The role of thioredoxin system in oxidative stress and cell aging

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by

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Declaration

I hereby declare that this thesis represents my own research work which has been done within the period from September 2003 to August 2005 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.

Levina Suk Mi Lam

April 2006

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List of Abbreviation

Abbreviation	Full Name
AchE	acetylcholinesterase
BSA	bovine serum albumin
CM	carboxylmethyl
ddH_2O	de-ionized distilled water
DNA	deoxyribonucleic acid
DTNB	5,5'-dithiobis-(2-nitrobenzoic) acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FAD	flavin adenine dinucleotide
H_2O_2	hydrogen peroxide
Hb	haemoglobin
HEPES	(N-[2-hydroxyethl]piperazine-N'-[2-ethanesulfonic acid])
HRP	horseradish peroxidase
IgG	immunoglobulin
kDa	kilodaltons
MCHC	mean cell haemoglobin concentration
mRNA	messenger ribonucleic acid
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

NADPH	nicotinamide adenine dinucleotide phosphate
NDP	nucleoside diphosphate
NF-κB	nuclear factor-kappaB
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCV	packed cell volume
PMSF	phenylmethanesulfonyl fluoride
PVDF	polyvinylidene fluoride
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
TEMED	N,N,N,N-tetramethylethylene diamine
TPBS	0.05% (v/v) Tween 20 in phosphate buffered saline
TR	thioredoxin reductase
Tris(HCl)	tris(Hydroxymethyl)aminomethane
Trx	thioredoxin
Tween	poly(oxyethylene) sorbitan monolaurate

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Abstract

Aging is an inevitable part of the life natural process that is governed by decreasing in physiological functions that ultimately result in mortality. Reactive oxygen species (ROS) are believed to be one of the casual factors in aging. Consequently, the ability to respond appropriately to oxidative challenge is likely to be an important factor in combating diseases and disabilities of aging. Unique amongst others, mammalian erythrocytes are continuously subject to oxidative damage but devoid of protein synthesis machinery, which implies the repairing mechanism is most important for cell survival. To defend the attack, there are quite a number of pathways and mechanisms. Thioredoxin system, comprising that of the enzyme thioredoxin (Trx), thioredoxin reductase (TR) and NADPH is one of the best representative systems for its protective function against oxidative stress in various cells. While the existence and importance of thioredoxin system in erythrocytes are least understood, it would therefore be informative to use erythrocytes as a study model to find out the relationship of cell aging and oxidative stress.

By means of their difference in density, young and old populations of red cells were separated, characterized and used in the analysis of thioredoxin system components. By immunoblotting, thioredoxin was found in young cells but not in old cells. The activity assay also revealed similar pattern that there was 0.19 U thioredoxin/mg total protein in young cells but no activity can be observed in old cells. Our findings suggest that the thioredoxin system may be involved in the aging process of erythrocyte. To further elucidate the role of thioredoxin system, young and old erythrocytes were subject

to oxidative challenge with hydrogen peroxide. We found an increase of 63.5% thioredoxin system activity in young cells challenged with 4 mM H_2O_2. Though the level and activity of thioredoxin in old cells were not detectable, an enhancement of the whole thioredoxin system activity was noticed after oxidative challenge. Such an enhancement of the system activity implies a regulatory system of the thioredoxin system may exist, which can respond to oxidative stress but *de novo* protein synthesis is not required. It therefore becomes important to extend our study to nucleated cells to investigate if endogenous regulatory system of thioredoxin system does exist universally.

HeLa and HT-29 cell cultures were employed to study the thioredoxin system activity upon oxidative challenge with and without prior treatment of cycloheximide, by which *de novo* protein synthesis of proteins would be inhibited. In both cell cultures, enhancement of thioredoxin system activity was observed upon oxidative challenge (8.2% and 19.7 % in HeLa cells; 13.5 % and 18.9 % increase in HT-29 cells, with and without the treatment of cycloheximide respectively). In this study, we have demonstrated enhancement of thioredoxin system activity upon oxidative challenge in both nucleated and anucleated cell models and that enhancement was not necessarily dependent on *de novo* protein synthesis. Our notion is that both *de novo* protein synthesis and a direct activation of the thioredoxin system may be involved in response to oxidative stress. Together with the yet to be characterized regulatory system, the full thioredoxin system appears to play important roles in cell aging and combat against oxidative stress.

I Introduction

1.1 Oxidative stress and aging

Aging is a highly emotive and health issue for human beings. It is the main underlying basis of most major human diseases such as atherosclerosis, cardiovascular defects, neurodegeneration, cataract and cancer (Ratten, 2005). Much evidence have shown that the maintenance and repairing capacities of cells determine the natural survival of a species. As a result, the maintenance of intrinsic homeodynamics characteristic becomes ultimately important. In addition, previous studies illustrated that wide ranges of biochemical pathways are actively involved in the aging process such as kinases, the transcriptional factors and the cell cycle pathways. By modulating the basic process of aging, the onset of those age-related diseases can be delayed.

The process of aging is not completely understood due to the involvement of an incalculable number of biological mechanisms and pathways (Barry, 2002). However, considerable progress has been made in explaining the aging process. Oxidative damage accumulation is one of the phenomena of aging in most cell types. Previous studies indicated that most of the age related alterations are generally related to oxidative stress with increased reactive oxygen species (ROS) formation due to aerobic metabolism (Kang, 2005). ROS are inevitable products in cells, which are relatively high energy, unstable free radicals produced by aerobic metabolism in cells. These include hydroxyl radicals, the superoxide radical anions, singlet oxygen, peroxyl radicals and hydrogen peroxide as well (Stuart, 2001). Indeed, the majority of intracellular ROS production is derived from mitochondria (Toren, 2000) as a result of electron escape from the electron

transport chain and the Fenton chemistry within the mitochondria matrix (Chen, 2005). Upon the generation of high levels of ROS from exogenous or endogenous sources, the increment of intracellular oxidant levels disturbs the redox balance and cells therefore undergo oxidative stress. Due to their high reactivity, the excessive ROS therefore cause damage to all major groups of biomolecules such as lipids, DNA and proteins. And finally results in preliminary cell dysfunction.

Despite of the damaging effects of ROS, they also play essential physiological role in various cells (Nordberg, 2001). It is found that the intracellular reactive oxygen species may act as signaling molecules and mediators in cell senescence. And mostly the reaction is transcriptional mediated (Ronata, 2005). ROS serve as second messengers of several cytokines, growth factors, hormones and neurotransmitters, thereby involving in the intracellular signal transduction (Nordberg, 2001). Taken together, the influence of ROS is therefore in a complex fashion (Toren, 2000).

Indeed, the ability of cells to defend, counteract and respond to oxidative stress becomes one of the factors affecting the lifespan of the organism. Numerous forms of ROS are generated by diverse cellular processes, which might have different reactivities and result in disastrous effects on cell viability. The survival is dependent on the antioxidant capacity of cells (Chen, 2005). As a result, controls and defences against ROS are of ultimately importance to cells. At the same time, the repairing and maintenance mechanisms also take important roles in the redox regulation.

ROS are known to oxidize, and therefore damage macromolecules. The effects are more pronounced on proteins. The oxidation of amino acid residues of proteins is one of the characteristics of oxidative damage (Stuart, 2001). Due to a less efficient removal through proteolytic cleavage in the aging cells, oxidized proteins are therefore accumulated. It finally leads to age-related diseases due to the structural alterations of proteins, the interferences of regulatory functions and the inhibitions of the enzyme activity (Barry, 2002). Progressive accumulation of molecular damage should be avoided to diminish the harmful effects onto cells. Despite the requirement of an efficient protein degradation system, repairing mechanisms are therefore essential to contribute to rescue the oxidized proteins, whereas the thioredoxin system is one of the best representatives in repairing proteins by a redox regulation.

1.2 Thioredoxin system

The thioredoxin system, which is comprised of thioredoxin (Trx), thioredoxin reductase (TR) and NADPH, is ubiquitous from Archea to man (Arnér, 2000). Trx was first described in 1964 as a small redox protein of *Escherichia coli* (Laurent, 1964). It is a ubiquitous protein with a molecular weight of 12 kDa, and is characterized as a heat stable protein that presents in cells, tissues and subcellular fragments (Nordberg, 2001). The presence of conserved dithol/disulphide active site, Trp-Cys_{32}-Gly-Pro-Cys_{35}-Lys, which consists of Cys_{32} and Cys_{35} catalytic residues that acts as a nucleophile for thiol-sulfide conversion reaction (Arnér, 2000). As a result, the conserved active site serves as the major cellular protein disulfide reductase. Such active center enables Trx to participate in thiol-dependent redox reactions. Thus the thioredoxin system becomes one

of the most important systems that facilitates the cellular thiol redox control and antioxidant defense (Nordberg, 2001). The most studied form is the cytosolic thioredoxin, Trx-1, which is ubiquitously expressed in mammalian cells (Haendeler, 2004).

Thioredoxin reductase, on the other hand, is a selenium dependent dimeric flavoprotein with a molecular size of 58 kDa. The mechanism of TR involves the transfer of the reducing equivalents from NADPH to a disulfide bond of TR within its conserved active site: -Cys-Ala-Thr-Cys- via FAD (Holmgren, 1995). The redox active site dithiol/disulphide of TR has a board range of substrate specificity, while Trx is one of the partners that work with TR. However, TR is the only enzyme that is known to be able to reduce the active site of Trx (Powis, 2001).

The reaction of the thioredoxin system is described as follows:

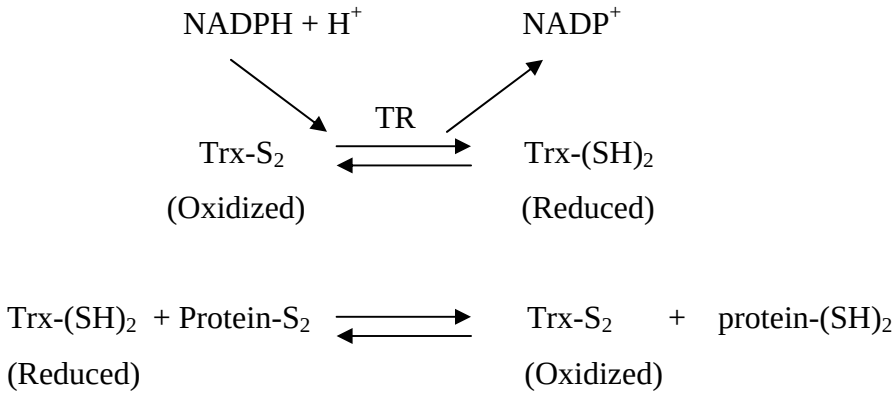

Fig. 1.2.1 Scheme of oxidoreductase activities of the thioredoxin system

The thioredoxin system is a NADPH-dependent protein disulfide reductase system (Nordberg, 2001). In the presence of NADPH (by acting as an electron donor), oxidized thioredoxin ($Trx\text{-}S_2$) is reduced to dithiol thioredoxin, $Trx\text{-}(SH)_2$ by thioredoxin

reductase. Reduced thioredoxin is highly efficient in reducing disulfides in proteins and peptides. As a result, Trx system plays a central role for redox control of cellular function.

1.3 Biological roles of thioredoxin system

The thioredoxin system is a highly conserved, ubiquitous system that plays an important role in the redox regulation of several intracellular processes, including DNA synthesis, antioxidation, hormone action and cytokine functions involvement, apoptosis and redox regulations. Some of these are described as follows:

1.3.1 DNA synthesis

One of the earliest known functions of Trx is that it serves as a source of reducing equivalents for ribonucleotide reductase. DNA synthesis requires deoxyribonucleotides. Trx acts as reducing equivalents for ribonucleotide reductase which is important for catalyzing the conversion of nucleotides to deoxynucleotides (Holmgren, 1989). Thus Trx is involved in the first unique step of DNA synthesis and an important step for cellular proliferation (Powis, 2001).

$$NDP + Trx\text{-}(SH)_2 \longrightarrow dNDP + H_2O + Trx\text{-}S_2$$

1.3.2 Antioxidation

The thioredoxin system is also involved directly in the antioxidation function. Most cells must keep their cell homeostasis for survival. At the time of aerobic

metabolism, inevitable by-products such as reactive oxygen species (ROS) are generated. ROS such as hydrogen peroxide, superoxide, singlet oxygen derived from the unwanted side reactions inflict oxidative damage to proteins, lipids and genetic materials in cells. Some kinds of damage are irreversible. Trx can maintain its function continuous by the help of TR to reduce the ROS concentration.

1.3.3 Regulation of apoptosis

ASK-1 activates the c-Jun amino-terminal kinase (JNK) and p38 MAP kinase pathways, and consequently apoptosis occurs. It is found that the reduced Trx forms inactive complex with apoptosis signal-regulating kinase (ASK-1), which prevents the downstream signaling for apoptosis. The findings also illustrated that the inhibition of ASK-1 by Trx is highly dependent on the redox status of Trx (Watson, 2003). Recent data also suggest that dissociation of Trx from ASK-1 does occur when Trx is oxidized by the stress-induced ROS and TNFα (Watson, 2003). Endogenous ASK-1 is therefore activated and it leads to an ASK-1 dependent apoptosis (Arnér, 2000).

1.3.4 Hormone actions and cytokine functions involvement

The thioredoxin system is involved in various aspects of hormone and cytokine actions. It is reported that the reduced Trx is secreted from cells extracellularly and it functions as an autocrine growth-factor synergizing with IL-1 and IL-2. The findings illustrate that the redox properties of Trx are involved in the autocrine stimulation process, yet its mechanism still needs to be investigated (Wakasugi, 1990).

