THE REGULATION OF THIOREDOXIN SYSTEM IN CELLS UNDER OXIDATIVE STRESS

YUNG MING HO MINGO

M.Phil

The Hong Kong Polytechnic University

2010

The Hong Kong Polytechnic University

Department of Applied Biology and Chemical Technology

The regulation of thioredoxin system in cells under oxidative stress

Yung Ming Ho Mingo

A thesis submitted

in partial fulfillment of the requirements for the degree of

Master of Philosophy

March 2010

Declaration

I hereby declare that this thesis represents my own research work which has been done within the period from September 2007 to March 2010 for the degree of Master of Philosophy. This thesis has not been previously included in a thesis, dissertation or report submitted to this or any other institutions for a degree, a diploma or other qualifications.

Yung Ming Ho Mingo

March 2010

Abstract

Aerobic organisms breathe fresh air instinctively. At the same time, they are exposed to the negative challenges and influences oxygen imposes on them. It can be expected that through selection forces, existing aerobic organisms should have developed effective anti-oxidative systems to help them survive under oxidative stress.

Aerobic cells have a variety of means that protect them from the harmful effects of reactive oxygen species. One such protective mechanisms in diverse organisms and cells is the thioredoxin system. It is a simple but multifunctional antioxidant system that consists of a small ubiquitous dithiol protein thioredoxin (Trx), a pyridine nucleotide-disulphide oxidoreductase named thioredoxin reductase (TR) and a hydrogen donor NADPH. The thioredoxin system participates critically in combating against oxidative stress. Although the thioredoxin system in cells has been known and studied quite extensively, how it is regulated during oxidative stress conditions is not understood in most cases.

To address this issue, the response of mammalian cells towards different levels of oxidative stress was studied to find out how the thioredoxin system was regulated. The levels and activities of the thioredoxin system in HeLa and HT-29 cells, were monitored upon challenged with various concentrations of hydrogen peroxide. Changes in cyctotoxicity, thioredoxin system proteins level, enzymatic activity and cell cycle were analysed. A decline in cell viabilities, both in HeLa cells and HT–29 cells, was observed

upon a concentration-dependent hydrogen peroxide challenge. However, some increases of the thioredoxin reductase activity and thioredoxin activity were observed. Using immunoglobulin as substrate, HeLa cell was found with a 29% increase in the protein disulphide reduction activity upon treatment with 2mM hydrogen peroxide. Similarly, a 28% increase in the protein disulphide reduction activity was observed in HT–29 cell treated with 0.5 mM hydrogen peroxide. Western blotting and RT-PCR analyses revealed that the enhancements of activities in thioredoxin reductase and thioredoxin were probably due to the induction of protein synthesis and mRNA expression.

In our previous investigations on erythrocytes, we found that a direct activation of the thioredoxin system could be involved in reaction to oxidative stress. In this study, we therefore looked into if regulations of the thioredoxin system do exist in cells generally without *de novo* protein synthesis. Cell cultures were pretreated with cycloheximide so as to inhibit *de novo* protein synthesis. The entire thioredoxin system activity, especially in cells treated with lower concentrations of hydrogen peroxide, was found functionally intact throughout the experiment. It was subsequently confirmed by the analyses of Western blot and RT-PCR which revealed insignificant changes in the cellular level of thioredoxin system proteins and mRNA respectively. Regulation of the thioredoxin system might therefore involve some endogenous factors instead of *de novo* protein synthesis.

Multiple cellular processes such as proliferation, cell cycle and prosurvival signalling cascades are regulated by redox sensitive signalling factors. Thioredoxin and thioredoxin

reductase, which are components of several redox regulated pathways, would exhibit their functional roles in the cellular defence against oxidative damage. To verify this notion, we looked into the relationship of the thioredoxin system and the changes in cell cycle stage in response to hydrogen peroxide treatment. HT–29 cells that had been exposed to hydrogen peroxide were subjected to fluorescence activated cell sorting analysis. A concomitant increase in thioredoxin system activity was observed in cells with an increase in the population of cells arrested at the G_2 phase. Apoptosis was undetectable.

We have provided some information on the protective effect of the thioredoxin system in cell viability and regulation of cell cycle in this study. A more in-depth understanding of the role of the thioredoxin system in fighting against oxidative stress will be further explored leading to potential applications of the system in the treatment of degenerated diseases and aging.

Acknowledgements

Our project has been supported by many people whose advice and encouragement are most appreciated; I am indebted to all of them.

The most important person that I would like to thank is my supervisor, Dr. K. S. Lee. On the one hand, he acted as a very good teacher who always provided kind guidance and useful discussion on my work. On the other hand, he was teacher who shared his valuable experience with us whether in work or in daily life and that helped me a lot when I faced different challenges in my life.

I would also like to grasp this great opportunity to thank my co-supervisor Dr. Leung Yun Chung Thomas for his encouragement and professional advices throughout the study journey.

I would like to express my appreciation to Dr. Zhong Liu and Dr. Alan Lam from the research laboratory of Dr. K. S. Lee for their support in the project. They helped me a lot by giving me inspirations and suggesting some useful ideas for improvement.

Besides, I would like to thank Ms. Mabel Yau, Ms. Sarah Yeung, Mr. Leung and Mr. Ken Cheng in the laboratory of the Department of Applied Biology and Chemical Technology for their kindness, patience and round the clock services without complaint. The work was also supported by Internal Grant of The Hong Kong Polytechnic University, Grant

No. A-PD96 awarded to K. S. Lee and partially supported by Internal Grant of The Hong Kong Polytechnic University with Grant No. 1-BD05 awarded to Andrew T. Chiu.

I lack the space here to acknowledge all the other individuals whose special efforts contributed to this project. I offer my sincere thanks and this finished work that they helped to complete.

Finally, I express my deepest appreciation to my parents, sister and grandmother for their encouragement and endurance with extraordinary grace the long evenings and holidays I devoted to this practical research.

Table of Contents

List of Abbreviations

Abbreviations	Full Names
ADF	Adult T-cell Leukemia-derived Factor
BSA	Bovine Serum Albumin
Da	Daltons
DHA	Dehydroascorbic acid
DNA	Deoxyribonucleic Acid
DTNB	5,5'-dithiobis-(2-nitrobenzoic) acid
FAD	Flavin adenine dinucleotide (Oxidized form)
GPx	Glutathione peroxidise
GR	Glutathione reductases
GSSG	Glutathione disulfide
GST	Glutathione-S-transferase
HRP	Horseradish peroxidase
IgG	Immunoglobulin
kDa	Kilodaltons
k_{cat}	Catalytic Constant
K_m	Michaelis – Menten Constant
M_r	Molecular Weight
MSR	Methionine Sulfoxide Reductase
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]
NADPH	Nicotinamide Dinucleotide Phosphate (Reduced Form)
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline

PMSF	Phenylmethylsulphonyl fluoride
PRDXs	Peroxiredoxins
PVDF	Polyvinylidene-difluoride
ROS	Reactive Oxygen Species
S.D.	Standard Deviation
SDS	Sodium dodecyl sulphate
SOD	Superoxide Dismutase
TBS	Tris Buffered Saline
TR	Thioredoxin Reductase
Trx	Thioredoxin
TTBS	Tween Tris Buffered Saline

List of Figures and Tables

List of Figures

List of Tables

1. Introduction

The earth is the only known planet in the Solar system that possesses the appropriate abundance of oxygen to support life. Most aerobic organisms need oxygen to provide energy for survival. Due to the oxidative respiration, different reactive oxidative species are formed [1] that will inflict damages to cells. In addition to this, different environmental oxidative species in the abundance of oxygen are generated. Nearly all organisms on earth are therefore exposed in and out to different kinds of reactive oxygen species which are generally recognized as oxidative stresses.

Oxidative stress is an unavoidable consequence of oxygen metabolism and takes place in the cells of most aerobic organisms [2]. An abnormal concentration of reactive oxygen species can be easily developed in our cells, at the same time, we, human, are all continuously exposed in a battery of reactive oxygen species. Reactive oxygen species are chemically very reactive and can damage intracellular components such as proteins, lipids, membrane particles and nucleic acids seriously [3-5], leading to many common biological problems such as aging [3], cancers [6], cardiovascular diseases, neurodegenerative diseases and numerous other chronic disorders [5].

1.1 Nature of reactive oxygen species (ROS)

Biological molecules such as proteins, lipids, carbohydrates and nucleic acids [7] are highly susceptible to oxidation by reactive oxygen species resulting in extensive damage. The majority of these reactive species are generally free radicals, even though some of them are not free radicals but are reactive lipid and carbohydrate species [8]. In fact, free radicals can be defined as reactive chemical molecules that have unpaired electron(s) in their outer atomic or molecular orbit which are capable of independent existence. The unpaired electron(s) contributes to the unstable configuration of the molecule and makes a significant extent of reactivity to the free radical [5,9,10]. The majority and most important types of free radicals that can cause damage to living systems are derived from oxygen [10] and they are generally known as reactive oxygen species (Table 1).

Table 1. The free radical reactive oxygen species [9].

Free Radicals	
Hydroxyl	$OH^{\cdot}$
Superoxide	$O_2^{\cdot-}$
Nitric Oxide	$NO^{\cdot}$
Thiyl	$RS^{\cdot}$
Peroxyl	$RO_2^{\cdot}$

Biological systems frequently encounter different reactive oxygen species which are derived from several pathways as summarized in Table 2. Reactive oxygen species can, on the one hand, be produced in nature through exposure to high energy electromagnetic waves such as ionising X-rays and gamma rays or exciting ultraviolet light irradiation [4,10-12]. In a cell, water, the main cellular component would generate free radicals through removal of the electrons by ionising irradiation. On the other hand, through the activation of protein-kinases signalling, reactive oxygen species may also be generated by phagocytic white blood cells such as neutrophils and macrophages and even non-phagocytic white blood cells during inflammatory states [7,10,11]. Besides these, oxygen free radicals can exist as noxious wastes in the atmosphere [3,4]. In normal metabolic processes, inevitable premature leaking of electrons to molecular oxygen in the mitochondrial electron transport chain reactions, in particular at Complex I (NADH dehydrogenase) and Complex III (ubiquinone-cytochrome b-c1 complex), results in the formation of unwanted free radicals by-products (Figure 1) [3,10,13,14].

Furthermore, hydrogen peroxide can be produced from the oxidation of fatty acids and other debris molecules. In peroxisomes, hydrogen peroxide can be converted to harmless form by the reaction of catalase. However, in certain situations, some of these hydrogen peroxide may escape elimination and go into cellular compartments, resulting in oxidative stress [3]. Reactive oxygen species are also generated by arginine metabolism and can be formed by the site-specific metal catalysed reactions with the participation of redox-active transition metals ions such as iron and copper via the Fenton Reaction (Figure 2) [3-5,14,15].

Different reactive species co-exist in the environment that makes them difficult to be recognized unequivocally which agent(s) should be the causative factor(s) for certain biological outcome. To gain a more in-depth understanding of the course and effect of the ROS, the development of new methods to ameliorate undesirable reactive oxygen species production may therefore be one of the pivotal issues in research on aging and oxidative stress-related diseases in the future.

Table 2. The various origins of reactive oxygen species *in vitro* and *in vivo* [3].

Causes	Reactive Oxygen Species
Atmosphere Pollution	CO, Ozone, NO_2, N_2O_2
By-Product Of Electron Transport Chain Reactions	$O_2^{\bullet-}$
Irradiation (X-, γ-, UV)	$O_2^{\bullet-}$
Site-specific Metal-catalysed Oxidation or Fenton Reaction.	$^{\bullet}OH$, H_2O_2, Ferryl Ion
Inflammation (Neutrophils and Macrophages)	OCl^-, H_2O_2, $O_2^{\bullet-}$, NO, $ONOO^-$
Oxidase	H_2O_2
Arginine Metabolism	NO

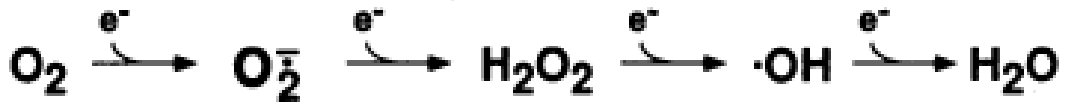


Figure 1. Oxidants from normal metabolism. The formation of $O_2^{\cdot-}$, H_2O_2, and $^{\cdot}OH$ occurs by successive additions of electrons to O_2. Cytochrome oxidase adds four electrons fairly efficiently during energy generation in mitochondria, but some of these toxic intermediates are inevitable by-products.

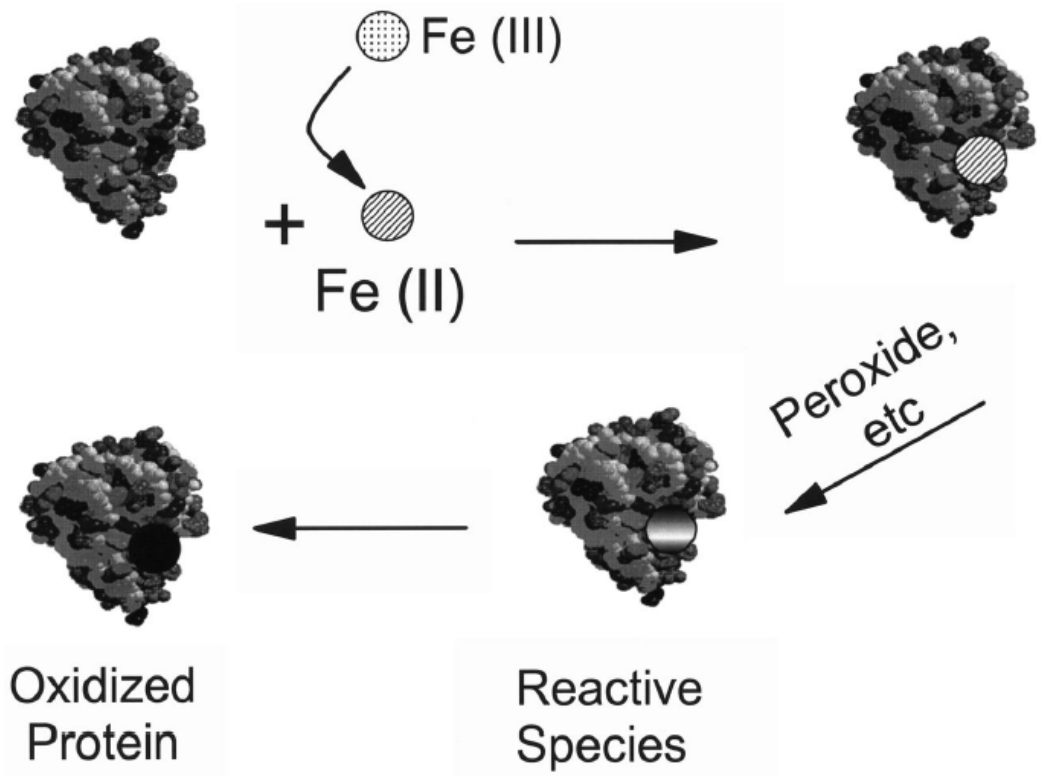


Figure 2. The metal catalyzed oxidation of proteins is a site specific process. Divalent cation binding sites will occasionally be occupied by redox – active cations such as Fe or Cu, rendering that site susceptible to oxidation by ROS generated *in situ*. Indeed, all proteins amino acid residues are potential targets for oxidation but some are more vulnerable such as cysteine and methionine residues [4].

1.2 Oxidation and damages in cellular proteins by reactive oxygen species (ROS)

Reactive oxygen species are highly reactive that they can oxidize the cellular components such as nucleic acids and intracellular proteins. ROS can even alter the structures and functions of enzymes irreversibly, with pathophysiological significance in most cases (Figure 3). While many mechanisms are known for repairing the damaged nucleic acids; similar mechanism that can repair damaged peptides and proteins may not always exist [4].

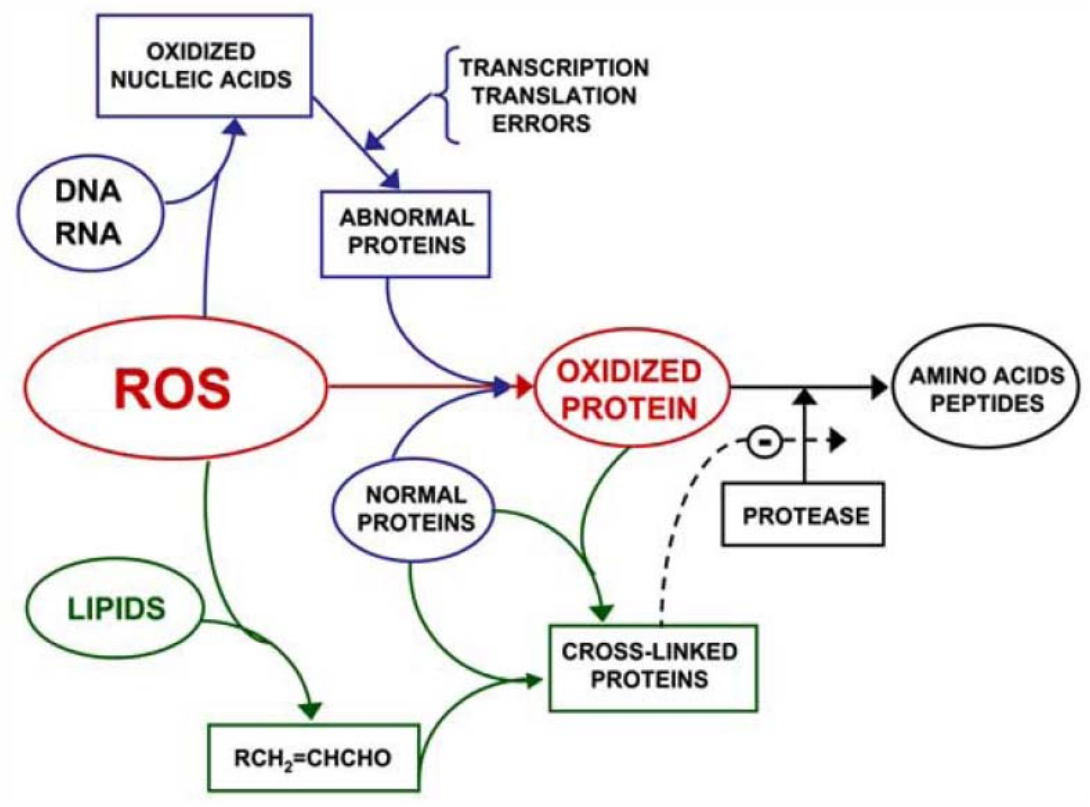


Figure 3. Reactive oxygen species can inflict irreversible damages to various biomolecules that subsequently lead to the formation of non-functional proteins [3].

In the investigations of the oxidation mechanism of proteins by reactive oxygen species, amino acids, simple peptides and proteins were exposed to ionizing radiations that resulted in the production of a mixture of hydroxyl radicals and superoxide radicals and subsequently a series of chain reactions [3,10,12,16]. In addition, polypeptide chains were also demonstrated to be vulnerable to redox oxidation by the electromagnetic waves, resulting in fragmentation. While ionized radiations are predominantly implicated in causing fragmentation of the polypeptide chains, they also play a major role in oxidative protein modifications. It is well-known that ionizing radiations would induce protein-protein cross links (Figure 4) through oxidation of amino acid residues side chains. Such protein cross linkages would initiate a series of physiological disorders. The amino acid residues that are most susceptible to oxidation are the surface-exposed cysteine and methionine. Upon such oxidative assault, protein disulfides, sulfenic acid, sulfinic acid and sulfonic acid can be resulted [3,10] (Table 3).

Under normal physiological situations, oxidatively damaged proteins are eliminated by proteolysis instead of being repaired. The level of oxidatively damage proteins in a cell would therefore increase if the efficiency of proteolytic degradation decreases in various aging and in some diseases. Accumulation of these oxidatively modified proteins is undesirable because this interrupts cellular functional roles either by loss of catalytic and conformational integrity or by disruption of regulatory pathways [4].

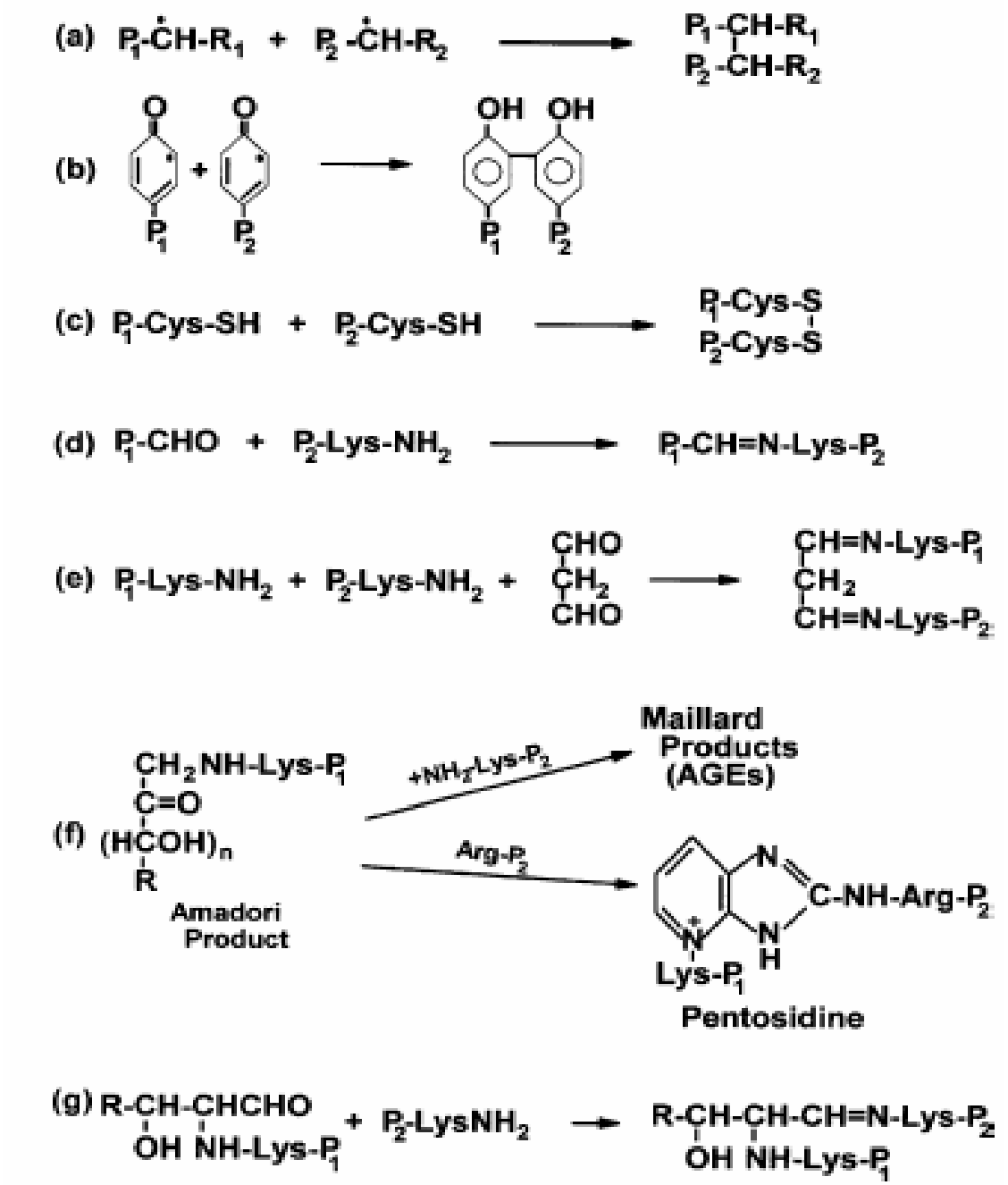


Figure 4. The production of different protein-protein cross linkage derivatives [16].

Table 3. The amino acids residues being modified to different compounds by reactive oxygen species [3, 12].

Amino acids residues	Products formed
Arginine	Glutamic semialdehyde
Cysteine	Disulfides: Cys-S-S-Cys, Cys-S-S-R
Glutamate	4-Hydroxy-glutamate, Pyruvate, · – Ketoglutatrate
Glutamic Acid	Oxalic Acid; Pyruvate Adducts
Histidine	2-Oxo-histidine; 4-OH-glutamate
Leucine	3-, 4- and 5-Hydroxy-leucine
Lysine	2-Aminoadipic- semialdehyde, 3-, 4-, and 5-hydroxy-lysine
Methionine	Methionine sulfoxide, Methionine sulfone
Phenylalanine	2-, 3-, and 4-hydroxy-phenylanine; 2, 3-dihydroxyphenylalanine
Proline	Glutamic semialdehyde, Pyroglutamic acid, 2-Pyrrolidone 4-hydroxy-proline
Threonine	2-Amino-3-keto-butyric acid
Tryptophan	N-Formyl-kynurenine, kynurenine, 2-, 4-, 5-, 6-, and 7-Hydroxy-tryptophan
Tyrosine	3-4-dihydroxy phenylanine A(DOPA), Tyr-Tyr cross-linked proteins, 3-Nitro-tyrosine, 3, 5-Dichloro-tyrosine
Valine	3-, 4-Hydroxy-valine

While sulfur-containing amino acid residues such as cysteine and methionine are susceptible to primary oxidation, fortunately the process can be reversed. In thiol transferases, for example, reduced glutathione or reduced thioredoxin can regenerate the oxidized disulfides bridges to protein sulfhydryl groups with the formation of disulfide oxidized glutathione or thioredoxin respectively [17].

On the one hand, oxidation of surface-exposed methionine to S- and R-epimers of methionine sulfoxide (MetO-S and Met-R) (Figures 5a and b) by oxidants would affect the biological activities of the proteins involved. On the other hand, the process can function as a protective mechanism to the cells in which reactive molecules are scavenged so that other functional, essential protein residues can be spared from the attack by these reactive oxygen species. This is attributed to a dithiol reduced thioredoxin dependent enzyme named methionine sulfoxide reductases (MsrA and MsrB) which can be found in many animals' tissues and in bacteria. The methionine sulfoxide reductase has the potential to convert methionine sulfoxide back to functional methionine with the formation of the disulfide oxidized thioredoxin which subsequently can be regenerated or reduced by the thioredoxin reductase and NADPH. This forms a helpful and essential cyclic oxidation and reduction antioxidant system in cells, especially in exposure to environments with high oxidative stress [4,16,18 19].

$Met + HOOH \rightarrow MetO + HOH$

$MetO + Trx(SH)_2 \rightarrow Met + TrxS\text{-}S$

$\underline{TrxS\text{-}S + NADPH + H^+ \rightarrow Trx(SH)_2 + NADP^+ + H_2O}$

Sum: $NADPH + HOOH + H^+ \rightarrow NADP^+ + 2\ H_2O$

Figure 5a. The cyclic oxidation and reduction of a protein Met residue by hydrogen peroxide (HOOH) can be described by the reactions.

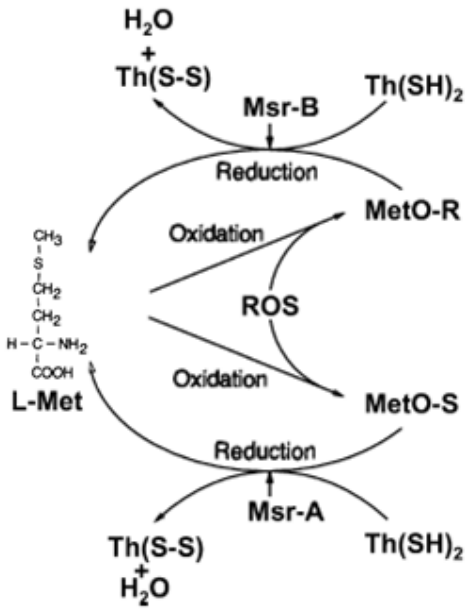


Figure 5b. Cyclic interconversion of Met and the R- and S-isomers of MeO. L- Met refers to the L-isomer of Met, MetO-R and MetO-S are R- and S-isomers of MetO respectively, MsrA and MsrB refer to the methionine sulfoxide reductases that are specific for the reduction of the S- and R-isomers of MetO respectively and $Th(SH)_2$ and Th(S-S) refer to the reduced and oxidized forms of thioredoxin respectively [18, 19].

1.3 The importance of maintaining homeostatic balance in the physiological level of reactive oxygen species

From a traditional point of view, reactive oxygen species seem to do more harm than benefit on the longevity and health of living organisms. Ironically, oxygen free radicals can be both detrimental and beneficial in the natural living systems in different situations. At appropriate physiological concentrations, reactive oxygen species exert many beneficial effects on the physiological functions of organisms. In addition to their role as bodyguards to defend against infectious agents, they also function in cellular signaling pathways which may influence the transcription factors to induce programmed cell death (apoptosis). Through apoptosis, damaged cells are eliminated that will protect the overall integrity and help in correct development of the organisms. Besides these, they also contribute to some gene expression events [5,10,11].

1.3.1 Redox and phosphorylative processes

The cascade of phosphorylation represents one of the most effective examples in modulating signaling regulatory processes associated with redox balance. Studies from Saitoh, M. *et al.* (1998) using the yeast two-hybrid system identified that thioredoxin (Trx) acted as an apoptosis signal regulating kinase (ASK1) interacting regulatory protein and the redox switch in Trx/ ASK1 complex resulted in a phosphorylative process. ASK1 belongs to the stress-activated protein kinase family that regulates the activation of different MAP kinase kinases (MKK). In the study, in stressed cells with an oxidizing

environment, disulfide bridge was formed on the thioredoxin moiety which thus caused the dimer dissociation between thioredoxin and ASK1. ASK1 thereafter could escape from thioredoxin inhibition and undergo multimerization to activate its kinase activity. Such interaction seemed to be modulated by the intracellular redox state and ultimately transcription of different genes implicated in the cell cycle regulation would be induced (Figure 6) [20].

The glutathione transferase π-1 (GST)/ JNK complex is another system which is associated with redox unbalance and phosphorylative events. Adler, V. *et al.* (1999) demonstrated that under resting condition in unstressed cells, the association between GST and JNK gave rise to a dimer GST/ JNK, which inhibited the phosphorylation of JNK [21]. While under oxidative stress condition induced by hydrogen peroxide or ultraviolet treatment, dissociation of the GST/ JNK complex occurred and phosphoactivation of JNK was allowed (Figure 6) [20].

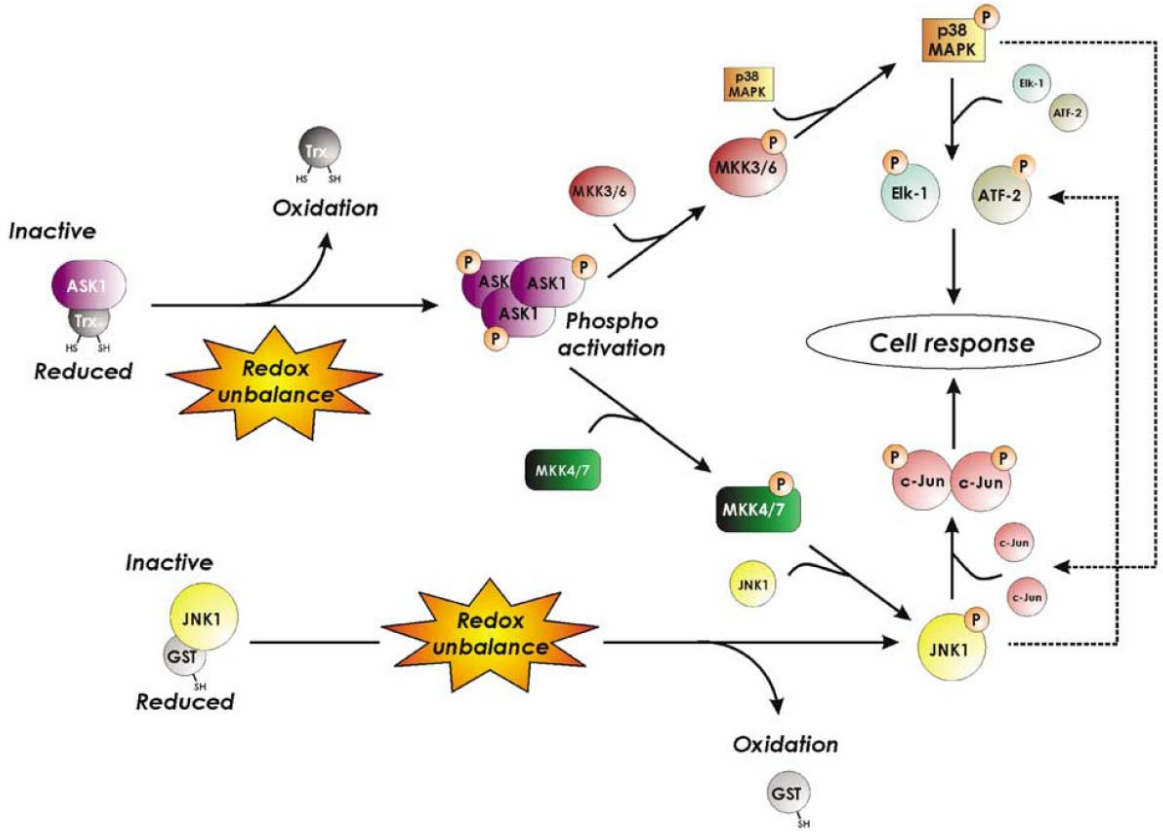


Figure 6. Redox regulation of cell response. The scheme represents the intimate relationship between phospho- and redox-mediated signals. The detachment of the redox-sensitive inhibitory subunits, Trx and GST, from ASK1 and JNK, respectively, allows transforming an oxidative stimulus in a phosphorylative cascade [20].

