Copyright © 2014 By IYPF
All rights reserved
Open Access Contents
Int. J. Drug Dev. & Res.
I July - September 2014 I Vol. 6 I Issue 3 I ISSN 0975-9344 I
www.ijddr.in

Full Length Original Research Paper

The influence of calreticulin on oxidative stress in MCF-7 cells

Sree Jaya S[1]

Sudha S[1]*

[1] Molecular Diagnosis and Drug Discovery Laboratory, Department of Biotechnology, Karpagam University, Coimbatore, India

Corresponding Authors:
Email:
sudhasellappa@gmail.com

Abstract:
Calreticulin (*CRT*), a multifunctional protein that regulates varied important cell functions, in addition CRT recently drawn notice that the function of oxidative stress induced apoptosis. At this point, the role of CRT through oxidative stress mediated apoptotic cell death is focused. Herein, we used mammary gland adenocarcinoma cell cells (MCF-7) *in vitro* to investigate the role CRT overexpression in cell death by promoting ROS induced apoptosis. Human CRT gene was isolated from blood, cDNA was synthesized, CRT was cloned to the XhoI/EcoRI restriction sites of a mammalian expression vector pcDNA 3.1 and plasmid was transfected in to MCF-7 cell line to promote apoptosis. After 24 h and 48 h transfection, cell proliferation, LDH leakage, lipid peroxidation, total protein, and glutathione concentrations were measured. CRT transfected cells expressed higher concentrations of lipid peroxidation and LDH leakage than control MCF -7 cells. There was a significant negative correlation between lipid peroxidation and cell proliferation. Glutathione did not appear to be a significant factor. Therefore, stimulation of CRT may modulate the growth inhibitory effects in human breast cancer cells. One mechanism of growth inhibition may be through increased lipid peroxidation.

Keywords: calreticulin, apoptosis, lipid peroxidation, oxidative stress

Introduction

Cancer is a widespread health problem with massive raise in recent times, and mortality rate in the developed countries have increased in spite of developments made in cancer diagnosis and therapy. At present, breast cancer is the main cause of cancer-related death in women [1]. Mammary homeostasis is depending upon establishing and maintaining a balance between cell proliferation and apoptosis. Augmented cell proliferation and down regulation of apoptosis direct to mutations that result in cancer [2,3]. Dysregulation makes ineffective changes of many normal checkpoint pathways that lead to expansion of neoplastic cells [1]. Oxidative stress has been concerned in the usual aging process and several disorders including cancer [4,5,6]. However, the connection between oxidative stress and patho-biology of cancer is not comprehensible, mainly due to a need of understanding of the mechanism by which ROS functions in both normal and disease states [7]. Endogenously generated ROS can be detected in cells undergoing apoptosis. Higher organisms have both ascorbate oxidation system and thiol oxidation system. These can be inhibited by catalase and glutathione synthetase, which protects them from ROS [8].

Mammary homeostasis is depending upon establishing and maintaining a balance between cell proliferation and apoptosis. CRT is a ubiquitous protein localized predominantly in the endoplasmic reticulum of eukaryotic cells and it plays a central role in intracellular calcium homeostasis and emerging as a vital mediator of physiological and pathological processes [3].

The function of CRT in cancer and the mechanistic pathway involved remains

© 2014 Sudha. S et al, publisher and licensee IYPF. This is an Open Access article which permits unrestricted noncommercial use, provided the original work is properly cited.

unidentified; while there are extensive reports about the role of calreticulin in immunogenic cell death of cancerous cells [9,10]. CRT exposure is potently triggered by pharmacological cell death inducers that provoke the production of reactive oxygen species (ROS) as well as an ER stress response. Furthermore, overexpression of CRT has been revealed to raise cell susceptibility to H_2O_2 induced cytotoxicity [11], demonstrating that CRT expression is a main reason influencing cellular susceptibility to oxidative stress induced apoptosis. However, the relationship between CRT and ROS-induced apoptosis in mammary cancer cells are still unknown. Hence, it is important to find out whether apoptosis can be induced by oxidative stress in mammary cancer cells under physical and/or pathological conditions needs to be investigated.

