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# Effects of tert-butyl hydroperoxide on $Ca^{2+}$ ATPase activity in isolated rat hepatocytes and its reversal by antioxidants

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**Abstract**

Calcium ions play an important role in various physiological processes such as nerve impulse transmission, muscle contraction, hormone action, blood clotting. They ions act as an intracellular second messenger, relaying information within cells to regulate their activity. To understand the mechanism of hepatotoxicity of t-BHP, studies were carried out using freshly isolated rat hepatocytes. The effect of t-BHP on $Ca^{2+}$ accumulation and $Ca^{2+}$ uptake by rat hepatocytes was monitored using $^{45}Ca^{2+}$. It caused decrease in 15% accumulation of $^{45}Ca^{2+}$ in comparison to the control group. t-BHP also significantly decreased the $Ca^{2+}$ ATPase activity in isolated hepatocytes .This decrease in $Ca^{2+}$ATPase activity by t-BHP was reversed 40% by naturally occurring antioxidant glutathione (GSH) and 20% by the synthetic antioxidant butylated hydroxy toluene (BHT). These results indicate that the hepatotoxic action of t- BHP involves oxidative stress as evident by the protection accorded by various antioxidants employed in the study as well as impairment of intracellular calcium homeostasis which can lead to liver cell injury.

**Key words**

Oxidative stress, Reactive oxygen species, Hepatotoxicity, Tert-butyl hydroperoxide, Calcium homeostasis.

## Introduction

Hepatocytes make up 60–65% of the cells in the liver and play a pivotal role in the metabolism of exogenous chemicals and toxins, thus making liver a target for toxic substances. Reactive oxygen and nitrogen species (ROS/RNS) are generated during detoxification which leads to oxidative stress. (Das *et al.,* 2004). tert-butyl hydroperoxide (t-BHP) is an organic lipid hydroperoxide analogue, used as pro-oxidant to evaluate mechanisms involving oxidative stress in cells and tissues. It has been shown to induce cell death in a variety of cells via apoptosis including U937 macrophages, PC-12 cells, SH-SY5Y neuroblastoma cells, cultured rat pulmonary microvascular endothelial cells and HepG2 cells (Amoroso *et al.*, 2002; Kanupriya *et al.*, 2007,Chen *et al.*, 2010).

Two distinctive pathways are involved in the metabolism of t-BHP in hepatocytes (Haidara *et al.,* 2001).Decreased GSH and oxidized NADPH contribute to altered $Ca^{2+}$ homeostasis, which is considered to be a major event in t-BHP-induced toxicity (Shimizu *et al.,* 1998). In situation where $Ca^{2+}$ overload is accompanied by a combination of other factors, most notably oxidative stress, high phosphate concentrations and low adenine nucleotide concentrations, the mitochondria undergo 'permeability transition' (MPT).This makes membrane nonspecifically permeable to any molecule up to 1.5 kDal (Crompton, 2004). This includes protons, which cause uncoupling of mitochondria, which no longer can maintain a pH gradient or mitochondrial membrane potential. Such defected mitochondria not only become incapable of ATP synthesis, but also now actively degrade ATP followed by cell death (Halestrap,

2006). The metabolism of t-butyl hydroperoxide has been shown to impair the ability of liver mitochondria to retain $Ca^{2+}$ (Aust *et al.,* 1993) and to cause $Ca^{2+}$ release from perfused liver (Tripathi *et al.,* 2009).

The generation of free radicals leading to membrane damage and alterations in calcium homeostasis seems to be the common unspecific mechanism in toxic responses which may lead to cell death but changes in the $Ca^{2+}$ ATPase activity of isolated rat hepatocytes due to chemical or oxidative stress gives mechanistic clues for the exploration of toxic effect of t-BHP. The current study was planned to observe the deleterious effects of the hepatotoxin t-BHP on isolated rat liver mitochondria and isolated rat hepatocytes, in terms of impairment of intracellular $Ca^{2+}$ homeostasis by observing the effect of t-BHP on $Ca^{2+}$ accumulation and $Ca^{2+}$ ATPase activity.