1.3.2 Redox control of gene expression

There is a growing amount of evidence which tells us that the intracellular redox state can modulate different kinds of gene expression. Thioredoxin is also known to be one such intracellular redox regulator.

AP-1 which is known to be an antioxidant-responsive transcription factor under redox regulation as it contains critical cysteine residues in the basic region of its DNA-binding domain. On the other hand, transient overexpression of thioredoxin can stimulate AP-1-dependent transcription. The increase in DNA binding activity of AP-1 is resulted from a direct association with an intranuclear redox factor, Ref-1, through the presence of critical cysteine residues in the catalytic site of the thioredoxin. Redox modulation of AP-1 activity with the participation of thioredoxin and Ref-1 is one of the examples of regulation of transcription factor activity by intracellular redox modification.

In the case of NF-κB, DNA binding activity is also known to be under oxidoreduction regulation [22]. Oxidizing conditions in the cytoplasm initially favor NF-κB transactivation and translocation into the intranuclear compartment because reactive oxygen species such as hydrogen peroxide can stimulate degradation and dissociation of the inhibitory subunit IκB from the inactive cytoplasmic NF-κB complex. Treatment of cells with radical scavengers and antioxidants inhibits nuclear translocation and activation of NF-κB. However, reducing environments seem to be important for the activation of NF-κB DNA binding within the nucleus since cysteine 62 in the DNA-binding loop of the p50 subunit

must be in reduced state in order to allow binding to promoter sites of target genes and thioredoxin again plays an important role in the redox-regulation of NF-κB [22,23]. Hirota, K. *et al.* (1999) demonstrated that inside the nucleus, the translocated thioredoxin associated to NF-κB potentiated NF-κB dependent transcription. Such two steps redox regulation with oxidation inside the cytoplasm and reduction within the nucleus has significant consequences. Not only the nuclear compartment is sheltered from oxidative challenge but gene transcription is also activated only upon reversible oxidation of cysteine residues. Given the effects of intracellular redox changes on the activity of the above transcription factors, it is feasible that regulation of the transcription of many genes is similarly affected.

On the other hand, at high concentrations, oxygen free radicals are proapoptoic and this leads to oxidative stress in cells by compromising the cellular components such as some skeleton proteins despite of the presence of antioxidant systems to counteract the reactive oxygen species. Similarly, the accumulation of these reactive oxygen species during a life cycle has been implicated in various pathological disorders like cardiovascular disease, cancer, neurological disorders, diabetes, stroke, other diseases and aging [5,10]. Therefore, well-tuned levels of antioxidants and reactive oxygen species are vital for a proper regulation of normal cellular functions.

1.4 Combating against oxidative stress

It is important to know how aerobic cells by the light of nature can on the one hand prevent cellular damage by the attacks of reactive oxygen species, and on the other hand how they respond and survive from oxidative stress. In most biological systems, they have already evolved many distinct defence mechanisms to turn reactive oxygen species into uncreative and less harmful products.

For instance, there are some co-factors like NADPH, NADH, lipoic acids, uric acid and vitamins that are utilised by many aerobic organisms in their cellular antioxidant system [4,7]. In addition to these, there are also antioxidants enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidise (GPx), glutathione-S-transferase (GST), glutathione, cystetine, methionine sulfoxide reductase (MSR) and last but not least the thioredoxin system which is the most well-known one in counteracting against reactive oxygen species [4,7].

1.4.1 Antioxidants

Antioxidants are especially important and essential for aerobic organisms to counteract the harmful effect of oxygen. In general, an antioxidant can be any matter or action and in any form that has the capability to delay, inhibit or eliminate oxidative damage in a target [24]. The antioxidants can be endogenous, *in vivo*, or exogenous, which are absorbed from meals and/or dietary supplements. Although some dietary components do not neutralize

oxidative damage, they can also be categorized as antioxidants because they are capable of enhancing endogenous antioxidant activities [5]. Endogenous antioxidants can naturally maintain the most favorable cellular functions and systemic health. However, under certain circumstances, in particular oxidative stress conditions, endogenous antioxidants may not be adequate and therefore exogenous antioxidants would be vital to sustain the normal cellular functions [5].

In both the endogenous and exogenous antioxidants, there are basically two kinds in terms of their proteinaceous or non-proteinaceous nature, namely enzymatic and non-enzymatic. Well-known enzymatic antioxidants include enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidise (GPx) and glutathione-S-transferase (GST). They are all of vital importance by far and away since each of them has specific functional role. For examples, superoxide dismutase detoxifies superoxide ions while catalase is responsible for the clearance of hydrogen peroxide. Glutathione peroxidase is in charge of the detoxification of cellular peroxides. Glutathione-S-transferase detoxifies endogenous peroxidised lipids and breakdowns xenobiotics by catalyzing the conjugation of reduced glutathione.

Non-enzymatic antioxidants include thiol antioxidants and some natural micronutrients such as selenium, polyphenols, carotenoids, vitamin C, vitamin E, flavonoids and other compounds [5,9,25]. Under normal situations, the maintenance of cellular redox homeostasis is tightly regulated by a balance between the activities and intracellular levels among antioxidants and reactive oxygen species. This balance is indisputably required for the

health and well-being of the organisms [10].

Among these antioxidant systems, the thiol antioxidants systems are of particular interest to us. One of the most well known thiol antioxidant is the thioredoxin system. It not only acts as an antioxidant system to reduce peroxides and reactive oxygen species to protect aerobic living organisms from oxidative stress, but also regenerates antioxidants. For instance, vitamin E is the major antioxidant in biological membranes and can react with free radicals to form vitamin E semiquinone, which in turn can be reduced back to vitamin E by ascorbic acid. In this reaction, ascorbyl free radicals are formed, two of which can spontaneously dismutate, generating one molecule of ascorbic acid and one molecule of dehydroascorbic acid (DHA). Thioredoxin reductase can efficiently reduce the latter to ascorbic acid. Thioredoxin reductase thus participates directly and actively in the conversion of the dehydroascorbic acid and ascorbyl free radical to functional ascorbic acid, which in turn control the recycling of vitamin E. In such way, the antioxidant functions of vitamin E are indirectly governed by thioredoxin system [26]. (Figure 7)

As a whole, thioredoxin system is very important and necessary in protecting organisms against oxidative stress through either its direct antioxidant effect or through its interactions with other key signaling molecules.

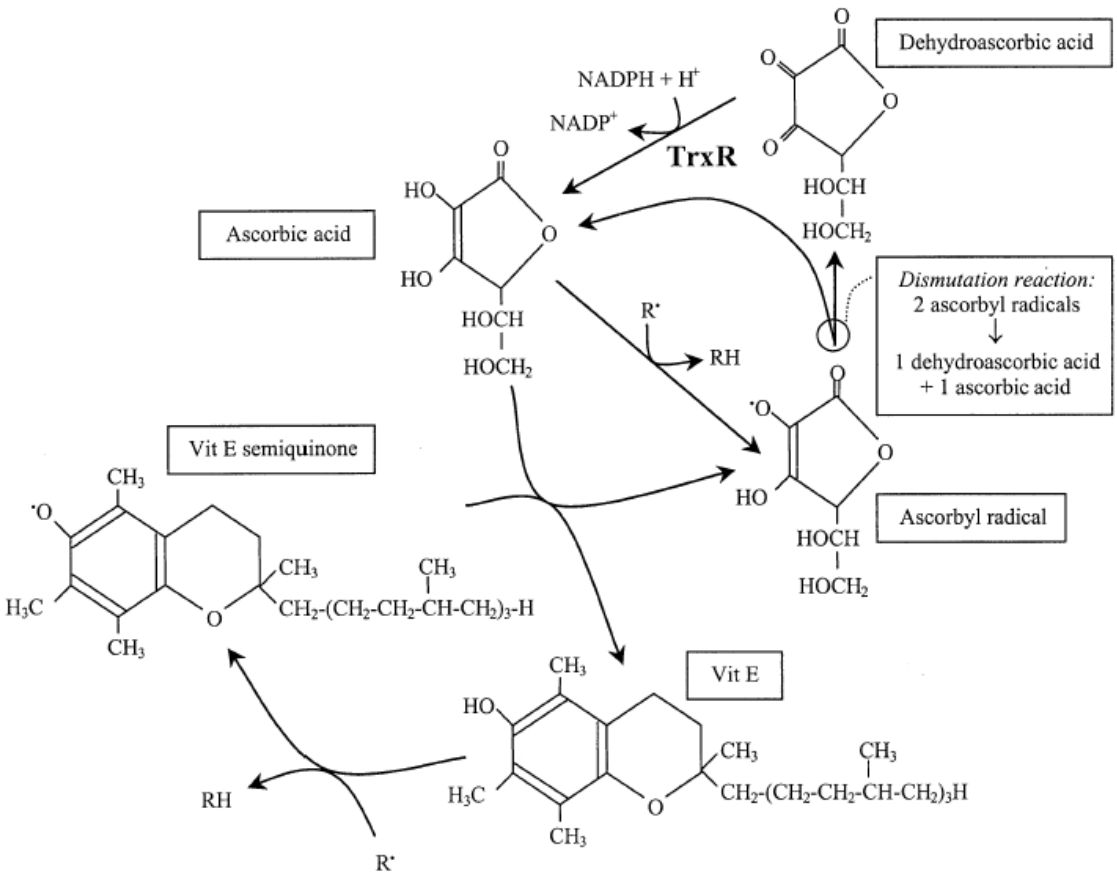


Figure 7. The interplay between different species of vitamin C and vitamin E in relation to TrxR [26].

1.5 The thioredoxin system

Living organisms produce reactive oxygen species such as H_2O_2 during physiological processes that may damage cellular proteins through oxidation of the amino acid residues in the active sites. These damages are cumulative that ultimately lead to functional disorders. To cope with these potentially destructive reactive oxygen species, cells have evolved antioxidant defenses such as the thioredoxin system.

The thioredoxin system is an important and essential conserved system for protection against oxidative stress, control of cellular redox balance, cells differentiation, cell morphogenesis and also cell fate [27,28]. It has been reported that it would be lethal in mouse embryos with thioredoxin gene knocked out [28].

The system is composed of thioredoxin, thioredoxin reductase and NADPH [2]. Through an interaction with the redox-active center of reduced thioredoxin, a general protein disulfide reductant, it can reduce the oxidized cellular proteins' disulfide cysteine groups back to the thiol groups with itself oxidized. This reaction is very important as some proteins are redox sensitive that they may only be functional when the corresponding amino acids residues are in the reduced state. In order to carry on such redox balancing reaction in cells so that activities of various redox-regulated pathways may not be affected, a continuous supply of reduced thioredoxin is very important. The regeneration of reduced thioredoxin is done by the thioredoxin reductase which can reduce oxidized thioredoxin with the help of the electron donor NADPH (Figure 8).

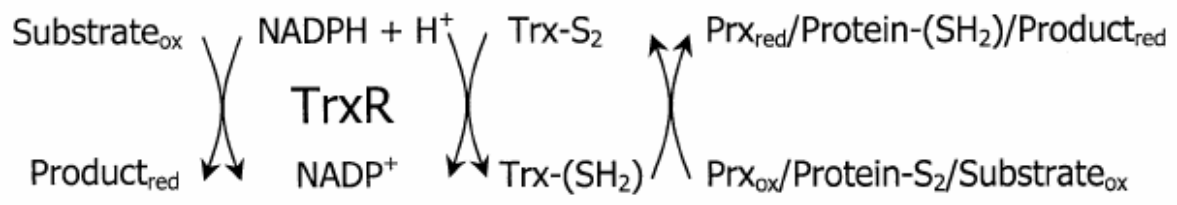


Figure 8. The enzymatic reactions of the thioredoxin system. Thioredoxin reductase reduces the active site disulfide in thioredoxin and several other substrates directly under consumption of NADPH [26].

1.5.1 Thioredoxin reductases

The thioredoxin reductases (TrxRs) participate in several crucial functions in sustaining the redox environment of cells. They are homodimeric flavoproteins [29,30] which belong to a family of pyridine nucleotide-disulphide oxidoreductases; members of this family also include glutathione reductase, lipoamide dehydrogenase, mercuric ion reductase, trypanothione reductase and NADPH peroxidise [26,31,32].

Different forms of thioredoxin reductases have been evolved in evolution (Table 4). Those in archaea, prokaryotes and lower eukaryotes are comparatively smaller with a subunit molecular weight of 35 kDa and without a interface domain; while those in higher eukaryotes have a subunit of 55 to 65 kDa and they show broader substrate specificity. Thioredoxin reductases of higher eukaryotes therefore not only have the ability to reduce oxidized thioredoxin from different spices, but also can reduce some low molecular weight substances that include selenite, vitamin K3, lipoic acid, lipid hydroperoxides, hydrogen peroxide and other non-disulfide physiological substrates, as well as proteins such as the cytotoxic peptide NK lysine, tumor-suppressor protein p53 and several exogenous compounds [6,26,29,30,33-36].

It is also noteworthy that thioredoxin reductase can catalyse an NADPH- dependent reduction on disulfide bond of the artificial substrate, 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB). This reaction has been extensively used as a rapid and simple assay of the activity of thioredoxin reductase (Figure 9 a and b) [33].

Table 4. Characterization of thioredoxin reductases [30].

Thioredoxin reductase (2 distinct types)
High molecular weight (Mr) type: A. Subunit Mr approximately 55 kDa (human and *Plasmodium falciparum*). B. Dimeric proteins with one FAD, one redox active disulfide and a third redox active group in each subunit. The third group is a selenenylsulfide in human TrxR and a disulfide in the *P. falciparum* enzyme. C. The enzymes from calf liver and thymus and rat liver were the first to be purified to homogeneity and characterized. [33]
Low molecular weight (Mr) type: A. Subunit Mr approximately 35 kDa (*E. coli* and other prokaryotes, yeast, mycoplasmas, *Giardia duodenalis, Arabidopsis thaliana and Methanococcus jannaschii*). B. Dimeric proteins with one FAD and one redox active disulfide in each subunit.

a.

$$NADPH + H^+ + DTNB \xrightarrow{\text{thioredoxin reductase}} NADP^+ + 2\,TNB$$

b.

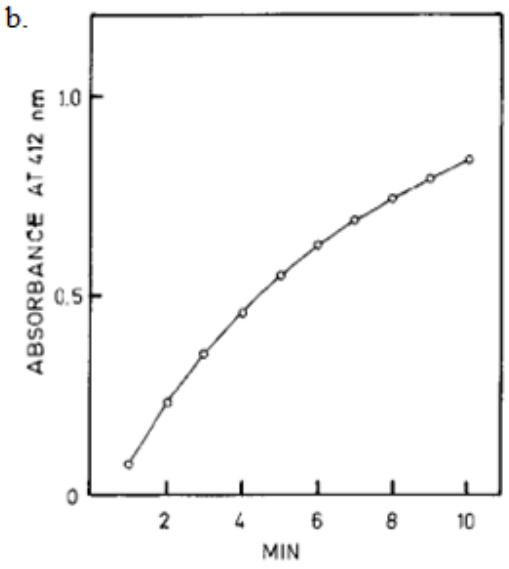


Figure 9. a. The reaction equation of DTNB activity assay and b. NADPH-dependent reaction of DTNB catalysed by thioredoxin reductase [33].

Both the prokaryotic and eukaryotic thioredoxin reductases can bring about their catalytic reactions initially by a FAD cofactor. It mediates the transfer of reducing equivalents from NADPH to the redox active disulfide [30]. For example, in *E. coli* thioredoxin reductase, it has been proposed that the nicotinamide ring of NADPH first bound to the enzyme does not get in direct contact with the isoalloxazine ring of FAD domains, until one of the NADPH binding domains of the enzyme is rotated 66 °toward the FAD domains. Then the bound NADPH nicotinamide ring is now shifted into close contact with the isoalloxazine ring. This permits electrons flow from NADPH to the FAD domain and via the latter to the redox active site disulphide of the enzyme. The enzyme active site once reduced would have its surface repositioned so that its reduced disulphide is reachable to the oxidized thioredoxin. It is important to note that the proposed mechanism is highly efficient without any large steric hindrance to prevent the conformational change during the reaction [6,32].

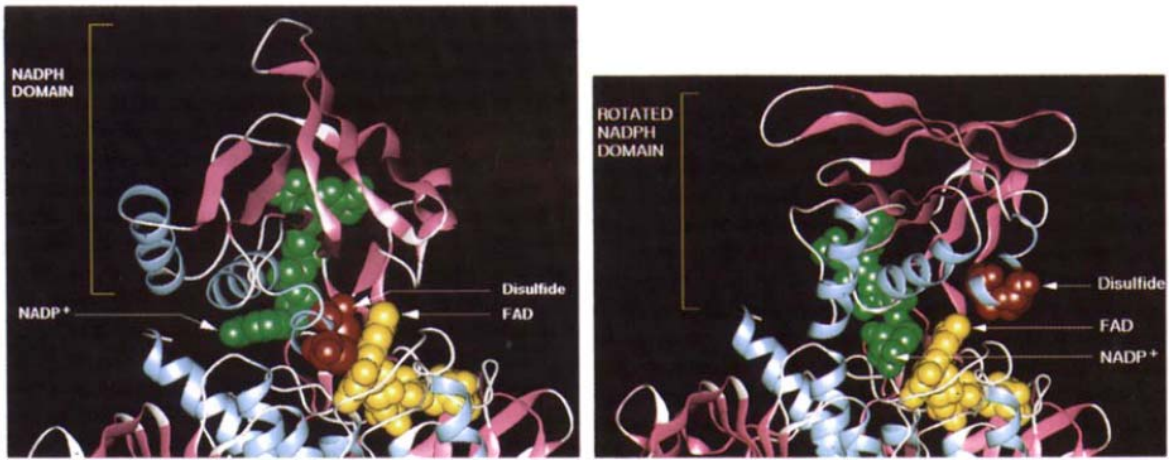


Figure 10. The proposed 66° conformational rotation of the NADPH domain and its interaction with FAD domain. The left one is before the conformational change and the right one after [32].

Mammalian thioredoxin reductases have been recognised for their diverse properties in comparison to prokaryotic thioredoxin reductases [6,34,37, 38]. The mammalian thioredoxin reductases are unique that they are members of the rare selenoenzymes [39]. The selenium is present in the form of selenocysteine residue, a natural selenium analogue of the cysteine which is therefore also known as the 21st amino acid. The selenocysteine residue appears in the penultimate position of the C-terminal, which is highly conserved with 16 residues in most mammalian thioredoxin reductase. The end sequence Gly-Cys$_{497}$-SeCys$_{498}$-Gly-COOH forms a selenenylsulfide in the oxidized enzyme. Upon reduction by NADPH, the selenenylsulfide is converted into selenolthiol [26,29,31]. Together with the adjacent cysteine, the selenocysteine in the conserved sequence has been suggested to be part of an open active site motif. This provides the enzyme a wider substrate specificity. Thioredoxin and thioredoxin reductases of different mammalian species are virtually interchangeable in terms of substrate specificities and activity, while prokaryotes thioredoxin reductase is only specific for its homologous thioredoxin. Some kinetic parameters of thioredoxin reductases are shown in Table 5.

Table 5. Kinetic parameters of *E.coli* and mammalian thioredoxin reductase [34].

		E. coli Thioredoxin Reductase	Mammalian Thioredoxin Reductase
E. coli Thioredoxin	K_m	3.0 μM	35 μM
	k_{cat}	2000 min^{-1}	3000 min^{-1}
Mammalian Thioredoxin	K_m	No activity	2.5 μM
	k_{cat}	–	3300 min^{-1}
DTNB	K_m	No activity	0.5 mM
	k_{cat}	–	4000 min^{-1}

The selenocysteine residue is necessary for the catalysis of thioredoxin reductase since either its deletion by carboxypeptidases' digestion or its alteration by alkylation brings about inactivation [29,31,40]. It has been demonstrated that replacement of the SeCys residue by a similar but redox-inactive Ser resulted in an inactive enzyme.

It has also been reported that growth of human cancer cells lines was enhanced with selenium supplement and thioredoxin reductase activity was correspondingly increased seven-fold. When selenium was depleted from the growth medium, animals and humans cell lines showed a major loss of thioredoxin reductase activity [26,29].

Furthermore, peptides sequencing, cloning and chemical modification demonstrated that cytosolic mammalian thioredoxin reductases share a close sequence identity and reaction mechanism with glutathione reductases. They all contain a disulfide in oxidized enzyme and a dithiol in NADPH reduced enzyme with the conserved –Cys_{59}-Val-Asn-Val-Gly-Cys_{64}- redox catalytic site sequence motif in the FAD domain [6,29,41]. Such high homology has also been further confirmed in a recent study. It showed that human thioredoxin reductase only shares 24% identity to *E. coli* thioredoxin reductase but it has a 44% identity to human glutathione reductase [42]. The conservation of the sequence in the two enzymes provide clue for the molecular evolution of mammalian thioredoxin reductase which may possibly have recently developed from GR rather than from the prokaryotic thioredoxin reductases [31].

Mammalian thioredoxin reductases are homodimeric proteins in which each 55 kDa

monomer contains a FAD prosthetic group binding domain with a tightly bound FAD cofactor, a NADPH binding site that has similar folds with FAD binding site [31], an interface domain and an active site containing a redox-active disulphide [29,31] (Figures 11a, b and c.).

11a.

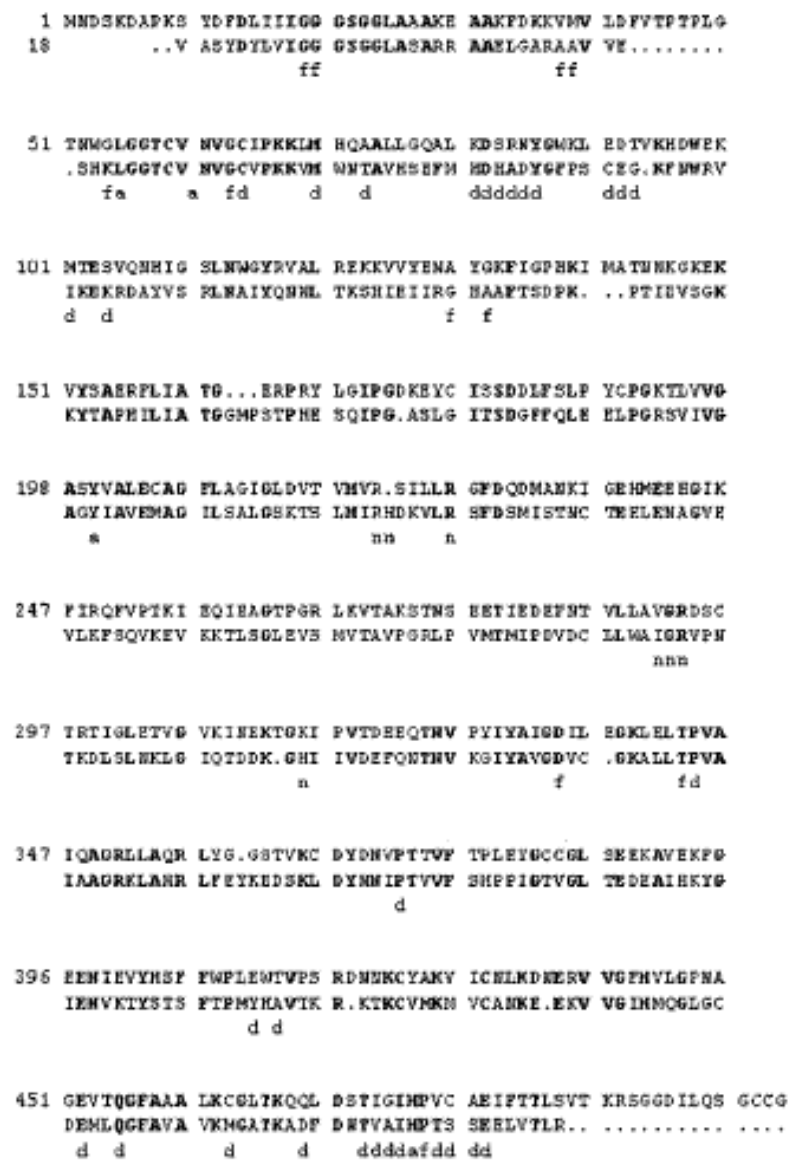


Figure 11a. The amino acids sequence of rat thioredoxin reductase (first row) and amino acids sequence of human glutathione reductase (second row). Conserved residues are shown in bold letters. Labels in the bottom line denote possible functions of the amino acids, in which f is flavin binding, n is NADPH binding, d is participation in dimer interface and a is active site [31].

11b.

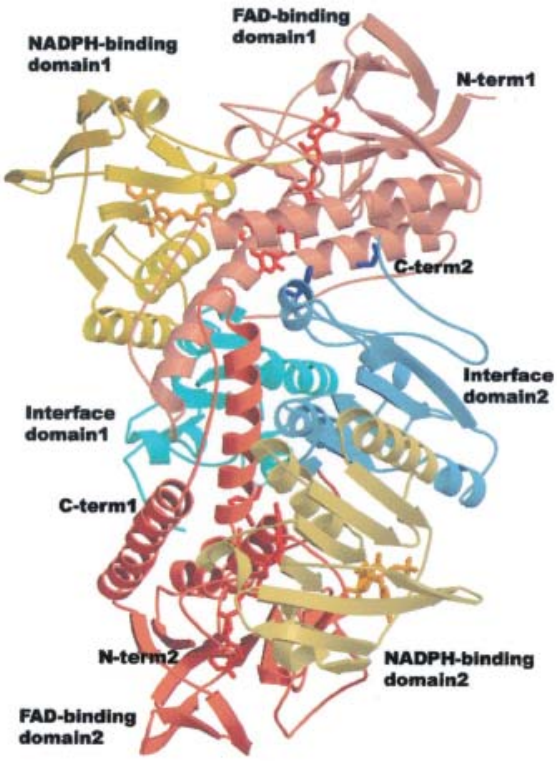


Figure 11b. The ribbon representation of the dimer of rat thioredoxin reductase [31].

11c.

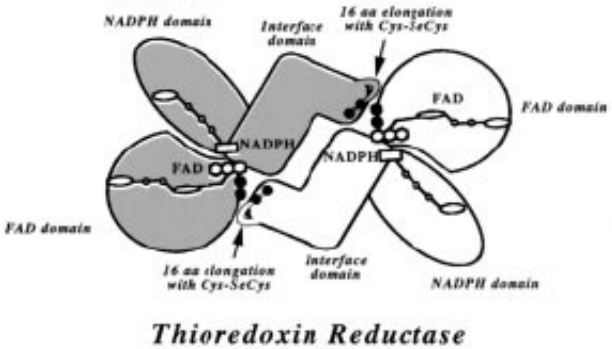


Figure 11c. The proposed structural model of mammalian Thioredoxin Reductase [29].

1.5.2 Reaction mechanism of mammalian thioredoxin reductase

TrxRs catalyse the NADPH-dependent reduction of the main substrates — the redox protein thioredoxin (Trx), as well as other endogenous and exogenous compounds [6]. The reaction involves the reversible transfer of electrons from NADPH through a redox-active disulfide system. Electrons are thought to be transferred from NADPH via FAD to the redox-active disulfide in the enzyme, which then reduces the thioredoxin or the substrate [37].

It has been revealed that the catalytic cycle of prokaryotic thioredoxin reductase involves a large conformational change of the NADPH and FAD binding motifs. In the case of mammalian thioredoxin reductase, a similar conformational change of the involved domains was found, but the movement was in a much less extent [31].

At the beginning of the catalysis, a charge-transfer intermediate is formed in the oxidized form of thioredoxin reductase by the reduction of NADPH at the FAD domain. Unique in the mammalian system, the selenenylsulfide bond between Cys_{497} and $SeCys_{498}$ in the oxidized enzyme is reduced upon addition of NADPH. The conserved Cys_{497}–SeCys-$_{498}$ motif thus performs as a second redox pivot in the mammalian TrxR. Electrons are then passed on from the redox-active disulfide via the redox center at the C terminal and eventually to the substrate, thioredoxin [31]. Figures 12 and 13 show the brief flow of the reaction mechanism and a proposed detailed reaction mechanism of thioredoxin reductase respectively.

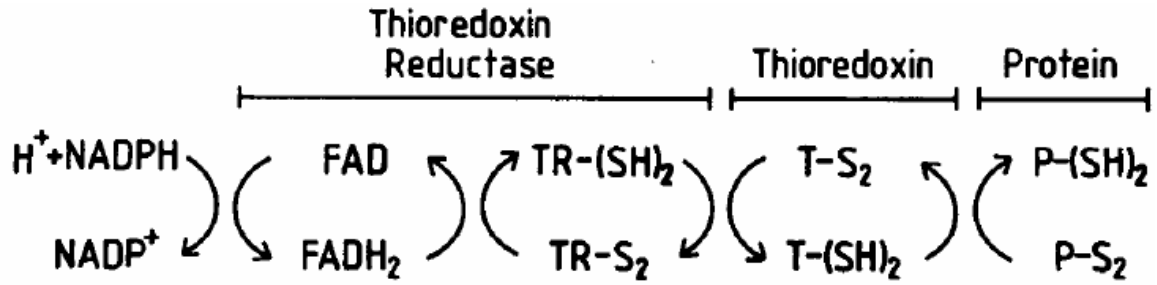


Figure 12. The brief mechanism of protein disulfide reduction catalyzed by the thioredoxin system [37].

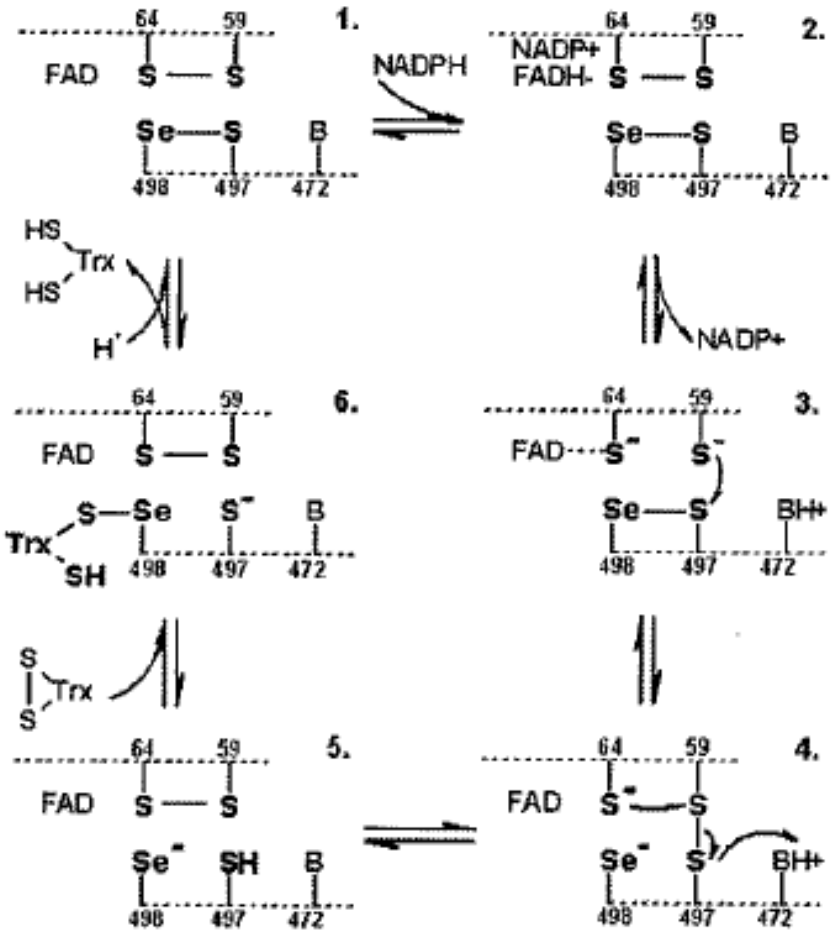


Figure 13. Proposed mammalian thioredoxin reductase reaction mechanism [31].

1.5.3 Isoforms of mammalian thioredoxin reductase

In addition to the cytosolic thioredoxin reductase, recently two more isoforms of the TrxRs have been discovered in mitochondria and testis. Similar to the cytosolic form, the two isoforms have the same general domain organization and carboxyterminal selenocysteine-containing motif. But different to the abundant cytosolic isoform, the mammalian testis thioredoxin reductase isoenzymes can reduce not only thioredoxin but also GSSG directly and therefore the testis isoform of TrxR is also known as thioredoxin and glutathione reductase (TGR). It is believed that the N-terminal end of the TGR enzyme is lengthened by an additional monothiol glutaredoxin domain that accepts electrons from the SeCys-containing centre in C-terminal [26,34]. On the other hand, a yeast mitochondrial thioredoxin system has just been revealed and these enzymes look very much alike the prokaryotic type thioredoxin reductase [34].

Although there are several thioredoxin reductases discovered, it is believed that many are yet to be explored.

1.5.4 Thioredoxin

Thioredoxin was first isolated in 1964 and had been recognized for more than forty years. It is found in various organisms, from simple lives such as T4 bacteriophage, filamentous phages like M13, prokaryotes such as *Escherichia coli*, and eukaryotes both in plant and animal kingdoms [38]. It exists in fact, commonly in different mammalian tissues and cells with different concentrations, e.g. in liver cells and in plasma at around 10 μmol L^{-1} and up to 6 nM respectively.

In general, there are multiple forms of thioredoxin encoded by different genes. The classical 12 kDa cytosolic and nuclear thioredoxin-1 are the most extensively studied. The other two isoforms are the mitochondria thioredoxin-2 with a special sixty amino acids N-terminal mitochondrial translocation signal and the SpTrx which is a thioredoxin variant highly expressed in spermatozoa [43,44]. (Unless otherwise specified, the term thioredoxin we describe afterwards is thioredoxin-1).