Materials and Methods

Cell lines

MCF-7 human breast cancer cells are obtained from the National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in Minimal Essential Medium (Gibco) supplemented with 10% FBS and antibiotic solution (Penicillin and Streptomycin) in a humidified CO_2 incubator (5%) at 37ºC [12,13].

Transfection

RNA was isolated from healthy human blood using RNeasy Mini kit (Qiagen) and cDNA of CRT was synthesized (cDNA synthesis kit, Axygen) using Super-Script II RNase H reverse transcriptase (Invitrogen). PCR primers were designed according to the DNA sequence of CRT (GenBank). The forward and reverse primers used were 5'-TCTCAGTTCCGGCAAGTTCT-3' and 5'-GTTGCTGAAAGGCTCGAAAC-3' respectively. Amplification of target gene was performed at 95 ℃ 3minutes, then 95 ℃ 30seconds, 58 ℃ 30seconds and 72 ℃ 45seconds for 35 cycles, and 72 ℃ for 5minutes. The product was finally held at 4 ℃. Amplified product was obtained at 1.2kb. This amplified target gene was ligated at XhoI and EcoRI restriction sites of mammalian expression vector pcDNA 3.1 (Invitrogen) [14,15]. The mock and CRT/pcDNA 3.1 recombinant plasmid was transfected to MCF-7 cells using Lipofectamine (Invitrogen) according to manufacturer's protocol. Stable transfectants were selected by treating the cells with 500 µg/ml of G418. The cloned G418-resistant lines were then screened for expression of CRT.

Cell proliferation assays

Determination of growth levels was performed using trypan blue exclusion method. MCF-7 cells cultured in 24-well plates at a density of 4×10^4 cells/ml were incubated for 24 - 48 hours with 2ng of CRT/pcDNA3.1 plasmid. Following incubation, cells were trypsinized and adherent cells were combined which was then washed with phosphate buffered saline (1X PBS: pH 7.4) to remove debris, stained with trypan blue and counted using an optical microscope and a Newbauer's chamber.

Histopathology of cells

Recombinant plasmid treated MCF 7 cells after 48 hours of post transfection were fixed in 70% methanol and 30% glacial acetic acid for 15 min at room temperature, washed with deionized water. After fixation cells were treated with dilute buffered giemsa and morphological changes were observed under inverted phase contrast microscope [16].

© 2014 Sudha. S et al, publisher and licensee IYPF. This is an Open Access article which permits unrestricted noncommercial use, provided the original work is properly cited.

LDH release assay

LDH assay was performed to assess the LDH release to the media following CRT plasmid transfection on MCF-7 cells for 24 and 48 hours respectively. Briefly, 1×10^4 cells/well of MCF-7 cells was transferred to 96-well plates. The plates were incubated overnight at 37°C to allow the cells to attach and proliferate. On the next day, 10 µl of lipofectamine with 240 µl fresh medium containing 2.2 ng plasmids were added to each well, and the plates were incubated at 37 °C in 5% CO_2. All treatments were tested at least in triplicate wells and the assays were repeated independently three times. After 48 hours, the plates were removed from the incubator and then 100 µl of medium from each well was carefully transferred to new plates. 100 µl of LDH substrate was added to each well. After 20 minutes shaking at room temperature, the enzymatic reaction was arrested by adding 50 µl of 1M hydrochloric acid. Lactate dehydrogenase activity was determined by change in absorbance at 490 nm. The percentage of LDH release was calculated as: (LDH activity in media)/ (LDH activity in media + intracellular LDH activity) ×100. For the purpose of calculating percent cytotoxicity values, background LDH release from culture cells was considered as low control and Lipofectamine treated cells as high control [17].

