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Inhibition of neuronal nitric oxide synthase prevents iron-induced cerebellar Purkinje cell loss in the rat

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Iron plays an important role in maintaining normal brain function. However, in many neurodegenerative diseases abnormal iron accumulation in specific brain regions has been consistently reported. In this study, we investigated the neurotoxic effect of the intracerebroventricularly injected iron on the cerebellar Purkinje cells in the rat and the role of nitric oxide (NO) in this process. The role of NO in rats administered iron ($FeCl_3 6H_2O$) was examined with the use of a donor of NO, L-arginine (L-Arg), and a central selective inhibitor of NO synthase, 7-nitroindazole (7-NI). For this reason, rats were divided into 5 groups: control, iron-injected, iron plus L-Arg, iron plus 7-NI, and iron plus L-Arg plus 7-NI. Means (value ± standard deviation) of the total numbers of Purkinje cells in the cerebellum were estimated as 337 ± 23, 209 ± 16, 167 ± 19, 305 ± 26, and 265 ± 14 thousands in the control, iron, iron plus L-Arg, iron plus 7-NI, and iron plus L-Arg plus 7-NI groups, respectively. Iron treatment alone and the combination of iron and L-Arg caused a significant reduction in the total number of cerebellar Purkinje cells. Therefore, L-Arg increased the Purkinje cell loss induced by treatment with iron. These data show that inhibition of the neuronal NOS by 7-NI can prevent some of the deleterious effects of iron on cerebellar Purkinje cells. Presence of L-arginine decreased the neuroprotective effect of 7-NI.

Key words: iron, neurotoxicity, Purkinje cell, nitric oxide, stereology

INTRODUCTION

Iron plays an important role in maintaining normal brain function and is the most abundant transitional metal in the brain (Moos 2002). Iron deposition is a characteristic feature of common neurodegenerative diseases such as Parkinson's and Alzheimer's diseases as well as the much rarer disorders such as aceruloplasminemia, Hallervorden-Spatz disease (neurodegeneration with brain iron accumulation), Friedreich's ataxia, and neuroferritinopathy (Zecca et al. 2004). It is still not clear whether abnormal accumulation of iron leads to neurodegeneration or the accumulation follows neuro degeneration occurring due to other reasons.

The neuronal nitric oxide synthase (nNOS) is constitutively expressed in a fraction of brain neurons (Brown and Bal-Price 2003). Nitric oxide (NO) is an important modulator of neuronal function in the central nervous system (CNS). However, under certain conditions its excessive formation may be an important mediator of the nervous tissue damage (Dawson and Dawson 1995). NO in high concentration is indirectly neurotoxic through various mechanisms, including iron-mediated lipid peroxidation. It also liberates iron from cell stores (Iadecola 1997, Bishop and Robinson 2001) and depletes cell energy by disruption of mitochondrial enzymes and nucleic acids. Thus, release of NO may also trigger neuronal apoptotic cell death (Iadecola 1997). Schulz and coworkers (1995) showed that a central selective inhibitor of nNOS, 7-nitroindazole (7-NI) produced significant neuroprotection against NMDA-mediated excitotoxic striatal lesions. In the rat model of focal cerebral ischemia, a significant neuroprotection was obtained with 7-NI. The effect was reversed by L-arginine (L-Arg) (Yoshida et al. 1994). Nevertheless, the mechanisms of NO neurotoxicity are still unclear.

Purkinje cells play a vital role in normal functioning of the cerebellum. They are highly susceptible to

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a variety of pathological conditions such as ethanol (Bonthius et al. 1996) and ischemia (Welsh et al. 2002). To our knowledge, current literature on neurotoxicity does not contain sufficient quantitative information on the influence of NO on the cerebellar Purkinje cell loss induced by iron. In the present study, we used the unbiased stereological techniques to investigate whether the intracerebroventricular (i.c.v.) injection of iron in Wistar rats' changes total numbers of cerebellar Purkinje cells and whether the NO precursor, L-Arg, and a nNOS inhibitor, 7-NI, have protective effects against the iron neurotoxicity.

