

Experimental Research



The Protective Effect of Nicardipine on Iron-Induced Purkinje Cell Loss in Rat Cerebellum: A Stereological Study

Ramazan KOZAN^{a1}, M. Ömer BOSTANCI², Fatih SEFİL², Faruk BAĞIRICI²

¹ Mustafa Kemal University, Faculty of Medicine, Department of Physiology, HATAY

² Ondokuz Mayıs University, Faculty of Medicine, Department of Physiology, SAMSUN

ABSTRACT

Objective: The aim of the present study is to investigate the effect of nicardipine, a calcium channel blocker, on the neurotoxicity induced by intracerebroventricular (i.c.v.) iron injection in rats.

Materials and Methods: Animals were divided into three groups; control, iron and iron+nicardipine groups. Rats in iron and iron+nicardipine groups received i.c.v. FeCl₃, while rats in control group received the same volume of saline. All animals were kept alive for ten days following the operation and animals in iron+nicardipine group were injected intraperitoneally nicardipine (10 mg/kg/day) once a day during this period. After ten days, all rats were perfused intracardially and cerebellar tissues were stained with Cresyl violet. Means of total Purkinje cells numbers in the cerebellum were estimated using the optical fractionator counting method.

Results: Means of total Purkinje cells numbers in the cerebellum as follows: 317182±9667, 209002±7836 and 265659±8291 in the control, iron and iron+nicardipine groups, respectively. Total number of Purkinje cells in iron and iron+nicardipine groups were significantly lower than control animals (p<0.05). However, comparison between iron and iron+nicardipine groups revealed that nicardipine significantly attenuates the iron-induced Purkinje cell loss (p<0.05).

Conclusion: It has been shown firstly in the present study that an excessive amount of iron has a toxic effect on cerebellar Purkinje cell in rats and this deleterious effect is protected by nicardipine, a calcium channel blocker. ©2008, Firat University, Medical Faculty

Key words: Iron, cell death, Purkinje cell, nicardipine, stereology

ÖZET

Siçan Serebellumunda Demirin İndüklediği Purkinje Hücre Kaybına Nikardipinin Koruyucu Etkisi: Stereolojik Bir Çalışma

Amaç: Bu çalışmanın amacı, siçanlarda, intraserebroventriküler (i.c.v.) olarak injekte edilen demirin indüklediği nörotoksisite üzerine bir kalsiyum kanal blokörü olan nikardipinin etkisini incelemektir.

Gereç ve Yöntem: Hayvanlar kontrol, demir ve demir+nikardipin olmak üzere üç gruba ayrıldı. Demir ve demir+nikardipin grubuna i.c.v. olarak FeCl₃, kontrol grubuna ise aynı hacimde salin verildi. Bütün hayvanlar operasyonu takiben on gün yaşatılırken bu esnada demir+nikardipin grubuna 10 mg/kg/gün dozunda nikardipin intraperitoneal olarak verildi. Onuncu günde, siçanların tamamı intrakardiyak olarak perfüze edildi ve serebellum dokuları kresil violet ile boyandı. Serebellumda ortalama toplam Purkinje hücre sayıları optik parçalama sayım metodu kullanılarak hesaplandı.

Bulgular: Hücre sayıları kontrol, demir ve demir+nikardipin gruplarında sırasıyla 317182±9667, 209002±7836 ve 265659±8291 olarak bulundu. Total Purkinje hücresi sayısı, demir ve demir+nikardipin gruplarında kontrol grubuna göre anlamlı olarak daha azdı (p<0.05). Ancak, demir ve demir+nikardipin karşılaştırıldığında, nikardipin demirin indüklediği Purkinje hücre kaybını anlamlı olarak önlediği bulundu (p<0.05).

Sonuç: Aşırı miktarda demirin siçan serebellar Purkinje hücrelerine toksik etkisinin olduğu ve bu zararlı etkinin bir kalsiyum kanal blokörü olan nikardipin tarafından önlediği sunulan çalışma ile ilk olarak gösterilmiştir. ©2008, Firat Üniversitesi, Tıp Fakültesi

Anahtar kelimeler: Demir, hücre ölümü, Purkinje hücresi, nikardipin, stereoloji

Iron is an essential mineral in humans and plays a crucial role in vital biochemical activities, such as oxygen sensing and transport, electron transfer, and catalysis. The human body contains approximately 45–55 mg/kg of body weight in adult women and men, respectively. Iron overload can result from either primary, genetic disorders that create an imbalance in iron metabolism, or secondary causes, factors that bypass normal iron homeostasis, such as repeated blood transfusions, or acute or chronic iron poisoning (1). When present in excess, iron poses a threat to cells and tissues, and therefore iron homeostasis has to be tightly controlled.

