The Protective Effect of N-acetyl Cysteine on Mitochondrial Copy Number of Salivary Glands after Induction of Oxidative Stress in Albino Rats

Saba I. Thanoon 1, Ghada A. Taqa2* and Muhammad A. Alkataan 3
1Ministry of Health, Nineveh Health Directorate, Mosul, Iraq
2Department of Dental Basic Sciences, College of Dentistry, University of Mosul, Mosul, Iraq
3Department of Biochemistry, College of Medicine, University of Nineveh, Mosul, Iraq

Background: Oxidative stress is defined as condition when reactive oxidative species generation exceed the physiological level, and overcome the antioxidant capacity which lead to biomolecules damage, excessive peroxidation of lipid, damage of DNA strands, impairs gene expression, mitochondrial dysfunction and play a main role in the pathophysiology of many diseases. Aim of the study: This study was aimed to investigate protective role of N-acetyl cysteine (NAC) as antioxidant on mitochondrial gene expression and copy number against oxidative stress damage in salivary glands Material and Methods: Forty adult male albino rats were used in this study. Animals were divided into 4 groups: Group1 (Control negative, n=10): Normal diet and tap water for drinking intraperitoneally for 4weeks. Group2: (Control positive, H2O2) (n=10) normal diet and drinking water contain 0.5% H2O2 daily to induce oxidative stress for 4weeks. Group3: N-acetyl cysteine (NAC, n=10) normal diet and tap water for drinking injected daily with NAC 150 mg /kg (i.p.) For 4weeks. Group4: (Protected group) (NAC+H2O2) (n=10) normal diet and drinking water contain 0.5% H2O2 daily to induce oxidative stress, injected daily with NAC 150 mg. /kg (i.p.) for 4weeks. Tissues were collected after 4 weeks of experiment, all animal groups were euthanized and salivary glands were removed for genomic and histopathologic study Result: The results showed that oxidative stress induced by H2O2 cause significant reduction in the mitochondrial copy number in salivary gland tissue and induce severe necrosis and degeneration in control positive group while protected group with NAC showed no significant changes in mitochondrial copy number and no necrosis or degeneration in salivary gland tissue Conclusion: N-acetyl cysteine protects the mitochondrial copy number of salivary glands from reduction by oxidative stress and prevents histopathological changes.

Keywords: N-acetyl cysteine, Mitochondrial copy number, Oxidative stress.

Introduction

Oxidative stress (OS) is defined as imbalance between the production of reactive oxygen species (ROS) and the ability of cells to neutralize or remove ROS by antioxidant systems, and lead to excess of ROS [1]. ROS chemically has high reactivity and result from the metabolism of oxygen or nitrogen. ROS are two forms: free radicals as superoxide radical (O2 ⋅−), hydroxyl radical (OH⋅), and nitric oxide (NO⋅) and non-free radicals as hydrogen peroxide (H2O2) and peroxynitrite (ONOO−) [2]. ROS are active
biomolecules that physiologically generated as byproducts of metabolic pathways and/or by immune cells. Low level ROS are physiologically important for many molecular pathways such as cellular signaling, defense mechanism against invading pathogens, cell to cell interactions for proper blood flow, important for normal activity of neurons, normal growth and apoptosis [3,4]. When ROS generation exceed the physiological level, it overcomes the antioxidant capacity of cells and lead to oxidative stress which consequently play a main role in the pathophysiology of many diseases, such as cancer, cardiovascular diseases, metabolic syndrome, inflammatory diseases, and neurodegenerative diseases [5,6]. Excessive ROS lead to biomolecules damage, excessive peroxidation of lipid and damage of DNA strands [7,8]. So ROS impairs gene expression, cytokine production, and cellular metabolism, change signaling pathways and promoting cellular injury and neuronal death, impairs lipids turnover, leads to mitochondrial dysfunction, and disturbs cross-communication in the neurovascular system. The dysfunction of receptors, ion channels, and other membrane proteins, can lead to impair cell membrane fluidity and permeability [9]. Mitochondria are organelles found in the cytoplasm of eukaryotic cells, having multiple functions including energy production by oxidative phosphorylation, intracellular calcium homeostasis, and production of endogenous reactive oxygen species (ROS) [10] also metabolism of amino acids, lipids, cholesterol, steroids and nucleotides in addition to their role in the regulation of the cell cycle, cell growth and apoptosis [11]. Mitochondrial DNA (MTDNA) consists of double stranded DNA molecule, encoding 2 ribosomal RNAs, 22 transfer RNAs, and 13 polypeptides of the respiratory chain [12]. Once MTDNA damage occurs by oxidative stress, the copy number level of mtDNA is altered in human [13]. Mitochondrial copy number can be measured by amplification of one of its gene as Mitochondrially encoded NADH (nicotinamide adenine dinucleotide NAD + hydrogen ) dehydrogenase subunit I (MT-ND1) [14]. The MT-ND1 gene provides instructions for making a protein called NADH dehydrogenase 1. This protein is part of a large enzyme complex known as complex I, which is involved in the first step of the electron transport chain of oxidative phosphorylation [15]. NAC : is a widely used antioxidant as free radical scavenger, it is used to treat acetaminophen overdose and as a mucolytic agent for many respiratory diseases[16]. This study was aimed to investigate protective role of NAC as antioxidant on mitochondrial copy number against H2O2 induced oxidative stress damage in salivary glands.