METHODS

Adult male rats were obtained from the Experimental Research Center of the Ondokuz Mayis University. Approval of Ethical Committee of the Ondokuz Mayis University was obtained prior to experiments and all animal work was performed according to the Experimental Animal Care Rules of the European Community Council. Thirty-five adult male Wistar rats (235–255 g) were housed individually on a 12-h light: 12-h dark cycle (lights on at 07:00 AM), at a temperature of 21 ± 2°C and 50% humidity. All animals were kept in constant laboratory conditions and supplied with food and water ad libitum. Before the experimental procedures, rats were randomly assigned to five groups, each consisting of seven rats: a control group receiving injections of normal saline, iron group receiving iron, iron plus L-Arg group receiving iron and L-Arg, iron plus 7-NI group receiving iron and 7-NI, and iron plus L-Arg plus 7-NI group receiving iron, L-Arg, and 7-NI.

Animals were deprived of food for 12 hours prior to surgery and weighed just before the operation. Anesthesia was induced by intraperitoneal (i.p.) injection of ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg). After the skin covering the skulls was shaved and animals were fixed in a stereotaxic apparatus, a rostro-caudal incision of 2 mm 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 1.5 mm lateral and 0.8 mm posterior to Bregma using a dental drill. All drugs were administered with a Hamilton microinjector introduced stereotactically into the left lateral ventricle through the hole drilled in the skull, that was lowered to a depth of 2.5 mm below the brain surface. Infusion rate was 0.5 µl/min. Rats in the control group received saline (2.5 µl, i.c.v.) while rats in iron, iron plus L-Arg, iron plus 7-NI, and iron plus L-Arg plus 7-NI groups received a single dose of 200 mM $FeCl_3$ (2.5 µl, i.c.v.) like in the experiments of Willmore and others (1983). Then the incision has been sutured and the suture area was cleaned using 10% povidon iodide before placement of the animals into their cages. All animals survived for ten days following the surgery and animals in the iron plus L-Arg, iron plus 7-NI, and iron plus L-Arg plus 7-NI groups were injected L-Arg (1 g/kg/day, i.p.) (cf. Shi et al. 2003) or/and 7-NI (40 mg/kg/day, i.p) (cf. Marangoz and Bagirici 2001) once daiyly during that period. The first doses of L-Arg and 7-NI were injected during the first five minutes following the surgical operation. Rats belonging to other groups received the same amount of i.p. saline injections throughout ten-day survival period.

After the survival period, all animals were perfused intracardially under deep urethane anesthesia (1.25 g/kg, i.p.) with 10% formaldehyde in saline, buffered for pH 7.2. After completion of the perfusion brains were removed and placed in the same fixative for postfixation. Cerebella were embedded in the paraplast embedding medium using the standard histological technique. Serial tissue sections were obtained using a rotary microtome (Leica RM 2135) in the 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. Iron ($FeCl_36H_2O$), L-Arginine, 7-NI, and Cresyl violet were obtained from Sigma Chemical Co. (St. Louis, Mo, USA); entellan, xylene and acetic acid were obtained from Merck (Darmstadt, Germany); formaldehyde, chloroform and others were obtained from Aklar Chemistry (Ankara, Turkey).

The cytoarchitectonic characteristics of the Purkinje cell layer were identified using the criteria of Gundersen (Gundersen et al. 1988). According to the pilot study, 12–15 sections were sampled in a systematic random fashion (ssf: 1/7) out of a total of 100–120 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.

Total Purkinje cell numbers in the cerebellum were estimated with the optical fractionator counting method, which is a combination of the fractionator