## Materials and Methods

**Isolation of hepatocytes**: Male albino Wistar strain rats weighing 100 –150 g maintained under standard conditions in the Department of Biochemistry, Dr. R.M.L. Avadh University, Faizabad. The rats were provided Lipton pellet diet and water *ad libitum*. The temperature was maintained at 25±5°C and humidity 65±5 %. The rats were maintained under standard animal house conditions. The study was conducted after obtaining Institutional Animal Ethical Committee clearance.

Rats were anesthetized with phenobarbital, and the peritoneal cavity was opened and a loose ligature was placed around the portal vein, an oblique incision was then made in the mesenteric part of the vein, and the canula was immediately inserted. The perfusion was initiated in situ at flow rate of 122 ml/ min. The liver was cut free, immersed in buffer at 37$^0$C. Liver was perfused for 5 min. with a modified Hanks buffer (pH 7.4) (136mM NaCl, 5mM KCl, 1mM $MgSO_4$, 0.3mM $NaHCO_3$) containing 0.5mM EGTA, 25mM HEPES (free acid), and 2% BSA. Subsequently liver were perfused with a modified Hanks buffer containing 0.12% collagenase, 4mM $CaCl_2$ and 2% BSA for an additional 10 min. At the end of perfusion, the liver which was swollen and pale was detached from canula and cell were dispersed in a Krebs – Henseleit buffer (118.1mM NaCl, 48mM KCl, 1mM $KH_2PO_4$, 2.9 mM $CaCl_2$, 23.8 mM $NaHCO_3$, 25mM HEPES) containing 2 mM glutamine, MEM amino acid and MEM vitamins, 1.7 mM glucose, 10% calf serum and 2% BSA (incubation medium). Cells were then filtered through a nylon mesh (150 mm) and allowed to settle after 5 min the excess medium was aspirated, cells were resuspended in 20 ml of fresh incubation medium and wash twice (200 g centrifugation). The hepatocytes were incubated at a concentration of 1 x $10^6$ cells per ml in Erlenmeyer flask at 37$^0$C and gassed with 95% oxygen and 5 % $CO_2$ ( Moldeus *et al.,* 1978).

**$Ca^{2+}$ ATPase activity :** The $Ca^{2+}$ ATPase was assayed as per method of Desaich *et al.* (1985) in a reaction mixture (1.5 ml) containing 40 mM Tris HCl buffer (pH 7.5), 5 mM $MgCl_2$, 0.05 mM $CaCl_2$, appropriate amount of hepatocyte membranes and 2.5 mM ATP, the magnesium ATPase activity was determined in presence of 1 mM EGTA and this was subtracted from the total calcium – magnesium ATPase activity in order to obtain net calcium ATPase activity. The reaction mixture was incubated at 37$^0$C for 15 min. and afterwards 0.1 ml of 50% w/v TCA was added to stop the reaction. The contents were centrifuged at 3000 g for 10 min. and the phosphorus released was estimated in the supernatant by the method of Fiske and Subbrarow (1925). To 1.6 ml of sample 0.15 ml of 2.5% ammonium molybdate in 3N sulphuric acid and 0.72 ml water were added, the contents were vortexed and 0.05ml of ANSA( 195 ml of 15% sodium bisulfite solution, 0.5 g of ANSA and 5 ml of 20 % sodium sulphite. were added. The sample were incubated for 10 min. at room temperature, the optical density was read at 660 nm. The results were expressed as µmole Pi/min/mg protein. A standard. curve (2-20 µg) for inorganic phosphate was plotted to calculate amount of inorganic phosphate in the sample .

**Calcium accumulation:** The freshly prepared isolated rat hepatocytes suspension was equilibrated at 25$^0$C for 10 min. Reaction was started by the addition of $^{45}Ca^{2+}$ – buffer (136 mM NaCl, 4.9 mM KCl, 0.1 µM $CaCl_2$, 10 mM Glucose in 20 mM Tris, pH 7.4). The sample was incubated for an appropriate amount of time. The incubation was stopped by addition of 3 ml of ice cold lanthanum medium (1.8 mM $LaCl_3$, 1 mM EGTA in 0.154 M of NaCl) and was filtered through Millipore filter under vacuum. Filters were washed three times using lanthanum medium. Filters were dried and the radioactivity was counted using Beckman liquid scintillation counter. (Ginsburg, 1991).

