 minutes at 4°C. The supernatant was placed into a clean tube intended to assay antioxidative enzymes immediately or froze at -80°C until analysis.

Estimation of antioxidative enzymes in the cell lysate

Glutathione reductase (GSH-R) was estimated in the cell lysate as described by Goldberg and Spooner (1983) with modification in which 3 ml of reaction mixture containing 2.6 ml phosphate buffer (0.12 M, pH 7.2), 0.1 ml EDTA (15 mM) and 0.1 ml GSSG (oxidized glutathione) (65.3 mM) was added. To this 10 µl of cell lysate was added and then the volume was made up to 2.95 ml with distilled water. After incubation at room temperature for 5 minutes, 0.05 ml of NADPH (9.6 mM) were added, and decrease in OD/minute was recorded at 340 nm for 3 minutes immediately. GSH-R activity was expressed as mM NADPH oxidized to NADP/mg protein/min.

Superoxide dismutase (SOD) was estimated in the cell lysate as described by Madesh and Balasubramanyam (1998) which involve the generation of superoxide by pyrogallol (100 µM) auto-oxidation and inhibition of superoxide dependent reduction of dye [(MTT 3-(4-5 dimethyl thiazol 2-xl) 2, 5 diphenyl tetrazolium bromide] to formazan. The reaction is terminated by addition of dimethyl sulfoxide (DMSO), which helps to solubilize the formazan. The colour evolved is stable for many hours. The optical density was read at 570 nm in spectrophotometer and SOD activity was expressed in U/mg of protein.

Catalase activity was assayed in the cell lysate by spectrophotometric method (Bergmeyer, 1983). The 1:100 dilution of cell lysate was prepared by addition of one part packed cell and 99 parts cell lysis buffer. In a test tube, 2 ml phosphate buffer and 10-µl of cell lysate was added, and the contents were transferred to the cuvette. Adding 1ml of H_2O_2 (10 mM) directly into the cuvette, the reaction was started and the optical density was recorded at every 30 sec. for three minutes at 240 nm against water as a blank in UV spectrophotometer. The activity of catalase was expressed as mM H_2O_2 utilized / minute/ mg protein. Soluble protein concentration in cell free extract was estimated by Folin-Phenol Reagent (Lowry *et al*., 1951) using bovine serum albumin (BSA) as standard.

Statistical analysis

The data obtained on the effect of different heat exposure duration on various enzyme activities were analyzed statistically using analysis of variance (ANOVA) (Snedecor, 1994).

Results

Mean±S.E. values of GSH-R, CAT, SOD activity and protein concentration at different incubation temperatures are presented in Table 1. Significant difference in the of enzyme activities and protein level was recorded at 37°C, 40°C and 43°C incubation temperatures. Significantly ($P \leq 0.01$) lower GSH-R and CAT activity was recorded at 40°C and 43°C as compared to 37°C incubation temperature whereas no significant ($P \geq 0.05$) difference occurred between GSH-R and CAT activities at 40°C and 43°C.

Table 1. Effect of different incubation temperatures on GSH-R, SOD, CAT activity and protein concentration in chicken embryo hepatocyte cell lysate

Incubation temperature	Enzyme Activity and Protein Concentration			
	GSH-R	SOD	CAT	Protein
37^0C	$0.214\pm0.015_x$	$0.112\pm0.008_x$	$21.31\pm0.74_x$	$0.234\pm0.012_x$
40^0C	$0.095\pm0.007_y$	$0.194\pm0.014_y$	$10.10\pm1.00_y$	$0.413\pm0.027_y$
43^0C	$0.082\pm0.007_y$	$0.179\pm0.031_y$	$10.30\pm1.74_y$	$0.476\pm0.020_y$
CD	0.046	0.064	0.570	0.094
Significance	1%	5%	1%	1%

Mean bearing subscript in a column differ significantly. All the samples were estimated in duplicate and the experiment was repeated four times. GSH-R activity- (mM NADPH/mg protein/min), SOD activity- (U/mg protein), Catalase activity- (mM H2O2 utilized/min/mg protein), Protein - (mg/ml)

Significantly ($P \leq 0.05$) higher SOD activity was recorded at 40°C and 43°C as compared to 37°C incubation temperature whereas no significant ($P \geq 0.05$) difference was observed in enzyme activity at 40°C and 43°C. Significantly ($P \leq 0.01$) higher protein concentration was recorded at 40°C and 43°C as compared to 37°C incubation temperature whereas no significant ($P \geq 0.01$) difference was observed in protein concentration at 40°C and 43°C.