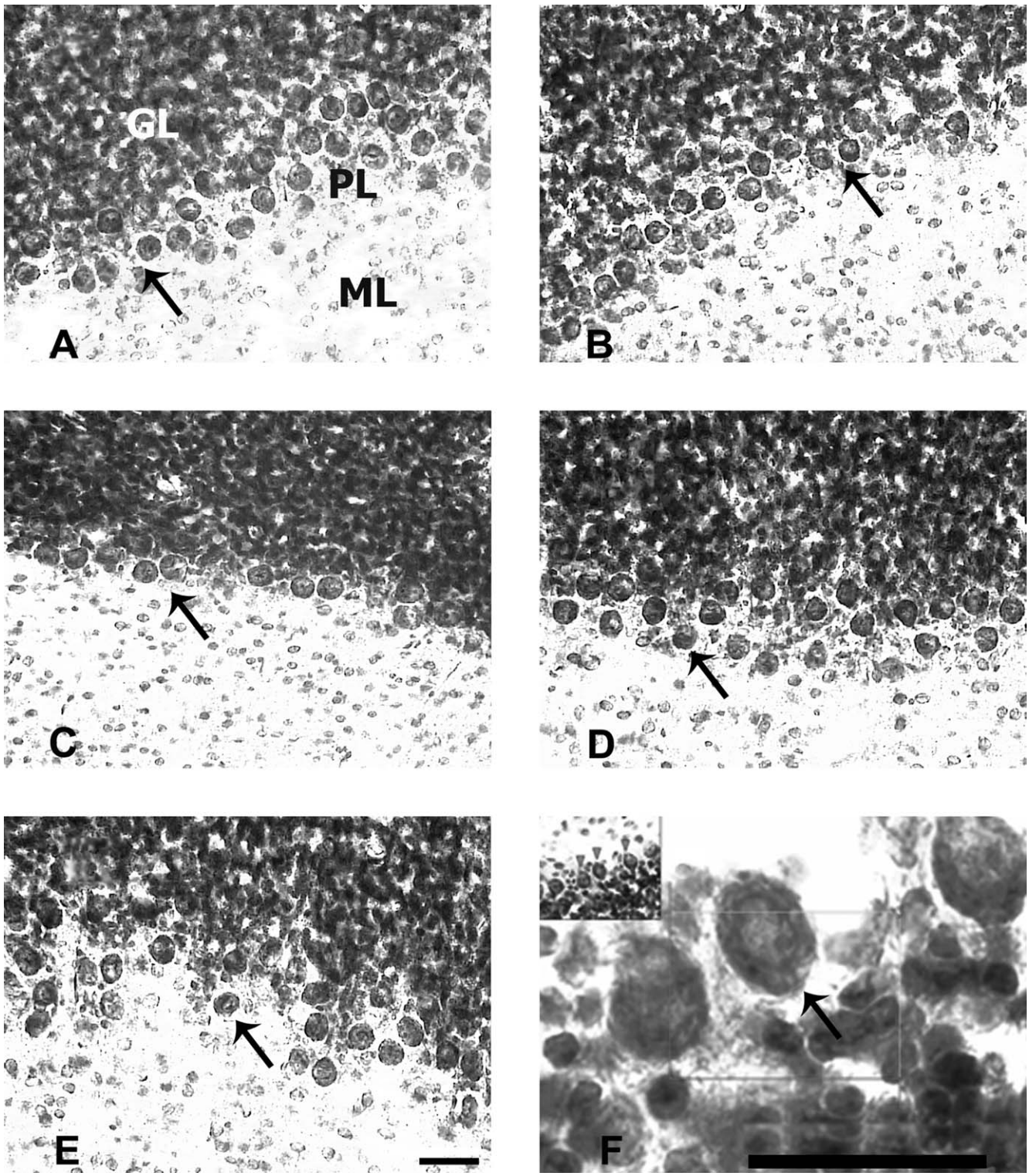

Fig. 1. Photomicrographs of the cerebellar Purkinje cells from the: (A) control (saline) group, (B) iron-injected group, (C) iron plus L-Arg-injected group, (D) iron plus 7-NI-injected group, (E) iron plus L-Arg plus 7-NI-injected group. (F) an larged picture of the Purkinje cells. At the photomicrograph (A) layers of the cerebellar cortex are indicated: (GL) granular cell layer; (PL) Purkinje cell layer; (ML) molecular layer. The length of the calibration bars equals to 40 μm. The bar in the photomicrograph (E) applies also to photomicrographs (A–D). The black arrows indicate the Purkinje cells.

sampling scheme and dissector counting technique (Gundersen 1986). 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 approximately 500 cell counts per individual. Randomly selected Purkinje sectional area was scanned automatically using consecutive steps of the 200 × 200-μm x-y size. Every step in this scanning was individually analyzed with optical dissector probes using 100× oil-objectives. When the total Purkinje cells number in a sample was estimated, the area of the "unbiased counting frame" was 587 μm^2. A counting frame was placed on sections in a systematic, uniform, randomized manner, and appropriately, sampled nuclei of neurons at the widest portion of the counter were counted (Gundersen 1977). Thus, the area sampling fraction (asf) is determined as (587/40 000) μm^2.

