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Investigation with the Oxidative Stress Theory (The Free Radical Theory) of Aging Neurobiology in the Experimental Spinal Cord Trauma Model: A Biochemical Study

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ABSTRACT

OBJECTIVE: Aging, expressing postmaturational changes, is a process that contains a decline in the organism's simultaneous and harmonic response, adaptation potential, and resistance ability to external and negative events (stressors and stimuli). The free radical theory of aging puts forward that aging and its accompanying diseases are the results of reactions between reactive free radicals and biomolecules. There is a high correlation between the hydroxyl radical forming rate and aging. Since pineal melatonin secretion decreases with aging, the toxic effects of free radicals are expected to increase secondary to decreased melatonin secretion. The pineal melatonin, also called the anti-aging hormone, attenuates hydroxyl radical-induced neuronal damage. The objective of our study was to create an experimental model for the free radical theory of aging in spinal cord trauma and to confirm lipid peroxidation levels in young and aging spinal cord segments after spinal cord trauma.

MATERIALS AND METHODS: In this study following experimental spinal cord trauma, the extent of oxidative neuronal injury was measured among different age groups in order to show its relation with aging. For this trial young (n:14, 3 months old, 80-110 gr), old (n:14, 18-22 months old, 350-450 gr, naturally aged) and pinealectomised albino-rats (n:14, 3 months old, 4 months wait, 80-110 g, artificially aged) were used. Lipid peroxidation was measured in all spinal cord segments obtained in every group of experiments.

RESULTS: Lipid peroxidation levels of both naturally and artificially aged groups were significantly higher than the group of young rats. There was no statistically significant difference between naturally aged and artificially aged groups. Lipid peroxidation levels increased during aging in the spinal cord of the animals.

CONCLUSIONS: One of the most important theories on the cause of spinal cord aging is the free radical mechanism. Pineal gland functions decrease by aging. Decreased melatonin function, decreased antioxidant capacity, and accumulation of hydroxyl radicals are the chief determinants of aging. It is necessary to carry out many studies on spinal cord aging especially regarding the long-term effects of the pineal gland and melatonin as they may be important agents for the recovery of spinal cord injury

KEY WORDS: aging, free radical theory, melatonin, pineal gland, pinealectomy, spinal cord trauma.

INTRODUCTION

Aging is associated with a progressive decline of a variety of physiological functions (11). The free radical theory of aging is based on highly reactive free radicals and their interactions with biomolecules (1, 3, 4, 11, 13, 20, 23). Since pineal melatonin secretion decreases with aging, the toxic effects of free radicals are expected to increase secondary to decreased melatonin secretion (6, 7, 9, 10, 13, 15, 17, 19, 21, 22, 24, 28). The pineal melatonin, suggested to be a natural anti-aging hormone, attenuates free radical-induced neuronal damage (12, 14, 15, 16, 18).

The objective of our study was to create an experimental model of the free radical theory of aging in the spinal cord trauma and to confirm lipid peroxidation levels in young and aging spinal cord segments after spinal cord trauma.

MATERIALS AND METHODS

This study was conducted at Hacettepe University Spinal Research Laboratories after Hacettepe University Ethical Committee approval. Male albino rats weighing 80-110 g (3 months old, young), 350-400 g (18-22 months, old, naturally old), and 80-110 g (three months old, 4 months wait, pinealectomized, artificially old) were used for the study.

Spinal cord trauma:

All rats were weighed before surgical procedures. They were not fed overnight and all surgical procedures were performed under general anesthesia induced by a combination of Ketamin HCl (Ketalar, 5%, Parke Davis Eczacibasi, Istanbul; 50 mg/kg) and Xylazine (Rompun, 2%, Bayer, Istanbul 8 mg/kg) administered intramuscularly. The rats were pinned in the prone position and left to self-respiration. After shaving and disinfection, a T5-T12 midline skin incision was carried out and the paravertebral muscles were dissected. The T7-T10 spinous processes and laminar arcs were removed under a surgical microscope. The dura was left intact. Trauma was produced by the method of Allen described in 1911 (25, 26, 29). The animals were subjected to a 50g/cm impact to the dorsal surface of the spinal cord that made them severely wounded and paraplegic. The force was applied via a stainless steel rod (3 mm tip, weighing 5 g) that was rounded at the surface, which made contact with the cord after being dropped vertically through a calibrated tube. The injury apparatus was a 10 cm guide tube that was positioned perpendicular to the center of the spinal cord. Postoperative early and 24-hour behavioral and motor capacities of rats were examined by inclined plane scoring explained by Gale and the Tarlow grade respectively (2, 5). Gale inclined plane score 0 and Tarlow grade 1 standard cord trauma were evaluated. After suturing muscle and skin, every six rats were placed in 20x35x40 cm cages. Urinary prophylaxis was performed by using gentamycin (Garamycin, Eczacibasi, Istanbul; 0.2 mg/100 gr/day). The bladders were emptied with the Crede maneuver every 12 hours. Feeding was with standard laboratory food while the rats were placed 14 hours in the dark and 10 hours in the light. The rats were kept under constant laboratory conditions of 23-25 °C room temperature.