In most cases, thioredoxin (Trx) is a small (M_r around 12,000 Da), acidic (pI 4.5), heat-stable [37], multifunctional and ubiquitous protein. It has a characteristic redox-active disulfide/ dithiol within the conserved active site sequence - Cys_{32}-X-X-Cys_{35}-, where X stands for any amino acid residue. The two cysteine molecules are essential in the redox regulatory function of the protein. They act as nucleophiles [43] in the protein disulfide oxidoreductase reaction. In general, thioredoxin appears in two forms; the oxidized form (Trx-S_2), which has a disulfide linkage between the two cystine residues in the active site,

and the reduced form, Trx-$(SH)_2$, which has a dithiol [37]. Accordingly, the conserved active site of thioredoxin works as the key cellular protein disulfide reductase and such catalytic site facilitates thioredoxin to contribute in thiol-dependent redox reactions.

There are a variety of extents of homology of thioredoxin's primary structure in different species. It is interesting to note that within prokaryotes there are about fifty percent similarity of the protein sequence but a high sequence homology of about ninety percent can be seen within mammals (Figure 14) [38]. From the close similarities of the sequences in mammals, it suggested that most thioredoxins have similar overall three-dimensional structure. The highly conserved property of the thioredoxin is well in accordance with its importance in a number of biological functions [45].

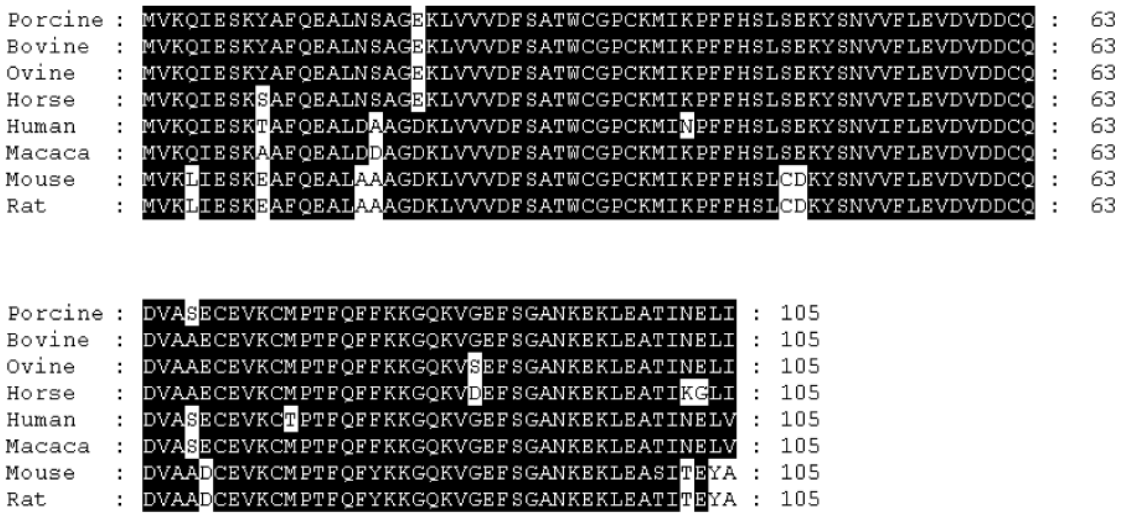


Figure 14. The comparison of thioredoxin protein sequences among different animals. The conserved amino acids residues are coloured. The sequence order from top to bottom are the porcine thioredoxin from pig liver, cytosolic (AF382821), bovine thioredoxin (AF104105), ovine thioredoxin (Z25864), horse thioredoxin (AB022431), human thioredoxin (AB022431), human thioredoxin (J04026), macaca thioredoxin (M84643), mouse thioredoxin (X77585) and rat thioredoxin (X14878) [45].

E. coli thioredoxin is the most extensively studied. It is an almost spherical molecule [38] containing a single polypeptide sequence with 108 amino acids residues. It contains a hydrophobic core formed by the single twisted β-sheet composed of five strands, flanked by four α-helical segments on the external surface [35,43]. Its active site sequence Trp-Cys-Gly-Pro-Cys- is positioned on the terminal tail of the β_2 strand and at the starting point of another α-helical segment [46]. This gives thioredoxin a very stable tertiary structure commonly known as 'Thioredoxin fold' [43] (Figure 15). In addition to the two cysteine residues at the catalystic site, human thioredoxin has three extra structural cysteine residues at the positions 62, 69 and 73 [43,44]. These structural cysteine residues impart unique biological properties to thioredoxin [44].

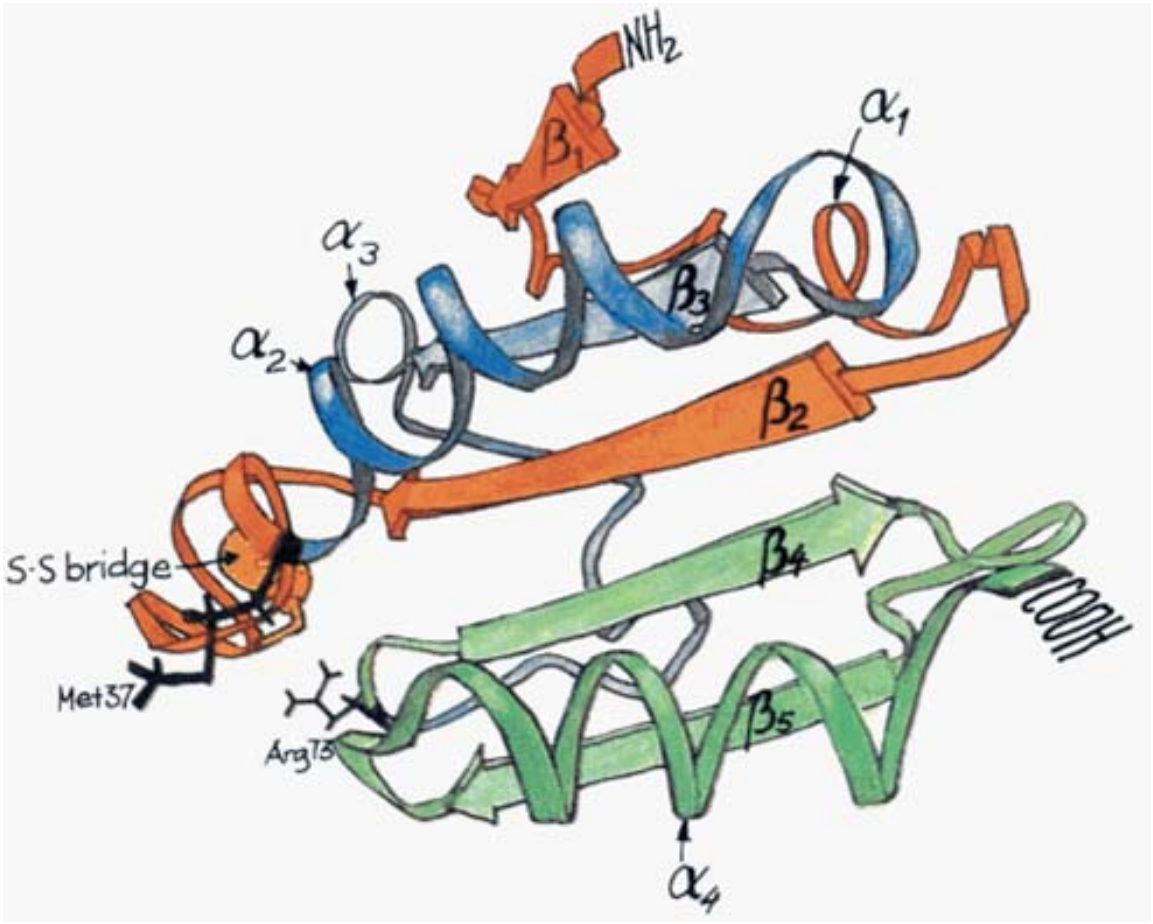


Figure 15. The three dimensional structure of *E. coli* thioredoxin-S_2 from X-ray crystallography at 2.8-Å resolution [37].

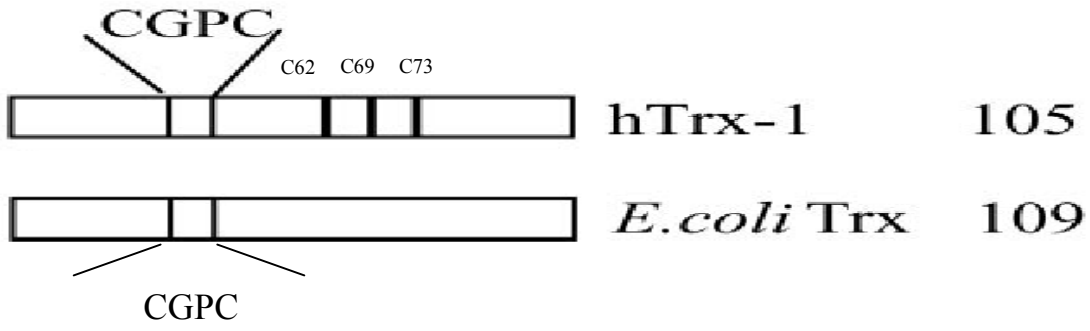


Figure 16. Thioredoxin family members - comparison between human thioredoxin-1 (hTrx-1) and *E. coli* thioredoxin. Thioredoxin exists both with and without the N-terminal methionine and numbering is from the N-terminal methionine. The dark lines show the position of cysteine residues in the protein sequence. Human and other mammalian thioredoxin contain, in addition to the two catalytic site cysteine residues -Trp-Cys32-Gly-Pro-Cys35-Lys, three other cysteine residues, Cys 62, Cys 69, and Cys 73, not found in bacterial thioredoxin [43].

1.5.4.1 The reaction mechanism of thioredoxin

The reaction mechanism of thioredoxin has been the subject of many investigations after the elucidation of the amino acid sequence and three-dimensional structure obtained in the past century. In the mechanism of reduction reaction of thioredoxin, one can observe a very systematic action in parallel with its orderly conformation. A covalently linked mixed disulfide bond (-Cys_{32}-S-S- protein) is formed at the beginning when the disulfide protein substrate binds to a preserved hydrophobic surface and is combined with the nucleophilic thiolate of cysteine 32. Ultimately, the covalently linked mixed disulfide bond –Cys_{32}-S-S- protein is assaulted by the deprotonated cysteine 35, immediately with the release of the reduced protein substrate and the formation of Trx-Cys_{32}-Cys_{35}-disulfide bond so that the dithiol of the two cysteine residues can be regenerated afterwards by thioredoxin reductase [43].

The active site surface in thioredoxin is apparently designed to fit many proteins substrates. Thioredoxin not only uses a chaperone-like mechanism of conformational changes to bind different proteins, but it also promote high rates of disulfide reduction by fasting thiol disulfide exchange chemistry in a hydrophobic environment. In addition to its reducing property, the thioredoxin system is also involved in a variety of cellular redox reactions such as protein folding, transcription regulation by acting as the subunit of the DNA polymerase of some viruses [37] and as growth factors for a variety of cells. Therefore, the thioredoxin system plays very important roles in the regulation of different cellular processes and they are discussed below.

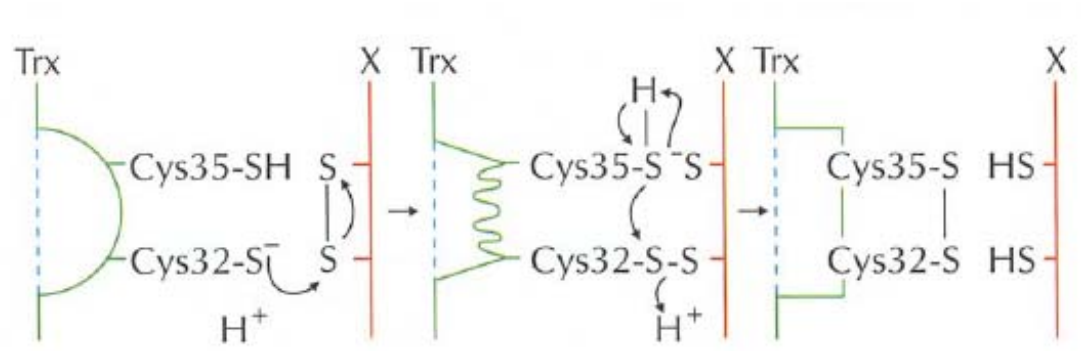


Figure 17. Postulated mechanism of thioredoxin-catalyzed protein disulfide reduction. Reduced thioredoxin binds to a target protein through its hydrophobic surface area. Nucleophilic attack by the thiolate of Cys 32 results in formation of a transient mixed disulfide, which is followed by a nucleophilic attack of the deprotonated Cys 35 generating Trx-S_2 and the reduced protein [47].

1.6 Functions of the thioredoxin system

Thioredoxin system has a large and growing number of functions. It acts as a protector protein against malicious oxidant-provoked intermolecular and intramolecular disulfide bond formation [48]. It is also known for its function as a growth factor, an antioxidant, an enzyme cofactor (particularly for ribonucleotide reductase), and in inhibiting or preventing apoptosis.

1.6.1 Synthesis of deoxyribonucleic acid (DNA)

It was first characterized as the hydrogen donor for the enzymatic synthesis of deoxyribonucleotides by ribonucleotide reductase, an essential biological catalyst which makes the deoxyribonucleotide precursors of DNA from the corresponding ribonucleotides and NADPH in *E. coli*. The S-S-bond of oxidized thioredoxin (thioredoxin-S_2) is reduced by NADPH and thioredoxin reductase.

$$\text{Thioredoxin-(SH)} + \text{rNDP} \xrightarrow[\text{reductase}]{\text{ribonucleotide}} \text{thioredoxin-}S_2 + \text{dNDP} + H_2O$$

The reduced thioredoxin (thioredoxin-$(SH)_2$) then serves as an efficient hydrogen donor for the reduction of ribonucleotides (rNDP) to deoxyribonucleotides (dNDP) by ribonucleotide reductase of *E. coli* and many other species [37].

1.6.2 Growth factor

Thioredoxin and its homologue, adult T-cell leukemia-derived factor (ADF) have been shown to promote cell growth [49]. Many cells such as lymphocytes, fibroblasts, hepatocytes and especially various human carcinoma cells overexpress and secrete thioredoxin. However, little is known about the over-expression and secretion mechanism [42,43]. The mechanism is believed to be different to the common endoplasmic-Golgi Body secretary pathway that it seems to be secreted by a leaderless pathway [28,43]. Subsequently, the released thioredoxin acts as a cytokine-like or chemokine-like factor [28].

Powis, G. *et al* had demonstrated that normal fibroblasts, lymphocytes and several human solid and leukemic tumor cells lines could still survive in a minimal amount of medium without the supply of serum. These cells were stimulated to grow and to proliferate by the addition of exogenous thioredoxin [42,43,50]. The study suggested that thioredoxin system is significant in helping cells to survive in stress environments by carrying out growth-promoting effects.

1.6.3 Protection from oxidative challenge

Oxidative challenges induced by reactive oxygen species are damaging to intracellular components and consequently lead to a number of pathological illnesses such as cardiovascular diseases, cancers, neurological disorders, diabetes, stroke, senile cataract formation, and aging [2,5,10,39].

Examinations of the serum thioredoxin level in patients suffered from oxidative stress such as those who are HIV-positive show an extraordinary elevation of the thioredoxin level [28,51]. Thioredoxin reductase is also abundantly expressed on the surface of human keratinocytes and melanocytes which have been proposed to offer the skin's first line of protection against free radicals caused in response to ultraviolet light irradiation [6].

Thus the thioredoxin system has long been known and believed to be a powerful system which plays a critical role in maintaining cells in a reduced state. It is able to remove H_2O_2, particularly when it is coupled with either methionine sulfoxide reductase by acting as a hydrogen donor or thioredoxin peroxidase systems by acting as an electron donor [46,48].

The thioredoxin system seems to exercise its antioxidant properties in organisms via thioredoxin peroxidase (Figure 18) [43]. The thioredoxin peroxidases, also known as peroxiredoxins (PRDXs), have been recently revealed to be members of the conserved antioxidant proteins capable of using the thyl groups as reducing equivalents to reduce

peroxides such as H_2O_2 and alky peroxides directly. In the process of scavenging oxidant species, the reduced monomeric form of thioredoxin peroxidase forms homo or heterodimer with other family members through disulfide bonds formation between the conserved Cys residues. Later on, reduced thioredoxin, in particular the mitochondrial specific Trx-2 specifically reduces the oxidized thioredoxin peroxidase back to the functional monomeric form [26,43,44].

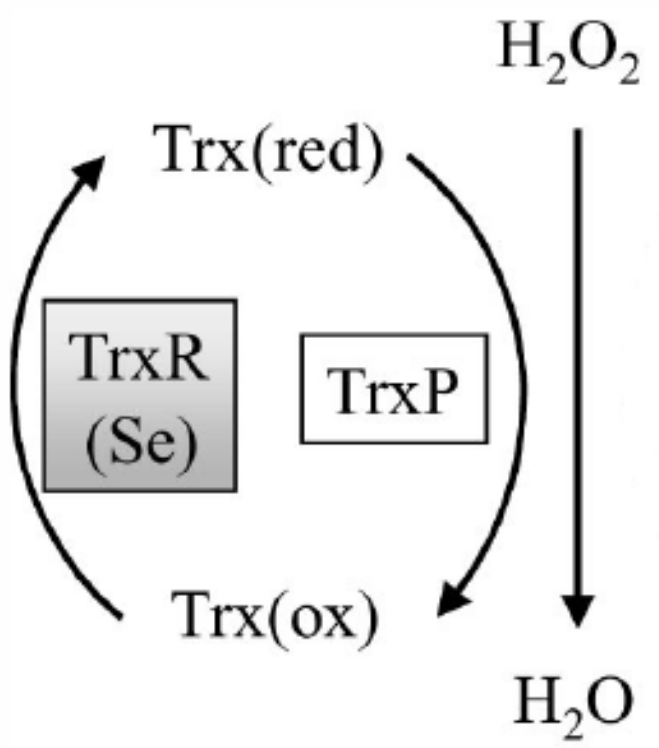


Figure 18. The catalytic cycle of thioredoxin peroxidase which involves reduced (red), thioredoxin oxidized (ox) thioredoxin, and the selenoproteins (shown shaded) thioredoxin reductase [43].

1.6.4 Inhibition and prevention of apoptosis

Apoptosis is a progression of programmed cell death which has its own specific and distinct morphological and biochemical hallmarks. It is characterized by distinct morphological changes that include cell rounding, membrane blebbing, phosphatidylserine (PS) exposure, cytoskeletal disassembly, formation of pyknotic bodies of condensed chromatin, nuclear condensation and DNA fragmentation [52,53]. These dramatic cellular alterations intervene various cellular processes such as normal cell turnover and tumor regression [42].

Exogenous thioredoxin was shown capable of preventing the apoptosis of lymphoid cells grown in culture medium without L-cysteine and glutathione [43]. Previous findings in mouse WEHI7.2 thymoma-derived cells transfected with human thioredoxin cDNA showed a 1.8 times increase in the amount of thioredoxin mRNA and also an increased level of thioredoxin. Such cells when artificially exposed to different apoptotic chemicals such as dexamethasone, staurosporine, etoposide and thapsigargin were shown to have less apopotosis [42,43].

On the other hand, when thioredoxin transfected cells were inoculated into severe combined immunodeficient (SCID) mice, the tumors that were formed grew more promptly and had a smaller amount of natural programmed cell death than the wild-type cell tumors and the anti-apopotosis oncogene bcl-2 transfected cells [42,43].

Thioredoxin system is essential for the maintenance of cellular thiol redox balance and is critical for cell survival [54]. It has been known to exert anti-apoptotic functions and extensively studied as regenerative machinery for many proteins inactivated by oxidative stress as severe oxidative stress has been implicated in the oxidation of proteins and cell death [55,56]. However, how the cellular thioredoxin system is regulated and its correlation to oxidants in cells is poorly understood. An investigation of the effects of different concentrations of oxidants on the thioredoxin system enzyme expression, activities and roles in cellular proliferation of mammalian cells should be helpful in providing more insights in the regulation of thioredoxin system and is therefore proposed in this study.

1.7 Objectives

Like other aspects of life, oxygen metabolism requires tradeoffs [13]. Cellular components such as DNA, protein, and lipid are highly susceptible to oxidation by one or more chemically very reactive oxidant by-products from normal oxygen metabolism and this leads to extensive damages to the morphologies and normal functional roles of cells. Such damages are the main contributors to aging and other pathological disorders such as cancers, cardiovascular diseases, immune-system decline, brain dysfunction, and cataracts [13]. Different antioxidant defenses therefore have been evolved in cells to combat against such damages. Thioredoxin system is one of these antioxidants defenses that is of particular interest due to its versatility in counteracting oxidative challenges. However, the mechanism by which the eukaryotic cell thioredoxin system senses and responds to redox conditions and how this system is regulated *in vivo* is not well understood.

In our previous investigation (unpublished data), the endogenous activity of the thioredoxin system of erythrocytes was found to be stimulated by hydrogen peroxide. A regulatory system that did not require *de novo* protein synthesis was implied. It is therefore worthy to broaden our examinations to other nucleated cells to see if same observation could be made in nucleated cells.

In my study, HeLa cells and HT–29 cells were challenged by different concentrations of hydrogen peroxide. Their viabilities were measured by MTS Tetrazolium reduction assay and the trend of the dosage-dependent toxicity that hydrogen peroxide inflicted onto the

cells was observed. Afterwards, they were harvested and their cytosolic extracts were prepared to check the expression and RNA levels of the thioredoxin reductase and thioredoxin. The functional role of the thioredoxin system of the cells after treatment with hydrogen peroxide was characterized and examined by carrying out the insulin dependent DTNB activity assay and the immunoglobulin reduction assay. In our notion, the thioredoxin system should participate a role in the response to oxidative challenge. Hence the aim of this study is to find out whether oxidative stress would influence the functional roles and also possibly exert a direct endogenous activation of the thioredoxin system in cells.

We hope that this study would contribute to a better understanding of the regulatory mechanism of the thioredoxin system and help pharmaceutical development in opening an area to intervene aging and to treat many degenerative and malignant diseases.

2. **Materials and Methods**

Trypsin and penicillin/ streptomycin were purchased from Gibco invitrogen. β-NADPH, Horseradish peroxidase-conjugated (HRP conjugated) anti-IgG antibody and mouse IgG antibody were purchased from Sigma. The enhanced chemiluminescence reagent solution (34080 Supersignal® West Pico Chemiluminescent substrate, 500 mL) was purchased from Thermo Fisher Scientific (Hong Kong) Limited. All buffer solutions were prepared in deionized water. All the chemicals and reagents were standard commercial products of analytical grade.

2.1 Study of thioredoxin system under oxidative stress

Fast growing cells [57] HeLa cells and HT–29 were chosen as the cell models to study the effects of thioredoxin system response under oxidative stress.

2.1.1 The culture of cell lines

The human cervical epithelial carcinoma cell line, HeLa, was grown in Gibco RPMI Medium 1640 (1X) purchased from Invitrogen, supplemented with 10% (v/v) heat-inactivated fetal calf serum and 100 units/mL penicillin/streptomycin. HT–29, a human colorectal carcinoma cell line, was grown in Dulbecco's Modified Eagle Medium (1X) purchased from Invitrogen, supplemented with 10% (v/v) heat-inactivated fetal calf serum and 100 units/mL penicillin/streptomycin. The cells were grown at 37℃ in an

incubator in a humidified atmosphere of 5% CO_2 and 95% air. The cell culture medium was changed every two days.

2.1.2 Oxidative stress treatment onto cells

To observe the influences of various concentrations of oxidants on cells' thioredoxin system, the cells were cultured to 90% confluence in cell culture flasks. They were then seeded in plates with their respective growing conditions with each well contained about 5000 viable cells in 96-well plates for the MTS tetrazolium assay and 0.4×10^6 viable cells in six-well plates for cell extract preparation. Afterwards, the cells were treated with different concentrations of hydrogen peroxide and incubated at 37℃ in a humidified atmosphere of 5% CO_2 and 95% air for three hours or eight hours.

2.1.3 Cell harvest and preparation of cytosolic extracts

Cell pellets were collected by a two-minute trypsinization and centrifugation at 1500 *g* for 5 minutes. The collected cell pellets were then washed with ice-cold phosphate buffered saline (PBS). Cell extracts were prepared using Total Protein Extraction Kit purchased from KeyGen Biotech, which contains cell lysis buffer, phosphatases inhibitor, protease inhibitor and phenylmethylsulphonyl fluoride (PMSF) at 4℃ for twenty minutes. Subsequently, they were centrifuged at 14000 *g* at 4℃ for fifteen minutes and supernatants were collected. The protein concentrations were determined by Bradford Method using bovine serum albumin (BSA) as standard with detailed procedures listed

below. The cell extracts were stored at -80℃ for future use.

Eight dilutions of the protein standard were prepared, which covered the linear range from 0 μg to 14 μg of protein. Protein solutions were assayed in duplicates or triplicates. 160 μL of each standard or sample solution were pipetted into separate microtiter plate wells. 40 μL aliquots of dye reagent concentrate were subsequently added to each well. The samples and reagents were mixed thoroughly using a microplate mixer and incubated at room temperature for at least 5 minutes. Full colour development usually took ten minutes at room temperature. The absorbance was then read at 595 nm using a multiplate reader from Bio-Rad (Model 680 Microplate Reader) within one hour of the assay (Table 6 and Figure 19).

Table 6. Bradford assay for microtiter plates.

Protein (µg)	**dd H_2O (µl)**	**BSA (0.1 mg/mL)** **Bovine Serum Albumin Acetylated**
0	160 µL	0 µL
2	140 µL	20 µL
4	120 µL	40 µL
6	100 µL	60 µL
8	80 µL	80 µL
10	60 µL	100 µL
12	40 µL	120 µL
14	20 µL	140 µL
16	0 µL	160 µL
Each sample were added with 40 µL Bradford solution concentrate to make up the final volume to 200 µL.		

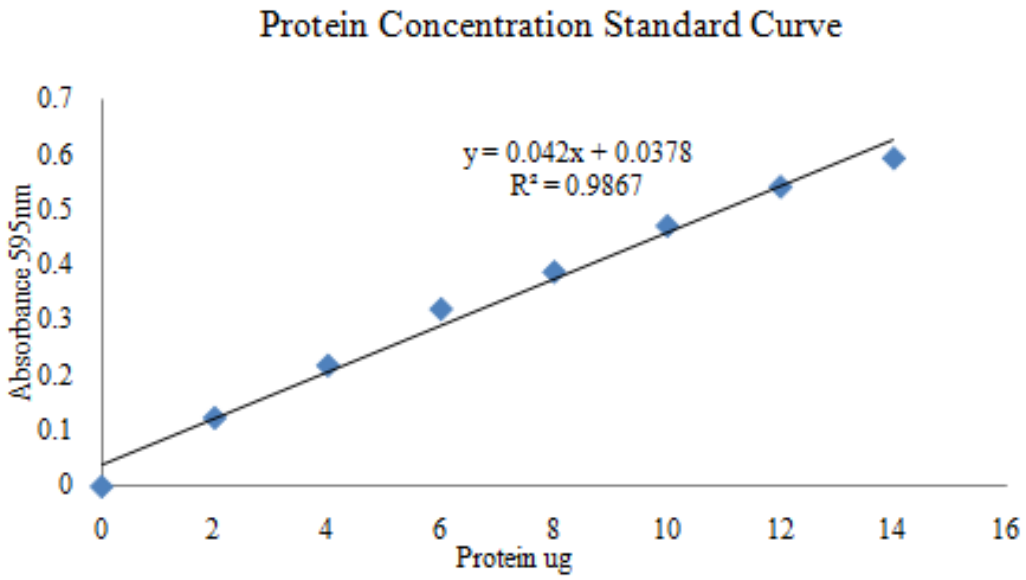


Figure 19. Bradford assay standard curve for microtiter plate format with bovine serum albumin as standard. The assay covered the protein determination from 0 µg to 14 µg in 200 µL used in this microtiter plate format.

2.1.4 Cell viability (MTS tetrazolium reduction) assay

[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) tetrazolium reduction assay is a colorimetric method for quantifying the number of viable cells in cytotoxicity assay. The yellow MTS Tetrazolium compound is converted by the mitochondrial dehydrogenase enzymes of viable cells at 37℃ into a purple coloured product, formazon which can be measured spectrophotometrically at 490nm with a 96-well plate reader. The amount of formazan produced is directly proportional to the number of viable cells present in the assay [58].

Briefly, 10 μL of MTS reagent were pipetted into each well containing the 5000 cells treated with various concentrations of hydrogen peroxide in 100 μL medium. After two hours of incubation at 37℃ in a humidified atmosphere of 5% CO_2 and 95% air, the quantity of the soluble aqueous formazan formed by viable cells was measured immediately at 490nm using a multiplate reader from Bio-Rad (Model 680 Microplate Reader). The results are expressed as relative viability by the equation: Relative viability (%) = $(A_{490nm\ of\ treated\ cells})/ (A_{490nm\ of\ Control}) \times 100\%$.

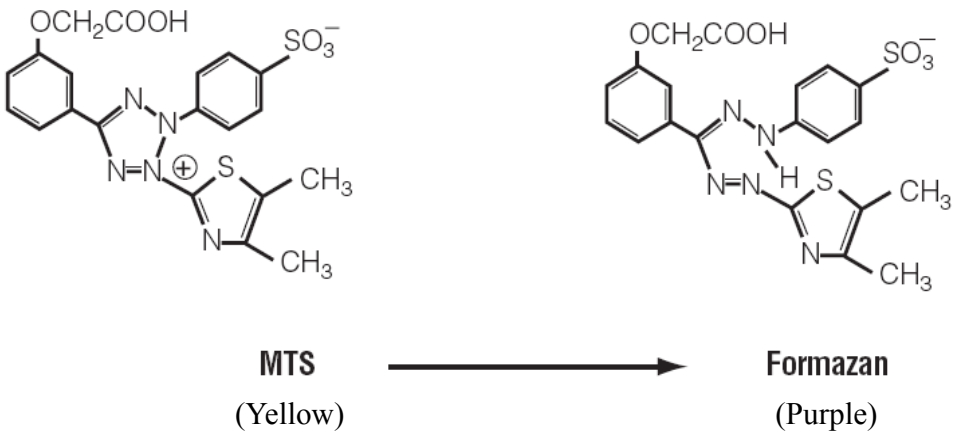


Figure 20. The structures and names of the MTS tetrazolium and the corresponding formazan product [58].

2.1.5 Western blot analysis of thioredoxin and thioredoxin reductase

Proteins in cells lysates were separated by 15% SDS-PAGE and transferred to polyvinylidene-difluoride (PVDF) membranes. The membranes were probed with rabbit anti-Trx or anti-TR antibodies diluted in 1:4000 in 1× TTBS buffer and then treated with goat anti-rabbit Ig G-hourseradish peroxidase purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Immunodetection was performed with enhanced chemiluminescent reagent solution (Supersignal® West Pico Chemiluminescent substrate) for ten minutes and then analyzed by imaging software.

2.1.6 Extraction of total RNA and detection of mRNA for thioredoxin reductase and thioredoxin by RT-PCR

Total mRNA was extracted from the cells with RNA Extraction mini kit (Favorgen) according to the manufacturer's protocol. In general, 0.5×10^6 cells were pelleted by centrifugation at 300 *g* for five minutes. All the supernatants were removed. Afterwards, 350 μL of FARB Buffer were added to the cell pellet and vortexed vigorously to lyse the cells, and then the sample mixtures were incubated for five minutes at room temperature. Filter columns were placed into collection tubes and the sample mixtures were applied onto the respective filter column, centrifuged at full speed (14,000 rpm or 10,000 *g*) for two minutes. The clarified supernatant from each collection tube was transferred to a new microcentrifuge tube and the volume of the clear lysate was adjusted. 1 volume of 70% ethanol was added to the clear lysate and mixed well by vortexing.

The tubes were briefly spun to remove drops from the inside of the lid. The samples (including any precipitate) were transferred to FARB Mini Columns that had been placed into respective Collection Tubes. They were centrifuged at full speed (14,000 rpm or 10,000 *g*) for 1 min and the flow-through were discarded. 500 μL of Wash Buffer 1 were added to the FARB Mini Columns and centrifuged at full speed (14,000 rpm or 10,000 *g*) for one minute and the flow-through were discarded. 750 μL of Wash Buffer 2 were added twice to the FARB Mini Column and centrifuged at full speed (14,000 rpm or 10,000 *g*) for one minute and the flow-through were discarded. Centrifugation at full speed (14,000 rpm or 10,000 *g*) was carried out for an additional three minutes to dry the

columns. FARB Mini Columns were placed into Elution Tubes. 50 μL of RNase-free ddH_2O were added at the center of the filter membrane and the FARB Mini Columns were left to stand for one minute to make sure that RNase-free ddH_2O was dispensed on the membrane center and was absorbed completely for effective elution. Centrifugation at full speed (14,000 rpm or 10,000 *g*) was carried out for two minute to elute RNA, which was then stored at -70℃ for future use.

Aliquots containing equal amounts (0.5 μg) of mRNA were reverse transcribed with Omniscript RT Kit (QIAGEN). The following primers were designed and synthesized to detect Trx, TR, and GADPH cDNA respectively.

Trx, (forward) 5'-CTGCTTTTCAGGAAG CCTTG-3',

Trx, (reverse) 5'-CGCAGATGGCAACTGGTTA-3';

TR, (forward) 5'- GCCCTGCAAGACTCTCGAAATTA-3',

TR, (reverse) 5'- GCCCATAAGCATTCTCATAGACGA-3';

GADPH, (forward) 5'-TGAACGGGAAGCTCACTGG-3',

GADPH, (reverse) 5'-TCCACCACCCTGTTGCTGTA-3'.

