

Oxidative Stress, Angiogenesis and Apoptosis in Relation to Aging

Nagwa S. Ahmed**, *Madeha M. Zakhary, *Reda S. Yousef**,
*Marwa shaban****

Biochemistry Department, Faculty of Medicine* Sohag,

**Assuit University

ABSTRACT

Apoptosis and aging are complementary and cooperative processes that reduce cell proliferation and promote resistance to tumor development. Cellular levels of oxidative stress increase during physiological aging and tend to induce apoptosis, thereby influencing cellular and tissue aging and life span. Aim of study: is to determine the relationship between aging and apoptotic indices in the form of s-Fas and oxidant-antioxidant axis by determination of Nitric oxide (NO) and Lipid peroxide (LP). Also, the role of angiogenesis by determination of PD/ECGF (Thymidine phosphorylase) is another aim of the present work. Subjects and Methods: Fasting serum samples from 50 healthy individuals divided into five groups with age range between 20-29; 30-39; 40-49; 50-59 and 60-69 from those who proved to be in a normal state of health and free from any signs of chronic diseases. Serum levels of NO, LP and thymidine phosphorylase were measured by chemical methods, while s-Fas was assayed by ELIZA kit. Results: There were significant higher levels of serum NO, lipid peroxide, s-Fas and thymidine phosphorylase in older persons than in young ones, and a significant positive correlation between NO, lipid peroxide, s-Fas and thymidine phosphorylase when each was correlated with age. Conclusion: it could be concluded that oxidative stress, apoptosis and angiogenic factor increase with aging and may play an important role in its pathogenesis. The question remains whether they are the initial pathogenic events of aging or might be a consequence of it.

INTRODUCTION

Aging is the process of change accumulation by age and it could be defined as a complex chronological and multifactor. General characteristics of the aging or senescence process are: a progressive, physiopathological deterioration with time, which leads to homeostasis impairment, with progressive decrease in physiological capacity, reduced

ability to respond adaptively to environmental stimuli with age, increased susceptibility and vulnerability to diseases and ultimately increased mortality of organisms⁽¹⁾.

There are several hypotheses to explain how aging occurs, considering complex physiological alteration in the organism described as: mitochondrial changes, accumulation of aberrant proteins in the cytosol⁽²⁾,

chemical damage to macromolecules, somatic mutations and enhanced or diminished transcription of specific genes.

Apoptosis is a vital component in the evolutionarily conserved host defense system. Apoptosis is the guardian of tissue integrity by removing unfit and injured cells without evoking inflammation⁽³⁾. Apoptosis is the process of programmed cell death that may occur in multicellular organisms. Biochemical events lead to characteristic cell changes and death. These changes include blebbing, loss of cell membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation the lysosomal compartment is rich in labile iron and, therefore, sensitive to the mild oxidative stress that cells naturally experience because of their constant production of hydrogen peroxide. Diffusion of hydrogen peroxide into the lysosomes results in Fenton-type reactions with the formation of hydroxyl radicals and ensuing peroxidation of lysosomal contents which endanger the stability of lysosomes⁽⁴⁾. For some time, the rupture of a limited number of lysosomes has been recognized as an early upstream event in many cases of apoptosis, particularly oxidative stress-induced apoptosis. Consequently, the regulation of the lysosomal content of redox-active iron seems to be essential for the survival of cells both in the short- and the long-term⁽⁵⁾.

Fas ligand (FasL) is a 40kDa type II transmembrane protein of the tumor

necrosis factor (TNF) family⁽⁶⁾. Its receptor, Fas, is a 45kDa type I transmembrane protein of the same family. Binding of the FasL to Fas induces apoptosis. The FasL–Fas interaction plays a critical role in the regulation of immune responses, killing of tumor cells

Two theories of the direct initiation of apoptotic mechanisms in mammals have been suggested: the TNF-induced (tumor necrosis factor) model and the Fas-Fas ligand-mediated model, both involving receptors of the TNF receptor (TNFR) family coupled to extrinsic signals. The Fas receptor (also known as Apo-1 or CD95) binds the FasL, a transmembrane protein part of the TNF family. The interaction between Fas and FasL results in the formation of the death-inducing signaling complex (DISC), which contains the Fas death domain (FADD), caspase-8 and caspase-10. In some types of cells (type I), processed caspase-8 directly activates other members of the caspase family, and triggers the execution of apoptosis of the cell. In other types of cells (type II), the Fas-DISC starts a feedback loop that spirals into increasing release of pro-apoptotic factors from mitochondria and the amplified activation of caspase-8⁽⁷⁾.

