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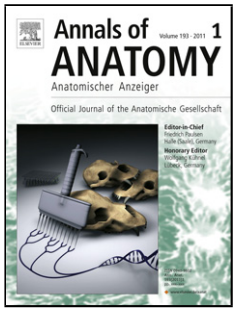

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Controlled ozone therapy modulates the neurodegenerative changes in the frontal cortex of the aged albino rat

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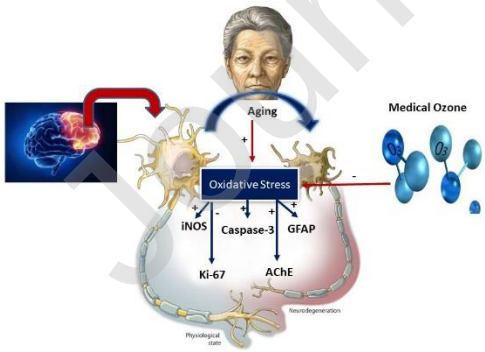
Graphical abstract



Abstract:

Aging is a normal process associated with neurodegenerative changes resulting in decline of cognitive and motor functions. Oxidative stress plays an important role. Controlled ozone (O_3) therapy has been proved to induce oxidative preconditioning thus reversing oxidative stress. To the best of our knowledge, this research is the first attempt to investigate whether the antioxidant properties of O_3 can ameliorate age-associated structural alterations of the cerebral cortex. Ozone administration (at a dose of 0.7mg/kg intraperitonially, three times a week for eight weeks) produced significant downregulation of tissue malondialdehyde (MDA) and upregulation of glutathione, superoxide dismutase (SOD) and catalase (CAT) within the frontal cortex of aged rats. Sections of the frontal cortex from adult and aged rats were stained with hematoxylin and eosin and analyzed using light microscopy. In addition, quantitative immunohistochemical assessments of the expression of inducible nitric oxide synthase (iNOS), caspase-3, glial fibrillary acidic protein (GFAP), Ki67 and acetylcholinesterase (AChE) were performed. Our results revealed the beneficial effect of O_3 in improving the neurodegenerative changes of the cerebral cortex of aged rats. Moreover, this study clarified that O_3 exerted its effects via reducing oxidative stress, apoptosis, gliosis as well as improving neurogenesis and cholinergic plasticity. This work added to the previously proved aging – associated neurodegenerative effects and provided a new insight into the promising role of O_3 to ameliorate these effects.

Key words: aged rat; frontal cortex; ozone; oxidative stress; histopathology; immunohistochemistry

1. Introduction

The modern development of medicine and medical research resulted in a considerable increase of the average human age. Unfortunately, this was not associated with a corresponding improvement in the quality of the aging process (Shehata et al., 2012). Aging is a normal process of gradual decline of the physiological functions involving various organs and tissues of the human body. Brain aging is a complex process manifested by decline in mental and cognitive functions (Apostolova et al., 2012; Taridi et al., 2014).

The mechanism of aging has been considered a matter of interest. Many theories have been implicated in this respect. One of the most accepted theories is the “oxidative stress theory”. This theory states that reactive oxygen species (ROS) are responsible for the functional changes that accompany aging. Moreover, the later changes constitute major risk factors for neurodegenerative diseases such as Alzheimer's disease. Among the most sensitive brain regions to oxidative stress is the cerebral cortex. The cerebral cortex, the major area of higher cognitive functions, is thus adversely affected by aging (Yoo et al., 2012).

One of the major challenges of modern gerontology is to reach healthy aging with minor or no cognitive dysfunction through the amelioration of age-related neurodegeneration. Traditionally, oxidative damage was interfered with through supplementation of antioxidant substances such as vitamins A, C and E, flavonoids and polyphenols. However, this is limited by the potential toxicity of excessive doses as well as the uncertainty of intestinal absorption with aging (Halliwell, 2001; Bocci, 2005).

Consequently, it becomes obvious that in chronic oxidative stress conditions, increasing the endogenous antioxidant capacity is much more effective than flooding the body with exogenous antioxidants. Medical O_3 is an ozone/oxygen mixture administered at low concentrations. Ozone (O_3) therapy has been employed for many decades in a variety of pathologies as orthopedic pathologies and vascular disorders (Bocci, 2005).

