



TERTIARY-BUTYL HYDROPEROXIDE (TBH), MEDIATED OXIDATIVE STRESS MAY LEAD TO ALTERATION IN NECROTIC AND APOPTOTIC GENE EXPRESSION IN N2A CELL LINES.

GOWTHAM PADMANABAN* AND DRAVIDA MANI ELUMALAI

*Department of Anatomy, Dr. ALM.PG. Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai, Tamilnadu, India.
Dr. ALM.PG. Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai -600 113, Tamilnadu, India.

ABSTRACT

Tertiary-butyl hydroperoxide (TBH) is a chemical that can cause oxidative stress in vitro through catalysis by glutathione peroxidase to form GSSG and tertiary butyl alcohol. N2a cell lines are a fast-growing mouse neuroblastomacell line and have been commonly used to study neurotoxicity. We aimed for present study to clarify whether; Tertiary-butyl hydroperoxide (TBH), mediated oxidative stress may lead to alteration in necrotic and apoptotic gene expression in N2a cell lines. The study involve following two groups: Group- 1: N2a without TBH exposure; Group -2: N2a with 45 min 62.5 micro molar concentration TBH exposure and reperfusion for 48 hrs. RT PCR analysis was done for the following gene; Degree of oxidative stress (TRX1&2, PRX); Type of cell death (PARP 1; Caspase 3), Cell defense (HSP 70, HO 1); Housekeeping (18 S RNA). We observed, serum deprivation for 45 min affects N2A cells to some extent as evident from "rounding-off" seen in them, while phase image did not show much difference between these and TBH treated cell. RT-PCR analysis showed increased in PARP-1, and decrease in HSP-70, Trx-2, and Prxgene expression which signifies cell death through necrosis. After 3 days of re-perfusion with normal growth medium (with serum), cells treated with TBH showed extensive loss. This could be due to the washing off of dead cells during medium changes (Cell death might be due to initial necrosis and also due to delayed apoptosis). Delayed apoptosis was evident from increased Caspase 3 expression from RT-PCR. We concluded that there was a gene expression change during the acute and sub-acute phase of the injury which mimics the in-vivo scenario of reperfusion after tissue insult and during the acute phase of stress the cell death occurs through necrosis and then gets shifted to apoptosis during sub-acute phase.

KEYWORDS: Apoptosis; Necrosis; N2a cell; Oxidative stress, TBH.



GOWTHAM PADMANABAN

**Dr. ALM.PG. Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai -600 113, Tamilnadu, India.*

INTRODUCTION

Oxidative stress represents an imbalance between the production and manifestation of reactive oxygen species¹. An excessive amount of reactive oxygen/nitrogen species (ROS/RNS) leading to an imbalance between antioxidants and oxidants can cause oxidative damage in vulnerable targets such as unsaturated fatty acyl chains in membranes, thiol groups in proteins, and nucleic acid bases in DNA². Aerobic metabolism induces the production of reactive oxygen species (ROS), which are able to induce oxidative stress that promotes cellular apoptosis³, characterized by an initial shrinkage of the cells and the ensuing break of the cell-to-cell contacts and in necrosis⁴, the degradation of the tissue starts with swelling within the cell and ultimately lead to an interruption of the membranes of the cell. Tertiary-butyl hydroperoxide (tBH), is a chemical that can cause oxidative stress in vitro through catalysis by glutathione peroxidase to form GSSG and tertiary butyl alcohol⁵. The heat shock proteins (Hsp70) are an important part of the cell's machinery for protein folding, and help to protect cells from oxidative stress⁶. Hemeoxygenase (HO-1) is an enzyme that catalyzes the degradation of heme and produces biliverdin, iron, and carbon monoxide⁷. The caspase-3 is crucial mediators of programmed cell death (apoptosis)⁸. Thioredoxin-2 (Trx-2) is a mitochondria-specific and has a crucial role in the signal transduction for apoptosis⁹. PARP is found in the cell's nucleus, the main role is to detect signal single strand DNA breaks¹⁰. N2a cell lines are a fast-growing mouse neuroblastoma cell line¹¹. It is possible to differentiate N2a cells into cells that have many properties of neurons, including neurofilaments. N2a cells have been used to study neurite outgrowth¹¹ and neurotoxicity¹². There is lacking of substantial data regarding growth factors to effect necrosis and apoptosis in n2a cell lines¹³⁻¹⁶. Hence we aimed for present study to clarify whether; Tertiary-butyl hydroperoxide (TBH), mediated oxidative stress may lead to alteration in necrotic and apoptotic gene expression in N2a cell lines.