Discussion

Generation of ROS is triggered by different types of stress, which include oxidative stress, heavy metal stress, hyperthermia, etc. (Droge, 2002). The unfavorable effects of ROS are neutralized by the antioxidant defense of the cells, which includes various antioxidant enzymes and ROS scavenging molecules. Of the various antioxidant enzymes, Glutathione reductase, a homodiameric flavoprotein disulphide oxidoreductase, which plays an indirect but essential role in the prevention of oxidative damage within the cell by helping to maintain appropriate levels of intracellular glutathione. Glutathione reductase together with cofactors, NADPH, catalyzes the reduction of oxidized glutathione to glutathione (Dringen and Gutterer, 2002). Superoxide dismutase is primarily responsible for the conversion of superoxide radicals ($O_2^{\cdot -}$) to hydrogen peroxide (H_2O_2), which is then degraded by catalase (a heme-containing enzyme) that catalyzes the conversion of H_2O_2 to H_2O and O_2 and prevents the accumulation of H_2O_2 (Halliwell and Gutteridge, 1999).

In present study incubation temperature of 40°C and 43°C to chicken embryo hepatocyte culture reduced the GSH-R activity, which may be due to excess lipid peroxidation and ROS production during heat stress (Li *et al*., 2010). To counteract lipid peroxidation and ROS generation, glutathione reductase activity could have increased initially but later on activity would have exhausted. Mild continuous hyperthermia (41°C) leads to oxidative stress and loss of cellular viability in a time-dependent man-

ner (Santos-Marques *et al.*, 2006). Hyperthermic conditions applied to isolated fresh hepatocyte not only increased lipid peroxidation and cell mortality but also caused severe oxidation of glutathione (Carvalho *et al*., 1997). Heat shock of cells has been shown to increase the flux of cellular free radicals (Flanagan *et al*., 1998).

In present study hyperthermia increased SOD activity in primary hepatocyte culture to protect the cells against free radical damage. Superoxide dismutase is responsible for approximately 90 % of the total cellular superoxide radical dismutation activity (Busuttil *et al*., 2005). Overexpression of SOD has been shown to be protective in neuronal cells and delays apoptosis triggered by many oxidative insults (Schwartz *et al*., 1998). It has been reported that heat stress produced moderate upregulation of SOD in cardiac myocytes conferring protection against hypoxia–reoxygenation injury (Yamashita *et al*., 1997). On the contrary, lack of increase in SOD activity in rat heart after hyperthermia has also been reported (Tekin *et al*., 2001). In present study, catalase activity decreased at incubation temperature of 40°C and 43°C, which may be attributed to excessive production of hydrogen peroxide and peroxide free radical, which is neutralized by catalase to water and oxygen. Upregulation of catalase activity has been reported by repetitive H_2O_2 stress (Sen *et al*., 2005), however, during chronic heat stress catalase upregulation is not observed (Mustafi *et al*., 2009). Varied response to hyperthermia for catalase activity may be due to variation in cellular response to different types of cells, stress duration and gene expression.

Heat stress at 40°C and 43°C to hepatocyte culture showed increase protein concentration in cell lysate. In response to wide variety of physiological and environmental stressors heat shock proteins are produced which accounts for the cytoprotective functions (Parcellier and Gurubaxani, 2003). Heat shock proteins are produced during extreme temperature changes and oxidative stress and are best known endogenous factors protecting cells from various stress conditions. In the present study the increased protein concentration may be an intrinsic protective mechanism of hepatocytes in response to extreme temperature conditions.

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