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Full Length Research Paper

Assessment of MCF-7 cells as an *in vitro* model system for evaluation of chemical oxidative stressors

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Studies have been carried out to establish an experimental *in vitro* model system for routine testing of oxidative stress inducers through biochemical analysis using human breast carcinoma (MCF-7) cell line. Hydrogen peroxide (H_2O_2) has been chosen as a test chemical oxidant to assess the level of induced glutathione (GSH), lipid peroxidation (LPO), superoxide dismutase (SOD), catalase, lactate dehydrogenase (LDH) release and cell viability using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and neutral red uptake (NRU) assays in MCF-7 cells. Cells were exposed with H_2O_2 in the range of 0.1 to 1.6 mM in MEM culture medium up to 24 h. The sensitivity of the system was examined by determining the dose response curve for induction of mitochondrial activity and growth inhibition. The concentrations of H_2O_2 above 0.5 mM were found to be cytotoxic, whereas, lower concentrations did not cause any significant decrease in cell viability. Results of the study showed a decrease in GSH level at 12 and 24 h (39 and 44% of control) and maximum increase (60% of control) in LPO at 24 h. In case of catalase and SOD, a concentration of 0.5 mM of H_2O_2 was found instantly effective and caused reduction in activity within 2 h, with which decreases significantly up to 24 h. The results indicate that the H_2O_2 induced oxidative stress mediated cytotoxicity in MCF-7 cells and usefulness of these cell types as sensitive biological system for routine testing of chemical oxidative stressors.

Key words: Hydrogen peroxide (H_2O_2), MCF-7 cells, Cytotoxicity oxidative stress.

INTRODUCTION

Oxidative stress induced cell damage has been implicated in a variety of diseases such as Alzheimer's disease (Su et al., 2008), Parkinson's disease (PD) (Zhou et al., 2008), cataractogenesis (Manikandan et al., 2010), cancer (Wei et al., 2010) and aging (Desai et al., 2010). It is mediated by reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide and hydroxyl radicals, which are generated as byproducts of normal and aberrant metabolic processes that utilize molecular oxygen. ROS can attack proteins, deoxynucleic acids and lipid membranes, thereby disrupting cellular function and integrity (Yamashita et al., 2008; Spencer et al., 2009). There are many types of chemicals and physiological inducers of oxidative stress, which are able to cause apoptotic cell death. For instance, H_2O_2 can induce apoptosis in many different cell types (Kang et al., 2008; Maheshwari et al., 2009). As the major component of ROS, H_2O_2 has been extensively used as an inducer of oxidative stress in various *in vitro* models (Cai et al., 2008; Hwang et al., 2008). The specific toxicological mechanism of such toxicant which induced the oxidative stress has also been investigated earlier (Salem et al., 2009; Wu et al., 2010). The experimental setup for *in vitro*

Abbreviations: PD, Parkinson's disease; **ROS,** reactive oxygen species; **MEM,** minimum essential medium; **LDH,** lactate dehydrogenase; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; **NRU,** neutral red uptake; **PBS,** phosphate buffered saline; **DMSO,** dimethyl sulfoxide; **TCA,** trichloroacetic acid; **EDTA,** ethylenediaminetetraacetic acid; **DTNB,** 5,5'-dithionitrobenzoic acid; **LPO,** lipid peroxidation; **TBARS,** thiobarbituric acid-reactive substances; **TBA,** thiobarbituric acid; **SOD,** superoxide dismutase; **PMS,** phenozine methosulphate; **NBT,** nitroblue tetrazolium; **NADH,** nicotinamide adenine dinucleotide; **GSH,** glutathione.

testing based on animal studies can be avoided and toxicological mechanism requires a more detailed analysis of change in biochemical pathways. Furthermore, a good reproducibility and high sensitivity have to be provided using a model system, which are time and cost effective and allow routine testing of multiple compounds. Several cell types such as testicular germ cells (Maheshwari et al., 2009), PC-12 (Kang et al., 2008; Siddiqui et al., 2010a) and MCF-7 cells (Kumar et al., 2009; Rong-Gua et al., 2010) have been used for assessment of chemical induced toxicity. The MCF-7 cells are metabolically active and are commonly used in toxicological investigations (Liu et al., 2008; Kumar et al., 2009; Rong-Gua et al., 2010). Therefore, these cells were employed in this study as a suitable model system for rapid and relatively inexpensive *in vitro* evaluation of chemical substances inducing oxidative stress, using H_2O_2, as a test oxidant, well known for generating free radicals in the cells.

