Journal of Biochemistry and Molecular Biology, Vol. 39, No. 2, March 2006, pp. 145-149

JOURNAL OF Biochemistry and Molecular Biology

Maltol Inhibits Apoptosis of Human Neuroblastoma Cells Induced by Hydrogen Peroxide

Yang Yang[1,#], Jian Wang[1,#], Caimin Xu[1,*], Huazhen Pan[1] and Zinan Zhang[2]

[1]National Laboratory of Medical Molecular Biology, Institute of Basic Medicine, Peking Union Medical College and Chinese Academy of Medical Sciences, Dong Dan San Tiao 5, Beijing, 100005

[2]Peking Union Medical College Hospital, Beijing, 100730, People's republic of China

Received 31 August 2005, Accepted 1 December 2005

To analyze the effect of Maltol on the apoptosis of Human Neuroblastoma Cells (SH-SY5Y) treated by free radical which was generated from Hydrogen Peroxide (H_2O_2), flow cytometry analysis on Phosphatidylserine (PS) inverting percentage was applied to determine the apoptosis. MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay was employed to analyze the cell viability. DNA electrophoresis was used to detect DNA fragmentation. Moreover intracellular calcium of concentration ($[Ca^{2+}]_i$) was measured by fluorescence emission. Flow cytometry analysis on the function of mitochondria and Western blto analysis of NF-κB. The results showed that the pretreatment with maltol for 2 hours could prevent the H_2O_2-induced apoptosis. Maltol could reduce the inverting percentage of PS, DNA fragmentation and $[Ca^{2+}]_i$, and enhance the cellular function of mitochondria. NF-κB activated by H_2O_2 is reduced. The experiments suggest that maltol could effectively inhibit the apoptosis induced by H_2O_2. As a novel anti-oxidant, maltol is a new promising drug in protecting the neurological cells from the damage by free radical.

Keywords: Apoptosis, Hydrogen peroxide, Maltol, Oxidative stress, SH-SY5Y

Introduction

The chemical name of Maltol is 3-hydroxy-2-methyl-4-pyrone with a molecular formula $C_6H_6O_3$ (molecular weight 126.11D). Maltol is always used as food additive in food products factory. In recent years, it was found that maltol has an excellent anti-oxidative activity (Hong *et al*., 1992). In this study, it was the first time to find that maltol is able to inhibit the apoptosis of human neuroblastoma cells, SH-SY5Y induced by oxidative damage generated by H_2O_2.

Oxidative stress and the damage have long been implicated in many age-associated diseases and neurodegenerative diseases, such as Alzheimer's Disease (AD) (Tan *et al*., 1998; Draczynska-Lusiak *et al*., 1998; Miranda *et al*., 2000). Many researchers have found that free radicals could induce DNA and protein damages (Smith *et al*., 1991; Mecocci *et al*., 1993). Recently many studies suggest that cumulative oxidative stress contributes to the pathogenesis and/or progression of AD and some other age-dependent diseases (Coyle and Puttfarcken, 1993; Markesbery, 1997; Halliwell, 2001). Therefore some anti-oxidants, such as Vitamin E and melatonin have been applied to prevent these diseases (Yatin *et al*., 2000; Pappolla *et al*., 2000). The results showed that maltol does have anti-oxidative activity and may have a promising application in the treatment of patients with degenerative or senile diseases inducing some types of dementias related to free radical damages.

Materials and Methods

Cells culture. SH-SY5Y were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 U/ml antibiotics. In the study, after treatment with 2 mmol/L maltol for 2 h, 100, 200, 300 μmol/L H_2O_2 were used to induce oxidative stress, respectively,. After 10 hours, we detected the effects of maltol.