Several neurogenerative disorders such as Parkinson's and Alzheimer's disease, or more rare conditions such as Huntington's disease and Hallervorden-Spatz syndrome have been associated with misregulation of iron metabolism in the central nervous system (2). In these disorders, iron-induced oxidative stress, combined with defective antioxidant capacities, promotes neuronal death and neurodegeneration. However, it is still controversial whether the extensive brain iron accumulation is the initial pathogenic event, or a secondary effect. Moreover, the involvement of iron in the aforementioned disorders, provides a rationale for the development of metal-binding drugs (chelators) as viable new

^a Corresponding Adress: Dr. Ramazan Kozan, Mustafa Kemal University, Faculty of Medicine, Department of Physiology, HATAY
Tel: +90 326 2142636 e-mail: drkozan@hotmail.com

therapeutic strategies.

Willmore et al. (3) reported that subpial injections of iron salts lead to free radical formation and these free radicals cause disintegration of plasma membrane by lipid peroxidation (4). As a result of such disintegration, ionic gradients cannot be preserved sufficiently and this eventually leads to an excessive calcium ion influx into the cells (5). Elevated intracellular Ca^{2+} levels in neurons are thought to mediate the oxidative cellular death (6). Gaasch et al. (7) reported that voltage-gated calcium channels (VGCC) are an alternate route for iron entry into neuronal cells under conditions that promote cellular iron overload toxicity. This role may be aggravated in pathophysiologic conditions of iron overload. Iron uptake (similar to calcium uptake) is inhibited by nimodipine, a specific L-type VGCC blocker, in a dose-dependent manner (7). In addition, it has been reported that nicardipine, a dihydropyridine calcium antagonist, decreased ischemic brain injury induced by the occlusion of the middle cerebral artery in rats (8) and cats (9). Signals for motor coordination and balance are coded in the rate and pattern of firing of cerebellar Purkinje neurons, the only neurons that project out of the cerebellar cortex. However, Purkinje cells are the most important targets in cerebellum for toxic substances such as ethanol (10) long-term nicotine exposure (11) and cadmium toxicity (12). There is no sufficient information in the current literature the effects of calcium channel blockers on quantitative aspects of cerebellar Purkinje cell loss induced by iron. In the present study we aimed to investigate the effects of nicardipine on iron-induced cerebellar Purkinje cell loss, using unbiased stereological techniques.

MATERIALS and METHODS

Animals

Twenty-one adult male Wistar albino rats (220- 250 g) were divided into three groups as control (n=7), iron (n=7) and iron+ nicardipine (n=7). All animals were obtained from Medical and Surgical Research Faculty of Ondokuz Mayıs University. All animals were kept in constant laboratory conditions and supplied with food and water ad-libidum.

Operation

Animals were kept away from food for 12 hours prior to surgery and all animals were weighted just before the surgical operation. Anesthesia was induced by i.p. injection of ketamine hydrochloride (100 mg/kg)+10 mg/kg xylazine. Animals were fixed to a stereotaxic apparatus and details of the surgical procedure can be seen in Bostanci et al. (13). Rats in the control group received 2.5 μ l saline while rats in iron group received 200 mM (2.5 μ l) $FeCl_3$ ($FeCl_3 \cdot 6H_2O$, Sigma, St. Louis, USA) (3). Rats in iron+nicardipine group received the same amount of $FeCl_3$ and i.c.v. nicardipine (Sigma, St. Louis, USA) (1 μ M, 2 μ l). Then, incisions were sutured and incision area was cleaned using 10% povidon iodide (Aklar Chemistry, Ankara, Turkey) just prior the placement of the animals to their cages. All animals were survived for ten days following the surgery. Only the rats belonging to iron+nicardipine group received additional i.p. nicardipine treatment as 10 mg/kg/day for ten days (12).