MATERIALS AND METHODS

MCF-7 cells cultured in minimum essential medium (MEM), supplemented with 10% fetal bovine serum (FBS), 0.2% sodium bicarbonate and antibiotic and antimycotic solution (100x, 1 ml/100 ml of medium, invitrogen, life technologies, USA). Cells were grown in 5% CO_2-95% atmosphere in high humidity at 37°C. Prior to experimental uses, cells were screened for viability (Pant et al., 2001). Batches showing more than 95% cell viability and passage number between 10 and 18 were used in the present studies.

Reagents and consumables

MEM culture medium, antibiotics, fetal bovine and horse serum were purchased from Gibco BRL, USA. Culture wares and other plastic consumables used in the study were procured from Nunc, Denmark. All the specified chemicals, reagents and diagnostic kits were purchased from Sigma Chemical Company Pvt. Ltd. St. Louis, MO, USA.

Experimental design

Cultured healthy cells were exposed to various concentrations (0.1 mM to 1.6 mM) of H_2O_2 for 24 h following the exposures of H_2O_2, cells were subjected to assess the cytotoxic response using standard endpoints including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), lactate dehydrogenase (LDH) and neutral red uptake (NRU) assays. Further, noncytotoxic concentration (0.5 mM) of H_2O_2 for 1 to 24 h was used to study the oxidative stress parameters that is, glutathione, lipid peroxidation, catalase and superoxide dismutase.

Mitochondrial activity by MTT assay

The MTT assay was done following the method of Siddiqui et al. (2008). In brief, cells ($1x10^4$ per well) were seeded in 96-well tissue culture plates and allowed to adhere for 24 h in CO_2 incubator at 37°C. The medium was then replaced with the fresh medium containing different concentrations (0.1 mM to 1.6 mM) of H_2O_2 for 24 h. Tetrazolium bromide salt (5 mg/ml of stock in phosphate buffered saline (PBS)) was added as 10 µl/well in 100 µl of cell suspension, 4 h prior to completion of incubation periods. At the end of incubation period, the reaction mixture was carefully taken out and 200 µl of dimethyl sulfoxide (DMSO) was added to each well by pippeting up and down several times unless the content gets homogenized. The plates were kept on rocker shaker for 10 min at room temperature and then, read at 550 nm using multiwell microplate reader (Synergy HT, Bio-Tek, USA). Untreated sets run under identical conditions served as basal control.

Neutral red uptake (NRU) assay

NRU assay was performed following the protocols of Siddiqui et al. (2008). Briefly, cells were exposed to various concentrations (0.1 mM to 1.6 mM) of H_2O_2 for 24 h. On completion of incubation periods, the test solution was aspirated and cells were washed with PBS twice. Cells were then incubated for 3 h in medium supplemented with neutral red (50 µg/ml). Then, medium was washed off rapidly with a solution containing 0.5% formaldehyde and 1% calcium chloride. The cells were further incubated for 20 min at 37°C in a mixture of acetic acid (1%) and ethanol (50%) to extract the dye. The plates were then read at 540 nm using microplate reader (Synergy HT, Bio-Tek, USA). The values were compared with control sets, run under identical conditions without the test compound.

Lactate dehydrogenase (LDH) release assay

LDH release is a method to measure the membrane integrity as a function of the amount of cytoplasmic LDH released into the medium. LDH assay was carried out using the commercially available kit for *in vitro* cytotoxicity evaluation (LDH-Assay kit, bio vision, CA, USA) following the exposure of various concentrations (0.1 mM to 1.6 mM) of H_2O_2 for 24 h. The assay was based on the measurement of activity of LDH released from damaged cells. In brief, following the treatment, plates were incubated as per the experimental schedule in CO_2 incubator for 24 h. The cells were centrifuged at 250 xg for 4 min and the supernatant of each well was transferred to a fresh flat bottom 96-well culture plate and preceded further for enzymatic analysis as per the standard manufacturer's protocol.

