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

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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, H_2O_2) (n=10) normal diet and drinking water contain 0.5% H_2O_2 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+H_2O_2) (n=10) normal diet and drinking water contain 0.5% H_2O_2 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 H_2O_2 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 (O_2 ·-), hydroxyl radical (OH·), and nitric oxide (NO·) and non-free radicals as hydrogen peroxide (H_2O_2) and peroxynitrite (ONOO-) [2]. ROS are active

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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 lipid 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 (MT.DNA) consists of double stranded DNA molecule, encoding 2 ribosomal RNAs, 22 transfer RNAs, and 13 polypeptides of the respiratory chain [12]. Once MT.DNA damage occurs by oxidative stress, the copy number level of mtDNA is altered In human [13]. Mitochondrial copy number can be measured by amplification one of its gene as Mitochondrially encoded NADH (nicotinamide adenine dinucleotide NAD + hydrogen) dehydrogenase subunit 1 (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 H_2O_2 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 cycle12-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 (H_2O_2): preparation solution extra pure (Scharlau®, Spain). This solution was diluted with distilled water to prepare 0.5% H_2O_2 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% H_2O_2 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 H_2O_2) (n=10) normal diet and drinking water contain 0.5% H_2O_2 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 ND1gene forward sequence primer 5'-AGGACCATTCGCCCTATTCT-3' and the reverse sequence 5'-GGGTAGGATGCTCGGATTCA-3' Fig.2. GAPDH forward sequence ACATGCACAGGGTACTTCGA and reverse sequence TTACCCCAGCCTTCTCCATG 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). ΔΔCt 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

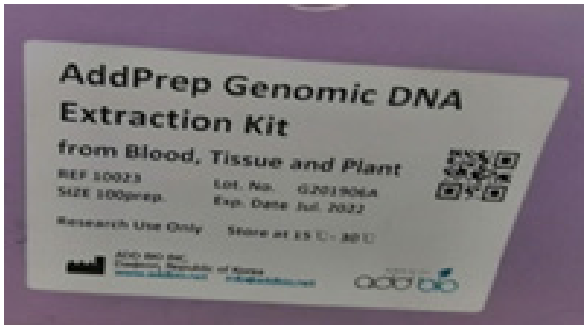
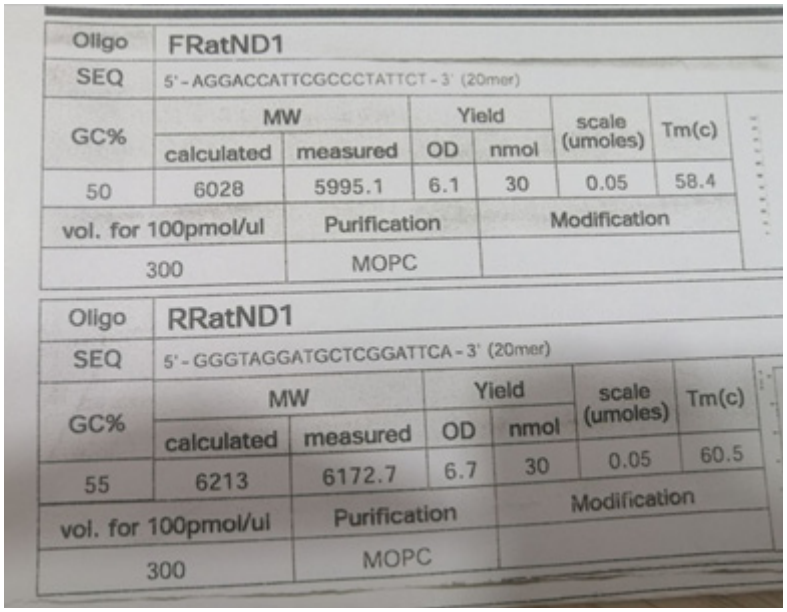


Fig.1. AddPrep® Genomic DNA Extraction Kit (Korea)



Oligo	FRatND1					
SEQ	5'-AGGACCATTCGCCCTATTCT-3' (20mer)					
GC%	MW		Yield		scale (umoles)	Tm(c)
	calculated	measured	OD	nmol		
50	6028	5995.1	6.1	30	0.05	58.4
vol. for 100pmol/ul		Purification		Modification		
300		MOPC				

Oligo	RRatND1					
SEQ	5'-GGGTAGGATGCTCGGATTCA-3' (20mer)					
GC%	MW		Yield		scale (umoles)	Tm(c)
	calculated	measured	OD	nmol		
55	6213	6172.7	6.7	30	0.05	60.5
vol. for 100pmol/ul		Purification		Modification		
300		MOPC				

Fig. 2. ND1gene forward and the reverse primer sequence

value. ΔΔCT was calculated to compare differences in gene expression between samples. The ΔCT value was calculated for each sample as the difference in CT between the gene of interest and the household gene. ΔΔCT was measured as the difference between the ΔCT values of the experimental sample and the control sample. The mitochondrial copy number in this study expressed by using a ΔCT 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 ΔΔCT 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 H_2O_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 H_2O_2 and NAC (0.04 ±0.32) which show mitochondrial protective effect of NAC drug against H_2O_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 (H_2O_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 (H_2O_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.