Annexin V assay. Treated cells were collected and washed twice with phosphate-buffered saline solution (PBS). Cells were re-suspended in reaction buffer (96 μL Hepes buffer, 2 μL Annexin-V-FITC, 2 μL 50 μg/mL Propidium iodide (PI) and then incubated in dark for 15 min at ambient temperature. Cells were centrifuged at

[#]The first two authors have contributed equally to this work

*To whom correspondence should be addressed.
Tel/Fax: 86-10-65296445
E-mail: caiminxu@yahoo.com.cn

1500 × g for 5 min and the pellet was re-suspended with 0.5 ml Hepes buffer (10 mmol/L Hepes/NaOH, 140 mmol/L NaCl, 5 mmol/$CaCl_2$, pH = 7.4). Percentage of cell apoptosis was analyzed with Becton Dickinson Flow cytometer. A minimum of 10^4 cells per sample was analyzed.

Analysis of cell viability. Cell viability was detected by MTT assay. At the end of treatment, cells were incubated with 50 μg/ml MTT for 1 h. Absorption at 570 nm was measured after solubilization of the formazan crystals with 0.04 mol/L HCl in 2-propanol. Cell viability was also analyzed by trypan blue exclusion method.

DNA electrophoresis. After treatment, cells were harvested with a cell scraper on ice. Following centrifugation at 4°C at 1500 × g for 5 min, cell pellets were incubated at 37°C for 2 h in a lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 20 mmol/L EDTA, 2% SDS, 20 μg/ml DNase-free RNase). Then, 200 μg/ml of proteinase K was added and the sample was further incubated overnight at 37°C. After 0.4 ml saturated NaCl was added to the sample prior to centrifugation at 12,000 × g for 40 min at 4°C. The supernatant was extracted with equal volumes of phenol, phenol: chloroform: isoamyl alcohol (25 : 24 : 1) and chloroform, respectively. The DNA solution was precipitated in equal volume of 100% ethanol overnight at −20°C and then centrifuged at 12,000 × g at 4°C for 10 min. The pellet was washed with 70% ethanol, air dried and then re-suspended in TE buffer containing 1 mmol/L EDTA and 10 mmol/L Tris-HCl, pH 7.4. The DNA concentration was determined from the absorbance at 260 nm. Ten micrograms each of DNA samples was subjected to electrophoresis on 2% agarose gel containing 0.1-0.5 μg/ml ethidium bromide(EB) and visualized under UV light. Electrophoresis was carried out in TE buffer for 1 h at 100V.

Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ was measured with the fluorescent indicator Fura-2/AM. Cells were collected washed with PBS twice and re-suspended in HBSS buffer (8 g/L NaCl, 0.4 g/L KCl, 1 g/L glucose, 0.06 g/L KH_2PO_4, 0.48 mg/L NaH_2PO_4, 0.19 g/L $CaCl_2$). Fura-2/AM was added to samples at 1mmol/L and cells were incubated at 37°C for 45 min. Then cells was washed with PBS and re-suspended in HBSS buffer. The suspension was maintained at 37°C during each experiment. At first 4-5 cells were selected randomly. The fluorescence emissions of these cells were measured with AUQA COSMOS image collection and analysis system, HAMAM ATSU Co. of Japan under 340 nm and 380 nm at 37°C. The ratio of fluorescences at 340 nm to 380 nm reflects the changes of $[Ca^{2+}]_i$. When the ratio didn't change, the $[Ca^{2+}]_i$ was stable. This was called baseline. Then H_2O_2 was added into samples and the curve was drawn with more than 300 data of ratios of fluorescences at 340 nm to 380 nm collected per 10s with 222mS exposure time.

The function of mitochondria assay. Treated cells were collected and washed twice with phosphate-buffered saline solution (PBS). Cells were re-suspended in 1 mL 10 μg/ml Rhodamine 123, and incubated in dark for 1 h at 37°C. Then, Cells were centrifuged at 1500 × g for 5 min and washed once with PBS. The pellet was re-suspended with 0.5 ml PBS, 2.5 μL 1 mg/mL PI, 12.5 μL 10 mg/mL RNAase. And then incubated in dark for 30 min at room temperature. Percentage of Rh123 positive was analyzed with Becton Dickinson Flow cytometer. A minimum of 10^4 cells per sample was analyzed.