1.3.5 Regulation of transcriptional factors

Redox regulation is one of the important systems in controlling cell function. Indeed, cysteines present in the active site of proteins are usually the main residues for activity. It is impossible to sustain the reducing cytosolic environment at all time, which implies that the formation of oxidized species is unavoidable. The thioredoxin system, on the other hand, helps redox-regulate those primary response mechanisms, such as the activation or deactivation of specific enzymes or transcription factors (Sun, 2002). Trx specifically activates a number of transcriptional factors involving DNA binding. One of the well known transcriptional factors, NF-κB, which takes part in the cell responses to oxidative stress, apoptosis and tumorigenesis as well, is directly redox-regulated by Trx (Powis, 2001). Trx catalyzes the reduction of Cys^{62} of the NF-κB p50 subunit, thus results in the enhancement of its DNA binding capability.

The transcription factor AP-1 (Fos and Jun homo- and heterodimers), with its activation closely correlated with increased cell growth, is also redox-regulated by Trx (Powis, 2001). The reduction of a single conserved Cys residue in the DNA binding domain of each of the homodimers results in an increment of the DNA binding of AP-1 (Jordan, 1998). It was shown that cells transfected with human Trx showed an increase in AP-1 activity measured by a reporter construct (Freemerman, 1999). However, Trx is not directly involved in the process. Trx does not reduce AP-1 directly but does so through another nuclear redox protein Ref-1 (37-kDa). Sequences in the N-terminal domain of Ref-1 are necessary for the redox activity, while C-terminal sequences are required for

the DNA repairing activity (Powis, 2001). Thereby, the N-terminal domain of Ref-1 is first reduced by the active Trx, then the reduced Ref-1 domain is able to reduce the Cys residue of AP-1, thus its DNA binding ability is further increased. It shows that Trx becomes the main thiol redox control center for cell functioning, with either the activation or inactivation of the transcription factors.

1.4 Local concentration, tissue distribution and subcellular localization of Trx and TR

It was reported that Trx and TR occur in all subcellular compartments. Trx exists in cells with concentration ranging from 1 to 20 μM. It can be up to 100 μM in some particular sites (Follmann, 1995). It was also shown that the concentration of TR in cells would be 1 μM, while the μM range of Trx and TR is thought to be in a functional physiological range (Nordberg, 2001). Due to the species difference and also deviations in different methods employed, the tissue specific expression of Trx and TR varies. However, the mRNA level and protein level should be taken into account for the evaluation of the distribution. Interestingly, it was found that Trx and TR have affinity towards all cellular membranes which implies they are membrane associated. Taken together, it is noteworthy that the abundance of the thioredoxin system with multifunctions implies the importance of the system in cells. However, the definitive localization and role of the thioredoxin system in nucleated and anucleated cells still need to be investigated.

1.5 Thioredoxin system and oxidative stress

In order to combat against oxidative challenge, various antioxidation mechanisms are present in cells. Cells have enzymatic and non-enzymatic systems to defend against oxidative stress (Stadtman, 1992). The thioredoxin system is thought to be involved in the cellular response to ROS. Previous studies showed that the thioredoxin system protects ordinary cells against oxidative stress (Powis, 2001). However, whether the thioredoxin system plays a similar protective role in specialized and/or anucleated cells is still largely unknown.

Erythrocytes offer a number of advantages for the study of the response of the thioredoxin system upon oxidative challenge. Circulatory mammalian erythrocytes are devoid of a protein synthesis machinery and continuously subject to oxidative damage. In many previous studies, evidence showed that increased oxidative stress would cause adverse effects on the proper functioning and integrity of the mammalian erythrocytes (Mauro, 1991). It would finally result in the oxidation of the protein sulfihydryl group (Snyder, 1988), enhancement of proteolysis (Daves, 1987) and erythrocytes membrane instability (Mauro, 1991; Deuticke, 1987). The survival of erythrocytes would therefore largely rely on effective cellular anti-oxidation and/or protein repairing systems. Amongst all these, the thioredoxin system, which comprises of the enzyme thioredoxin reductase (TR), thioredoxin (Trx) and NADPH has been known to be one of the essential protective systems in various cells (Arnér, 2000; Mustacich, 2000). Yet its importance and existence in erythrocytes are not very well understood. It would therefore be

informative to use erythrocytes as a study model to find out the relationship between cell aging and oxidative stress.

It was reported that repair of proteins inactivated by oxidative challenge requires the help of the thioredoxin system (Fernando, 1995). The thioredoxin system assists the repair of proteins in a direct or indirect manner. Protein refolding is one of the well known functions of the thioredoxin system. Many eukaryotic proteins are produced in *Escherichia coli* as insoluble aggregates, thus undesirable products with abnormal functions are formed. Recent findings suggested that Trx acts as a chaperone to help refolding of various proteins. The findings also illustrated that Trx can refold proteins without disulfide bridges, which implies an active site of Trx may not be involved in the refolding process (Yu, 2003). Despite the refolding function, Trx and TR can also serve to recycle antioxidants (Nordberg, 2001). TR assists the recycle of dehydroascorbate to ascorbate, which is a water soluble antioxidant in human plasma. It helps reduce peroxides and ROS such as superoxides (Nordberg, 2001).

Surprisingly, the system involves more than 50 % of the repairing function (Gromer, 2004). The thioredoxin system is therefore one of the most important protein repairing mechanisms from prokaryotes to eukaryotes. Taken together, the thioredoxin system is essential in defending against oxidative stress. However, the distinct role of the thioredoxin system in erythrocytes is still not well understood. A further investigation on the biochemical and biophysical changes, level, activity and integrity of the thioredoxin system in both dividing and non-dividing cells will then be the most essential and urgent

in elucidating the role of the system in the cell aging process. The understanding of the cellular response to oxidative challenge will in the end provide important insight into cellular aging.

1.6 Potential applications of thioredoxin system in medical aspects

The application of the thioredoxin system in medical science has been far-reaching in the past several years. The thioredoxin system takes part in various cellular mechanisms which implies potential medical applications. Indeed, the thioredoxin system plays an important role in fighting against infectious diseases and non-infectious disease. These applications of the thioredoxin system have led to more research in the treatment of inflammatory disease and virus disease.

Transmittable pathogens are the main cause of infectious disease. In order to survive in a hostile environment, an effective defense mechanism such as the thioredoxin system is necessary to defend against the host immune system. It was shown that Trx is able to inactivate immunoglobulins to some degree (Magnusson, 1997). Indeed, thioredoxin system should be investigated to fight against infectious disease. Due to the species difference of the thioredoxin system, it is possible to make an antiparasitic drug with few side effects.

The thioredoxin system may play a role of defense against non-infectious diseases such as cancer. The thioredoxin system employs its antioxidative properties to reduce

ROS formed by carcinogens (Clark, 1996). It was found that with the supplement of selenium in the therapy, the activity of selenium dependent TR increases, which in turn enhances the formation of a tumor preventing compound methyselenol, which can penetrate membrane easily and helps in detoxifying ROS. Such applications imply the importance of studying the thioredoxin system in response to oxidative challenge.

1.7 Objectives

The thioredoxin system, which comprises of the Trx, TR and NADPH, is a multifunctional proteins with a well-known protective mechanism in many cells. In some previous studies, this ubiquitous system was shown to exhibit an oxidoreductase activity with a redox regulatory role. Moreover, the anti-oxidation function of the thioredoxin system in dividing cells has been previously described. Recent findings suggested that TR could be expressed at elevated levels under oxidative stress and the process could be transcription factors mediated. Nevertheless, the direct relationship between the thioredoxin system and ROS is still not well understood.

Such findings prompt us to investigate the true role of the whole system in defense against oxidative stress and cell aging. In this research project, two cell types: non-dividing cells and dividing cells would be investigated. Porcine erythrocytes were used as a non-dividing cell model. The thioredoxin system of young and old erythrocytes was investigated by western blotting analysis, insulin DTNB activity assay and immunoglobulin reduction assay. Furthermore, hydrogen peroxide was used as oxidative challenge agent to erythrocytes to study the action of the thioredoxin system in the red cell model. At the same time, the level and activity of TR and Trx were studied in HeLa and HT-29 cells challenged with H_2O_2 and a prior treatment of cycloheximide. The aim of the comparative study between the dividing and non-dividing cells is to find out whether *de novo* protein synthesis and/or possibly a direct activation of the thioredoxin system are involved in response to oxidative stress. As a long term goal, the findings will not only provide some basic understandings of the role of the thioredoxin system in

combating against oxidative stress but also opening up new areas for potential applications. We hope that this study contributes to the understanding of the aging problems.

II Methodology

Section I Methods and Materials

Biological samples

Porcine blood was freshly collected from slaughterhouse. The blood samples were collected in heparin containing bottles.

Cell cultures

Human carcinoma cell lines (HeLa and HT-29) were a gift kindly provided by Dr. N. S. Wong of the Department of Biochemistry, The University of Hong Kong.

Materials

Thioredoxin, thioredoxin reductase and antibody against thioredoxin reductase were previously prepared by Dr. K.S. Lee's research group. Antibody against thioredoxin was obtained from Bio-Synthesis Corp. Trypsin-ethylenediaminetetraacetic acid (EDTA), Penicillin-Streptomycin-Glutamine, Dulbecco's modified Eagle's medium and fetal bovine serum were obtained from Gibco. 4-Chloro-1-naphthol, 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB), NADPH, bovine insulin and horseradish peroxidase-linked antibody against IgG were from Sigma. CM Sephadex™ C-50 and dithiothreitol (DTT) were purchased from Amersham Biosciences. Protein A Sepharose® CL-4B was obtained from Pharmacia Biotech. SuperSignal® West Pico Chemiluminescent Substrate was purchased from Pierce. Micro Biospin® 6 chromatography column was obtained from BioRad.

Ultrafiltration membranes with 1000 Da cut-off, Immobilon™ transfer polyvinylidene fluoride (PVDF) membranes and Amicon® Ultra were purchased from Millipore.

All buffer reagents were prepared in deionized water. All other chemicals were of analytical reagent grade.

Section II Preparation of young and old erythrocytes

Erythrocytes experience continuous oxidative insults by being exposed to reactive oxygen species. Although there are various protective systems in erythrocytes, oxidative damage of proteins is one of the most prominent features of erythrocytes senescence. To protect them from premature destruction erythrocytes depends on various repairing mechanisms. Though the thioredoxin system is one of the important repairing mechanisms in various cells, the level and activity of the thioredoxin system in erythrocytes are least understood. In this study, erythrocyte was therefore employed as an anucleated cell model for the study of the thioredoxin system in cell aging. Since porcine blood is readily available and that the cell's morphology and physiology are comparable with that of human, pig erythrocyte therefore serves as a good model for this study.

In order to study the role of the thioredoxin system in cell aging, young and old erythrocytes were first separated from porcine blood by means of centrifugation. The separation of young and old cells was then assessed by various haematological and

biochemical methods including red blood cells counting, mean cell haemoglobin concentration, mean corpuscular volume and membrane acetylcholinesterase activity.

Part 2.1 Separation of young and old erythrocytes from porcine whole blood

2.1.1 Separation of erythrocytes into young and old cell populations

Erythrocytes were separated into young cells and old cells by density gradient centrifugation (Murphy, 1973)**.** Fresh heparinized pig whole blood was centrifuged at 4,000 g at 4 ºC for 10 min to obtain packed red cells, which were resuspended to 90 % haematocrit, and then centrifuged at 39,000 g at 30 ºC for 1 h with a JA 20 rotor (Beckman). The young and old cells were separated as the upper 10 % packed red cells and bottom 10 % cells respectively. Cells were then re-suspended and washed three more times with phosphate buffered saline (PBS). The young and old cells were divided into three aliquots, each of them challenged with different concentrations of H_2O_2 (0, 4 and 8 mM) for 60 min at 37 ºC. After the challenge, cells were immersed in an ice bath for 60 s. Subsequently, 10 % haemolysates were prepared by the addition of a defined volume of PBS with saponin. Membranes of the red cells were separated from lysed erythrocytes by centrifugation at 39,000 g at 4 ºC for 30 min. Membranes were then washed 2 times with 50 mM Tris-HCl with 1 mM EDTA at pH 7.4 and 3 more times with 50 mM Tris-HCl at pH 7.4 while 10 % haemolysates (membrane-free) were collected and concentrated by ultrafiltration with Amicon® Ultra at 3,500 g for 30 min.

Part 2.2 Assessment of the separation of young and old erythrocytes

To verify the successful separation of the young and old cells, red blood cell counting, haemoglobin concentration, mean corpuscular volume as well as membrane acetylcholinesterase activity of the young cells and old cells were determined. This ensured good yield of the young and old cells samples for further investigation of the thioredoxin system in different aged group of erythrocytes.

2.2.1 Red blood cell counting

Red blood cell counting was performed by using an improved Neubauer hemocytometer (Chanarin, 1989). Cell suspensions (20 µL) were transferred to 4 mL cell count diluents. The cell number within a definite area of known depth was counted and cell concentrations were derived from the counting.

2.2.2 Determination of haemoglobin concentration of young and old erythrocytes

The mean cell haemoglobin concentration is one of the parameters to differentiate the structural difference in the two erythrocytes fractions. Haemoglobin concentration was determined by the Cyanomethaemoglobin method (Dacie & Lewis, 1975). Cell suspensions (20 µL) were mixed with 4 mL Drabkin's reagent (200 mg potassium ferricyanide, 50 mg potassium cyanide and 140 mg potassium dihydrogen phosphate/L). Absorbance of the mixture at 540 nm was determined after standing for 20 min with cyanomethaemoglobin (57.2 mg/100 mL) as standard.