**Statistical analysis** : The data are the mean ± SD of three separate observations employing one way analysis of variance (ANOVA) followed by Newman Keuls Multiple Comparison Test for comparison between groups. Values having $p<0.05$ were considered significant.

## Results and Discussion

Mitochondrial $Ca^{2+}$ accumulation by isolated rat hepatocytes was studied using $^{45}Ca^{2+}$. It was found that in control calcium accumulation increased significantly ($p<0.05$) with time and with the addition of t-BHP, the accumulation also increased with time but decreased in comparison to the control group. This suggests that t-BHP acted by enhancing membrane permeability to $Ca^{2+}$ ions and inducing functional alterations in isolated rat hepatocytes (Singh *et al.,* 2006). t-BHP caused decrease in $Ca^{2+}$ATPase activity in isolated rat hepatocytes which was reversed by naturally occurring antioxidant(GSH). GSH plays an important role in the maintenance of critical cellular and mitochondrial protein and non thiols in the reduced state. Hence, the inhibition of $Ca^{2+}$ATPase activity involves the oxidation of SH groups of the enzyme.

Ruthenium red which is a known calcium cycling inhibitor protected against the t-BHP induced impairment of calcium homeostasis(Kakkar *et al.,* 1996). With the addition of ruthenium red (0.05mM), $Ca^{2+}$ accumulation decreased significantly ($p<0.05$) in contrast to t-BHP alone. This suggests that decreased $Ca^{2+}$ accumulation by mitochondria induced by t-BHP involves inhibition