Western blot analysis of NF-κB. Cells were harvested and washed twice in PBS, and lysed in the buffer A (10 mmol/L HEPES, PH7.9, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 10 mmol/L KCL, 1.5 mmol/L $MgCL_2$, 1 mmol/L DTT, 1 mmol/L PMSF, 1 μg/mL Leupeptin and Aproptinin). Then Nonidet P-40 (0.625%) was added, incubated on ice for 10 min with intermittent vortexing, and the tubes were centrifuged (2,000 g, for 5 min).Cell pellet was resuspended with buffer C (20 mmol/L HEPES, pH 7.9, 0.4 mol/L NaCL, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L PMSF). After incubation on ice for 15 min with intermittent vortexing, nclear proteins were collected by centrifugation (12,000 g for 15 min). 50-100 μg of nuclear proteins were separated by 15% SDS-PAGE, transferred to nitrocellulose membranes, and blocked for 3h at room temperature in TBST (0.1% Tween-20, 5% nonfat dry milk). The membrane was incubated with a rabbit anti human NF-κB, followed by incubation with a 1 : 5000 dilution of horseradish peroxidase conjugated goat anti rabbit secondary antibody. The immunoblot signal was visualized using enhanced chemiluminescence (ECL).

Results

PS inverting analysis. Usually PS was one of the indicators of early phase of apoptosis. We carried out Annexin V experiment, so cells were stained with PI and Annexin V to detect PS on the cell surface. The flow cytometric results indicated that when cells were treated with H_2O_2 the apoptosis occurred in a dose-dependent manner. After maltol protection, the percentage of apoptosis was decreased remarkably. (shown in Fig. 1.)

Cell viability. The cell viability was assessed by MTT assay (shown in Fig. 2.). When cells were treated with 200 μmol/L H_2O_2 the viability decreased to 60.5% of the control; when cells were treated with 500 μmol/L H_2O_2 the viability decreased to 40% of the control. The treatment with 2 mmol/L maltol for 2 h could protect cells from death and the cell viability was increased as shown in Fig. 2.

DNA fragmentation. DNA fragmentation became more apparent along with the increase of H_2O_2 concentrations. This again confirmed that apoptosis induced by H_2O_2 was dose-dependent. After protection by maltol, the DNA fragmentation became decreased as compared to cells without protection. (shown in Fig. 3.)

Measurement of intracellular $[Ca^{2+}]i$. We measured H_2O_2-induced intracellular Ca^{2+} mobilization (shown in Fig. 4.). H_2O_2 induced a rapid increase in intracellular Ca^{2+} concentration in SH-SY5Y cells. When the baseline is stable, H_2O_2 was added and it's found that $[Ca^{2+}]_i$ was increased immediately and the increases were H_2O_2-dose dependent. After pre-treatment with 2 mmol/L maltol for 2 h, the increases of $[Ca^{2+}]_i$ induced by H_2O_2 were reduced greatly.