The sizes of the amplification products of Trx, TR, and GADPH were 392, 337, and 306 bp respectively. Equal amounts of synthesized cDNA were amplified by PCR with *Taq* DNA polymerase (QIAGEN) to detect mRNA for Trx, TR, and GADPH. The conditions used for PCR were 94°C for 0.5 minute, 55°C for 0.5 minute, and 72°C for 1 minute for 29 cycles. Aliquots were taken from PCR mixtures and analyzed by 1% agarose gel electrophoresis.

2.1.7 Enzyme assays

2.1.7.1 Determination of TR activity by end – point insulin dependent DTNB activity assay in biological samples

This was determined by the insulin dependent reduction assay as described by Arnér, E. S. and Holmgren, A. (2000) [34]. The principle of the activity assay is the fast reaction between reduced form of thioredoxin (Trx-$(SH)_2$) and protein disulfides. In the assay, insulin is used as the substrate in which the three disulfide bridges are involved (Figure 21a). By utilizing the full components of the thioredoxin system, *i.e.* NADPH, thioredoxin and thioredoxin reductase, the reducing equivalent of NADPH can be passed to insulin disulfides via the reaction of the thioredoxin system yielding split chains of insulin with free thiol groups (Figure 21b). The reaction is stopped by adding guanidine hydrochloride and DTNB. The former denatures proteins and inactivates the thioredoxin system activity. The DTNB reagent will then react with free thiol groups formed earlier in the reaction to give yellow colored TNB. When NADPH and thioredoxin are in excess, with the enzyme thioredoxin reductase being the limiting factor, the reaction would then be dependent on the amount of thioredoxin reductase in the system. Thus the thioredoxin reductase amount can be assayed. The scheme of reactions that outlined the basis of the insulin dependent DTNB activity assay is shown in Figure 21b. The assay is based on the net increase of free sulfhydryl groups in the reaction. In cell extracts, there are sulfhydryl containing proteins or other sources of SH-groups, which need to be subtracted by running blank determinations.

In the assay, stock reaction mixture (2000 μL) was prepared by mixing 1295 μL 200 mM HEPES buffer (85 mM final), 625 μL 1.6 mM insulin (0.3 mM final), 40 μL 50 mM NADPH (660 μM final) and 40 μL 0.2 M EDTA (3 mM final). To each sample, 33 μL reaction mixture, Trx (15 μM final) and 40 μg lysate protein were mixed and incubated at 37 ºC for 30 min in 96-well plate. The reaction was stopped by the addition of 200 μL 10 mM (4 mg/mL) DTNB [5,5' –dithio-bis(2-nitrobenzoic acid) in 6 M Guanidine HCl. Absorbance at 405 nm was measured with multiplate reader from Bio-Rad (Model 680 Microplate Reader) while enzyme activity in the assay was expressed as μmol/min/ml of formation of TNB by using the extinction coefficient of 6.35 mM^{-1}. One unit is defined as the NADPH-dependent production of 2 μmol of 2-nitro-5-thiobenzoate per minute.

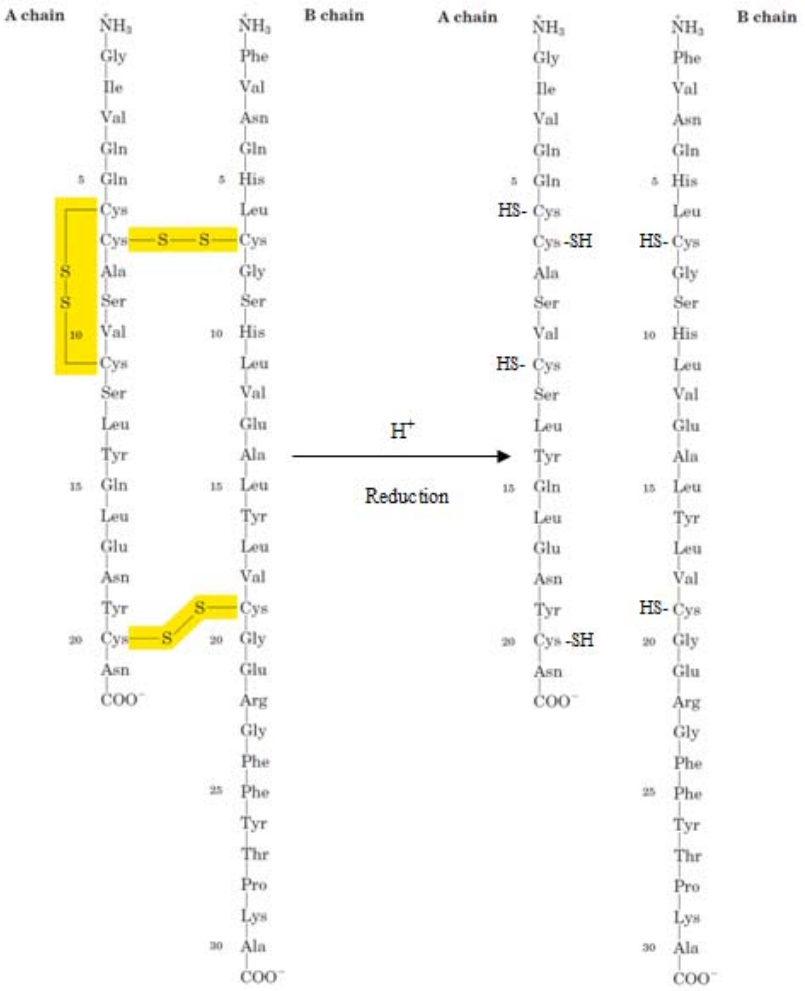


Figure 21a. Amino acid sequence of bovine insulin. The two polypeptide chains are joined by disulfide crosslinkages [59].

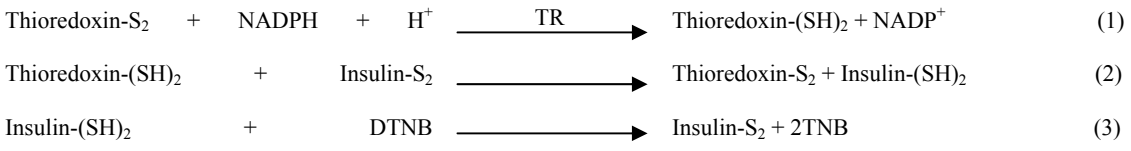


Figure 21b. Reactions sequence of the insulin dependent DTNB activity assay [34].

2.1.7.2 Determination of Trx activity by end – point insulin dependent DTNB activity assay in biological samples

This was determined by the insulin dependent reduction assay as described by Arnér, E. S. and Holmgren, A. (2000) with some minor modifications. The principle of the assay is similar with the one measuring thioredoxin reductase with the only difference in using an excess amount of pure thioredoxin reductase so that thioredoxin becomes the limiting factor to be measured (Figure 21b). Samples from cell lysate were allowed to incubate at 55 ºC for ten minutes to inactivate other sulfhydryl containing proteins in cell extracts since thioredoxin is heat stable protein that can withstand up to 70 ºC and thioredoxin reductase is also stable up to between 60 and 70 ºC. Then, 2 μL of DTT activation buffer (50 mM HEPES, 1 mM EDTA, 1 mg/ml BSA and 2 mM DTT) was added onto each sample. The mixture was incubated at 37 ºC for ten minutes in 96-well plate. Afterwards, 33 μL of reaction mixture (200 mM HEPES at pH 7.6, 20 mM EDTA, 50 mM NADPH, 1.6 mM insulin) was added. The reaction was started by the addition of TR (~50 nM final) and was incubated at 37 ºC for thirty minutes. The reaction was terminated by the addition of 200 μL of 10 mM DTNB/6 M Guanidine HCl, and the absorbance was measured at 405 nm with multiplate reader from Bio-Rad (Model 680 Microplate Reader).

2.1.8 Determination of thioredoxin system activity by immunoglobulin G reduction assay

The principle of the immunoglobulin reduction activity assay (IgG assay) is based on the ability of the thioredoxin system to break down disulfides bonds in immunoglobulin G. In the presence of NADPH, samples with intact thioredoxin system can reduce the protein disulphide bonds between the heavy chains and light chains of IgG molecules during incubation. By subsequent Western blotting analysis, the amount of split chains can be detected and measured. This assay is used to demonstrate whether the samples studied have intact thioredoxin system and how effective they are in the function of reducing large substrate proteins.

2.1.8.1 Mouse IgG is substrate for the intact thioredoxin system

An intact thioredoxin system is composed of thioredoxin which is a 12 kDa protein with two redox-active cysteine residues, thioredoxin reductase and also NADPH. [2,38,51,60] Through an interaction with the redox-active center of thioredoxin which is a general protein disulfide reductant, it can effectively reduce disulfides in small cellular proteins. This property can therefore be further extended and utilized as a molecular probe measurement for the activity of the thioredoxin system [51], for example, in cooperation with large protein molecules like immunoglobulins.

In fact, all immunoglobulins are potential substrates for the reduced thioredoxin because their arrangements are maintained by many interchain and intrachain disulfides between

the heavy and light chain (Table 7) [51]. Previous studies had demonstrated that human monoclonal and polyclonal IgG were substrates for both prokaryotic and eukaryotic thioredoxin system. The thioredoxin system could directly reduce human IgG inter-heavy-light chain and inter-heavy-heavy chain disulfides in a time (Figure 22) and dose-dependent manner with apparent catalytic speed $2 \times 10^4\ M^{-1}sec^{-1}$ [51,57]. Large IgG molecule can therefore be catalyzed by the thioredoxin system as effective as other small molecule such as insulin and employed in activity assay.

Table 7. The characteristics of different human IgG [51].

IgG subclass	Molecular weight	Number of S-S Bridge/ IgG Molecule		
		Inter-H-H chain	Inter-H-L chain	Total
IgG_1	150000	2	2	4
IgG_2	150000	4	2	6
IgG_3	160000	11	2	13
IgG_4	150000	2	2	4

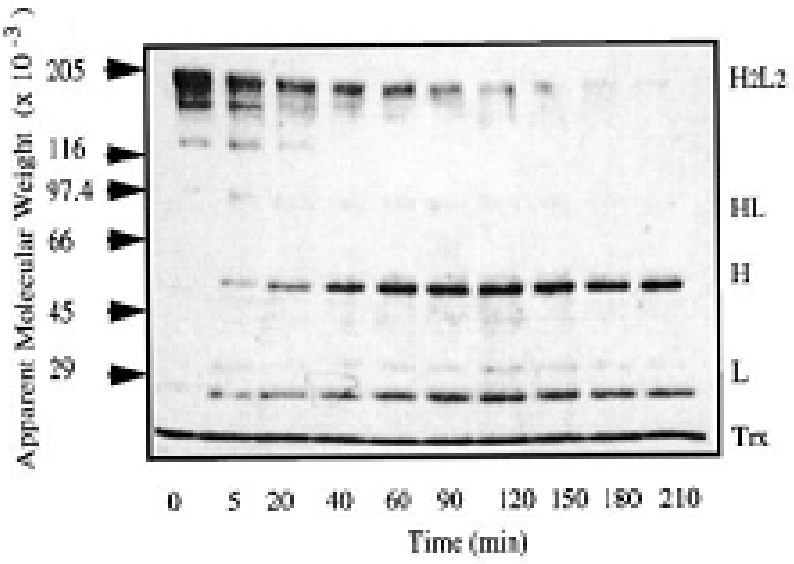


Figure 22. Reduction of polyclonal human IgG by the thioredoxin system [47].

2.1.8.2 IgG reduction assay on cell lysates

The assay was generally performed by mixing and incubating 10 μg protein of cell lysates, 5 μg mouse IgG and 500 μM NADPH at 37°C for one hour. To terminate the reaction, samples were then mixed with the SDS sample buffer without 2-ME with the same volume of the sample mixture and boiled at 100°C in a water bath for 10 minutes, followed by the separation of proteins on a 15% non-reducing SDS-PAGE with dimensions 7cm (L) × 8 cm (W) × 1 mm (T) using mini-PROTEAN II Bio-Rad Gel Electrophoretic Set (Bio-Rad) with constant voltage at 200V for one hour.

Proteins were then transblotted from the 15% SDS gel to a polyvinylidene-difluoride (PVDF) membrane purchased from Bio-Rad for one and half hours at 128V before it was blocked with 5% BSA or skim milk powder overnight. Afterwards, the PVDF membrane was washed at least three times for 5 minutes, each using TTBS and then incubated at 4°C for three hours with horseradish peroxidase-conjugated (HRP conjugated) anti-IgG at a dilution of 1:4000 in 1× TTBS containing 2 % BSA as the secondary antibody. Prior to the visualization of the immunolabeled bands of split IgG heavy chains, the PVDF membrane was washed at least three times for 5 minutes each using TBS and subsequently it was incubated with enhanced chemiluminescence reagent solution (Supersignal® West Pico Chemiluminescent substrate) for ten minutes and finally analyzed by imaging software. The intact thioredoxin system activity of the cells is corresponded to the intensity of split chains of IgG by densitometric measurement [46].

2.1.9 Fluorescence activated cell sorting analysis

Cell cycle analysis of HT–29 cells was carried out to elucidate the stage affected by the oxidative stress in cells. We aimed to see if there could be a correlation of the change of level and activity of the thioredoxin system components with cell cycle. HT–29 cells were seeded into six-well plates at a density of 0.5×10^6 viable cells per well and incubated at 37˚C in an incubator with a humidified atmosphere of 5% CO_2 and 95% air in the presence of a complete medium with different concentrations of hydrogen peroxide (0 mM, 0.05 mM, 0.25 mM, 0.5 mM, 1 mM and 2 mM) for eight hours. Afterwards, the cells were trypsinized, centrifuged at 1500 *g* for 5 minutes to collect the cell pellets, washed twice in PBS and fixed in 70% ethanol.

The fixed samples were stored at -20˚C until analysis, and then centrifuged for one more cycle to collect the cell pellets. The samples were incubated in PI/RNase staining buffer (BD Pharmingen) for 15 minutes at room temperature and protected from light before analysis. Cells containing the respective DNA states were analysed by a flow cytometer (BD Biosciences) and the number of cells in each phase of the cell cycle quantitated by Modfit software.

3 Results

3.1 Culture of cell lines

The cells were cultured under the condition described in section 2.1 of materials and method. In general, when the cells concentrations in each well reached 0.5×10^6, the cultures would be employed for experiments. In the investigation, populations of cells were treated with or without different concentrations of hydrogen peroxide and afterwards cells extracts were isolated for further analysis such as finding out the effect of hydrogen peroxide on the activity or the expression level of thioredoxin reductase and thioredoxin.

3.2 Susceptibility of HeLa cells and HT-29 cells to oxidative stress as determined by MTS tetrazolium assay

In a 96-well plate, each well was seeded with approximately 5000 cells and allowed to grow overnight. Cells were then exposed to different concentrations of hydrogen peroxide (0 mM, 0.05 mM, 0.125 mM, 0.25 mM, 0.5 mM, 0.75 mM, 1 mM, 2 mM and 4 mM) for one, three, five and eight hours in serum containing medium. Cells viabilities were measured by MTS tetrazolium assay.

3.2.1 HeLa cells and HT–29 cells viability upon oxidative stress

To study the effect of oxidative challenge and response of thioredoxin system in nucleated cells, two cell lines, HeLa and HT–29 were investigated. Hydrogen peroxide induced a dose-dependent decline in the relative cell survival rate. The cell viability of the two cell lines reduced slightly in low concentration of oxidative stress (0.05 mM) and markedly in the 0.125 mM to 0.5 mM hydrogen peroxide. In HeLa cells, unusual results were observed at low concentrations of hydrogen peroxide (0.05 – 0.5 mM) with lengthened oxidative challenge (8 hours) that cells survived better than the groups with shorter period of oxidative stress. A general decrease of cell viability was observed at higher concentrations of hydrogen peroxide treatment (1 mM to 4 mM) in both cell lines at all time intervals tested.

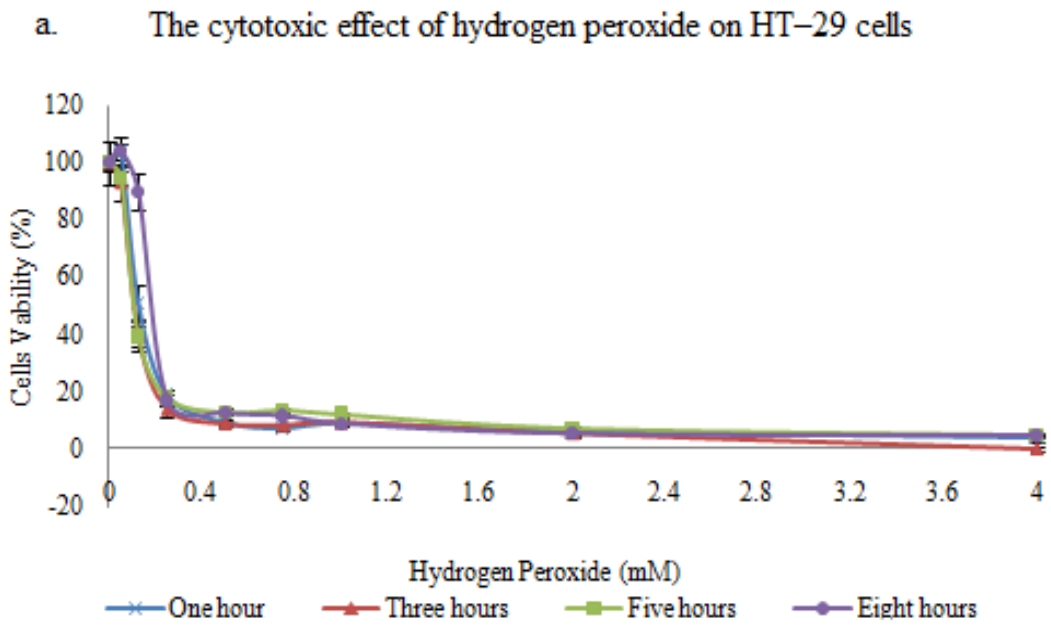


Figure 23a. HT–29 cells survival assessed by MTS tetrazolium assay after treatment with different concentrations of hydrogen peroxide at different time intervals. Data were represented as means ± SEM with n=4.

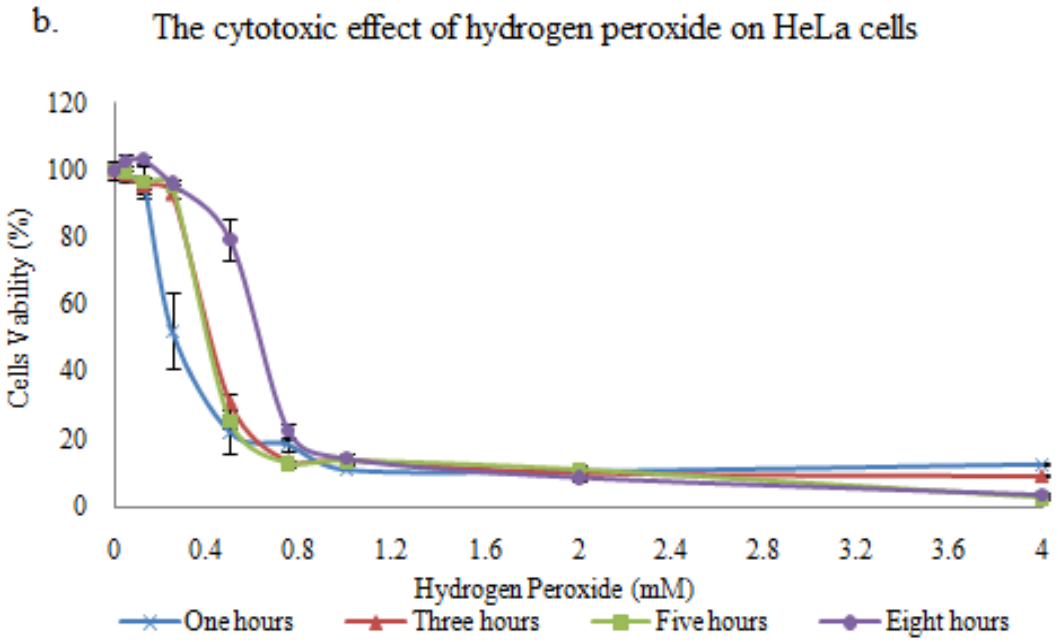


Figure 23b. HeLa cells survival assessed by MTS tetrazolium assay after treatment with different concentrations of hydrogen peroxide at different time intervals. Data were represented as means ± SEM with n=4.

3.2.2 Viability of HeLa cells and HT–29 cells with prior treatment of cycloheximide upon oxidative stress

The viability of HT-29 cells treated with prior treatment of cycloheximide and various concentrations of hydrogen peroxide was measured by the MTS tetrazolium reduction assay. Cycloheximide is an antibiotic that inhibits *de novo* protein synthesis. Cells with such prior treatment should have no transcription though they may be demanded to respond to stimuli. The results illustrated a dose dependent trend of death of cycloheximide treated HT-29 cells towards hydrogen peroxide toxicity (Figure 24a).

However, viability of HT-29 cells, with or without the prior treatment of cycloheximide seemed not to be dependent on the intervals of exposure tested but mainly on the concentrations of hydrogen peroxide.

In addition, with the same concentration of hydrogen peroxide, cell survival was less in cycloheximide treated HT-29 cells. Consequently, it was believed that HT-29 cells with treatment of cycloheximide were more sensitive to the cytotoxic effects of hydrogen peroxide under oxidative stress environment.

On the other hand, the viability of HeLa cells treated with cycloheximide and various concentrations of hydrogen peroxide were also monitored. The results illustrated a general dose dependent trend of cell death of HeLa towards hydrogen peroxide toxicity. However, similar unusual phenomena were observed with lengthened oxidative challenge

that cells seemed to survive better with longer periods of exposure to oxidative stress. Such effect was even more pronounced in cycloheximide treated HeLa cells at higher concentrations of hydrogen peroxide (Figure 23b and Figure 24b). This indicated that cycloheximide treated HeLa cells were not more sensitive to the cytotoxic effect of hydrogen peroxide and *de novo* protein synthesis might not be required for HeLa cell survival under oxidative stress.

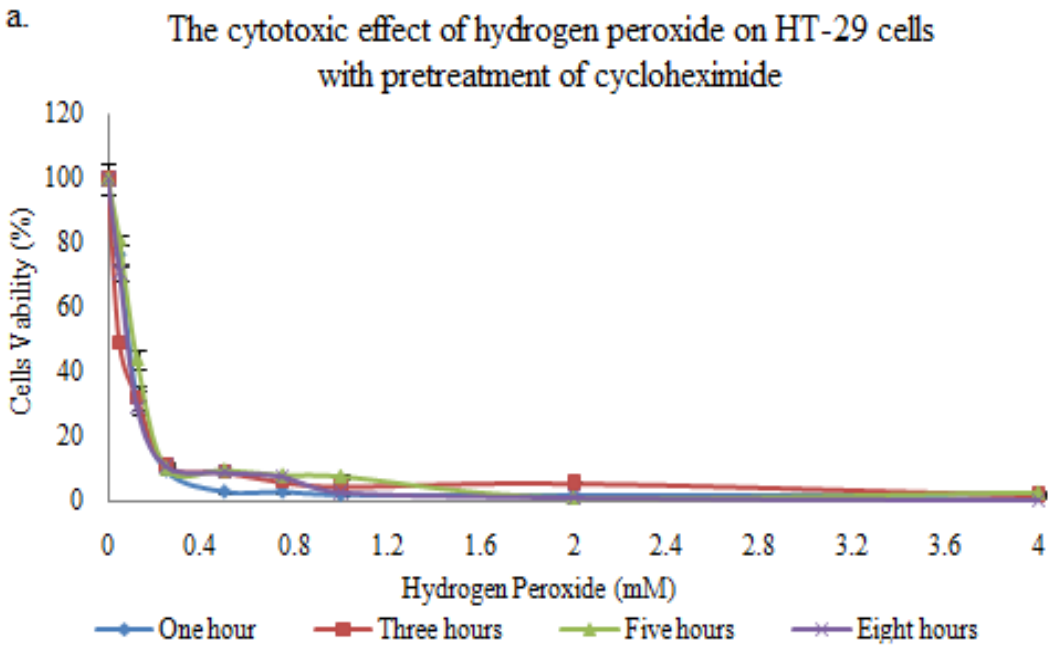


Figure 24a. Cycloheximide pretreated HT-29 cells viability assessed by MTS tetrazolium assay after treatment with different concentrations of hydrogen peroxide at different time intervals. Data were represented as mean ± SEM with n=4.

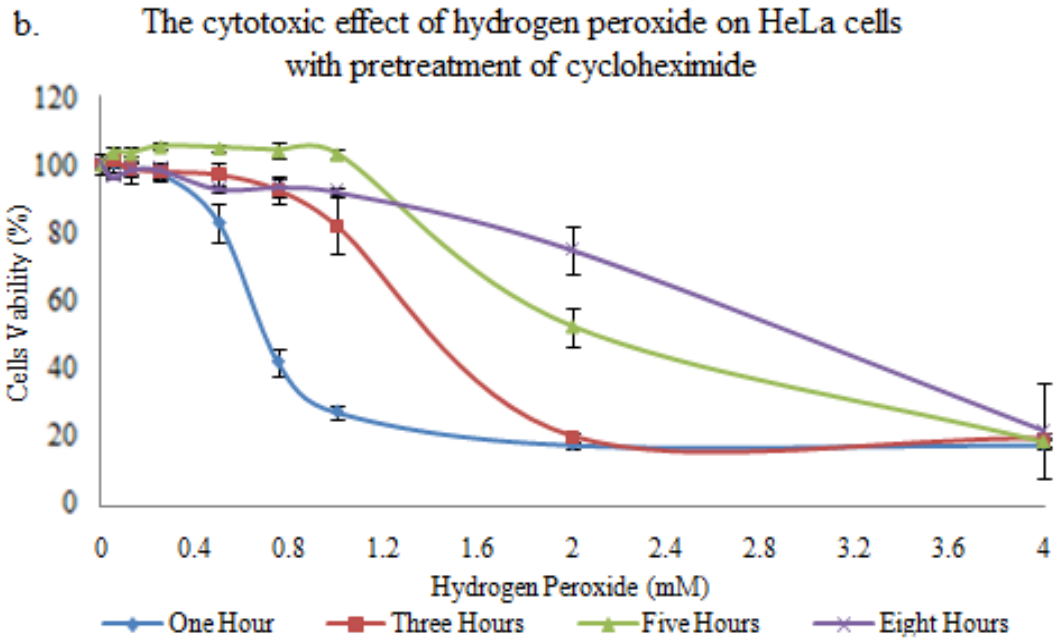


Figure 24b. Cycloheximide pretreated HeLa cells viability assessed by MTS tetrazolium assay after treatment with different concentrations of hydrogen peroxide at different time intervals. Data were represented as mean ± SEM with n=4.

3.2.3 Microscopic investigations of HT-29 cells

To monitor the cytotoxic effect of hydrogen peroxide on HT-29, cell cultures with and without the prior treatment of cycloheximide were examined under inverted light microscope with photomicrographs taken for comparison. Compared with the MTS assay, a similar dose dependent trend of hydrogen peroxide toxicity was confirmed by the morphological observation.

Normal control HT-29 cells were confluent and closely adhered to the substratum. The cells therefore showed a healthy morphology (Figure 25). However, cells exposed to 8 hours of hydrogen peroxide became unhealthy and produced significant morphological changes in size, shape, and elongation of the cells. More than 70% of the cultures without cycloheximide pretreatment became rounded up and detached in cells exposed to 1 to 2 mM hydrogen peroxide for eight hours (Figure 25). A dose dependent trend of hydrogen peroxide toxicity was shown in the study. This phenomenon was more severe in HT-29 cells treated with cycloheximide at all concentrations of hydrogen peroxide tested (Figure 26) as compared with HT-29 cells without cycloheximide treatment. It implied that HT-29 cells with treatment of cycloheximide were more sensitive to the cytotoxic effects of hydrogen peroxide under oxidative stress conditions.

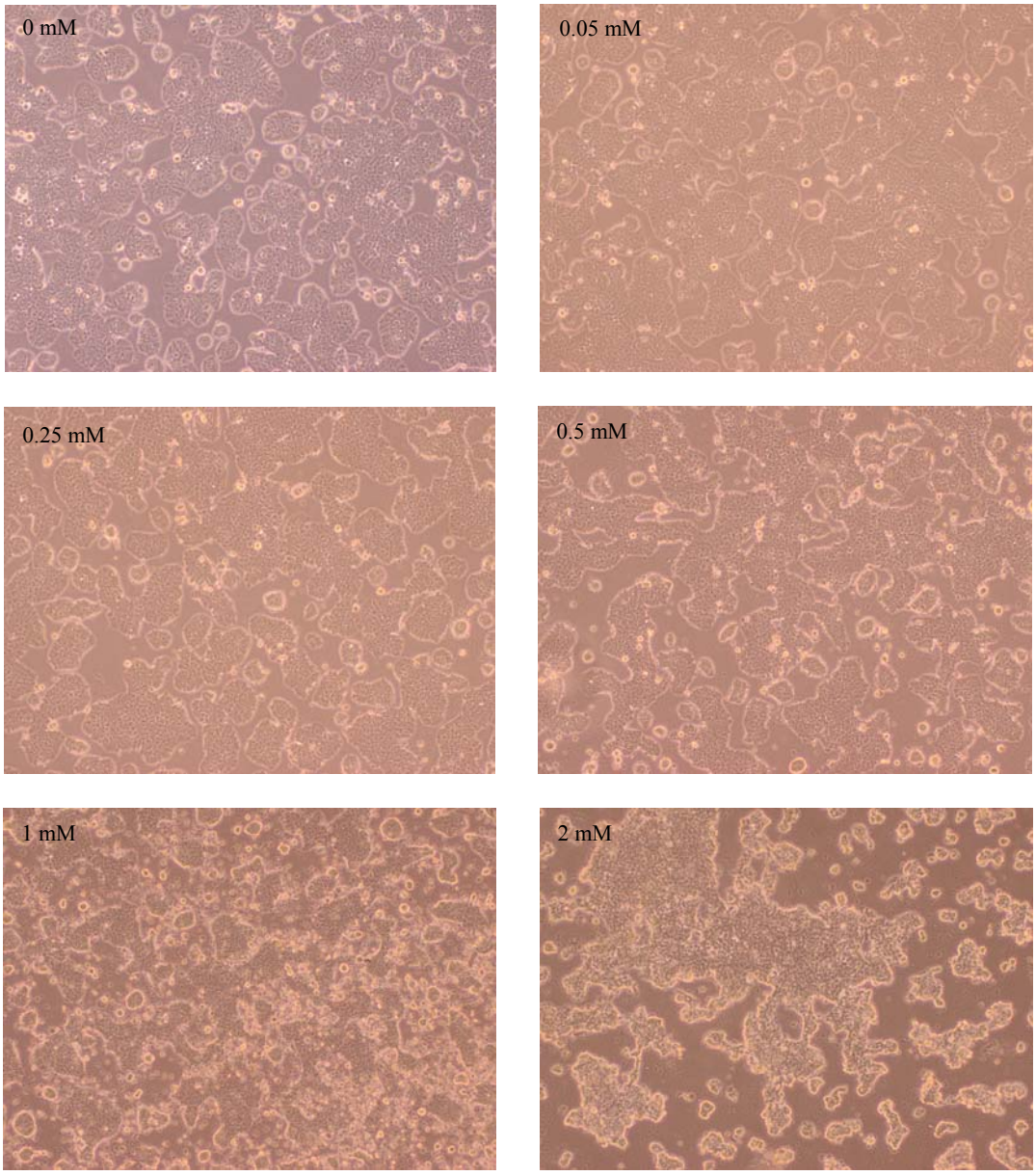


Figure 25. Photomicrographs of HT-29 cells exposed to different concentrations of hydrogen peroxide (0 mM, 0.05 mM, 0.25 mM, 0.5 mM, 1 mM and 2 mM as indicated in the respective micrographs) without prior treatment of cycloheximide. The data were from one representative experiment.