2.2.3 Determination of mean corpuscular volume in young and old erythrocytes

The mean corpuscular volume (MCV) is one of the best age parameters to assess the age of the red cells. The MCV of red cells is known to decrease progressively with age. Erythrocytes in young and old cell suspension were counted while haematocrit of the young and old cells was determined. Then, mean corpuscular volume of young and old cells could be calculated (Dacie & Lewis, 1975).

2.2.4 Determination of membrane acetylcholinesterase activity of young and old erythrocytes

During the *in vivo* aging process, contents of the erythrocytes membranes change with age. The membrane protein, acetylcholinesterase is one of the aging markers. The membrane acetylcholinesterase activity of young and old cell membrane was determined using a previously described method with some minor modifications (Ellman, 1961). Membrane (40 µL) was added to 3 mL 0.1 M sodium phosphate buffer at pH 8.0. 20 µL of 75 mM acetylthiocholine iodide, and 100 µL DTNB reagent (10mM DTNB/10mM sodium hydrogen carbonate in 0.1 M sodium phosphate buffer at pH 7.0 were subsequently added. Absorbance was measured and monitored at 412 nm for 10 min. Membrane protein concentration was determined by the Lowry method with some minor modifications (Lowry, 1951).

Part 2.3 Preparation of young and old red cell lysates for analysis

In the study, the interfering protein, haemoglobin, was removed by cation exchange chromatography. The young and old cell lysates were then subjected to further analysis.

Both young and old haemolysates were treated as follows.

2.3.1 Removal of haemoglobin from red cell haemolysates by cation exchange chromatography

This method was developed in our laboratory. A column of 3 cm × 10 cm, bed volume (70 mL) was packed with CM Sephadex^{TM} C-50 previously swollen in equilibrating buffer of 25 mM Tris-HCl, pH 6.8. The packed column was then further washed with at least four bed volume of the equilibrating buffer until the column was well equilibrated to pH 6.8. The capacity of CM Sephadex^{TM} C-50 for haemoglobin is 140 mg/mL drained gel.

Before loading onto the column, haemolysates were concentrated and extensively dialysed against the equilibrating buffer. Then 5 mL of haemolysate was added onto the column. After the sample was loaded, the column was eluted with four bed volume of the equilibrating buffer to elute the unbound proteins. Thus, the target proteins (Trx and TR) together with other unbound proteins were collected. The column was washed with 4 bed volume of the equilibrating buffer. The unbound proteins were collected at 2 mL per fraction. Eluted proteins were concentrated to 2 mL by ultra-filtration. Activity assays and western blots were performed for characterization.

2.3.2. Concentration of young and old sample by ultrafiltration

All the eluents prepared in the previous step were subjected to ultrafiltration with Amicon® Ultra and YM-1 membranes (1000 MWCO) for subsequent studies. 4 Ba pressure was applied to it. All steps were carried out at 4 ºC.

Part 2.4 Analysis of young and old sample

2.4.1 Western Blot analysis of Trx in young and old sample

The existence of Trx was detected by the immunoblotting method. 30 µg of concentrated haemoglobin deficient cell lysates of the young and old samples were heated in the reducing sample buffer at 100 ºC for 5 min and subjected to SDS PAGE analysis. After electrophoresis, the separated proteins were then electroblotted onto the polyvinylidene difluoride (PVDF) membrane at 100 V for 2 hours. The membranes were blocked with 5 % skimmed milk in 1 × TPBS (1 × PBS + 0.05 % Tween 20) for 1 hr. Then, the membrane was incubated overnight with custom-made Trx antibody (1: 4000) in 1 × TPBS containing 2 % BSA. The Trx antibodies were polyclonal, probed for the whole molecule of Trx. After washing three times with 1 × TPBS, the membrane was incubated in horseradish peroxidase (HRP) conjugated goat against rabbit secondary antibody (1: 5000) which was in conjunction with chemiluminescent substrate. Following 1 hr incubation, the membrane was washed three times with 1 × TBS. The detection was performed by the addition of Supersignal® West Pico Chemiluminescent substrate and visualized by Lumicap 3.1 software.

2.4.2 Western Blot analysis of TR in young and old sample

The steps in western blotting analysis of TR were the same as the above mentioned (2.4.1). The existence of TR was detected by the immunoblotting method. 30 µg of concentrated haemoglobin deficient cell lysates of the young and old samples were heated in the reducing sample buffer at 100 ºC for 5 min and subjected to SDS PAGE analysis. After electrophoresis, the separated proteins were then electroblotted onto polyvinylidene difluoride (PVDF) membrane at 100 V for 2 hours. The membranes were blocked with 5 % skimmed milk in 1 × TPBS (1 × PBS + 0.05 % Tween 20) for 1 h. Then, the membrane was incubated overnight with TR antibodies previously raised in our laboratory at 1: 4000 in 1 × TPBS containing 2 % BSA. After washing three times with 1 × TPBS, the membrane was incubated in HRP conjugated secondary antibody (1: 5000) which was in conjunction with chemiluminescent substrate. Following 1 h incubation, the membrane was washed three times with 1 × TBS. The detection was performed by the addition of Supersignal® West Pico Chemiluminescent substrate and visualized by Lumicap 3.1 software.

2.4.3 Determination of Trx activity by insulin dependent DTNB activity assay

Trx activity was determined by the insulin dependent DTNB activity assay as described by Holmgren & Björnstedt (1995) with some minor modifications. Active Trx is capable of reducing disulphide bonds in insulin. Sample (68 µL) was allowed to incubate at 80 ºC for 10 min. Then, 2 µL DTT activation buffer (50 mM HEPES, 1 mM EDTA, 1 mg/mL Bovine serum albumin, 2 mM DTT) was added onto the samples. The mixture was incubated at 37 ºC for 10 min. Afterwards, 40 µL reaction mixture

(260 mM HEPES at pH 7.6, 10 mM EDTA , 2 mg/mL NADPH , 6 mg/mL insulin) was added. The reaction was started by the addition of 30 µL TR and was incubated at 37 ºC for for 20 min. Free thiols generated were assayed by the addition of 500 µL DTNB (0.4 mg/mL) in 6 M Guanidine HCl. Absorbance at 412 nm was measured while enzyme activity in the assay was expressed as µmol of TNB formation by using the molar extinction coefficient of 13600 $M^{-1}cm^{-1}$.

2.4.4 Determination of TR activity by insulin dependent DTNB activity assay

This was determined by the insulin dependent reduction assay as described by Luthman & Holmgren (1982). A stock reaction mixture was prepared by mixing 200 µL 1.0 M HEPES at pH 7.6, 40 µL NADPH (40 mg/mL), 40 µL 0.2 M EDTA and 500 µL insulin (10 mg/mL). To each microtubes, 40 µL reaction mixture, 10 µL 0.36 mM Trx and 70 µL sample were mixed and incubated at 37 ºC for 20 min. The reaction was stopped by the addition of 500 µL 0.4mg/mL DTNB in 6 M Guanidine HCl. Absorbance at 412 nm was measured while enzyme activity in the assay was expressed as µmol of TNB formation by using a molar extinction coefficient of 13600 $M^{-1}cm^{-1}$.

2.4.5 Functional studies of thioredoxin system by IgG reduction assay

The assay measures the capability of the thioredoxin system in breaking down IgG light chains from heavy chains (Magnusson, 1997; Windle, 2000). Concentrated haemolysates from the young and old cell population were first incubated with 1 µL 0.1 M DTT at 37 ºC for 30 min. DTT was then removed by a Bio-spin column. Mouse IgG (5 µg), 500 µM NADPH and DTT activated concentrated haemolysates were incubated at 30 °C for two hours. After incubation, 15 µL (50 % gel) Protein A Sepharose was added into the sample mixture. Binding buffer (50 mM Tris-HCl at pH 7.5) was added onto it. It was then centrifuged at 8000 g for 20 seconds to spin down the gel. Supernatants were discarded, and the washings were repeated twice. Samples were then run in a non-reducing electrophoresis system using 10 % polyacrylamide gel. Western blotting was then applied with horseradish peroxidase-conjugated anti-IgG as antibody. Split IgG heavy chains were visualized with the addition of 4-chloro-1-naphthol. Intensity of intact IgG and split chains were measured which reflected the thioredoxin system activity of the tested samples.

Section III Preparation of HeLa and HT-29 lysates

To further delineate the role of the thioredoxin system, it is crucial to use other cell model to compare with results obtained from that of a specialized anucleated cell, erythrocyte. HeLa and HT-29 cells were chosen as the nucleated cell model for the studying of the thioredoxin system. These were used to investigate the activity of the thioredoxin system upon oxidative challenge.

Part 2.5 Culture of HeLa and HT-29 cell line

The cervical cancer cells HeLa were kept at the laboratory in Dulbecco's MEM supplemented with 10 % fetal calf serum, 1% penicillin/streptomycin. They were incubated at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air. The culture medium was changed every 2 days.

Part 2.6 Oxidative challenge on HeLa and HT-29 cell line

HeLa cells were seeded in 6-well plates with the growing conditions as described above. Medium was changed one day before the experiment. HeLa cells were plated at 0.4×10^6 cells per well in a 6-well plate for 48 h. Afterwards, cells were treated with cycloheximide (CHX) at 1 mM for 4 hr to block *de novo* protein synthesis followed by incubation with the addition of H_2O_2 at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air for 8 h. After incubation, the viability of cells was determined by the Trypan Blue dye exclusion assay and MTS tetrazolium assay.

Part 2.7 Cell viability by Trypan Blue dye exclusion assay

Cell viability was evaluated by the Trypan Blue dye exclusion assay. Briefly, cell suspensions were mixed with an equal volume of 0.4 % Trypan Blue, and the percentage of viable cells (ratio of cells excluding trypan blue to the total number of cells present) was determined by counting with a haemacytometer.

Part 2.8 Cell proliferation by MTS tetrazolium assay

The MTS assay for cell viability is based on the conversion of a tetrazolium salt into a coloured, aqueous soluble formazan product by the mitochondrial activity of living cells at 37 ºC (Malich & Boban, 1997). MTS activity assays were performed on cells treated with hydrogen peroxide for 8 h and also prior to treatment with cycloheximide. Cells were cultured overnight at 5,000 cells/well in a 96-well plate. Cycloheximide (1 mM) was added into the cell medium for 4 h followed by the addition of hydrogen peroxide for 8 h. The tetrazolium assay reagent (10 µL/well) was added immediately after the treatment, and the assay plates were incubated at 37 ºC. Absorbance at 490 nm was then recorded.

Part 2.9 Cell extracts preparation

Adherent cells were washed once with Dulbecco's PBS. They were harvested by trypsinization. Cell pellets were lysed in lysis buffer (20 mM Tris-HCl containing 1 % Triton X-100, 1 mM EDTA, 1 mM PMSF) in an ice bath for 20 min. Then, it was centrifuged at 13 000 g for 15 min at 4 °C. Afterwards, supernatants were collected and protein concentration was determined by the Bio-Rad Dc Protein Assay using bovine serum albumin as standard. Cell lysates were stored at -80 °C and thawed immediately before use.

Part 2.10 Analysis of cell lysates after oxidative challenge

2.10.1 Western Blot analysis of Trx in cell lysates after oxidative challenge

The level of Trx was detected by the immunoblotting method. Cell lysates (10 µg) were heated in the reducing sample buffer at 100 ºC for 5 min and subjected to SDS PAGE analysis. After electrophoresis, the separated proteins were then electroblotted onto the polyvinylidene difluoride (PVDF) membrane at 100 V for 2 hours. The membranes were blocked with 5 % skimmed milk in 1 × TPBS (1 × PBS + 0.05 % Tween 20) for 1 h. Then, the membrane was incubated overnight with custom-made Trx antibody (1: 4000) in 1 × TPBS containing 2 % BSA. The Trx antibodies were polyclonal, probed for the whole molecule of Trx. After washing three times with 1 × TPBS, the membrane was incubated in the horseradish peroxidase (HRP) conjugated goat against rabbit secondary antibody (1: 5000) which was in conjunction with chemiluminescent substrate. Following 1 h incubation, the membrane was washed three times with 1 × TPBS. The detection was performed by the addition of Supersignal® West Pico Chemiluminescent substrate and visualized by Lumicap 3.1 software.

2.10.2. Western Blot analysis of TR in cell lysates after oxidative challenge

The steps in western blotting analysis of TR were the same as the above mentioned (2.10.1). The level of TR was detected by the immunoblotting method. 10 µg of cell lysates were heated in the reducing sample buffer at 100 ºC for 5 min and subjected to SDS PAGE analysis. After electrophoresis, the separated proteins were then electroblotted onto the polyvinylidene difluoride (PVDF) membrane at 100 V for 2 hours. The membranes were blocked with 5 % skimmed milk powder in 1 × TPBS (1 × PBS +

0.05 % Tween 20) for 1 h. Then, the membrane was incubated overnight with TR antibodies previously raised in our laboratory at 1: 4000 in 1 × TPBS containing 2 % BSA. After washing three times with 1 × TPBS, the membrane was incubated in HRP conjugated secondary antibody (1: 5000) which was in conjunction with chemiluminescent substrate. Following 1 h incubation, the membrane was washed three times with 1 × TPBS. The detection was performed by the addition of Supersignal® West Pico Chemiluminescent substrate and visualized by Lumicap 3.1 software.

