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Hematological Disorders in Ibotonic Acid-Induced Rat Model of Alzheimer's Disease

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Abstract: The present research was conducted in order to investigate the effects of right-unilateral lesion of substantia nigra neurons by means of Ibotonic acid, a cholinergic-selective neurotoxin, on hematological parameters in rats. Thirty male Wistar rats weighing 200±50 g at the start of the experiment were used. The substantia nigra was right-unilateral lesioned by stereotaxic microinjections of 8 μg (free base) ibotonic acid, dissolved in 4 μL physiological saline containing 0.1% ascorbic acid, administered through the Hamilton microsyringe over 4.50 min. Seven days after neurosurgery, we assessed the total number of White Blood Cells (WBC), the total number of Red Blood Cells (RBC), Red cell Distribution (RDW), platelet and hemoglobin level and the erythrocyte indexes, Mean Cell Volume (MCV), Mean Cell Hemoglobin (MCH), Mean Cell Hemoglobin Concentration (MCHC). Ibotonic acid treatment induced a highly significantly decrease of white blood cells ($p<0.001$), followed by significant decrease in red blood cells ($p = 0.02$) and hemoglobin level ($p = 0.03$) comparative with sham-operated rats. Also, in the ibotonic acid-lesioned rats the erythrocyte indexes, Mean Cell Volume (MCV) ($p = 0.04$); Mean Cell Hemoglobin (MCH) ($p = 0.02$)) were significantly decreased comparative with sham-operated rats. By contrast, in the ibotonic acid-lesioned rat's platelet (0.34), Mean Cell Hemoglobin Concentration (MCHC) ($p = 0.41$) and Red blood cell Distribution Width (RDW) ($p = 0.03$) were significantly enhanced comparative with sham-operated rats. On the whole, the obtained data indicate the important role of the central nerves system in the regulation of erythrocyte dynamics.

Key words: Substantia nigra, ibotonic acid, hematological parameters, hemoglobin, regulation

INTRODUCTION

Alzheimer's Disease (AD) is characterized by degenerative changes in the brain (Etienne *et al*., 1986) and the impairment of learning and memory function (Miranda *et al*., 2003). It is widely accepted that learning and memory are deeply associated with the functional activity of the cholinergic system in the Central Nervous System (CNS). The Basal Fore brain (BF) provides the major source of cholinergic in put to the neocortex and hippocampus (Miranda *et al*., 2003) and the cholinergic neurons in the Nucleus Basalis of Mynert (NBM) are markedly degenerated in AD (Etienne *et al*., 1986). Because, the Nucleus Basalis Magnocelluralis (NBM) in the rat is analogous to the NBM in humans, the NBM-lesioned rat has been regarded as an animal model for cholinergic dysfunction in the cerebral cortex of patients with AD (Wu *et al*., 2005). The NBM-lesioned rat shows decreases in cholinergic markers, such as Acetylcholine (Ach) release (Meyer *et al*., 1987) and Choline Acetyltransferase (ChAT) activity (Arendash *et al*., 1987), in the cerebral cortex and shows learning and memory impairment (Salamone *et al*., 1984). In AD, the principal neurochemical abnormality is the alteration of the cholinergic system in the Central Nervous System (CNS) (Zatta *et al*., 2002; Kaizer *et al*., 2005).

Acetyl Cholinesterase (AChE, E.C. 3.1.1.7) is an important regulatory enzyme, which rapidly hydrolyses the neurotransmitter Acetylcholine (ACh) found mainly in the brain, muscles, erythrocytes and cholinergic neurons (Ahmed *et al*., 2006). Hematopoiesis, the dynamic process of blood cell production and development, is characterized by a continuous, robust turnover of cells. The hematopoietic system, which consists of the Bone

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Marrow (BM), liver, spleen, lymph nodes and thymus, provides leukocytes, erythrocytes and platelets through a complex network of tissues, organs, stem cells and regulatory factors (Huang and Liu, 2009).