Materials and Methods

Experimental Model

Forty Albino male rats were obtained from Animal house, College of Veterinary Medicine, University of Mosul, Mosul, Iraq. Rats’ age (10-12) weeks and weight (190-220)gm. They were kept in separated plastic cages with wood shavings bed, free access to stander diet and water. Standard housing conditions were kept for rats: temperature of air 20-21°C, light cycle 12-hours dark/12 hours light, humidity according to external conditions of environment [17]. Animals checked by veterinary physician before experiment. This study was done according to the guidelines of the institutional animal research and approval of ethical committee (UoM.Dent/ A.L.8/22).

Medications

N-Acetyl cysteine (NAC): Ampule 300mg/3ml. Asist/Bilim®, Turkey). Dose of NAC in this study was 150 mg/kg, injected intra-puritanically (i.p.) [18], daily for 4weeks [17].

Hydrogen peroxide (H2O2): preparation solution extra pure (Scharlau®, Spain). This solution was diluted with distilled water to prepare 0.5% H2O2 working solution daily and used as drinking water for induction of oxidative stress for 4 weeks [19].

Study design

Animals were divided to 4 groups: Group1: (Negative control) (n=10): normal diet and tap water for drinking, injected daily with 1ml/kg D.W (i.p.) for 4weeks. Group2: (Positive control) (n=10) normal diet and drinking water contain 0.5% H2O2 daily to induce oxidative stress [19]. Injected daily with 1ml/kg N.S (i.p.) for 4weeks. Group3: (NAC only) (n=10) normal diet and tap water for drinking injected daily with NAC 150 mg. /kg (i.p.) for 4weeks. Group4: protected group (NAC+ with H2O2) (n=10) normal diet and drinking water contain 0.5% H2O2 daily to induce oxidative stress, injected daily with NAC150 mg /kg i.p.[18], for 4weeks[17].

Salivary glands tissue preparation

Salivary glands tissues collected after 4 weeks of experiment, all animal groups were euthanized by inhalation of Diethyl ether and salivary glands

were extracted. Part of salivary glands species for each animal were kept in phosphate buffer saline for genomic study and other salivary glands species were kept in 10% formaldehyde for histopathological study.

Genomic study

Includes measurement of mitochondrial DNA copies number (mt-DNA). DNA extraction from salivary gland tissues by using AddPrep® Genomic DNA Extraction Kit (Korea) Figure (1) and Nano photometer used to evaluate the quality and quantity of extracted mt-DNA. ND1 gene primers were designed used NCBI https://www.ncbi.nlm.nih.gov/ software and synthesized as follow ND1 gene forward sequence 5’-AGGACCATTCCGCTATTCT-3’ and the reverse sequence 5’-GGGTAGGATGCTCCTCATG -3’.