Angiogenesis is one of the major vascular processes which play a major role in vascular tissue repair and development, and this complex process generates new capillaries from pre-existing vessels. In aging, angiogenesis is critical in wound-healing, in the development of collateral blood flow in ischemic disease, and in tumor growth and the

development of metastatic conditions. The effect of aging on angiogenesis has not extensively been explored. Knowing advanced age is a major risk for various vessel-associated diseases; angiogenesis is likely compromised during aging⁽⁸⁾.

Thymidine phosphorylase (TP) is an enzyme which catalyses two reactions: (i) the reversible phosphorylation of thymidine to thymine and 2-deoxy-D-ribose-1-phosphate, and (ii) deoxyribosyl transfer between pyrimidines. Overexpression of the enzyme has been associated with the development of various cancers⁽⁹⁾. TP-platelet derived-endothelial cell growth factor (PD-ECGF) has been shown to possess angiogenic activity *in vivo*⁽¹⁰⁾, and chemotactic activity *in vitro*⁽¹¹⁾. The enzymatic activity of TP was required for the generation of ROS⁽¹²⁾.

Oxidative stress has been increasingly recognized as a contributing factor in aging and in various forms of pathophysiology generally associated with aging⁽¹³⁾.

ROS are oxygen-derived metabolites that have higher reactivity than molecular oxygen. ROS include unstable oxygen radicals such as superoxide radical and hydroxyl radical and non radical molecules like hydrogen peroxide. These reactive species and others, with nitrogen and sulphur atom in the chemical composition, are continually produced as consequences of normal aerobic metabolism as well as taken up from the external environment⁽¹⁴⁾.

Nitric oxide (NO), derived from the vascular endothelium and platelets, has an important role in the physiological regulation of blood

flow. It is generated from the amino acid L-arginine via NO synthase (NOS)⁽¹⁵⁾, human aging was associated with an increase in NO Synthase (NOS) activity, a decrease in basal cyclic GMP levels in human platelets, and an increase in thiobarbituric acid-reactant substances (TBARS) in erythrocytes⁽¹⁶⁾.

Reactive oxygen and nitrogen species are the most common electrophiles formed during lipid peroxidation and lead to the formation of both stable and unstable lipid peroxide (LP). Of the LP formed, highly reactive aldehydes are a well-recognized causative factor in aging and age-associated diseases⁽¹⁷⁾. The level of lipid peroxides is an index of cellular membrane damage caused by the action of free radicals. The membranes of the organelles within the cells (mitochondria, lysosomes, peroxisomes) can also be damaged. Membrane proteins, membrane lipids and cholesterol can be damaged due to an insufficiency of antioxidants to deal with the level of oxidative stress/free radicals. The elevation of lipid peroxides serves as an early warning of the potential long-term effects of oxidative stress. The outcome of long-term oxidative stress is chronic degenerative disease, an example being the peroxidation of low-density lipoproteins contributing to atherosclerosis⁽¹⁾.

AIM OF THE STUDY

The study was done to determine the relationship between aging and apoptotic indices in the form of s-Fas and oxidant-antioxidant axis by determination of NO and Lipid

peroxide. Also, the role of angiogenesis by determination of PD/ECGF (Thymidine phosphorylase) would be evaluated.

SUBJECTS & METHODS

A. SUBJECTS:

This study was conducted on 50 healthy individuals included into five groups and each group had ten with age range 20-29; 30-39; 40-49; 50-59 and 60-69 chosen among a group of volunteers. Only those who proved to be in a normal state of health and free from any signs of chronic disease by:

1. Full history taking and clinical examination including blood pressure
2. Routine laboratory investigation in the form of, fasting blood sugar.