The last sampling level in the 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. Virtual movement of a section plane (the focal plane) through the section thickness forms this height. Generally, a narrow upper "guard zone" was passed before the actual optic disector counting was started in order to avoid the possible irregularities of the sectional surface. We were leaving a 5-μm upper guard zone, then applied particle counting through 10-μm dissector height, and afterwards 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 (disector 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. After completing sampling throughout all sampled sections, properly sampled Purkinje cells were counted as dissector particles (Q-). Total number of the cerebellar Purkinje cells (N) were then calculated using the following formulation: $N = (1/ssf) \times (1/asf) \times (1/tsf) \times \sum Q^-$

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 with the one-way ANOVAs with *post-hoc* Tukey test. The P value <0.05 was considered to be statistically significant.

RESULTS

The mean values of the eaverage section thickness, coefficient of variation (CV) and coefficient of error (CE) in all groups were respectively 27.52 ± 1.86 μm, 0.068 and 0.071. Figure 1A–F presents the representative light micrographs of sections through a rat cerebellum in each study group. Figure 2 shows percentage of reduction in the number of Purkinje cells in the experimental groups. The means (value ± standard deviation) of the total numbers of Purkinje cells in the cerebellum were estimated as 337 041 ± 23 081, 209 402 ± 15 840, 166 639 ± 19 050, 304 654 ± 25 786, and 265 002 ± 14 203 in the control, iron, iron plus L-Arg, iron plus 7-NI, and iron plus L-Arg plus 7-NI groups, respectively. Statistical analysis showed a significant Purkinje cell loss in the cerebellum of animals in the iron group (–37.8 ± 3.6%, $P<0.05$), iron plus L-Arg group (–50.8 ± 10.5%, $P<0.01$), iron plus 7-NI group (–9.6 ± 5.8%, $P>0.05$), and in the iron plus L-Arg plus 7-NI group (–21.4 ± 10.8%, $P<0.05$) in comparison to the control group. The number of Purkinje cells in the iron group was significantly higher than that in the iron plus L-Arg group ($P<0.05$). Therefore, L-Arg increased the Purkinje cell loss induced by the treatment with iron ions ($P<0.05$).

There was no difference in the Purkinje cell number between the iron plus 7-NI group and controls ($P>0.05$). Most importantly, statistical analysis showed that the mean number of the Purkinje cells in the iron plus 7-NI group was significantly higher than that in the iron only group (+28.2%, $P<0.05$), iron plus L-Arg group (+41.2%, $P<0.05$, Fig. 2), and iron plus L-Arg plus 7-NI group (+11.8%, $P<0.05$, Fig. 2). The mean numbers of the Purkinje cells in the iron plus L-Arg plus 7-NI group was significantly higher than those of the iron group (+16.4%, $P<0.05$) and the iron plus L-Arg group (+32.4%, $P<0.05$), and lower than that of the control group (–21.4%, $P<0.05$). The numbers of Purkinje cells in the iron plus 7-NI group was significantly higher than that of the iron plus L-Arg plus 7-NI group ($P<0.05$). The number of Purkinje cells in the