Glutathione (GSH) content

Intracellular GSH content was estimated following the protocol of Siddiqui et al. (2010a) with modifications. In brief, sonicated cell suspension (1ml) was treated with 1 ml trichloroacetic acid (TCA) (10%) and placed on ice for 1 h to get the complete protein precipitation and then, centrifuged at 3000 rpm for 10 min. The supernatant was added to 2 ml of 0.4 M Tris buffer (pH 8.9) containing 0.02 M ethylenediaminetetraacetic acid (EDTA) followed by addition of 0.01 M 5,5'-dithionitrobenzoic acid (DTNB) and diluted with 0.5 ml distilled water to a final volume of 3 ml. The tubes were incubated for 10 min at 37°C in water bath with shaking. The absorbance of yellow colour developed was read at 412 nm using multiplate reader (Synergy HT, Bio-Tek, USA).

Lipid peroxidation (LPO)

Lipid peroxidation was performed using thiobarbituric acid-reactive substances (TBARS) protocol (Buege and Aust, 1978). Briefly, cells were collected by centrifugation and sonicated in ice cold potassium chloride (1.15%) and centrifuged for 10 min at 3000 xg. The resulting supernatant (1 ml) was added to 2 ml of thiobarbituric

acid (TBA) reagent (15% TCA, 0.7% TBA and 0.25N HCl) and heated at 100°C for 15 min in a boiling bath. The sample was then placed in cold and centrifuged at 1000 xg for 10 min. Absorbance of the supernatant was measured at 535 nm.

Catalase activity

The activity of catalase in cells was assayed following the protocol of Sinha (1972) using H_2O_2 as a substrate. Reaction mixture in a final volume of 1 ml consisted of phosphate buffer (pH 7.0), 0.08 µmol of H_2O_2 and enzyme protein. The enzyme activity was measured following disappearance of H_2O_2 at 570 nm using UV-Vis Spectrophotometer.

Superoxide dismutase (SOD) activity

The SOD activity was determined using the protocol described by Kakkar et al. (1984). In brief, in a final volume of 3 ml containing 0.052 M sodium pyrophosphate buffer (pH 8.3), 186 µM phenozine methosulphate (PMS), 300 µM nitroblue tetrazolium (NBT), 780 µM NADH, sonicated enzyme preparation and water, the reaction was started with the addition of nicotinamide adenine dinucleotide (NADH) followed by incubation at 37°C for 90 s. Then, the reaction was stopped by adding 1.0 ml of glacial acetic acid and the content was rigorously shaken with 4.0 ml of n-butanol. The mixture was allowed to stand for 10 min, centrifuged and butanol layer was separated. The colour intensity of chromogen in butanol was measured at 560 nm against butanol using a spectrophotometer. A mixture devoid of enzyme containing cell suspension served as control.

Protein estimation

Protein estimation of each sample was done following the method of Lowry et al. (1951) using bovine serum albumin as a reference standard.

Statistical analysis

The results are expressed as mean and standard error of means (SEM). One way analysis of variance (ANOVA) using Dunnet posthoc test was employed to detect differences between the groups of treated and control. $P < 0.05$ was taken to indicate significant differences.

RESULTS

MTT and NRU assays

The sensitivity of the system was evaluated by the determination of concentration response of H_2O_2 on MCF-7 cells by the MTT assay (Figure 1) and the results were further confirmed by NRU assay (Figure 2). Figure 1 shows that H_2O_2 induced statistically significant decrease in cell viability of MCF-7 cells in a concentration dependent manner. Cells were exposed to various concentrations in the range of 0.1 to 1.6 mM H_2O_2 for 24 h. MCF-7 cells exposed with 0.5 mM H_2O_2 and higher concentrations for 24 h were found to be cytotoxic. Cell viability was found to be 75% at 0.6 mM, whereas, maximum reduction in cell viability at 1.6 mM was found to be 14% (Figure 1). Similar kind of effects in case of NRU assay was also observed in H_2O_2 exposed MCF-7 cells (Figure 2).