However, the mechanism causing anemia associated with autonomic dysfunction is not well explained. Catecholamines and their corresponding receptors are widely distributed in both the central and peripheral nervous system. Besides, their vasoactive effect (Siarakas *et al*., 1997), catecholamines have been known to be involved in different forms of learning and memory (Hefco *et al*., 2003; Spreng *et al*., 2001). Immune cell types associated with innate immunity such as natural killer cells, neutrophils and macrophages are the potential subjects to be regulated by catecholamines because, these cells express functional, β2- and/or α-adrenergic receptors (Dent *et al*., 2001).

The processes of hematopoiesis in the bone marrow are regulated by a variety of factors including those produced by the stromal elements, the developing hematopoietic cells themselves and by exogenous substances, such as hormones and neurotransmitters, like acetylcholine. In particular, cutting nerves, which enter the hip bones influenced erythropoiesis, while injecting nicotine inside the bone induced changes in the processes controlled by the autonomic nervous system (Desforges, 1984). These data demonstrated the role of the bone marrow innervation in hematopoiesis and the presence of nicotinic acetylcholine receptors within the bone marrow. This view was further supported by the recent studies of nicotine-stimulated changes in hematopoiesis (Khaldoyanidi *et al*., 2001; Serobyan *et al*., 2005).

Nicotinic acetylcholine receptors (nicotinic receptors) are ligand-gated ion channels mediating synaptic transmission in nerve and muscle cells. They are composed of several types of alpha and beta subunits forming either homomeric or heteromeric functionally distinct receptor subtypes (Paterson *et al*., 2000). Nicotinic receptors are also present in many non-excitable cells, such as skin keratinocytes (Arrendolo *et al*., 2003), respiratory tract epithelial cells, vascular endothelium (Conti-Fine *et al*., 2000) and most of the blood cells: leukocytes Cormier *et al*. (2004), lymphocytes (Kawashima and Fujii, 2003), macrophages (Wang *et al*., 2003) and erythrocytes (Bennekou, 1993), where their functions are quite different from those in muscles or neurons. In summary, the primary goal of this study was to evaluate whether disorders hematopoiesis regulation via Nucleus Basalis Magnocelluralis (NBM) neuron lesion may induce hematological disorders.

MATERIALS AND METHODS

This research was conducted from June 2008-2009.

Animals and surgery: Thirty male Wistar rats, weighing 250-300 g and housed with free access to food and water. They were maintained in a 12-h light/dark cycle with lights on at 6: 00 am at constant temperature (25°C). The animals were divided into two groups: Sham Operated (ShO) and Ibotenic acid (IBO), containing 15 and 15 rats, respectively. The rats were not found to have other causes of anemia, the nutritional state was adequate and no evidence of any hemorrhagic and thrombotic disorder.

Neurosurgery and drug administration: On the day of surgery, the animals were anesthetized with ketamine/xylazine (50 mg kg^{-1}, i.p.) and placed in a stereotaxic apparatus (Kopf). The incisor bar was set-0.16 mm posterior and ±0.40 mm lateral to the bregma and -0.55 mm below the top of the skull (Ahmed *et al*., 2004; Paxinos and Watson, 2007; Yamamoto *et al*., 2003) to reach the nucleus basalis magnocellularis. Rats received bilateral infusions of 0.5 μL volume of vehicle (NaCl 0.9%) or ibotenic acid (8 μg μL^{-1}) using a 5 μL Hamilton syringe. The injection was given over a period of 5 min and the needle was left in the injection site for a further 5 min. After behavioral tasks, rats were sacrificed for neurochemical analysis. On the 7th day: 15 rats were evaluated, from the ShO and IBO groups, respectively and on the 28th day: 10 and 10 rats were evaluated, from both ShO and IBO groups, respectively. The protocol concerning this research is in accordance with the guidelines of the Committee on the Care and Use of Experimental Animal Resources, School of Medicine, Ahwaz Jondishapour University of Medical Sciences, Ahwaz, Iran. Hematological parameters were assayed one week after the neurosurgery.