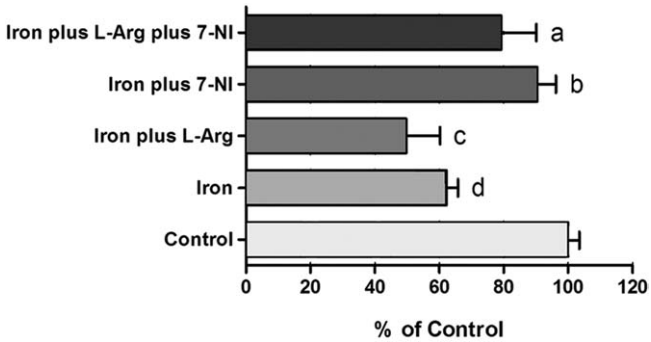

Fig. 2. The percentage of surviving cerebellar Purkinje cells in the iron-treated, iron plus L-Arg-treated, iron plus 7-NI-treated and iro plus L-Arg and 7-NI-treated rats, in comparison to controls. Data are presented as means ± SD (n=7 in each group). *Post-hoc* Tukey test revealed a significant Purkinje cell loss in the cerebellum of animals of all treated groups (except iron plus 7-NI group). Significance of differences was as follows: [a] $P<0.05$ vs. control, iron, iron plus L-Arg, and iron plus 7-NI groups; [b] $P<0.05$ vs. iron and iron plus L-Arg groups; [c] $P<0.05$ vs. all other groups except of control, where $P<0.01$; [d] $P<0.05$ vs. all other groups.

iron only group was significantly lower than those of the iron plus 7-NI and iron plus L-Arg plus 7-NI groups. Therefore, 7-NI exerted a significant neuroprotective effect against iron toxicity ($P<0.05$) and L-Arg decreased that effect ($P<0.05$).

DISCUSSION

We showed that animals that received the intraventricular injections of iron had significantly fewer Purkinje neurons in the cerebellum than animals in the control group (Fig. 1A–B and Fig. 2). These findings suggest that the Purkinje cells are sensitive to the damaging effects of iron, that is similar to the effects of nicotine (Chen et al. 2003) and cadmium (Bagirici et al. 2001).

We also showed that the intraventricular injections of iron combined with injections of L-Arg resulted in an even greater loss of Purkinje cells than in the group treated with iron only. These results show that L-Arg increased the neurotoxic effect of iron and increased the iron-induced Purkinje cell loss (Fig. 2). Excessive NO production is known to cause the oxidative stress to neurons that ultimately impairs their function and may result in cell death (Dawson and Dawson 1995). The aggravating effect of L-Arg might be due not only to NO produced out of it by nNOS but also by other isoforms of the enzyme. It may also depend on still another mechanism. It is likely that the neurotoxic action of NO is mediated by the peroxynitrite and/or superoxide anion and by perturbing iron metabolism (Reif and Simmons 1990).

In addition, comparison of the Purkinje cell percentages among the study groups revealed that 7-NI alone and L-Arg and 7-NI in combination significantly attenuate the iron-induced Purkinje cell loss from 37.8% to 9.6% and 21.4%, respectively (Fig. 2). Therefore, 7-NI protects the rat Purkinje cells against the iron toxicity. This result is in agreement with previous studies showing that 1-methyl-4-phenylpyridinium ion (MTP^+)-induced cell death in primary cultures of the cerebellar granule (CCG) cells was abolished by cotreatment with 7-NI (Gonzalez-Polo et al. 2004). Primary cultures of CGC are frequently used in neurodegeneration and neuroprotection studies (Zieminska and Lazarewicz 2006). The protective effect of 7-NI could be explained by its blocking the mediator effect of NO on iron toxicity. NO diffuses into neurons and other cells following its release from neurons and macrophages. During early postnatal development, both nNOS and, surprisingly, iNOS (in macrophages) are expressed constitutively at several-fold higher levels than in the adults (Zhu et al. 2005). On the other hand, Ciani and others (2002) showed that inhibition of the NO production induced by a wide spectrum of NOS inhibitors, like N^{ω}-nitro-L-arginine methyl ester, also results in the apoptotic cell death of CGC. Increased levels of NO may have variable effects on lipid peroxidation (Blomgren and Hagberg 2006) and reasons for these effects are as yet unclear, but in part they may be attributed to differences in the experimental design, e.g., the drug dose, duration of exposure or differences among strains of the tested animals.

CONCLUSION

Iron treatment alone and the combination treatment of iron plus L-Arg caused a significant reduction in the total number of cerebellar Purkinje cells. Inhibition of neuronal nitric oxide synthase by 7-NI had a significant neuroprotective effect against iron and iron plus L-Arg toxicity on cerebellar Purkinje cells in the rat.

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