Original article:

Interplay between Nitric oxide, mitochondrial thiols and thioredoxin system in cancers of ovary and cervix.

Joshi P.N.*, Joshi N.G. Bulakh P.M.†**

*Associate Professor, Dept of Biochemistry, B. J. Medical College, Pune, Maharashtra.

**Associate Professor, Dept of Biochemistry, Dr. V. M. G. Medical College, Solapur, Maharashtra.

† Ex-professor of Biochemistry, B. J. Medical College, Pune. Maharashtra

Corresponding author : Joshi P.N. ; Email: drpoojanitinjoshi@gmail.com

Abstract:

Introduction: Nitrosative stress in mitochondria which is mainly created by nitric oxide, nitrothiol and nitrotyrosine may alter thiol status in cancers of ovary and cervix. As an antioxidant defence Thioredoxin system may be playing role in restoring the functions.

Methods: Mitochondria were lysed and lysate was used for estimation of Trx, TR, nitrothiols, total and membrane thiol concentrations. The cell lysate was used for estimation of nitrotyrosine. NOx (nitrate and nitrite) levels were estimated in plasma samples.

Observation: We found that the levels of total mitochondrial and membrane protein thiols were decreased significantly ($p<0.05$). Levels of NO were not much affected and nitrothiols were not detected. However nitrotyrosine was detected which may be contributing to thiol modification. The levels of thioredoxin and thioredoxin reductase were elevated significantly ($p<0.05$).

Result and Conclusion: NO, nitrothiols and nitrotyrosine might be responsible for reduction in mitochondrial thiols. This modification of thiols may be counteracted by thioredoxin system.

Keywords: Nitric oxide, nitrotyrosine, nitrothiols, Thioredoxin system

Introduction:

Cancer is fundamentally a disease of failure of regulation of tissue growth and is characterised by inflammation and release of cytokines. Inflammation induced carcinogenesis is caused by many factors; and reactive oxygen species (ROS) and reactive nitrogen species (RNS) are major contributors. The ROS/RNS damage bio-molecules like lipids, DNA and proteins in their close vicinity.

Nitric oxide (NO°) is mainly responsible for generation of RNS. This molecule is synthesized from L-Arginine in the reaction catalysed by nitric oxide synthase (NOS). At low concentration it has protective role. At higher concentrations it causes damages to biomolecules. At equimolar concentration, NO° formed reacts with superoxide to form peroxynitrite[3]. Peroxynitrite is also responsible for nitration of free and protein bound tyrosine residue forming nitrotyrosine. It is thought that nitrotyrosine is footprint of increased NO° production. In addition to this NO° can react rapidly in the intracellular environment to form nitrite and nitrate, S-nitroso-thiols etc[4].Thus thiol containing proteins more vulnerable to the action of NO° and peroxynitrite . Mitochondrial proteins can be thus modified by nitration, S-nitrosylation, S-glutathionylation, or

www.ijbamr.com P ISSN: 2250-284X E ISSN :2250-2858

carbonylation. NO° is oxidizing as well as nitrating agent which can traverse biological membranes. Protein oxidation and nitration results in altered function of many enzymes[5, 6].

Oxidized proteins are reduced back by various systems in the body. Thioredoxin system is one of them. It consists of thioredoxin reductase (TR), thioredoxin (Trx) and NADPH. Trx in its active form (reduced) provides reducing equivalents to target molecules including ribonucleotide reductase (involved in DNA synthesis), peroxiredoxins (cellular antioxidants) and various transcription factors. During these reactions Trx gets oxidised. This oxidised Trx is reduced back to its active form in presence of TR. Trx exists in two isoforms: Trx1 (cytosolic) and Trx2 (mitochondrial). Similarly, the TrxR, occurs both as cytosolic form (TrxR1) and mitochondrial form (TrxR2) that act upon Trx1 and Trx2, respectively [7, 8] Constitutive Trx and TrxR expression has been observed in several cell types of the mammalian body, including keratinocytes of the skin, placental cells, liver cells, secretory cells, and leukocytes [9,10] Physiological stimuli, including UV light, hydrogen peroxide and mitogens can induce the expression of Trx and TrxR pointing at an important role in protection against oxidative stress and in regulating cell growth and cell death [11].

Urbanization leading to changes in lifestyle made life more stressful and this may be one of the contributory factors for various disorders; Cancer is one of them. Prevalence of cancers of ovary and cervix is increasing in India. Keeping these facts in mind, the present work was designed to study the effects of NO° and Trx system on mitochondrial thiol levels in ovarian and cervix cancer. The present study was planned to know the levels of mitochondrial total and membrane protein thiols in cancers of ovary and cervix and to find the levels of plasma NOx levels and nitrothiols and nitrotyrosine from the cell lysate.