Blood sampling protocol: One week after neurosurgery, blood samples were withdrawn via the Biotrol sampling catheter from 15 sham operated and 15 ibotenic acid-treated rats. Blood samples (0.5 mL approximately/sample) were collected in vials containing EDTA for hematological investigations. Hematological parameters were assayed by a COULTER® Ac•T 5diff™ CP-precision instrument for hematology research. In order to rule out, the iron deficiency anemia, the standard discrimination indices were calculated by using red blood cell indices as in our previous study (Keikhaei *et al*., 2007).

Histological ShO: At the end of the experiment, all rats were sacrificed with an overdose of sodium pentobarbital (100 mg kg^{-1} i.p.) followed by a transcardial infusion of 0.9% saline and a 10% formalin solution. The brains were removed and placed in a 30% sucrose/formalin solution.

The brains were frozen and cut into coronal sections (50 μm) using a freezing microtome and stained with crystal violet for verification of the point of the syringe needle. Only experimental data from lesions correctly located in the substantia nigra were used for statistical analysis.

Data presentations and statistical analysis: Results were expressed as mean±SEM. Because, the data were not normally distributed, the non-parametric statistic Mann-Whitney U test was employed. Results were considered significant if $p<0.05$. The number of observation was 30.

RESULTS AND DISCUSSION

Experimental data were registered one week after the ibotenic acid administration (Table 1). We have calculated the different indices for ibotenic acid-lesioned rats individually. The differential value for each discrimination index in differentiation between non iron deficiency and iron deficiency anemia are shown in Table 2. In the ibotenic acid-lesioned rats, we observed a significant decrease in the total number of white blood cells ($4.36\pm0.5\ 10^3\ mm^{-3}$ vs. $8.9\pm0.26\ 10^3\ mm^{-3}$, U = 34.5 $p<0.001$), Mean Cell Volume (MCV) (47.79±1.2 fL vs. 62.27±0.33 fL, U = 27.5 $p<0.05$), red blood cells ($7.40\pm0.1\ 10^6\ mm^{-3}$ vs. $9.05\pm0.26\ 10^6.mm^{-3}$, U = 50.5 $p<0.05$) and hemoglobin level ($11.09\pm0.32\ g\ L^{-1}$ vs. $9.9\pm0.3\ g^{-1}L$, U = 41.5 $p<0.05$) (Fig. 1) compared with the sham-operated groups. Ibotenic acid significantly increased Red cell Distribution Width (RDW) (15.92±0.28 fL vs. 13.16±0.29 fL, U = 28.5 $p<0.05$), platelet ($719.3\pm25.8\ 10^3\ mm^{-3}$ vs. $702.9\pm12.7\ 10^3\ mm^{-3}$, U = 35.5 $p<0.05$) and Mean Cell Hemoglobin Concentration (MCHC) ($32.93\pm0.61\ g\ dL^{-1}$ vs. $31.2\pm0.4\ g\ dL^{-1}$, U = 50.5 $p<0.05$) compared with sham-operated groups (Fig. 2).

It is well recognized that the immune response is under the influence of a variety of neural or neuroendocrine mechanisms. Much less studied is the possible influence of these mechanisms on hematopoiesis. Previous studies reported that the central dopaminergic system has a crucial role in regulation of the immune processes as well as hematopoiesis (Nanda *et al*., 2005; Pacheco-Lopez *et al*., 2003). In our present study, we used a procedure of chemical sympathectomy by lesioning the basalis magnocellularis with ibotonic acid. By means of this particularly electrolytic lesion, we observed a significant decrease in hematological parameters registered one week after ibotonic acid administration, tested by the total number of leukocytes, erythrocytes, hemoglobin level and the erythrocyte indexes (MCV and MCH). Interestingly, we observed increase in platelet numbers, Red Dell Width distribution (RDW) and other erythrocyte index (MCHC) in ibotenic acid-lesioned rats comparing to sham operated group. We demonstrated that rats treated with ibotonic acid showed anemia. In addition, since the WBC significantly decreased during ibotonic acid-induced anemia, the effect of ibotonic acid in this experiment may be specific for erythropoiesis, as well as for bone morrow suppression. The regulation of the hematopoietic system is achieved at three levels: at the cellular level of bone marrow stroma, at the humoral level by cytokines and by catecholamines and other neuroendocrine factors. Sympathetic nerve endings and bone marrow cells are the main source of bone marrow catecholamines (Felten, 1993; Maestroni, 1998; Hoogduijn *et al*., 2006). Among the catecholamines, a substantial amount of dopamine was detected in bone marrow (Marino *et al*., 1997). Bone marrow catecholamines originate from sympathetic nerve fibers and from hematopoietic cells directly. Catecholamines of neural origin show a circadian rhythmicity. Adrenoceptors present on bone marrow cells include the 1-subtype, which seems to mediate the catecholaminergic ShO of hematopoiesis.