After ten days, all animals were perfused intracardially under deep urethane (Sigma, St. Louis, USA) anesthesia (1.25 g/kg, i.p.) with 10% formaldehyde (Aklar Chemistry, Ankara, Turkey) and saline, buffered for pH=7.6. After the completion of the perfusion process all animals were decapitated, 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 (Sigma, St. Louis, USA) embedding media. Serial tissue sections obtained using a rotary microtome (Leica RM 2135) in horizontal plane with a section thickness of 40 μ m. The slides were stored overnight in the oven (60 °C) and stained with Cresyl violet (Sigma, St. Louis, USA) staining. Approval of Ethical Committee of Ondokuz Mayıs University has been obtained prior to experiments and all animal work was performed according to the Experimental Animal Care Rules of European Community Council.

Section sampling and the determination of total Purkinje number

The cytoarchitectonic characteristics of the Purkinje cell layer were identified using the criteria of Gundersen (14). According to the pilot study, 14-18 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 7 sections containing the cerebellum and then the consecutive samples were selected with a fixed interval of 7 sections.

The counting and analysis of Purkinje cells were performed with a stereology workstation using the optical fractionator counting method (CAST-GRID-Computer Assisted Stereological Toolbox-Olympus, Denmark) (15). Cell counts were done using a sampling scheme optimized for a total of approximately 500 cell counts per individual. Determined Purkinje sectional areas were scanned automatically using consecutive steps with 200x200 μ m x-y size. Every step in this scanning was individually analyzed with optical dissector probes using 100x oil-objectives. During optical dissector application, an unbiased counting frame comprising the 20% of the total step area was used for particle sampling and counting. Thus, the area sampling fraction (asf) is determined as (587/40.000) μ m².

According to previous pilot studies, a fixed dissector height of 10 μ m was predetermined and used throughout the study. It was left a 5 μ m for upper guard zone, applied particle counting through a 10 μ m dissector height and measured the section thickness. All such measurements were done using a digital microcator (Hidenhein, 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 29 \pm 2 μ m among all animals. After completing a throughout sampling for 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: $N = [1/ssf] \times [1/asf] \times [1/tsf] \times \sum Q'$

Statistical analysis

Statistical analysis was performed using SPSS statistical software package (version: 12.0). Differences between groups, the estimated number of the Purkinje cell, were analysed with one way ANOVA and Post Hoc Tukey tests. The P-level was set at P < 0.05.

BULGULAR

Representative light micrographs of sections through the cerebellum from the different groups of rats are shown in Fig.1 A–C. The mean±SEM total number of Purkinje cells, coefficient of variation (CV) and coefficient of error (CE) of control and experimental groups are presented in Table 1.

Table 1. Total Purkinje cell number in rat cerebellum.

Groups	Total Purkinje cell number (N±SEM)	CV	CE
Control (n=7)	317 182 ± 9667	0.067	0.064
Iron (n=7)	209 002± 7836*	0.062	0.077
Iron + Nicardipine (n=7)	265 659 ± 8291*‡	0.072	0.068

SEM; standard error of the mean, CV; coefficient of variation, CE; coefficient of error. *P<0.05 compared with control, ‡ P<0.05 compared with iron- treated alone.

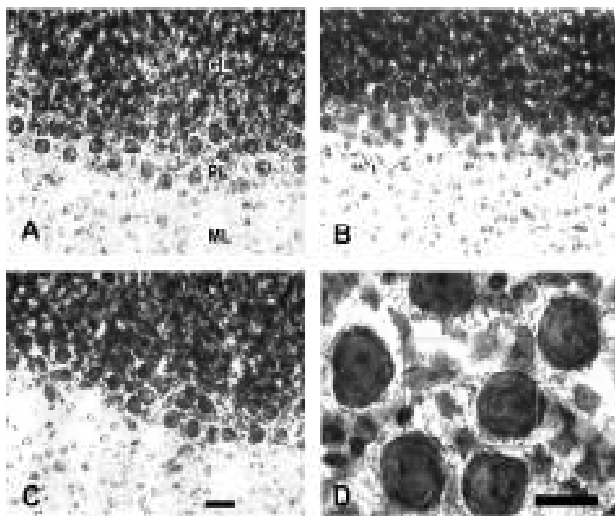


Figure 1. The photomicrographs of cerebellar Purkinje cells. (A) Control, (B) Iron treatment, (C) Iron+nicardipine treatment and (D) a larged photomicrograph of Purkinje cells. The layers of the cerebellar cortex is showed on the photomicrograph A. Bar: 40 µm (C) and 25 µm (D). GL: Granular cell layer; PL: Purkinje cell layer; ML: Molecular layer.

