Study of Cerebellar Tissue Following Induction of Aging in Mice By Di-Galactose

Estudio del Tejido Cerebeloso tras la Inducción del Envejecimiento en Ratones por D-Galactosa

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SUMMARY: The cerebellum is a crucial area of the hindbrain that plays an essential role in balancing, excitement control, and subtle and accurate functions. Studies have shown that long-term use of D-galactose in mice, as with the symptoms of aging, causes morphological and functional disorders in the brain. This study was performed to evaluate the changes in the cerebellum cortex tissue and the measurement of reactive oxygen species (ROS) in the cerebellum following the induction of aging in mice by D-galactose. Accordingly, subjects were randomly assigned into two groups: Normal saline group and Aging group (D-galactose). To create an aging model, D-galactose, and saline solution (sodium chloride 0.9 %) were used. After completing the preparation and passage of the tissue, the cerebellum specimens were cut in 5 microns thickness and then stained with hematoxylin-eosin stain and finally examined under a Nikon microscope. Quantitative variables were analyzed by SPSS software using T-test. In the observations of cerebellum tissue samples, in the aged induced group by D-galactose, the most changes were observed in the Neuron purkinjense (Purkinje cells) layer. In the observations of the cerebellum tissue samples of aging group induced by D-galactose, the most changes were observed in the Neuron purkinjense, and the arrangement and placement of these cells were disorientated. The nucleus positioning was not central, and the Neuron purkinjense induced by aging were seen in different morphological forms. Necrosis, Chromatolysis, and Pyknosis were found. Based on the results, D-galactose (induction of aging) causes pathological changes in the cerebellar cortex, especially in the Neuron purkinjense layer.

KEY WORDS: Aging; Cerebellum; D-galactose; Mice; Neuron purkinjense (Purkinje cells).

INTRODUCTION

Aging is a complicated time-dependent process in which progressive loss of physiological integrity reduces the function and increases mortality. D-galactose is widely used to create aging models and to investigate its properties in rodents, including the oxidative stress, which chronic and systemic infusion of D-galactose by inducing oxidative stress can imitate aging properties, because high concentrations of D-galactose in the form of Galactitol accumulate in cells and increase the level of reactive oxygen species (ROS). Also, D-galactose increases the level of Malondialdehyde (MDA) and antioxidant capacity (TAC) and decreases the activity of the enzyme superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), monoamine oxidase, as well as catalase, all of which the cases increase the amount of oxidative stress in the brain. Furthermore, evidence suggests that increased oxidative damage can significantly result in premature cognitive impairment and pathological aging. This study aimed to evaluate the possible changes in possible changes in cerebellar tissue, ROS measurements of reactive oxygen species in the cerebellum after D-galactose induction of aging in mice.

D-galactose. It has been demonstrated that chronic administration of D-galactose (D-gal) is capable of causing morphological and functional impairments in the brain resembling symptoms of normal aging insults, and thus, it is considered an effective paradigm for establishing aging brain models (Cui *et al*., 2006; Chen *et al*., 2010; Park & Choi, 2012; Gu *et al*., 2013). The neural impairments underlying the D-gal exposure are presumed to result from increased oxidative stress and disrupted neurotransmitter balance in nervous tissues (Cui *et al*., 2006; Marosi *et al*., 2012; Gu *et al*., 2013).

ROS (reactive oxygen species). ROS contains anion superoxide and hydrogen peroxide. When the amount of ROS produced from these sources increases, antioxidants are used

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to balance. Thus, in nerve abnormalities that oxidative stress increase, the application of antioxidants is used as a therapeutic approach (Babaei-Abraki & Chavoshi-Nejad, 2014).