Kaizer *et al*. (2008) have suggested that AChE activity in erythrocytes may be considered a marker of easy access of the central cholinergic status. Many researches showed that the bone marrow acetylcholine can be released by the stromal elements affecting generation and differentiation of blood cells. In the cited research nicotine affected hematopoiesis supportive stromal microenvironment, interfering with the stem cell

Table 1: Demographic variables of study groups with their U and p-values

Variables	IBO	ShO	U-value	p-value
Hemoglobin ($g\ dL^{-1}$)*	11.09±0.32	14.17±0.15	41.5	0.030
Mean Cell Hemoglobin (MCH) (pg)*	15.99±0.21	21.6±0.36	50.5	0.020
Mean Cell Volume (MCV) (fl)*	47.79±1.2	62.27±0.33	27.5	0.040
Mean Cell Hemoglobin Concentration (MCHC) ($g\ dL^{-1}$)£	32.93±0.61	31.2±0.4	50.5	0.470
White blood cell count ($10^3/mm^3$)*	4.36±0.5	8.9±0.26	34.5	0.001
Platelet count ($10^3/mm^3$)©	719.3±25.8	702.9±12.7	35.5	0.340
Red Blood Cell (RBC) ($10^6/mm^3$)*	7.40±0.1	9.05±0.26	50.5	0.020
Random Distribution of RBC Weight (RDW)£	15.92±0.28	13.16±0.29	28.5	0.030

©: Significant increase in values of variable among the ibotenic acid exposed group (IBO); *: Significant decrease in values of variable among the sham operated group (ShO); £: Non significant difference

Table 2: The differential value for each discrimination index in differentiation between non iron deficiency and iron deficiency anemia

	Calculated values of different discriminate indices											
Ibotonic-leisoned study groups	Mentzer Index (MI)		England and Fraser index (E and F)		Srivastava Index (SI)		Green and King index (G and K)		Shine and Lal index (S and L)		Red blood cell Distribution Index (RDWI)	
	IDA>13	NIDA<13	IDA>0	NIDA<0	IDA>3.8	NIDA<3.8	IDA>65	NIDA<65	IDA>1530	NIDA<1530	IDA>220	NIDA<220
Case 1	-	6.40	-	-23.62	-	2.044	-	41.3	-	378.49	-	90.14
Case 2	-	7.50	-	-15.97	-	2.47	-	42.01	-	405.09	-	116.94
Case 3	-	6.45	-	-24.48	-	2.13	-	46.69	-	380.28	-	105.06
Case 4	-	6.39	-	-12.36	-	2.11	-	38.52	-	417.5	-	97.82
Case 5	-	7.36	-	-21.79	-	2.36	-	63.87	-	509.27	-	125.12
Case 6	-	6.34	-	-21.62	-	2.10	-	39.22	-	326.572	-	105.98
Case 7	-	5.61	-	-18.22	-	2.00	-	31.22	-	321.12	-	87.57
Case 8	-	5.89	-	-21.42	-	2.14	-	31.98	-	287.10	-	96.05
Case 9	-	7.29	-	-9.79	-	2.18	-	38.41	-	390.71	-	111.61
Case 10	-	5.51	-	-26.82	-	2.04	-	33.64	-	270.82	-	93.74
Case 11	-	6.38	-	-23.62	-	2.08	-	41.30	-	388.69	-	90.14
Case 12	-	6.42	-	-15.97	-	2.48	-	42.01	-	425.09	-	116.94
Case 13	-	7.49	-	-24.48	-	2.17	-	46.69	-	350.27	-	105.06
Case 14	-	6.44	-	-12.36	-	2.13	-	38.25	-	417.5	-	97.82
Case 15	-	6.37	-	-21.79	-	2.36	-	63.87	-	509.26	-	125.12