Exclusion criteria: Included smokers, alcohol drinking and who received any drug on a long-term basis. Only those who proved to be in a normal state of health and free from any signs of chronic diseases were included in the study.

B. Methods

Blood sample: Fasting blood samples were collected from each of the 50 participants and then serum was separated after centrifugation and kept in aliquots at -20°C till analysis.

Study protocol: The study protocol was approved by Institutional Ethical Committee on Human Research, Faculty of Medicine, Sohag University; written consent was obtained from each subject before the study.

- 1- **Measurement of lipid peroxides:** Lipid peroxides were determined by colorimetric

method according to **Buege and Aust** ⁽¹⁸⁾.

- 2- **Measurement of Nitric oxide (NO):** Nitric oxide production was assayed by Griess reaction which measures the combined oxidation products for NO nitrites and nitrates after reduction with nitrate reductase ⁽¹⁹⁾.
- 3- **Measurement of s-Fas:** Human Fas kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human Fas in serum, Cat No. ELH-Fas-001 ⁽²⁰⁾
- 4- **Assay of thymidine phosphorylase:** Thymidine phosphorylase was determined by a spectrophotometric method ⁽²¹⁾.

Statistical analysis:

Data was analyzed using STATA intercooled version 9.2. Quantitative data was analyzed using student "t" test. Mann-Whitney test was used as the data was not normally distributed. Kruskal-Wallis rank test was used to compare more than two groups. Spearman rank correlation test was used to test the relation between age and each of the investigated parameters. P value was considered significant if it was less than 0.05

RESULTS

The present study included 50 healthy individuals, 27 female (54%) and 23 male (46%), and their ages ranged 20–72 years, with a mean \pm SD of 45.06 ± 15.06 years.

Table (1) represents the clinical and biochemical criteria of the studied individuals.

Table (2) represents the comparison between groups 1, 2 and 3

as regard serum levels of s-Fas, NO, lipid peroxides and thymidine phosphorylase. Group 1 individuals showed significantly lower levels of serum s-Fas, NO, lipid peroxides and thymidine phosphorylase compared to that of groups 2 and 3. Also, group 2 individuals showed significantly lower levels of serum s-Fas, NO, lipid peroxides and thymidine phosphorylase compared to that of group 3.

Table (3): represents the comparison between groups 1,2,3,4 and group 5 as regard serum levels of s-Fas, No, lipid peroxides and thymidine phosphorylase. Group 1 individuals showed significantly lower levels of serum s-Fas, NO, lipid peroxides and thymidine phosphorylase compared to that of groups 4 and 5. Also, group 2

individuals showed significantly lower levels of serum s-Fas, NO, lipid peroxides and thymidine phosphorylase compared to that of groups 4 and 5. Group 3 individuals showed significantly lower levels of serum s-Fas, NO, lipid peroxides and thymidine phosphorylase compared to that of group 4 and 5. Group 4 individuals showed significantly lower levels of serum s-Fas, NO, lipid peroxides and thymidine phosphorylase compared to that of group 5

Table (4) showed that the serum level of s-Fas, NO, lipid peroxides and thymidine phosphorylase showed highly significant positive correlation when compared in all age groups with each other.

Figures 1, 2, 3 show the mean levels of NO, LP, TP and s-Fas.

Table 1: Clinical and biochemical criteria of studied individuals

Characteristics	Values (mean \pm SD)
Age (years)	45.06 \pm 15.06
Sex	
Female No.	27 (54.00)
Male %	23 (46.00)
Systolic blood pressure (mmHg)	118.5 \pm 5.99
Diastolic blood pressure (mmHg)	75.7 \pm 5.15
Fasting blood sugar (mg/dl)	92.76 \pm 8.17
s-Fas (ng/ml)	1.73 \pm 0.61
Nitric oxide (μ mol/L)	21.15 \pm 4.98
Lipid peroxides (μ mol/L)	6.52 \pm 3.65
Thymidine phosphorylase (nmol/L)	47.91 \pm 18.92

Table 2: Comparison between all the parameters as (mean±SD) between group 1 and group2