MATERIALS AND METHODS

N2A cell line was obtained from National Center for Cell Lines, Pune, India.

Plating Neuro2A cell line

The cryopreserved cell line was thawed until it reached temperature of 37 degree Celsius. The cell line was then centrifuged at 1000 rpm for 5 minutes and supernatant discarded to remove DMSO. The pellet collected was then washed twice in HBSS. The final pellet was then triturated in medium well and homogeneous suspension

was obtained. 400 micro liters from the mixture was plated in each well of a 24 well plate.

TBH – Oxidative stress

The lethal dose 50 (LD₅₀) was obtained by MTT assay: Keeping the exposure time constant as 45-minutes varying concentration of TBH (500 micro molar – 31.25 micromolar) was added. After 45-minutes of TBH exposure, The TBH was removed and 0.5 ml of medium was added and incubated at 37 degree Celsius for 2 days. The medium was removed and 0.3 ml of working solution was added to each well and incubated for 3 hours at 37 degree Celsius. Isopropyl alcohol was added ten minutes prior to taking OD readings (520 and 540 nm). The OD was measured using ELISA plate reader.

The study involve following two groups

Group- 1: N2a without TBH exposure

Group-2: N2a with 45 min 62.5 micro molar concentration TBH exposure and reperfusion for 48 hrs.

RNA isolation

Trizol were used 1 ml for 100 mm petriplates (0.5 ml for 60 mm and 40 mm petriplates). The petriplates were rocked to detach cells. The cell lysate was taken in a 2ml eppendorf tube and then 200 microliter of chloroform was added to the cell lysate and vortexed. The mixture was spinned at 12000 rpm for 15 min at 4 degree C. The aqueous part was taken in a fresh 2 ml eppendorf tube. Equal volume of isopropyl alcohol was added. The tube was incubated for 30 minutes at 4 degree Celsius. The supernatant was discarded. 1ml of 70% ethanol was added and spinned at 7500 rpm for 10 min at 4 degree Celsius. The supernatant was discarded and the pellet was dried. 50 microliter of DEPC treated water was added. 10 microliter of the above mixture was taken and 990 microliter of distilled water was added to it. The content was then read at 260 and 280 nm in a spectrometer to quantify the amount of RNA present.

List of analyzed genes

Degree of oxidative stress (TRX1&2, PRX); Type of cell death (PARP 1; Caspase 3), Cell defense (HSP 70, HO 1); Housekeeping (18 S RNA).

Reverse Transcriptase—Polymerase Chain Reaction (RT PCR analysis)

Reverse transcription polymerase chain reaction (RT-PCR) is a variant of polymerase chain reaction (PCR). In RT-PCR, an RNA strand is first reverse transcribed into its DNA complement (complementary DNA, or cDNA) using the enzyme reverse transcriptase, and the resulting cDNA is amplified using traditional PCR. Amplification by PCR requires primer for the initiation. Forward and reverse primers were used.

Gene	Species	Forward	Reverse
HO1	Mouse	CACGCATATACCCGCTACCT	CCAGAGTGTTTCATTTCGAGCA
HSP-70	Mouse	GACAAGTGCCAGGAGGTCAT	CCCACGTGCAATACACAAAG
Casp 3	Mouse	ACGGAGCTGGACTGTGGCAT	TTGTGCGCGTACAGCTTCAG
PARP-1	Mouse	CGACATGGTGTCAAAAGTG	TGTCTATGGAGCTGTGGCTG
Trx-1	Mouse	TGGATCCATTTCCATCTGGT	CCACACCACGTAGCAGAGAA
Trx-2	Mouse	CATTTCTCTCCTGCCTCTG	TGTTACAGCTCCTCCTCCT
Prx	Mouse	GTGTACAGGTCACAGAGGT	CTGCAGACAGGGAAGTGCA

Gel Electrophoresis

The amplified product was run on agarose gel for visualization and the intensity of the band was measured using a gel documentation system.