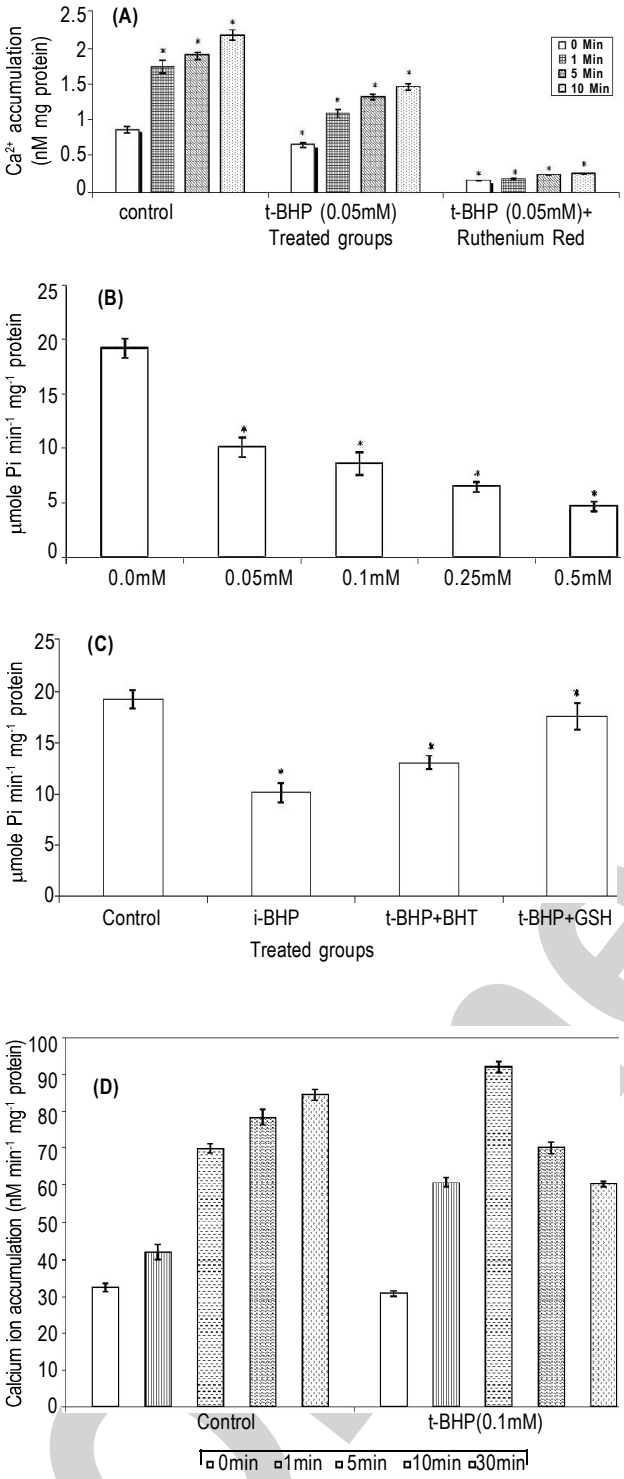


**Fig. 1:** (A) Effect of t-BHP on mitochondrial $Ca^{2+}$ accumulation using $^{45}Ca^{2+}$ treated groups were compared with control group, (B) Effect of varying concentration of t-BHP on $Ca^{2+}$ ATPase activity. All groups were compared with control group (0.0 mM), (C) Effect of GSH and BHT on Ca ATPase activity. t-BHP treated group was compared with control group, t-BHP(0.1mM)+BHT(0.1mM) and t-BHP(0.1mM)+GSH(0.1mM) treated groups were compared with t-BHP treated groups, (D) Effect of t-BHP on $Ca^{2+}$ ion uptake by hepatocytes using $^{45}Ca^{2+}$. Values are mean±SD. *p<0.05

of mitochondrial calcium pump resulting in elevation of intracellular $Ca^{2+}$ (Fig.1a).

This fact is further corroborated by the results obtained on the effect of t-BHP on $Ca^{2+}$ ATPase activity. Increased concentration of t-BHP decreased significantly ($p<0.05$) $Ca^{2+}$ ATPase activity in isolated rat hepatocytes. These results suggest that impaired $Ca^{2+}$ ATPase activity contributes to the decreased ability of hepatocytes to pump out calcium. This may involve oxidation of critical -SH groups present in the enzyme by t-BHP (Fig.1b).

Results obtained with reduced glutathione (-SH agent) and antioxidant (BHT) support this view. The effect of -SH agents and antioxidants on calcium ATPase activity in isolated rat hepatocytes was studied and found that GSH and BHT accorded protection against t-BHP induced cell death. Out of antioxidants and -SH agents studied, GSH was found to be more effective than BHT (Fig.1c). This confirms that inhibition of $Ca^{2+}$ ATPase involves oxidation of -SH groups of enzyme. $Ca^{2+}$ uptake by hepatocytes using $^{45}Ca^{2+}$ was measured. It is clear from the results that $^{45}Ca^{2+}$ uptake increased with time from 0–30 min. At 0 minute t-BHP exposure there was higher $^{45}Ca^{2+}$ uptake as compared to control animals till 5 min. However, longer incubation resulted in less $^{45}Ca^{2+}$ uptake. (Fig.1d).

Previous studies of our group on the toxic effect of t-BHP on isolated rat hepatocytes and isolated liver mitochondria have reported decrease in hepatocytes viability ,increased lipid peroxidation of hepatocytes ,decreased GSH content of hepatocytes ,increased mitochondrial swelling and decreased mitochondrial GSH content in a dose dependant manner . Also protection was accorded by various synthetic and natural antioxidant against the toxic effects of t-BHP(Singh *et al.*, 2006; Mehrotra *et al.,* 2007). In this experimental study toxic effect of t-BHP on $Ca^{2+}$ ATPase activity of hepatocytes have been shown. Our results suggest that t-BHP causes impairment of calcium homeostasis and $Ca^{2+}$ ATPase activity by causing a decrease in calcium accumulation of mitochondria and decrease in $Ca^{2+}$ ATPase activity of hepatocytes. Decrease in $Ca^{2+}$ ATPase activity affected the $Ca^{2+}$ fluxes of hepatocytes. The disturbance in $Ca^{2+}$ ATPase activity causes elevation of intracellular $Ca^{2+}$ concentration. $Ca^{2+}$ ions are biologically very active, and their accumulation in dead or dying cells may actually contribute to morphologic transformations characterizing coagulative necrosis. Thus, the influx and accumulation of calcium ions and the resultant morphologic changes of coagulative necrosis can account for the common morphology of cell death (Buja, 2005; Singh *et al.*, 2010) proposed that a disturbance in intracellular $Ca^{2+}$ homeostasis is an early event in the genesis of lethal injury produced by an acute oxidative stress. This disturbance in $Ca^{2+}$ homeostasis were paralled by a depletion of glutathione resulting from the reduction of either peroxide by glutathione peroxidase and the oxidation of pyridine nucleotide accompanying the reduction of GSSG by glutathione reductase. On the basis of these observations a model of oxidative injury was proposed whereby the ultimate loss of viability of the liver