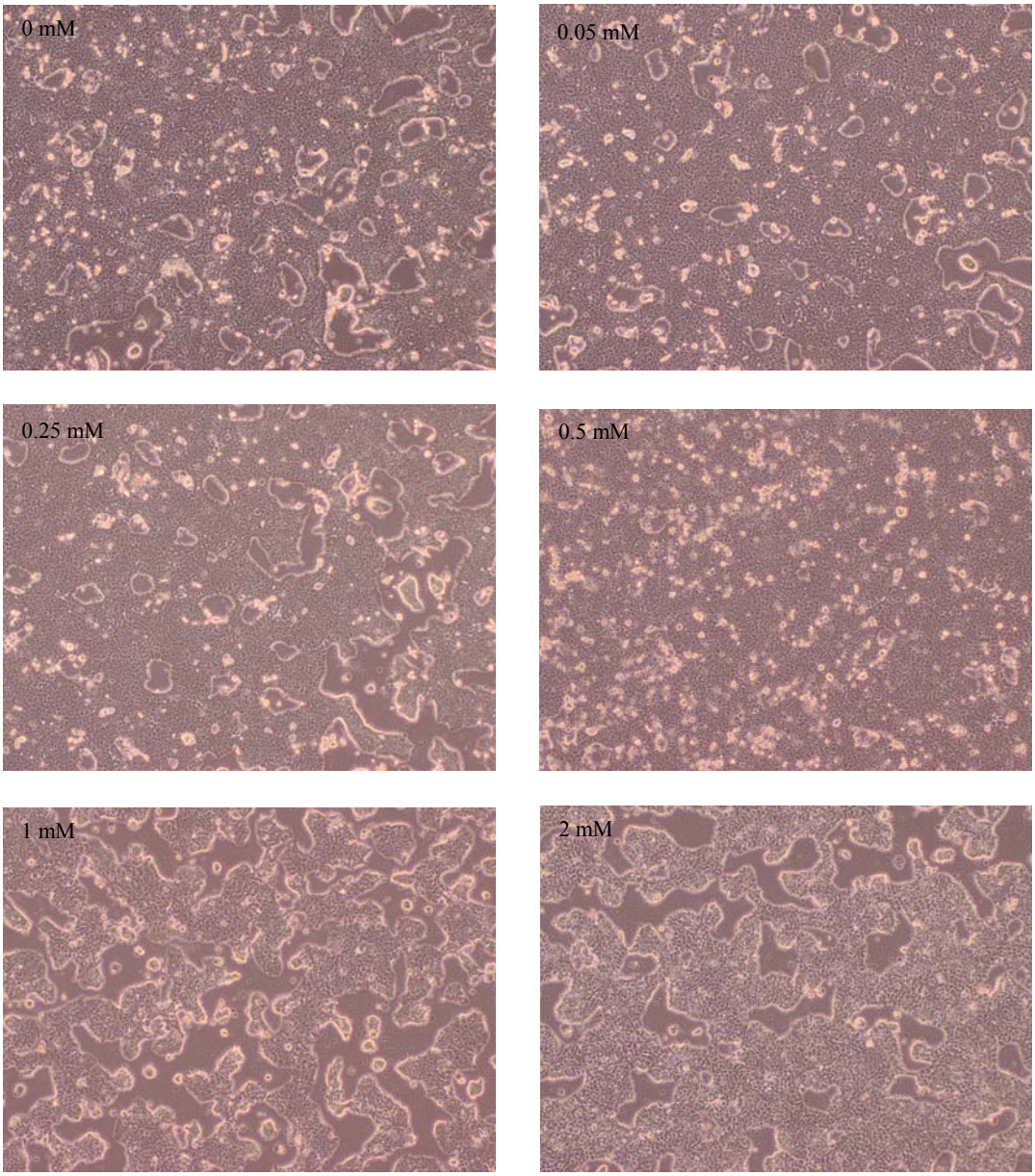


Figure 26. Photomicrographs of HT-29 cells exposed to different concentrations of hydrogen peroxide (0 mM, 0.05 mM, 0.25 mM, 0.5 mM, 1 mM and 2 mM as indicated in the respective micrographs) with prior treatment of cycloheximide. The data were from one representative experiment.

3.2.4 Microscopic investigations of HeLa cells

Compared with the MTS assay, a dose dependent trend of hydrogen peroxide toxicity in the results was confirmed by the morphological observation. The cells treated with 0.5 to 4 mM hydrogen peroxide without cycloheximide illustrated reduction of cell bodies, loss of neuritis and damaged network (Figure 27), while the control group still presented great vigor, with obvious cell bodies, smooth and long progressions linked in a network.

Unusual results observed again in cycloheximide pretreated cells exposed to 0.25 to 1 mM hydrogen peroxide (Figure 28). They appeared healthier than “normal control group” with obvious cell bodies and networks.

With reference to the results of cell viability, it was believed that HT–29 cell line would be a more rational model for our proposed study instead of HeLa. We therefore focused our work on HT-29 and used the range of 0 mM to 0.5 mM hydrogen peroxide for the study of oxidative challenge, in which HT-29 cells would receive considerable amount of oxidative stress but still be able to survive and respond towards the challenges.

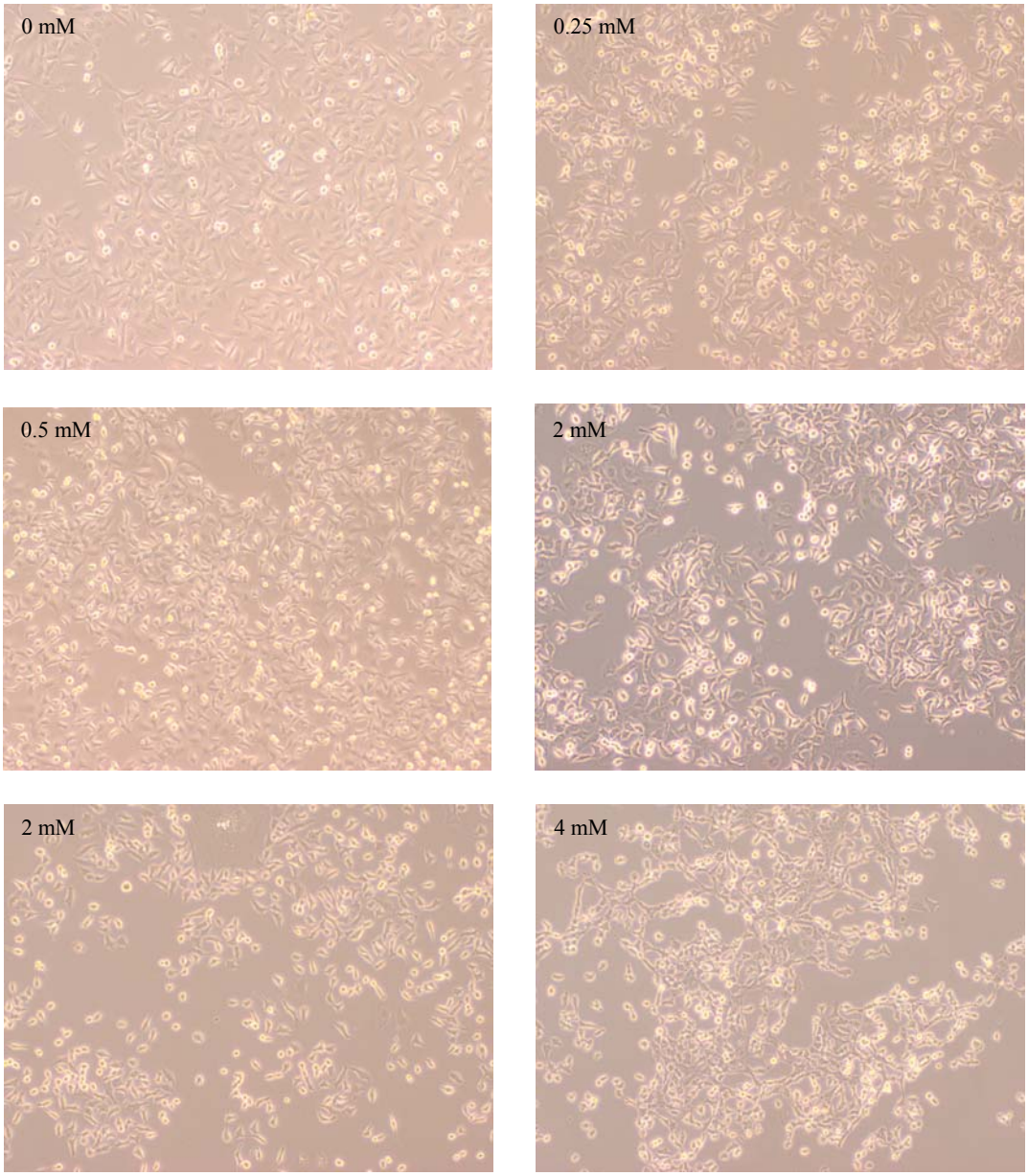


Figure 27. Photomicrographs of HeLa cells exposed to different concentrations of hydrogen peroxide (0 mM, 0.25 mM, 0.5 mM, 1 mM, 2 mM and 4 mM as indicated in the respective micrographs) without prior treatment of cycloheximide. The data were from one representative experiment.

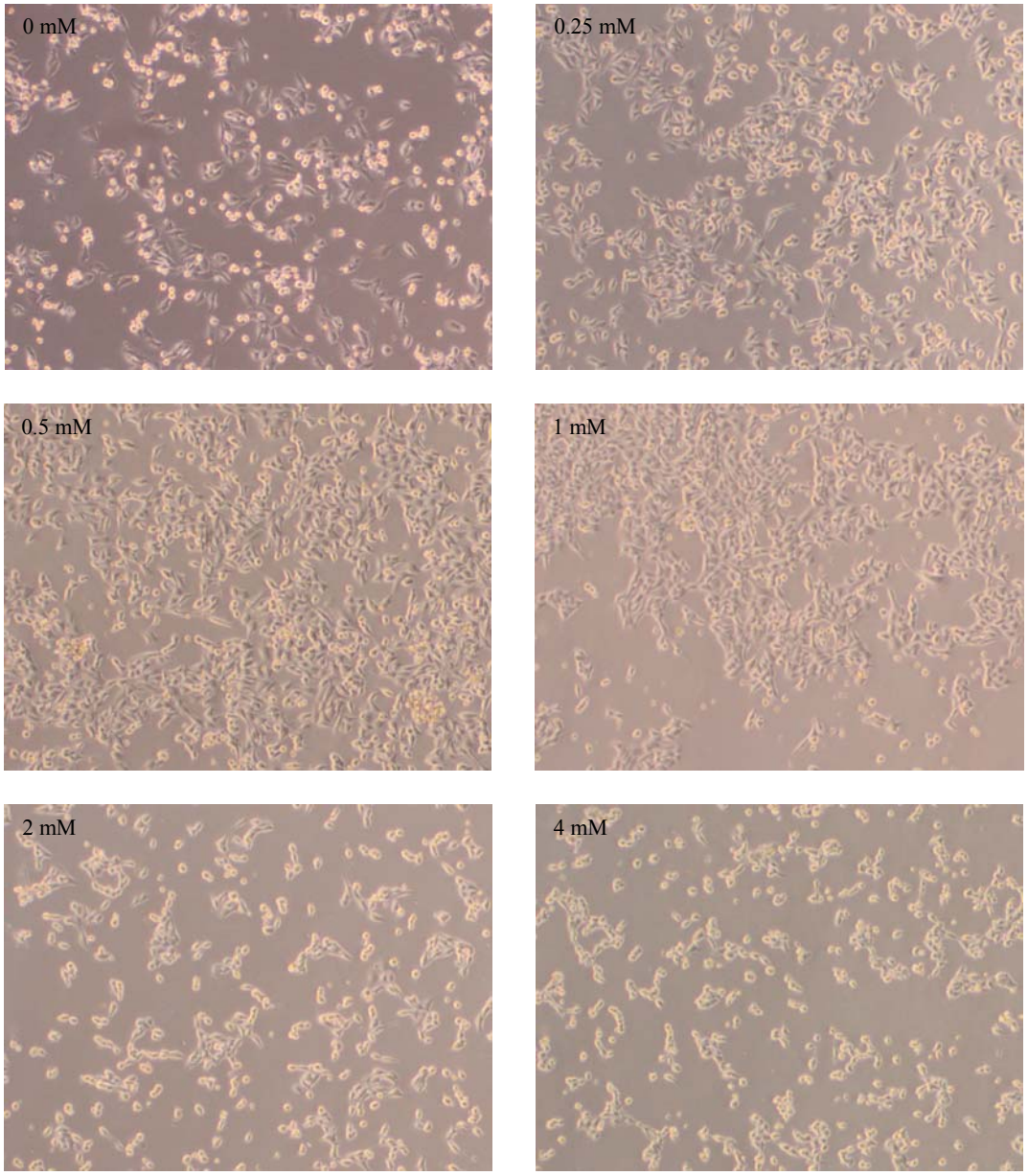


Figure 28. Photomicrographs of HeLa cells exposed to different concentrations of hydrogen peroxide (0 mM, 0.25 mM, 0.5 mM, 1 mM, 2 mM and 4 mM as indicated in the respective micrographs) with prior treatment of cycloheximide. The data were from one representative experiment.

3.3 Western blot analysis of thioredoxin reductase and thioredoxin in HT-29 cells upon oxidative challenge

To investigate the expression of thioredoxin reductase and thioredoxin upon oxidative stress, the level of thioredoxin reductase and thioredoxin in HT-29 cell challenged with various concentrations of hydrogen peroxide was determined.

Thioredoxin expression increased in 0.05 to 0.5 mM hydrogen peroxide treated cells without the prior treatment of cycloheximide. Cells treated with higher concentrations of hydrogen peroxide (1 and 2 mM), however showed a general decrease in the level of thioredoxin. On the other hand, there was an insignificant increase in thioredoxin reductase expression in 0.05 to 0.5 mM hydrogen peroxide treated cells and a decline in the thioredoxin reductase level in those cells treated with higher concentrations of hydrogen peroxide (1 – 2 mM). The steady beta actin expression indicated an equal amount of proteins used in this analysis.

In cells pretreated with cycloheximide, thioredoxin and thioredoxin reductase expression remained constant with control in 0.05 to 0.5 mM hydrogen peroxide and then slightly decreased in 1 to 2 mM hydrogen peroxide. Again, the steady beta actin expression indicated an equal amount of proteins used in this analysis.

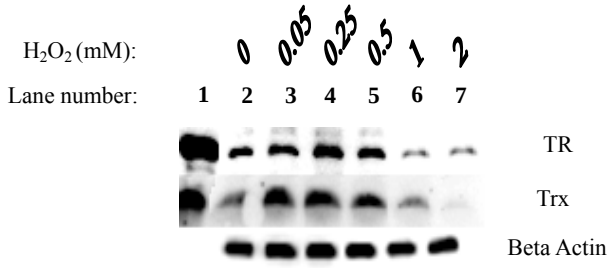


Figure 29a. Western Blot Analysis of TR, Trx in HT-29 cells upon oxidative stress without prior treatment of cycloheximide. Beta Actin was used as the internal control.

Lane 1: Standard TR and Standard Trx;

Lane 2 to 7: Lysates from HT-29 cells treated with 0 mM, 0.05 mM, 0.25 mM, 0.5 mM, 1 mM and 2 mM hydrogen peroxide respectively.

Figure 29b. Western Blot Analysis of TR, Trx in HT-29 cells upon oxidative stress with prior treatment of cycloheximide. Beta Actin was used as the internal control.

Lane 1: Standard TR and Standard Trx;

Lane 2 to 7: Lysates from HT-29 pretreated with cycloheximide then with 0 mM, 0.05 mM, 0.25 mM, 0.5 mM, 1 mM and 2 mM hydrogen peroxide respectively.

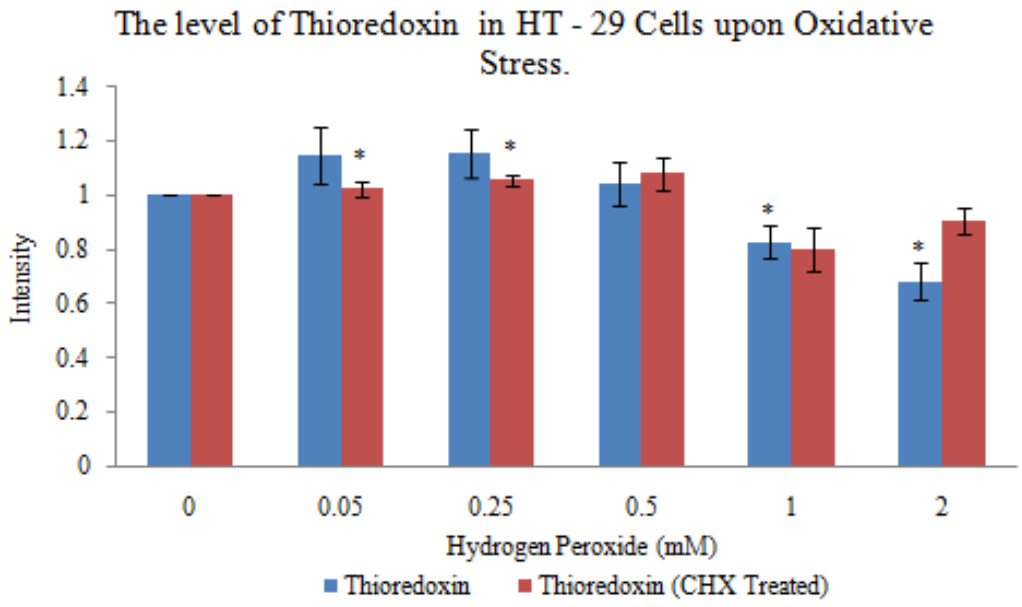


Figure 30. The effect of hydrogen peroxide on the level of Trx in HT-29 cells. HT-29 cells were divided into two groups. One group was treated with different concentrations (0 mM, 0.05 mM, 0.25 mM, 0.5 mM, 1 mM and 2 mM respectively) of hydrogen peroxide and the other was treated in the same way but with an extra one hour pretreatment with cycloheximide. Total soluble fractions of the cells lysates from each group were obtained after eight hours incubation and were subjected to immunoblot analysis with antibody specific to thioredoxin. Data were the average of three determinations ± SEM. Statistical calculations were done using the student's t test with (* p value < 0.05) was considered statistically significant when the hydrogen peroxide treated samples were compared with control.

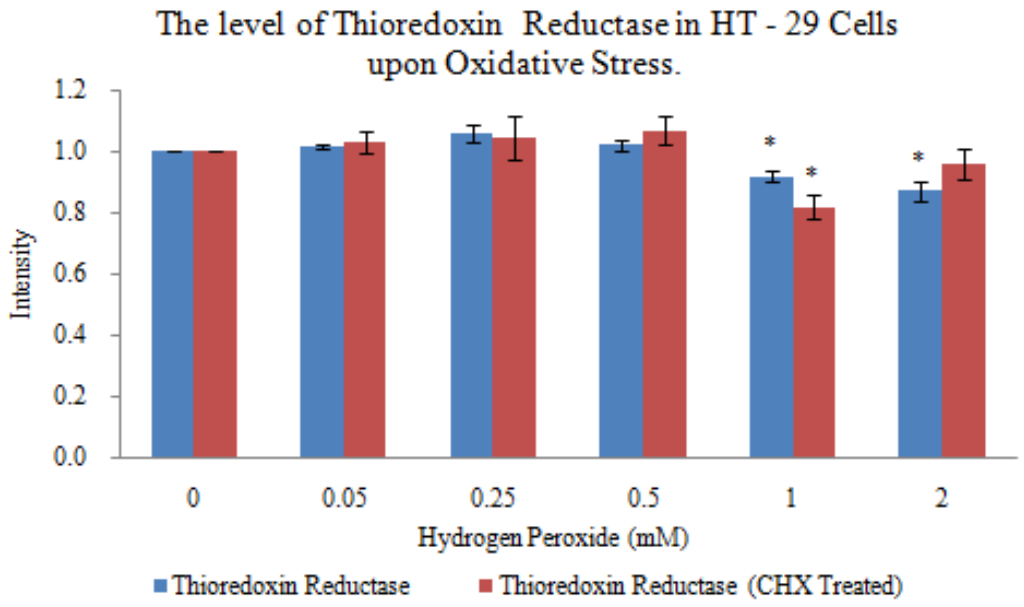


Figure 31. The effect of hydrogen peroxide exerted on the level of TR in HT-29 cells. HT-29 cells were divided into two groups. One group was treated with different concentrations (0 mM, 0.05 mM, 0.25 mM, 0.5 mM, 1 mM and 2 mM respectively) of hydrogen peroxide and the other was treated in the same way but with an extra one hour pretreatment with cycloheximide. Total soluble fractions of the cells lysates from each group were obtained after eight hours incubation and were subjected to immunoblot analysis with antibody specific to thioredoxin reductase. Data were the average of two determinations ± SEM. Statistical calculations were done using the student's t test with (* p value < 0.05) was considered statistically significant when the hydrogen peroxide treated samples were compared with control.

3.4 Effect of H_2O_2 on thioredoxin and thioredoxin reductase mRNA expression in HT-29 cells

The oxidative stress conditions induced some changes to the mRNA levels of TR and Trx in the eight hours of incubation. TR mRNA level in the 0.05 to 0.5 mM hydrogen peroxide treated cells without cycloheximide was upregulated. It declined back to basal level at higher concentrations of hydrogen peroxide. Furthermore, there were no significant changes in the mRNA levels of thioredoxin in cells treated with low concentrations of hydrogen peroxide whereas there was a slight upregulation in cells treated with 0.5 to 1 mM hydrogen peroxide. In cells treated with higher concentrations of hydrogen peroxide, the Trx mRNA fell to below basal level as compared with control. On the other hand, in cycloheximide pretreated group, the TR mRNA level remained unchanged in all cells exposed to different levels of hydrogen peroxide. The pattern of Trx mRNA level, however, differed from that of TR. Upon treatment with hydrogen peroxide, Trx mRNA levels decreased in all cases. More remarkable decreases were observed in cells treated with higher concentration of hydrogen peroxide, *i.e.* 1 and 2 mM.

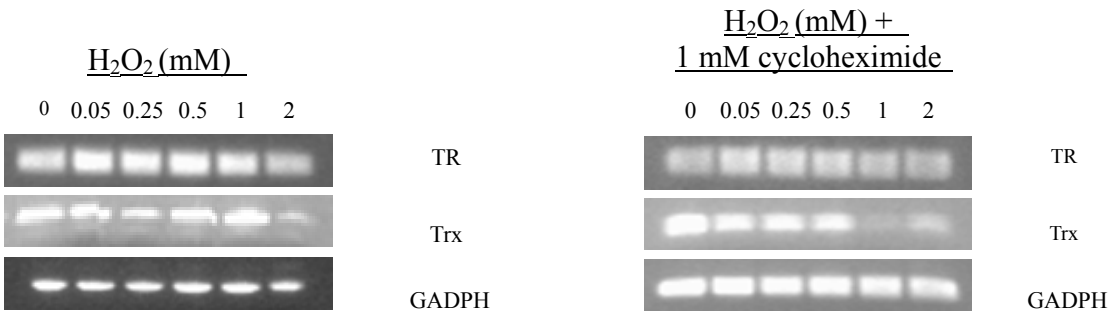


Figure 32. The effect of H_2O_2 on thioredoxin reductase and thioredoxin mRNA level in HT-29 cells with (right) or without (left) cycloheximide pretreatment. HT-29 cells were incubated in the presence of 0 mM, 0.05 mM, 0.25 mM, 0.5 mM, 1 mM and 2 mM H_2O_2 respectively for 8 hours. HT-29 cells were removed at the indicated times and thoroughly rinsed with PBS. Total RNA was extracted and reverse transcribed. Primers were designed and synthesized to detect Trx, TR, and GADPH. Equal amounts of synthesized cDNA were amplified by PCR with primers. PCR products were analyzed by agarose gel (1%) electrophoresis. GADPH was amplified as the internal control. The data were from one representative experiment.

3.5 Activity of thioredoxin system in HeLa and HT-29 cells upon oxidative challenge

3.5.1 Effect of hydrogen peroxide on thioredoxin reductase activity in HeLa cells

The end-point insulin dependent DTNB activity assay was carried out to measure the activity of thioredoxin reductase in HeLa cells upon oxidative challenge. Thioredoxin reductase activity in HeLa cells treated with 0.25 to 0.5 mM hydrogen peroxide marginally increased but decreased to 90%, 80% and 65% of the basal activity in cells treated with 1 mM, 2 mM and 4 mM of hydrogen peroxide respectively (Figure 33).

On the other hand, in cycloheximide pretreated cells, thioredoxin reductase activity decreased in all cells inflicted with oxidative damage in a dose-dependent manner. At the highest concentration of hydrogen peroxide tested (4 mM), the activity decreased to 45% of the basal activity.

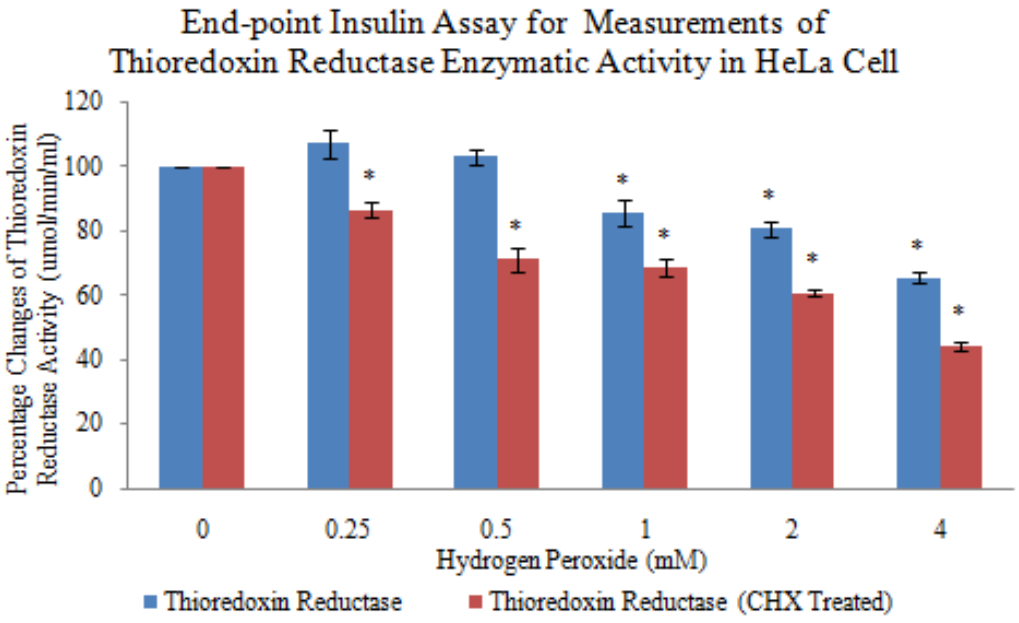


Figure 33. The effect of hydrogen peroxide on thioredoxin reductase activity in HeLa cells. Cells were treated with different concentrations (0 mM, 0.25 mM, 0.5 mM, 1 mM, 2 mM and 4 mM respectively) of hydrogen peroxide with or without prior treatment of cycloheximide. The lysates of cells were obtained after inflicted with oxidative challenge and analyzed for thioredoxin reductase activity. Data were the average of three determinations ± SEM. Statistical calculations were done using the student's t test with (* p value < 0.05) was considered statistically significant.

3.5.2 Effect of hydrogen peroxide on thioredoxin activity in HeLa cells

The end-point insulin dependent DTNB activity assay was carried out to measure the thioredoxin activity in HeLa cells upon oxidative challenge (Figure 34 and 35). Thioredoxin activity stayed unchanged in control cells with and without cycloheximide. In comparison with the control, thioredoxin activities in HeLa cells without blockage of *de novo* protein synthesis treated with 0.25 to 0.5 mM hydrogen peroxide decreased but when treated at 1 mM and 4 mM, the activities were the same as basal.

On the other hand, in cycloheximide pretreated cells, they all had increased activities of thioredoxin after oxidative stress. The highest respond was noted in cells treated with 1 mM hydrogen peroxide.

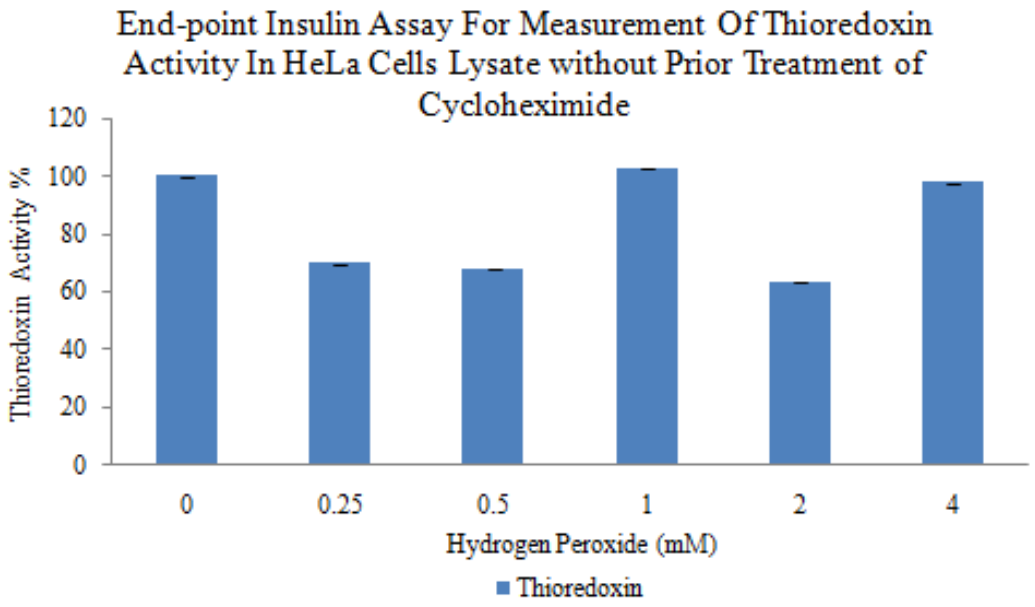


Figure 34. The effect of hydrogen peroxide on thioredoxin activity in HeLa cell. Cells were treated with different concentrations (0 mM, 0.25 mM, 0.5 mM, 1 mM, 2 mM and 4 mM respectively) of hydrogen peroxide. The lysates of cells were obtained after inflicted with oxidative challenge and analyzed for thioredoxin activity. Data were the average of two determinations ± SEM.

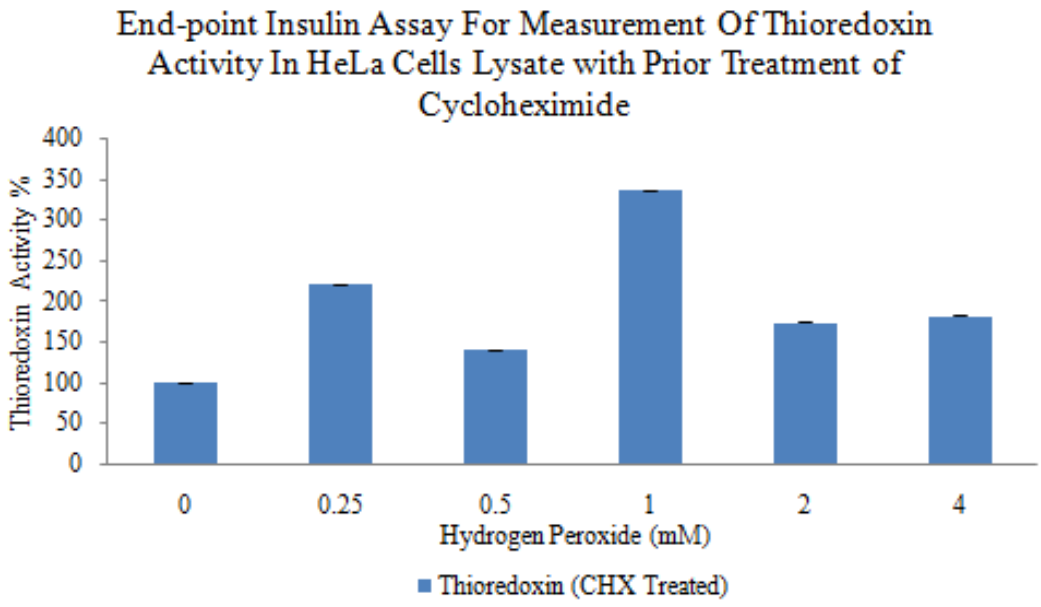


Figure 35. The effect of hydrogen peroxide on thioredoxin activity in HeLa cell. Cells were treated with different concentrations (0 mM, 0.25 mM, 0.5 mM, 1 mM, 2 mM and 4 mM respectively) of hydrogen peroxide and prior treatment of cycloheximide. The lysates of cells were obtained after inflicted with oxidative challenge and analyzed for thioredoxin activity. Data were the average of two determinations ± SEM.

3.5.3 Effect of hydrogen peroxide on thioredoxin reductase activity in HT-29 cells

HT-29 cells incubated with 0.05 mM hydrogen peroxide had a large increase of 40% in thioredoxin reductase activity compared to the basal. The highest response of 50% increase was found in cells treated with 0.25 mM hydrogen peroxide. At higher concentrations, the activity became lower and went down to 50% of the basal in cells treated with 2 mM hydrogen peroxide.

For HT-29 cells with prior treatment of cycloheximide, there were no significant changes in 0.05 mM and 0.25 mM hydrogen peroxide treatment. Increase in the thioredoxin reductase activity was noted in cells with 0.5 mM hydrogen peroxide treatment. The activity then declined in cells treated with higher concentrations of hydrogen peroxide of 1 and 2 mM (Figure 36).

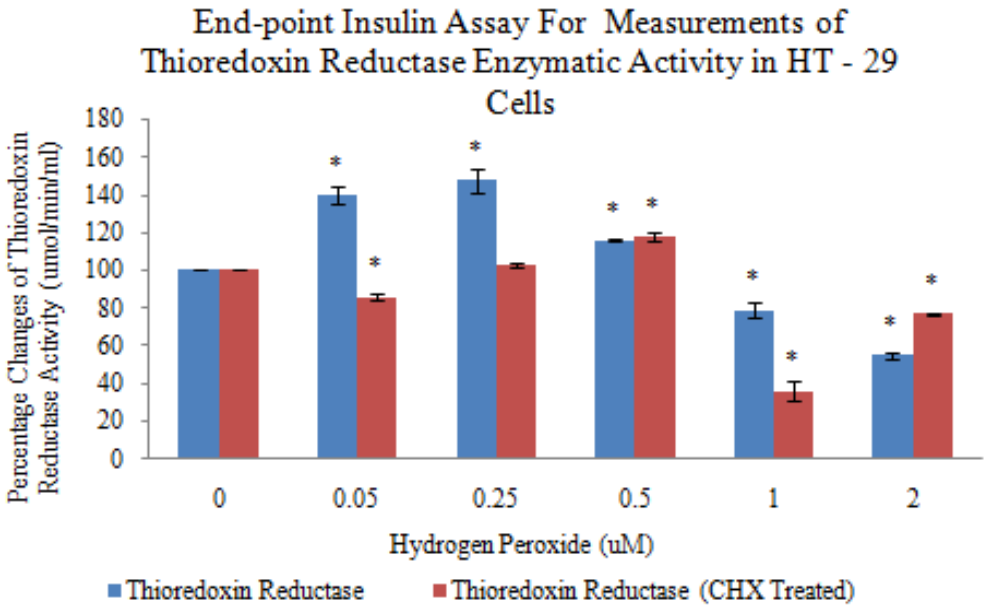


Figure 36. The effect of hydrogen peroxide on thioredoxin reductase activity in HT-29 cells. HT-29 cells were divided into two groups. Group one cells were treated with different concentrations (0 mM, 0.05 mM, 0.25 mM, 0.5 mM, 1 mM and 2 mM respectively) of hydrogen peroxide for eight hours and group two cells were treated in the same way but with a prior treatment of cycloheximide. The lysate of cells from each group was obtained after inflicted with oxidative challenge and analyzed for thioredoxin reductase activity. Data were the average of the best two determinations ± SEM. Statistical calculations were done using the student's t test with (* p value < 0.05) was considered statistically significant when the hydrogen peroxide treated samples were compared with control.