RESULTS

MTT assay

The result is summarized in (Figure 1): The MTT assay showed TBH concentration for lethal dose 50 was found to be 62.5 micromolars.

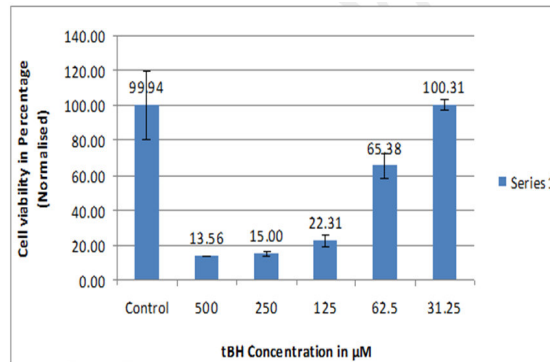


Figure 1
MTT assay for TBH concentration

Effect of TBH induced oxidative stress in neuro 2a cell after 45 minutes reperfusion

The result are précised in (Figure 2; 3a-b): Serum deprivation for 45 min affects N2A cells to some extend as evident from “rounding-off” (withdrawal of processes)

seen in them, While phase image did not show much difference between these and TBH treated cells. RT-PCR analysis showed increased in PARP-1, and decrease in HSP-70, Trx-2, and Prxgene expression which signifies cell death through necrosis.

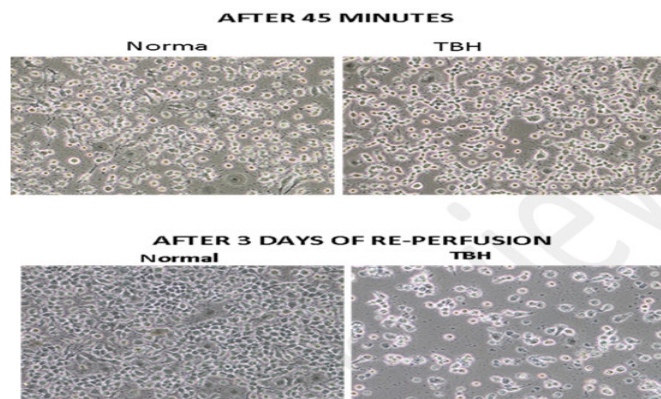


Figure 2
Phase image of effect of TBH in N2a cell after 45-minutes and 3-days reperfusion

Figure 3a

Figure 3b

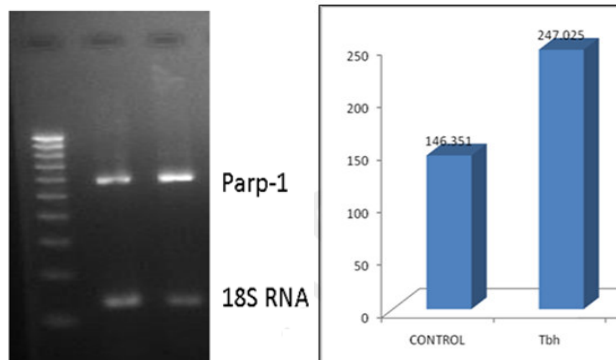


Figure 3a-b
RT PCR analysis of PARP 1; after 45-minutes reperfusion

Effect of TBH induced oxidative stress in neuro 2a cell after 3-days reperfusion

The result are précised in (Figure 2; 4a-b; 5a-b): After 3 days of re-perfusion with normal growth medium (with serum), cells treated with TBH showed extensive loss.

This could be due to the washing off of dead cells during medium changes (Cell death might be due to initial necrosis and also due to delayed apoptosis). Delayed apoptosis was evident from increased Caspase 3 expression from RT-PCR.