The lipid peroxidation level in young and old (naturally aged) groups was evaluated on cord segments resected 24 hours after the spinal cord trauma. The lipid peroxidation level in the pinealectomized (artificially aged) group was evaluated on traumatic cord segments resected 4 months after the pinealectomy. The purpose of this procedure was to evaluate and compare difference between naturally and artificially aged groups.

Pinealectomy

The rats were pinealectomized by the model described by Palaoglu (8). Following general anesthesia, the rats were shaved from vertex to suboccipital region. Polyvinylpyrrolidone iodine (Polyod, 10 % solution, Drugsan, Ankara) was applied for field disinfection. Percutaneous alpha-n-propylaminopropion-O-toluidehydrochloride 20mg NaCl 6mg (Cytanest, 2 % solution, Astra, Ssodertage-0.5 cc) was applied subcutaneously to the periosteum to decrease scalp bleeding and to make the periosteal dissection easier. A 2 cm long longitudinal incision was performed. The periosterum was dissected bilaterally from the area where temporal muscles adhered to the temporal bone. Sagittal and lambdoid sutures were clarified. Using a dental round drill, 1x1 craniectomy was performed by placing lambda centrally. The distal part of the sagittal sinus was located anteriorly and both transverse sinuses laterally by this process. The dura was incised by a parallel incision to the right transverse sinus. The sinus was incised from the center by bipolar cauterization and a dural flap was created. The superficial part of the pineal gland below the confluent sinium was excised by the aid of

left angled microalligator. Surgicel (sterilized oxidised cellulose BP, Ethicon Ltd) was used for venous hemorrhage. The skin was sutured by 4/0 prolene following hemostasis.

Study Protocol

To assess the alteration of lipid peroxidation after trauma and evaluate the response of different age groups, two main groups of control and trauma each of which contained young, naturally aged and artificially aged rats was designed.

Experiment Groups

Control groups

Control groups were composed of 3 months old, 80-110 g, young (n=7/group); 18-22 months old, 350-400 g (naturally aged, n=7/group) and pinealectomized (artificially aged, n=7/group) albino rats. These were accepted to be controls of similar age groups and the resected spinal segments were examined for the level of lipid peroxidation.

Trauma groups

Similar groups (n=7/group) underwent experimental spinal trauma and the lipid peroxidation levels were evaluated in spinal segments resected 24 hours after the trauma.

Sample preparation and determination of lipoperoxides

The exposed cord segments were removed in all groups of animals 24 hours after the trauma. The samples were thoroughly cleaned of blood with a scalpel, and the meninges were carefully removed. The samples were immediately frozen and stored in a –70°C freezer for assays of malondialdehyde (MDA). The levels of lipid peroxides were evaluated by the thiobarbituric acid (TBA) method of Uchiyama and Mihara at the Department of Biochemistry of Hacettepe University Faculty of Medicine. Tissue samples were weighed and 10% homogenates were prepared in 10- fold 25 mM cold Tris HCl (pH:7) using glass Teflon homogenizer of 1500 spin/min for 30 seconds. 0.5 ml of this homogenate was mixed with 3 ml 1% H3PO4 TBA of 6% and incubated in a boiling water bath for 45 minutes. 4 ml of n-butanol was added, mixed and butanol and water phases were separated by centrifugation. The intensity of the color of n-butanol phase was evaluated by spectrophotometry at 532 nm (Shimadzu UV 120-02 Spectrophotometry). Lipid peroxide levels were evaluated by molar absorbtivity of the colour formed by malondialdehyde with TBA (?=1.56 X 105 M-1 cm-1) as nanomole (nmol) gram wet tissue (27).