IDA: Iron Deficiency Anemia; NIDA: Non Iron Deficiency Anemia

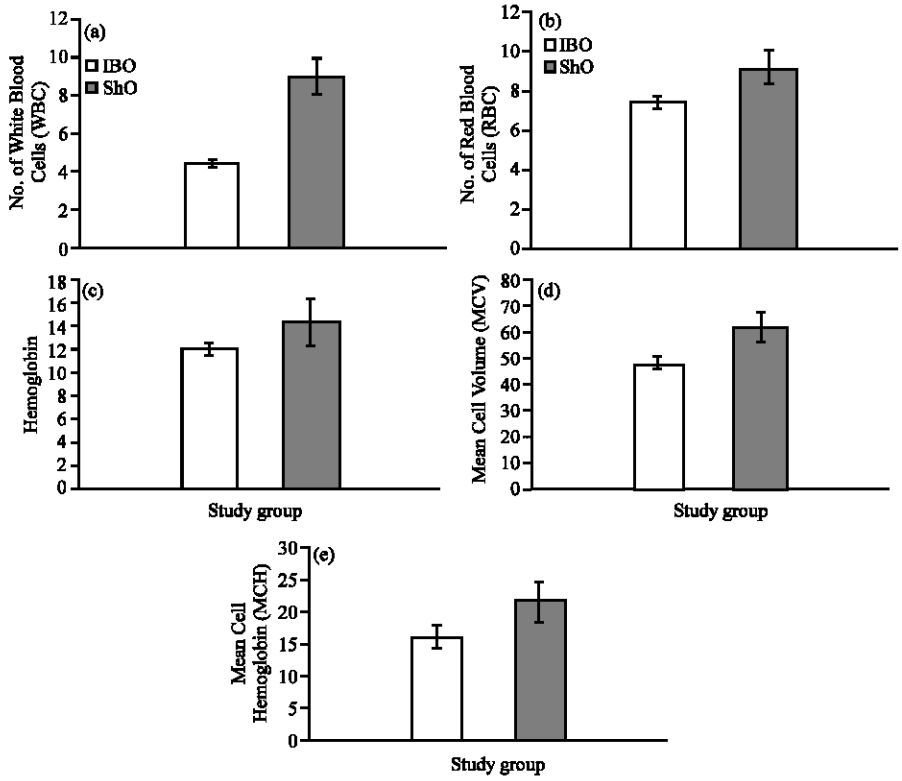

Fig. 1: a) The effect of the chemical lesion with ibotonic acid on total number of leukocytes ($p<0.03$ vs. sham-operated group); b): Total number of erythrocytes ($p<0.01$ vs. sham-operated group); c): Hemoglobin level ($p<0.02$ vs. sham-operated group); d): Mean cell volume ($p<0.04$ vs. Sham-Operated (SO) group) and E, Mean cell volume ($p<0.04$ vs. sham-operated group) all tested one week after lesion. Values are means±SEM (n = 15 per group)

homing. Later, it was shown that nicotinic receptors are expressed in the very hematopoietic cell precursors (Serobyan *et al*., 2007; Koval *et al*., 2008).