LDH release assay

The results from LDH release assay are presented in Figure 3. A concentration dependent increase in the LDH release was observed in MCF-7 cells exposed to H_2O_2 for 24 h. The increase in LDH release was started at 0.5 mM of H_2O_2, and was found to be significantly high at concentrations 0.6 to 1.6 mM of H_2O_2. The H_2O_2 at concentrations of 0.5 mM and when lower did not cause any effect in LDH release (Figure 3). The non-cytotoxic concentration (0.5 mM) of H_2O_2 was selected to investigate further oxidative stress parameters, that is, glutathione (GSH), lipid peroxidation (LPO), superoxide dismutase (SOD) and catalase activities in MCF-7 cells.

Glutathione level

Influence on the level of GSH in the cultured MCF-7 cells exposed to 0.5 mM concentration of H_2O_2 for 1 to 24 h is depicted in Figure 4. The result indicates that, 0.5 mM of H_2O_2 decreased the GSH levels in a time dependent manner. At 12 and 24 h of exposure, the level was brought down significantly to 39 and 44% of the control, respectively. There was no significant decrease observed in the GSH level in cells exposed upto 6 h. (Figure 4).

Lipid peroxidation

Figure 5 shows the trend of LPO which is similar to that of SOD and catalase. However, the increase in the level of LPO was found to be 11% after 4 h exposure of H_2O_2, which continuously increases to 17, 21 and 45% of control at 6, 8 and 12 h, respectively. At the end of 24 h exposure period, the level of LPO was observed to be the maximum (60% of the control).

Catalase activity

The results of the catalase activity are shown in Figure 6. A noticable reduction (10% of control) in the level of catalase was observed in treated cultured MCF-7 cells within 1 h of incubation. However, the effect of exposure on catalase level decreases as functions of time increase that is, 2 h (16%), 4 h (17%), 6 h (22%), 8 h (37%), 12 h (44%) and 24 h (48%) of incubation.

Superoxide dismutase level

Figure 7 shows the pattern of SOD levels at different time

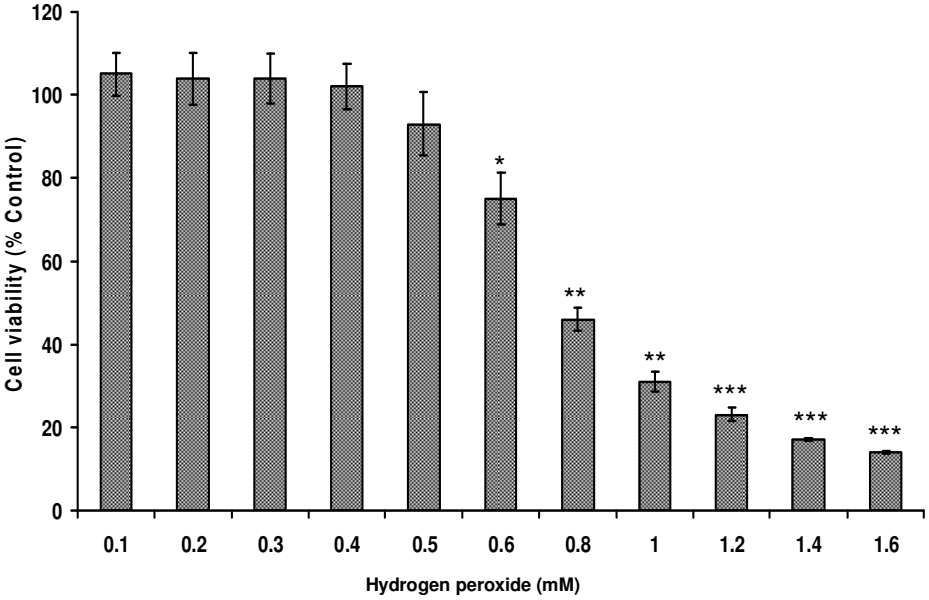

Figure 1. Hydrogen peroxide induced alteration in mitochondrial activity in MCF-7 cells by MTT assay. The values are presented as percent cell viability. The data are the mean ± SE of three independent experiments and values were taken from at least six wells from each experiment (*p < 0.05, **p < 0.01, ***p < 0.001 versus control).