Fig. 1. AddPrep® Genomic DNA Extraction Kit (Korea)

Fig. 2. GAPDH forward sequence ACATGCACAGGATCTTCA and reverse sequence TTACCCAGCCTCTCAG as housekeeping gene. The mitochondrial copy number change was determined by quantitative polymerase chain reaction (qPCR using the Go-Taq qPCR master mix from Promega (A6000) [20]. and PCR max Eco machine. Replication reactions for each gene of interest and household genes were performed for each sample (3 technical replicates was used). ΔACT calculated to compare differences in gene copy number between the tested samples. Replication reactions for each gene of interest (ND1) and household genes were performed for each sample. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene was used as an internal control in the calculation of the ΔCT.
value. \( \Delta \Delta CT \) was calculated to compare differences in gene expression between samples. The \( \Delta CT \) value was calculated for each sample as the difference in CT between the gene of interest and the household gene. \( \Delta \Delta CT \) was measured as the difference between the \( \Delta CT \) values of the experimental sample and the control sample. The mitochondrial copy number in this study expressed by using a \( \Delta CT \) method.

**Histopathological studies**

Include salivary glands dissection and processing [21]. Two pathology specialists for investigation the histological changes using light microscope (Mix Olympus CX 21) examination by using (Omax* China) digital camera, which is connected to the microscope. The scores are descriptive expression of severity of pathological changes of salivary glands for rats of each group done by pathologist then the scores data analyzed statistically by descriptive statistics by Kruskal Wallis and post hoc Dunn’s test for comparison of mean of scores of severity between groups . Table 1.

**Statistical Analysis**

Statistical Analysis was performed by SPSS program version 21 for Windows software. Descriptive statistics of data expressed as mean ± standard deviation (SD). The data of 4 groups were statistically analyzed by t-test for genomic study and Kruskal Wallis and post hoc Dunn’s test for histopathology results[22].

**Results**

**Results of genomic study**

The results of ND1 gene used to calculate mitochondrial copy number as \( \Delta \Delta CT \) value, t-test showed that there is significant elevation in mitochondrial copy number in rats treated with NAC for 4 weeks (1.39 ±0.44, p <0.01) compared to negative control group. While 4 weeks treatment with \( \text{H}_2\text{O}_2 \) lead to significant reduction in mitochondrial copy number (-2.46±0.52). No significant change in mitochondrial copy number in protected group treated for 4 weeks with \( \text{H}_2\text{O}_2 \) and NAC (0.04 ±0.32) which show mitochondrial protective effect of NAC drug against \( \text{H}_2\text{O}_2 \) induced oxidative stress. Fig. (3,4)

**Results of histopathology**

The microscopic examination of salivary glands specimens from control negative and NAC groups showed no evidence of histological abnormalities and show regular cells and intact acini and ducts of salivary glands and no pathological changes was observed. Control negative Fig. 5, NAC Fig. 6. while positive control (\( \text{H}_2\text{O}_2 \)) group, many pathological changes included severe necrosis of epithelial cells lining acini, granular convoluted tubules and striated duct, increase fibrous tissue surrounding interlobular ducts, present of edema between lobules and congested blood vessels Figure(7) Finally, in the protected group (\( \text{H}_2\text{O}_2 \)+NAC) intact mucous and serous acini and interlobular ducts with degeneration of epithelium lining granular convoluted tubules was observed in Figure(8). Severity scores for pathological changes of salivary glands analyzed statistically by descriptive statistics by Kruskal Wallis and post hoc Dunn’s test for comparison of mean of scores of severity between groups[23]. Table (3).

### TABLE 1. Ordinal descriptive severity scoring system of salivary gland histopathological changes of all groups of study.

