Correlation Between nDNA and mtDNA Fragmented by Reactive Oxygen Species in Human Uroepithelial Cells

Sabah S. Abdul-Saheb **Osama Bassim abdul Khaliq**
Madenat Al-elemUniv. College,Biology Dept. , Al-Kadhmiya
Baghdad - Iraq

Abstract

Fifty mid-stream urine specimens were collected from Urinary Tract Infections (UTI) patients. Patients were divided into five groups (A, B, C, D and E) according to the pus cells level in their urine specimens. The results showed that the level of Reactive Oxygen Species was significantly ($P<0.05$) increased as the level of pus cells increased. Therefore, group E showed high level of ROS (9.08 nmol/l) in comparison with other groups in this study.The correlation between ROS level and fragmentation DNA in nuclei (n) and mitochondria (mt) was estimated. In general, increasing level of ROS induced either nDNA or mtDNA fragmentation. nDNA fragmentation in group E was was noticed in high percentage (65.14%) and associated with a high level of ROS (9.08 nmol/l). Similar results were obtained fom mtDNA fragmentation. Nevertheless, ROS significantly ($P<0.05$) affected mtDNA more than nDNA.

Key words: DNA , Oxygen , Urine , Cells and Human physiology .

العلاقة بين الدنا النووي والدنا المايتوكنديري المتجزئة بفعل الاوكسجين المنشط في الخلايا الطلائية البولية للانسان

صباح سعد عبد الصاحب **اسامة باسم عبد الخالق**
كلية مدينة العلم الجامعة – قسم علوم الحياة – الكاظمية
العراق - بغداد

الخلاصة

جمعت خمسون عينة ادرار من مرضى يشكون من اخماج المجاري البولية ،وقسمت هذه العينات الى خمس مجموعات (A,B,C,D,E) اعتمادا على اعداد الخلايا الالتهابية الموجوده فيها. قد اكدت النتائج ان مستوى MDA قد ازداد معنوياً ($P<0.05$) طبقاً لزيادة الخلايا الالتهابية، لذا لوحظ ان المجموعة E قد اظهرت زيادة في Malondialdehyde (MDA)(9.08 نانومول \ مل) مقارنةً بالمجاميع الاخرى . تمت دراسة العلاقة بين مستوى ROS والدنا المتجزء في النواة والمايتوكندريا. وعموماً، وجد ان الزيادة في مستوى ROS يحث على تجزئة الدنا سواء الدنا الموجود في النواة (nDNA) او المايتوكندريا (mtDNA). لذا اشارت النتائج الى ان النسبة المئوية nDNA المجزء في المجموعة E كانت عالية و بلغت (65.14%) نظراً لارتفاع مستوى MDA (9.08 نانومول\مل). وقد اشارت النتائج ايضا الى انر المستوى العالي من ROS اثر معنويا على mtDNA ايضا.

الكلمات المفتاحية: دنا ، اوكسجين ، يوريا ، خلايا وعلم ترشيح الانسان .

Introduction

Reactive oxygen species (ROS) are deleterious in excess. They are naturally produced by aerobic metabolism and are a permanent threat to living organisms(Droge, 2002). All organisms have developed ways of protecting themselves against ROS, including specific defences and global responses that enable cells to survive periods of oxidative stress. Both types of protection are regulated and responded to the environment-associated oxidative threat (Raju, and Madala, 2005).Reactive oxygen species instability and inability to permeate lipid membranes usually provide an effective shield against propagating damage. However, through reactions with polyunsaturated fatty acids, they generate lipid hydroperoxides and unsaturated aldehydes, which are highly electrophilic, stable, readily propagating between cellular compartments, and capable of reacting with proteins and nucleic acids. This chain reaction of lipid peroxidation accounts for the role played by ROS in the pathogenesis of atherosclerosis, ischemia, reperfusion injury, and other diseases(Sano,and Fukuda, **2008**).

It well established that mitochondria is the main site of the generation of oxygen radicals, such as, superoxide anion, hydroxyl radical, singlet oxygen and hydrogen peroxide (Burtke, and Sandstrom, 1994). It is estimated that 1–4% of oxygen reacting with the respiratory chain leads to the formation of superoxide radicals ($O_2^{\cdot -}$)**.**The other sources of reactive oxygen species include radiation, cytotoxic chemicals and antibiotics (Raju, and Madala, 2005).