2.10.3 Functional studies of thioredoxin system in HeLa and HT-29

The steps involved in the functional assay were the same as described in the study of erythrocytes. The assay measures the capability of the thioredoxin system in breaking down IgG light chains from heavy chains (Magnusson, 1997; Windle, 2000). 10 µg cell lysates from samples inflicted with different degree of oxidative challenge were incubated with 5 µg mouse IgG, 500 µM NADPH at 37 °C for two hours. Samples were incubated at 90 °C for 3 min with the non-reducing buffer. Samples were then run in a non-reducing electrophoresis system using 10 % polyacrylamide gel. Western blotting was then applied with horseradish peroxidase-conjugated anti-IgG as antibody. Split IgG heavy chains were visualized with the addition of 4-chloro-1-naphthol. Intensity of intact IgG and split chains were measured which reflected the thioredoxin system activity of the tested samples.

III Results

A. Study of thioredoxin system in young and old erythrocytes

3.1 Separation of young and old erythrocytes from porcine whole blood

The young and old cell separation was performed by the centrifugation method. The achievement of separation was checked by red blood cell counting, mean cell haemoglobin concentration (MCHC), mean corpuscular volume (MCV) as well as membrane acetylcholinesterase activity, which are the established parameters for the assessment of the age of red cells.

3.1.1 Red blood cell counting

Red blood cell counting is an estimation of the number of red blood cells per litre of blood. It is one of the parameters to differentiate between the young and old cells. Both young and old erythrocytes were counted by means of a hematocytometer. The value illustrated that the samples of old cells had a higher number than that of young cells (by 32 %) per unit volume.

Table 3.1.1

Red blood cell counting of young and old erythrocytes

	Young cells	Old cells
Red blood cell counting (no./L)	$4.47 \times 10^{12} \pm 0.3$	$5.9 \times 10^{12} \pm 0.2$

Results are the mean ± SD of four experiments. Data are analyzed as the percentage difference between the young and old erythrocytes.

3.1.2 Haemoglobin concentration determination

The mean corpuscular haemoglobin concentration (MCHC) is another parameter to characterize the separation of the young and old cells. The MCHC determines the average haemoglobin concentration in a given volume of packed red cells. The result obtained from a representative separation clearly illustrated that there was a higher MCHC value in the old cells than that of the young one (by 16.2 %). The percentage difference was better than the findings of human as described by Yalouris (2003).

Table 3.1.2

Mean cell haemoglobin concentration of young and old cells

	Young cells	Old cells
Mean cell haemoglobin concentration (g/L)	27.8 ± 1.1	32.3 ± 1.8
Literature reference value (g/L) (Yalouris, 2003)	30.4 ± 1.3	33.5 ± 1.6

Results are the mean ± SD of four experiments. Data are analyzed as the percentage difference between the young and old erythrocytes.

3.1.3 Mean corpuscular volume determination

The mean corpuscular volume was the average volume of the erythrocytes. It could be found from the haematocrit (%) and erythrocytes counting analysis. The results obtained from a representative separation indicated the average size of the young erythrocytes was larger than the old one (by 32.6 %). The percentage difference was in good agreement with the literature reference range (Fausta, 2003).

Table 3.1.3

Mean corpuscular volume of young and old erythrocytes

	Young erythrocytes	Old erythrocytes
Mean corpuscular volume (fL)	61 ± 0.5	46 ± 0.2
Literature reference value (fL) (Fausta, 2003)	93 ± 0.7	81 ± 0.5

Results are the mean ± SD of four experiments. Data are analyzed as the percentage difference between the young and old erythrocytes.

3.1.4 Membrane acetylcholinesterase activity determination

Acetylcholinesterase is a glycoprotein on the erythrocytes membrane. The results indicated a higher activity in the young than that of old (by 15 %) which implied that the old erythrocytes are more vulnerable to osmotic shock. In comparison with the literature reference value, it showed a good separation between the young and old erythrocytes.

Table 3.1.4

Specific activity of acetylcholinesterase in young and old cells membrane

	Young cell membrane	Old cell membrane
Specific activity (U/mg)	0.6 ± 0.13	0.51 ± 0.12
Literature reference value (U/mg) (Fausta, 2003)	4.9 ± 1.2	3.1 ± 0.9

Results are the mean ± SD of four experiments. Data are analyzed as the percentage difference between the young and old erythrocytes.

3.2 Partial purification and concentration of young and old haemolysates

The oxygen carrying protein, haemoglobin, constitutes 95 % of the erythrocytes proteins. The heme groups on haemoglobin present the characteristic intense red colour. It is therefore difficult to analysis the young and old erythrocytes samples with methods involving colorimetric measurement. However, haemoglobin can be removed by means of chromatography. The young and old haemolysates were subjected to cation exchange chromatography for the partial purification of the thioredoxin system components. The cation exchanger was used to remove the most interfering protein, haemoglobin. After entrapment of haemoglobin from each cell systems on a cation exchange column, the flow throughs were further concentrated by ultrafiltration. Presumably about 97 % of haemoglobin was removed by the cation exchanger.

3.3 Analysis of young and old sample

3.3.1 Western blotting analysis of TR in young and old sample

In a quantitative measurement of TR present in young and old sample, western blotting analysis was performed.

After removing haemoglobin from the young and old haemolysate, all the eluents prepared in the previous step were subjected to ultrafiltration with Amicon® Ultra and YM-1 membranes (1000 MWCO) for concentration purposes. From each sample, they were concentrated to approximately 2 mL and the protein concentration was checked by the Lowry assay (Lowry, 1951). Western blot analysis was then performed. 30 μg protein was applied onto each lane (Lane 2 and Lane 3). As shown in the results below, TR was found in both young and old samples, while porcine TR was used as a positive control (Fig.3.3.1).

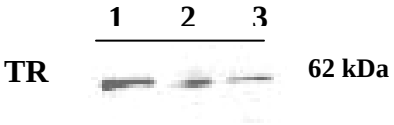

Fig.3.3.1 Western blotting analysis of TR in young and old sample

Lane 1: control TR

Lane 2: young sample

Lane 3: old sample

The densitometric measurement showed that the TR protein levels decreased by 32.6 % in the old cells compared with that of the young. Results are the mean ± SD of two experiments. Data are analyzed by comparing the percentage difference between the young and old erythrocytes.

3.3.2 Western blotting analysis of Trx in young and old sample

Determination of Trx in young and old cell population by Western Blotting

To examine the presence and the level of Trx in the young and old cells, the immunoblotting of Trx was carried out. Custom made anti-Trx was used as primary antibody while HRP conjugated secondary antibody was used in conjunction with chemiluminescent substrate (Supersignal®). Sample from the young cells showed one band (11.6 kDa) while it showed no bands in the old sample (Fig.3.3.2).

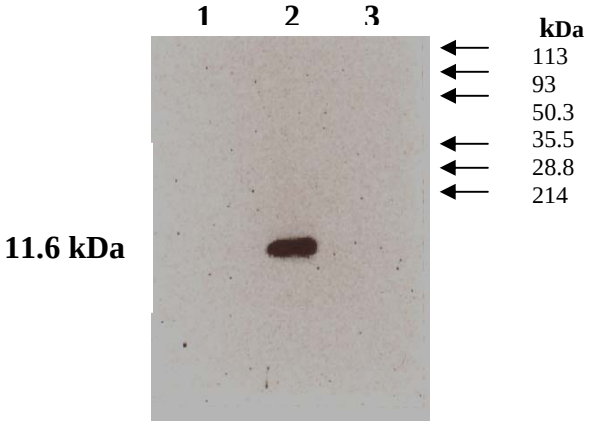

Fig.3.3.2. Western blotting analysis of Trx in young and old sample

Lane 1: old cell sample

Lane 2: young cell sample

Lane 3: prestained SDS-PAGE Standards (not shown in chemiluminescent detection)

The results revealed that Trx was present in the young but not in the old sample. It clearly showed that the amount of Trx was substantially higher in the young sample while there would be no Trx in the old sample or the concentration was beyond the detection limit. Results are the mean ± SD of two experiments.

3.3.3 Thioredoxin and thioredoxin reductase activity assay

Determination of thioredoxin and thioredoxin reductase activity by insulin dependent reduction assay.

To further investigate the individual components of the thioredoxin system, two key components, the Trx and TR activities were monitored.

Table 3.3.3

Insulin-dependent DTNB assay of Trx and TR on young and old erythrocytes

	Unit of enzyme (U)	Specific activity (U/mg)
Trx from young sample	0.028 ± 0.002	0.19 ± 0.01
Trx from old sample	N.D.	N.D.
TR from young sample	0.039 ± 0.014	0.27 ± 0.14
TR from old sample	0.029 ± 0.010	0.19 ± 0.07

*N.D. Not detectable

The insulin dependent DTNB reduction assay was carried out. The results showed the age-related changes in the activity of both Trx and TR in the young and old erythrocytes (Table 3.3.3). The results as shown above, illustrated that both Trx and TR activity in the young cells were higher than that of the old cells. Trx in the young sample showed some activity while no activity in the old sample was shown. The findings were consistent with the result that no Trx was detected by the western blotting analysis (Fig.3.3.2).

The findings were in good agreement with previous findings which showed an age-related decrease in the Trx activity in other tissues (Cho, 2003). Results are the mean ± SD of two experiments. Data are analyzed by comparing the percentage difference between the young and old erythrocytes.

3.3.4 IgG reduction assay of young and old haemolysates upon oxidative challenge

Functional studies of thioredoxin system of erythrocytes after oxidative stress by IgG reduction assay

To investigate the effect of age on the susceptibility of the thioredoxin system in the young and old erythrocytes towards H_2O_2 oxidative challenge, erythrocytes separated from young and old cells were exposed to various concentrations of H_2O_2 (4 and 8 mM). IgG reduction assay was carried out to monitor the response of the thioredoxin system upon oxidative challenge.

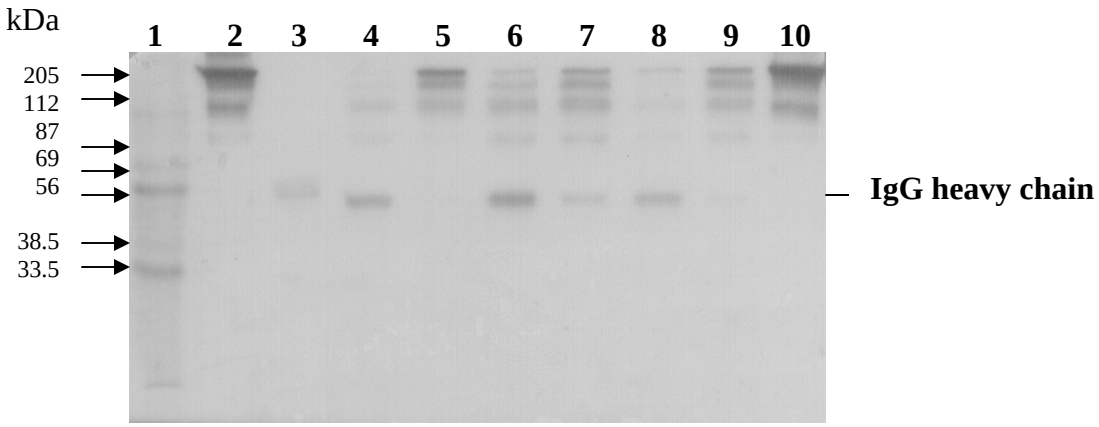

Fig.3.3.4 Reduction of mouse IgG by the thioredoxin system in young and old samples after oxidative stress. IgG reduction was monitored and determined by immunblotting. Blot development was performed by adding HPR conjugated Anti-IgG and was visualized by 4-chloro-1-naphthol.

Lane 1: SDS-7B prestained molecular weight marker

Lane 2: Non-reduced IgG

Lane 3: Reduced IgG

Lane 4: YH_o + NADPH + Non-reduced IgG

Lane 5: OH_o + NADPH + Non-reduced IgG

Lane 6: YH_4 + NADPH + Non-reduced IgG

Lane 7: OH_4 + NADPH + Non-reduced IgG

Lane 8: YH_8 + NADPH + Non-reduced IgG

Lane 9: OH_8 + NADPH + Non-reduced IgG

Lane 10: NADPH + Non-reduced IgG

(H_n represents hydrogen peroxide concentration in mM)

Results are the mean ± SD of two experiments. Data are analyzed by comparing the percentage difference between the young and old erythrocytes. Lane 4 and 5 show the thioredoxin system activity of the young and old cells respectively with no addition of H_2O_2. Lane 6 and 7 indicate the result of the thioredoxin system activity of Y and O cells after applying 4 mM H_2O_2 at 37ºC for 1 hour. We found an increase of 63.5 % ± 11.3 % thioredoxin system activity in the young cells challenged with 4 mM H_2O_2. Though the level and activity of thioredoxin in the old cells were not detectable, an enhancement of the whole thioredoxin system activity was observed after oxidative challenge. Both results showed an increase in the the IgG reduction capability of the thioredoxin system, while the capability was further decreased when the samples were treated with 8 mM H_2O_2.

B. Study of thioredoxin system in nucleated cell model

3.4 Culture of HeLa and HT-29 cell lines

The cells were cultured under the condition as described in part 2.2.2. The experiment would be carried out until HeLa cells were grown to 10^6 cells per well. All cultures used in the experiment exhibited similar viabilities of about 94 %. In the study, cell lysates were isolated from the control and hydrogen peroxide treated HeLa cell populations. In addition, samples were analyzed for the thioredoxin system activity by the IgG reduction assay. Equal concentration of protein was used as sample for analysis.