3.5.4 Effect of hydrogen peroxide on thioredoxin activity in HT-29 cells

Thioredoxin activity in HT-29 cells treated with 0.05 mM hydrogen peroxide had no observable change when compared with control. However, thioredoxin activity in cells treated with 0.5 mM hydrogen peroxide reached its maximum of 50% higher than the basal. The activity declined in cells treated with higher concentrations of hydrogen peroxide of 1 and 2 mM.

On the other hand, in cycloheximide treated cells, there was a slight increase in the thioredoxin activity at 0.05 mM hydrogen peroxide though it may not be significant. Again, the highest response was noted in the cells treated with 0.5 mM hydrogen peroxide, that the activity was 150% higher than the basal. Similar to the non-cycloheximide treated group, the thioredoxin activity declined in cells treated with higher concentrations of hydrogen peroxide of 1 and 2 mM (Figure 37).

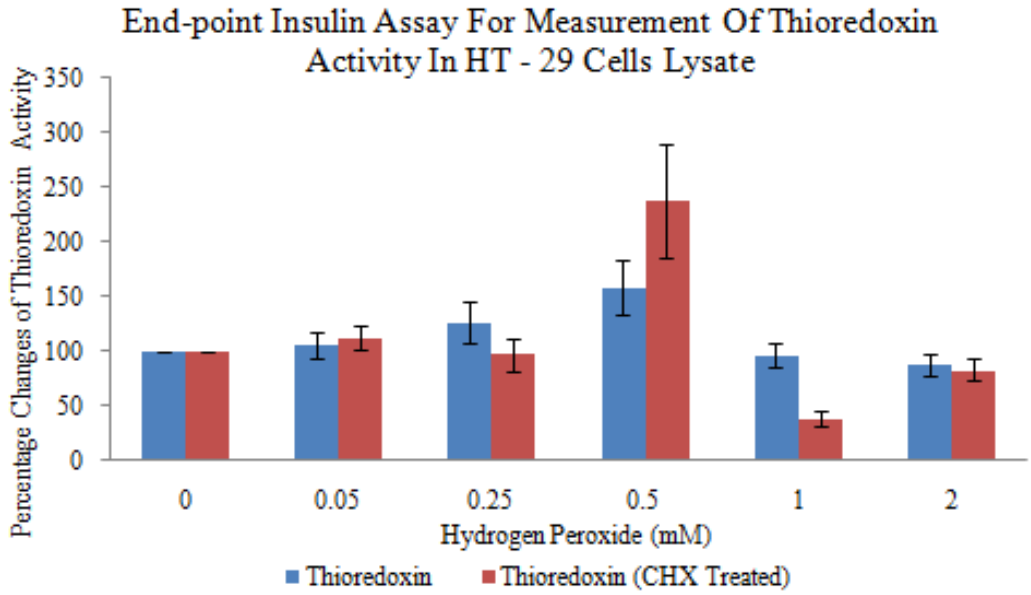


Figure 37. The effect of hydrogen peroxide on thioredoxin activity in HT-29 cells. HT-29 cells were divided into two groups. Group one cells were treated with different concentrations (0 mM, 0.05 mM, 0.25 mM, 0.5 mM, 1 mM and 2 mM respectively) of hydrogen peroxide and group two cells were treated in the same way but with an extra one hour prior treatment of cycloheximide. The lysate of cells from each group was obtained after inflicted with oxidative challenge and analyzed for thioredoxin activity. Data were the average of two determinations ± SEM.

3.5.5 Functional investigations of thioredoxin system in cells by IgG reduction activity assay

3.5.5.1 Reduction of mouse whole IgG protein by the thioredoxin system

The immunoglobulin reduction activity assay (IgG assay) was confirmed to be a specific assay for measuring the functional activity of the whole thioredoxin system in particular for investigating cell lysates samples which contain many other cellular contents. With the mouse polyclonal IgG as the substrate, different combinations and amounts of the constituents of the thioredoxin system could be tested. Results obtained were in agreement with published data [51] that the effective reduction of mouse IgG relies on the presence of all the components (thioredoxin, thioredoxin reductase and NADPH) of the thioredoxin system especially the reduced thioredoxin. In the tests without the complete system of all the components; that either thioredoxin reductase or NADPH was omitted, no effective breakdown of the disulfides bonds in IgG could be observed (Figure 38).

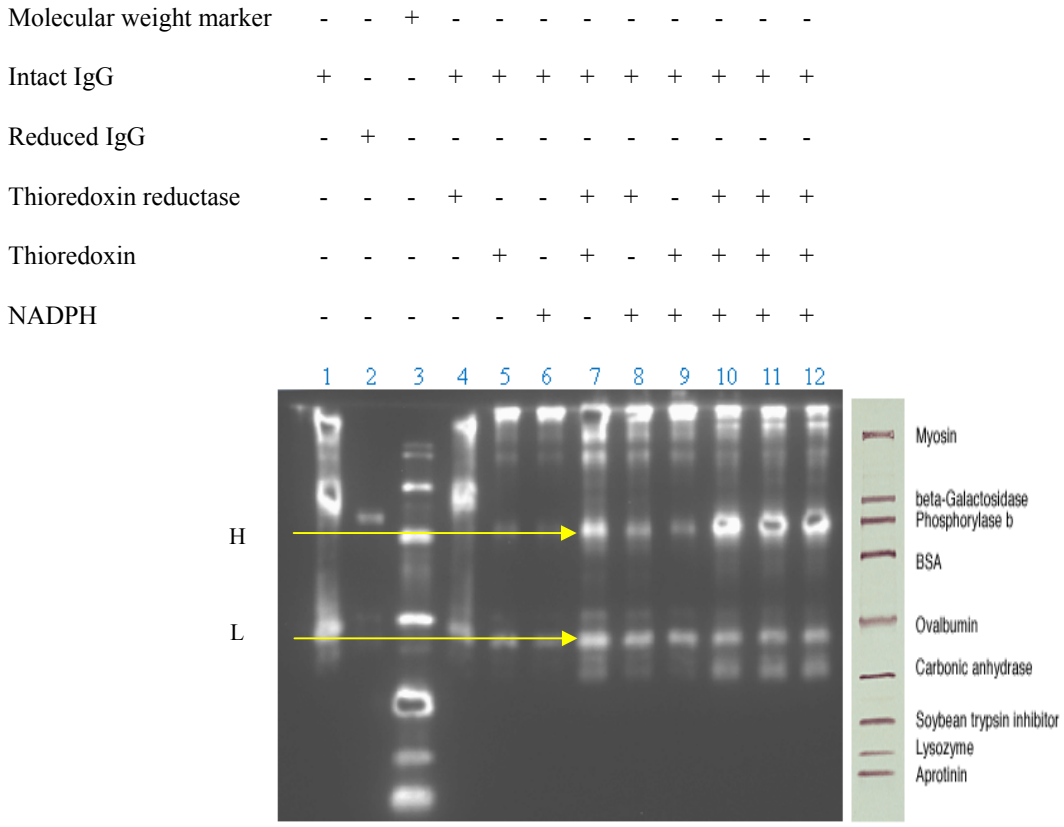


Figure 38. The Western Blotting analysis of the reduction of IgG by thioredoxin system. IgG samples were incubated with an incomplete thioredoxin system (Lane 4 to 9) and an intact thioredoxin system which contains thioredoxin, thioredoxin reductase and NADPH (Lane 10 to 12). There are differences in the sizes of heavy chains and light chains generated by mercaptoethanol in the gel buffer system to that by the reduction of biological thioredoxin system. The molecular weight markers used were myosin (M_r = 205000), beta-Galactosidase (M_r = 116000), bovine serum albumin (M_r = 66000), ovalbumin (M_r = 45000), carbonic anhydrase (M_r = 29000), soybean trypsin inhibitor (M_r = 20100), lysozyme (M_r = 14313) and aprotinin (Mr = 6517). (Refer to next page for details of components in each sample.)

Lane 1: 5 μg non-reduced mouse IgG treated with SDS buffer without 2-ME;

Lane 2 : 5 μg mouse IgG reduced fully by 2-ME;

Lane 3 : High molecular weight broad range biotinylated standards;

Lane 4 : 5 μg mouse IgG treated with thioredoxin reductase and Tris-HCl buffer for thirty minutes followed by mixing with 50 μL SDS buffer without 2-ME;

Lane 5 : 5 μg mouse IgG treated with 21 μM thioredoxin and Tris-HCl buffer for thirty minutes followed by mixing with 50 μL SDS buffer without 2-ME;

Lane 6 : 5 μg mouse IgG treated with 500 μM NADPH and Tris-HCl buffer for thirty minutes followed by mixing with 50 μL SDS buffer without 2-ME;

Lane 7 : 5 μg mouse IgG treated with thioredoxin reductase, 21 μM thioredoxin and Tris-HCl buffer for thirty minutes followed by mixing with 50 μL SDS buffer without 2-ME;

Lane 8 : 5 μg mouse IgG treated with thioredoxin reductase, 500 μM NADPH and Tris-HCl buffer for thirty minutes followed by mixing with 50 μL SDS buffer without 2-ME;

Lane 9 : 5 μg mouse IgG treated with 21 μM thioredoxin, 500 μM NADPH and Tris-HCl bluffer for thirty minutes followed by mixing with 50 μL SDS buffer without 2-ME and Lane 10 to 12 : triplicates of the positive control containing the intact thioredoxin system, i.e. 5 μg Mouse IgG, thioredoxin reductase, 21 μM thioredoxin and 500 μM NADPH incubated for thirty minutes followed by mixing with 50 μL SDS buffer without 2-ME.

3.5.5.2 Intactness of IgG as revealed by the SDS-PAGE

It seemed that even in samples with the omission of either thioredoxin reductase or NADPH, some splittings of heavy and light chains could still be observed. However, it was believed that such cleavages were not due to any of the reducing system but a small amount of background naturally occured in our mouse IgG purchased. The degree of intactness of the commercial “non-reduced” IgG was therefore also tested and confirmed by the analysis using 15% SDS-PAGE on the mouse IgG sample treated with non-reducing SDS buffer.

From the above findings, it was not surprising to see that the commercial IgG contained very trace amount of antibodies in the reduced from (squared area) and this little amount of reduced IgG was considered as background and would not affect the overall result of the IgG reduction assay.

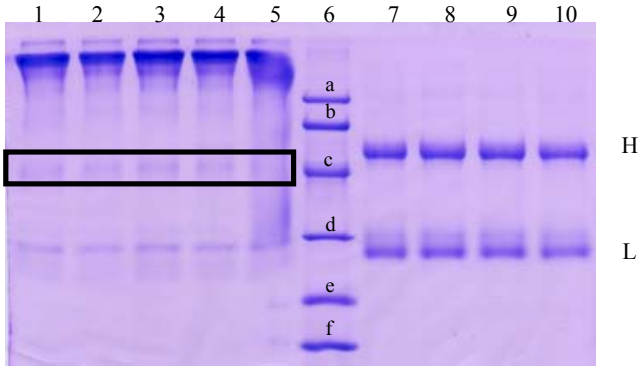


Figure 39. The SDS-PAGE of mouse IgG.

Lane 1 to 5: 5 μg mouse IgG and 10 μL non-reducing buffer;

Lane 6: Molecular markers - (a) rabbit muscle phosphorylase b (97400Da), (b) bovine serum albumin (66200Da), (c) hen egg white ovalbumin (45000Da), (d) bovine carbonic anhydrase (31000Da), (e) soybean trypsin inhibitor (21500Da) and (f) hen egg white lysozyme (14400Da) respectively. The data were from one representative experiment.

Lane 7 to 10: controls, 5 μg mouse IgG chemically reduced in 10 μL reducing buffer

3.5.5.3 Optimization of IgG reduction assay for the functional studies of thioredoxin system

In order to obtain the best performance, some optimizations were tried in this newly developed assay. In Figure 40, it showed that when too much IgG were used (Lane 3 – 6), they could not be fully and effectively broken down by the thioredoxin system that they appeared as some high molecular weight bands (size larger than heavy chain). High quality results could be obtained generally by using 10 μg of cell lysates proteins from HeLa cells and 3 μg of IgG with incubation time one hour (Lane 8) because it had a discrete and clear heavy chain of IgG without any other interferences.

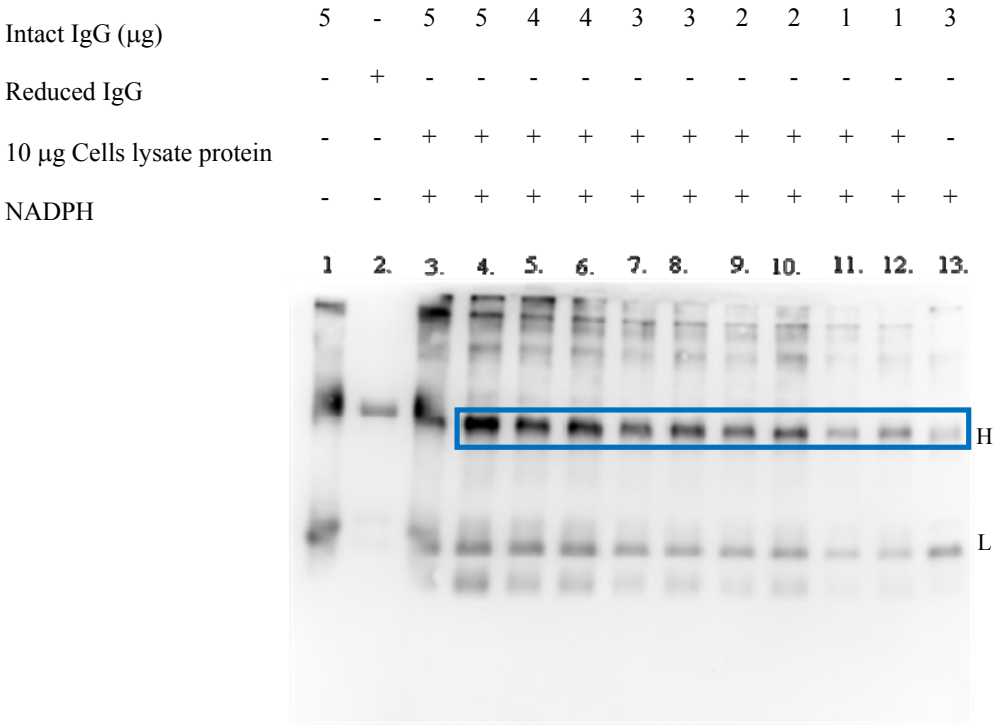


Figure 40. Optimization of the reduction of mouse IgG by the thioredoxin system in HeLa cells. The data were from one representative experiment. (Refer to next page for details of components in each sample.)

Lane 1: 5 μL 1 μg/uL non- reduced mouse IgG and 45 μL Tris-HCl buffer treated with 50 μL SDS buffer without 2-ME;

Lane 2: 5 μL 1 μg/μL mouse IgG fully reduced by 2-ME;

Lane 3 and 4: 5 μL 1 μg/μL non- reduced mouse IgG treated with 10 μL 1 μg/μL $(H_2O_2)_{0mM}$ cell lysate protein, 5 μL 5000 μM NADPH and 30 μL Tris-HCl buffer incubated for thirty minutes and sixty minutes respectively, followed by mixing with 50 μL SDS buffer without 2-ME;

Lane 5 and 6: 4 μL 1 μg/μL non- reduced mouse IgG treated with 10 μL 1 μg/μL $(H_2O_2)_{0mM}$ cell lysate protein, 5 μL 5000 μM NADPH and 31 μL Tris-HCl buffer incubated for thirty minutes and sixty minutes respectively, followed by mixing with 50 μL SDS buffer without 2-ME;

Lane 7 and 8: 3 μL 1 μg/μL non- reduced mouse IgG treated with 10 μL 1 μg/μL $(H_2O_2)_{0mM}$ cell lysate protein, 5 μL 5000 μM NADPH and 32 μL Tris-HCl buffer incubated for thirty minutes and sixty minutes respectively, followed by mixing with 50 μL SDS buffer without 2-ME;

Lane 9 and 10: 2 μL 1 μg/μL non- reduced mouse IgG treated with 10 μL 1 μg/μL $(H_2O_2)_{0mM}$ cell lysate protein, 5 μL 5000 μM NADPH and 33 μL Tris-HCl buffer incubated for thirty minutes and sixty minutes respecively, followed by mixing with 50 μL SDS buffer without 2-ME;

Lane 11 and 12: 1 μL 1 μg/μL non- reduced mouse IgG treated with 10 μL 1 μg/μL $(H_2O_2)_{0mM}$ cell lysate protein, 5 μL 5000 μM NADPH and 34 μL Tris-HCl buffer incubated for thirty minutes followed by mixing with 50 μL SDS buffer without 2-ME

and

Lane 13: negative control with 3 μL 1 μg/μL non-reduced mouse IgG, 5 μL 5000 μM NADPH and 42 μL Tris-HCl buffer incubated for thirty minutes followed by mixing with 50 μL SDS buffer without 2-ME.

3.5.5.4 Functional studies of thioredoxin system of HeLa and HT-29 cells by IgG reduction assay.

Recent investigations revealed that the thioredoxin system activity in erythrocytes could be enhanced when they were exposed to suitable oxidative stress in short term [57]. It was of our interest to find out whether the complete thioredoxin system could still carry out its normal function upon oxidative challenge and to see if such harsh condition would further stimulate the response of the whole thioredoxin system in cells.

In Figure 41, Lane 2 illustrated the basal functional activity level of the thioredoxin system in HeLa cells. Lane 3 to 7 indicated that there were slight increases of the thioredoxin system activity level upon challenged with 0.25 mM to 4 mM hydrogen peroxide. Table 8 and Figure 42 summarised the reduction capabilities of the thoioredoxin system of HeLa cells upon different concentrations of oxidative stress.

In the results, thioredoxin system activity in HeLa cells had a slight increase after treatment with the various concentrations of hydrogen peroxide. However, the enhancements were not very significant until challenged up to 2 mM of hydrogen peroxide, which induced a 29% increase of the reducing activity.

3.5.5.4.1 Whole thioredoxin system activity of HeLa cells by the IgG reduction assay

Molecular weight marker	-	-	-	-	-	-	-	-	-	-	-	+
Intact IgG	+	+	+	+	+	+	+	+	-	-	-	-
Reduced IgG	-	-	-	-	-	-	-	-	-	-	+	-
Cells lysate protein (μg)	-	10	10	10	10	10	10	-	-	-	-	-
H_2O_2 (mM) treatment to cells	-	0	0.25	0.5	1	2	4	-	-	-	-	-
NADPH	-	+	+	+	+	+	+	+	-	-	-	-

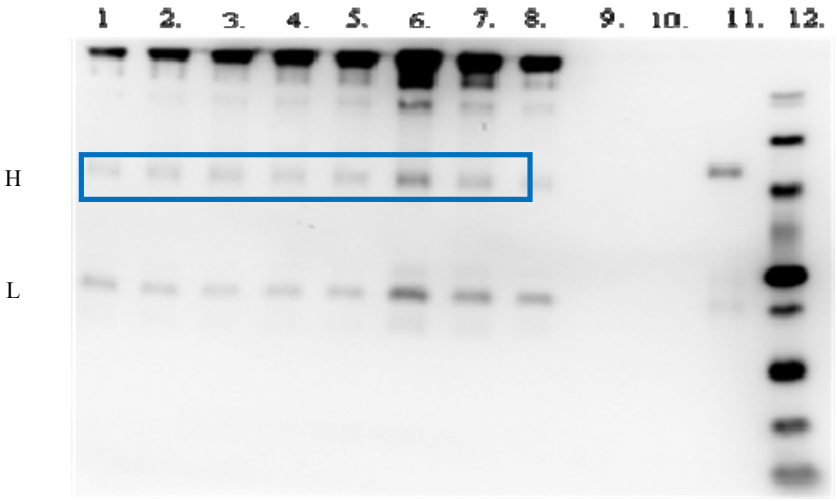


Figure 41. The reduction of mouse IgG by the thioredoxin system in HeLa cells after oxidative stress. The concentration of IgG and cell lysates used were 1 μg/μL and 2 μg/μL respectively. The subscript under $H_2O_{2,\ (n\ mM)}$ denotes concentration of hydrogen peroxide that the cells were exposed to. The molecular marker used were the same as previous membrane. The data were from one representative experiment. (Refer to next page for details of components in each sample.)

Lane 1: 3 μL 1 μg/μL non-reduced mouse IgG and 47 μL Tris-HCl buffer treated with 50 μL SDS buffer without 2-ME;

Lane 2 to 7: 3 μL 1 μg/μL non-reduced mouse IgG treated with 5 μL 2 μg/μL $(H_2O_2)_{0\ mM}$, $(H_2O_2)_{0.25\ mM}$, $(H_2O_2)_{0.5\ mM}$, $(H_20_2)_{1\ mM}$, $(H_2O_2)_{2\ mM}$ and $(H_2O_2)_{4\ mM}$ cell lysate protein respectively, 5 μL 5000 μM NADPH and 37 μL Tris-HCl buffer for sixty minutes followed by mixing with 50 μL SDS buffer without 2-ME;

Lane 8: negative control with 3 μL non-reduced mouse IgG, 5 μL 5000 μM NADPH and 42 μL Tris-HCl buffer for sixty minutes followed by mixing with 50 μL SDS buffer without 2-ME;

Lane 9 and 10: Blanks;

Lane 11: 3 μL 1 μg/μL mouse IgG reduced fully by 2-ME with 47 μL Tris-HCl to act as reference and

Lane 12: High molecular weight broad range biotinylated standard.

Table 8. The reduction capabilities of HeLa cells exposed to various concentrations of hydrogen peroxide.

Concentrations of H_2O_2. (mM)	Percentage changed in reduction capabilities of Thioredoxin System
0	0%
0.25	11.08%
0.5	16.26%
1	19.98%
2	29.26%
4	24.22%

Reduction capabilities of cell lysates were assessed by densitometric measurement and calculated by using the reduction capability of untreated cells (control) as the basal activity. Results presented were the means ± S.E.M. of four experiments.

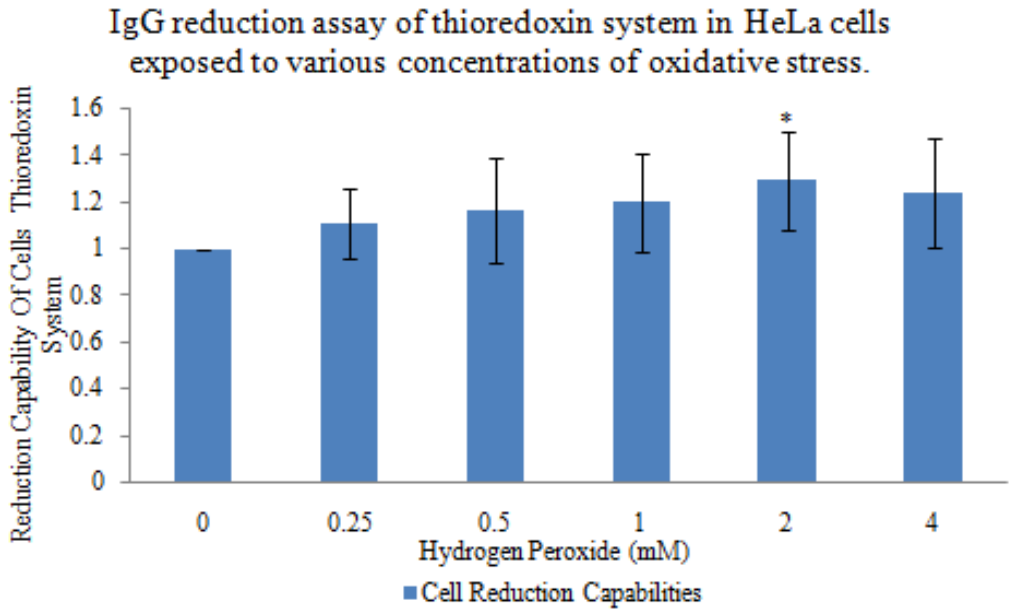


Figure 42. Graphic presentation of the reduction capability of the thioredoxin system in HeLa cells upon treatment with hydrogen peroxide for three hours. Data expressed were the means ± SEM of four independent experiments performed in duplicate. * $p < 0.05$ vs control.

3.5.5.4.2 Whole thioredoxin system activity of HeLa cells with prior treatment of cycloheximide by the IgG reduction assay

To further elucidate whether this phenomenon in the thioredoxin system activity was indeed due to thioredoxin expression or with other enhancement pathways, HeLa cells were treated with 1 mM cycloheximide to inhibit the *de novo* protein synthesis prior to the oxidative challenge. The blockage of *de novo* protein synthesis allowed us to have a better understanding on how the thioredoxin system response upon oxidative stress.

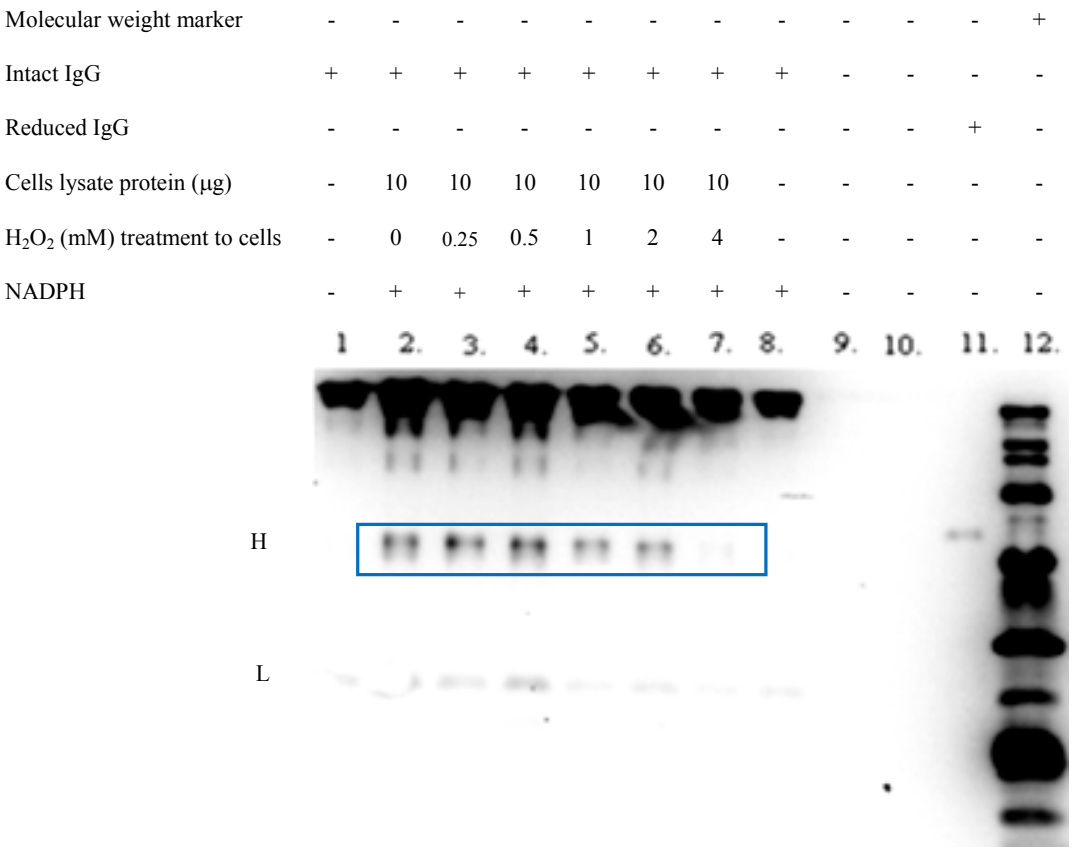


Figure 43. The reduction of mouse IgG by the thioredoxin system in HeLa cells with prior treatment of cycloheximide and then three hours of oxidative challenge. The concentration of IgG and cell lysates used were 1 μg/μL and 2 μg/μL respectively. The subscript under $H_2O_{2,\ (n\ mM)}$ denotes concentration of hydrogen peroxide that the cells were exposed to. The molecular marker used were the same as previous membrane. The data were from one representative experiment. (Refer to next page for details of components in each sample.)

Lane 1: 3 μL 1 μg/μL non- reduced mouse IgG and 47 μL Tris-HCl buffer treated with 50 μL SDS buffer without 2-ME prior to SDS-PAGE;

Lane 2 to 7: 3 μL 1 μg/μL non-reduced mouse Ig G treated with 10 μg $(H_2O_2)_{0\ mM}$, $(H_2O_2)_{0.25\ mM}$, $(H_2O_2)_{0.5\ mM}$, $(H_20_2)_{1\ mM}$, $(H_2O_2)_{2\ mM}$ and $(H_2O_2)_{4\ mM}$ cell lysate protein respectively, 5 μL 5000 μM NADPH and 37 μL Tris-HCl buffer for sixty minutes followed by mixing with 50 μL SDS buffer without 2-ME;

Lane 8: negative control with 3 μL mouse IgG, 5 μL 5000 μM NADPH and 42 μL Tris-HCl buffer for sixty minutes followed by mixing with 50 μL SDS buffer without 2-ME;

Lane 9 and 10: Blanks;

Lane 11: reference with 47 μL Tris-HCl buffer and 3 μL 1μg/μL mouse IgG reduced fully by 2-ME and

Lane 12: High molecular weight broad range biotinylated standard.

In Figure 43, Lane 2 illustrated the basal functional activity level of the thioredoxin system in HeLa cells. Lane 3 to 4 indicated that thioredoxin system activity level was maintained at 0.25 mM hydrogen peroxide and even had a very slight increase upon treatment with 0.5 mM hydrogen peroxide. The thioredoxin system activity then further decreased upon higher level of challenges with 1 to 4 mM hydrogen peroxide. Figure 44 summarised the reduction capabilities of the thoioredoxin system of HeLa cells upon different concentrations of oxidative stress and prior treatment of cycloheximide.

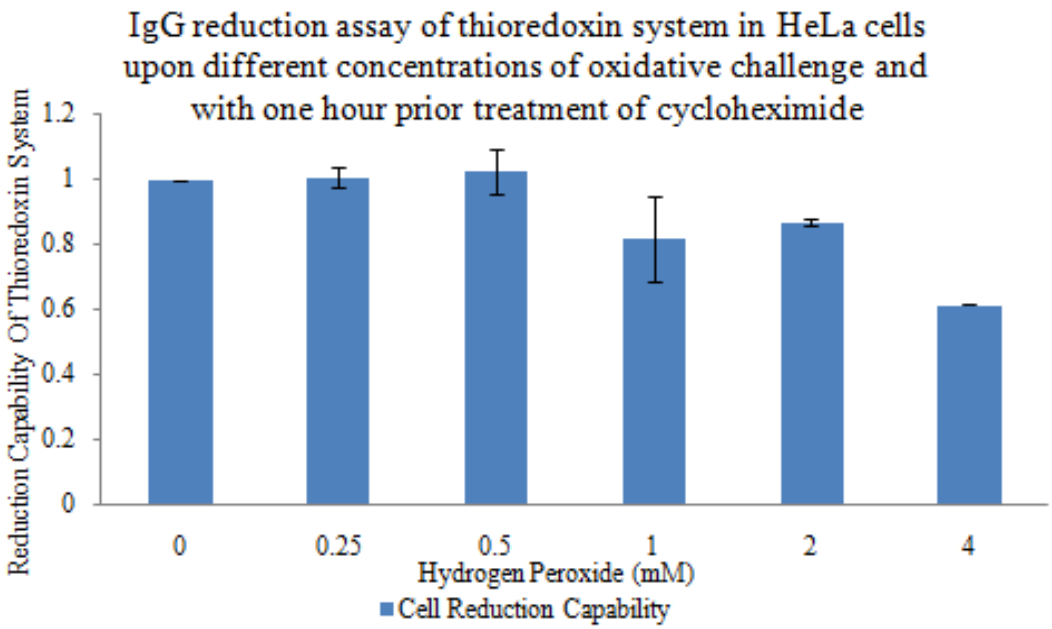


Figure 44. Graphic presentation of the reduction capability of the thioredoxin system in HeLa cells upon treatment with 0-4 mM hydrogen peroxide and prior treatment of cycloheximide. Reduction capabilities of exposed cells were assessed by densitometric measurement and calculated by using the reduction capability of untreated cells (control) to be 1 as basal for comparison and results represented the means ± standard error of mean (S.E.M.) of two experiments.