Intracellular glutathione measurement

Intracellular glutathione was determined according to Ellman's method [18]. Cells at 24h, 48h and 72h post transfection with CRT were trypsinized and washed with phosphate buffered saline. Cells were resuspended in the same buffer and lysed by diluting it with 4% sulfosalicylic acid with rapid mixing, and the samples were centrifuged at 10,000 x g for 10 min in a refrigerated centrifuge. Aliquots of 1 ml of resulting supernatants were added to 2 ml of Ellman's reagent (8 mg/100 ml of dinitro 5-thiobenzoic acid in 0.1 M sodium phosphate buffer, pH 8.0), and the absorbance was recorded at 412 nm after 5 min. The cellular homogenate was taken to determine the amount of intracellular glutathione and results were expressed as nmole GSH reduced/mg protein.

Lipid peroxidation measurement

The degradation of lipids was determined by measuring malondialdehyde (MDA), which is the end product of lipid peroxidation, using the thiobarbituric acid reactive substance (TBARS) assay protocol [19]. Briefly, after 24h, 48h and 72h post transfection MCF-7 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 was added to 2 ml of thiobarbituric acid (TBA) reagent and heated at 100°C for 15 min. The sample was then placed in cold and centrifuged at 1000xg for 10 min. Absorbance of the supernatant was measured at 535 nm. By considering the molar extinction coefficient of the MDA-TBA complex to be 1.49×105/M.cm [20], the amount of TBARS was calculated in terms of mol MDA equivalents that were formed per mg of cell protein.

Protein estimation

Protein estimation of each sample was done following the method of Lowry *et al.* [21] 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

© 2014 Sudha. S et al, publisher and licensee IYPF. This is an Open Access article which permits unrestricted noncommercial use, provided the original work is properly cited.

employed to detect differences between the groups of treated and control. P < 0.05 was taken to indicate significant differences.

Results

Inhibition of growth and proliferation of human breast cancer cells by CRT after 48 hours post transfection was determined. Measurement of cell viability in MCF-7 cell line was determined using trypan blue assay. 48 hours post transfection showed a significant increase in cell death than 24 hours post transfection with 20 % and 55 % of cells were viable as shown in Figure 1.

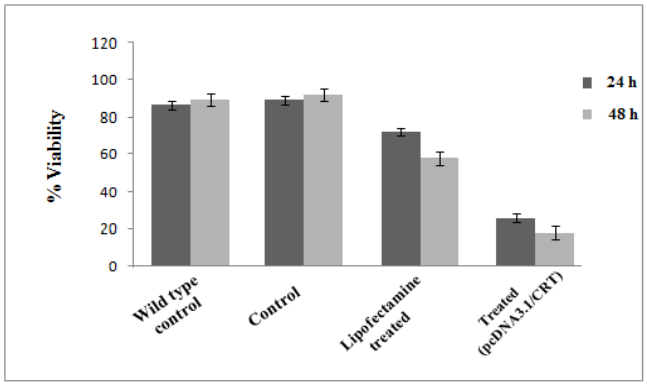

Figure 1: Percentage of cell death on MCF -7 cells treated with CRT/pcDNA3.1, at 24 hours and 48 hours post transfection (Trypan blue exclusion assay).

The morphological changes of MCF-7 cells treated with CRT vector were also observed using Axiovert-25 inverted microscope (Soft-ware: Axiovision 4.0) at 24 hours and 48 hours post transfection. Results revealed a typical apoptosis with morphological changes like chromatin condensation and cell shrinkage. These changes were apparent at 24 hours, and became 50 % or more after 48 hours. The majority of cells at 48hours post transfection lead to shrink, round up and detach from the culture dish (Figure 2).