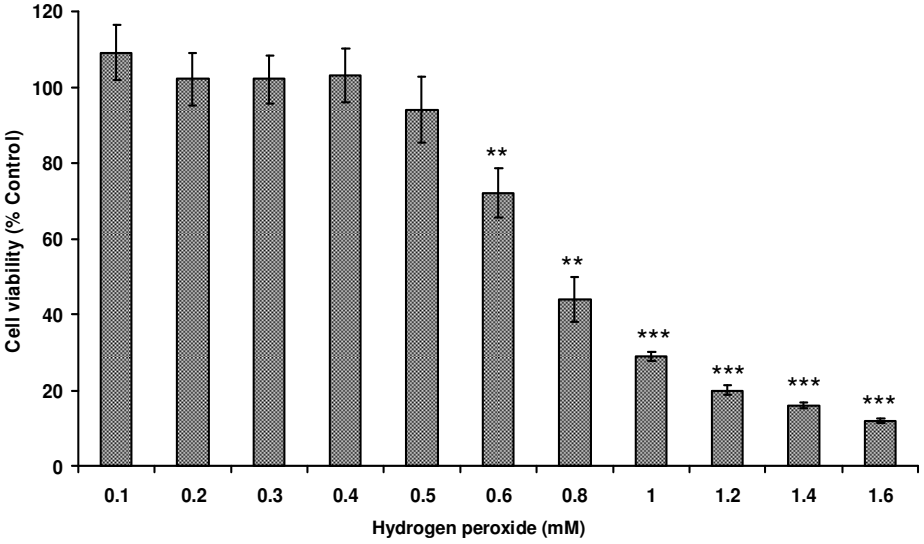

Figure 2. Hydrogen peroxide induced alteration in the lysosomal activity in MCF-7 cells by NRU assay. The values are presented as percent cell viability. The data are the mean ± SE of three independent experiments and values were taken from at least six wells from each experiment (*p < 0.05, **p < 0.01, ***p < 0.001 versus control).

intervals upto 24 h following the exposure with 0.5 mM H_2O_2 in MCF-7 cells. A time dependent decrease in the level of SOD was observed. The decrease in SOD level occurred within 2 h of exposure with H_2O_2, which further decreased to 22% of control at 6 h followed by a continuous decline to 45 and 47% of control at 8 and 12 h, respectively. The maximum decrease in the level of SOD was observed as 48% of control at 24 h of the end incubation period.

DISCUSSION

The aim of the present study was to investigate if the

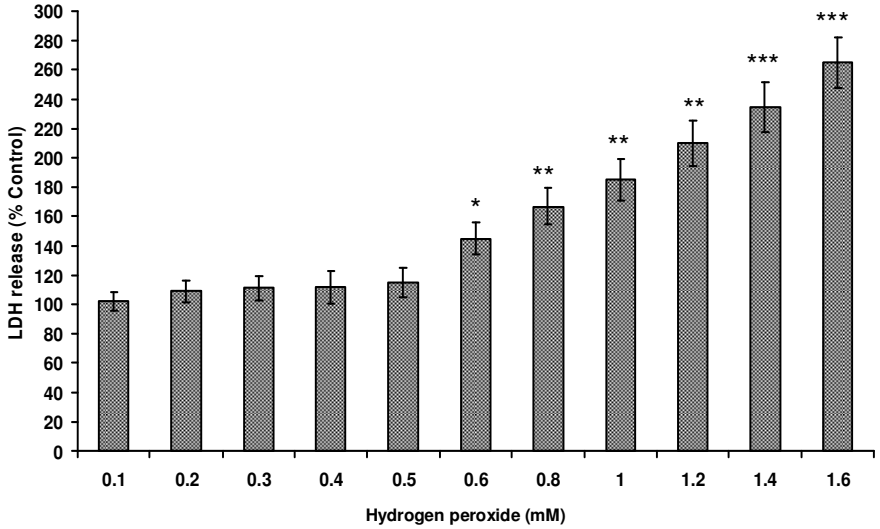

Figure 3. Hydrogen peroxide induced loss in membrane integrity in MCF-7 cells by LDH release assay. The data are the mean ± SE of three independent experiments and values were taken from at least six wells from each experiment (*p < 0.05, **p < 0.01, ***p < 0.001 versus control).