cell is a consequence of a sequence of events proceeding from GSH depletion to an elevated cytosolic $Ca^{2+}$ concentration and finally to membrane injury (Lash, 2008). It seems that the action of t-BHP on isolated rat liver hepatocytes may involve oxidation of critical -SH groups present in $Ca^{2+}$ ATPase enzyme. Results obtained with various thiol agents and antioxidants support this view.

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## References

Amoroso, S., A. D'Alessio, R. Sirabella, G. Di Renzo and L. Annunziato: $Ca^{2+}$ independent caspase-3 but not $Ca^{2+}$dependent caspase-2 activation induced by oxidative stress leads to SH-SY5Y human neuroblastoma cell apoptosis. *J.Neurosci. Res.,* **68**, 454–462(2002).

Aust, S.D., C.F.Chignell, T.M. Bray, B. Kalyanaraaman and R.P. Mason: Contemporary issues in toxicology: Free radicals in toxicology. *Toxicol. Appl. Pharmacol.*, **120**, 168, (1993).

Boelsterli, U.A., H.K. Ho, S. Zhou and K.Y. Leow: Bioactivation and hepatotoxicity of nitroaromatic drugs. *Curr. Drug Metab*.**, 7**, 715-727(2006).

Buja, L.M.: Myocardial ischemia and reperfusion injury. *Cardiovasc. Pathol*.**, 14**, 170-175 (2005).

Cervinkova, Z., H. Lotkova, P. Krivakova, T. Rousar, O. Kucera, L. Tichy, M. Cervinka and Z. Drahota: Evaluation of mitochondrial function in isolated rat hepatocytes and mitochondria during oxidative stress. *Altern. Lab. Anim.*, **35**, 353-61 (2007).

Cervekova, Z., H. Rauchova and Z. Krivakova: Inhibition of palmityl carnitine oxidation in rat liver mitochondria by tert-butyl hydroperoxide. *Physio*l. Res., **57**, 133-136 ( 2008).

Chen, C.R., Y.W. Liao, L. Wang, Y.H. Kuo, H.J. Liu, W.L. Shih, H.L. Cheng and C.I. Chang: Cucurbitane triterpenoids from *Momordica charantia* and their cytoprotective activity in tert-butyl hydroperoxide-induced hepatotoxicity of HepG2 cells. *Chem. Pharm. Bull.,* **58**, 1639-42 (2010).

Crompton, M.: Mitochondria and aging: A role for the permeability transition? *Aging Cell*, **3**, 3–6 (2004)

Das, D.K., N. Maulik and R.M. Engelman: Redox regulation of angiotensin II signaling in the heart. *J. Cell Mol. Med*., **8**, 144–152(2004).

Desaich, D., C.H. Chetty and K.S. Prasada Rao: Chlordicane inhibition of calmodulin activated calcium ATPase in rat. *J. Toxicol. Env. Health*., **16**, 189-196 (1985).

Dhalla, N.S., R.M. Temsah and T. Netticadan: Role of oxidative stress in cardiovascular diseases. *J. Hypertens*., **18**, 655-664 (2000).

Drahota, Z., P. Krivakov and Cervinkova: Tert-butyl hydroperoxide selectively inhibits mitochondrial enzymes in isolated rat hepatocytes. *Physiol. Res.,* **54**, 67-72 (2005).

Dubuisson, M.L., B. De Wergifosse, A. Trouet, F. Baguet, J. Marchand-Brynaert and J.F. Rees: Antioxidative properties of natural coelenterazine and synthetic methyl coelenterazine in rat hepatocytes subjected to tert-butyl hydroperoxide-induced oxidative stress. *Biochem. Pharmacol*, **60**, 471-478( 2000).

Fiske, C.H. and Y. Subbarow: Colorimetric determination of phosphorus. *J. Biol. Chem.,* **66**, 375-400 (1925).

Ginsburg, H. and R. Kramer: Calcium transport and compartment analysis of free and exchangeable calcium in *Plasmodium falciparum* infected red blood cells. *J. Protozool*., **38**, 594-601 (1991).