<table>
<thead>
<tr>
<th>Histopathological changes</th>
<th>Description</th>
<th>Score</th>
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<tbody>
<tr>
<td>-Cell injury</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-Degeneration</td>
<td>No lesions</td>
<td>0</td>
</tr>
<tr>
<td>2- Necrosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-Circulatory disturbances</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1- Edema</td>
<td>Mild lesions</td>
<td>1</td>
</tr>
<tr>
<td>2- Congestion of blood vessels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Cell adaptation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1- Atrophy</td>
<td>Moderate lesions</td>
<td>2</td>
</tr>
<tr>
<td>2- Hyperplasia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Inflammation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-Increase fibrous connective tissue</td>
<td></td>
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</tr>
</tbody>
</table>

The scores are descriptive expression of severity of pathological changes of salivary glands for rats of each group done by pathologist then the scores data analyzed statistically by descriptive statistics Kruskal Wallis and post hoc Dunn’s test for comparison of mean of scores of severity between groups.

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Fig. 3. Amplification of ND1 gene

Fig. 4. Expression of normalized mitochondrial copy number as ΔΔCT(mean ±SD)

Fig. 5. Photomicrograph of rat salivary gland of control negative group shows intact mucous and serous acini (A), granular convoluted tubules (B), straight ducts (C) and blood vessel (D). H&E stain. 400X

Fig. 6. Photomicrograph of rat salivary gland of NAC group shows intact mucous and serous acini (A), granular convoluted tubules (B), straight ducts (C). H&E stain. 400X.

Fig. 7. Photomicrograph of rat salivary gland of H₂O₂ group shows severe degeneration (A) and necrosis with atrophy (B) of epithelial cells lining granular convoluted tubules and atrophy of acini (C). H&E stain. 400X.

Fig. 8. Photomicrograph of rat salivary gland of protected group (NAC + H₂O₂) shows intact mucous and serous acini (A) and interlobular ducts (B) with degeneration of epithelium lining granular convoluted tubules (C). H&E stain. 400X.
Table 3. Scores of ordinal descriptive severity of salivary gland histopathological changes of all groups of study

<table>
<thead>
<tr>
<th>Pathological changes</th>
<th>Control group</th>
<th>NAC group</th>
<th>H$_2$O$_2$ group</th>
<th>NAC + H$_2$O$_2$ group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell injury</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Degeneration</td>
<td>0.5 ± 0.25 A</td>
<td>0.25 ± 0.25 A</td>
<td>2.75 ± 0.25 B</td>
<td>1.25 ± 0.28 A</td>
</tr>
<tr>
<td>Necrosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Circulatory disturbances</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Edema</td>
<td>0.75 ± 0.47 A</td>
<td>0.75 ± 0.47 A</td>
<td>2.5 ± 0.28 B</td>
<td>2 ± 0.4 AB</td>
</tr>
<tr>
<td>Congestion of blood vessels</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cell adaptation</td>
<td></td>
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<tr>
<td>Atrophy</td>
<td>0.0 ± 0.0 A</td>
<td>0.0 ± 0.0 A</td>
<td>2.25 ± 0.47 B</td>
<td>1.25 ± 0.25 C</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Inflammation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0 ± 0.0 A</td>
<td>0.0 ± 0.0 A</td>
<td>2.28 ± 0.4 B</td>
<td>0.14 ± 0.28 A</td>
<td></td>
</tr>
<tr>
<td>Increase fibrous tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0 ± 0.0 A</td>
<td>0.0 ± 0.0 A</td>
<td>1.5 ± 0.25 B</td>
<td>0.5 ± 0.25 A</td>
<td></td>
</tr>
</tbody>
</table>

Data expressed as Mean ± standard error. The difference letters mean there are significant differences between groups at p ≤ 0.05. Score 0: No lesions, Score 1: Mild lesions, Score 2: Moderate lesions, Score 3: Severe lesions. Different letters indicate statistical difference at p ≤ 0.05