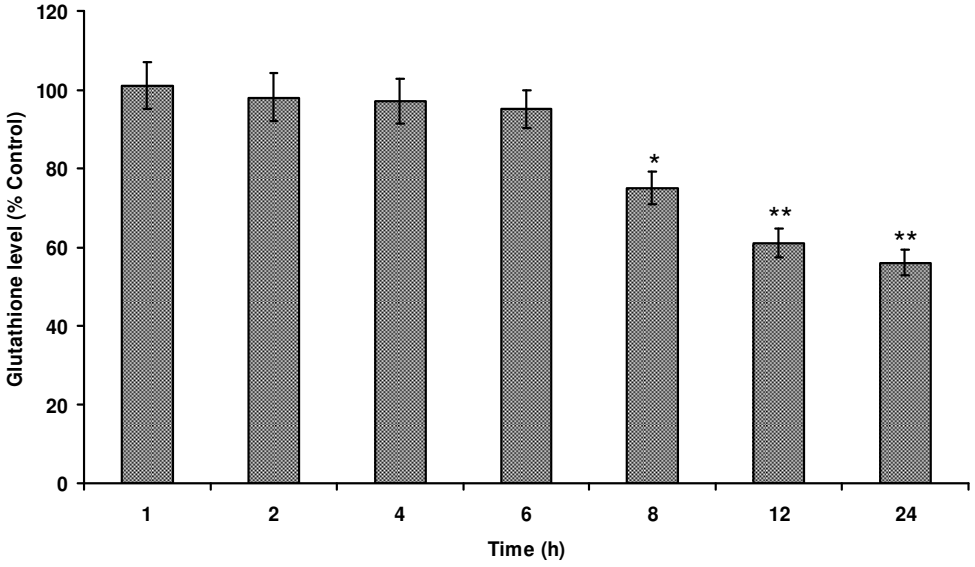

Figure 4. Glutathione depletion in MCF-7 cells following the exposure of 0.5 mM concentrations of hydrogen peroxide for various time periods. All values represent the mean ± SE (*p < 0.01, **p< 0.001 versus control).

MCF-7 cells, a human breast adinocarcinoma cell line could be exploited as an *in vitro* model system for detecting the levels of oxidative stress induced by any chemical substance with sufficiently high sensitivity and reproducibility. The sensivity and reproducibility are the critical factors, in case the biological system is used as an indicator system for the routine testing for toxicological purposes. Therefore, a well known oxidizing agent H_2O_2 has been chosen for inducing oxidative cell damage in cultured cells because oxidative stress is believed to be an important mediator of cell death and has been postulated to contribute to the pathogenesis of various diseases (Kaminsky and Kosenko, 2008; Hwang et al., 2008). H_2O_2 is a precursor of highly oxidizing, tissue-damaging radicals such as hydroxyl radicals and is known to be toxic to many systems. Among a great variety of reactive oxygen species, H_2O_2 plays a pivotal role because it is generated from nearly all sources of oxidative stress and exogenous H_2O_2 can enter the cells and induce cytotoxicity due to its high membrane