Histopathological changes	Description	Score
-Cell injury 1-Degeneration 2- Necrosis	No lesions	0
-Circulatory disturbances 1- Edema 2-Congestion of blood vessels	Mild lesions	1
- Cell adaptation 1- Atrophy 2- Hyperplasia	Moderate lesions	2
- Inflammation -Increase fibrous connective tissue	Severe lesions	3

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**.**

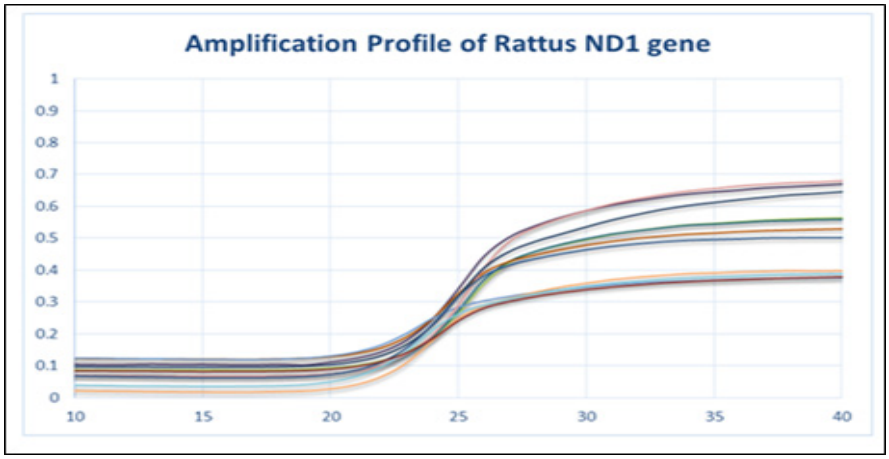


Fig. 3. Amplification of ND1 gene

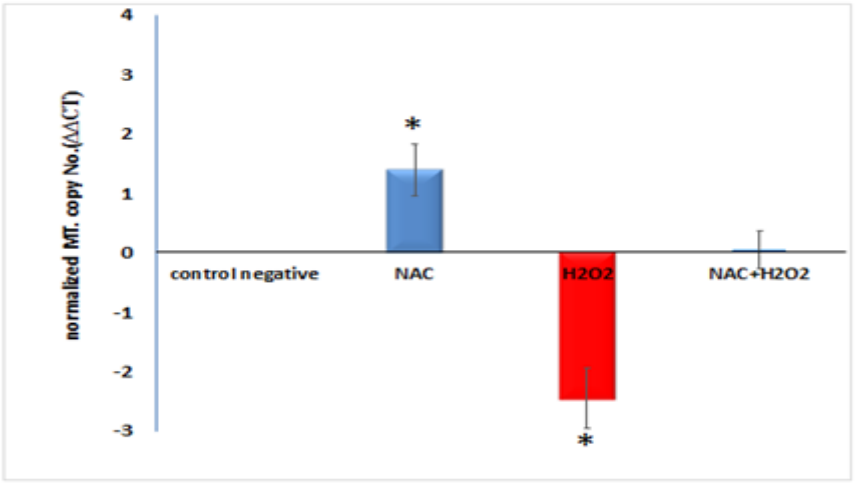


Fig. 4. Expression of normalized mitochondrial copy number as ΔΔCT(mean ±SD)

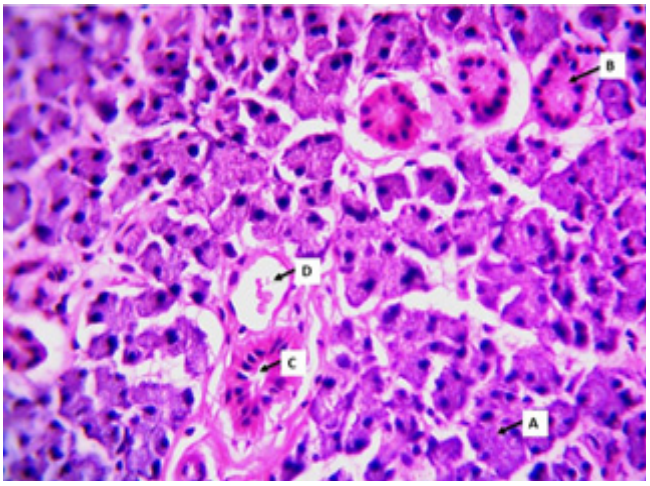


Fig. 5. Photomicrograph of rat salivary gland of control negative group shows intact mucous and serous acini (A), granular convoluted tubules (B), straight ducts (C) and blood vessel (D). H&E stain. 400X