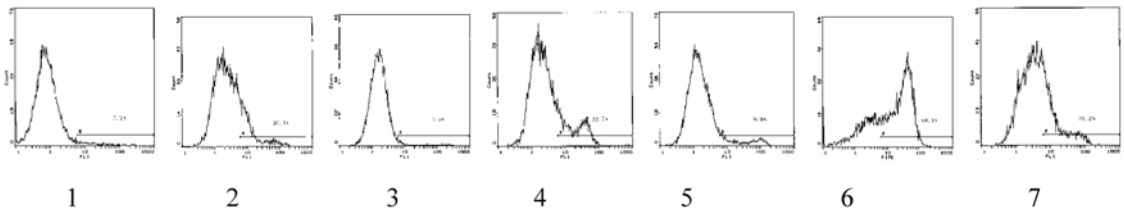

Fig. 1. Percentage of apoptosis when treated with H_2O_2 with and without protection of maltol. 1: control (3.2%); 2: 100 μmol/L H_2O_2 (20.4%); 3: 100 μmol/L H_2O_2 + 2 mmol/L maltol (3.9%); 4: 200 μmol/L H_2O_2 (22.7%); 5: 200 μmol/L H_2O_2 + 2 mmol/L maltol (9.8%); 6: 300 μmol/L H_2O_2 (68.1%); 7: 300 μmol/L H_2O_2 + 2 mmol/L maltol (31.2%). The values in the brackets represent the percentage of apoptosis.

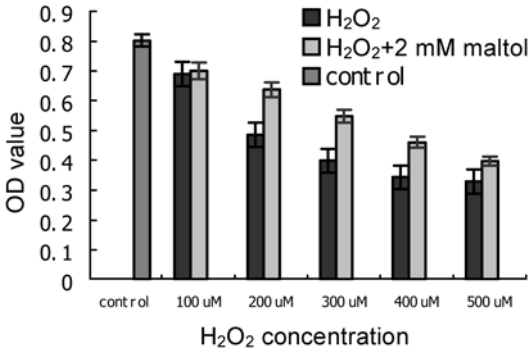

Fig. 2. cell viability with and without treatment of maltol when induced by H_2O_2 ($\bar{x} \pm s$, $n = 3$), $p < 0.05$.

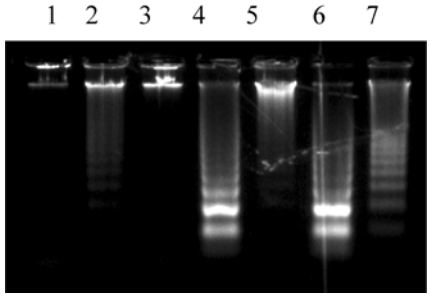

Fig. 3. The protection of maltol on SH-SY5Y cell DNA. DNA fragmentation identified as typical ladder pattern of apoptosis on 1.2% agarose gel. 1: control; 2: 100 μmol/L H_2O_2; 3: 100 μmol/L H_2O_2 + 2 mmol/L maltol; 4: 200 μmol/L H_2O_2; 5: 200 μmol/L H_2O_2 + 2 mmol/L maltol; 6: 300 μmol/L H_2O_2; 7: 300 μmol/L H_2O_2 + 2 mmol/L maltol. The gel was stained with ethidium bromide and examined under UV illumination.

The function of mitochondria analysis. Cells were stained with PI and Rh123 to detect green fluorescence on the cell. The flow cytometric results indicated that when cells were treated with H_2O_2 the the function of mitochondria decreased in a dose-dependent manner. After maltol protection, the percentage of apoptosis was decreased remarkably. (show in Fig. 5.) The decrease of the mitochondrial membrane potential in cells pretreated by maltol is lee than that in control cells, indicating maltol is protective on cells from the damage of H_2O_2.

Western blot analysis of NF-κB. NF-κB is a homo- or heterodimer. At inactive state, NF-κB binds to a inbitory molecule, I κB, which retains NF-κB in plasma. Once activated, I κB is degraded and NF-κB enters into muclei to regulate the expression of genes. In the present experiment, the subunit P65 extracted from the nuclei is used to indicate the extend of the activation of NF-κB. SH-SY5Y cells are pretreated with 2 mmol/L maltol for 2 h, followed by treatment of 200 μmol/L H_2O_2 for 10 h, and the nuclei protein is extracted for the Western Blotting detection and the result is shown in Fig. 6. After the cells are pretreated with maltol, the NF-κB activated by H_2O_2 is reduced. (show in Fig. 6.)