The effect of O_3 therapy in neutralizing oxidative stress has been observed in many pathologies in different animal models like cisplatin induced nephrotoxicity (Borrego et al., 2004), hepatic and renal ischemia reperfusion injury (Ajamieh et al., 2004; Chen et al., 2008), diabetic nephropathy (Morsy et al., 2010) as well as coronary artery disease (Martínez-Sánchez et al., 2012). Recently, the effect of O_3 therapy on reversing certain biochemical alterations in aged rat cerebral cortex has been studied (Shehata et al., 2012). However, its effect on the accompanying histological changes hasn't been studied.

Consequently, the present study was designed to investigate, for the first time to the best of our knowledge, the possible effect of O_3 therapy on aging related oxidative stress reflected in the histopathological and immunohistochemical alterations in the rat cerebral cortex.

2. Materials and methods

2.1. Animals

Twenty adult (three months old) and twenty aged (20 months old) male albino rats obtained from the National Center of Researches (Cairo, Egypt) were used in this study. They had an average weight of 200-250 g and were maintained in the animal house of the Faculty of

Medicine, Menoufia University. They were allowed free access to chow and water. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures were conducted in accordance with the guidelines approved by the Committee of Animal Research Ethics, Menoufia University, Faculty of Medicine.

2.2. Experimental design:

According to age, rats were divided into two main groups: adult and aged. Each of these groups were subdivided into two equal subgroups (ten rats each): control and ozone (O_3) treated. Rats of O_3 treated groups were administered O_3 intraperitonially at a dose of 0.7mg/kg three times a week for eight weeks. Ozone was generated by an ozone generator (Ozone Longevity Resources Dwyermade, Canada) at ozone unit, Faculty of Medicine, Menoufia University. Ozone was produced from medical grade oxygen, used immediately upon generation and represented only about 3% of the O_3/O_2 gas mixture. The O_3 concentration was measured using a built-in UV spectrophotometer set at 254 nm.

At the end of the experimental period (8 weeks), rats were anesthetized by inhalation of pentobarbital overdose (200mg/kg) followed by rapid cervical dislocation and decapitation. Craniotomy was performed to dissect out the intact brains. The left hemisphere was selected for studying the cerebral cortex.

2.3. Evaluation methods:

2.3.1. Tissue biochemical study:

Cerebral cortical tissue was immediately frozen at −70°C. The tissue sample was then homogenized in 5-10 ml cold buffer (i.e. 50 mM potassium phosphate, pH 7.5. 1 mM EDTA). Homogenates were centrifuged at 10000 × g for 15 minutes at 4°C. The supernatant was used for the measurement of tissue superoxide dismutase (SOD), and catalase (CAT) as antioxidant markers according to the methods of (Kono, 1978) and (Aebi, 1984) respectively. Meanwhile, the concentration of Malondialdehyde (MDA) was measured as an index of lipid peroxidation and oxidative stress (Uchiyama and Mihara, 1978). Moreover, glutathione (GSH) level of cerebral cortex was determined by the methods of (Ellman, 1959).

2.3.2. Histological and immunohistochemical studies:

Specimens were fixed in 10% neutral formalin and processed for light microscopic study. Paraffin sections (5-µm thick) were prepared and subjected to hematoxylin and eosin (H&E) staining.

For immunohistochemical staining, paraffin sections were deparaffinized in xylene for 1–2 minutes and then rehydrated in descending grades of ethanol (100%, 95%, and 70%ethanol) two changes five minutes each, then brought to distilled water for another five minutes. Sections were rinsed with PBS, blocked for 30 minutes in 0.1% H_2O_2 as inhibitor for endogenous peroxidase activity. After rinsing in PBS, sections were incubated for 60 minutes in blocking solution (10% normal goat serum) at room temperature (22°C). The sections were then incubated with the primary antibody (inducible nitric oxide synthase (iNOS), 1:500, Labvision; Caspase-3, 1:500, Labvision; glial fibrillary acidic protein (GFAP), 1:300, Labvision; Ki67, 1:500, Labvision, and acetylcholinesterase (AChE), 1:300, Genetex) at room temperature for an hour.

