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Iron-Induced Cerebellar Purkinje Cell Loss Is Ameliorated by Flunarizine*

Ramazan KOZAN[1]
M. Ömer BOSTANCI[2]
Tuncer NACAR[3]
Ali ASLAN[3]
Faruk BAĞIRICI[3]

[1] Department of Physiology, Faculty of Medicine, Mustafa Kemal University, Hatay - TURKEY

[2] Health School, Hitit University, Çorum - TURKEY

[3] Department of Physiology, Faculty of Medicine, Ondokuz Mayıs University, Samsun - TURKEY

Aim: In this study, we investigated the effect of intracerebroventricular-injected iron neurotoxicity on the total number of cerebellar Purkinje cells in rats and the possible neuroprotective effect of flunarizine, a piperazine-derived calcium channel blocker.

Materials and Methods: Rats were divided into four groups: control, flunarizine, iron, and iron + flunarizine groups. Rats in the iron and iron + flunarizine groups received intracerebro-ventricular iron ($FeCl_3 6H_2O$, 200 mM, 2.5 µl), while those in flunarizine and iron + flunarizine groups were intraperitoneally injected with flunarizine (10 mg/kg/day) once a day after the operation for 10 days. After 10 days, all rats were perfused intracardially and then sacrificed. Brain tissues were removed and standard histological techniques were performed. The total numbers of Purkinje cells were estimated using unbiased stereological techniques.

Results: Means of the total numbers of Purkinje cells in the cerebellum were estimated as 310441 ± 6558, 298658 ± 9636, 200201 ± 6822 and 282658 ± 6327 in the control, flunarizine, iron, and iron + flunarizine groups, respectively. Comparison between iron and iron + flunarizine groups revealed that flunarizine significantly attenuates the iron-induced neuron loss from 35.5% to 8.9% ($P < 0.05$).

Conclusions: Findings of the present study suggest that flunarizine has a neuroprotective effect on iron-induced Purkinje cell loss in the rat cerebellum via blocking influx of calcium ions into neurons.

Key Words: Flunarizine, iron, neurotoxicity, Purkinje cell, stereology

Demirin İndüklediği Serebellar Purkinje Hücre Kaybının Flunarizin Tarafından İyileştirilmesi

Amaç: Bu çalışmada, intraserebroventriküler olarak verilen demirin, sıçan serebellar Purkinje hücrelerinde oluşturduğu nörotoksisiteyi ve bir piperazin derivesi kalsiyum kanal blokörü olan flunarizinin muhtemel nöroprotektif etkisini araştırdık.

Yöntem ve Gereç: Sıçanlar, kontrol, flunarizin, demir ve demir + flunarizin grupları olmak üzere dört gruba ayrıldı. Demir ve demir + flunarizin gruplarındaki sıçanlara intraserebroventriküler olarak demir ($FeCl_3 6H_2O$, 200 mM, 2.5 µl) verildi. Flunarizin ve demir + flunarizin gruplarındaki sıçanlara operasyonu takiben on gün süreyle 10 mg/kg/gün dozunda flunarizin intraperitoneal olarak verildi. On günün sonunda, hayvanlar intrakardiyak yolla perfüze edildikten sonra dekapite edildiler. Beyin dokuları çıkarılarak standart histolojik doku takibi uygulandı. Toplam Purkinje hücre sayıları tarafsız sayım metodu olan stereolojik yöntemle hesaplandı.

Bulgular: Ortalama toplam Purkinje hücre sayıları, kontrol, flunarizin, demir ve demir + flunarizin gruplarında sırasıyla 310441 ± 6558, 298658 ± 9636, 200201 ± 6822 ve 282658 ± 6327 olarak bulundu. Demir ve demir + flunarizin grupları karşılaştırıldığında, flunarizin demirin indüklediği Purkinje hücre kaybını % 35.5'den % 8.9'a anlamlı olarak gerilettiği bulundu ($P < 0.05$).

Sonuç: Bu çalışmanın sonuçları, sıçan serebellumunda demirin indüklediği Purkinje hücre kaybı üzerine flunarizinin nöroprotektif etkisinin, kalsiyumun nöron içine girişinin bloklanması yoluyla olabileceğini göstermektedir.