Characteristics	Group1	Group2	Group3	P value
s-Fas (ng/ml)	0.83 ± 0.28	1.48 ± 0.19	1.80 ± 0.22	P ₁ = 0.0002 P ₂ = 0.0002 P ₃ = 0.006
Nitric oxide (µmol/L)	15.81 ± 0.49	17.54 ± 0.77)	19.28 ± 0.66	P ₁ =0.0002 P ₂ =0.0002 P ₃ =0.0005
Lipid peroxides (µmol/L)	3.03 ± 0.74	3.89 ± 0.56	5.39 ± 0.57	P ₁ =0.02 P ₂ =0.0002 P ₃ =0.0002
Thymidine phosphorylase (nmol/L)	21.41 ± 4.73	36.25 ± 5.89	50.21 ± 6.44	P ₁ =0.0002 P ₂ =0.0002 P ₃ =0.0007

P₁: Compare the means between group 1 and 2. P₂: Compare the means between group 1 and 3.
P₃: Compare the means between group 2 and 3.

Table 3: Comparison between group 1, 2, 3 and group 4, 5

Characteristics	Group1	Group2	Group3	Group4	Group5	P value
S-Fas(ng/ml)	0.83 (0.28)	1.48 (0.19)	1.80 (0.22)	2.05 (0.05)	2.51 (0.30)	P ₁ =0.0002 P ₂ =0.0002 P ₃ =0.002 P ₄ =0.0002 P ₅ =0.0002 P ₆ =0.0002 P ₇ =0.0003
Nitric oxide (µmol/L)	15.81 (0.49)	17.54 (0.77)	19.28 (0.66)	24.11 (1.55)	28.99 (1.79)	P ₁ =0.0002 P ₂ =0.0002 P ₃ =0.0002 P ₄ =0.0002 P ₅ =0.0002 P ₆ =0.0002 P ₇ =0.0002
Lipid peroxide (µmol/L)	3.03 (0.74)	3.89 (0.56)	5.39 (0.57)	8.03 (1.44)	12.24 (2.76)	P ₁ =0.0002 P ₂ =0.0002 P ₃ =0.0006 P ₄ =0.0002 P ₅ =0.0002 P ₆ =0.0002 P ₇ =0.0009
Thymidine phosphorylase (nmol/L)	21.41 (4.73)	36.25 (5.89)	50.21 (6.44)	59.51 (6.39)	72.15 (8.32)	P ₁ =0.0002 P ₂ =0.0002 P ₃ =0.007 P ₄ =0.0002 P ₅ =0.0002 P ₆ =0.0005 P ₇ =0.002

P₁: Compares between the means of groups 1 and 4. P₂: Compares between the means of groups 2 and 4.
P₃: Compares between the means of groups 3 and 4. P₄: Compares between the means of groups 1 and 5.
P₅: Compares between the means of groups 2 and 5. P₆: Compares between the means of groups 3 and 5.
P₇: Compares between the means of groups 4 and 5.

Table 4: Correlation between age and different parameters

Relation of the factor listed related to age	Correlation Coefficient	P value	Regression co-efficient	P value
S-Fas, (ng/ml)	0.98	<0.0001	0.04	<0.0001
Nitric oxide, (µmol/L)	0.99	<0.0001	0.31	<0.0001
Lipid peroxide, (µmol/L)	0.97	<0.0001	0.22	<0.0001
Thymidine phosphorylase, nmol/L	0.98	<0.0001	1.19	<0.0001