MATERIAL AND METHOD

Mature male Balb/c mice were prepared with an average initial weight of 25-30 g from the Pasteur Institute of Iran and transferred to the laboratory environment and got familiar with the new environment, weighed and were randomly categorized into double cages with 5 g weight difference. Since animal transfer would have induced stress on them and thus had an effect on the final results of the experiment, the animals were kept under new conditions for two weeks after transfer to the laboratory, and then, according to the purpose of the research, aging modeling was implemented on all of them. Accordingly, Mice were randomly assigned to two groups, including standard group and D-gal-induced aging group. D-galactose has been reported, due to the prolonged injection of D-galactose, a process similar to that of normal aging in mice is produced.D-gal-induced aging group (D-gal,Sigma, St Louis, MO, USA) dissolved in 0.9 % saline, 100 mg/ml/kg each day was injected intraperitoneally for eight weeks (Lee *et al*., 2014). Each group consisted of 12 adult mice, four of which were exposed to clear polycarbonate cages and maintained at a temperature of 20 to 24 ° C, the humidity of 45-55 %, and a dark cycle of 12:12 hours. During the research period, the mice used standard pellet feeds provided to them freely. The animal's water was also freely available to the subjects through 500 ml bottles for experimental animals. All ethical principles of the present study were conducted following the principles of working with laboratory animals. At the end of the aging induction period in mouse, the cerebellum was isolated from each of the subjects after cervical dislocation. The samples were put in a 10 % buffer formalin solution, and 6-mm thick paraffin blocks were colored with the general method of hematoxylin and Eosin (H&E) after passing the autotechnicom steps (Bancroft & Gamble, 2008). The cross-section samples were measured underneath a Nikon optical microscope (E200 Eclipse made in Japan) having a linear gradient and a 40 ¥ lens. The height and diameter of the Neuron purkinjense (Purkinje cells), the molecular layer, and the granular layer thickness were considered in this study. To investigate the potential of the mitochondrial membrane (MMP) was measured by the lipophilic cationic probe JC-1 staining was performed. A lipid peroxidation kit (manufactured by Navand Health Co.) was used to measure Reactive oxygen species(ROS). Quantitative variables will be evaluated and analyzed by SPSS software using T-test, and the significance level of the test was considered $p < 0.05$.

Information analysis method: The mean of quantitative variables was evaluated and analyzed by SPSS software using T-test, and the significance level of the tests was considered $p < 0.05$.

RESULTS

Results of the average reactive oxygen species ROS: There were significant differences between the two groups in standard and interventional groups ($p < 0.01$). Fig. 1.

Mitochondrial Membrane Potential Results: The results of the mitochondrial membrane potential showed a significant difference between standard and intervention groups ($p < 0.01$). Fig. 2.

The average results of the granular layer of the cerebellar cortex: There was a significant difference between the standard and interventional groups in the granular layer of the cerebellar cortex ($p < 0.01$) (Table I).

The average results of the molecular layer of the cerebellar cortex: There was a significant difference between the standard and interventional groups in the molecular layer of the cerebellar cortex ($p < 0.01$) (Table I).
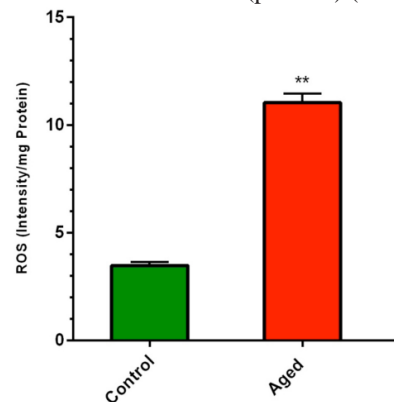

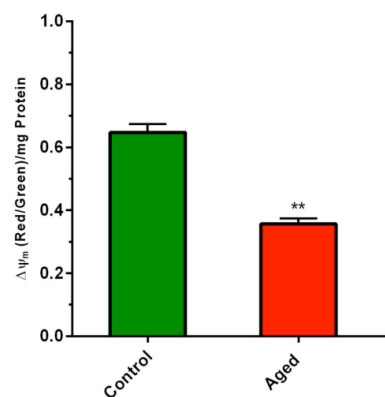Fig. 1. Reactive oxygen species Mean ± SD comparison in normal and treatment group.

Fig. 2. Mitochondrial Membrane Potential Mean ± SD comparison in normal and treatment group.