Anahtar Sözcükler: Flunarizin, demir, nörotoksisite, Purkinje hücresi, stereoloji

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Correspondence
Ramazan KOZAN
Department of Physiology,
Faculty of Medicine,
Mustafa Kemal University,
31100 Hatay – TURKEY

drkozan@hotmail.com

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Introduction

Iron (Fe) is an essential trace metal involved in numerous cellular processes. However, excessive levels of iron in the brain can contribute to the formation of free radicals, leading to lipid peroxidation and neurotoxicity (1). Implication of iron in some severe neurodegenerative diseases (Friedreich's ataxia, Parkinson's disease, Alzheimer's disease, Wilson's disease, multiple sclerosis) by oxidative stress implication (2) is now well established (3).

Subpial injections of iron salts lead to free radical formation (4) and free radicals also lead to disintegration of the plasma membrane by causing lipid peroxidation (5). As a result of such disintegration, ionic gradients cannot be preserved sufficiently and this eventually leads to an excessive ion influx, including calcium, into the cells (6). Intracellular calcium accumulation in neuronal cells is the main factor thought to be responsible for cellular death (7). Therefore, calcium channel blockers can protect the cell from toxicity by preventing the peroxidation of the lipid bilayer.

Cerebellar Purkinje cells are the only cortical neurons whose axons extend through the white matter to synapse with the central nuclei of the cerebellum. They are involved in the processing of efferent impulses that come from the motor cortex. Purkinje cells may receive hundreds of thousands of inhibitory and excitatory impulses to process and integrate. However, Purkinje cells are the most important targets in the cerebellum for toxic substances such as ethanol (8), long-term nicotine exposure (9) and cadmium toxicity (10).

In particular, the current literature on neurotoxicity does not contain sufficient information on the effects of calcium channel blockers on quantitative aspects of cerebellar Purkinje cell loss induced by iron. The aim of this study was to investigate the effects of the potent calcium channel antagonist flunarizine on $FeCl_3$-induced cerebellar Purkinje cell loss, using unbiased stereological techniques.

Materials and Methods

Animals

Adult male rats were obtained from the Experimental Research Center of Ondokuz Mayıs University. Approval of the Ethical Committee of Ondokuz Mayıs University was obtained prior to experiments and all animal work was performed according to the Experimental Animal Care Rules of the European Community Council. Twenty-four adult male Wistar rats (300g) were housed individually on a 12-h light: 12-h dark cycle (lights on at 07.00 h), at a temperature of 20-22°C and 50% humidity. All animals were kept under constant laboratory conditions and supplied with food and water ad libitum. Before the study procedure, rats were randomly assigned to four groups, each consisting of 6 rats (n = 6): control group receiving normal saline, flunarizine group receiving flunarizine, iron group receiving iron, and iron + flunarizine group receiving iron and flunarizine.

Operations and Histological Processing

Animals were kept away from food for 12 hours prior to surgery and all animals were weighed just before the surgical operation. Anesthesia was induced by intraperitoneal (i.p.) injection of ketamine hydrochloride (100 mg/kg). After the skin covering the skulls was shaved and animals were fixed to a stereotaxic apparatus, a rostrocaudal incision 2 mm in length was made using an electric cutter (Ellman Surgitron). After the Bregma line was exposed clearly, a hole with a diameter of 1 mm was drilled at the point located 2 mm left and 0.6 mm posterior to Bregma using a dental drill. All drugs administered were given through this hole to a depth of 4.2 mm using a Hamilton microinjector. Rats in the control group received 2.5 µl saline while rats in iron group received 200 mM (2.5 µl) $FeCl_3$ (4). Rats in the iron + flunarizine group received the same amount of $FeCl_3$ and intracerebroventricular (i.c.v.) flunarizine (1 µM, 2 µl) (11). Then, incisions were sutured and the incision area was cleaned using 10% povidone iodide just prior to the placement of the animals in their cages. All animals survived for 10 days following the surgery. Rats in the flunarizine and iron + flunarizine group received additional i.p. flunarizine treatment as 10 mg/kg/day for 10 days. The first dose of flunarizine was administered during the first five minutes following the surgical operation. Rats in the other groups received no additional treatment during their 10-day survival period.