Sections were rinsed with PBS, followed by 20 minutes of incubation at room temperature with secondary biotinylated antibody. After rinsing the sections in PBS, enzyme conjugate "Streptavidin-Horseradish peroxidase" solution was applied to the sections for 10 minutes. Secondary antibody binding was visualized using 3,3-diaminobenzoic acid (DAB) dissolved in PBS with the addition of $H_2O_2$to a concentration of 0.03% immediately before use. Finally, sections were PBS rinsed and counterstaining of slides was done using two drops of hematoxylin. Slides were washed in distilled water until the sections turned blue. Finally, slides were dehydrated in ascending grades of ethanol (70%, 95%, and100%) for 5 min each and were cleared in xylene and finally cover-slipped using histomount mounting solution (Kabiraj et al., 2015).

For quantitative assessment, five non-overlapping fields (20 x) per section were randomly taken using a Leica DML B2/11888111 microscope equipped with a Leica DFC450 camera. The examined parameters were calculated using ImageJ software version K1.45

For histological quantitative assessment, the percentage of degenerated cells was calculated (number of degenerated cells/number of total cells x 100).

For immunohistochemical quantitative assessment, the following parameter were calculated:

-percent of Caspase-3 immunopositive cells

-percent of iNOS immunopositive cells

-percent of Ki67 immunopositive cells

-percent of AchE immunopositive cells

-number of GFAP immunopositive cells

2.3.3. Statistical analysis:

The collected data were presented as mean ± SD. Data analysis was performed using SPSS (Inc., Chicago, IL, USA) version 23 on IBM compatible computer. The obtained data were analyzed using one way-ANOVA followed by post hoc Bonferroni test. The results were considered statistically significant and nonsignificant when the p-values were <0.05 and >0.05 respectively (Dawson and Trapp, 2004).

3. Results

There was no significant difference between the adult control and adult control+O3 rats in all the outcomes; therefore, these two groups were pooled in one control group.

3.1. Biochemical results:

The levels of malondialdehyde (MDA), glutathione, superoxide dismutase (SOD) and catalase (CAT) within the frontal cortex homogenate were assessed biochemically in the different experimental groups and the values were statistically analyzed. In the aged group, there was a significant increase in the MDA level (2.89±0.22 vs 1.02±0.12) compared with the adult control group ($P<0.001$), however, O_3 treated group showed a significant decrease in its level (1.66±0.25 vs 2.89±0.22) compared with the aged one ($P<0.001$). On the other hand, there was a significant decrease in the level of glutathione, SOD and CAT in the aged frontal cortex

(0.78±0.15 vs 1.54±0.29 for glutathione, 3.94±0.21 vs 6.16±0.50 for SOD and 0.10±0.03 vs 0.26±0.03 for CAT) compared with those of the adult control group (P<0.001).This was ameliorated in O_3 treated group via a significant increase in these levels (1.14±0.10 vs0.78±0.15 for glutathione, 5.21±0.36 vs 3.94±0.21 for SOD and 0.20±0.02 vs 0.10±0.03 for CAT) compared with the aged group (P>0.001 for glutathione- P<0.001 for SOD and CAT) (Fig. 1).

3.2. Histological results:

Hematoxylin and eosin (H&E) stained sections of the different experimental groups were examined under light microscope. In the adult control group, the pyramidal cells, granular cells, and perineural neuroglia represented the main cellular components of the frontal cortex. These cells were scattered in an eosinophilic background (neuropil). The pyramidal cell showed an open face nucleus, a basophilic cytoplasm and an apical process. The granular cells were rounded in shape and showed large rounded vesicular nuclei with prominent nucleoli. Glial cells appeared smaller in size with small deeply stained nuclei. Sections of the aged group revealed alterations of the cortical structure in the form of neuronal degeneration with hyperchromatic pyknotic nuclei and vacuolated cytoplasm embedded in an extensively vacuolated neuropil. In O_3 treated group, there was restoration of the normal histological structure of the frontal cortex except for the presence of a few degenerated cells with pyknotic nuclei and vacuolated cytoplasm. Statistically, there was a significance increase in the percentage of degenerated cells in the frontal cortex of the aged rats (59.60±3.33 vs 3.71±0.74) compared with the adult control (P<0.001). on the other hand, the percentage of degenerated cells was decreased in the aged

rats treated with O_3 (13.56±0.97 vs 59.60±3.33) compared with the aged untreated rats (P<0.001) (Fig 2)