Material and Method:

Mitochondria were isolated[12] from 35 tissues of ovarian cancer, 31 tissues of cervix cancer and 38 non-malignant tissues. Mitochondria were lysed and lysate was used for estimation of Trx, TR, nitrothiols, total and membrane thiol concentrations. The cell lysate was used for estimation of nitrotyrosine. NOx levels were estimated in plasma samples.

Table 1: Shows parameters estimated in the present study and the methods used for the estimation [13-19].

Parameters	**Method**
Plasma NOx concentration	Moshage et al and Granger et al.
Nitrotyrosine	Crow and Ischiropoulos
Nitrothiols	Cook et al
Thioredoxin (Trx)	Holmgren and Bjornstedt
Thioredoxin Reductase TR	Luthman and Holmgren
Mitochondrial total thiol	Modified Habeeb
Mitochondrial membrane protein thiol	Kowaltowski et al

www.ijbamr.com P ISSN: 2250-284X E ISSN :2250-2858

The samples were run in duplicate and for each sample; the mean of the two values was taken. The statistical significance was calculated by Mann –Whitney U test by using NCSS-PASS statistical software. Statistical significance was chosen as $p<0.005$.

In the present study the levels of mitochondrial total and membrane protein thiols were found to be decreased significantly ($p<0.05$). No significant increase or decrease in the levels of NO° was observed. Nitrothiols were not detected. However nitrotyrosine was detected. The levels of thioredoxin and thioredoxin reductase were elevated significantly ($p<0.05$).

Observation and Results:

	Plasma NOx (µmol/L) Mean ± SD	Nitrotyrosine µmol/L) Mean ± SD	Total thiol level (nmol/mg) Mean ± SD	Membrane protein thiol level(nmol/mg) Mean ± SD	Trx level (pmol/mg) Mean ± SD	TR level (pmol/mg) Mean ± SD
Controls (n=38)	35.61±5.94	Not detected	111.35±11.6	56.35±5.9	367.5±103	61.66±5.9
Ovarian cancer (n=35)	34.61±7.44	11.34*±3.67	62.3*±15.2	31.64*±6.4	3054*±1134	695.77*±6.5
Cervix cancer (n=31)	35.71±8.09	10.74*±3.93	62.4*±13.2	33.86*±7.1	3443*±1125	696.74*±7.1

[Table 2 showing results of plasma NOx, Nitrotyrosine, Nitrothiols, Trx, TR, Total and membrane thiol levels in control and experimental groups , *= $p<0.05$ statistically significant. (Nitrothiol was not detected in control and experimental group)]

Discussion: In the present study the levels of mitochondrial total and membrane protein thiols were found to be decreased significantly ($p<0.05$). This decrease could be due to its oxidative and/or nitrative modification. To know whether NO° has any role in the process, plasma NOx levels were determined. When these results were compared with controls, no significant increase or decrease in the levels of NO° was observed. So there are various possibilities like, NO° synthesised was diffused in plasma and diluted or this NO° was modified by reaction with other species in the vicinity. In the present study, nitrotyrosine was detected. This indicates that NO° was generated in large amount and reacted with

www.ijbamr.com P ISSN: 2250-284X E ISSN :2250-2858

superoxide to form peroxynitrite which further formed nitrotyrosine and this was detected in patients with ovarian and cervix cancer. [20]This could be one of the factors modifying mitochondrial thiols.

NO° might also react with thiols to form nitrothiol. However nitrothiol was not detected in experimental as well as control group. The fact can be explained by the role of TR in degrading nitrothiols mainly GSNO. [21]This means that nitrothiols, the modified proteins as and when formed were degraded by TR. Also the levels Trx and TR were found to be elevated significantly ($p<0.05$). The increase in the Trx and TR, might be to restore the function of modified thiol groups. As a major intracellular reducing agent, these are upregulated in certain tumours, to protect cancer cells from oxidative stress [22]. Since cancer cells are often under high oxidative and hypoxic stress it is not surprising that they also express high levels of antioxidant proteins [23]. However these effects are no longer solely beneficial for the patient once the tumour is established [24].This probably could be the reason why levels of mitochondrial total thiols and membrane protein thiols were found to be decreased in presence of elevated levels of Trx and TR. The increased levels of Trx and TR as observed in the present study might be playing role in tumour progression by various mechanisms like increasing supply of reducing equivalents to ribonucleotide reductase for DNA synthesis, activation of transcription factors that regulate cell growth and an increase in the sensitivity of cells to other cytokines and growth factors [25]or inhibiting apoptosis [26].

Conclusion: It can be concluded from the present study, that the RNS generated in the mitochondria of tissues of cervix and ovarian cancer have modified thiol groups of mitochondria forming nitrothiols and nitrotyrosine which may further affect mitochondrial function. Antioxidant defence system responds to it by elevating the levels of TR and Trx initially .However once the tumour is established elevated levels of these two proteins help in tumour progression and might not play restoring mitochondrial thiols. Further research using inhibitors of NOS to control production and effects of NO° will help in treating cancers.