After the survival period, all animals were perfused intracardially under deep urethane anesthesia with 10% formaldehyde and saline, buffered for pH = 7.6. After the completion of the perfusion process, all animals were decapitated, and brains were removed immediately and

placed in the same fixative for postfixation. After the cerebra and cerebelli were separated physically, cerebelli were processed using the standard histological techniques and embedded in paraplast embedding media. Serial tissue sections were obtained using a rotary microtome (Leica RM 2135) in horizontal plane with a section thickness of 40 µm. The slides were stored overnight in the oven (60°C) and stained with Cresyl violet staining.

Section Sampling and the Stereologic Analysis

The cytoarchitectonic characteristics of the Purkinje cell layer were identified using the criteria of a previous study (12). According to the pilot study, 13-16 sections were sampled in a systematic random fashion (ssf: 1/7) out of a total of 120 horizontal sections per individual cerebellum. First sections were chosen randomly from the first set of seven sections containing the cerebellum and then the consecutive samples were selected with a fixed interval of seven sections. Cerebellar Purkinje cells were counted using the optical fractionator counting method, which is a combination of fractionator sampling scheme and dissector counting technique (12).

All counting and analysis were performed using a modified computer-assisted stereological analysis system. Areas for cell counting were determined and delineated using CAST Grid stereological analysis software (Olympus, Denmark). Cell counts were done using a sampling scheme optimized for a total of approximately 500 cell counts per individual. Randomly selected Purkinje sectional areas were scanned automatically using consecutive steps with 200x200 µm x-y size. Every step in this scanning was individually analyzed with optical dissector probes using 100x oil-objectives. For optical dissector counting (or sampling), an unbiased counting frame comprising 20% of the total step area was used for particle sampling and counting. Thus, the area sampling fraction (asf) was determined as 587/40,000 µm^2.

The last sampling level in optical fractionator applications is the thickness sampling stage. Optical dissector counting requires a virtual vertical scanning of the section of interest in order to count stacks of particles. According to previous pilot studies, a fixed dissector height of 10 µm was predetermined and used throughout the study. This height is formed by virtual movement of a section plane (the focal plane) through the section thickness. Generally, a narrow upper “guard zone” passed before the actual optic dissector counting in order to avoid the possible irregularities of the sectional surface. Here we left a 5 µm upper guard zone, applied particle counting through a 10 µm dissector height and measured the section thickness. All such measurements were done using a digital microcator (Heidenhain, Germany), incorporated in the stereological analysis system. Thus, the final sampling stage, generally called the thickness sampling fraction (tsf), was calculated by [Dissector Height]/[Mean Section Thickness]. Average section thickness was estimated for each section by measuring the thickness of every 10th field of counting with a random start and by averaging the measured thickness values for each section. The total average of section thickness was 26.75 ± 1.28 µm among all animals.

After completing sampling throughout all sampled sections, properly sampled Purkinje cells were counted as dissector particles (Q^-). Total number of cerebellar Purkinje cells (N) was then calculated using the following formulation:

$$N = [1/ssf] \times [1/asf] \times [1/tsf] \times \Sigma Q^-$$

Statistical Analysis

Data were analyzed using a commercially available statistics software package (SPSS® for Windows, version: 12.0). Group means and standard deviations were calculated for all the study groups. The estimated number of the Purkinje cells of the study groups was analyzed by one-way ANOVAs with post hoc Tukey test. *P* values <0.05 were considered to be statistically significant.

Chemicals

Flunarizine, Cresyl violet and $FeCl_3 6H_2O$ were obtained from Sigma Chemical Co. (St. Louis, Mo, USA); Entellan, xylene and acetic acid were obtained from Merck (Darmstadt, Germany); and formaldehyde, chloroform and others were obtained from Aklar Chemistry (Ankara, Turkey).