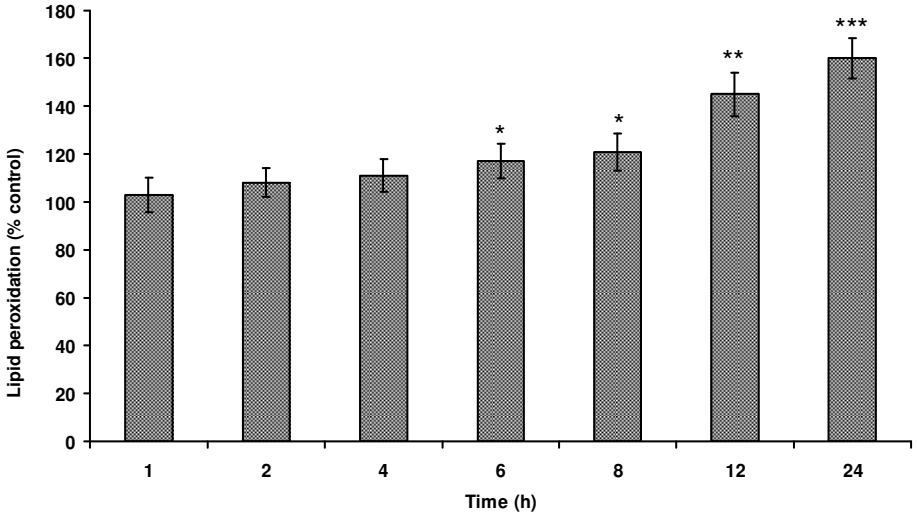

Figure 5. Lipid peroxidation in MCF-7 cells following the exposure of 0.5 mM concentrations of hydrogen peroxide for various time periods. All values represent the mean ± SE (*p < 0.05, *p < 0.01, ***p< 0.001 versus control).

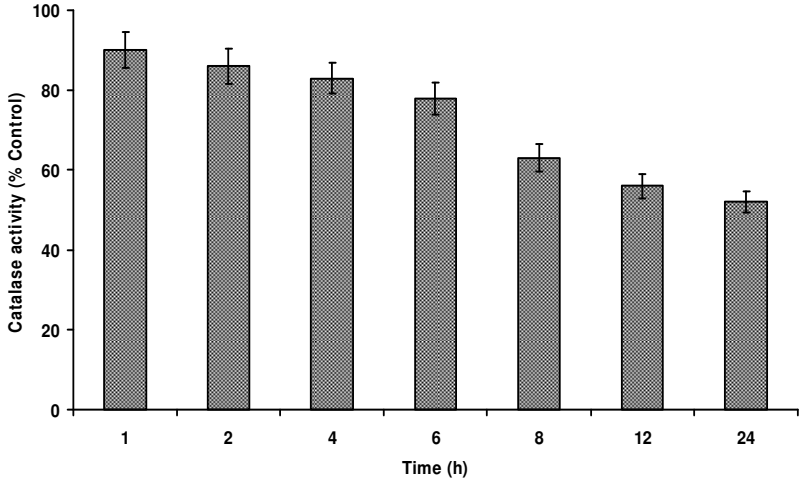

Figure 6. Catalase activity in MCF-7 cells following the exposure of 0.5 mM concentrations of hydrogen peroxide for various time periods. All values represent the mean ± SE (*p < 0.01, **p < 0.001 versus control).

permeability (Margittai et al., 2008; Pedroso et al., 2009). It is well documented in the literature that, the short term exposure of low to moderate concentrations of exogenous H_2O_2 increases cell proliferation and growth in hamster, rat and a variety of mammalian cell types in culture system (Burdon et al, 1990; Kim et al., 2001). However, the high doses of H_2O_2 pose severe oxidative and cytotoxic effects in the mammalian culture cells (Kim et al., 2001; Cai et al., 2008; Hwang et al., 2008). In the present study, we found that H_2O_2 significantly reduced cell viability in MCF-7 cells in a concentration dependent manner. The concentrations 0.6 mM and above of H_2O_2 were found to be cytotoxic, whereas, concentrations at 0.5 mM and below could not exhibit any significant adverse effects in MCF-7 cells. Siddiqui et al. (2010b) have also demonstrated cytotoxicity of H_2O_2 in PC-12 cells at concentrations above 0.5 mM after 24 h exposure whereas, the concentration of 0.2 mM and less were found to be non-cytotoxic in PC-12 cells. Thus, our results of dose response on the cell viability are in the agreement with previous findings. H_2O_2 causes cell death by reacting with the cell membrane, resulting in lipid peroxidation of the membrane. It can easily cross the cell membrane and exert detrimental effects on tissues by a number of different mechanisms, such as perturbing intracellular calcium homeostasis (Edwards et al., 2008) decreasing intracellular ATP (Sukhanov et al., 2006) inducing DNA damage (Joyce et al., 2009) and inducing