Results obtained from the above study indicated that insignificant increases of reduction capability of the thioredoxin system were observed in both cell groups, with or without the prior treatment of cycloheximide, then challenged with lower concentration of hydrogen peroxide up to 0.5 mM. In cells challenged with higher concentrations (2 mM), the activity did increase in fact in non-treated cells (Figure 42) compared to cycloheximide treated cells (Figure 44). The results suggested that increase of thioredoxin system activity upon oxidative challenge might be related to *de novo* protein synthesis.

In order to further elucidate better the phenomenon of oxidative stress and the activity level of the thioredoxin system in nucleated cells, another cell line HT–29 which had been reported to have a good response in the TR promoter site was investigated.

3.5.5.4.3 Whole thioredoxin system activity of HT–29 cells by the IgG reduction assay

Our previous studies showed that the thioredoxin system activity level was activated upon oxidative stress in HeLa cells. To examine whether the induction was cell type independent, another nucleated cell, HT–29 cells were studied.

In Figure 45, Lane 2 illustrated the basal functional activity level of the thioredoxin system in HT–29 cell. Lane 3 to 7 indicated that there were increases of the thioredoxin system activity level upon challenged with 0.05 mM to 2 mM hydrogen peroxide. Table 9 and Figure 46 summarise the reduction capabilities of the whole thoioredoxin system in HT–29 cell upon different concentrations of oxidative stress. In the results, there was an increase in the activity of the thioredoxin system in HT–29 cells with treatment at 0.05 to 0.25 mM hydrogen peroxide and a maximum response at 0.5 mM hydrohen peroxide oxidative stress.

Intact IgG	+	+	+	+	+	+	+	+	-	-	-	-
Reduced IgG	-	-	-	-	-	-	-	-	-	-	+	-
Cells lysate protein	-	+	+	+	+	+	+	-	-	-	-	-
H_2O_2 (mM) treatment to cells	-	0	0.05	0.25	0.5	1	2	-	-	-	-	-
NADPH	-	+	+	+	+	+	+	+	-	-	-	-

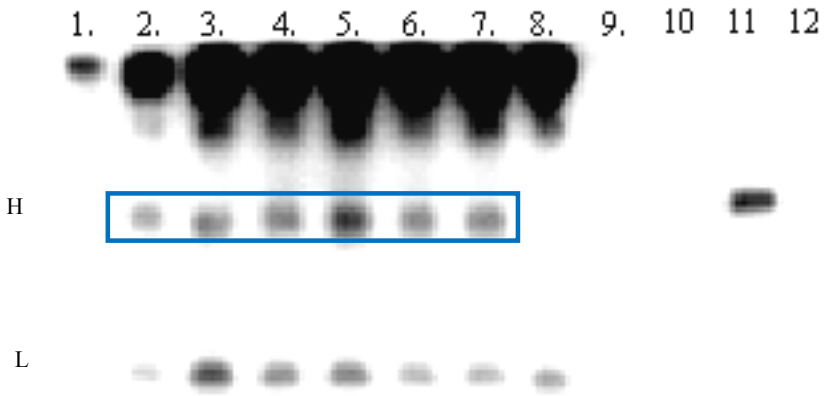


Figure 45. The reduction of mouse IgG by the thioredoxin system in HT–29 cells after eight hours of oxidative stress. The subscript under $H_2O_{2,\ (n\ mM)}$ denotes concentration of hydrogen peroxide that the cells were exposed to. The data were from one representative experiment. (Refer to next page for details of components in each sample.)

Lane 1: 3 μL 1 μg/μL non- reduced mouse IgG and 47 μL Tris-HCl buffer treated with 50 μL SDS buffer without 2-ME;

Lane 2 to 7: 3 μL 1 μg/μL non-reduced mouse IgG treated with $(H_2O_2)_{0\ mM}$, $(H_2O_2)_{0.05\ mM}$, $(H_2O_2)_{0.25\ mM}$, $(H_202)_{0.5\ mM}$, $(H_2O_2)_{1\ mM}$ and $(H_2O_2)_{2\ mM}$ cell lysate protein respectively, 5 μL 5000 μM NADPH and 37 μL Tris-HCl buffer for sixty minutes followed by mixing with 50 μL SDS buffer without 2-ME;

Lane 8: negative control with 3 μL mouse IgG, 5 μL 5000 μM NADPH and 42 μL Tris-HCl buffer for sixty minutes followed by mixing with 50 μL SDS buffer without 2-ME;

Lane 9 and 10: Blanks and

Lane 11: 3 μL 1 μg/μL mouse IgG reduced fully by 2-ME with 47 μL Tris-HCl buffer to act as reference.

Table 9. The reduction capabilities of HT–29 cells exposured to various concentrations of hydrogen peroxide without prior treatment of cycloheximide.

Concentrations of H_2O_2. (mM)	Percentage changed in reduction capabilities of Thioredoxin System
0	0%
0.05	17.84%
0.25	16.61%
0.5	28.19%
1	12.67%
2	10.47%

Reduction capabilities of cell lysates were assessed by densitometric measurement and calculated by using the reduction capability of untreated cells (control) to be the basal activity. Results presented were the means ± S.E.M. of two experiments.

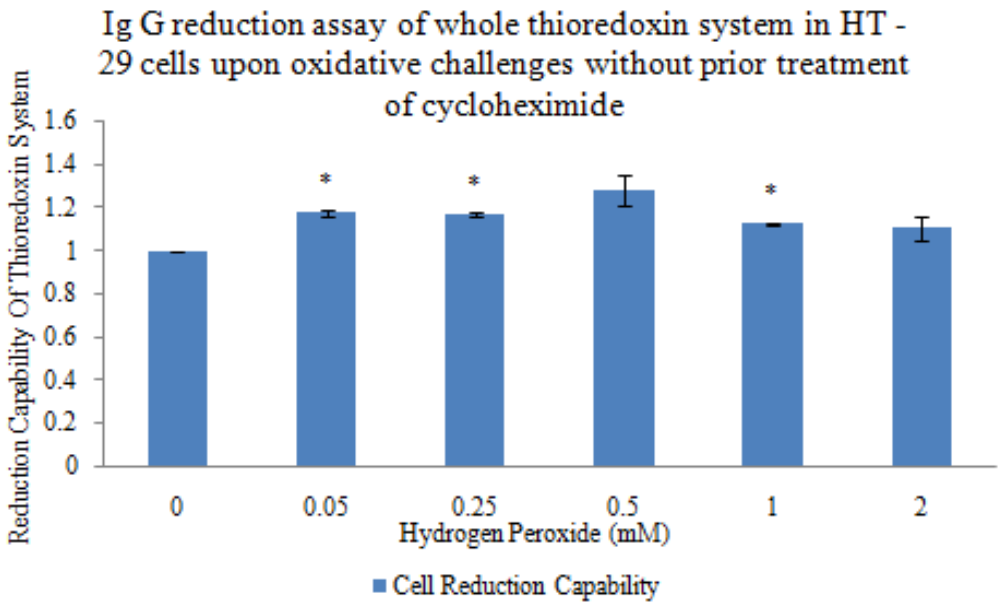


Figure 46. Graphic presentation of the reduction capability of the thioredoxin system in HT–29 cell upon treatment with 0-2 mM hydrogen peroxide. Reduction capabilities of exposed cells were assessed by densitometric measurement and calculated by using the reduction capability of untreated cells (control) to be 1 as basal for comparison and results represented the means ± standard error of mean (S.E.M.) of two experiments. Statistical calculations were done using the student's t test with (* p value < 0.05) was considered statistically significant when the hydrogen peroxide treated samples were compared with control.

3.5.5.4.4 Whole Thioredoxin system activity of HT–29 cells with prior treatment of cycloheximide by the IgG reduction assay

Intact IgG	+	-	+	+	+	+	+	+	-	-	-	-
Reduced IgG	-	-	-	-	-	-	-	-	-	-	-	+
Cells lysate protein	-	-	+	+	+	+	+	-	-	-	-	-
H_2O_2 (mM) treatment to cells	-	-	0	0.05	0.25	0.5	2	-	-	-	-	-
NADPH	-	-	+	+	+	+	+	+	-	-	-	-

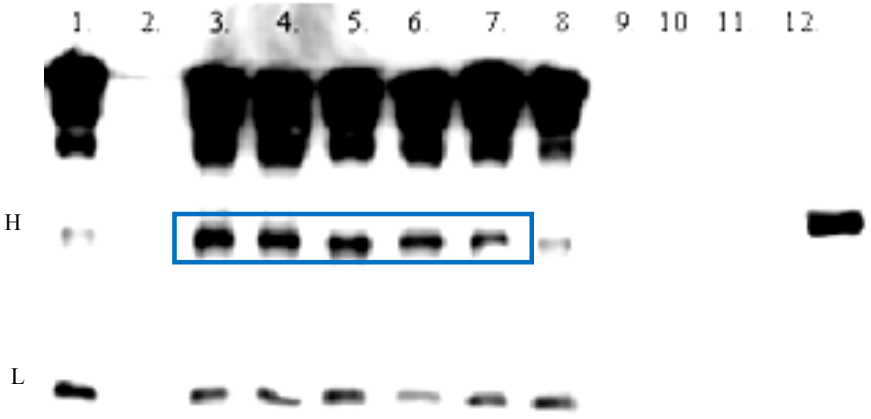


Figure 47. The reduction of mouse IgG by the thioredoxin system in HT–29 cells with prior treatment of cycloheximide and then eight hours of oxidative challenge. The subscript under $H_2O_{2,\ (n\ mM)}$ denotes concentration of hydrogen peroxide that the cells were exposed to. The data were from one representative experiment. (Refer to next page for details of components in each sample.)

Lane 1: 3 μL 1 μg/μL non-reduced mouse IgG protein and 47 μL Tris-HCl buffer treated with 50 μL SDS buffer without 2-ME;

Lane 2: Blank;

Lane 3 to 7: 3 μL 1 μg/μL non-reduced mouse IgG treated with $(H_2O_2)_{0\ mM}$, $(H_2O_2)_{0.05\ mM}$, $(H_2O_2)_{0.25\ mM}$, $(H_20_2)_{0.5\ mM}$ and $(H_2O_2)_{2\ mM}$ cell lysate protein respectively, 5 μL 5000 μM NADPH and 37 μL Tris-HCl buffer for sixty minutes followed by mixing with 50 μL SDS buffer without 2-ME;

Lane 8: negative control with 3 μL mouse IgG, 5 μL 5000 μM NADPH and 42 μL Tris-HCl buffer for sixty minutes followed by mixing with 50 μL SDS buffer without 2-ME;

Lane 9 to 11: Blanks and

Lane 12: 3 μL 1 μg/μL mouse Ig G reduced fully by 2-ME with 47 μL Tris-HCl buffer to act as reference.

In Figure 47, Lane 3 illustrated the basal functional activity level of the thioredoxin system in HT–29 cells. Lane 4 to 6 indicated that thioredoxin system activity level was about the same and maintained at 0.05 to 0.5 mM mM hydrogen peroxide. The thioredoxin system activity then decreased upon higher level of challenges with 2 mM hydrogen peroxide. Figure 48 summarised the reduction capabilities of the thoioredoxin system in HT–29 cells with prior cycloheximide treatment then different degree of oxidative stress.

Results obtained from the study indicated that insignificant increases of reduction capability of the thioredoxin system were observed in cells with the prior treatment of cycloheximide (Figure 48). However, in cells without cycloheximide pretreatment (Figure 46), the activity did enhance when compared to cycloheximide treated cells at hydrogen peroxide concentrations less than 0.5 mM (Figure 48). The results interestingly suggested that increase of thioredoxin system activity upon oxidative challenge might be related to *de novo* protein synthesis.

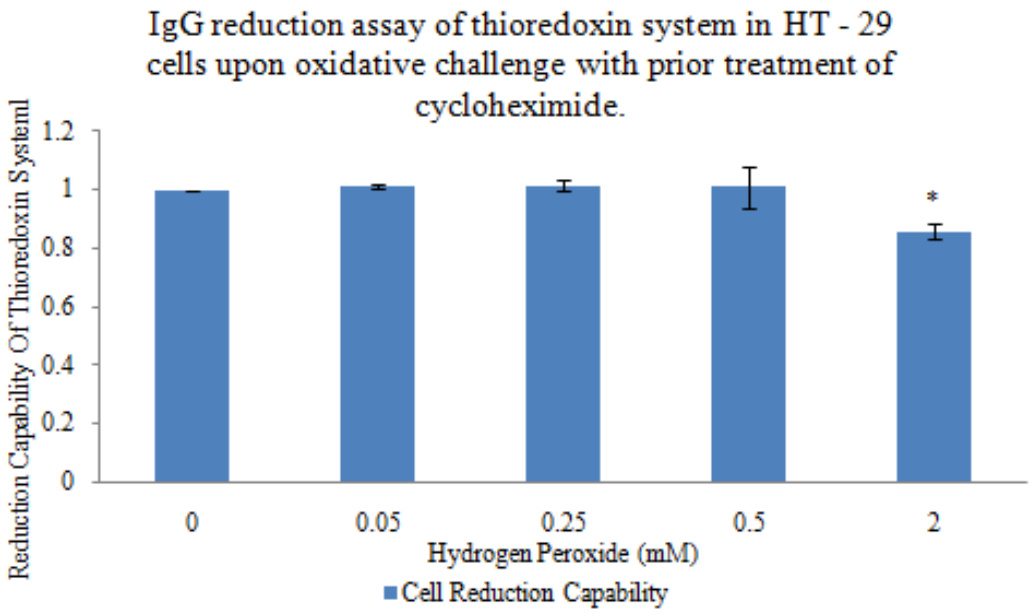


Figure 48. Graphic presentation of the reduction capability of the thioredoxin system in HT–29 cells with prior treatment of cycloheximide, then treated with 0-2 mM hydrogen peroxide. Reduction capabilities of exposed cells were assessed by densitometric measurement and calculated by using the reduction capability of untreated cells (control) to be 1 as basal for comparison and results represented the means ± standard error of mean (S.E.M.) of three experiments. * $p < 0.05$ vs control.

3.6 Altered cell cycle distribution in HT-29 cells upon oxidative stress by fluorescence activated cell sorting analysis

It is a well recognized observation that redox pathways participate crucial functions in the regulation of numerous intracellular processes, including apoptosis and cell progression. In this regard, we would like to determine whether changes in the activity of the thioredoxin system in cells in response to hydrogen peroxide induced oxidative stress would lead to altered cell cycle properties.

HT-29 cells were harvested and their cell cycle profiles were assessed via FACS analysis. In general, there was a gradual increase in the number of cells in the G_2 phase of the cell cycle when the cells were exposed to progressively increased concentrations of hydrogen peroxide.

The data showed that cells exposed to 0.25 mM hydrogen peroxide found with increased thioredoxin system activity in results obtained earlier, were also found a marked increase in the number of cells at the G_2 phase of the cell cycle. Whereas cells exposed to 2 mM hydrogen peroxide showed more remarkable response that nearly two folds of the cells were in the G_2 phase of the cell cycle when compared with control.

In parallel with these, the flow cytometry profiles histograms had shown undetectable apoptotic sub-G_1 DNA content after propidium iodide staining throughout the experiment in most hydrogen peroxide treated samples which also had increase in thioredoxin system

activity. It suggested that there could be a protective effect of the thioredoxin system proteins in cells by inhibiting hydrogen peroxide induced apoptosis.

In summary, our results suggested a relationship of the activity of the thioredoxin system in HT-29 cells with the viability of cells and regulation of progression through the cell cycle checkpoints under oxidative stress.

Table 10. Cell cycle phase analysis. HT-29 cells were grown in complete medium with different concentrations of hydrogen peroxide (0 mM, 0.05 mM, 0.25 mM, 0.5 mM, 1 mM and 2 mM) for eight hours. Harvested cells were stained with PI/RNase staining buffer and quantitated in each phase of growth cycle by a flow cytometer. Phases of the growth cycle, G_1, S and G_2/M were shown and the values given represented the percentage of the total cell population.

Cell Samples treated with different concentrations of H_2O_2 (mM)	% of Total Cell Population		
	G_1 Phase %	S Phase %	G_2/M Phase %
0	67	23.0	10
0.05	66.6	21.9	11.5
0.25	58.8	26.4	14.9
0.5	62.3	25.6	12.1
1	65.6	22.6	11.9
2	65.4	17.8	16.8

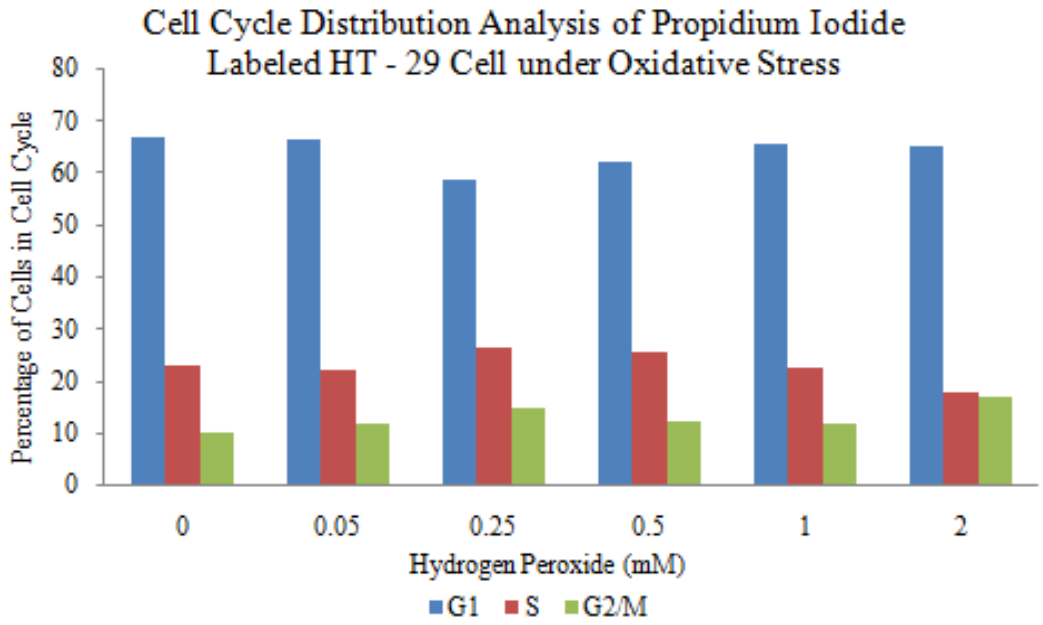
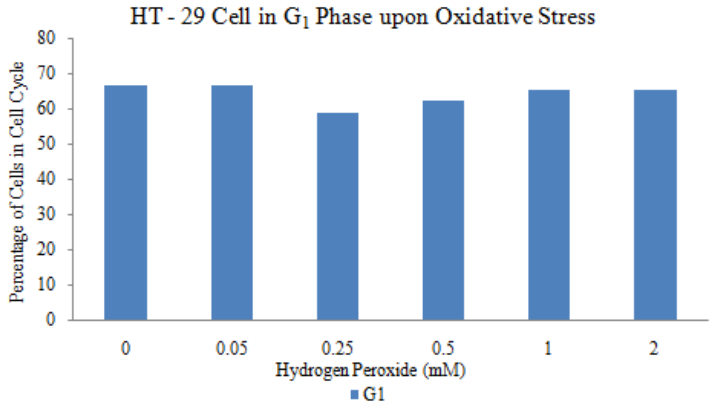
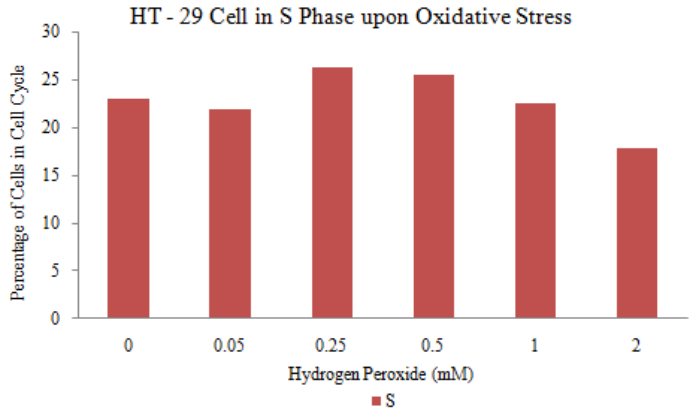


Figure 49. HT-29 cells were plated at a density of 0.5×10^6 viable cells per well in six wells plates, treated with different concentrations of hydrogen peroxide (0 mM, 0.05 mM, 0.25 mM, 0.5 mM, 1 mM and 2 mM) and harvested after eight hours. Cell cycle phase distribution was assessed by analysis of propidium iodide labeled cells using a flow cytometer. Oxidative stress affects cells proliferation and regulates cells progression through the G_2 cell cycle checkpoint.





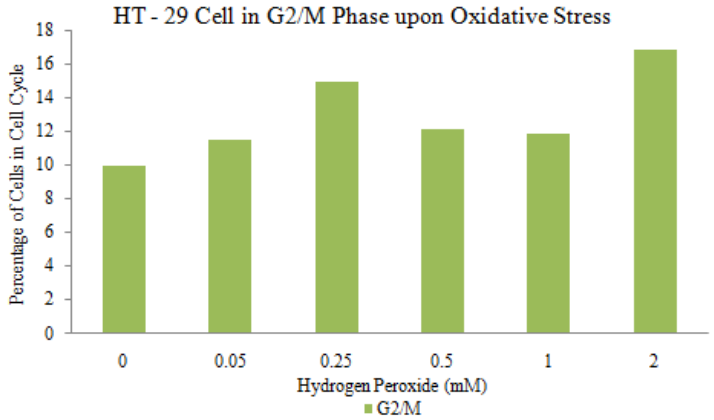


Figure 50. Effect of different concentrations of H_2O_2 in the percentage of cells in each cell cycle.

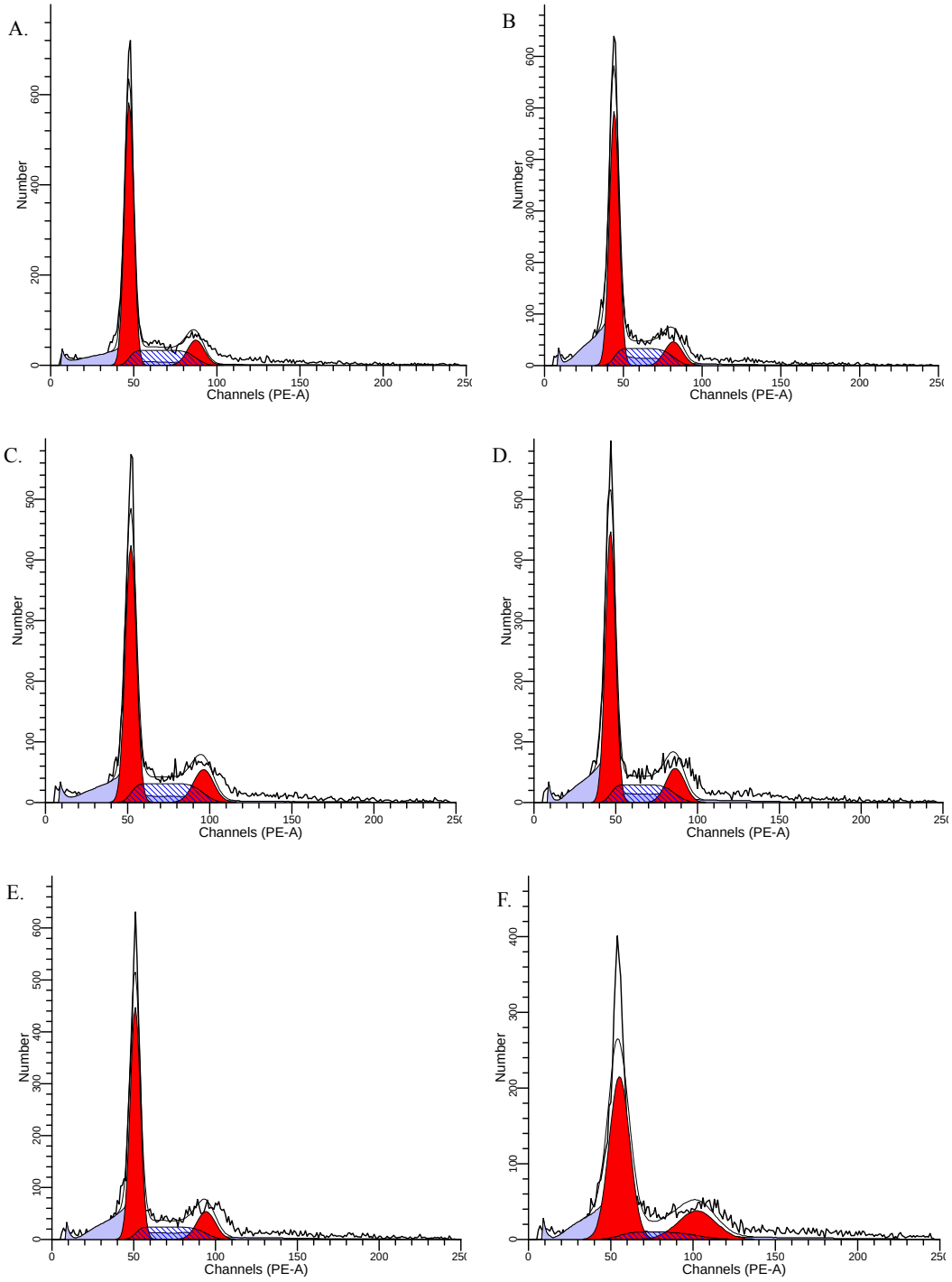


Figure 51. DNA content histogram of fixed cells treated with different concentrations of

hydrogen peroxide (From A to F are 0 mM, 0.05 mM, 0.25 mM, 0.5 mM, 1 mM and 2 mM respectively). Cells were fixed with 70% ethanol and stored at -20°C. Cells were stained with 0.5 ml PI/RNase staining buffer for 15 minutes at room temperature and cell cycle phase distribution analyzed by flow cytometry. The data were from one representative experiment.

4. Discussions

Reactive oxygen species are produced as natural byproducts during the natural aerobic metabolism. It is believed that physiological level of reactive oxygen species especially hydrogen peroxide is significant in the regulation of eukaryotic cell signal transduction via thiol residues [61]. Nevertheless, the use of such a cytotoxic chemical as a signaling molecule obviously has potential risks and if these reactively oxygen species are dramatically increased during pathologic conditions, they would pose harmful effects to the structures and normal functional roles of the cells such as modifying the cysteine residues of cellular proteins. Changes in the reversible oxidation state of cysteine residues can change other posttranslational modifications, affect protein turnover and enzymatic activity, adjust protein- protein interactions and vary subcellular trafficking. All these issues produced by oxidative stress are important causes of cell damage associated with the initiation and progression of many diseases. Consequently, it is not surprising that cells usually have developed efficient defensive ability to tightly regulate and fight against these reactive oxygen species and maintain themselves in redox balanced status through the participation of different antioxidant systems [44,61]. Unique amongst others, thioredoxin system is one of the best representative antioxidant systems, which is very important and powerful in the area of redox regulation and it may lead to direct antioxidant properties for example the elimination of hydrogen peroxide [44].

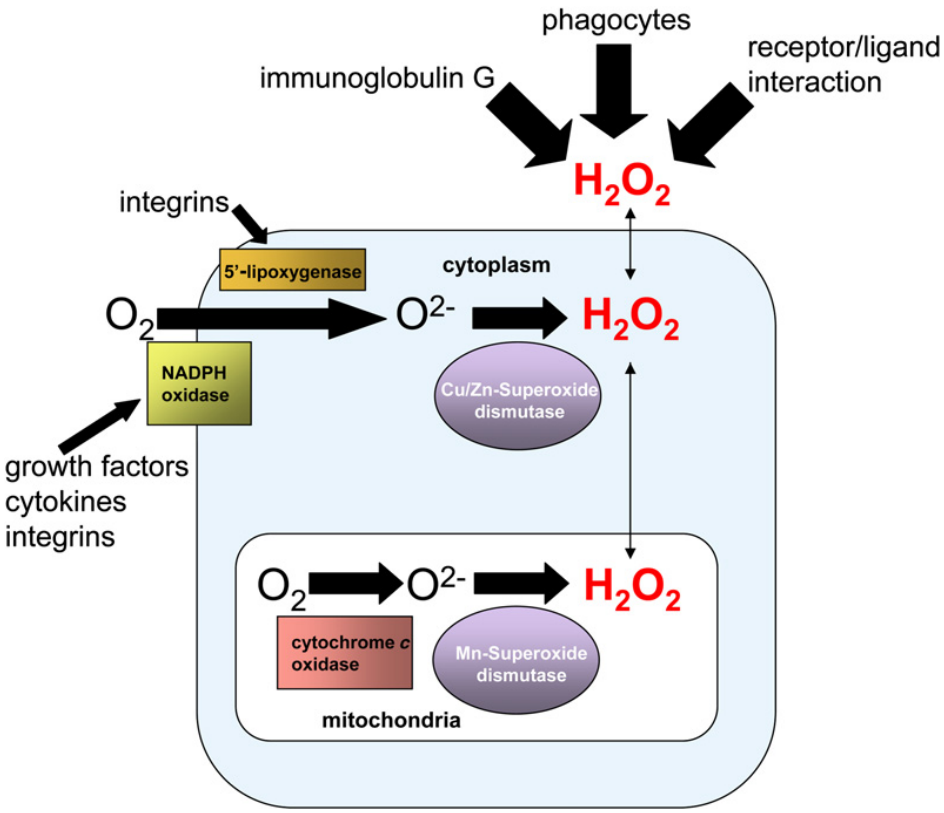


Figure 52. Eukaryotic cells are exposed to different sources of hydrogen peroxide. Hydrogen peroxide can be produced extracellularly, for example by the immunoglobulin G-catalyzed oxidation of water, by receptor/ ligand interactions, and by phagocytic immune cells. Superoxide anions, which are produced by the partial reduction of oxygen by cytochrome c oxidase in mitochondria, by membrane associated NADPH oxidase, or by 50-lipoxygenase in the cytoplasm, are rapidly converted to hydrogen peroxide by the action of cytoplasmic and mitochondrial superoxide dismutase enzymes. Growth factors, cytokines, and integrins stimulate the activation of NADPH oxidase and/or 50-lipoxygenase. Hydrogen peroxide can diffuse across membranes as indicated by the finer arrows [61].

Previous studies have confirmed that oxidative stress like hydrogen peroxide could exert a time and dose dependent induction of apoptosis in cultured cells and also increase in the thioredoxin system activity due to the increased transcriptional mediated expression of thioredoxin system enzymes [39]. However, finding [57] suggested that the increase of the thioredoxin system activity upon oxidative stress might not be related to *de novo* protein synthesis because they observed such increase in thioredoxin system activity even in the mature erythrocytes which were devoid of protein synthesis machinery. In spite of this, the existence and mechanism of the endogenous regulatory system in the increased activity of the thioredoxin system in response to oxidative stress still have not been thoroughly understood and reported. It is also believed that this repairing mechanism is the real determinant factor in maintaining cell homeostasis and most significantly the cell viability. In view of this, we would like to further extend our study in nucleated cells to examine the importance and contribution of the thioredoxin system upon oxidative stress.

In this study, we attempt to investigate the endogenous regulation of the thioredoxin system in cells under oxidative challenge. In search of the probable regulator(s) in cells, HeLa and HT–29 were employed as the cell models since these are more typical cells (compared to anucleated erythrocytes) that they can respond to external challenges by transcriptional and/or translational changes. Accordingly, by studying the thioredoxin system in the two cell models with and without *de novo* protein synthesis inhibited, it would help to verify our notion that whether there exists a universal regulatory system of the thioredoxin system.

HeLa cells are human cervical epithelial carcinoma cells which are fast growing monolayer adherent epithelial cell with high quantity of endogenous thioredoxin (15.2 ng/ug of cell protein) when compared with other cell lines such as SH-SY5Y, U-87MG and A549 [48]. In contrast, HT–29 is a human colorectal carcinoma cell line that is also a monolayer adherent epithelial cell and has been reported to have a good response in the thioredoxin reductase promoter site. HeLa and HT–29 for these reasons were chosen to look into the functional roles of the thioredoxin system [48,57].

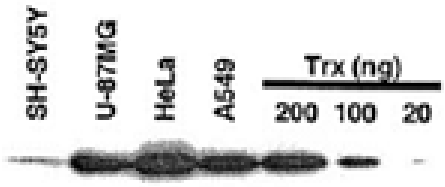


Figure 53. The different level of endogenous Trx in various human cell lines. The results of SH-SY5Y, neuroblastoma (2.3 ng/ug of protein); U-87MG, astroglioma (10.3 ng/ug of protein); HeLa, cervical carcinoma (15.2 ng/ug of protein) and A549, lung carcinoma (10.9 ng/ug) were compared with human Trx standards (20–200 ng) [48].

Experiments on oxidative stress in cells were implemented by the treatment of HeLa and HT-29 cells with different concentrations of hydrogen peroxide. Among most of the reactive oxygen species, cytotoxic hydrogen peroxide was chosen in our experiments because hydrogen peroxide could diffuse through cellular membranes easily and is a very common, natural and mild physiological oxidant by-product (compared with others, such as superoxide anion and hydroxyl radicals) of aerobic metabolism generated naturally by the superoxide dismutases in the dismutation of superoxide anion [14].

In our investigations, sub-lethal dosage of hydrogen peroxide was used to act as a stimulant to the cells and it was ensured that there were sufficient cells viable for the subsequent experiments on the response of the thioredoxin system. Consequently, we needed to verify the survivals of control and different concentrations of hydrogen peroxide treated cells, by using the MTS tetrazolium reduction assay and confirmed by the morphological observation.