3.3. Immunohistochemical results:

Inducible nitric oxide synthase (iNOS), a marker for oxidative stress, was significantly up-regulated in the frontal cortex of the aged group (80.80±2.60 vs 12.79± 2.02) compared with the adult control group (P<0.001). On contrast, a significant downregulation was observed in O_3 treated group (27.06±2.33 vs 80.80±2.60) compared with the aged one (P<0.001) (Fig 3 A-C). This was associated with significant increase in caspase-3 immunoreaction, a marker for apoptosis, in the aged group (87.82±3.29 vs 15.06±1.75) compared with the adult control group (P<0.001) that was dramatically decreased in O_3 treated rats (38.95±4.71 vs 87.82±3.29) compared to the aged group (P<0.001) (Fig 3 D-F).

Moreover, in the aged group, Glial Fibrillary Acidic Protein (GFAP) positive immunoreaction, a marker for astrocyte, was significantly upregulated (122.90±7.14 vs 22.60±2.22) compared with the adult control group (P<0.001). On the other hand, its significant downregulation in O_3 treated rats (30±10 vs 122.90±7.14) compared to the aged group (P<0.001) was observed (Fig 3 G-I).

A significant down-regulation in Ki-67 immunoreactive cells, a marker for neurogenesis, in the aged group (29.37±2.27 vs 90.07±2.14) compared with the adult control (P<0.001) was observed, however its expression was dramatically upregulated in O_3 treated rats (76.06±4.39 vs 29.37±2.27) compared to the aged group (P<0.001) (Fig 2 J-L). In addition, acetylcholine

esterase (AChE), a marker for of cholinergic plasticity affection, was significantly expressed in the aged rats (88.72±3.54 vs 10.99±1.49) compared with the control group (P<0.001) and this was dramatically downregulated in the O_3 treated group (40.06±2.59 vs 88.72±3.54) compared to the aged group (P<0.001) (Fig 3 M-O).

4. Discussion

Aging is a complex physiological event affecting several essential processes and is characterized by behavioral, physiological and neurochemical alterations (Al-qudah, 2012) **.** One of the most significant socioeconomic problems affecting the quality of life of the aged people is the decline of both cognitive and motor skills (Hedden and Gabrieli, 2004)**.** Therefore, improving the aging process to achieve healthy aging and delay the onset and progression of multiple age-related diseases is one of the major challenges nowadays.

Several trials have demonstrated the validity of using medical ozone (O_3) as a complementary therapeutic agent for amelioration of some disorders as Judicious O_3 doses are able to counteract oxidative stress (Hernández et al., 1995)**.** However, very little is known about the application of O_3 therapy for age - associated changes in various tissues including nervous tissue. EI-Sawalhi et al. (2013) has shown that O_3 therapy can ameliorate age-related changes in oxidative stress markers and energy status in the rat hippocampus. Moreover, Elkholy and Al-Gholam, (2018) showed that O_3 therapy can attenuate age-related changes in the rat cerebellum by histological and immunohistochemical studies. A recent study proved the ameliorative effect of O_3 therapy on age-related cerebrocortical biochemical alterations (Shehata et al., 2012)**.** Though it is the first time to show that O_3 therapy can ameliorate age-

related oxidative stress reflected in the histopathological and immunohistochemical alterations in the rat cerebral cortex.

Our data has shown severe neurodegenerative changes in the frontal cortex of the aged rats in the form of structural disorganization and cellular degeneration in the form of presence of darkly stained hyperchromatic neuronal cells with vacuolated cytoplasm that were embedded in a disorganized vacuolated neuropil. This was in accordance with Singh et al. (2011) who found disorganization of various cell layers as well as shrinkage of neuronal cells in the cerebral cortex of aged rats. Conde and Streit, (2006) clarified that the brain atrophy occurring with age, which affects both grey and white matter, was presumed to result from a loss of neurons and myelinated axons. Moreover, Bordiuk et al., 2014 attributed the loss of cognitive, motor and analytical potency of the aged brain to the loss of its neurons.