Statistical evaluation:

Kruscal Wallis variance analysis was used to compare the groups. There was a difference of lipid peroxidation levels between the groups (x2=61.530, p=0.0001 or p<0.0001). Paired groups were compared with the Mann-Whitney U test.

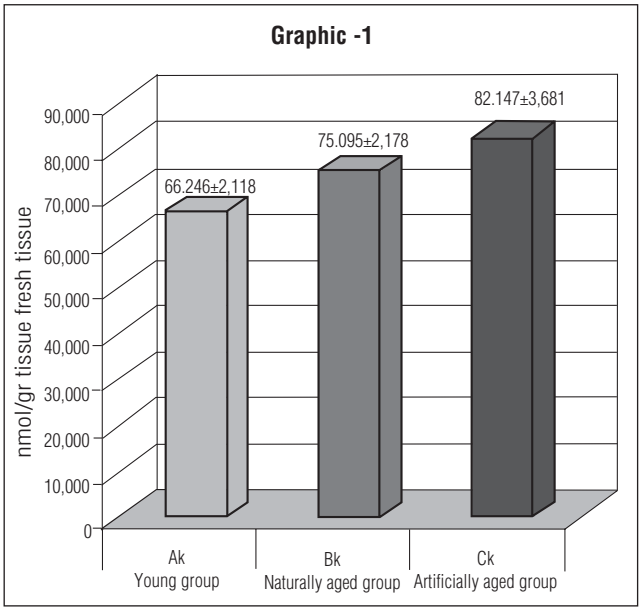
Findings

Control groups

Graph 1 depicts the lipid peroxidation levels detected in cord segments of three different groups of 3 months (young), 18-22 months (naturally aged/elderly population) and 3 months pinealectomized (artificially aged/artificial old) rats. Lipid peroxidation levels of both naturally and artificially aged groups were significantly higher than the group of young rats (p=0.022, p=0.005). There was no statistically significant difference between naturally aged and artificially aged groups (p=0.180). Lipid peroxidation levels increased during aging in the spinal cord of the animals. Although there was a statistical difference between young and naturally aged and artificially aged groups, there was no difference between naturally and artificially aged groups regarding lipid peroxidation.

Trauma groups

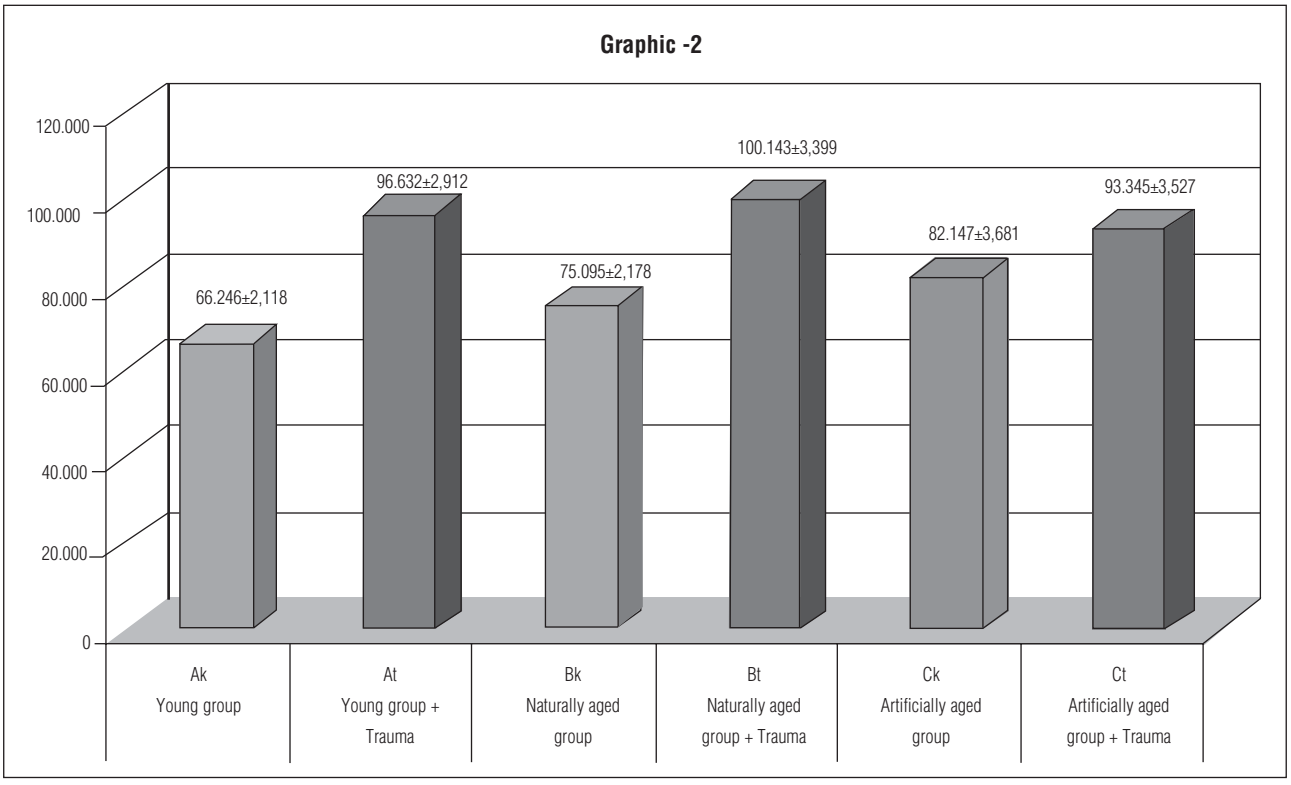
There was a statistically significant increase in lipid peroxidation in the group where standard spinal cord trauma was performed at the level of