Acknowledgement: To clinical staff of department of gynaecology and surgery for providing samples and laboratory staff of biochemistry department of BJMC.

References:

1. Jancov R.P.Negus A., Transwell A.K. (2001) Antioxidants as therapy in the newborn: some words of caution. Pediatric research.50 (6).

2. Welming XU, Li Zhi Liu, Marilena Loizidou, Mohamed A., Ian G Charles(2002) Therapy of Human Ovarian Cancer by Transfection with the Murine Interferon β Gene: Role of Macrophage-Inducible Nitric Oxide Synthase. Cell Research; 12(5-6):311-320

3. Cadenas E, Davies K J.(2000) Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic Biol Med.;* 29: 222 - 230.

4. Navarro A. (2004) Mitochondrial enzyme activities as biochemical markers of aging.*Mol Aspects Med.*; 25: 37 - 48.

5.Gasdaska PY, Gasdaska JR, Cochran S, Powis G. (1995). Cloning and sequencing of a human thioredoxin reductase**.** FEBS Lett; 373:5–9.

6. Gasdaska PY, Berggren MM, Berry MJ, Powis G. (1999). Cloning, sequencing and functional expression of a novel human thioredoxin reductase. FEBS Lett; 442: 105–11.

7. Rozell, B., Hansson, H. A., Luthman, M., and Holmgren, A. (1985). Immunohistochemical localization of thioredoxin and thioredoxin reductase in adult rats. Eur. J. Cell Biol., *38:* 79–86, 13.

8. Schallreuter, K. U., Pittelkow, M. R., and Wood, J. M. (1986) Free Radical Reduction by Thioredoxin Reductase at the Surface of Normal and Vitiliginous Human Keratinocytes J. Invest. Dermatol, *87:* 728–732.

9. Nakamura, H., Nakamura, K., and Yodoi, J. (1997) . Redox regulation of cellular activation.
Annu. Rev. Immunol., *15:* 351–369.

10. Mattoon J. R.,Sherman F. (1966). Reconstitution of phosphorylating electron transport in mitochondria from a cytochrome c-deficient yeast mutant J.Biol. Chem.241:4330-4332.

11. Moshage H,Kok B, Huizenga J.R.,Jansen P.L.M. (1995) Nitrite and nitrate determinations in plasma: a critical evaluation. Clin.Chem. 41/6: 892-896.

12. Crow J. P.,Ischiropoulos H.(1996). Detection and quantitation of nitrotyrosine in proteins: in vivo marker of peroxynitrite.Methods Enzymol.269: 185-191.

13.Cook J. A.,Kim S. M.,Teang D.,Krishna M. C., Paulli R., Mitchell J. B., Nims R. W. Christodoulou D., Miles A. M., Grisham M. B., Wink D. A. (1996).Anal Biochem.238: 150-154.

14. Holmgren A.,Bjornstedt M.(1995). Thioredoxin and thioredoxin reductase. Methods Enzymol., 252 : 199-208

15. Luthman M.,Holmgren A.(1982) Rat liver thioredoxin and thioredoxin reductase: purification and characterization. Biochemistry. 21:6628

16. Habeeb A.F.S.A. (1972) Reaction of protein sulfhydryl groups with Ellman's reagent. Methods Enzymol XXV: 457-461.

17. Kowaltowski A.J., Castilho R. F., Vercesi A. E. (2001). Mitochondrial permeability transition and oxidative stressFEBS Lett.495 (1-2):12-15.

18. Jancov R.P..Negus A., Transwel lA. K. (2001) Antioxidants as therapy in the newborn: some words of caution. Pediatric Research. 50(6)

19. Nordburg J., Arner E. S. J. (2001). Reactive oxygen species, antioxidants, and the mammalian thioredoxin systemFree radical Biology and Medicine. 31:1287-131.

20. Mukherjee and S G MARTIN (2008). The thioredoxin system: a key target in tumour and endothelial cells
The British Journal of Radiology, 81, S57–S68

21. Therese Christina Karlenius and Kathryn Fay Tonissen(2010). Thioredoxin and Cancer: A Role for Thioredoxin in all States of Tumor Oxygenation. *Cancers*, *2*, 209-232.

22. Katja Becker, Stephan Gromer, R. Heiner Schirmer and Sylke Muller (2000). Thioredoxin reductase as a pathophysiological factor and drug target.Eur.J.Biochem. 267, 6118-6125

23. Soderburg A, Sahaf B., Rosen A. (2000) Lymphocyte surface thiol levels. Cancer Research 60:2281-2289.24. Garth Powis, Debbie Mustacich[a], Amy Coon (2000). The role of the redox protein thioredoxin in cell growth and cancer.Free Radical Biology and Medicine Volume 29, Issues 3–4, Pages 312–322

Date of submission: 22 Aug 2013 Date of Provisional acceptance: 29 September 2013
Date of Final acceptance: 12 October 2013 Date of Publication: 04 December 2013
Source of support: Nil; Conflict of Interest: Nil