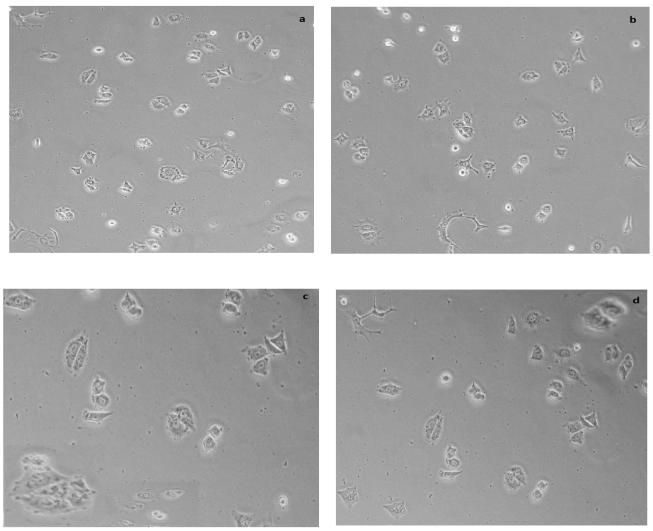

Figure 2: Representative photomicrograph shows morphological changes in MCF-7 cells treated with pcDNA3/CRT, at 24 hours and 48 hours post transfection (a & b) and MCF-7 cells treated with empty pcDNA3 at 24 hours and 48 hours (c & d), imaged by inverted phase contrast microscope at a magnification of 200x.

Measurement of LDH release following 24 hours and 48 hours transfection with CRT plasmid, the release of LDH was measured as percentage of LDH release. The percentage of cell viability and inhibition of cell proliferation was calculated considering control cells as 100 %. In all the cases, error bars indicate SD from three independent experiments. P values ($p<0.05$) denoted by asterisk were calculated comparing mean control cells with mean treated cells (*$p<0.05$).

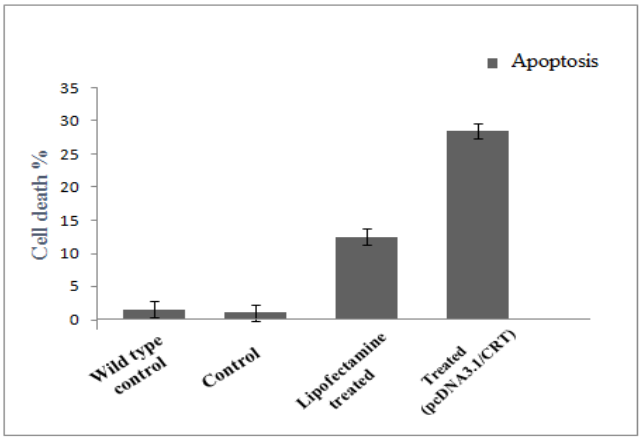

Figure 3: Effect of CRT overexpression on LDH activity in MCF-7 cells treated with CRT/pcDNA3.1. LDH activity was measured by changes in optical

© 2014 Sudha. S et al, publisher and licensee IYPF. This is an Open Access article which permits unrestricted noncommercial use, provided the original work is properly cited.

densities due to NAD+reduction which were monitored at 490 nm, as described in the text, the experiments were performed in triplicates; data shown represent mean + SD of three independent experiments. *P < 0.05 as compared with untreated cells.

The result indicates that, CRT transfection decreased in a time dependent manner. At 48 h and 72 h post transfection, the GSH level was significantly brought down. There was no significant decrease observed in the GSH level in cells exposed up to 24 h when compared to controls. However, the increase in the level of TBARS was determined after 24 h exposure of CRT, which continuously increases than that of controls at 48 and 72 h. At the end of 72 h exposure period, the level of TBARS was observed to be the maximum (Table 1).