Graph 1 depicts the lipid peroxidation levels detected in cord segments of three different groups of 3 months (young), 18-22 months (naturally aged/elderly population) and 3 months pinealectomized (artificially aged/artificial old) rats.

Gale inclined plane score 0 and Tarlow grade 1. The lipid peroxidation levels of these 3 groups after trauma are depicted in Graph 2.

The lipid peroxidation level of the young group increased from 66.246 ± 2.118 to 96.632 ± 2.912 (p=0.001); 75.095 ± 2.178 increased to 100.143 ± 3.399 (p=0.001) for the elderly group; and 82.147 ± 4.681 increased to 93.345 ± 3.527 (p=0.038) in the artificially aged groups.



Graph 2: Depicts the lipid peroxidation levels detected in cord segments of three different groups of young, naturally aged and artificially aged rats after trauma.

DISCUSSION

Aging, expressing postmaturational changes, is a process that contains a decline in the organism's simultaneous and harmonic response, adaptation potential, and resistance ability to external and negative events (stressors and stimuli) (11). Approaches related to the neurobiology of aging help us to understand aging and its process. The increased life expectancy and the elderly population have led to a number of new concepts and theories about the basics of aging neurobiology. Theories of central nervous system aging are molecular crosslinking, changes in immunological or neuroendocrine functioning, DNA damage and senescence genes and damage by free radical reactions (1, 3, 4, 11, 13, 20, 23). The free radical theory of aging, one of the aging theories, puts forward that aging and its accompanying diseases are the results of reactions between reactive free radicals and biomolecules. Aging has been also described as an internal desynchronization, dysdifferentiation and degeneration due to cumulative oxidative damages (11).

It is known that the oxidation accelerator factors existed before the adaptation of biologic systems and that they had a rapid change. If these factors reach a definite size in a short period of time, they keep the antioxidative capacity of the biological system under pressure. If these factors are chronically higher than that of the functions of the organism that help it to repair and survive, temporary or permanent damages occur. Because of these reasons, antioxidative mechanisms controlling life are very important (10, 11, 13). Living organisms specifically describe some pathways to avoid the cytotoxic products of intracellular oxidative reactions. These pathways also support living a long period of time. They include the reactions of DNA repair and

reactions that decrease intracellular toxic effects, naturally present antioxidants, free radical preventive enzymes, selective and other protein degrading systems. They also include endogenous substances that are sensitive to oxygen derivatives such as arachidonic acid, prostaglandins, cyclooxygenase and guanylate cyclase that produce cGMP. It is put forward that cGMP levels therefore increase in respect to cAMP levels and this hinders the cell (1, 3, 4).

There is a high correlation between the hydroxyl radical forming rate and aging (1, 13). Organisms that keep the concentration of hydroxyl radical at a low level or have better enzymatic or non-enzymatic antioxidative mechanisms have a higher chance of living. It has also been shown that the oxidative damage occurring due to hydroxyl radicals increases exponentially with age (1, 13). Three major molecular mechanisms are defined related to the irreversible situation of neuronal death during the aging period. These are glutamate-mediated excitotoxicity, intracellular calcium loading and hydroxyl radical-mediated peroxidation of biomolecules that result in oxidative damage.