Table I. Mean ± SD comparison of quantitative parameters in normal and D-galactose treated group

Variable	Normal group Mean±SD n=7	D-galactose treated group Mean±SD n=7
Granular layer thickness (μ)	115.8 ± 10.11	86.06 ± 9.61 *
Molecular layer thickness(μ)	151.3 ± 14.96	109.4 ± 13.82*
Purkinje cell height (μ)	16.58 ± 1.3	10.41 ± 2.02 *
Purkinje cell's width (μ)	10.8 ± 1.67	8.713 ± 1.313*

* Indicates a significant difference between the groups (p <0.01)

Results of the average height of Neuron purkinjense from the cerebellar cortex: There was a significant difference between the standard and interventional groups in the height of the Neuron purkinjense in the cerebellar cortex (p <0.01) (Table I).

Results of the average width of Neuron purkinjense from the cerebellar cortex: There was a significant difference between the standard and interventional groups of Neuron purkinjense in the cerebral cortex (p <0.01) (Table I).

Histological results. In the observations from the cerebellum cortex of the regular group, three layers of tissue were distinguishable. The outer layer is the molecular layer. This layer was identified under a microscope with a pale red under the meninges. In this layer, in addition to the scaffold of neuron fibers, it also contained neurons. In the surface area of this ring, star-shaped cells known as II Golgi cells were observed. In the depths of this layer, the cell-baskets were visible with the accumulation of dendrites. On the Purkinje layer, which contained a single, separate cell line of Purkinje, was observed at close distances with a conical shape. The cone head of these cells was toward the environment so that the head of these cells branched out from several dendrites. Neuron purkinjense are the most prominent and largest cerebellar cells and are very sensitive to the changes that have been made. These cells have the central, round, and coarse nucleus with evident nucleoli and acidophilic cytoplasm, which were recognizable
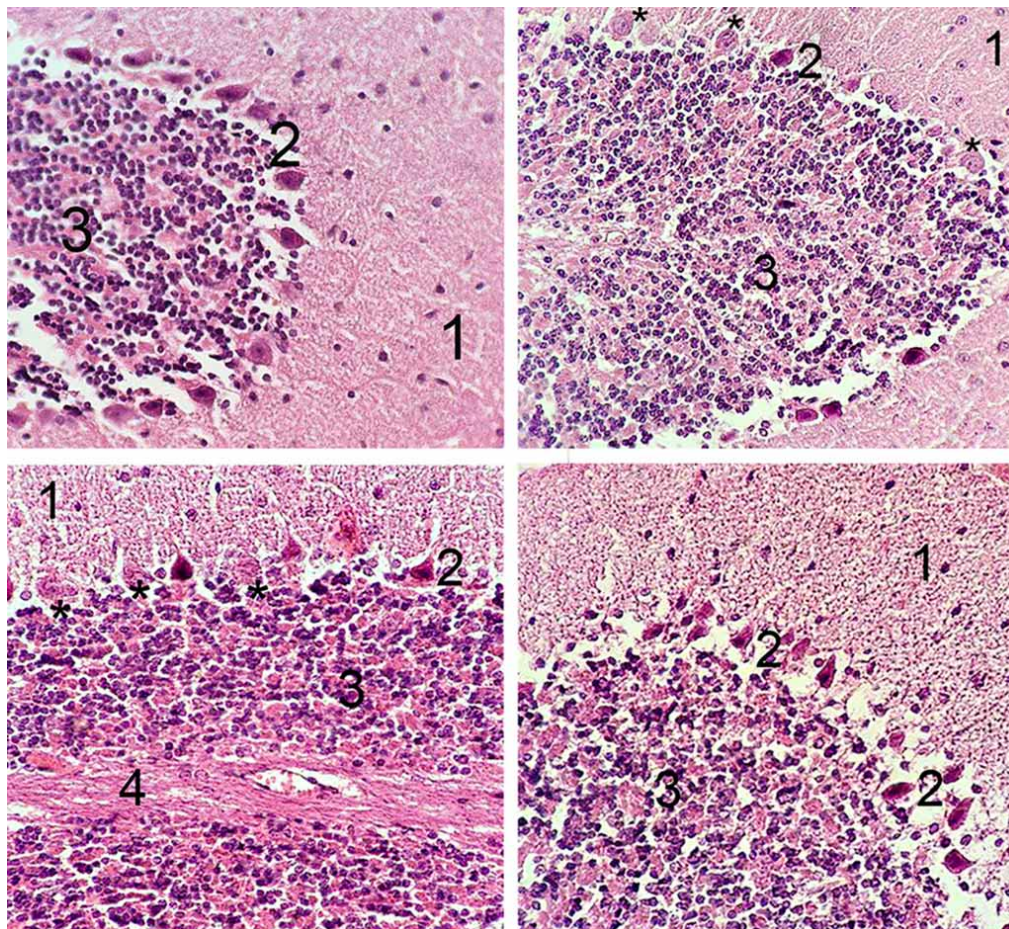