Table 1: Effect of CRT on the level of intracellular GSH concentration and TBARS formation in MCF 7 cells

Hours of treatment	Wild type control		Positive control		CRT Treated	
	GSH[a]	TBARS[b]	GSH[a]	TBARS[b]	GSH[a]	TBARS[b]
24 h	11. 06 ± 0.01	0.02 ± 0.03	14. 02 ± 1.53	0.18 ± 0.01	13. 3 ± 1.10	1.33 ± 0.02
48 h	11. 59 ± 0.03	0.03 ± 0.08	15. 92 ± 1.53	0.19 ± 0.03	7.9 ± 0.97*	1.42 ± 0.07*
72 h	12.03 ± 0.04	0.06 ± 0.05	15. 23 ± 1.53	0.19 ± 0.01	5.4 ± 1.01*	1.57 ± 0.05*

[a](nmole glutathione reduced/mg protein)
[b](nmole malondialdehyde /mg protein)
P < 0.001

Discussion

Apoptosis, a well recognized biological response displayed by cells after suffering DNA damage and is a valuable means for developing potential anticancer agents. The on time elimination of apoptotic cells by phagocytes is important for maintaining tissue homeostasis. The detection and disposal of apoptotic cells normally promote an anti-inflammatory response at the tissue level. There are number of genes in charge for the protection of tissue homeostasis, by which CRT engages in many biological processes including regulation of cell homeostasis and apoptosis. CRT also reported for molecular chaperones which enhances radiation-induced apoptosis through reduction of ROS production [22].

CRT was reported to be a helpful marker for mammalian apoptotic cells to be recognized by phagocytes [23]. The prostate cancer cells over expressing exogenous CRT produced smaller quantity of colonies support the hypothesis that CRT inhibits growth and metastasis of cancer cells [24]. Cells deficient in CRT are relatively resistant to [9,25] and over expression of CRT causes differentiation induced apoptosis in H_9C_2 cells [26].

In the present study, we found that CRT significantly reduced cell viability in MCF-7 cells in a time dependent manner. These findings established that CRT overexpression significantly inhibited the proliferation of breast cancer cells in vitro by inducing apoptosis compared to untreated cells. Thus, the results of the cell viability are in the agreement with previous findings [15] that CRT can exert detrimental effects on tissues by a number of different mechanisms, such as disturbing intracellular calcium homeostasis [27] decreasing intracellular ATP [28] inducing DNA damage [29] and inducing apoptosis [30].

In our study, the remarkable increase in the levels of LPO and LDH and decreases in the levels of GSH was noticed following the CRT transfection. The level of GSH, LPO and LDH shows variation towards the oxidative damage in most of the parameters studied during 24 and 48hours incubation period. This might be due to the oxidative stress.

© 2014 Sudha. S et al, publisher and licensee IYPF. This is an Open Access article which permits unrestricted noncommercial use, provided the original work is properly cited.

LDH is a ubiquitous enzyme that functions in a regulatory manner at a key point in normal intermediary metabolism. Several investigators have reported an elevation in the amount of LDH release in cancer cells against variety of anticancer agents, which is inline with our observations. GSH is an anti-oxidant and decreased intracellular levels of GSH are related with better susceptibility to ROS mediated apoptosis [31]. In our present study we have observed a significant reduction in the intracellular GSH levels in MCF7 after treatment with recombinant CRT plasmid. These observations suggest that, at reduced intracellular GSH levels, which are known to scavenge the generated free oxygen radicals, tumour cells are sensitized to apoptotic death. The CRT overexpression depleted the intracellular glutathione concentration in MCF 7 cells which was associated with an increase in the level of lipid peroxidation products. Therefore our study suggests that CRT could induce apoptosis in breast cancer cells, which require additional in vitro and in vivo studies for examining the role of CRT mediated apoptosis against breast cancers.

In conclusion, the present study indicated that the overexpression of CRT inhibited the growth and proliferation of mammalian breast cancer cells and it confirms the induction of apoptosis in a time dependent manner. A significant variation in biological end-points between exposed of unexposed MCF 7 cells was observed in our experiments which reveals that, the human breast cell line MCF-7 cells as model system for the routine analysis of substances inducing oxidative stress by using biochemical end points like GSH, LPO, and LDH concentration which gives an altered redox status.

Acknowledgements

The authors thank Department of Biotechnology, New Delhi for the grants provided to carry out this work (No.Bio-CARe/08/58/2010-11). We are indebted to the authorities of Karpagam University for providing the facilities and constant encouragements to carry out the work.