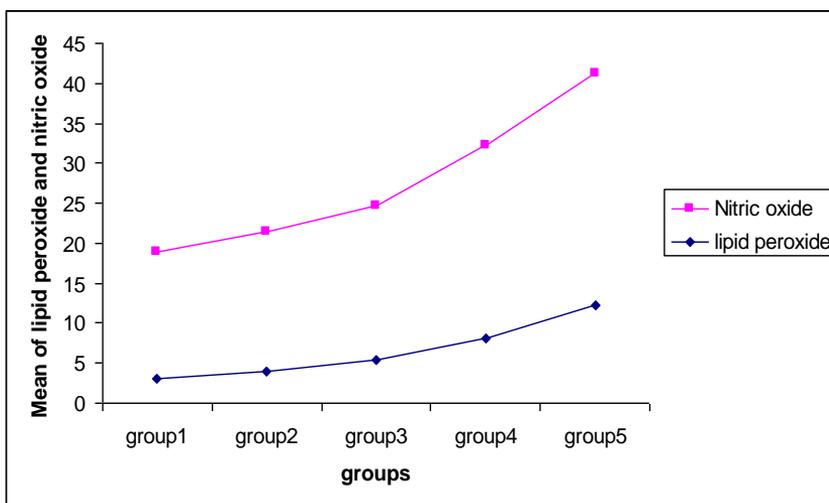


Figure1: The mean levels of lipid peroxide and NO (µmol/L) in all groups

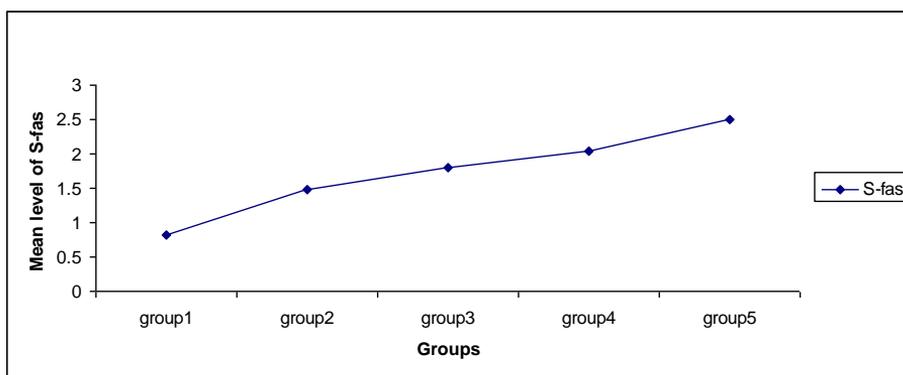


Figure2: The mean levels of S-fas (ng/ml) in all groups

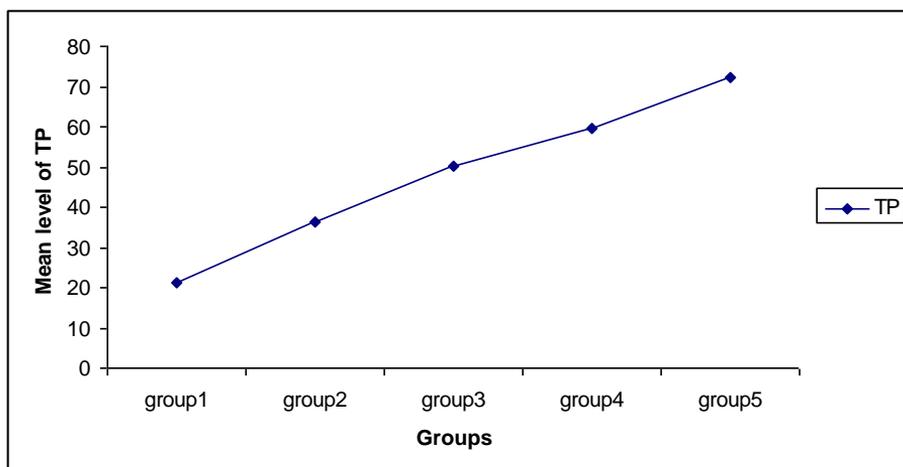


Figure3: The mean levels of TP (nmol/L) in all groups

DISCUSSION

Aging is a physiologic state in which a progressive decline of organ functions is accompanied with the development of age-related diseases. The cause(s) of aging remain unknown, probably being related to a multifactorial process. The free radical and mitochondrial theories seem to be the two most prominent theories on aging. Such theories claim that oxidative stress within mitochondria can lead to a vicious cycle in which damaged mitochondria produce increased amounts of reactive oxygen species, leading in turn to progressive augmentation in damage⁽²²⁾. Also, mitochondrial theory⁽²³⁾, cellular senescence theory⁽²⁴⁾ and molecular inflammatory theory have been postulated⁽²⁵⁾ Despite much investigation in recent years, no single theory has been completely successful in explaining the aging process but in general there are two

widely recognized and equally important aspects of the aging process: 1) aging is characterized as a progressive decline in biological functions with time, and 2) aging results in a decreased resistance to multiple forms of stress, as well as an increased susceptibility to numerous diseases⁽²⁶⁾.