Reactive oxygen species are generated at very low levels during mitochondrial respiration under normal physiologic conditions. Oxidative damage to mtDNA by ROS may lead to DNA strand breaks and the occurrence of somatic mtDNA mutations. Accumulation of these mtDNA mutations may result in dysfunction of the cells, leading to increased ROS production in mitochondria and subsequent accumulation of more mtDNA fragmentation (Addabbo *et al.,* 2009).

The aims of this study were:

- Determination of produced ROS.
- Study the correlation between ROS and DNA fragmentation in uroepithelial cells.
- Determination of fragmented mtDNA and comparison with nuclear DNAfragmentation.

Materials and Methods

- **Patients and urine samples**

A total of 50 of midstream urine specimens from patients with UTI only were collected in 5 ml of sterile container, with symptoms suggesting acute UTI. Inclusion criteria were dysuria, frequency, urgency, and abdominal flank pain with or without fever.

Level of pus cells in UTI patients

The specimens were divided into 5 groups according to the level of pus cells as represented in table 1.

Determination of MDA (Indirect method for ROS).

Measuring the malondialdehyde (MDA) by thiobarbituric acid (TBA) reactivity is the most widely used method for assessing lipid peroxidation. Malondialdehyde was estimated according to the modified method by Hunter 1985. The pink color which

produced in this method is due to the formation of an adduct between the thiobarbituric acid (TBA) and malondialdehyde under acidic conditions (Halliwell, 1985).

MDA levels were measured by a spectrophotometer. The reaction mixture contained 0.1ml urine sample, 0.2ml of 8.1%sodium dodecyl sulfate (SDS), 1.5ml of 20% acetic acid, and 1.5ml of 0.8% aqueous solution of thiobarbituric acid. The mixture pH was adjusted to 3.5 and the volume was finally made up to 4.0ml with distilled water and 5.0ml of the mixture of n-butanol was added. The mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 minutes, the absorbance of the organic layer was measured at 532 nm. MDA level was expressed as nmol/l (Hunter 1985).

Quantitation of DNA fragmentation with Diphenylamine (John, **2006).**

The protocol includes the lysis of cell and release of nuclear DNA, a centrifugation step with the generation of two fraction (corresponding to intact and fragmented DNA, precipitation of DNA, hydrolysis and colorimetrical quantitation upon staining with Diphenylamine (DPA),which binds to deoxyribose (John, 2006).

0.5 ml pellet suspension of urine was put in tubes labeled A. Cells were centrifuged at 500 rpm for 10 min at 4°C. Then supernatants were carefully transferred to a new tube labeled B (supernatant).

Tris-HClTriton X-100EDTAsolution (1.0 ml) was added to the pellets in tubes labeled A and vortex vigorously. To separate fragmented DNA from intact chromatin, tubes A were centrifuged at 14000 rpm for 20 min at 4°C. The supernatants were carefully transferred in new tubes labeled C. TTE solution (1.0 ml) was added to the small pellets in tube A. Then, 1.0 ml of 25% TCA was added to tubes (C, A and B), vortexed vigorously, and allowed the precipitation to proceed overnight at 4°C. After incubation, precipitated DNA was recovered by pelleting for 20 min at 14000 rpm at 4°C, the supernatants discarded by aspiration. Nuclear DNA hydrolyzed by adding 320µl of 5% TCA to each pellet and heating for 15 min at 90°C in water bath. Thereafter, to each tube (A,B,C) , 640µl of freshly prepared Diphenylamine solution was added, then vortex. The color was allowed to develop for about 4 h at 37°C or overnight at room temperature. Aliquots of colored solution (ignoring dark particles) was transferred from crystal tube to read by spectrophotometer. The optical density was read at 600 nm and blank was set to 0. Then percentage of fragmented DNA can be calculated using the formula:

$$\%\, fragmentedDNA = \frac{B+C}{A+B+C} * 100$$

Determination of DNA fragmentation in mitochondria

1-Isolation of mitochondria

It was done as described by DeSalle *et al.,* (1993)and Bloom *et al.*(1996)

1- Mitochondrial Isolation Buffer (MIB) (2.5ml) was added to 1 ml of urine samples and homogenized on ice. The mixture was centrifuged at 3000 rpm, 4°C for 5 min. The resulting supernatant was transformed to a new centrifuged tube on ice and submitted to another run of centrifugation (10000 rpm,4°C for 5 min). The supernatant was discarded and the mitochondria were resuspended in 1.0 ml

of Mitochondrial Lysis Buffer (MLB). The resultant solution was used for preparing mitochondrial DNA.