3.4.1 Cell viability of HeLa after oxidative challenge by Trypan Blue dye exclusion assay

Cell viability was determined by the Trypan Blue dye exclusion assay in which cells were treated with different defined concentrations of H_2O_2 for 8 hr (0-0.4 mM). The experiments were carried out in duplicates while the results were expressed as the mean ± S.D. Statistical calculations were done using the student's *t* test. A p value < 0.05 was considered statistically significant.

Determination of cell viability of HeLa by Trypan Blue dye exclusion assay

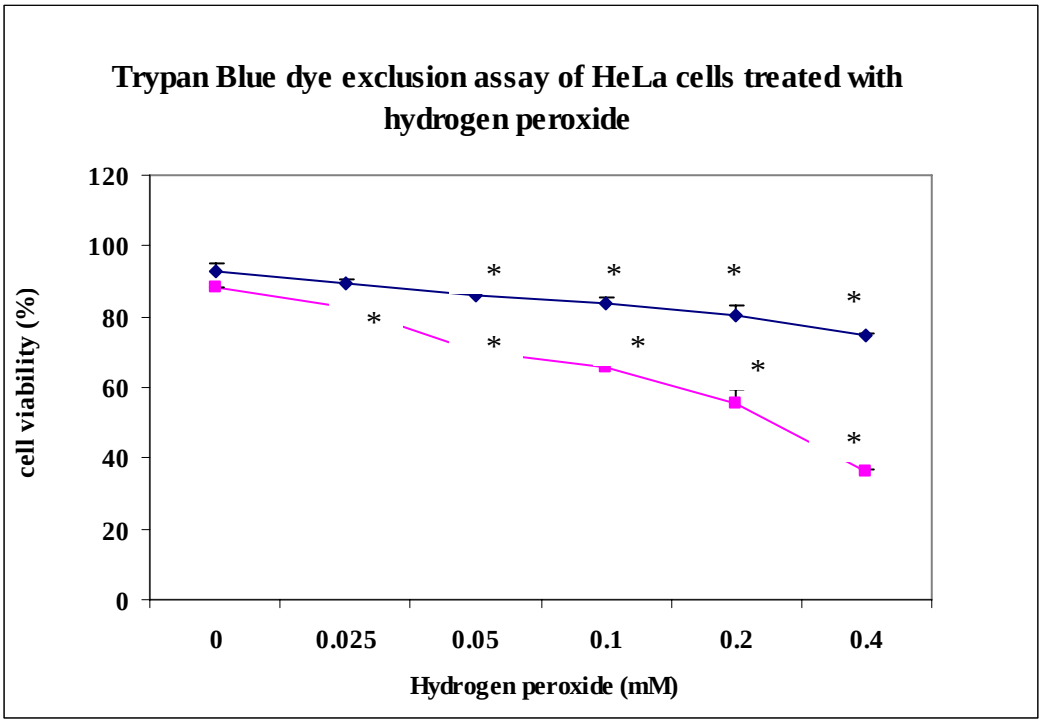

Fig.3.4.1 Trypan Blue dye exclusion assay by HeLa cells treated with 0-0.4 mM hydrogen peroxide for 8 hr with (■) or without (♦) prior treatment of cycloheximide

* $p < 0.05$ when it was compared with H_2O_2 treated cells and untreated control.

The results showed a dose-dependent trend of hydrogen peroxide toxicity, that was comparable with the later MTS assay. However, the findings also illustrated that the cycloheximide treated cells were significantly more sensitive to the cytotoxic effect of H_2O_2, which were not shown by the MTS assays, indicating that *de novo* protein synthesis is essential for cell survival under oxidative stress.

3.4.2 Cell viability of HeLa after oxidative challenge by MTS tetrazolium assay

MTS tetrazolium was reduced to formazan by HeLa cells treated with 0-0.4 mM hydrogen peroxide for 8 h and a prior treatment with cycloheximide for 4 h. HeLa cells were cultured overnight at 5,000 cells/well in a 96-well plate. Cycloheximide (1 mM) was added into the cell medium for 4 h followed by the addition of hydrogen peroxide for 8 h. The tetrazolium assay reagent (10 µL/well) was added immediately after the treatment, and the assay plates were incubated at 37 ºC prior to the recording of absorbance at 490 nm. The absorbance value for the control of culture medium without cells was 0.38 ± 0.05 at 490 nm (not shown).

Determination of cell viability of HeLa by MTS tetrazolium assay

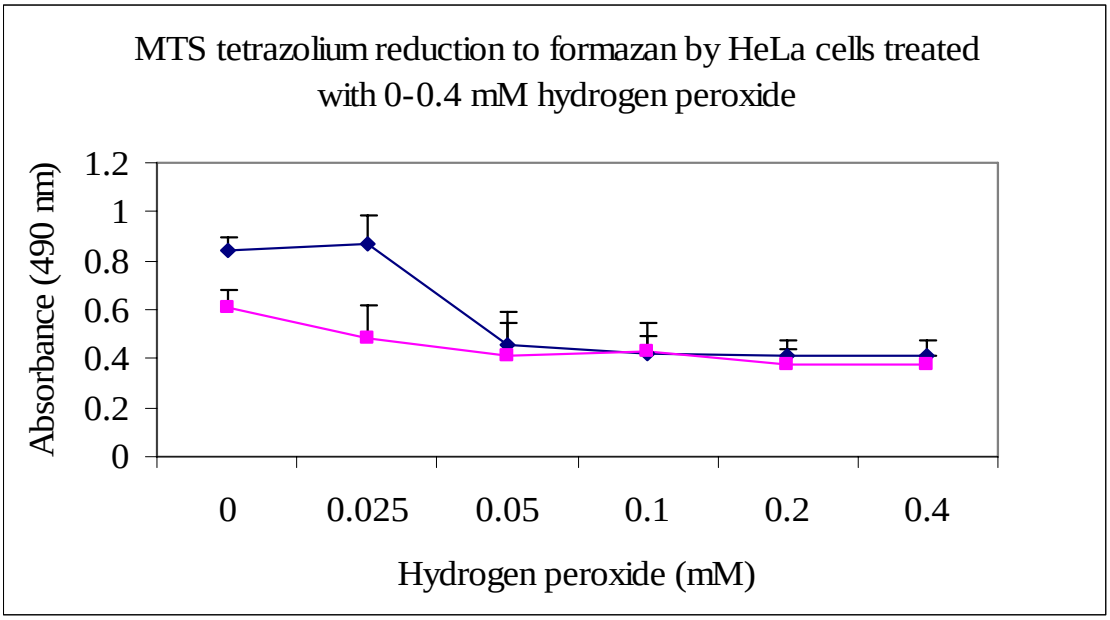

Fig.3.4.2 MTS tetrazolium reduction to formazan by HeLa cells treated with 0-0.4 mM hydrogen peroxide for 8 h with (■) or without (♦) prior treatment of cycloheximide for 4 h

*p< 0.05 when it was compared with H_2O_2 treated cells and untreated control.

The MTS tetrazolium reduction assay was used to measure the cell viability of HeLa cells treated with hydrogen peroxide in the presence or absence of cycloheximide. The results showed a dose-dependent trend of hydrogen peroxide toxicity, implying the validity of this method for measuring viability comparing with the Trypan Blue dye exclusion assay.

Because of the necessity for viable cells to reduce the tetrazolium compound to a water soluble formazan product, a 3 hr incubation period is needed. It is interesting to point out that there was a slight increase in absorbance at 490 nm at 0.025 mM hydrogen peroxide for the 8 hr exposure period. Such interesting phenomenon is consistent with previous observations (Haendeler, 2004). However, a significant decrease of cell viability was observed in cycloheximide treated HeLa cells at the same concentration of hydrogen peroxide, indicating cycloheximide treated cells were more sensitive to the cytotoxic effect of hydrogen peroxide.

3.4.3 Western blotting analysis of Trx and TR in HeLa cells upon oxidative challenge

Determination of Trx and TR in HeLa cells upon oxidative challenge by Western Blotting

To address the possible effect of transcription of Trx and TR upon oxidative challenge, the level of Trx and TR in HeLa cells challenged with various concentrations of H_2O_2 was determined. Results are the mean ± SD of two experiments.

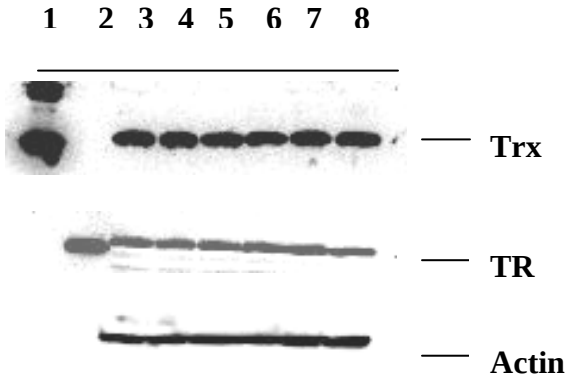

Fig.3.4.3 Western blotting analysis of Trx and TR in HeLa cells upon oxidative challenge

Lane 1: standard Trx

Lane 2: standard TR

Lane 3: HeLa lysate (control)

Lane 4: HeLa lysate (pretreated with 0.025 mM H_2O_2)

Lane 5: HeLa lysate (pretreated with 0.05 mM H_2O_2)

Lane 6: HeLa lysate (pretreated with 0.1 mM H_2O_2)

Lane 7: HeLa lysate (pretreated with 0.2 mM H_2O_2)

Lane 8: HeLa lysate (pretreated with 0.4 mM H_2O_2)

3.4.4. Western blotting analysis of Trx and TR in HeLa cells upon oxidative challenge and with prior treatment of cycloheximide

Determination of Trx in HeLa cells upon oxidative challenge with prior treatment of CHX by Western Blotting

To examine the level of Trx in HeLa cells upon oxidative challenge with prior treatment of cycloheximide, the immunoblotting of Trx and TR was carried out. Results are the mean ± SD of two experiments.

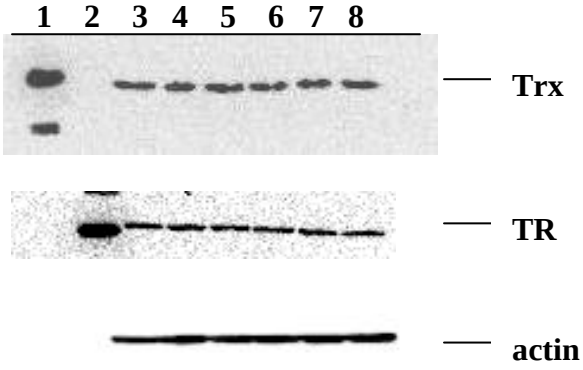

Fig.3.4.4 Western blotting analysis of Trx and TR in HeLa cells upon oxidative challenge and with prior treatment of cycloheximide

Lane 1: standard Trx

Lane 2: standard TR

Lane 3: HeLa lysate (pretreated with cycloheximide)

Lane 4: HeLa lysate (pretreated with cycloheximide and 0.025 mM H_2O_2)

Lane 5: HeLa lysate (pretreated with cycloheximide and 0.05 mM H_2O_2)

Lane 6: HeLa lysate (pretreated with cycloheximide and 0.1 mM H_2O_2)

Lane 7: HeLa lysate (pretreated with cycloheximide and 0.2 mM H_2O_2)

Lane 8: HeLa lysate (pretreated with cycloheximide and 0.4 mM H_2O_2)

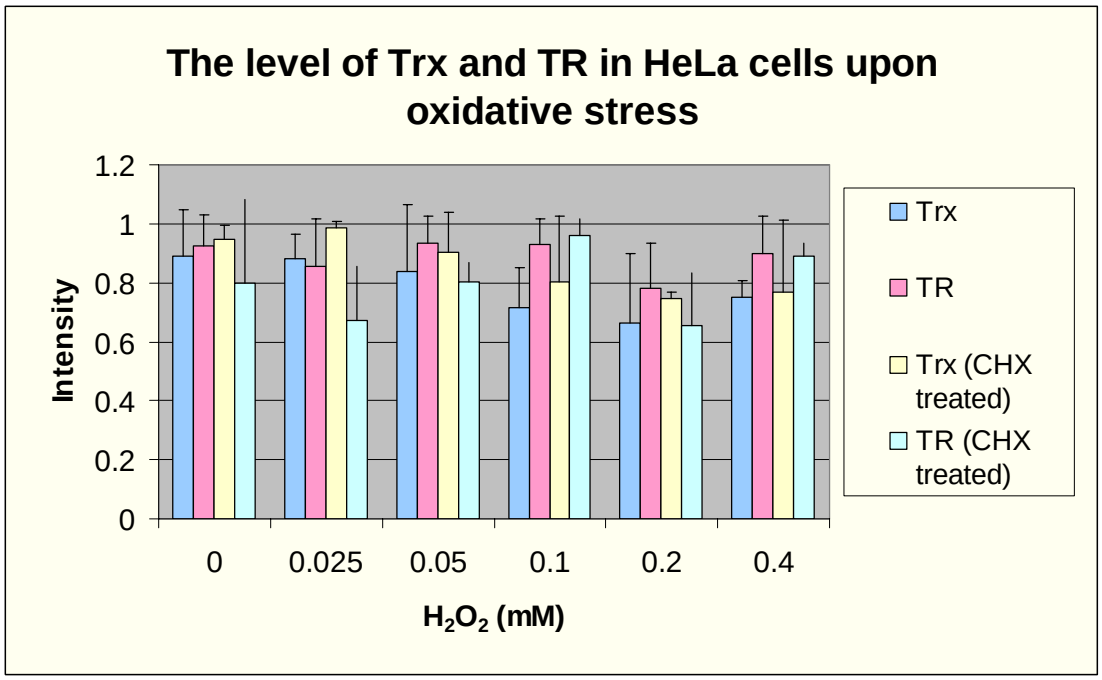

Fig.3.4.4.1 The level of Trx and TR in HeLa cells upon oxidative stress with or without prior treatment of cycloheximide

The results of these experiments showed that there was no significance difference in the level of Trx and TR in HeLa cells upon oxidative challenge with and without the prior treatment of cycloheximide.