Haidara, K., L. Morel, V. Avalea, M. Gascon-Barre and F. Denizeau: Mechanism of tert-butyl hydroperoxide induced apoptosis in rat hepatocytes: Involvement of mitochondria and endoplasmic reticulum. *Biochem. Biophys.* Acta., **15**,173–185(2001).

Halestrap, A.P.: Calcium, mitochondria and reperfusion injury: A pore way to die. *Biochem. Soc*. Trans., **34**, 232–237(2006).

Han, D., M. Shinohara, M.D. Ybanez, B. Saberi and N. Kaplowitz: Signal transduction pathways involved in drug-induced liver injury. In: Adverse Drug Reactions (Ed.: J. Uetrecht). Handbook of Experimental Pharmacology. Springer-Verlag Berlin Heidelberg. p. 267-310 (2010).

Kakkar, P. and B. Singh: Mitochondria: A hub of redox activities and cellular distress control. *Mol. Cell Biochem*.**, 305**, 235-253 (2007).

Kakkar, P., S. Mehrotra and P.N. Vishwanathan: t-BHP induced *in-vitro* swelling of rat liver mitochondria. *Mol. Cellular Biochem*., **154**, 39-45 (1996).

Kaasik, A., D. Safiulina, A. Zharkovsky and V. Veksler: Regulation of mitochondrial matrix volume. *Amer. J. Cell Physiol*., **292**, 157-163 (2007).

Kanupriya, A., D. Prasad, M. Sai Ram, R.C. Sawhney, G. Ilavazhagan and P.K. Banerjee: Mechanism of tert-butylhydroperoxide induced cytotoxicity in U-937 macrophages by alteration of mitochondrial function and generation of *ROS. Toxicol. In-Vitro*, **21**, 846–854 (2007).

Lash, L.H.: Mitochondrial GSH transport and intestinal cell injury: A commentary on "contribution of mitochondrial GSH transport to matrix GSH status and colonic epithelial cell apoptosis. *Free Radi. Biol. Med*.**, 44**, 765-767(2008).

Mauro, S., V. Battaglia, A.M. Brunati, N.L. Rocca, E. Tibaldi, P. Pietrangeli, L. Marcocci, B. Mondovi, C.A. Rossi and A. Toninello: Catalase takes part in rat liver mitochondria oxidative stress defense. *J. Biol. Chem.,* **282**, 24407–24415 ( 2007).

Mayevsky, A. and E. Barbiro-Michaely: Use of NADH fluorescence to determine mitochondrial function *in-vivo*. Int. *J. Biochem. Cell Biol.*, **41**, 1977–1988 (2009).

Mehrotra, S., S. Singh, A. Gupta and R. Sandhir: Tert-butyl hydroperoxide induced structural and functional alterations in rat hepatocytes and rat liver mitochondria and protection by antioxidants. *Toxicol. Int.,* **14**, 25-31(2007).

Moldeus, P., J. Hogberg and S. Orrenius: Isolation and use of liver cells. In Methods in Enzymology, Academic Press, New York 60-71 (1978).

Singh, S., S. Mehrotra and A. Gupta: Mechanistic aspects of hepatotoxicity of tert-butyl hydroperoxide (t-BHP) and protection by antioxidants. *Environ. Degrad. Manag*., **2**, 39-48 (2006).

Shimizu, S., Y. Eguchi, W. Kamiike, Y. Funahashi, A. Mignon, V. Lacronique, H. Matsuda and Y: Tsujimoto: Bcl-2 prevents apoptotic mitochondrial dysfunction by regulating proton flux. *Proc. Natl. Acad. Sci*., **95**, 1455–1459 (1998).

Singh, B.K., M. Tripathi, P.K. Pandey and P. Kakkar: Nimesulide aggravates redox imbalance and calcium dependant mitochondrial permeability transition leading to disfunction. *In-vitro*. Toxicol., **10**, 1016 (2010).

Tripathi, M., B.K. Singh and P. Kakkar: Glycyrrhizic acid modulates t-BHP induced apoptosis in primary rat hepatocytes. *Food and Chemical Toxicol.*, **47**, 339–347(2009).