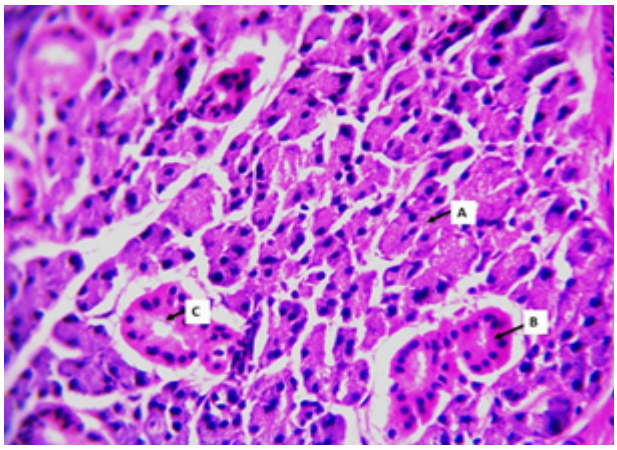


Fig. 6. Photomicrograph of rat salivary gland of NAC group shows intact mucous and serous acini (A), granular convoluted tubules (B), straight ducts (C). H&E stain. 400X.

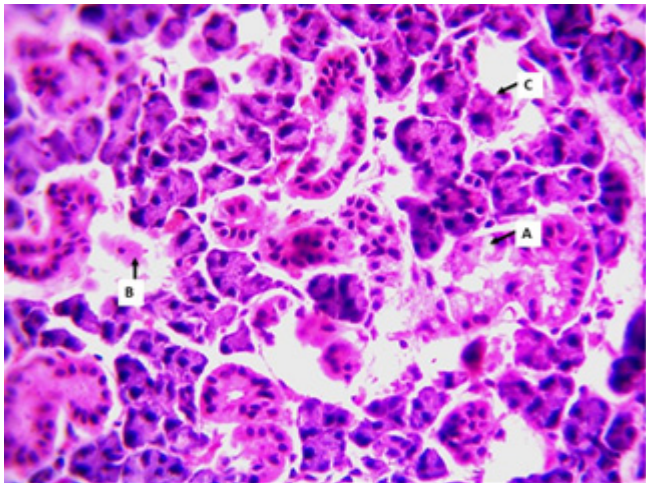


Fig. 7. Photomicrograph of rat salivary gland of H2O2 group shows severe degeneration (A) and necrosis with atrophy (B) of epithelial cells lining granular convoluted tubules and atrophy of acini (C). H&E stain. 400X.

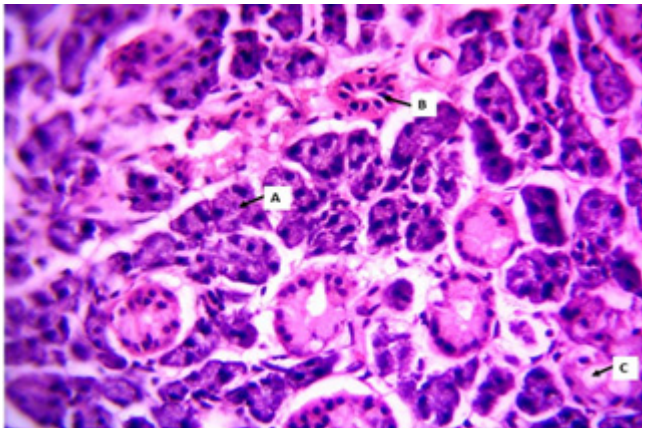


Fig. 8. Photomicrograph of rat salivary gland of protected group (NAC +H2O2) shows intact mucous and serous acini (A) and interlobular ducts (B) with degeneration of epithelium lining granular convoluted tubules (C). H&E stain. 400X.

TABLE 3. Scores of ordinal descriptive severity of salivary gland histopathological changes of all groups of study

Pathological changes		Control group	NAC group	H_2O_2 group	NAC + H_2O_2 group
Cell injury	Degeneration	0.5 ± 0.25 A	0.25 ±0.25 A	2.75 ± 0.25 B	1.25 ± 0.28 A
	Necrosis				
Circulatory disturbances	Edema	0.75 ± 0.47 A	0.75 ±0.47 A	2.5 ±0.28 B	2 ± 0.4 AB
	Congestion of blood vessels				
Cell adaptation	Atrophy	0.0 ± 0.0 A	0.0 ± 0.0 A	2.25 ± 0.47 B	1.25 ± 0.25 C
	Hyperplasia				
Inflammation		0.0 ± 0.0 A	0.0 ± 0.0 A	2.28 ± 0.4 B	0.14 ± 0.28 A
Increase fibrous tissue		0.0 ± 0.0 A	0.0 ± 0.0 A	1.5 ± 0.25 B	0.5± 0.25 A

Data expressed as Mean ± stander 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_2O_2 which is a powerful oxidizing gent and inducer of oxidative stress [25]**.** H_2O_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 re-manufacture 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 ND1gene 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_2O_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+ H_2O_2) 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]. H_2O_2 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+H_2O_2) 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 aminoacylase 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 H_2O_2 induced oxidative stress and ameliorates histopathological changes.