Figure4a

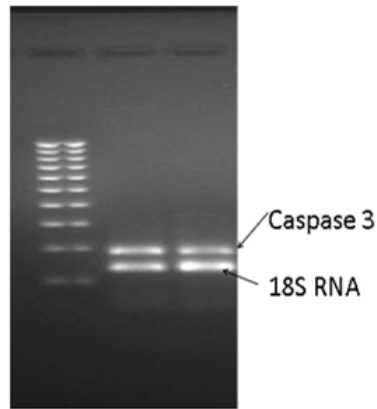


Figure 4b

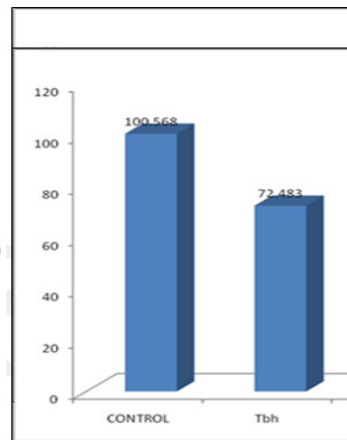


Figure 4a-b
RT PCR analysis of Caspase 3; after 3-days reperfusion

Figure 5a

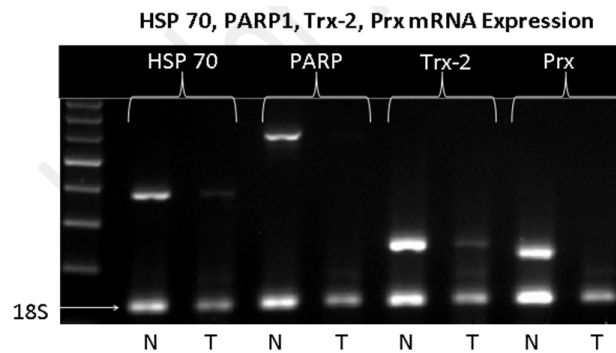


Figure 5b

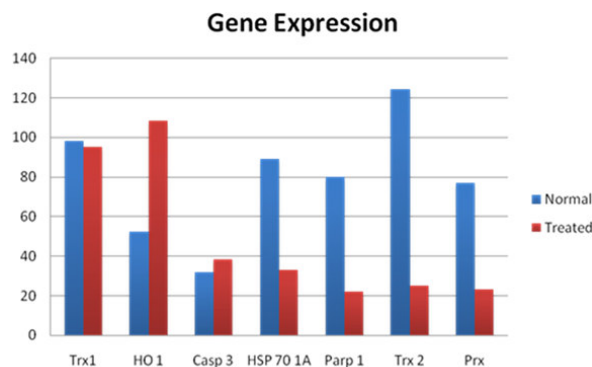


Figure 5a-b: RT PCR analysis of degree of oxidative stress (TRX1&2, PRX); Type of cell death (PARP 1; Caspase 3), Cell defense (HSP 70, HO 1); Housekeeping (18 S RNA) after 45- minutes reperfusion.

DISCUSSION

Neuronal cell death is typically discussed dichotomously as either apoptosis or necrosis¹⁷. Apoptosis is an active, programmed process of autonomous cellular dismantling that avoids eliciting inflammation¹⁸. Necrosis is a passive, accidental cell death resulting from environmental perturbations with uncontrolled release of inflammatory cellular contents¹⁹. We observed increase in caspase gene expression level as evident from the results of reperfusion and this increase signify cell death occurring by apoptosis²⁰. Results from acute phase show increase in parp gene expression which signifies cell death through necrosis²¹. The comparison of gene expression analysis during acute and sub-acute phase of oxidative stress clearly shows cell which has survived initial stage of necrosis if could not defend itself and recover from the stress goes for apoptosis as the time lapses²². Thus initial periods of oxidative stress are marked by cell necrosis²³ as there is no enough ATP available for the cell to go through programmed cell death (apoptosis) cascade²⁴ as it requires energy for the synthesis of caspase and other factors involved in cascade. During sub-acute phase TBH was removed and serum was added; this depicts in-vivo scenario where the blood supply to cells may be suspended temporarily due to injury or block and after treatment (clot lysing agent such tissue plasminogen activator) there is reperfusion of blood²⁵. This sub-acute phase is marked by apoptotic cell death in vitro; this suggest the same scenario may also possible in-vivo where initial period of the stress is marked by the necrosis of tissue and once the condition becomes more stable and less stress full cells which has survived the necrosis but not recovered completely may opt for apoptosis²⁶⁻²⁸. As we know apoptosis and necrosis are counter active a drug given to inhibit cell death by apoptosis during acute phase may drive cell for necrosis and make the condition worse²⁹. This study shows that during the acute phase of cell death the primary treatment strategy should be to remove the oxidative stress and provide necrosis inhibitors^{30,31} and once the