Discussion

Recently, many reports suggest that oxidative stress might play an important role in the pathogenesis of some degenerative neurologic disorders. There is much evidence suggesting that free radicals might be the key factor causing cerebral ischemia-

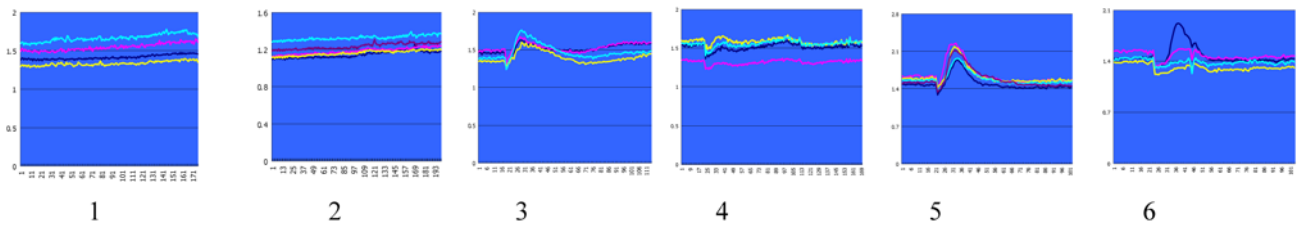

Fig. 4. Effect of maltol on intracellular Ca^{2+} concentration induced by H_2O_2. 1: 100 μmol/L H_2O_2; 2: 100 μmol/L H_2O_2 + 2 mmol/L maltol; 3: 200 μmol/L H_2O_2; 4: 200 μmol/L H_2O_2 + 2 mmol/L maltol; 5: 300 μmol/L H_2O_2; 6: 300 μmol/L H_2O_2 + 2 mmol/L maltol.

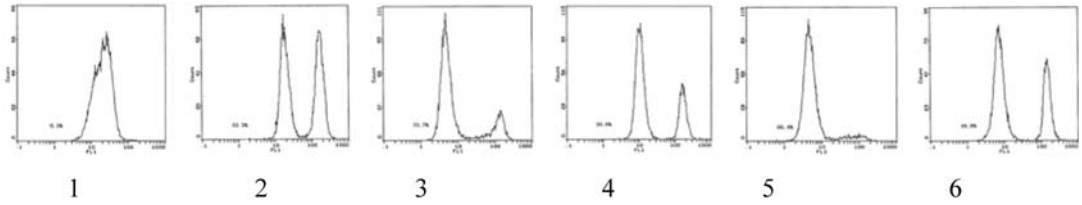

Fig. 5. The function of mitochondria assay. 1: control (0.3%); 2: 100 μmol/L H_2O_2 (52.3%); 3: 100 μmol/L H_2O_2 + 2 mmol/L maltol (20.3%); 4: 200 μmol/L H_2O_2 (73.7%); 5: 200 μmol/L H_2O_2 + 2mmol/L maltol (36.6%); 6: 300 μmol/L H_2O_2 (86.4%); 7: 300 μmol/L H_2O_2 + 2 mmol/L maltol (46.8%).

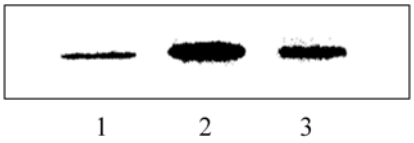

Fig. 6. Western blot analysis of NF-κB. 1: control; 2: 200 μmol/L H_2O_2; 3: 200 μmol/L H_2O_2 + 2 mmol/L maltol.

reperfusion syndrome, neurological damage after head injury, Parkinson's diseas and Alzheimer's disease. As a result, the trial of vitamin E(8) in Alzneimer's Diseases was launched in the US.