References

1) Lu J, Steeg PS, Price JE, Krishnamurthy S, Mani SA, Reuben J. Breast cancer metastasis: challenges and opportunities, *Cancer Research* 2009; 69: 4951.

2) Parton M, Dowsett M, Smith I. Studies of apoptosis in breast cancer. *BMJ* 2001; 322: 1528.

3) Kasibhatla I, Tseng B. Why target apoptosis in cancer treatment, *Molecular Cancer Therapy* 2003; 2: 573.

4) Sha H, Huang AC, Farris M, Vazarian M, De Vellis J, Chen W. Thioredoxin peroxidase (natural killing enhancing factor) regulation of activator protein-1 function in endothelial cells. *Biochemical and Biophysical Research Communications* 1988; 249: 683-86.

5) Sarasfian TA, Huang C, Kim A, et al (1998). Expression of the antioxidant gene NKEF in the central nervous syatem. *Molecular and Chemical Neuropathology*, 34, 39-51.

6) Valko M, Leibfritz D, Moncol J. Free radicals and antioxidants in normal physiological functions and human disease," *International Journal of Biochemistry and Cell Biology* 2007; 39: 44–84.

7) Yong S, Yong-Kwan C, Nam HK, Chung HT, Kang DG, Pae HO. Mitogen-Activated Protein Kinases and Reactive Oxygen Species: How Can ROS Activate MAPK Pathways? *Journal of Signal Transduction* 2011, 2011, 6.

8) Baker A, Payne CM, Briehl MM, Powis G. Thioredoxin a gene found overexpressed in

© 2014 Sudha. S et al, publisher and licensee IYPF. This is an Open Access article which permits unrestricted noncommercial use, provided the original work is properly cited.

human cancer, inhibits apoptosis *in vitro* and *in vivo*. *Cancer Research* 1997; 57: 5162-67.

9) Nakamura K, Bossy-Wetzel E, Burns K, et al (2000). Changes in endoplasmic reticulum luminal environment affect cell sensitivity to apoptosis. *The Journal of Cell Biology*, 150, 731-740.

10) Chapu N, DeBotton S, Obeid M. Molecular determinants of immunogenic cell death: surface exposure of calreticulin makes the difference. *Journal of Molecular Medicine* 2007; 85: 1069-1076.

11) Ihara Y, Urata Y, Goto S, Kondo T. Role of calreticulin in the sensitivity of myocardiac H_9C_2 cells to oxidative stress caused by hydrogen peroxide. *American Journal Physiology Cell Physiology* 2006; 290: 208-221.

12) Osborne CK, Hobbs K, Trent JM. Biological differences among MCF-7 human breast cancer cell lines from different laboratories. *Breast Cancer Research and Treatment* 1987; 9: 111-21.

13) Sandra VV, McLeskey Junichi K, Susan FH, James Z, Marc EL, Robert BD, Francis GK. Fibroblast growth factor 4 transfection of mcf-7 cells produces cell lines that are tumorigenic and metastatic in ovariectomized or tamoxifen-treated athymic nude mice. *Cancer Research* 1993; 53: 2168-77.

14) Prathyumnan S, Sellappa S, Joseph S. Enhanced Calreticulin Expression Triggers Apoptosis in the MCF-7 Cell Line. *Asian Pacific Journal of Cancer Prevention* 2010; 11: 1133-1136

15) Prathyumnan S, Sellappa S, Keyan KS, Joseph S. Cloning of CRT cDNA from human blood and its over-expression in MCF-7 cells. *International Journal of Current Research and Review* 2011; 1: 120-124.

16) Vidhya N, Niranjali Devaraj S. Induction of apoptosis by eugenol in human breast cancer cells. *Indian Journal of Experimental Biology* 2011; 49: 871-878.

17) Wacker EC, Warren D, Ulmer L, Valle. Metalloenzymes and myocardial infarction. II. Mallic and lactate dehydrogenase activities and zinc concentration in serum, *The New England Journal of Medicine* 1956; 225: 449.