2-Isolation of Mitochondrial DNA (mtDNA)

DeSalle *et al.,* (1993) and Bloom *et al.*,(1996) method for isolation mtDNA as following :

1. Mitochondrial suspension (0.5 ml) was aliquot into 10 ml centrifuge tube and 200 µl of 2% Sodium dodecyl sulfate/0.4 M NaOH was added to centrifuge tube. The contents were mixed by inverting tube 10 times. The mitochondrial suspension should be changed from an opaque brown to a more transparent amber color. This indicates that the mitochondrial membrane have been solubilized by SDS, and the tube was put on ice for 5 minutes.

2. KOAc/acetic acid solution (300 µl) was added to centrifuge tube. The content of tube were mixed by inverting each tube 10 times. The contents should become cloudy as proteins and lipids precipitate due to the high ionic strength (5 M acetate and 3 M potassium) of the KOAc solution. The tube was let on ice for 5 minutes; the centrifugation at 14000 rpm for 5 min at 4°C was done.

3. The resulting supernatants were transferred into 3 new 1.5 ml micro tubes, and 1.0 ml of phenol/chloroform (TE-saturated) was added to the tube. The contents were mixed by inverting each tube 20 times, to ensure thorough mixing of the aqueous and organic (phenol/chloroform) solutions. Centrifugation at 14,000 rpm for 5 minutes at 4°C was done. This resulted in 2 layers in the centrifuge tube: bottom layer - phenol/chloroform, and top layer - aqueous, contain mtDNA.

4. The top layer was carefully pull out of each tube and transferred into a new 10 ml centrifuge tube. Then, 1000 µl of ice-cold 100% ethanol were added to the tube, mixed well and put in ice for 5 minutes. A centrifugation tube at 14,000 rpm for 10 minutes at 4°C was done.

5. After centrifugation, each tube was appeared to contain a small white pellet of mitochondrial DNA. Ethanol was removed from supernatant layer, then, 1000 µl of 70% ethanol were added to each tube, mixed well for 10 times and centrifugated tubes at 10000 rpm for 5 min at 4°C, then removed ethanol (supernatant) was removed. Finally TE buffer was added to pellet of mtDNA which came free of the wall of tubes.

6. mtDNA fragmentation was calculated by equation according to the method previously mentioned (John, 2006).
Data were statistically analysed by student "t" test and correlation coefficient (Daniel, 1983).

Results and Discussion

The pus cells gave an indication to the severity of urinary tract infections, (Huostin, **1963**).So, according to WHO standards, less than 6 pus cells in a urine specimen, it will be considered as a healthy (table 1.). The result indicated in table (1) that group A has a level of pus cells ranged between (6-10 c.mm) because if pus cells were less than 6 its considered as healthy group (WHO,2003).

The relationship between ROS level and DNA fragmentation in UTI patients classified to five groups according to the degree of infection. The result in table 2 showed that the value of DNA fragmentation in pus and epithelial cells was correlated with MDA level. As seen, the low percentage of DNA fragmentation in group A (55.62 %) was correlated with low level of MDA production (4.75 nmol/l). Then, the increasing percentage of DNA fragmentation in group B, C, D, and E (59.14%, 61.95%, 63.49%, and 65.14%) was significantly ($P<0.05$) related to the increasing level of MDA in the cells production (5.76, 7.21, 8.03, and 9.08 nmol/l respectively).Therefore results clearly showed that there was significant ($p<0.05$)differences between DNA fragmentation in healthy epithelial cells and those in patients with UTI.

Indeed, ROS induces DNA fragmentation in the form of modification of all bases (primarily guanine via lipid peroxyl or alkoxyl radicals), production of frame shifts mutation, cross-links of DNA through covalent binding to ROS, and chromosomal rearrangements (Aitken, and Krausz, 2001). Oxidative stress has also been associated with high frequencies of single and double DNA strand breaks. Reactive Oxygen Species can also induced oxidation of critical -SH groups in proteins and DNA, which will alter structure and function of cells with an increased susceptibility to attack by macrophages. Conclusive evidence suggests that high levels of ROS mediate the DNA fragmentation commonly (Twigg, *et al.*,1998).

-Mitochondrial DNA (mtDNA) fragmentation associated with ROS

The results in table 3 indicated the association between the level of MDA and mtDNA fragmentation. In group A, the mean of mtDNA fragmentation was 64.07% and the mean of MDA in this group was 4.75 nmol/l. In general, the increasing in the mean of mtDNA fragmentation was significantly ($P<0.05$) increased in all groups under study corresponding to the increasing of the mean of the MDA in uroepithelial cells (table 3).