Fig. 3. Microscopic view of the cerebellum section in Mice. A-B) Normal group. C-D) Treatment group. 1. Molecular layer 2. Purkinje layer 3. Granular layer. Necrotic change of purkinje cell (star: C), Atrophic change (Star: D), (H&E staining, 100×).

with this feature (Fig. 3A-B). On the third layer of the cerebellum, the granulosa was located deep in the cerebellum. The cells were found in the second layer with high density. The characteristics of these cells were purple and circular nucleus with very low cytoplasm, and they were detectable by this (Fig. 3A-B).

In the observations of the cerebellum specimens of the age-induced group by D-galactose, the most changes were observed in the Purkinje cell line. The order and arrangement of these cells were disrupted. The position of nuclei in the cell did not have a central position. Neuron purkinjense induced by aging were found to be changed in different morphological forms. Some of these cells were observed with a lack of staining of the nucleus (nuclei fading) so that the nucleus could not be separated from the cytoplasm, indicating the occurrence of necrosis (Star:C). In some of these cells, a condensed and shrunken nucleus was observed indicating the occurrence of cellular damage of Pyknosis. Disrupted morphology of Purkinje was seen with atrophy of the cell. Cellular atrophy led to cellular deformity (shrinkage), and the conic point of the Purkinje was not detected by the presence of dendrites (Star:D). Decreasing the density of the Neuron purkinjense was evident compared to the standard group sample (Fig. 3C-D). In the evaluation of the molecular layer in aging group, a decrease in cell density was observed in comparison with the standard group, and the astroglia of this layer in the aging group was associated with reduced staining. In the granular layer of the aging group, pathological changes were not detected in comparison with the standard group.

DISCUSSION

The cerebellum is an essential area of the brain that plays a crucial role in balancing, controlling excitement, and subtle and accurate functions (Timmann *et al.*, 2010). The axon of the Purkinje cerebellar cortex cells forms an integral part of the output of the cerebellum, which contributes to the process of motor control and discipline (Ito, 2002). There are several neurological diseases that affect the death of neurons (Kiessling *et al.*, 2013). In studies by Cui *et al.* (2006), Park & Choi (2012) and Gu *et al.* (2013) has been shown that long-term use of D-galactose in mice causes morphological and functional disorders in the brain, similar to the symptoms of natural aging. In studies by Cui *et al.* (2006), Marosi *et al.* (2012) and Gu *et al.* (2013) has been shown that D-galactose in nerve tissue causes an increase in oxidative stress and distribution the balance of nervous transmission balance. The results of Song *et al.* (1999), which was first reported in China, indicated that low dose. D-galactose injection induces