Apoptosis is a vital component in the evolutionarily conserved host defense system. Apoptosis is the guardian of tissue integrity by removing unfit and injured cells without evoking inflammation. However, apoptosis seems to be a double-edged sword since during low-level chronic stress, such as in aging, increased resistance to apoptosis can lead to the survival of functionally deficient, post-mitotic cells with damaged housekeeping functions⁽³⁾.

In the present study, serum levels of s-Fas, as an evidence of apoptosis were significantly higher in old persons than in younger ones. This may suggest that increased apoptosis

may play a role in pathogenesis of aging, and the serum s-Fas level showed significant positive correlation with age, this is in agreement with those of **Ryo et al.**⁽²⁷⁾ who showed significant positive correlation between serum s-Fas and aging.

Angiogenesis is the formation of new capillaries from existing blood vessels. It is a multi step process that involves degradation of the surrounding extracellular matrix (ECM) by proteases, endothelial cell migration, proliferation, and differentiation into mature blood vessels. Cytokines, growth factors, growth factor receptors, enzymes (like TP), components of the ECM, and adhesion molecules each have their own specific role in this well-coordinated process⁽²⁸⁾. The equilibrium between angiogenic and angiostatic proteins, the so-called angiogenic balance, in the microenvironment controls the rate of new blood vessel formation⁽²⁹⁾. Alteration of this angiogenic balance, for example by the uncontrolled release of angiogenic regulators, can lead to several pathological conditions including inflammation, tumor growth, and metastasis⁽³⁰⁾.

It was found that both the transcriptional activity, and the protein expression, of TP were enhanced by DNA damage-inducing agents⁽³¹⁾, also the enzymatic activity of TP was required for the generation of ROS⁽¹²⁾. It was also demonstrated that TP transfected cell lines are more resistant to various apoptosis inducing stimuli such as Fas and cisplatin⁽³²⁾.

In the present study, serum levels of thymidine phosphorylase, as an

evidence of angiogenesis were significantly higher in old persons than in younger ones. Also, there is significant positive correlation when correlated with age.

Oxidative stress in a physiological setting can be defined as an excessive bioavailability of ROS, which is the net result of an imbalance between production and destruction of ROS (with the latter being influenced by antioxidant defenses)⁽²³⁾. It has long been recognized that high levels of ROS can inflict direct damage to macromolecules, such as lipids, nucleic acids, and proteins⁽³³⁾.

It has been clearly established that ROS and ROS-modulated participate in both intrinsic and extrinsic apoptotic pathways⁽³⁴⁾. One possible explanation could be that the enzymes are modified in old tissues and that their efficiency is lowered and they are modified with age⁽³⁵⁾. Another explanation may be: If in vivo oxygen radical generation is depressed in old animals, the decreases of the antioxidant defenses should be interpreted as a physiological compensatory down regulation instead of a deleterious change leading to additional oxidative damage. Finally, most of the reports concentrate on a single or a few antioxidants. This can complicate the interpretation of the results since it is known that cellular antioxidants are under homeostatic control⁽³⁶⁾.

In the present study, serum levels of NO, as an evidence of oxidative stress, were significantly higher in older persons than in younger ones. This finding is consistent with that of **Gordon**⁽³⁷⁾ in his study of an older group of 25 individuals (61 to 79

years, median 72 years) and a younger group of 23 individuals (21 to 30 years, median 24 years), who reported significant increase of exhaled NO. in older group .

In the present study, serum levels of lipid peroxides, as an evidence of oxidative stress, were significantly higher in older persons than in younger ones, and consistent with those of **Kasapoglu and Ozben**⁽³⁶⁾ in their study on a sample of 100 healthy men and women ranging in age from 20 to 70 years. They reported significant increase of serum lipid peroxides in older persons. This may suggest that oxidative stress resulting in generation of free radicals may play a role in pathogenesis of aging. Also, our results agree with those of **Barja de Quiroga et al.**⁽³⁸⁾ who showed that the lipid peroxidation increased with aging when expressed as thiobarbituric acid reactive substances. In the present study, the parameters of oxidant levels including serum NO and lipid peroxides showed significant positive correlations when each correlated with age. In agreement with our results **Gordon**⁽³⁷⁾ and **Kasapoglu and Ozben**⁽³⁶⁾ showed significant positive correlation between NO and lipid peroxides when correlated with age. Several studies showed that the nitric oxide pathway deteriorates with age. **Reckelhoff et al.**⁽³⁹⁾ found that the execution of NO metabolites, nitrate/nitrite as marker for NO, decreased progressively with age; urinary nitrate/nitrite decreased by 50 and 80% in male Sprague–Dawley rats, aged 12 and 17 months respectively.