Results

The mean values of average coefficient of variation (CV) and coefficient of error (CE) in all groups were 0.07 and 0.05, respectively. Representative light photomicrographs of sections through the cerebellum from all groups of rats are shown in Figure 1 A–D.

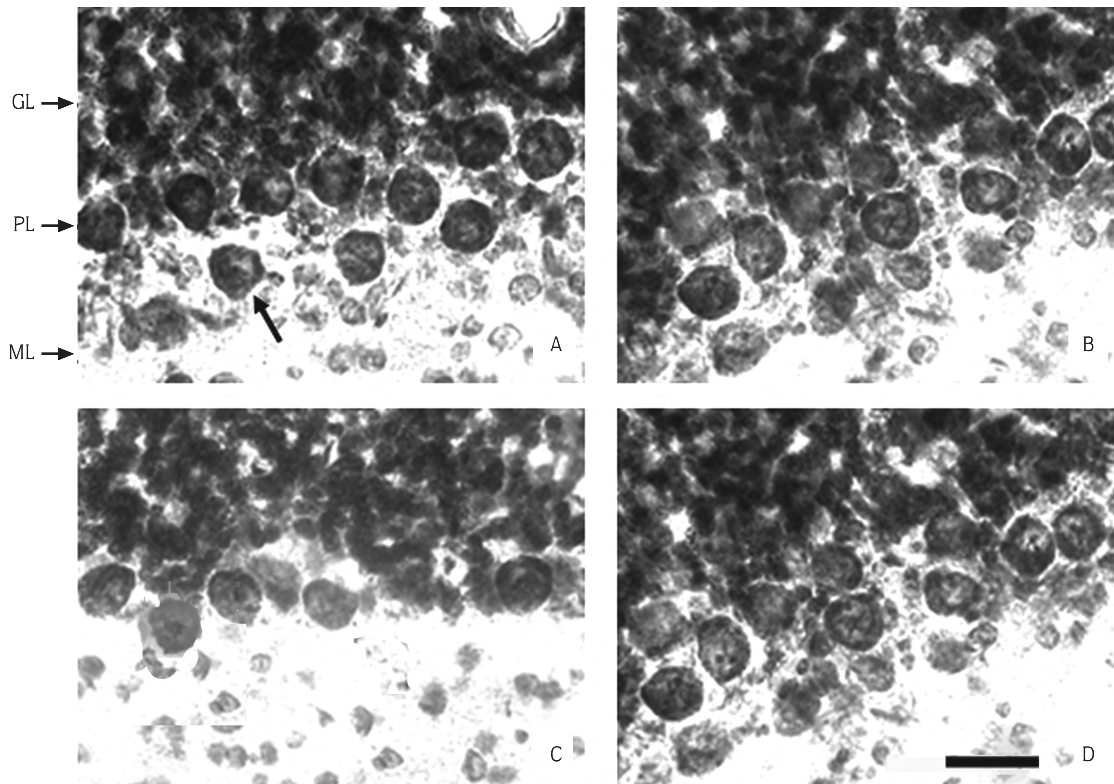

Figure 1. The photomicrographs of cerebellar Purkinje cells (A) from control, saline, (B) flunarizine-treatment, (C) iron-treatment and (D) iron + flunarizine treatment groups. Arrowhead on the photomicrograph A indicates a Purkinje cell, and the three arrows on the left of the photomicrograph A are used to indicate the layers of the cerebellar cortex (GL: granular cell layer; PL: Purkinje cell layer; ML: molecular layer). The length of the bar (D) represents 40 µm for all photomicrographs.

The means (value ± SEM) of the total numbers of Purkinje cells in the cerebellum were estimated as 310441 ± 6558, 298658 ± 9636, 200201 ± 6822 and 282658 ± 6327 in the control, flunarizine, iron, and iron + flunarizine groups, respectively. There was no significant difference in the total numbers of Purkinje cells in the flunarizine group compared with the control group ($P > 0.05$, Figure 2). Rats in the iron group were determined to have lower numbers of Purkinje cells (by 35.5 ± 3.1%) than rats in the control group, and this difference was statistically significant ($P < 0.05$). Rats in the iron + flunarizine group had 8.9 ± 3.8% lower Purkinje cell numbers with respect to controls ($P > 0.05$, Figure 2). Comparison between iron and iron + flunarizine groups revealed that flunarizine significantly attenuated the iron-induced neuron loss from 35.5% to 8.9% and protected Purkinje cells against iron toxicity ($P < 0.05$, Figure 2).