18) Ellman GI. Tissue sulfhydryl groups. *Arch. Biochem. Biophysics* 1959; 82: 70-77.

19) Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods Enzymmology* 1978; 52: 302-310.

20) Draper HH, Hadley M. Malondialdehyde determination as index of lipid peroxidation. *Methods Enzymol* 1990; 186: 421- 431.

21) Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J. Biol. Chem 1951; 193: 265- 275.

22) Liu H, Bowes RC, van de Water B, Sillence C, Nagelkerke JF, Stevens JL. Endoplasmic reticulum chaperones GRP78 and calreticulin prevent oxidative stress, Ca^{2+} disturbances, and cell death in renal epithelial cells. *The Journal of Biological Chemistry* 1997; 272: 21751-21759.

23) Kuraishi T, Manaka J, Kon M, Ishii H, Yamamoto N, Koizumi K, Shiratsuchi A, Lee BL, Higashida H, Nakanishi Y. Identification of calreticulin as a marker for phagocytosis of apoptoticcells in Drosophila. *Experimental Cell Research* 2007; 313: 500-10.

24) Alur M, Nguyen MM, Eggener SE, Jiang F, Dadras SS, Stern J, Kimm S, Roehl K, Kozlowski J. Suppressive roles of calreticulin in prostate cancer growth and metastasis. *American Journal of Pathology* 2009; 175: 882-90.

25) Pinton P, Ferrari D, Rapizzi E, Di Virgilio F, Pozzan T, Rizzuto R. The Ca2+ concentration of the endoplasmic reticulum is a key determinant of ceramide-induced apoptosis: significance for the molecular mechanism of Bcl-2 action. *EMBO Journal* 2001; 20: 2690-701.

26) Kageyama K, Ihara Y, Goto S, Urata Y, Toda G, Yano K, Kondo T. Overexpression of caleticulin modulates protein kinase B/Akt signaling to promote apoptosis during cardiac differentiation of cardiomyoblast H9c2 cells. *The Journal of Biological Chemistry* 2002; 277: 19255-64.

© 2014 Sudha. S et al, publisher and licensee IYPF. This is an Open Access article which permits unrestricted noncommercial use, provided the original work is properly cited.

27) Edwards MJ, Talelli P, Rothwell JC. Clinical applications of transcranial magnetic stimulation in patients with movement disorders. *Lancet Neurology* 2008; 7: 827-840.

28) Sukhanov S, Higashi Y, Shai SY, Itabe H, Ono K. Delafontaine P. Novel effect of oxidized low-density lipoprotein: cellular ATP depletion via downregulation of glyceraldehyde-3-phosphate dehydrogenase. *Circulation Research* 2006; 99: 191-200.

29) Joyce NC, Zhu C, Harris DL. Relationship between oxidative stress, DNA damage, and proliferative capacity in human corneal endothelium. *Investigative Ophthalmology & Visual Science* 2009; 0: 2116-2122.

30) Seo M, Nam HJ, Kim SY, Juhnn YS. Inhibitory heterotrimeric GTP-binding proteins inhibit hydrogen peroxide-induced apoptosis by up-regulation of Bcl-2 via NF-kB in H1299 human lung cancer cells. *Biochemical and Biophysical Research Communications* 2009; 381: 153-158.

31) Beaver JP, Waring P. A decrease in intracellular GSH concentration precedes the onset of apoptosis in murine thymocytes. *European Journal of Cell Biology* 1995; 68: 47-54.

Article History: ----------

Date of Submission: 16-08-2014

Date of Acceptance: 29-08-2014

Conflict of Interest: NIL

Source of Support: NONE

SJR SCImago Journal & Country Rank

Powered by SCOPUS™

© 2014 Sudha. S et al, publisher and licensee IYPF. This is an Open Access article which permits unrestricted noncommercial use, provided the original work is properly cited.