It has been well documented that there are α- and β-adrenergic receptors on the surface of the erythrocytes. However, β-receptors are more important in

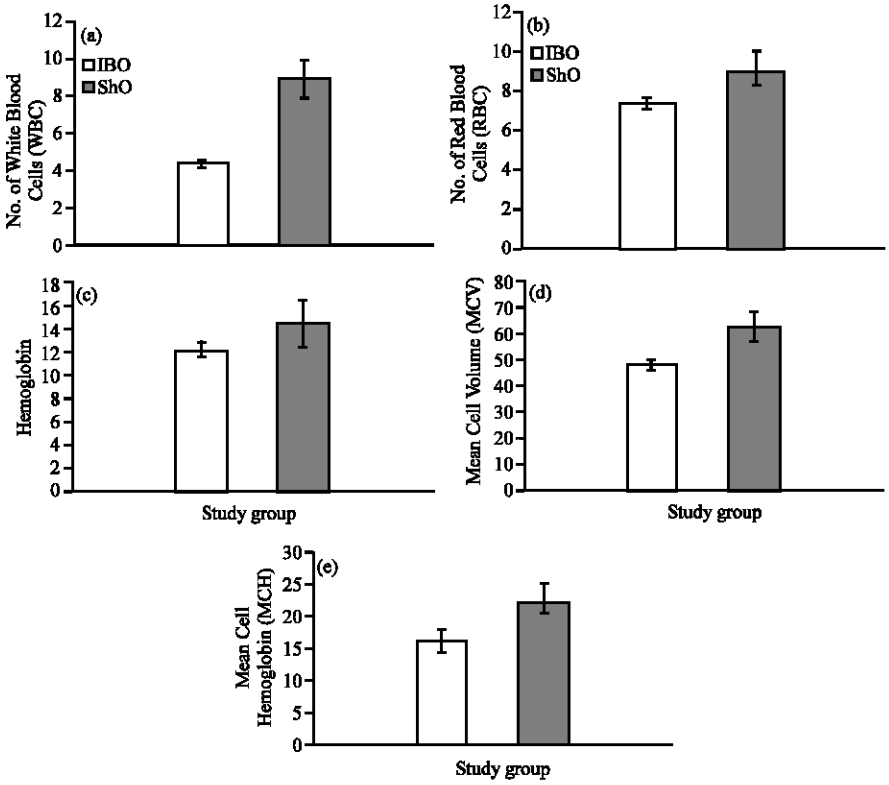

Fig. 2: a) The effect of the chemical lesion with ibotonic acid on increased mean cell volume ($p<0.04$ vs. sham-operated group); b): Platelet ($p<0.04$ vs. sham-operated group) and c): Red cell distribution width ($p<0.03$ vs. sham-operated group) all tested one week after lesion. Values are means±SEM (n = 15 per group)

erythropoiesis. Administration of an α-adrenergic stimulant did not elicit the erythropoietic effect, whereas a, β-adrenergic stimulant induced erythropoiesis in an *in vitro* culture of erythroid progenitor cells (Mladenovic and Adamson, 1984). Since, β-adrenergic receptors are abundant on the surface of erythrocytes, the effect of, α-adrenergic blocking agents has often been investigated using erythrocytes (Miklavc *et al*., 1989; Hritcu, 2006). In accordance with these findings, in the present study, we observed some abnormalities of hematopoiesis after electrolytic lesion of the central dopaminergic neurons from the substantia nigra by means of ibotonic acid. Ibotonic acid is a useful chemical agent for inducing neurogenic anemia.

Serum levels of methylmalonic acid rise in B_{12} deficiency, whereas homocysteine levels rise in both folate and B_{12} deficiency (Andrès *et al*., 2008). Hematological features of B_{12} and folate deficiencies include anemia, macrocytosis, thrombocytopenia, neutropenia and neutrophil hypersegmentation. Recently, elevated serum homocysteine in patients with Alzheimer's Disease (AD) has been described (McCaddon *et al*., 1998). Other studies have confirmed this observation and suggested that elevated homocysteine is a risk factor for cognitive decline and dementia (McCaddon *et al*., 2001, 2002; Seshadri *et al*., 2002; Seshadri, 2006; Schulz, 2007).

CONCLUSION

This study confirms earlier observations of increased plasma homocysteine and reduced serum folate levels in patients with clinically diagnosed AD Hb and platelet counts fell only slightly with increasing dementia duration, also there were other changes in hematological indices; macrocytosis and RDW in particular were related to disease duration and there were anemic subjects.

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