3.4.5 Activity of thioredoxin system in HeLa cells upon oxidative challenge without prior treatment of of cycloheximide

Recent studies have indicated that short term exposure of low doses of H_2O_2 induced Trx mRNA (Haendeler, 2004). To find out the relationship between mRNA expression and Trx system activity, human epithelial carcinoma, HeLa cells were exposed to 0-0.4 mM H_2O_2 for 8 hr. Trx system activity was then demonstrated by the IgG reduction assay. Results are the mean ± SD of two experiments. Statistical calculations were done using the student's *t* test. A p value < 0.05 was considered statistically significant.

Functional studies of thioredoxin system of HeLa cells after oxidative stress by IgG reduction assay

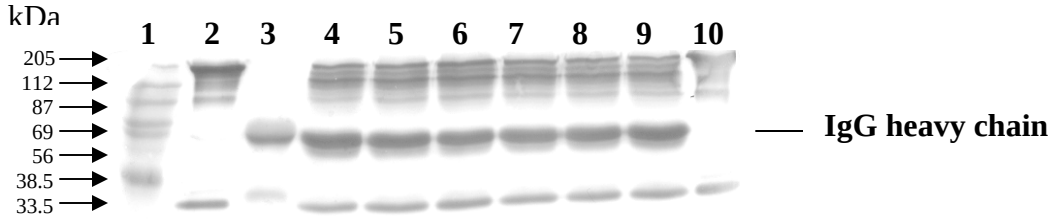

Fig.3.4.5 Reduction of mouse IgG by the thioredoxin system in HeLa cells after oxidative stress

* Equal loading according to its protein concentration

Lane 1: SDS 7B Markers

Lane 2: Non-reduced IgG

Lane 3: Reduced IgG

Lane 4: H_0 + Non-reduced IgG + NADPH

Lane 5: $H_{0.025}$ + Non-reduced IgG + NADPH

Lane 6: $H_{0.05}$ + Non-reduced IgG + NADPH

Lane 7: $H_{0.1}$ + Non-reduced IgG + NADPH

Lane 8: $H_{0.2}$ + Non-reduced IgG + NADPH

Lane 9: $H_{0.4}$ + Non-reduced IgG + NADPH

Lane 10: Non-reduced IgG + NADPH

(H_n represents hydrogen peroxide concentration in mM)

In Fig.3.4.5, Lane 4 illustrated the basal functional activity of the thioredoxin system in HeLa cells. Lane 5 to 8 indicated that there were a decrease of the thioredoxin system activity upon challenge with 0.025-0.2 mM H_2O_2. Lane 9 showed the thoredoxin system activity increased by 19.7 % upon a higher level of challenge with 0.4 mM H_2O_2.

Fig.3.4.5.1 summarizes the reduction capability of the thioredoxin system of HeLa upon different levels of oxidative stress. Results were obtained from 2 sets of experiments.

Reduction capability of thioredoxin system of HeLa cells after oxidative stress

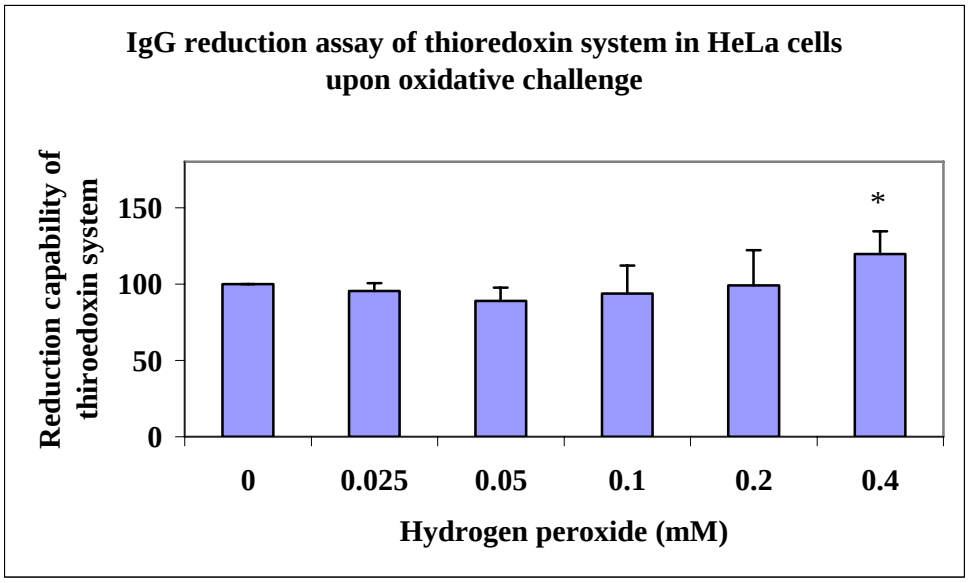

Fig.3.4.5.1 Reduction capability of thioredoxin system in HeLa cells upon treated with 0-0.4 mM hydrogen peroxide for 8 h.

Results shown are the means ± SD for two experiments. *$p<0.05$ comparing with H_2O_2 treated cells and untreated control.

(It was assessed by densitometric measurement, in which 0 mM hydrogen peroxide was represented as 100 for comparison)

3.4.6 Activity of thioredoxin system in HeLa cells upon oxidative challenge with prior treatment of cycloheximide

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

Functional studies of thioredoxin system of HeLa cells after oxidative stress by IgG reduction assay

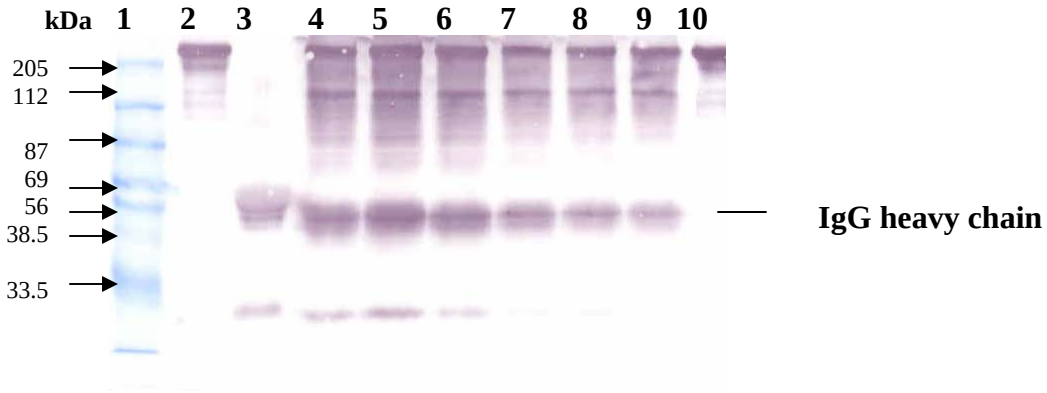

Fig.3.4.6 Reduction of mouse IgG by the thioredoxin system in HeLa cells after oxidative stress with prior treatment of cycloheximide

* Equal loading according to its protein concentration

Lane 1: SDS 7B Marker

Lane 2: Non-reduced IgG

Lane 3: Reduced IgG

Lane 4: H_0 + Non-reduced IgG + NADPH

Lane 5: $H_{0.025}$,+ Non-reduced IgG + NADPH

Lane 6: $H_{0.05}$ + Non-reduced IgG + NADPH

Lane 7: $H_{0.1}$ + Non-reduced IgG + NADPH

Lane 8: $H_{0.2}$ + Non-reduced IgG + NADPH

Lane 9: $H_{0.4}$ + Non-reduced IgG + NADPH

Lane 10: Non-reduced IgG + NADPH

(H_n represents hydrogen peroxide concentration in mM)

As shown in Fig.3.4.6, Lane 4 showed the basal functional activity of the thioredoxin system in HeLa cells. Lane 5 indicated that there was a 8.2 % increase of the thioredoxin system activity upon challenge with 0.025 mM H_2O_2. The thioredoxin system activity then further decreased upon higher level of challenge with 0.05-0.4 mM H_2O_2.

Fig.3.4.6.1 summarizes the reduction capability of HeLa cells after oxidative stress with prior treatment of cycloheximide. As compared with the results obtained from Fig.3.4.5.1, the enhancement of the thioredoxin system activity by 8.2 % occurred at lower concentration of H_2O_2 (0.025 mM). Since cycloheximide acted as inhibitors for *de novo* protein synthesis, the results interestingly suggest that the thioredoxin system activity is enhanced during oxidative challenge which is not related to translation.

Reduction capability of thioredoxin system of HeLa cells after oxidative stress with prior treatment of cycloheximide

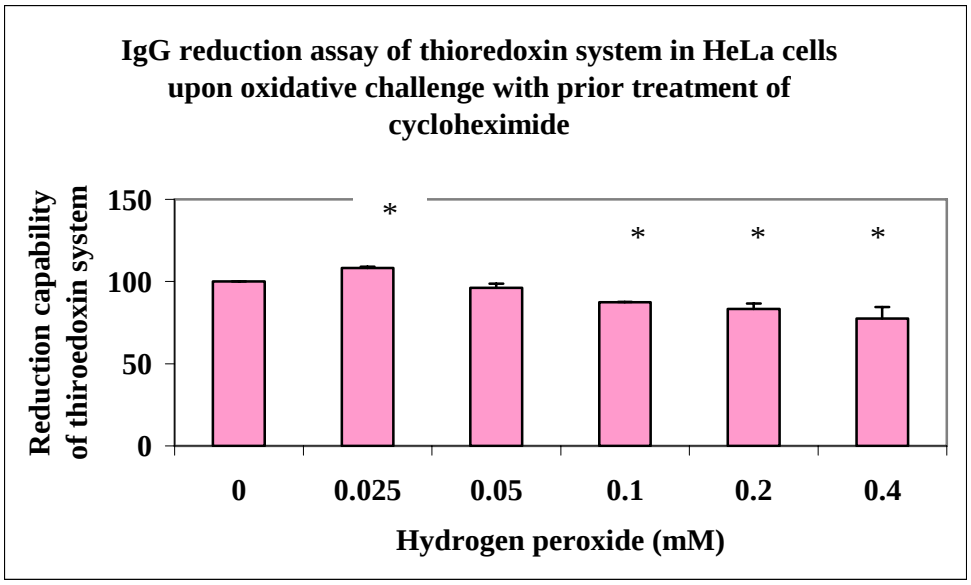

Fig.3.4.6.1 Reduction capability of thioredoxin system in HeLa cells upon treated with 0-0.4 mM hydrogen peroxide for 8 h and prior treatment with cycloheximide for 4 h

Results shown are the means ± SD for two experiments. * $p < 0.05$ when it was compared with H_2O_2 treated cells and untreated control.

3.4.7 Cell viability of HT-29 after oxidative challenge by Trypan Blue dye exclusion assay

To further study the effect of oxidative challenge and response of the thioredoxin system in nucleated cells, another cell line HT-29 was investigated. Cell viability was determined by the Trypan Blue dye exclusion assay in which HT-29 cells were treated with different defined concentrations of H_2O_2 for 8 hr (0-1 mM). The experiments were carried out in duplicates while the results were expressed as the mean ± S.D.

Determination of cell viability of HT-29 by Trypan Blue dye exclusion assay

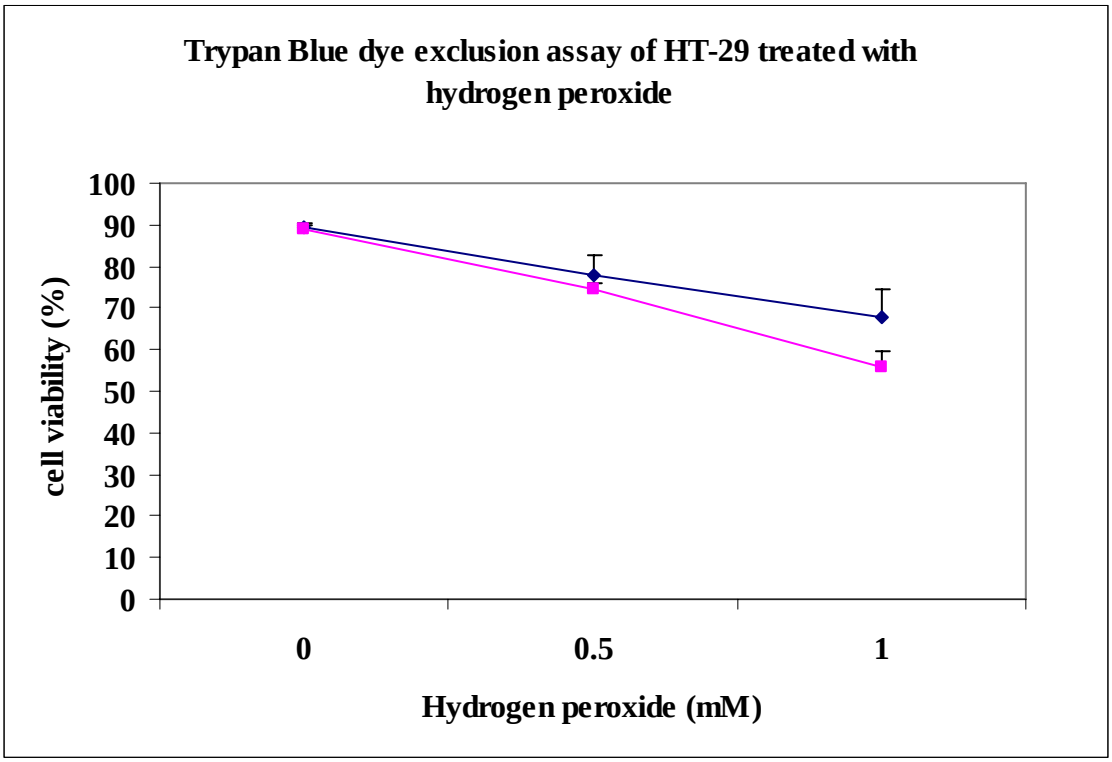

Fig.3.4.7 Trypan Blue dye exclusion assay by HT-29 cells treated with 0-1 mM hydrogen peroxide for 8 h with (■) or without (♦) prior treatment of cycloheximide

The results showed a dose dependent trend of hydrogen peroxide toxicity, that is comparable with the MTS assay. However, the findings also illustrated that cycloheximide treated cells were significantly more sensitive to the cytotoxic effect of H_2O_2, and that *de novo* protein synthesis is essential for cell survival under oxidative stress.