It could be concluded that aging is a complex process involving a

multitude of factors, the oxidative stress and mitochondrial dysfunction and two important factors contributing to the aging process. Chronic inflammation is associated with the aging process, inflammation aggravates the microenvironment in aging tissues by secreting 1) proteinases that degenerate the extracellular matrix and 2) cytokines and growth factors that can even enhance apoptotic resistance. A better understanding of response to oxidative stress and mitochondrial dynamics will lead to new therapeutic approaches for the prevention or amelioration of age associated degenerative disease.

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وطىء الاكسدة ، والتكونات الوعائية الجديدة وموت الخلايا المبرمج وعلاقتهم بالشيخوخة

نجوى سيد أحمد^١ ، مديحه محروس زخارى^٢ رضا صلاح يوسف^١ مروة شعبان هلشم^١
قسم الكيمياء الحيوية الطبية ، كلية الطب جامعتى أسيوط و^١سوهاج،

الشيخوخة هى تغيرات تتراكم مع تقدم العمر مما يؤدى الى اختلال التوازن وانخفاض تدريجى فى القدرات الفسيولوجيه وانخفاض قدره على الاستجابة للمحفزات البيئيه وزياده الحساسيه والتعرض للأمراض وفى نهاية المطاف زيادة الوفيات . هناك نظريات عدة لشرح كيفية حدوث الشيخوخة

التغيرات فى الميتوكوندريا وتراكم البروتينات الشاذة فى العصارة الخلويه والاضرار الكيمياء للجزيئات والطفرات الجسميه والنسخ المحسنه او تقلص من جينات معينة ، وقد تم تقسيم نظريات الشيخوخة الى نظريتين: العشوائيه والتطور الجينى. النظرية الاولى يحدث طفرة جسيديه واصلاح للحمض النووى والخطا الكارثى تعديل البروتين والجذور الحرة (الاكسدة) الميتوكوندريا الحمض النووى). اما فى النظرية الثانية وهى التطور الجينى يتم تعريف الشيخوخة انها تكون جزء من عملية التواصل المبرمج وراثيا . والتى تسيطر على النمو .وعند اختلال توازن الاكسده فان الشيخوخة تحدث نتيجة تراكم الضرر للجزيئات.

هذه الدراسة التى أجريت على ٥٠ شخصا أصحاء مقسمة إلى خمس مجموعات تبعاً الفئة العمرية ما بين ٢٠ - ٢٩ ؛ ٣٠ - ٣٩ و ٤٠ - ٤٩ و ٥٠ - ٥٩ و ٦٠ - ٦٩ سنة من أولئك الذين ثبت أنهم اصحاء وخالين من أي علامات الأمراض المزمنة ، من خلال اخذ عينة الدم لتقدير مستوى اكسيد النيتريك ، بروكسيداز الدهون ، وفاس الذائب وثيميدين فوسفوريلاز .

أظهرت هذه الدراسة تركيزا اعلى وذات دلالة إحصائية لذي كبار السن عنها فى الصغار لكل من اكسيد النيتريك ، ، بروكسيداز الدهون ، والفاس الذائب وثيميدين فوسفوريلاز ، وان هناك علاقة ارتباط إيجابي ذات دلالة إحصائية بين العمر واي من هؤلاء .

وبذلك يمكن الاستنتاج ان الاكسدة قد تلعب دورا هاما فى التسبب فى الشيخوخة ، وان زيادة عامل الثيميدين فوسفوريلاز قد تكون السبب الرئيسى [او على الاقل احد المسببات] للشيخوخة.