The excessive production of free radicals, which causes an imbalance between the levels of free radicals and antioxidant defenses in an organism, is proposed to be responsible for age-associated structural and functional deterioration (Hamezah et al., 2017). This was confirmed in our results by the significant increase in the level of MDA and decrease in the level of SOD, CAT and glutathione in the cortical tissue of aged rats. This could explain the significant upregulation of iNOS, a marker of oxidative stress (Gürpinar et al., 2012), in the cortical specimens of aged rats in this work. Aging has been previously reported to induce a significant increase in iNOS expression in rat brain (Panarsky et al., 2012; Thangnipon et al., 2015). When a large amount of nitric oxide (NO) is produced from activated microglia, it reacts with

superoxide anion to produce peroxynitrite which is a toxic radical to tissue (Kankuri et al., 2003).

Furthermore, Chen et al. (2018) postulated that oxidative stress and apoptosis were involved in the aging process. This was confirmed in our research by the significant increase in caspase-3 expression in the brain sections of the aged rats. Oxidative stress causes depolarization of the inner mitochondrial membrane with subsequent release of cytochrome c into the cytosol leading to induction of caspase mediated apoptosis (Gürpinar et al., 2012). Similarly, Tatarkova et al. (2016) detected remarkable increase of TUNEL +ve cells in the cortex of aged rats and suggested significant neurodegenerative effect of aging in the neuronal cells. Fukudome et al. (2008); Nishikawa et al. (2000); Paradies et al. (2011) explained that NO, which showed significant upregulation in this study, induced oxidative damage to cellular proteins, lipids, or DNA and subsequently disturbed homeostatics within the neuron, ultimately leading to cell death via apoptosis.

Our results showed a significant increase in GFAP positive cells within the studied brain sections of the aged rats. This agreed with Nichols et al. (1993) who noted an increase in GFAP mRNA in both humans and rats with age and with Chepkova et al. (2015) who demonstrated GFAP overexpression in the aged mice brain. This may be attributed to the occurrence of reactive gliosis induced by an increase in ROS as supported by Kaneko et al. (2002); Pekny and Nilsson, (2005) who stated that glial cells respond to the oxidative insult by producing GFAP and S100B. Brahmachari, (2006) suggested that astroglial activation and gliosis during neurodegeneration was represented by overexpression of GFAP. Moreover,

Koellhoffer et al. (2017) postulated that one of the hallmarks of the aging brain was microglial activation and this was coincident with age-related neurodegeneration and cognitive decline. In addition, Phatnani and Maniatis, (2015) suggested that astrocytes were implicated in the onset and progression of different neurodegenerative diseases and they referred these changes either to loss of astrocytes' normal function or toxic gain of function or both.

Brahmachari, (2006) demonstrated that NO played an important role in the induction of GFAP expression in the astrocytes through the guanylate cyclase (GC)–cGMP–cGMP-activated protein kinase (PKG) signaling pathway. This goes in line with our results which showed significant upregulation of both iNOS and GFAP within the cortical specimens of the aged rats.

Galvan and Jin, (2007) revealed that neurogenesis continues throughout the adult life and that adult neurogenesis is necessary for the stability of the brain functions. They believed that this process declines with advanced age. Kee et al. (2002) clarified that immunohistochemical detection of Ki-67, a nuclear protein expressed in different phases of the cell cycle, might be used as a safe non-expensive alternative procedure to 5'-bromo-2-deoxyuridine (Brdu) for detection of adult neurogenesis.

In the last decade, cortical adult neurogenesis and its neural stem cells (NSCs), which are self-renewing and can generate neurons, astrocytes and oligodendrocytes, and neural progenitor cells (NPCs), which tend to differentiate into certain types of neurons, have been found in the cerebral cortex of adult mammals (Ohari, 2011). In this study, a significant decrease in the ki-67 immuno-expression was observed in the brain sections of the aged

rats. This was in agreement with Kempermann et al. (2002) who noted a decline in neurogenesis in the aged rodents. Bernal and Peterson, (2011) attributed the decrease in neurogenesis with age to the changes occurred in the astrocytes that led to a decrease in vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) signaling that in turn affected the maintenance of the progenitor cells and hence led to decreased neurogenesis and a decline in brain functions.

One of the most important neurotransmitters involved in regulation of cognitive functions is acetylcholine (Nath et al., 2002) which is involved in the neuronal and cholinergic plasticity (Rasmusson, 2000). Neural plasticity is a cardinal process for neuronal adaptation (Liu et al., 2017) and it has been subjected to a great deal of research in the last century (Cohen et al., 2017). Acetylcholinesterase (AChE) is an enzyme of brain cholinergic system that hydrolyses the neurotransmitter acetylcholine to choline and acetate in the synaptic cleft (Soreq, 2001).