Discussion

Reactive oxygen species (ROS) result as by-products of energy-producing reactions that are largely occur in the mitochondria by oxidative metabolism. Low levels of ROS act as signaling molecules act to balance cell differentiation, self-renewability, and proliferation; while highly elevated ROS levels induce cell damage due to the interaction frequency with molecules such as proteins, RNA and DNA; thus resulting in diseases and this condition is defined as oxidative stress [24]. One of these ROS is H$_2$O$_2$ which is a powerful oxidizing agent and inducer of oxidative stress [25]. H$_2$O$_2$ leads to depletion of GSH and decrease NADPH production via inhibition of (Pentose phosphate shunt) which is source of NADPH necessary to activate glutathione reductase enzyme which is required to remanufacture of GSH from oxidized GSH[26]. Mitochondrial DNA is more vulnerable to reactive oxygen species (ROS) damage than nuclear DNA due to two factors, first MT.DNA attached to the mitochondrial inner membrane are susceptible to series of oxidation products generated within the membrane and modified by them. Secondly because, it lacks protection and repair mechanisms [27].

Expression of mitochondrial genes, and activity of mitochondria itself, is proportional to MT.DNA copy number. The MT.DNA copy number reflect the level of MT.DNA damage; mitochondrial dysfunction considered an important pathogenesis of many diseases. Therefore, the MT.DNA copy number can be used as a biological indicator of mitochondrial damage such as in case of chemotherapeutic therapy or diseases [28].

This study focused on evaluation of change of ND1 gene as indicator for changes in mitochondrial copy number due to OS and protecting antioxidant mechanism of NAC on mitochondria from such changes. MT.DNA showed significant decrease in mitochondrial copy number in control positive (H$_2$O$_2$) group compared to negative control group this can be explained by that oxidative stress lead to MT.DNA damage and enhance inflammatory response [29]. This lead to reduced ND1 levels which cause defects in the function and activity of complex-I enzymes then respiratory defect,
diminished mitochondrial ATP production, and decreased mitochondrial membrane potential, in addition to increased production of mitochondrial ROS [30].

Protected group (NAC+ H₂O₂) showed no significant changes in mitochondrial copy number compared to control negative group and this indicate the protective effect of mitochondrial dysfunction from oxidative stress, while NAC group show increase mitochondrial copy number compared to control group negative. This result agreed with other studies as [31,32]. Mitochondrial complexes are essential for energy production, respiratory chain enzymes are proteins having active thiol groups for sensing redox status in the cell. The activity of these enzymes is inhibited in oxidative stress status in which balance is disturbed by free sulfhydryl groups that are a ready source of reducing equivalents and radical species. NAC most likely prevents the reduction of mitochondrial complexes by protecting sulfhydryl groups from oxidation. In addition, thiol groups provided by NAC can preserve the tertiary structure of mitochondrial enzymes by serving as a donor for weak hydrogen bonds [33].

Histopathological result of this study showed normal tissue of salivary gland in both negative control group and NAC group. Oxidative damage of the salivary glands lead to dysfunction of the salivary glands of rats and its salivary secretion, Saliva has a crucial role in maintaining good health, not only in the oral cavity but also in whole body by control homeostasis, moisturizing and cleaning of mucous membranes and teeth. It participates in the initial stage of carbohydrate digestion as well as facilitates the formation and swallowing of food pieces. Buffer systems of saliva maintain constant pH, protecting teeth against decay and erosion. From the point of view of redox balance, saliva contains very effective antioxidant systems that constitute the first line of defense of the gastrointestinal tract against ROS. Damaging salivary gland by oxidative stress are disrupting all these function [34].