changes that are similar to early aging. The developed aging model indicates a neurological disorder, decreased activity of antioxidant enzymes, and weakened immune response. In a study by Lee *et al.* (2014), long-term infusion of a D-galactose solution leads to oxidative damage, memory loss, and a process similar to that of normal aging in rats. Based on this, rodents that have been infused with long-term D-galactose are used as an aging animal model for anti-aging research. Accordingly, in this study, D-galactose was used to create an aging model. ROS (Reactive oxygen species) plays a role in various processes such as cell growth, signaling pathways, synthesis of biological molecules, immune response, and blood pressure. Reactive oxygen species can be created from internal sources such as mitochondria, macrophages, peroxisomes, and plasma membranes, and external sources such as immune cells, drugs, and stress (Babaei-Abraki & Chavoshi-Nejad, 2014). ROS contains superoxide anion and hydrogen peroxide. When the amount of ROS produced from these sources increases, antioxidants are used to balance this. For this reason, in nerve abnormalities that oxidative stress is increased, the application of antioxidants is used as a therapeutic approach (Babaei-Abraki & Chavoshi-Nejad, 2014). To prevent oxidative stress, there are enzymatic and non-enzymatic systems inside the cells. In physiological conditions, the level of formation of ROS is in equilibrium with the antioxidant capacity of the cell. If the cell is exposed to environmental stresses such as heat, UV, etc., for long periods of time, or if an antioxidant deficiency occurs in the body, the balance will be disrupted, and the formation of ROS will be higher than the antioxidant capacity of the body. The result of this condition will be oxidative stress (Ames & Shigenaga, 1992; Alaluf, 2000). ROS production in mitochondrial dysfunction is the primary cause of intracellular damage. The excessive production of ROS with the defense of the antioxidant that is disintegrated results in oxidative damage to mitochondria. Therefore, the use of antioxidants is considered as a useful treatment for mitochondrial damage caused by ROS (Edeas *et al.*, 2010). Parameshwaran *et al.* (2010) found that natural aging is accompanied by a gradual decrease in cognitive and motor activity. However, in abnormal nerve disorders, cognitive decline is rapid and leads to severe abnormalities in ordinary life. Pathology of the disease usually involves standard processes, including disorders of mitochondrial function. Mitochondrial dysfunction seems to be an indication of the onset of aging by decreasing the oxidative phosphorylation and increasing the relative production of radical oxygen (ROS). The results obtained in this study after induction of aging by D-galactose in mice resulted in a significant increase in the production of reactive oxygen species (ROS), which is consistent with previous studies. Therefore, in this study, following the induction of aging by D-galactose in rats, a significant increase was found in the

production of reactive oxygen species (ROS), which is consistent with previous studies.

Also, in the present study, there is a significant increase in the mitochondrial membrane permeability, which has an inverse relationship with the reduction of reactive oxygen species of ROS. In the results of Bakalian *et al*. (1991), the population of Neuron purkinjense lost in the very old rats was reduced by 9.5 %; however, it did not report statistically significant. In other studies, such as Sturrock (1989), it has been shown that aging results in loss of 44 % of Neuron purkinjense in a monkey, as well as in a study by Woodruff *et al*. 2010 in rabbits 25-20 % of Neuron purkinjense have been reported. Andersen *et al*. (2003) also estimate that in an old human cerebellum, the size of the Purkinje cell pericrion is significantly associated with a decrease of 33 %. The cause of Purkinje soma decrease is related to the reduction of the nucleus elements of the Purkinje cell at the old age (Ogata *et al*., 1984) or to the degeneration of other Organelles, along with the loss of the matrix of Purkinje cell pericrion(Monteiro, 1991). Nandy (1981) in his study on Sekanstria monkeys in the number of Purkinje, granular layer and lipofuscin substitution in Neuron purkinjense during an aging period, reported that the distribution of lipofuscin in old Purkinje was significantly increased. The number of Neuron purkinjense decreased significantly, while the number of cells in the granulosa cells of the old cerebellum did not change. These changes are not exactly evident in Purkinje, but may be associated with aging motor activity changes. In a study by Torvik *et al*. (1986), it has been reported that atrophy occurs in the three cerebellum cortical layers, accompanied by a decrease in the number of Neuron purkinjense and a decrease in the thickness of the molecular and granular layers of the cerebellum cortex. According to Andersen *et al*. (2003), Neuron purkinjense have a steady population in most cerebellar parts, with the exception of the anterior cerebellar lobe, which is significantly declined in the aging period by 40.9 % reduction in the number of Purkinje. In Zhang *et al*. (2006), it was reported that the thickness of the molecular layer and the whole cerebellar mass in the old cats was significantly lower than the young cats. In old cats, the density of neurons in each layer decreased significantly. In the study of Sturrock (1989), cerebellar cell mass ratios in the rats from 6 months to 31 months of age were reported to increase with granular cells compared to other cerebellar layers. Since the number of granulosa cells in adults does not increase, it is related to the increase in the ratio of these cells to Purkinje due to the loss of Neuron purkinjense with aging. Zhang *et al*. (2010), in their study, indicated a significant connection between the reduction of Neuron purkinjense and aging. Hall *et al*. (1975) reported that about 2.5 % of the Neuron purkinjense disappeared during each decade of age. However, there was no significant difference in the age and reduction