The above result in agreement with recent study(Thompson,2007), and confirmed the relationship between ROS and mutation of mtDNA, it suggested that the ROS responsible for mutation mtDNA in mice, and the ROS increased the induction of mutation in mtDNA.

-Relationship between nuclear DNA (nDNA) and mtDNA fragmentation

The results in table 4 revealed the relationship between nDNA and mtDNA fragmentation. In general, the mean mtDNA fragmentation was significantly ($P<0.05$) higher than nDNA fragmentation in all studied groups. In group A, the mean of mtDNA fragmentation was 64.07%, which was significantly higher than nDNA fragmentation (55.62%). The mean mtDNA fragmentation for groups B,C,D, and E was also significantly ($P<0.05$) increased (67.47, 70.34, 74.99 and 77.26%) in comparison with nDNA fragmentation (59.14, 61.95, 63.49, and 65.14%).The rate of mtDNA fragmentation was significant and it was the result of direct impact of ROS on this DNA, in addition the mitochondria were a

source of ROS, while the nDNA has been less affected and was more complicated in structure and the presence of proteins made it less vulnerable to ROS (Shokolenko *et al.*, 2009).

Kang and Naotaka,(2005), suggested that the damage in mtDNA increased during exposure to ROS and caused mtDNA fragmentation they reported that the mtDNA was more sensitive to ROS than nuclear DNA for many reasons such as simply structure compared with nuclear DNA.

Table (1) Grouping level of pus cells in urine sample from patients with UTI

Groups	Pus Cell Level (c/mm)
A	**(6-10)**
B	**(11-20)**
C	**(21-30)**
D	**(31-40)**
E	**(Over 40)**

Table (2) The relationship between MDA and nDNA fragmentation in UTI patients.

Groups	Mean MDA (nmol/l)± SD	Mean nDNA fragmentation in patients (%)± SD
A	4.75 ±0.60 a	55.62 ±2.95 a
B	5.76 ±0.59 b	59.14 ±1.92 b
C	7.21 ±0.54 c	61.95 ±1.46 c
D	8.03 ±0.53 d	63.49 ±1.29 d
E	9.08 ±0.30 e	65.14 ±1.45 e
Controls	2.76 ±0.17 f	18.50 ±1.60 f

-The identical small letters refer to non- significant differences between mean of nDNA fragmentation in each row at p<0.05 level. ;± SD: Standard Deviation

Table (3) The relationship between mtDNA fragmentation and MDAin UTI groups.

Groups	Mean MDA (nmol/l)± SD	Mean mtDNA fragmentation in patients (%)± SD
A	4.75 ± 0.60 a	64.07 ±0.98 a
B	5.76± 0.59 b	67.46 ±0.94 b
C	7.21±0.54 c	70.34 ±1.30 c
D	8.03±0.53 d	74.99 ±1.82 d
E	9.08 ± 0.30 e	77.26 ±2.47 e
Control	2.76 ± 0.17 f	31.20 ±3.20 f

-The identical small letters refer to non- significant differences between mean of mtDNA fragmentation in each (group) row at p<0.05 level.; ± SD: Standard Deviation

Table (4) Fragmentation of nuclear nDNA and mtDNA in UTI patients

Groups	Mean MDA (nmol/l) in groups ± SD	Mean nDNA fragmentation in groups(%)± SD	Mean mtDNA fragmentation in groups(%)± SD
A	4.75 ± 0.60 A a	55.62 ± 2.95 B a	64.07 ± 0.98 C a
B	5.76 ± 0.59 A b	59.14 ± 1.92 B b	67.46 ± 0.94 C b
C	7.21 ± 0.54 A c	61.95 ± 1.46 B c	70.34 ± 1.30 C c
D	8.03 ± 0.53 A d	63.49 ± 1.29 B d	74.99 ± 1.82 C d
E	9.08 ± 0.30 A e	65.14 ± 1.45 B e	77.26 ± 2.47 C e
Controls	2.76 ± 0.17 A f	18.50 ± 1.60 B f	31.20 ± 3.20 C f

-The identical Large letters refer to non- significant correlation between mean of mtDNA fragmentation in each row at p<0.05 level. -The identical small letters refer to non- significant differences between mean of ROS, nDNA fragmentation, and mtDNA fragmentation in each (group) row at p<0.05 level. ± SD : Standard Deviation

The conclusion of this study was as follow :A correlation was found between the level of ROS and the nDNA, mtDNA fragmentaion. mtDNA fragmentaion in uroepithelial cells was higher than nDNA fragmentaion.

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