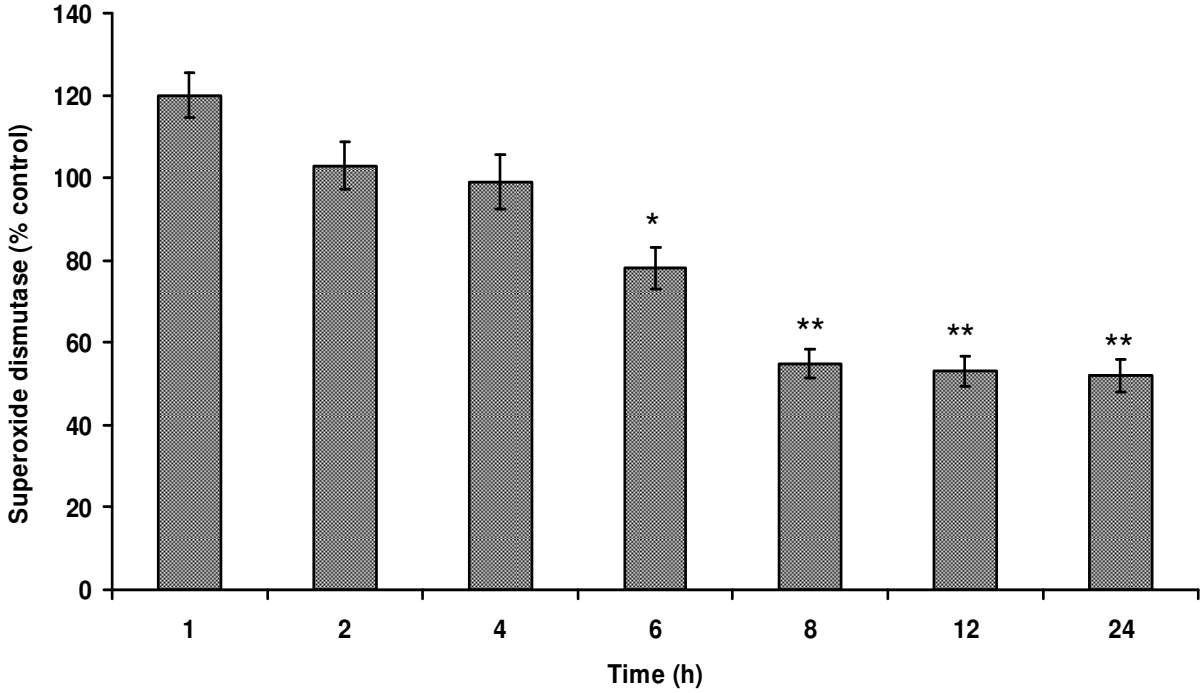

Figure 7. Superoxide dismutase in MCF-7 cells following the exposure of 0.5 mM concentrations of hydrogen peroxide for various time periods. All values represent the mean ± SE (*p < 0.01, **p < 0.001 versus control).

apoptosis (Seo et al., 2009).

In our study, the remarkable increase in the levels of LPO and LDH and decreases in the levels of antioxidant enzyme, GSH content, SOD and catalase activities at early time points were noticed following the exposure of H_2O_2. During cellular exposure, H_2O_2 mimicking the burden of oxidative stress leads to a fast oxygenation of DNA nucleotide (Collins et al., 1993). This gives rise to the formation of 8-hydroxyguanosine and disintegration of DNA, as demonstrated by comet assay (Singh et al., 1988). This method revealed that, DNA damage occurs during the time of cells being exposed to H_2O_2 (Dusinska and Collins, 1996). Therefore, when in our experiments, cells were exposed to H_2O_2, the level of GSH contents, LPO, SOD, catalase and LDH shows severe alteration towards the oxidative damage in most of the parameters studied during late hours which was continued upto the end of incubation period (24 h). This might be due to the oxidative stress to a certain extent.

Conclusions

A significant variation in biological end-points between exposed of unexposed cultured cells was observed in our experiments which reveals that, the human breast cell line MCF-7 can be a suitable choice as *in vitro* model system for the routine analysis of substances inducing oxidative stress by using biochemical end points like GSH, LPO, SOD and catalase activities.

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