Acknowledgement

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Conflicts of interest

The authors declared no competing interests.

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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.45/22).**

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التأثير الوقائي للان اسيتيل سيستين على عدد نسخ الميتوكوندريا في الغدد اللعابية بعد تحريض الإجهاد التأكسدي في الجرذان البيضاء

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الخلفية: يُعرَّف الإجهاد التأكسدي بأنه حالة عندما يتجاوز توليد الأنواع المؤكسدة التفاعلية للمستوى الفسيولوجي ، ويتغلب على القدرة المضادة للأكسدة التي تؤدي إلى تلف الجزيئات الحيوية ، والأكسدة المفرطة للدهون ، وتلف خيوط الحمض النووي ، وإعاقة التعبير الجيني ، وخلل الميتوكوندريا ، ويلعب دورًا رئيسيًا في الفيزيولوجيا المرضية للعديد من الأمراض. هدف الدراسة: تهدف هذه الدراسة إلى التحقق من الدور الوقائي لـلان اسيتيل سيستين كمضاد للأكسدة في التعبير الجيني للميتوكوندريا ورقم نسخة الميتوكوندريا ضد أضرار الإجهاد التأكسدي في الغدد اللعابية. المواد والطرق: استخدم في هذه الدراسة أربعون من ذكور الجرذان البيضاء بمدى وزن (١٩٠-٢٢٠) جرام. قسمت الحيوانات إلى ٤ مجموعات: المجموعة ١ (سلبي الضبط) (ن = ١٠): غذاء عادي ومياه الصنبور للشرب ، تحقن يومياً ب١ مل/ كغم ماء مقطر لمدة ٤ أسابيع داخل الغشاء البيريتوني. المجموعة ٢: (التحكم الإيجابي)(بيروكسيد الهيدروجين) (ن = ١٠) يحتوي النظام الغذائي العادي ومياه الشرب تحتوي على ٠,٥٪ بيروكسيد الهيدروجين يوميًا للحث على الإجهاد التأكسدي ، ويتم حقنها يوميًا ب١ مل/ كغم ماء مقطر لمدة ٤ أسابيع داخل الغشاء البيريتوني. المجموعة ٣ (ان اسيتيل سيستسن) (ن = ١٠) نظام غذائي عادي ومياه الصنبور للشرب يتم حقنها يوميًا بـ١٥٠ ملغ/كغم لمدة ٤ أسابيع داخل الغشاء البيريتوني المجموعة ٤: (المجموعة المحمية: ان اسيتيل سيستين)ن = ١٠) :يحتوي النظام الغذائي العادي ومياه الشرب تحتوي على ٠,٥٪ بيروكسيد الهيدروجين يوميًا للحث على الإجهاد التأكسدي ، ويتم حقنها يوميًا ب ١٥٠ ملغ / كغم لمدة ٤ أسابيع داخل الغشاء البيريتوني . تم جمع الأنسجة بعد ٤ أسابيع من التجربة ، وتم قتل جميع المجموعات الحيوانية بشكل رحيم واستخراج الغدد اللعابية. تم حفظ جزء من أنواع الغدد اللعابية لكل حيوان في محلول فوسفاتي عازل للدراسة الجينية وأنواع أخرى من الغدد اللعابية تم حفظها في ١٠٪ فورمالدهايد للدراسة النسيجية. النتيجة: أظهرت النتائج أن الإجهاد التأكسدي الناجم عن بيروكسيد الهيدروجين سبب انخفاضًا كبيرًا في عدد نسخة الميتوكوندريا في أنسجة الغدد اللعابية ويؤدي إلى نخر شديد وانحلال في المجموعة الإيجابية الضابطة بينما لم تظهر المجموعة المحمية مع ان اسيتيل سيستين أي تغييرات معنوية في عدد نسخة الميتوكوندريا وعدم وجود نخر أو تنكس في أنسجة الغدد اللعابية. الخلاصة : ان اسيتيل سيستين يحمي عدد نسخ الميتوكوندريا من الغدد اللعابية من الاختزال بواسطة الإجهاد التأكسدي ويمنع التغيرات النسيجية المرضية.

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