condition stabilize (treatment with clot lysing agent) the next treatment strategy is to prevent apoptosis of the surviving cells. Stem cells are biological cells that can divide through mitosis and the potential of stem cells in regenerative medicine relies upon removing them from their natural habitat, propagating them in culture, and placing them into a foreign tissue environment³². Bone marrow stem cell are said to rescue surviving neurons after injury by producing growth factors³³. Some of the previous studies has been shown that growth factors prevents cell from apoptosis³⁴⁻³⁷ but there not much substantial data available to show effect of growth factors to stop necrosis^{38,39}. Therefore it is important to know the optimum time for transplant of stem cells as well as it should be timed such that transplantation takes place at the end of necrosis and before the initiation of apoptosis.

CONCLUSION

The study shows there is a gene expression change during the acute and sub-acute phase of the injury which mimics the in-vivo scenario of reperfusion after tissue insult. It has been shown that during the acute phase of stress the cell death occurs through necrosis and then gets shifted to apoptosis during sub-acute phase. The future direction of this study is to find the time at which the cell shift from necrosis pathway to initial phase of apoptosis so that treatment can be timed specific to prevent cell death by both mechanisms.

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CONFLICT OF INTEREST

The author declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

REFERENCES

- Mittler R. Oxidative stress, antioxidants and stress tolerance. *Trends in plant science*. 2002;7(9):405-10.
- Kalyanaraman B. Teaching the basics of redox biology to medical and graduate students: Oxidants, antioxidants and disease mechanisms. *Redox biology*. 2013;1(1):244-57.
- Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB. Oxidative stress, inflammation, and cancer: how are they linked? *Free Radical Biology and Medicine*. 2010;49(11):1603-16.
- Apel K, Hirt H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* 2004;55:373-99.
- Gardiner CS, Reed DJ. Glutathione redox cycle-driven recovery of reduced glutathione after oxidation by tertiary-butyl hydroperoxide in preimplantation mouse embryos. *Archives of biochemistry and biophysics*. 1995;321(1):6-12.
- Goldberg AL. Protein degradation and protection against misfolded or damaged proteins. *Nature*. 2003;426(6968):895-9.
- Ryter SW, Alam J, Choi AM. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiological reviews*. 2006;86(2):583-650.
- Lakhani SA, Masud A, Kuida K, et al. Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. *Science*. 2006;311(5762):847-51.
- Watanabe R, Nakamura H, Masutani H, Yodoi J. Anti-oxidative, anti-cancer and anti-inflammatory actions by thioredoxin 1 and thioredoxin-binding protein-2. *Pharmacology & therapeutics*. 2010;127(3):261-70.
- Bouchard VJ, Rouleau M, Poirier GG. PARP-1, a determinant of cell survival in response to DNA