Oxidative stress is related to change of potential of the mitochondrial membrane leads to hyper production of ROS[35] . ROS can trigger nuclear kappa B factor (NF-κB); a redox-sensitive transcription factor that trigger production of further inflammatory chemical mediators [36]. H₂O₂ causes mitochondrial inhibition by disruption plasma membrane and lead the occurrence of necrosis [37] . This results was compatible with histopathological result which showed necrosis, edema and congestion in blood vessels in salivary gland section in control positive group compared to control negative group. Oxidative stress induced salivary gland damage agree with other studies as [38]. protected group (NAC+H₂O₂) showed much fewer of such degeneration changes compared to control positive group, this indicate anti-inflammatory and antioxidant effect of NAC.N-Acetyl cysteine prevents activation of NF-κB by removal of ROS, N-Acetyl cysteine also inhibited the synthesis of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and TNFα [39]. The direct antioxidant activity of NAC is due its free thiol group that reacts with reactive oxygen(ROS) and reactive nitrogen species (RNS) and act as scavenger of free radicals. While indirect antioxidant activity is due to that NAC is precursor of Cysteine ( by a deacetylation reaction catalyzed by aminoaoylase I) which is the building block of glutathione [40]. glutathione is considered a powerful antioxidant that protects cell from damage [41]. Another mechanism linked to the indirect antioxidant activity exerted by NAC is related to its reducing capacity by restoring systemic store of thiols and reduced protein sulfhydryl groups, which are involved in the regulation of the redox state [42].

Conclusion

N-acetyl cysteine protects salivary glands mitochondrial copy number from reduction by H₂O₂ induced oxidative stress and ameliorates histopathological changes.

Acknowledgement

Great thanks to the College of Dentistry, University of Mosul, Department of Dental Basic Sciences for their support to conduct this study.

Conflicts of interest

The authors declared no competing interests.

Funding/Support

None.

Ethical Approval

All procedures involving animals in this study followed the National Institutional Health Principles of Laboratory Animal Care guidelines. The authors disclosed that this work received institutional ethical approval REC reference no. (UoM.Dent/A.L.8/22).
References


التأثير الوقائي لان اسيتيل سيستين على عدد نسخ الميتوكوندريا في الغدد اللعابية بعد تحميل الإجهاد التأكسدي

الخلفية: يُعرَّف الإجهاد التأكسدي بأنه حالة عندما يتجاوز توليد الأنواع المؤكسدة التفاعلية للمستوي الفسيولوجي، ويغلب على القدرة المضادة للأكسدة التي تؤدي إلى تلف الخلايا والأنظمة. وحالة الإجهاد التأكسدي تتنوع بشكل رئيسي في الفيزيولوجيا المرضية لعدة أمراض. تهدف هذه الدراسة إلى التحقق من الفعالية في العلاج الريادي لإن اسيتيل سيستين كمضاد للإجهاد التأكسدي في التجربة臨 الزمنية لعدد نسخ الميتوكوندريا.

المادة واللوبي: استخدم في هذه الدراسة أربع مجموعات من الذكور، كل مجموعة مكونة من 20 جرذانًا، تتراوح أعمارهم بين 10-14 أسابيع، وتوزع في عاملات بيئة مراقبة في غرفة مغلقة. تُعطى المجموعة المحمية ان اسيتيل سيستين (10 مل/كغم ماء) يوميًا، وتشتمل المجموعة المحمية (ال kontrol الإيجابي) على بيروكسيد الهيدروجين (0.5% ماء) يوميًا.

النتيجة: أظهرت النتائج أن الإجهاد التأكسدي الناجم عن إجهاد التأكسدي (بيروكسيد الهيدروجين) يؤدي إلى تراجع عدد نسخ الميتوكوندريا في أنسجة الغدد اللعابية. ومع ذلك، لم يؤدي الإجهاد التأكسدي المحمي من إن اسيتيل سيستين إلى تغيرات معنوية في عدد نسخ الميتوكوندريا.

الخلاصة: إن اسيتيل سيستين يحمي عدد نسخ الميتوكوندريا من الغدد اللعابية من الإجهاد التأكسدي ويمنع التغييرات النسيجية المرضية.

الكلمات المفتاحية: إن اسيتيل سيستين، رقم نسخة الميتوكوندريا، الإجهاد التأكسدي.