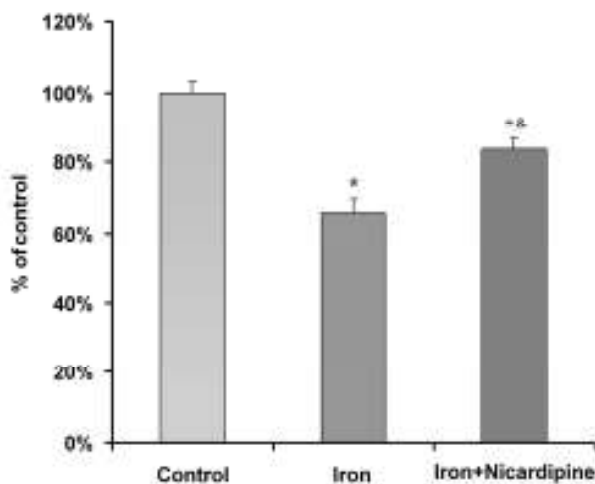


Figure 2. The percentage changes in Purkinje cell numbers in iron-treated and iron+nicardipine treated rats compared with control rats. Each vertical line represents the standard error of the mean (S.E.M.), (n=7). *p<0.05 compared with control, & p<0.05 compared with iron-treated alone.

After comparing iron group with control rats in iron group were appeared to have 34±4% less number of Purkinje cell than rats in control group and this difference was statistically significant (p<0.05), (Figure 2). Rats in iron+nicardipine group had 16±3% lower Purkinje cell numbers with respect to controls (p<0.05) (Fig.2).

In addition, comparison between iron and iron+nicardipine groups revealed that nicardipine significantly attenuates the iron-induced neuron loss from 34 % to 16% and protects Purkinje cells against iron toxicity (p<0.05), (Fig.2).

DISCUSSION

The present study focused on the effect of a dihydropyridine (DHP) calcium antagonist, nicardipine, on intracerebro-ventricular applied iron-induced Purkinje cell loss, using unbiased stereological techniques. This is the first study that demonstrates the neuroprotective effect of nicardipine against iron toxicity on cerebellar Purkinje cells.

We estimated that there are about 300-330 thousand Purkinje cells in the normal rat cerebellum using an unbiased stereological technique. 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 (11,16). Purkinje cells play a vital role in the normal function of the cerebellum. However, it has been demonstrated that Purkinje cells are highly susceptible to a variety of pathological conditions and toxic substances such as ischaemia (17), ethanol (10), long-term nicotine exposure (11) and cadmium toxicity (12). Similarly, this study is exhibited also that intracerebroventricular iron administration has a neurotoxic effect for Purkinje cells in rat cerebellum.

Although essential for cell function, increased tissue iron can promote tissue oxidative damage to which the brain is especially vulnerable (18). It has been reported that the subpial injection of iron salts causes the transient formation of free radicals (3). When the concentrations of free radicals exceed the normal levels, they lead to membrane peroxidation and membrane disintegration which start to bind the unsaturated bonds of fatty acids and cholesterol (4). This interruption of membrane integrity threatens the transmembrane differences of ionic concentrations and cations, resulting in an influx of extracellular ions, especially calcium begin to enter the cell (5). Thus, resulting excessive calcium influx is generally held responsible for triggering of epileptic discharges and cellular death (19). We have also demonstrated in previous studies that intracortically administrated iron causes hippocampal neuronal loss and a calcium channel blocker, flunarizine, attenuates the neurotoxic effects of iron (13).

Strong evidence from studies in humans and animal models suggests that nicardipine increases cerebral blood flow (20), and it is used for the treatment of cerebral vasospasm (21). Purkinje cells have at least three types of voltage-gated

calcium channels; T-type, DHP-sensitive L-type, N-type. Nicardipine also prevents calcium ion influx into neurons by blocking L-type channels outside the membrane. Furthermore, in previous studies showed that nicardipine depressed penicillin induced epileptiform activity (22) and the Purkinje cell density was 16-23% less in nicardipine-treated group than control and cells were partially protected significantly from toxic effects of cadmium (12). Our results confirm the previous studies and demonstrate that the neuroprotective effects of nicardipine against iron toxicity on Purkinje cells. According to our findings, nicardipine attenuates significantly the iron-induced Purkinje cell loss from 34% to 16%.

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