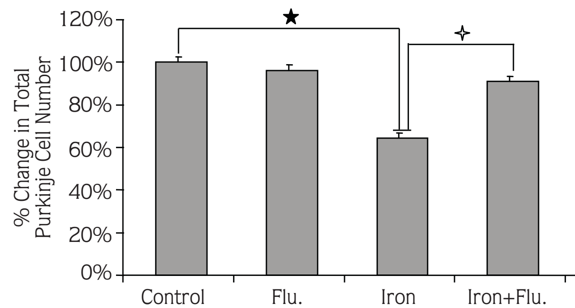

Figure 2. The percentage change in total numbers of cerebellar Purkinje cells in flunarizine-, iron- and iron + flunarizine-treated rats. Data represents mean ± S.E.M. of 6 animals. $P < 0.05$ vs. control; $P < 0.05$ vs. iron + flunarizine-treated rats. Flu: Flunarizine.

Discussion

This study dealt with the effects of flunarizine on iron-induced cerebellar Purkinje cell loss, using unbiased

stereological techniques. Iron is known to induce neurotoxicity by means of oxidative stress, most likely via excessive calcium influx into the cells. We aimed to determine the effect of flunarizine, a calcium channel blocker, on the iron-induced neurotoxicity, and concluded that there was a Purkinje cell loss of 35.5% in the iron group. However, this percentage was reduced to 8.9% in the iron + flunarizine group. Therefore, flunarizine has a neuroprotective effect on iron-induced Purkinje cell loss in the rat cerebellum.

In this study, it has been estimated that there are 310441 ± 6558 Purkinje cells in the control rats' cerebellum. This value is in good agreement with other researchers who have used similar advanced, relatively unbiased, stereological procedures as those employed in the present study (12,13). Administration of iron to rats in the iron group displayed a significant decrease in Purkinje cells numbers when compared with controls (Figure 2). According to the results, iron mediated Purkinje cell loss in rats. Iron is a frequently used metal for inducing lipid peroxidation and cellular damage. Ferro (Fe^{2+}) and ferric (Fe^{3+}) iron produce hydroxyl radicals via Fenton and Haber-Weiss reactions and cause cellular damage (14). When the concentrations of free radicals exceed the normal levels, they start to bind the unsaturated bonds of fatty acids and cholesterol, leading to membrane peroxidation and membrane disintegration (5). This disruption of membrane integrity threatens the transmembrane differences of ionic concentrations and cations, and especially calcium begins to enter the cell (6). Elevated intracellular Ca^{2+} levels in neurons are thought to mediate the oxidative cellular death (7,15).

We also showed that iron + flunarizine treatment results in a smaller loss of Purkinje cells as a result of iron injection into the brain of rats (Figure 2), and flunarizine significantly protects neurons against iron toxicity. Flunarizine blocks L-type calcium channels as well as T-type channels in certain tissues (16). T-type calcium channels are widely distributed in the body and are more concentrated in neuronal cells (17). Flunarizine has a high affinity for the membrane binding regions of nitrendipine, a dihydropyridine compound, and it has been hypothesized that flunarizine exerts its channel blocking activity through those binding sites. The neuroprotective effect of flunarizine may be due to the large spectrum of effects on different types of Ca^{2+} channels such as N, T and P-type voltage-gated calcium channels.

In conclusion, results of the present study show that a piperazine-derived calcium channel blocker flunarizine attenuates the iron-induced Purkinje cell loss in the rat cerebellum from approximately 35% to 9%. This neuroprotective effect of flunarizine may be due to the prevention of lipid peroxidation as well as the prevention of excessive calcium influx into neurons.

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