The pineal gland or functional units of this gland give the most hope among the new concepts regarding aging. It has been shown that the pineal gland and its products directly or indirectly contribute to slowing down of aging and prevent the diseases associated with aging (9, 10, 11, 13, 17, 19, 21, 22, 28). Because of these reasons, melatonin is also called as anti-aging hormone. Experimental animal and human studies have shown that decreased melatonin function accelerates the process of aging (11, 17, 19, 21, 22, 28). It has been also shown that aging accelerates after pinealectomy. On the other hand the circadian rhythm of melatonin release is corrupted with aging and ceases in neurodegenerative diseases like dementia (10, 11, 21, 22, 28). Melatonin's oncostatic, immunostimulant and antioxidant properties are important factors for its effects as an anti-aging hormone (11). It has been shown that pinealectomized subjects have shorter median lives (11). However, these studies cannot be regarded as the proof of correlation between melatonin and aging.

Decreasing antioxidant capacity, melatonin insufficiency and accumulation of hydroxyl radicals are the chief determinants of aging (11). Aging is characterized by decreased synthesis and secretion of melatonin which results in insufficiency of endogenous activation of non-enzymatic defense mechanisms, lack of prevention of oxidative stresses and finally cellular dysdifferentiation, degeneration and death (10, 11, 17, 21, 22). Almost all organisms improve antioxidant defense mechanisms against destroying effects of free radicals especially the hydroxyl radical. Melatonin is a very potent and efficient endogenous radical scavenger (12, 14, 15, 16, 18). The pineal indolamine reacts with the highly toxic hydroxyl radical and provides on-site protection against oxidative damage to biomolecules within every cellular compartment due to its lipophilic character. The pineal gland is the main source of melatonin. Melatonin is almost exclusively synthesized and secreted during darkness at night. The 24-hr rhythm of melatonin is a very robust in young animals and humans (9, 10, 19, 28). This cycle frequently deteriorates during aging and is totally abolished in neurodegenerative diseases.

Up to now, the studies about the free radical theory, one of the main determinants of aging, have been performed excluding the medulla spinalis, one of the most important parts of the central nervous system. There are also no studies on the relationship between the spinal cord, aging, and trauma. Aging is very important determinant of the spinal cord response to trauma. Although there is a large amount of data in the literature about spinal cord trauma and hydroxyl radicals, the effect on spinal cord trauma of natural and artificial aging has not been investigated. We decided to create a spinal cord aging model. The rats were chosen as the subjects in the research because their circadian rhythm resembles that of humans. The rats were easily accessible and cheap and the experimental model was standard and durable. In our study we used Tarlow's grade that is used to score spinal cord injuries in rats and Gale's score. All subjects were made to be grade 1 with standard cord trauma with an inclined plane score of 0. Lipid peroxidation starts by the free radicals' effects on plasma membranes and it is a cascade that continues with function loss and cell death. Lipid peroxidation levels are determined to show free oxygen radical levels indirectly in many studies on free radicals because the method is easy to use. In this experimental study, free oxygen radical levels in traumatized spinal cord were determined indirectly by measuring lipid peroxidation levels.

The control group of the study was composed of naturally and artificially aged subjects. Their lipid peroxidation levels in spinal cords were found to be higher than that of young subjects and this was statistically significant (p=0.022; p=0.005). This shows that spinal cord faces oxidizing factors during the aging process. The organism is unable to overcome these factors due to a decrease in melatonin and pinealectomy, suddenly removing melatonin, accelerates the aging process.

When these increases in lipid peroxidation after trauma was evaluated as percentages and compared, there was a statistically significant increase of 45% in young subjects while this figure was 33% in the naturally aged group and 13% in the artificially aged group. After trauma, lipid peroxidation levels increased in all animals and ultrastructurally there was severe injury of the spinal cord. This shows that normally expected oxidative damage increases and becomes significant as the subjects get older.

CONCLUSION

One of the most important theories causing spinal cord aging is the free radical mechanism. Pineal gland functions are decreased by aging. Decreased melatonin function, decreased antioxidant capacity, and accumulation of hydroxyl radicals are the chief determinants of aging. Lack of prevention of toxic effects of hydroxyl radicals results in cellular dysdifferentiation, degeneration and death. It is necessary to carry out many studies on spinal cord aging especially regarding the long-term effects of the pineal gland and melatonin as they may be important agents for the recovery of spinal cord injury.

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