In the present study, H_2O_2 was used to generate free radicals in order to damage human neuroblastoma cell line-SH-SY5Y. The apparent characters of apoptosis were detected. After treatment with 2 mmol/L maltol for 2 h, cell apoptosis was inhibited. It indicated that maltol had anti-oxidatve effects. DNA fragmentation, a characteristic biochemical feature of cell apoptosis, was evaluated by DNA electrophoresis. The DNA degradation occurring during apoptosis, resulting in accumulation of typical mono- and oligonucleosomal-sized fragments forming a "ladder" during electrophoresis, was observed in our study.

We furthermore investigated the possible mechanism of maltol inhibiting cell apoptosis induced by oxidative damage. Mitochondria were important during cell's apoptosis and the change of its function relates to apoptosis, such as releasing apoptosis inducing factors, the excessive production of reactive oxygen species, the defect of the energy produciton, and the imbalance of intracellular calcium. The apoptosis inducing factor (AIF) results in many alteration of cells, for example, the change of cell morphology, the fragmentation of DNA, the decrease of mitochondrial transmembrane potential ($\Delta\psi_m$), the inversion of phosphotidylserine (PS). The decease or elimination of $\Delta\psi_m$ is closely correlated with the permeability of permeability transition pore (PTP). PTP substantially consists of voltage-dependent anion channel (VDAC), adenylate transferase (ANT), hexokinase (HK), wherein ANT locates in intra-membrane and is sensitive to calcium. ANT thiol group is readily oxidized by reactive oxygen species (ROS) to facilitate the apoptosis. Through analyzing the function of mitochondria, maltol was found to be able to sustain its function and restrain the apoptosis.

Calcium is an important element in human bodies, which presents in cells and body fluids. Under normal conditions, the concentration of intracellular calcium is much less than that of extracellular calcium. The free intracellular calcium is a member involving in the signaling pathway and correlates with the early signals in the apoptosis. Calcium is an important message molecule in apoptosis (Ermak and Davies, 2002; Kanno *et al*., 2004; Barlow *et al*., 2005). We measured intracellular Ca^{2+} concentration when cells were treated with H_2O_2 with or without pretreatment with maltol. The results showed that matol could effectively inhibit the increase of $[Ca^{2+}]_i$ induced by H_2O_2. It indicated that maltol may affect $[Ca^{2+}]_i$ so as to inhibit apoptosis. After protected by maltol, the results are shown: the fact that after the cells are stimulated by ROS the magnitude of the concentration of the intracellular calcium is reduced indicates that maltol inhibits the increase of the concentration of the intracellular calcium potentially, reducing the physiological change of cells induced by calcium; the analysis of membrane potential shows that maltol also inhibits the opening of PTP, resulting in the alleviation of the damage of the mitochondrial functions thereby inhibiting the apoptosis induced by the mitochondrial pathway.

The increase of ROS is accompanies by the increase of the concentration of the calcium in the plasma and activate the nuclear factor NF-κB, which enters into the nuclei to up-regulate certain genes (15,16). The increase of the concentration of calcium causes the alteration of mitochondrial functions, the collapse of the mitochondrial potential, and the releasing of AIP to induce apoptosis.

Some anti-oxidative drugs had been used to treat neurodegenerative diseases, such as AD, clinically. Now there do not exist very good drugs for AD. As a novel anti-oxidant, maltol is possibly a new drug in preventing human from AD clinically. This result isn't disclosed before .Of course more researches needed to discover the precise mechanism of inhibiting apoptosis featured by maltol and the possibility of maltol as an agent to prevent or slow down the progress of AD. Further studies are also required to explain the protection of normal neurons from oxidative damage by maltol.

Acknowledgments This research is supported by grant from National Major Basic Research and Development Program of China (973 Program) (No. 2005CB522507), the Major State Basic Research Program of China (No. 2003DIBIJ077) and

the National Natural Science Foundation of China (No. 30430200).

References

Barlow, C. A., Shukla, A., Mossman, B. T. and Lounsbury, K. M. (2005) Oxidant-mediated CREB activation: Calcium regulation and role in apoptosis of lung epithelial cells. *Am. J. Respir. Cell Mol. Biol.* **34**, 7-14.