3.4.8 Cell viability of HT-29 after oxidative challenge by MTS tetrazolium assay

The results also illustrated a dose dependent trend of HT-29 cells towards hydrogen peroxide toxicity as compared with the HeLa cells. A significant decrease of cell viability was observed in cycloheximide treated HeLa cells at the same concentration of hydrogen peroxide, indicating cycloheximide treated cells were more sensitive to the cytotoxic effect of hydrogen peroxide under oxidative stress.

Determination of cell viability of HT-29 by MTS tetrazolium assay

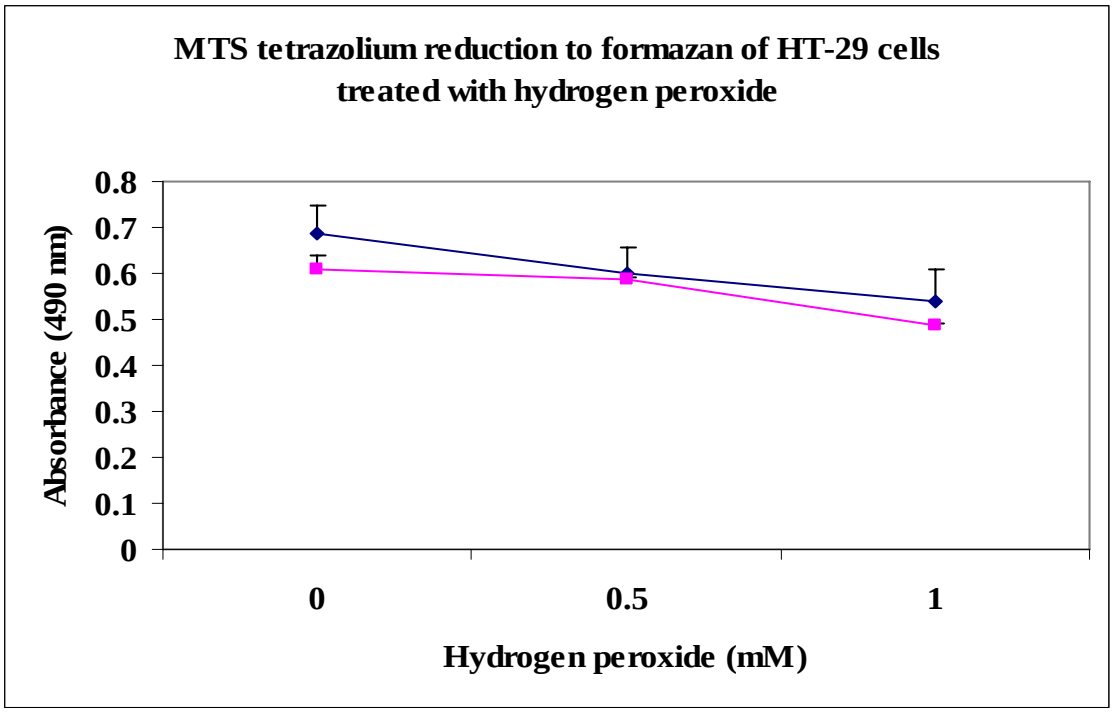

Fig.3.4.8 MTS tetrazolium reduction to formazan by HT-29 cells treated with 0-1 mM hydrogen peroxide for 8 h with (■) or without (♦) prior treatment of cycloheximide for 4 h

3.4.9 Activity of thioredoxin system in HT-29 cells upon oxidative challenge with or without prior treatment of cycloheximide

Previous studies showed the activation of the thioredoxin system activity upon oxidative challenge in HeLa cells (Fig.3.4.5.1 and Fig.3.4.6.1). To examine whether the induction was cell type independent, another nucleated cell line, HT-29 cells were examined and pretreated with 1 mM cycloheximide to inhibit the *de novo* protein synthesis. As compared with the HeLa cells, higher concentrations of H_2O_2 (0-1 mM) were used in the oxidative challenge. The experiments were carried out in triplicate while the results were expressed as the mean ± S.D.

Functional studies of thioredoxin system of HT-29 cells after oxidative stress by IgG reduction assay

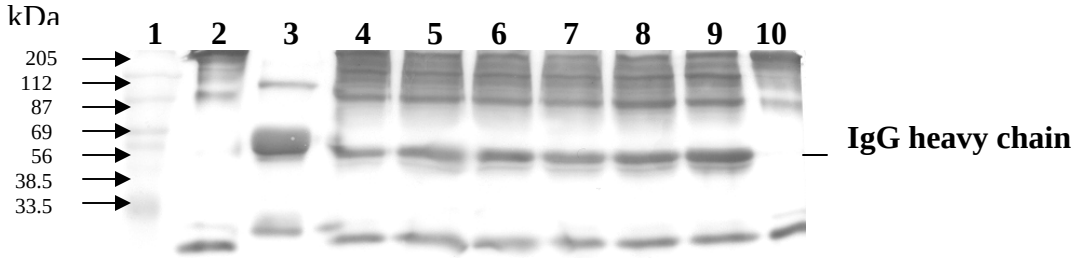

Fig.3.4.9 Reduction of mouse IgG by the thioredoxin system in HT-29 cells after oxidative stress with or without prior treatment of cycloheximide

* Equal loading according to its protein concentration

Lane 1: SDS 7B Marker

Lane 2: Non-reduced IgG

Lane 3: Reduced IgG

Lane 4: H_0 + Non-reduced IgG + NADPH

Lane 5: $H_{0.5}$ + Non-reduced IgG + NADPH

Lane 6: H_1 + Non-reduced IgG + NADPH

Lane 7: H_0 + Non-reduced IgG + NADPH (CHX treated)

Lane 8: $H_{0.5}$ + Non-reduced IgG + NADPH (CHX treated)

Lane 9: H_1 + Non-reduced IgG + NADPH (CHX treated)

Lane 10: Non-reduced IgG + NADPH

(H_n represents hydrogen peroxide concentration in mM)

We examined the reduction capability of the thioredoxin system in HT-29 cells upon oxidative challenge. Fig.3.4.9.1 showed that the thioredoxin system activity increased by 16.3 % and 18.5 % after addition of 0.5 and 1 mM H_2O_2 respectively. Such progressive increase of the thioredoxin system activity could also be observed in the cells pretreated with cycloheximide with a smaller increment of 8.1 % and 13.9 % respectively. Since cycloheximide acted as inhibitors for *de novo* protein synthesis, the results also revealed that the thioredoxin system activity was interestingly enhanced during oxidative challenge that was not necessarily related to translation.

Reduction capability of thioredoxin system of HT-29 cells after oxidative stress with or without prior treatment of cycloheximide

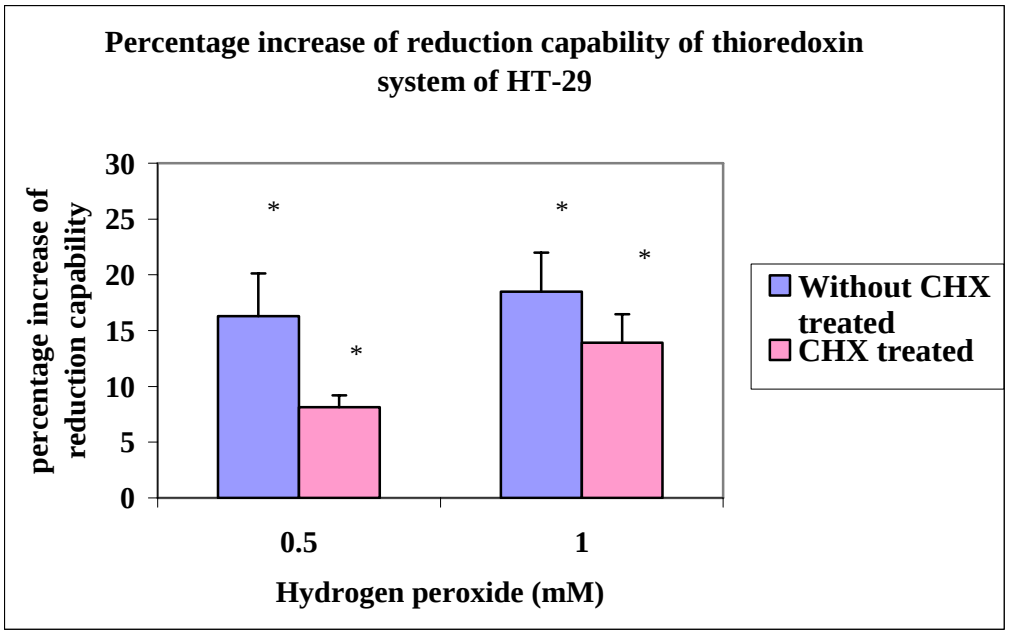

Fig.3.4.9.1 Reduction capability of thioredoxin system in HT-29 cells upon treated with 0-1 mM hydrogen peroxide for 8 h with or without prior treatment of cycloheximide for 4 h

Results shown are the means ± SD for three experiments. *p< 0.001 when it was compared with H_2O_2 treated cells and untreated control, with or without prior treatment of CHX.

V Discussion

Aging is an inevitable part of the life natural process that is governed by the decreasing physiological functions which ultimately results in mortality. Previous studies showed that ROS induced apoptosis. In addition, ROS are believed to be one of the causal factors of aging. The balance between ROS generation, antioxidant defenses and repairing mechanisms is therefore of paramount importance. There are numerous biochemical pathways and mechanisms involved in the defence against oxidative stress. Unique amongst others, the thioredoxin system is one of the best representative systems for its protective function against oxidative stress. It can be found in different cell types ranging from prokaryotes to eukaryotes. However, the existence of the thioredoxin system in erythrocytes has not been reported. Previous studies illustrated that Trx involves in the first unique step of DNA synthesis and an important step for cellular proliferation (Powis, 2001). However, the anucleated feature of erythrocytes implies no DNA synthesis in the mature state. From our studies, Trx and TR were found surprisingly high in erythrocytes. It is noteworthy that besides the involvement in DNA synthesis, the thioredoxin system has other roles and functions. Oxidative damage to erythrocytes has known to cause destructive events in many haematological disorders and aging. Erythrocytes encounter high oxidative stress due to its oxygen carrying function. Indeed, the anucleated feature of erythrocytes implies no protein synthesis. The repairing mechanism is therefore becoming the determinant factor in keeping cell homeostasis and most importantly the cell viability.

Consequently, erythrocyte can be used as a cell aging model. We attempted to find out the level of the thioredoxin system components in erythrocytes, the role they played, and in particular how they might vary in the aging process and how they responded to oxidative challenge. Through this study, it is hoped that a better understanding on the role of the thioredoxin system and its relationship with aging in erythrocytes can be obtained.

During cellular aging, erythrocytes become smaller and denser. Young and old populations of cells were first separated by means of density, while the success of the separation was further verified by the difference in erythrocytes counting, mean haemoglobin concentration, mean corpuscular volume and membrane acetylcholinestase activity between the young and old erythrocytes. The findings showed an age related decrease in MCV and AchE compared with that of the corresponding young cells. The results were in agreement with published data (Fausta, 2003). The results obtained were consistent with the nature of the young and old erythrocytes. Previous studies illustrated the significant structural and membrane deviation between the young and old human erythrocytes (Fausta, 2003). In the present study, pig erythrocytes were employed and comparable results to that of human erythrocytes were obtained. It indicates clearly that pig red cells serve as a good substitution for human erythrocytes.