- damage. *Experimental hematology*. 2003;31(6):446-54.
11. Salto R, Vílchez JD, Girón MD, et al. β -Hydroxy- β -methylbutyrate (HMB) promotes neurite outgrowth in neuro2a cells. *PloS one*. 2015;10(8):e0135614.
 12. LePage KT, Dickey RW, Gerwick WH, Jester EL, Murray TF. On the use of neuro-2a neuroblastoma cells versus intact neurons in primary culture for neurotoxicity studies. *Critical Reviews™ in Neurobiology*. 2005;17(1).
 13. Liu X, Turbyville T, Fritz A, Whitesell L. Inhibition of insulin-like growth factor I receptor expression in neuroblastoma cells induces the regression of established tumors in mice. *Cancer Research*. 1998;58(23):5432-8.
 14. Pignatelli M, Rosario L-M, Pérez-Rendón A, Santos A, Perez-Castillo A. The transcription factor early growth response factor-1 (EGR-1) promotes apoptosis of neuroblastoma cells. *Biochemical Journal*. 2003;373(3):739-46.
 15. Roth W, Wagenknecht B, Klumpp A, et al. APRIL, a new member of the tumor necrosis factor family, modulates death ligand-induced apoptosis. *Cell Death & Differentiation*. 2001;8(4).
 16. Schätzl HM, Laszlo L, Holtzman DM, et al. A hypothalamic neuronal cell line persistently infected with scrapie prions exhibits apoptosis. *Journal of virology*. 1997;71(11):8821-31.
 17. Graeber MB, Moran LB. Mechanisms of cell death in neurodegenerative diseases: fashion, fiction, and facts. *Brain pathology*. 2002;12(3):385-90.
 18. Taylor RC, Cullen SP, Martin SJ. Apoptosis: controlled demolition at the cellular level. *Nature reviews Molecular cell biology*. 2008;9(3):231-41.
 19. Festjens N, Berghe TV, Vandenaebelle P. Necrosis, a well-orchestrated form of cell demise: signalling cascades, important mediators and concomitant immune response. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*. 2006;1757(9):1371-87.
 20. Ferrer I, Planas AM. Signaling of cell death and cell survival following focal cerebral ischemia: life and death struggle in the penumbra. *Journal of Neuropathology & Experimental Neurology*. 2003;62(4):329-39.
 21. Fink SL, Cookson BT. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infection and immunity*. 2005;73(4):1907-16.
 22. Ng F, Berk M, Dean O, Bush AI. Oxidative stress in psychiatric disorders: evidence base and therapeutic implications. *International Journal of Neuropsychopharmacology*. 2008;11(6):851-76.
 23. Elmore S. Apoptosis: a review of programmed cell death. *Toxicologic pathology*. 2007;35(4):495-516.
 24. Levine B, Yuan J. Autophagy in cell death: an innocent convict? *The Journal of clinical investigation*. 2005;115(10):2679-88.
 25. Ramos A, Visozo A, Piloto J, Garcia A, Rodriguez C, Rivero R. Screening of antimutagenicity via antioxidant activity in Cuban medicinal plants. *Journal of ethnopharmacology*. 2003;87(2):241-6.
 26. Bugge TH, Flick MJ, Danton M, et al. Urokinase-type plasminogen activator is effective in fibrin clearance in the absence of its receptor or tissue-type plasminogen activator. *Proceedings of the National Academy of Sciences*. 1996;93(12):5899-904.
 27. Bugge TH, Flick MJ, Daugherty CC, Degen JL. Plasminogen deficiency causes severe thrombosis but is compatible with development and reproduction. *Genes & Development*. 1995;9(7):794-807.
 28. Kalyan NK, Lee SG, Wilhelm Ja, et al. Structure-function analysis with tissue-type plasminogen activator. Effect of deletion of NH2-terminal domains on its biochemical and biological properties. *Journal of Biological Chemistry*. 1988;263(8):3971-8.
 29. Kroemer G, El-Deiry W, Golstein P, et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. *Cell Death & Differentiation*. 2005;12:1463-7.
 30. Dumont RJ, Okonkwo DO, Verma S, et al. Acute spinal cord injury, part I: pathophysiologic mechanisms. *Clinical neuropharmacology*. 2001;24(5):254-64.
 31. Nel A, Xia T, Mädler L, Li N. Toxic potential of materials at the nanolevel. *science*. 2006;311(5761):622-7.
 32. MACHERLA S, MURAHARI NK. Stem Cell Therapy-An Overview. *International Journal of Pharma and Bio Sciences*. 2012;3(1):66-75.
 33. Zhang L, Zhang H-T, Hong S-Q, Ma X, Jiang X-D, Xu R-X. Cografted Wharton's jelly cells-derived neurospheres and BDNF promote functional recovery after rat spinal cord transection. *Neurochemical Research*. 2009;34(11):2030-9.
 34. Lum JJ, Bauer DE, Kong M, et al. Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell*. 2005;120(2):237-48.
 35. Nagata S. Apoptosis by death factor. *cell*. 1997;88(3):355-65.
 36. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science*. 1995;267(5203):1456.
 37. Yao R, Cooper GM. Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science*. 1995;267(5206):2003.
 38. Kang SW, Chae HZ, Seo MS, Kim K, Baines IC, Rhee SG. Mammalian peroxiredoxin isoforms can reduce hydrogen peroxide generated in response to growth factors and tumor necrosis factor- α . *Journal of Biological Chemistry*. 1998;273(11):6297-302.
 39. Kraupp BG, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R. In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. *Hepatology*. 1995;21(5):1465-8.