in the number of Neuron purkinjense in Drüge *et al*. (1986) and Bakalian *et al*. (1991). The results of histomorphometry in this study showed a significant decrease in cerebellum cortex thickness. So that the thickness of the molecular layer, Neuron purkinjense and granular cells in the age induction group was less than that of the standard group, which is consistent with the results of other studies in older animals (physiological aging). From the microscopy results of this study, the occurrence of atrophy, cell necrosis and cell death in Neuron purkinjense or chromatolysis in cerebellar molecular cells was followed by D-galactose induction of aging, and this was consistent with the process of cerebellar disorders in the physiological aging of mammals.

CONCLUSION

D-galactose, a drug with aging induction in the animal model, can create the aging process in cerebellar tissue through cellular atrophy, necrosis, apoptosis, and also the increase of active oxygen species.

HEJAZI, S. Estudio del tejido cerebeloso tras la inducción del envejecimiento en ratones por Di-galactosa. *Int. J. Morphol., 41(3)*:825-830, 2023.

RESUMEN: El cerebelo es un área crucial del rombencéfalo que desempeña un papel esencial en el equilibrio, el control de la excitación y las funciones sutiles y precisas. Los estudios han demostrado que el uso a largo plazo de D-galactosa en ratones, al igual que con los síntomas del envejecimiento, provoca trastornos morfológicos y funcionales en el cerebro. Este estudio se realizó para evaluar los cambios en el tejido de la corteza del cerebelo y la medición de especies reactivas de oxígeno (ROS) en el cerebelo luego de la inducción del envejecimiento en ratones por D-galactosa. En consecuencia, los sujetos fueron asignados aleatoriamente a dos grupos: grupo de solución salina normal y grupo de envejecimiento (D-galactosa). Para crear un modelo de envejecimiento, se utilizaron D-galactosa y solución salina (cloruro de sodio al 0,9 %). Después de completar la preparación y el paso del tejido, las muestras de cerebelo se cortaron en un grosor de 5 μm y luego se tiñeron con tinción de hematoxilina-eosina y finalmente se examinaron bajo un microscopio Nikon. Las variables cuantitativas se analizaron mediante el software SPSS utilizando la prueba T. En las observaciones de muestras de tejido de cerebelo, en el grupo envejecido inducido por D-galactosa, la mayoría de los cambios se observaron en la capa de neuronas purkinjenses (células de Purkinje). En las observaciones de las muestras de tejido del cerebelo del grupo de envejecimiento inducidas por D-galactosa, la mayoría de los cambios se observaron en las neuronas purkinjenses, y la disposición y ubicación de estas células estaban desorientadas. El posicionamiento del núcleo no era central y las neuronas purkinjenses inducidas por el envejecimiento se observaban en diferentes formas morfológicas. Se encontró necrosis, cromatólisis y picnosis. Según los resultados,

la D-galactosa (inducción del envejecimiento) provoca cambios patológicos en la corteza cerebelosa, especialmente en la capa de neuronas purkinjenses.

PALABRAS CLAVE: Envejecimiento; Cerebelo; D-galactosa; Ratones; Neuronas purkinjenses (Células de Purkinje).

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