In this research, there was a significant upregulation in AChE immunoreaction in the examined brain sections of the aged rats. This was in agreement with (Bronfman et al., 2000) who noted massive cholinergic deafferentation in the hippocampus of aged mice. Several studies have reported that the increase in AChE activity in the diabetic brain was associated with cognitive deficits (Schmatz et al., 2009; Sherin et al., 2012).

Moreover, Yufu et al. (1994) demonstrated an extensive depletion of AChE as one of various biochemical cholinergic markers in the aged rat brain. Chacón et al. (2003) contributed the AChE in the pathogenesis of neurodegeneration as they noted a toxic effect on the hippocampal neurons with neuronal loss with the injection of AChE in the dorsal

hippocampus. In addition, Talesa, (2001) postulated that AChE had a role in the neurodegeneration and cognitive deficits observed in Alzheimer's disease. Melo et al. (2003) referred the increase of AChE activity that affected the acetylcholine synaptic levels to the presence of oxidative stress.

Our results revealed that medical O_3 was able to exert a neuroprotective effect against aging induced neurodegeneration. This beneficial effect was mediated by O_3 ability to down-regulate aging-induced oxidative distress, apoptosis and gliosis in the aged frontal cortex, in addition to its ability to improve suppressed neurogenesis and cholinergic plasticity. Administration of O_3 showed regeneration of the neurons in a marvelous manner with preservation of neuropil and decrease of astrocyte activation. Similarly, previous studies showed that antioxidants could protect neurons against experimental neurodegenerative conditions (Baluchnejadmojarad and Roghani, 2011; Bhutada et al., 2011; Suge et al., 2012; Liu et al., 2013).

In the present work, medical O_3 treatment significantly downregulated iNOS and caspase-3 expression in cortical sections of aged rats. This proves that O_3 treatment can ameliorate aging-induced oxidative stress and apoptosis. This was in accordance with Liu, (2016) who noted reduction of the apoptotic cells of the diabetic retina treated with O_3. Moreover, Safwat et al. (2014) clarified that O_3 had a beneficial effect on the aging induced liver and kidney damage through its antioxidant property. Güçlü et al. (2016) found that the anti-apoptotic role of O_3 occurred via decreased expression of caspase 1,3 and 9 as confirmed in our study by demonstration of caspase-3.

As proved in this study, Morsy et al. (2010) referred the antioxidant effect of O_3 to the reduction of MDA and increase in CAT and SOD in the diabetic nephropathy. In addition, Morelli et al. (2018) stated that O_3 activated the mitochondrial antioxidant system and facilitated the elimination of cell catabolites.

Our data showed that O_3 treatment ameliorated aging-induced gliosis as proved by down-regulation of GFAP expression in brain specimens. This goes in line with previous studies which showed that the beneficial effects of antioxidants against reactive gliosis were attributed to their free radical scavenging properties (Baydas et al., 2003; Duarte et al., 2012). Our findings suggested that O_3 treatment antagonizes age – induced suppression of neurogenesis as shown by upregulation of Ki-67 expression in brain specimens of aged rats following O_3 treatment. Moreover, treatment with O_3 was able to significantly decrease AChE activity in brain sections of aged rats. Many antioxidant substances have been found to restore the cognitive deficits by reducing AChE activity (Peeyush Kumar et al., 2011; Weinstock et al., 2013). Accordingly, we can suggest that O_3 might be useful for improving the age – related cognitive and memory deterioration by increasing cholinergic communication.

Ozone acts by inducing cellular adaptation to oxidative damage through the production of certain physiological messengers that activate numerous biological pathways (Bocci et al., 2009). Moreover, these messengers can stimulate the bone marrow to produce super-gifted erythrocytes which are able not only to upregulate antioxidant defenses, but also activate glycolysis and pentose phosphate pathways and have great abilities to deliver O_2 to tissues (Bocci, 1996). In addition, those messengers could stimulate the release of bone marrow stem

cells for regeneration of infarcted or degenerated organs (Bocci, 2006). Ohira, (2018) clarified that activation of endogenous NSCs and NPCs in the cerebral cortex might reduce the loss of the cortical functions with aging. Therefore, it is possible to manipulate the proliferation, suppression, and/or differentiation of endogenous NSCs and NPCs by administering substances, such as drugs and nutrients. The results of this work add to the known neurodegenerative effects of aging and provide a new insight into the possible use of medical O_3 therapy to ameliorate these effects.