The successfully separated cells were then used in the subsequent analysis of the thioredoxin system. It has been the first attempt to compare the thioredoxin system in the young and old erythrocytes. In the study, western blotting analysis showed the presence

of Trx in the young but not in the old cells. The molecular weight of Trx was found to be 11.6 kDa. The findings were consistent with the molecular weight of the mammalian Trx (Arnér, 2000). Such findings indicated the existence of Trx in porcine erythrocytes while no Trx could be found in the old erythrocytes or the concentration of Trx was beyond the detection limit. Immunoblotting of TR was also carried out in the young and old cells. The level of TR in the young cells was higher than that of the old cells by 32.6 %. It is worth noting that there was still some TR found in the old erythrocytes. The findings were consistent with the relatively stable nature of TR when compared with Trx. In addition, the enzyme activity study of Trx of the young and old erythrocytes was also consistent with such findings. The results illustrated that there was 0.19 U thioredoxin / mg total protein in young cells but no activity could be observed in the old cells. Only the concentrated young sample, but not the old sample, was able to reduce the disulfide-containing protein, insulin. The results showed the age-related changes in the activity of both Trx and TR in the young and old erythrocytes. In general, both level and activity of Trx and TR in the young cells were substantially higher than that of the old cells. Such deviations illustrated that the thioredoxin system may be essential in the cell aging process of erythrocytes.

To further confirm our preliminary hypothesis, immunoglobulin (IgG) reduction assay was employed to study the functional role of the whole thioredoxin system in both cell populations. Mouse immunoglobulin was chosen as the substrate, which contains interchain and intrachain disulphide bridges between the heavy and light chain of immunoglobulin to maintain the conformation of the whole structure. Previous studies

showed that functioning thioredoxin system is able to reduce the interchain disulphide bridges between the heavy chain and light chain of IgG (Magnusson, 1997). The advantage of using the assay was to illustrate the disulfide reducing capacity of the whole thioredoxin system, which overcomes the limitations of the above insulin dependent DTNB assay that can only measure either component in the whole thioredoxin system independently. Indeed, with the excessive supplement of NADPH, sufficient amount of hydrogen ions could be supplied to the whole system, thus the system was kept in an active form for carrying its oxidoreductase function continuously.

In the experiment, with the supplement of NADPH, the results clearly illustrated that both TR and Trx in the young haemolysates could reduce the interchain disulfide bridges of IgG. TR activity in the old cells, though measurable by the insulin reduction activity assay, was not detected in the IgG reduction activity assay. The result was consistent with no Trx activity and a lower TR activity found in the old cells in the previous experiments.

The findings indicated the difference of Trx and TR activity between the young and old cells in erythrocytes. Such deviations were significantly pronounced in the different ages of cells, thereby suggesting an interesting relationship between the thioredoxin system and cell aging in erythrocytes. To further elucidate the role of thioredoxin system in erythrocytes, the young and old erythrocytes were exposed to different levels of oxidative stress. In this experiment, hydrogen peroxide acted as oxidative challenge which mimicked the ROS attack on cells. Previous studies showed

that H_2O_2 is one of the most important molecules triggering response to oxidative stress in animals (Buchanan, 2005). The results indicated that an enhancement of the thioredoxin system activity was clearly observed after the addition of H_2O_2 at 4 mM in both young and old erythrocytes. We found in particular an increase of 63.5 % thioredoxin system activity in the young cells. Though the Trx level and activity were not detectable in the old cells, an activation of the whole thioredoxin system activity was also noticed in old cells after oxidative challenge. It is worth noting that the thioredoxin system activity in both young and old cells was enhanced, and the activation could be observed in the old cells, from originally undetectable to now some detectable activity. However, addition of H_2O_2 higher than 4 mM led to gradual decrease in the thioredoxin system activity in both young and old cells. The results clearly showed that thioredoxin system in old cells subjected to 8 mM H_2O_2 challenge significantly lost its capability in reducing IgG. It might be due to the oxidation of the thioredoxin system in such adverse environment (Watson, 2003).

Circulatory mammalian erythrocytes are devoid of a protein synthesis machinery and continuously subject to oxidative challenge. Their survival is therefore largely dependent on the cellular effective anti-oxidation and/or protein repairing mechanisms. And most importantly, this is the first report to compare the difference between the thioredoxin system in the young and old erythrocytes. Such valuable findings suggested that the thioredoxin system may play a role in response to oxidative stress in erythrocytes, and the relationship may be age related. In the study, the thioredoxin system activity in the young cells was found much higher than that in the old when NADPH was

supplemented. In addition, both Trx and TR activity were declining in the old cells. However, the results also illustrated clearly that at the appropriate level, oxidative challenge might enhance the Trx system activity in both populations. Such findings strongly suggested that the activation of the system may not be related to *de novo* protein synthesis since the mature erythrocytes are devoid of protein synthesis machinery. And it raises a possibility that a regulatory system of the thioredoxin system may exist, which can respond to oxidative stress but most importantly, *de novo* protein synthesis is not involved. In search of this plausible regulator(s), we therefore extended our study to HeLa and HT-29 cell culture to investigate the importance and involvement of the thioredoxin system upon oxidative challenge.

In this study, the thioredoxin system in dividing cells: HeLa and HT-29 was investigated. Various findings showed time and dose dependent induction of apoptosis when H_2O_2 was added (Wang, 1998). In comparison with the thioredoxin system of the anucleated mammalian erythrocytes, nucleated feature of HeLa cells implied transcriptional mediated changes may be involved in any alterations. As a result, by comparing the response of the thioredoxin system in both nucleated and anucleated cell models, it helps confirm our notion that if a regulatory system of the thioredoxin system does exist universally.

HeLa is a human cervical epithelial cancer cells. It is an ideal nucleated cell line for studying the biochemical pathways due to its fast growth rate. The level of Trx in HeLa cells is relatively high compared with that found in other cell lines (Tsuguobu,

2002). On the other hand, HT-29, a human colorectal carcinoma cell line, which is an adherent epithelial cell growing as a monolayer and in large colonies, has been reported to have a good response in the TR promoter site . HeLa and HT-29 cells therefore serve as good tools for exploring functions of the thioredoxin system.

In this study, oxidative stress elicited by the addition of H_2O_2 to HeLa cells was performed. We employed IgG as the substrate to assay the full thioredoxin system activity of HeLa cells after different degree of oxidative stress, both with and without prior treatment of cycloheximide. In the cell viability assay, the MTS assay showed a dose dependent trend of H_2O_2 toxicity, which was in good agreement with the results in the Trypan Blue dye exclusion assay. It is however interesting to note that there was an increase in absorbance at 490 nm at a low H_2O_2 concentration treatment (0.025 mM) in the MTS assay. Such interesting phenomenon had also been demonstrated in other workers' observations which suggested that upon challenges at low concentrations of oxidants, cells may respond with a stimulated mitochondrial activity. In addition, a significant decrease of cell viability was also observed in cycloheximide treated HeLa cells at the corresponding concentration of H_2O_2, indicating that the cycloheximide treated cells were more sensitive to the cytotoxic effect of H_2O_2. In our subsequent studies, we therefore employed a sub-lethal dose of hydrogen peroxide to ensure sufficient cell viability for the investigation of the thioredoxin system response under oxidative challenges, which also acted as a stimulant to the cells.

We found an increased thioredoxin system activity in cells challenged with low levels of H_2O_2 both with and without the prior addition of cycloheximide. There was a 19.7 % increased in the thioredoxin system activity in the cells treated with 0.4 mM H_2O_2. Similarly but at a lower level, an 8.2 % increase in the thioredoxin system activity could also be observed in the cells pretreated with cycloheximide. It was found that the cycloheximide treated cells showed an enhancement of the thioredoxin system activity at a lower concentration of H_2O_2 (i.e. 0.025 mM) than that of the control cells, implying a higher sensitivity of the cycloheximide treated cells towards oxidative stress. It is plausible that cycloheximide inhibits *de novo* protein synthesis; so that it is more likely that any increase in the thioredoxin system activity is due to the enhancement of the system *per se*. While comparing results from the control cells without treatment of cycloheximide, it further substantiated the protective role of the thioredoxin system in combating against oxidative stress that the system could still function after treatment with relatively high concentrations of hydrogen peroxide. The cells also responded to the oxidative challenge with an enhanced thioredoxin system activity when the stimulant was at a sufficiently high concentration.

Through the use of another cell model, HT-29 cells, we have addressed the relatively high robustness of HT-29 cells upon oxidative challenge with H_2O_2 in comparison with HeLa cells. The findings supported the general hypothesis that oxidative challenge varies from cells to cells. It is worth noting that ROS such as H_2O_2 induce different cellular effects which are concentration and cell type dependent (Haendeler, 2004). It is necessary to perform the same experiment with more cell lines and therefore,

HT-29 was employed. In the study, higher concentrations (0.5 and 1 mM) of H_2O_2 were employed to act as oxidative challenge onto HT-29 cells. In agreement with the HeLa cells findings, an enhancement of the thioredoxin system activity could also be observed in cells challenged with H_2O_2 both with and without the prior addition of cycloheximide. There were a 16.3 % and 18.5 % increase in the thioredoxin system activity when 0.5 and 1 mM H_2O_2 were added respectively, while in the cells pretreated with cycloheximide, an 8.1 % and 13. 9 % increased in the thioredoxin system activity could be observed after the addition of 0.5 and 1 mM H_2O_2 respectively. Results from both HeLa and HT-29 cells support the notion that thioredoxin system may play active role in defense against oxidative challenge. And interestingly, the enhancement was not necessarily dependent on *de novo* protein synthesis.

In many previous studies, ROS were shown to act as stress signals that activated specific redox sensitive signaling pathways to respond to such oxidative challenge (Toren, 2000). The initiating events leading to the activation of the pathways upon oxidative challenge are still not completely understood. Recent findings suggested that TR would be expressed at elevated levels and the process could be transcriptional mediated (Smart, 2004). However in our studies, both nucleated and anucleated cells showed an enhancement of the thioredoxin activity that was not necessarily dependent on transcription and indeed we have also demonstrated that there were no significant changes of expression and level of Trx and TR upon oxidative challenge. The findings supported a very important role of the thioredoxin system in cells. Taken together, the results of these experiments not only showed the involvement of the thioredoxin system

in combating against oxidative stress, but also suggested the presence of a potential regulatory system in the regulation of the thioredoxin system. It seems logical that upon an oxidative challenge, a regulatory system may contribute to the activation of the thioredoxin system. We therefore hypothesize that both *de novo* protein synthesis and a direct activation of the thioredoxin system may be involved in response to oxidative stress. In addition, there may exist an endogenous regulatory system of the thioredoxin system that can respond to oxidative stress.

Taken together, the thioredoxin system can be found in both anucleated and nucleated cells, which play a role in defending against oxidative stress. From our findings in the anucleated cell model, the decline of the thioredoxin system activity and consequently the loss of protein repairing and related anti-oxidative capabilities during cell aging is largely due to alterations of thioredoxin. However, thioredoxin reductase is the only enzyme that can reduce thioredoxin, which also implies the significant role of thioredoxin reductase. Our findings also raise a plausible notion that an endogenous regulatory system of the thioredoxin system may exist which can respond to oxidative stress. It is interesting to find that the enhancement of the thioredoxin system activity is actively involved in response to oxidative challenge. It is postulated that either or both transcription and/or activity enhancement can be the immediate response to oxidative stress. Consequently, understanding the interactions of the system and its plausible regulators upon oxidative challenge are of importance for future rational therapeutic approach in relation to ROS induced age-related diseases such as cancers, which is the most promising field for the thioredoxin system. To further confirm our notion, a

comparison of the activity enhancement and the transcriptional mRNA level of the thioredoxin system may provide a better understanding of the true role of the thioredoxin system in cell aging and oxidative stress. The search for the regulatory system(s) should also be actively pursued.

VI Conclusion

The thioredoxin system activity in the young erythrocytes was found substantially higher than that in the old ones when NADPH was supplemented. Both Trx and TR activity were found declining in the old erythrocytes. Moreover, TR in old cells still showed some degrees of activity as illustrated by the insulin assay while it showed no Trx activity in the old cells. In addition, the thioredoxin system activity of the H_2O_2-treated erythrocytes increased with an increasing H_2O_2 level at up to 4 mM H_2O_2. Beyond this level, the thioredoxin system activity progressively decreased. As a result, we propose that low concentrations of ROS may exert a stimulating effect on the signaling, receptors and enzymatic response. The addition of oxidative stress exceeding the maximum tolerance level would lead to gradually degeneration of the thioredoxin system due to excessive insults. The decline of the thioredoxin system activity and consequently loss of the protein repairing and related anti-oxidative capabilities during cell aging thus largely look attributed to the decline of the function of the important protective protein co-factor of the system, which is thioredoxin.

In conclusion, our study confirms the ability of the thioredoxin system in combating against oxidative stress in both erythrocytes and cell culture (HeLa and HT-29 cells). The enhancement of the thioredoxin system activity, observed after oxidative stress, is in concert with the data describing the role of the thioredoxin system. Oxidative stress elicited by H_2O_2, which mimics the conditions of cellular aging process, trigger cells to initiate apoptotic signaling. Recently, the term hormesis has been widely discussed. It is postulated that low level of stress can exert beneficial effects to cell, while

it has stimulatory effects on the signaling, receptor stimulatory and enzyme stimulatory effects (Radak, 2004). In this study, we demonstrated that there was an enhancement of the thioredoxin system activity upon oxidative challenge in both young and old erythrocytes, and such an activation could also be observed in nucleated HeLa and HT-29 cell models. The results clearly illustrated that the enhancement was not necessarily dependent on *de novo* protein synthesis. Our notion is that both *de novo* protein synthesis and a direct activation of the thioredoxin system may be involved in response to oxidative stress. Together with the yet to be characterized regulatory system, the full thioredoxin system appears to play important roles in cell aging and the combat against oxidative stress. To confirm our hypothesis, further studies concerning the regulators of the thioredoxin system may provide a better understanding on the response and involvement of the thioredoxin system upon oxidative challenge. Our findings have provided more information on the regulation of redox balance and hopefully a step forward to unravel the mystery of aging.

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