In the MTS assay, it showed that hydrogen peroxide induced a dose-dependent decline in the relative survival rate in the cells, which was generally in good agreement with the results in the morphological observation. In addition, with the same concentration of hydrogen peroxide, cell survival was less in cycloheximide pretreated HT-29 cells and this indicated that HT-29 cells undergone treatment with cycloheximide would become more sensitive to the cytotoxic effects of hydrogen peroxide under oxidative stress condition. It is interesting to note that although HeLa cells demonstrated a dose-dependent response towards hydrogen peroxide toxicity, an unusual phenomenon was

observed in our study. The cycloheximide pre-treated HeLa cells apparently survived better than non-treated cells at all concentrations and exposure intervals of hydrogen peroxide tested. This indicated that cycloheximide treated HeLa cells might not be more sensitive to the hydrogen peroxide cytotoxic effect and *de novo* protein synthesis might not be necessary for HeLa cell survival under oxidative stress. Such phenomena may need further investigation but it was not pursued in our present study since we focused on the response of viable cells only.

In order to find out the influence on thioredoxin system activity in the cells subsequent to different levels of oxidative challenge; the insulin dependent DTNB reduction assay was employed and the IgG reduction assay was carried out for further confirmation.

It has been shown in the Western blotting study and the insulin dependent DTNB reduction assay that there was an upregulation of the protein expression and the thioredoxin activity in cells without the prior treatment of cycloheximide under medium stress conditions, which was in good agreement with the results in the IgG reduction assay. In our present study, 0.5 mM to 2 mM hydrogen peroxide treatment on HeLa cells and 0.25 mM to 0.5 mM hydrogen peroxide treatment on HT–29 cells were considered to be medium stress conditions on the respective cells. The variation between the two cell lines in response to oxidative stress in general indicated a cell type dependence and the concentrations range of hydrogen peroxide applied in the *in vitro* experiment therefore may not reflect the real oxidative stress conditions in human body.

The IgG reduction assay findings revealed slight increases of the thioredoxin system

activity in HT–29 cells and HeLa cells exposed to 0.05 mM and 1 mM hydrogen peroxide respectively. The increase of activity then became more significant with 28% in HT–29 cells treated with 0.5 mM H_2O_2 and 29% in HeLa cells treated with 2 mM H_2O_2. Conversely, treatment with higher concentrations of hydrogen peroxide showed decline in the activity of the thioredoxin system to reduce the IgG. It might be due to the unfavorable condition for the cells to continue to grow in such harsh oxidative environment that could also be confirmed from the findings in cells survival experiments.

Actually, it is reasonable to anticipate that when cells are facing prolonged oxidative stress, they *per se* would not had adequate oxidative defense capability, such as thioredoxin to remove hydrogen peroxide sufficiently in time and to regenerate oxidized thiols of target proteins to maintain the cellular redox homeostasis. Under such circumstances, gene and protein expression would be enhanced to compensate for the increased workload. However, upon persistent and high oxidative stress conditions, the thioredoxin system might be severely inactivated resulting in a decline in both activity and expression.

Although our present data cannot substantiate the claim that *de novo* protein synthesis was involved solely in the regulation of the thioredoxin system, an increase of the activity of the entire thioredoxin system was indeed detected in the cells after oxidative stress by the IgG reduction assay. Such results implied that the thioredoxin system in cells participates a role in the defence against oxidative challenge.

We also found a significant increase in the thioredoxin reductase activity in both HeLa cells and HT-29 cells challenged with medium levels of hydrogen peroxide without the prior treatment of cycloheximide. It was intriguing to find out that the induction of the thioredoxin reductase gene expression and activity in general was much more sensitive towards the stress than that of the thioredoxin. It was possible that the earlier the induction of such oxidation sensor; the more adequate reducing power it would give to sustain reduced form of thioredoxin so that the cells could maintain their redox balance by reducing the oxidized target proteins and enzymes [39].

On the other hand, in HT-29 cells pretreated with cycloheximide, it has also been shown that there was a slight increase of the thioredoxin and thioredoxin reductase activity under medium stress conditions. In case of HeLa, the increase was observed only in the activity of thioredoxin but not in thioredoxin reductase. These findings suggested that there are always considerable cell type dependant variations in the response towards oxidative stress. For instance, depending on the cell type, the concentration of hydrogen peroxide required to cause apoptotic cell death of mammalian cells could vary as much as twenty folds [61]. Nevertheless, it was very interesting to see that there were no significant changes of protein expression and mRNA level of the thioredoxin and thioredoxin reductase upon the challenge pretreated with cycloheximide, yet activity of both thioredoxin and thioredoxin reductase could be maintained or in some cases even higher in most of the oxidative challenged cells. These finding suggested that the thioredoxin system activity might not be solely dependent on transcription only.

In the IgG reduction activity assay, we found the whole thioredoxin system activity in both HeLa and HT-29 cells with the prior treatment of cycloheximide were generally maintained in low and medium level of oxidative stress. It would be fascinating to note that the thioredoxin system gene expression should have been inhibited that the amount of thioredoxin proteins and thus the activity in cells should gradually decrease throughout the course of oxidative challenge. However, the thioredoxin system activity after the challenge was sustained without significant changes. It was possibly that the thioredoxin system might participate in active role in protection against oxidative stress and also regulation of the thioredoxin system activity was not necessarily dependent on *de novo* protein synthesis. Again, it also indicates that the thioredoxin and thioredoxin reductase are relatively stable proteins that can withstand the adverse oxidative environment.

Thioredoxin system bears numerous cellular functions and it is widely involved in redox cellular processes. Elevated thioredoxin and thioredoxin reductase under oxidative stress would be expected to help cells to recover from oxidative damage since thioredoxin has the ability to regenerate oxidatively damaged proteins or enzymes by breaking the disulfide bonds and eliminating poisonous hydrogen peroxide through donation of electrons for peroxiredoxins to hydrolyze hydrogen peroxide.

In the cell cycle analysis, approximately seventy percent of the cells were retained in the G_0G_1 phase, the quiescent state in the control group. In the parallel tests on cells undergone oxidative stress, the percentage of cells in the apoptotic sub-G_1 content was undetectable and this represented cell apoptosis remained low even in severe oxidative

stress environment. Several lines of evidence indicated that apoptotic cells have reduced DNA stainability with propidium iodide or other fluorochromes. Therefore, the appearance of cells with low DNA stainability, lower than that of G_1 cells (sub-G_1 peak) in cultures have been considered to be the marker of cell death by apoptosis [62]. The diminished DNA stainability in apoptotic cells is probably due to DNA degradation and its subsequent leakage from the apoptotic cells. At the early stage of apoptosis, activation of apoptosis-specific endonucleases occurs and they cleave cellular DNA between nucleosomes. Consequently, free, single nucleosomes or small oligonucleosomes may diffuse into the cytoplasm and these nucleosomes would no longer be retained in the cells when the cells are rinsed after their permeabilization. The portion of DNA fragments still remain in the nuclear matrix of apoptotic cells after their fixation would then be stained by DNA specific fluorochromes and so apoptotic cells are recognized by their reduced stainability [62].

The low apoptosis in our results might be due to the stimulatory effects of exposing dividing mammalian cells in culture to low concentrations of oxidants. Study on the role of thioredoxin in protection again oxidative stress-induced apoptosis in neuroblastoma cells have implicated the nature of hormesis, which is the beneficial effects derived from low doses of potentially harmful substances, could prevent apoptosis and induce cell proliferation [48]. Although hydrogen peroxide is cytotoxic, in moderate concentrations range, it might not bring about a serious oxidative stress to the cells we studied. Instead, it might act as a mitogenic signaling molecule and stimulated cell growth and division; similar effects that could also be seen in a study on fibroblast [63]. One plausible

explanation for this hormesis effect could be that hydrogen peroxide and other apoptotic cell death oxidative stress generating chemicals such as Parkinsonian-producing neurotoxin 1-methyl-4-phenylpyridinum (MPP^+) [48], would cause the elevation of the endogenous concentration and activity of the oxidants consuming enzymes especially those in the thioredoxin system. Elimination of the oxidants would be therefore quicker and this provides improvement to the culture conditions allowing cell proliferation.

We therefore hypothesize that the major antioxidant system being involved in the protection of cell against oxidative stress, is the thioredoxin system. Cells exposed to moderate concentrations of hydrogen peroxide would be stimulated and respond by an increase in the activity and expression of the thioredoxin system proteins. Thioredoxin has been known to induce cell proliferation. Also, cancer cell lines such as SH-SY5Y, U-87MG, HeLa and A549 when supplemented with thioredoxin were resistant to various apopotosis-inducing agents including reactive oxygen species [48]. Recently it has been shown that overexpression of wild type thioredoxin reductase gene in parental HeLa cells caused accelerated growth and apparent decrease in the number of cells at the G_1 phase compared with HeLa cells having Cys-Ser mutant thioredoxin reductase. The cells with the mutant thioredoxin reductase indeed had a significant increase in the number of cells in the G_1 phase and a substantially decreased rate of proliferation [1].

In addition, Smart, D. K. *et al* (2004) showed that pre-treatment with 1-methyl-1-propyl-2-imidazolyl disulfide (IV-2), a pharmacologic inhibitor of thioredoxin reductase, on HeLa cells before exposure to hydrogen peroxide resulted in a significant increase in

hydrogen peroxide induced tumor cell death. Similar to the results with permanent cell lines which overexpressed the mutant thioredoxin reductase gene, inhibition of thioredoxin reductase not only couldn't protect the cells against oxidative stress induced apoptosis, but it also sensitized cells to oxidative stress and might function as either a major or minor mechanism of cell death [1].

Thioredoxin has also been identified as a physiological interacting partner of apoptosis signal-regulating kinase 1 (ASK1) and formed Trx-ASK1 complexes to inhibit the activity of ASK1 and its subsequent ASK1- dependent apoptosis [64, 65]. Whereas there were also other findings showing that after treatment of hydrogen peroxide on fibroblast and MCF-7 breast cancer cells, none of these hydrogen peroxide consuming enzymes such as catalases, glutathione peroxidases, phospholipid hydroperoxide glutathione peroxidase and total superoxide dismutase activities or mRNA levels were increased [66,67]. All these findings suggested some other enzymes and/or systems, and most likely the thioredoxin system proteins should be the candidate for the study in search of the mechanism of protection against oxidative stress-induced apoptosis in cells.

On the other hand, hydrogen peroxide is an oxidant and could be genotoxic [68]. In our present study, when cells were treated with hydrogen peroxide, there was a significant increase in the percentage of cells arrested in the G_2 phase of the cell cycle. This oxidative stress responding growth arrest occurred in many different cell lines such as human lung cells [69], human lens epithelial B-3 cells [70], colorectal cancer cells [71], human hepatocellular carcinoma cells [72], mammalian fibroblasts [73,74] and malignant mouse DT

cells [75]. The reason behind probably was the toxic outcomes of oxidant exposure that hydroxyl radicals might attack DNA, causing strand breakages and formation of oxidized bases which eventually produced oxidative DNA damage [68]. Under conditions of modest genomic damage, mammalian cell may appear to enter a sustained cell cycle arrest. The most prominent arrest is in the G_2 phase of the cell cycle in order to prevent mitotic entry with damaged DNA (Figure 54).

With reference to the results obtained from cell challenged with low to medium level of hydrogen peroxide, one may note that there was a general increase in the activity of both the thioredoxin and thioredoxin reductase accompanied with an increase in the population of cells arrested in the G_2 phase of the cell cycle. While any correlation of these may not be established from the present study, it would be interesting to explore further the involvement if any of the thioredoxin system proteins in the cell cycle progression under oxidative stress environments in the future.

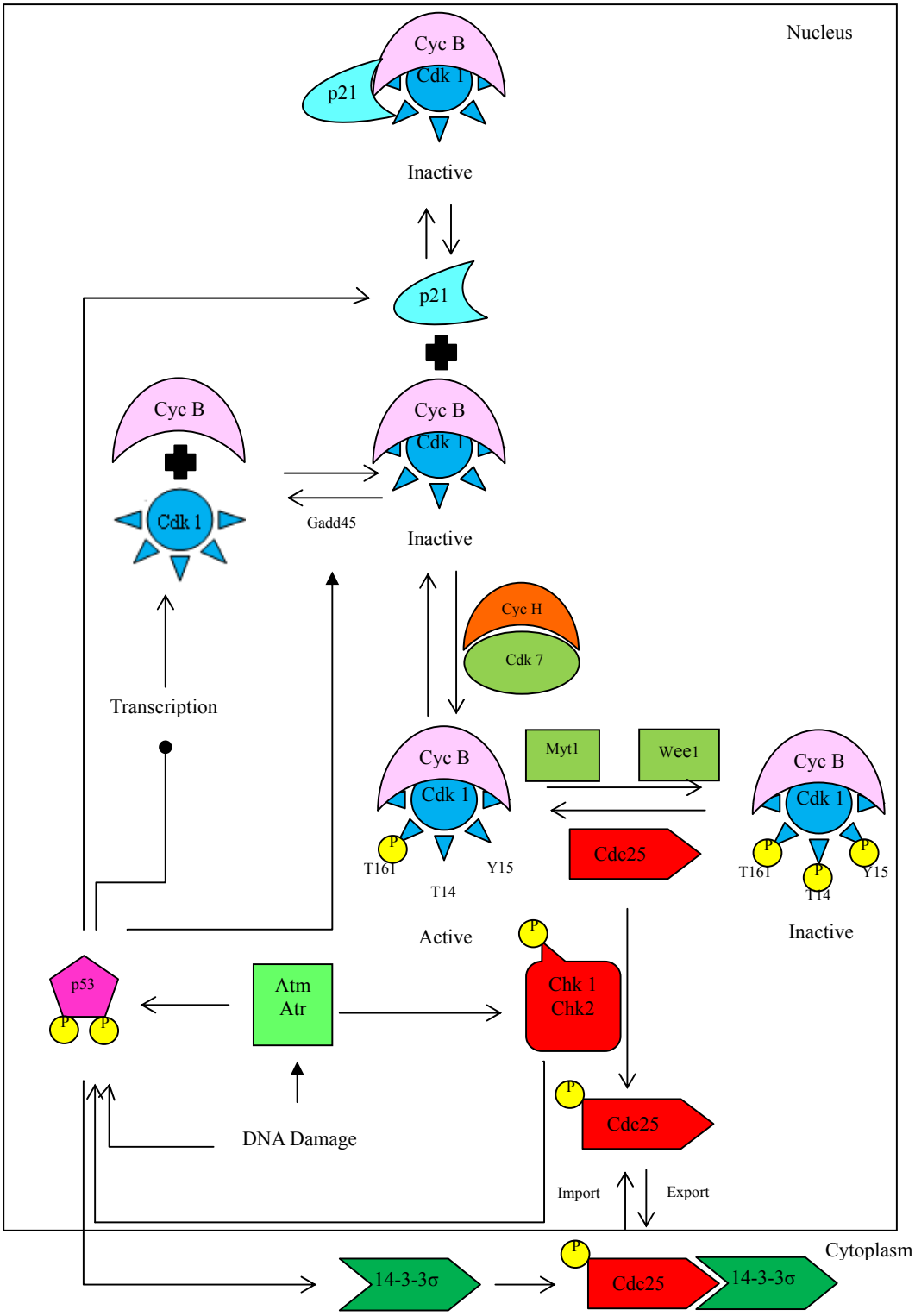


Figure 54. Participation of multiple levels of regulations of the G_2/M transition in

response to DNA damage. G_2 progression to mitosis in cells requires the formation of a complex of active Cdk1 (Cdc2) and catalytic partner cyclin B1. The Cdk1 in the complex is then phosphorylated on its threonine 161 (T161) by Cdk-activating kinase (CycH/Cdk7 complex) and dephosphorylated on threonine 14 (T14) and tyrosine 15 (Y15) by Cdc25C protein phosphatase. In response to DNA damage, members of phosphatidylinositol 3-kinase (PI3K) family protein ATM/ATR kinases are activated and they pass the DNA damage signal to the phosphorylated effector kinase Chk1 and Chk 2. Subsequent to this, Chk1 and Chk 2 can phosphorylate Cdc25C at serine 216 so that the phosphorylated Cdc25C is then bound by 14-3-3σ and sequesters into the cytoplasm. The Cdc25C cannot dephosphorylate the T14 and Y15 residues of Cdk1/ cyclin B1 complex and the complex therefore continue to be inactive due to its phosphorylation on T14 and Y15 by the Myt1 and Wee1 kinases respectively in the nucleus [76,77]. Following the initiation of the G_2 checkpoint, cells would continue to stay in the G_2 phase until they recover. p53 has been attributed the role in the maintenance of the cells in G_2 [76-78]. DNA-damaging agents such as hydrogen peroxide could induce p53 protein in fibroblasts as low as 75 μM. An increase in p53 protein is not only related with the arrest of cells in the G_1 phase of the cell cycle [68,79], but it has also been shown to mediate the maintenance of G_2 arrest by inducing the transcription of various targets, e.g. cyclin-dependent kinase inhibitor p21 which directly inhibits Cdk1, 14-3-3σ which anchors Cdk1 in the cytoplasm so that it cannot induce mitosis and Gadd45 which dissociates Cdk1 from cyclinB1. With these multiple levels of regulations, entry into mitosis is blocked by the G_2 checkpoint mechanism when DNA is damaged.

5. Future Prospects

Increased levels of hydrogen peroxide in cells can result in oxidative stress and even cause cellular damage [61]. The correlation of oxidative challenge with different diseases and aging has attracted enormous attentions in exploiting antioxidants to protect against oxidative stress induced damage. As one of the most important and protective cellular antioxidants system, thioredoxin system has great contributions in protecting against damages and malignancies [80].

Our findings support this notion that the activity of the thioredoxin system in nucleated cells is maintained and in some cases increased after oxidative stress. We believe that when cells are facing oxidative challenge, in order to keep surviving, cells themselves at once, must have adequate oxidant defense ability to response and defend against the challenge as soon as possible. Therefore, besides the comparatively slower transcriptional process, it is logical to think that there may be involvement of some other potential factors or pathways which can directly and immediately regulate the increase of the thioredoxin system to control the cellular reduction/ oxidation status in cells upon oxidative stress. To further confirm our hypothesis, the finding and deeper understanding of the associations of the thioredoxin system and its plausible regulators upon oxidative stress will ultimately be required, which would be a promising field in drugs development for intervention of oxygen species induced diseases and aging [57].

Redox biology of the cell cycle and its regulation with the antioxidants enzymes is

another horizon with many unexplored areas. The regulatory and protective roles of thioredoxin and thioredoxin reductase in normal cell cycle progression seem to be unclear and it is believed that these enzymes may play essential roles in the cell cycle phase transitions. In order to address this issue in the future, we can inflict oxidative damage onto some transformed cell lines with over-expression of either wild type or mutant thioredoxin and thioredoxin reductase genes and monitor the differences if any in the growth and proliferation rates. The results of these experiments will not only demonstrate the role of thioredoxin and thioredoxin reductase in the regulation of cell cycle transition, but they would also throw light on drug development in employing thioredoxin and thioredoxin reductase as the targets for curing malignancies. Future investigations of the complex relationships between the regulation of thioredoxin and thioredoxin reductase are therefore required for better understanding of how cells appropriately respond to oxidative stress.

6. Conclusion

In our investigation, thioredoxin and thioredoxin reductase were found able to withstand and combat oxidative stress and their activities were increased upon low to medium level of oxidative stress conditions. Survived cells with increased thioredoxin system activity were found with a higher population of cells shifted to the G_2 phase of the cell cycle.

7. References

[1] Smart, D. K. et al., Thioredoxin reductase as a potential molecular target for anticancer agents that induce oxidative stress. *Cancer Res* **64** (18), 6716 (2004).

[2] Ross, S. J., Findlay, V. J., Malakasi, P., and Morgan, B. A., Thioredoxin peroxidase is required for the transcriptional response to oxidative stress in budding yeast. *Mol Biol Cell* **11** (8), 2631 (2000).

[3] Stadtman, E. R., Role of oxidant species in aging. *Curr Med Chem* **11** (9), 1105 (2004).

[4] Stadtman, E. R. and Levine, R. L., Protein oxidation. *Ann N Y Acad Sci* **899**, 191 (2000).

[5] Rahman, K., Studies on free radicals, antioxidants, and co-factors. *Clin Interv Aging* **2** (2), 219 (2007).

[6] Mustacich, D. and Powis, G., Thioredoxin reductase. *Biochem J* **346 Pt 1**, 1 (2000).

[7] Federico, A. et al., Chronic inflammation and oxidative stress in human carcinogenesis. *Int J Cancer* **121** (11), 2381 (2007).

[8] Gracy, R. W., Talent, J. M., Kong, Y., and Conrad, C. C., Reactive oxygen species: the unavoidable environmental insult? *Mutat Res* **428** (1-2), 17 (1999).

[9] Somogyi, A. et al., Antioxidant measurements. *Physiol Meas* **28** (4), R41 (2007).

[10] Valko, M. et al., Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* **39** (1), 44 (2007).

[11] Poli, G., Leonarduzzi, G., Biasi, F., and Chiarpotto, E., Oxidative stress and cell signalling. *Curr Med Chem* **11** (9), 1163 (2004).

[12] Stadtman, E. R., Protein oxidation and aging. *Free Radic Res* **40** (12), 1250 (2006).

[13] Ames, B. N., Shigenaga, M. K., and Hagen, T. M., Oxidants, antioxidants, and the degenerative diseases of aging. *Proc Natl Acad Sci U S A* **90** (17), 7915 (1993).

[14] Lopaczynski, W. and Zeisel, S. H., Antioxidants, programmed cell death, and cancer. *Nutr Res* **21** (1-2), 295 (2001).

[15] Winterbourn, C. C., Toxicity of iron and hydrogen peroxide: the Fenton reaction. *Toxicol Lett* **82-83**, 969 (1995).

[16] Stadtman, E. R. and Levine, R. L., Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids* **25** (3-4), 207 (2003).

[17] Stadtman, E. R., Protein oxidation in aging and age-related diseases. *Ann N Y Acad Sci* **928**, 22 (2001).

[18] Vogt, W., Oxidation of methionyl residues in proteins: tools, targets, and reversal. *Free Radic Biol Med* **18** (1), 93 (1995).

[19] Stadtman, E. R. et al., Methionine oxidation and aging. *Biochim Biophys Acta* **1703** (2), 135 (2005).

[20] Filomeni, G., Rotilio, G., and Ciriolo, M. R., Disulfide relays and

phosphorylative cascades: partners in redox-mediated signaling pathways. *Cell Death Differ* **12** (12), 1555 (2005).

[21] Adler, V. et al., Regulation of JNK signaling by GSTp. *EMBO J* **18** (5), 1321 (1999).

[22] Esposito, F. et al., Redox control of signal transduction, gene expression and cellular senescence. *Neurochem Res* **29** (3), 617 (2004).

[23] Hirota, K. et al., Distinct roles of thioredoxin in the cytoplasm and in the nucleus. A two-step mechanism of redox regulation of transcription factor NF-kappaB. *J Biol Chem* **274** (39), 27891 (1999).

[24] Halliwell, B., Biochemistry of oxidative stress. *Biochem Soc Trans* **35** (Pt 5), 1147 (2007).

[25] Sies, H., Stahl, W., and Sevanian, A., Nutritional, dietary and postprandial oxidative stress. *J Nutr* **135** (5), 969 (2005).

[26] Nordberg, J. and Arner, E. S., Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic Biol Med* **31** (11), 1287 (2001).

[27] Soerensen, J. et al., The role of thioredoxin reductases in brain development. *PLoS One* **3** (3), e1813 (2008).

[28] Nishiyama, A. et al., Redox regulation by thioredoxin and thioredoxin-binding proteins. *IUBMB Life* **52** (1-2), 29 (2001).

[29] Zhong, L., Arner, E. S., and Holmgren, A., Structure and mechanism of mammalian thioredoxin reductase: the active site is a redox-active selenolthiol/selenenylsulfide formed from the conserved cysteine-selenocysteine sequence. *Proc Natl Acad Sci U S A* **97** (11), 5854

(2000).

[30] Williams, C. H. et al., Thioredoxin reductase two modes of catalysis have evolved. *Eur J Biochem* **267** (20), 6110 (2000).

[31] Sandalova, T. et al., Three-dimensional structure of a mammalian thioredoxin reductase: implications for mechanism and evolution of a selenocysteine-dependent enzyme. *Proc Natl Acad Sci U S A* **98** (17), 9533 (2001).

[32] Waksman, G., Krishna, T. S., Williams, C. H., Jr., and Kuriyan, J., Crystal structure of Escherichia coli thioredoxin reductase refined at 2 A resolution. Implications for a large conformational change during catalysis. *J Mol Biol* **236** (3), 800 (1994).

[33] Holmgren, A., Bovine thioredoxin system. Purification of thioredoxin reductase from calf liver and thymus and studies of its function in disulfide reduction. *J Biol Chem* **252** (13), 4600 (1977).

[34] Holmgren, A., Measurement of Thioredoxin and Thioredoxin Reductase. *Current Protocols in Toxicology*, 7.4.1 (2000).

[35] Jung, H. I. et al., Differential thioredoxin reductase activity from human normal hepatic and hepatoma cell lines. *Yonsei Med J* **45** (2), 263 (2004).

[36] Kang, H. J. et al., Effects of heterologous expression of thioredoxin reductase on the level of reactive oxygen species in COS-7 cells. *Mol Cells* **22** (1), 113 (2006).

[37] Holmgren, A., Thioredoxin. *Annu Rev Biochem* **54** (237) (1985).

[38] Holmgren, A. , Thioredoxin and glutaredoxin systems. *J Biol Chem* **264** (24)

(1989).

[39] Moon, S., Fernando, M. R., and Lou, M. F., Induction of thioltransferase and thioredoxin/thioredoxin reductase systems in cultured porcine lenses under oxidative stress. *Invest Ophthalmol Vis Sci* **46** (10), 3783 (2005).

[40] Lee, S. R. et al., Mammalian thioredoxin reductase: oxidation of the C-terminal cysteine/selenocysteine active site forms a thioselenide, and replacement of selenium with sulfur markedly reduces catalytic activity. *Proc Natl Acad Sci U S A* **97** (6), 2521 (2000).

[41] Kuriyan, J. et al., Convergent evolution of similar function in two structurally divergent enzymes. *Nature* **352** (6331), 172 (1991).

[42] Powis, G., Kirkpatrick, D. L., Angulo, M., and Baker, A., Thioredoxin redox control of cell growth and death and the effects of inhibitors. *Chem Biol Interact* **111-112**, 23 (1998).

[43] Powis, G. and Montfort, W. R., Properties and biological activities of thioredoxins. *Annu Rev Pharmacol Toxicol* **41**, 261 (2001).

[44] Maulik, N. and Das, D. K., Emerging potential of thioredoxin and thioredoxin interacting proteins in various disease conditions. *Biochim Biophys Acta* **1780** (11), 1368 (2008).

[45] Yu, G. W., Xu, J. Y., Xu, L., and Lee, K. S., cDNA sequence of porcine thioredoxin. *DNA Seq* **13** (2), 113 (2002).

[46] Arner, E. S. and Holmgren, A., Physiological functions of thioredoxin and thioredoxin reductase. *Eur J Biochem* **267** (20), 6102 (2000).

[47] Holmgren, A., Thioredoxin structure and mechanism: conformational

changes on oxidation of the active-site sulfhydryls to a disulfide. *Structure* **3** (3), 239 (1995).

[48] Andoh, T., Chock, P. B., and Chiueh, C. C., The roles of thioredoxin in protection against oxidative stress-induced apoptosis in SH-SY5Y cells. *J Biol Chem* **277** (12), 9655 (2002).

[49] Nakamura, H. et al., Expression and growth-promoting effect of adult T-cell leukemia-derived factor. A human thioredoxin homologue in hepatocellular carcinoma. *Cancer* **69** (8), 2091 (1992).

[50] Gasdaska, J. R., Berggren, M., and Powis, G., Cell growth stimulation by the redox protein thioredoxin occurs by a novel helper mechanism. *Cell Growth Differ* **6** (12), 1643 (1995).

[51] Magnusson, C. G., Bjornstedt, M., and Holmgren, A., Human IgG is substrate for the thioredoxin system: differential cleavage pattern of interchain disulfide bridges in IgG subclasses. *Mol Immunol* **34** (10), 709 (1997).

[52] Kurokawa, M. and Kornbluth, S., Caspases and kinases in a death grip. *Cell* **138** (5), 838 (2009).

[53] Zhivotosky, B. and Orrenius, S., Assessment of apoptosis and necrosis by DNA fragmentation and morphological criteria. *Curr Protoc Cell Biol* **Chapter 18**, Unit 18 3 (2001).

[54] Myers, C. R. and Myers, J. M., The effects of acrolein on peroxiredoxins, thioredoxins, and thioredoxin reductase in human bronchial epithelial cells. *Toxicology* **257** (1-2), 95 (2009).

[55] Haendeler, J. et al., Low doses of reactive oxygen species protect endothelial cells from apoptosis by increasing thioredoxin-1 expression. *FEBS Lett* **577** (3), 427 (2004).

[56] Fernando, M. R. et al., Thioredoxin regenerates proteins inactivated by oxidative stress in endothelial cells. *Eur J Biochem* **209** (3), 917 (1992).

[57] Lam, S. M., The role of thioredoxin system in oxidative stress and cell aging. (2006).

[58] Corporation, Promega, CellTiter 96® AQueous One Solution Cell Proliferation Assay Instructions For Use Of Products G3580, G3581 AND G3582. *Technical Bulletin* (© 1996-2005).

[59] Nelson, David L. and Cox, Michael M., *Lehninger principles of biochemistry*, 4th ed. (W.H. Freeman, New York, 2005).

[60] Windle, H. J., Fox, A., Ni Eidhin, D., and Kelleher, D., The thioredoxin system of Helicobacter pylori. *J Biol Chem* **275** (7), 5081 (2000).

[61] Veal, E. A., Day, A. M., and Morgan, B. A., Hydrogen peroxide sensing and signaling. *Mol Cell* **26** (1), 1 (2007).

[62] Darzynkiewicz, Z. et al., Features of apoptotic cells measured by flow cytometry. *Cytometry* **13** (8), 795 (1992).

[63] Davies, K. J., The broad spectrum of responses to oxidants in proliferating cells: a new paradigm for oxidative stress. *IUBMB Life* **48** (1), 41 (1999).

[64] Saitoh, M. et al., Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J* **17** (9), 2596 (1998).

[65] Mochizuki, M., Kwon, Y. W., Yodoi, J., and Masutani, H., Thioredoxin

Regulates Cell Cycle via the ERK1/2-Cyclin D1 Pathway. *Antioxid Redox Signal* **11** (12), 2957 (2009).

66 Chua, P. J., Yip, G. W., and Bay, B. H., Cell cycle arrest induced by hydrogen peroxide is associated with modulation of oxidative stress related genes in breast cancer cells. *Exp Biol Med (Maywood)* **234** (9), 1086 (2009).

67 Wiese, A. G., Pacifici, R. E., and Davies, K. J., Transient adaptation of oxidative stress in mammalian cells. *Arch Biochem Biophys* **318** (1), 231 (1995).

68 Chen, Q. M. et al., Molecular analysis of H2O2-induced senescent-like growth arrest in normal human fibroblasts: p53 and Rb control G1 arrest but not cell replication. *Biochem J* **332 (Pt 1)**, 43 (1998).

69 Chung, Y. W. et al., H(2)O(2)-induced AP-1 activation and its effect on p21(WAF1/CIP1)-mediated G2/M arrest in a p53-deficient human lung cancer cell. *Biochem Biophys Res Commun* **293** (4), 1248 (2002).

70 Seomun, Y. et al., Induction of p21Cip1-mediated G2/M arrest in H2O2-treated lens epithelial cells. *Mol Vis* **11**, 764 (2005).

71 Habold, C. et al., Trichostatin A causes p53 to switch oxidative-damaged colorectal cancer cells from cell cycle arrest into apoptosis. *J Cell Mol Med* **12** (2), 607 (2008).

72 Gan, L., Yang, X. L., Liu, Q., and Xu, H. B., Inhibitory effects of thioredoxin reductase antisense RNA on the growth of human hepatocellular carcinoma cells. *J Cell Biochem* **96** (3), 653 (2005).

73 Barnouin, K. et al., H2O2 induces a transient multi-phase cell cycle arrest in

mouse fibroblasts through modulating cyclin D and p21Cip1 expression. *J Biol Chem* **277** (16), 13761 (2002).

[74] Kang, K. A. et al., Cytoprotective effect of tectorigenin, a metabolite formed by transformation of tectoridin by intestinal microflora, on oxidative stress induced by hydrogen peroxide. *Eur J Pharmacol* **519** (1-2), 16 (2005).

[75] Yoo, M. H. et al., Targeting thioredoxin reductase 1 reduction in cancer cells inhibits self-sufficient growth and DNA replication. *PLoS One* **2** (10), e1112 (2007).

[76] Plesca, D., Crosby, M. E., Gupta, D., and Almasan, A., E2F4 function in G2: maintaining G2-arrest to prevent mitotic entry with damaged DNA. *Cell Cycle* **6** (10), 1147 (2007).

[77] Taylor, W. R. and Stark, G. R., Regulation of the G2/M transition by p53. *Oncogene* **20** (15), 1803 (2001).

[78] Bunz, F. et al., Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* **282** (5393), 1497 (1998).

[79] Draus, J. M. et al., p53 gene transfer does not enhance E2F-1-mediated apoptosis in human colon cancer cells. *Exp Mol Med* **33** (4), 209 (2001).

[80] Mukherjee, A. et al., A cellular and molecular investigation of the action of PMX464, a putative thioredoxin inhibitor, in normal and colorectal cancer cell lines. *Br J Pharmacol* **151** (8), 1167 (2007).