Author's contribution

Both authors have contributed equally to this work.

They are allowed free access to chow and water. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures were conducted in accordance with the guidelines approved by the Committee of Animal Research Ethics, Menoufia University, Faculty of Medicine.

Conflict of interest

The author declares that he has no conflict of interest, commercial association, or intention of financial gain regarding this research

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Figure caption:

Fig (1): Mean level of MDA, glutathione, SOD and CAT within the frontal cortex homogenate in the different studied groups. ᵒᵒᵒ P<0.001, compared with the adult control group; *** P<0.001, ** P>0.001 compared with the aged group.

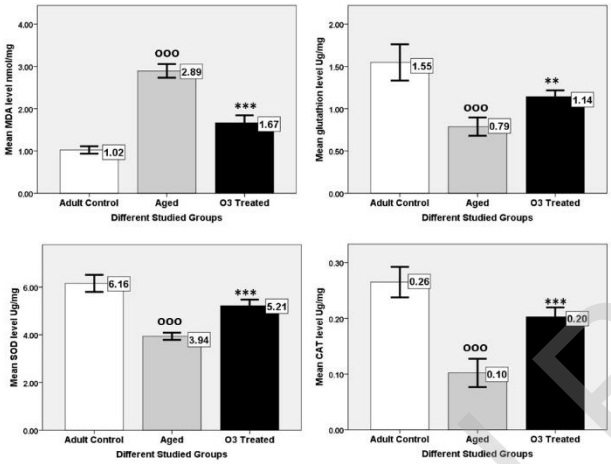

Fig (2): [A, B & C]: Representative micrographs of coronal sections in the frontal cortex of the different experimental groups. A: Adult control group showing different neuronal cells; pyramidal cells (P), granular cells (G) and neuroglia cells (Ng) embedded in a well-organized neuropil (asterisk). B: Aged group showing disorganization of the cortical architecture with presence of degenerated cells (Dc) with hyperchromatic pyknotic nuclei (arrow) and vacuolated cytoplasm (V) in a disrupted vacuolated neuropil (asterisk). C: Ozone treated group showing more or less well-organized cortical structure with normal neuronal cells (arrow) and well-organized neuropil (asterisk), however a few cells appear with pyknotic nuclei and vacuolated

cytoplasm (V). **(H&E, scale bar 50 µm 20 x).** D: A histogram revealing a significant increase in the % of degenerated cells within the frontal cortex in the aged group compared to the control group that is significantly decreased in the O3 treated group. ooo P<0.001, compared with the adult control group; *** P<0.001, compared with the aged group.

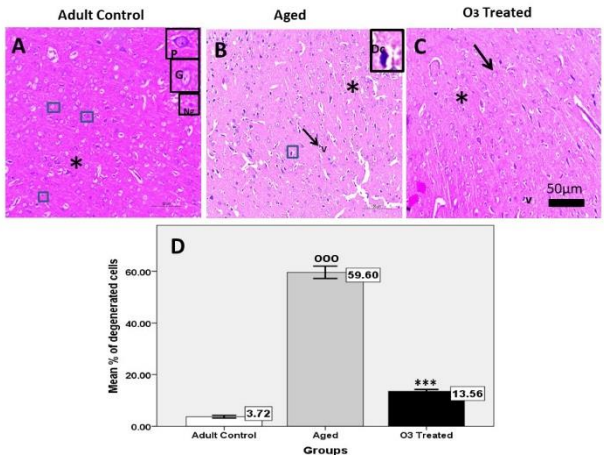

Fig (3): Representative immunostaining of rat frontal cortex of the different experimental groups revealing that O3 treatment significantly downregulates the iNOS (A-C), Caspase-3 (D-F), GFAP (G-I), AChE (M-O) immunoreactions and upregulates Ki-67 expression (J-L) comparing to the aged group. Inserts show a higher magnification (40x) of the boxed regions. ooo P<0.001, compared with the adult control group; *** P<0.001, compared with the aged group. **(Scale bar 50 µm 20 x).**