Coyle, J. T. and Puttfarcken, P. (1993) Oxidative stress, glutamate and neurodegenerative disorders. *Science* **262**, 689-695.

Draczynska-Lusiak, B., Dough, A. and Sun, A. Y. (1998) Oxidized lipoproteins may play a role in neuronal cell death in Alzheimer disease. *Mol. Chem. Neuropathol.* **33**, 139-148.

Egan, L. J., Eckmann, L., Greten, F. R., Chae, S., Li, Z. W., Myhre, G. M., Robine, S., Karin, M. and Kagnoff, M. F. (2004) IkappaB-kinasebeta-dependent NF-kappaB activation provides radioprotection to the intestinal epithelium. *Proc. Natl. Acad. Sci. USA* **101**, 2452-2457.

Ermak, G. and Davies, K. J. (2002) Calcium and oxidative stress: from cell signaling to cell death. *Mol. Immunol.* **38**, 713-721.

Greten, F. R., Eckmann, L., Greten, T. F., Park, J. M., Li, Z. W., Egan, L. J., Kagnoff, M. F. and Karin, M. (2004) IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell* **118**, 285-296.

Halliwell, B. (2001) Role of free radicals in the neurodegenerative diseases. *Drugs Aging* **18**, 685-716.

Hong, Y. L., Pan, H. Z., Scott, M. D. and Meshnick, S. R. (1992) Activated oxygen generation by a primaquine metabolite: inhibition by antioxidants derived from Chinese Herbal remedies. *Free Rad. Bio. Med.* **12**, 213-218.

Kanno, T., Sato, E. E., Muranaka, S., Fujita, H., Fujiwara, T., Utsumi, T., Inoue, M. and Utsumi, K. (2004) Oxidative stress underlies the mechanism for Ca(2+)-induced permeability transition of mitochondria. *Free Radic Res.* **38**, 27-35.

Markesbery, W. R. (1997) Oxidative stress hypothesis in Alzheimer's disease. *Free Rad. Bio. Med.* **23**, 134-147.

Mecocci, P., MacGarvey, U., Kaufman A. E., Koontz, D., Shoffner, J. M., Wallace, D. C. and Beal, M. F. (1993) Oxidative damage to mitochondrial DNA shows marked age-dependent increases in human brain. *Ann. Neurol.* **34**, 609-616.

Miranda, S., Opazo, C., Larrondo, L. F., Muñoz, F. J., Ruiz, F., Leighton, F. and Inestrosa, N. C. (2000) Inestrosa NC. The role of oxidative stress in the toxicity induced by amyloid beta-peptide in Alzheimer's disease. *Prog. Neurobiol.* **62**, 633-648.

Pappolla, M. A., Chyan, Y. J., Poeggeler, B., Frangione, B., Wilson, G., Ghiso, J. and Reiter, R. J. (2000) An assessment of the antioxidant and the antiamyloidogenic properties of melatonin: implications for Alzheimer's disease. *J. Neural. Transm.* **107**, 203-231.

Smith, C. D., Carney, J. M., Starke-Reed, P. E., Oliver, C. N., Stadtman, E. R., Floyd, R. A. and Markesbery, W. R. (1991) Excess brain protein oxidation and enzyme dysfunction in normal aging and Alzheimer disease. *Proc. Natl. Acad. Sci. USA* **88**, 10540-10543.

Tan, S., Wood, M. and Maher, P. (1998) Oxidative stress induces a form of programmed cell death with characteristic of both apoptosis and necrosis in neuronal cells. *J. Neurochem.* **71**, 95-105.

Yatin, S. M., Varadarajan, S. and Butterfield, D. A. (2000) Vitamin E prevents alzheimer's amyloid β-peptide (1-42)-induced neuronal protein oxidation and reactive oxygen species production. *J. Alzheimer's Disease* **2**, 123-131.