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Protective effects of glucose-6-phosphate dehydrogenase on neurotoxicity of aluminium applied into the CA1 sector of rat hippocampus

Marina D. Jovanović, Ankica Jelenković*, Ivana D. Stevanović, Dubravko Bokonjić**, Miodrag Čolić, Nataša Petronijević† & Danica B. Stanimirović††

*Military Medical Academy, Institute for Medical Research, *University of Belgrade, Institute for Biological Research, **Military Medical Academy, National Poison Control Centre, †University of Belgrade, School of Medicine, Institute of Biochemistry, Belgrade, Serbia & ††Institute for Biological Sciences, National Research Council, Ottawa, Canada*

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***Background & objectives*: Aluminum (Al) toxicity is closely linked to the pathogenesis of Alzheimer's disease (AD). This experimental study was aimed to investigate the active avoidance behaviour of rats after intrahippocampal injection of Al, and biochemical and immunohistochemical changes in three bilateral brain structures namely, forebrain cortex (FBCx), hippocampus and basal forebrain (BF).**

***Methods*: Seven days after intra-hippocampal (CA1 sector) injection of $AlCl_3$ into adult male Wistar rats they were subjected to two-way active avoidance (AA) tests over five consecutive days. Control rats were treated with 0.9% w/v saline. The animals were decapitated on the day 12 post-injection. The activities of acetylcholinesterase (AChE) and glucose-6-phosphate dehydrogenase (G6PDH) were measured in the FBCx, hippocampus and BF. Immunohistochemical staining was performed for transferrin receptors, amyloid β and tau protein.**

***Results*: The activities of both AChE and G6PDH were found to be decreased bilaterally in the FBCx, hippocampus and basal forebrain compared to those of control rats. The number of correct AA responses was reduced by $AlCl_3$ treatment. G6PDH administered prior to $AlCl_3$ resulted in a reversal of the effects of $AlCl_3$ on both biochemical and behavioural parameters. Strong immunohistochemical staining of transferrin receptors was found bilaterally in the FBCx and the hippocampus in all three study groups. In addition, very strong amyloid β staining was detected bilaterally in all structures in $AlCl_3$-treated rats but was moderate in G6PDH/$AlCl_3$-treated rats. Strong tau staining was noted bilaterally in $AlCl_3$-treated rats. In contrast, tau staining was only moderate in G6PDH/$AlCl_3$-treated rats.**

***Interpretation & conclusions*: Our findings indicated that the G6PDH alleviated the signs of behavioural and biochemical effects of $AlCl_3$-treatment suggesting its involvement in the pathogenesis of Al neurotoxicity and its potential therapeutic benefit. The present model could serve as a useful tool in AD investigations.**

Key words Active avoidance - aluminium - Alzheimer's disease - brain - glucose-6-phosphate dehydrogenase - neurotoxicity

Aluminium (Al) neurotoxicity has been implicated in the pathogenesis of Alzheimer's disease (AD)[1,2]. The first recognition of Al neurotoxicity in humans was recorded as encephalopathy in haemodialysis patients[3]. Individuals with impaired renal function are particularly vulnerable due to their poor ability to excrete Al, as the kidneys are a major route of Al elimination. Epidemiological studies have demonstrated that the continued consumption of potable water with a high Al concentration is a risk factor for developing Alzheimer-type dementia[4].

β-amyloid (Aβ) deposition and neurofibrillary tangles, the main neuropathological features of AD, occur in selectively vulnerable brain regions such as the hippocampus and the forebrain cortex (FBCx). Related changes are found in the same regions of brains exposed to Al[5]. Aluminum has also been found in senile plaques in post-mortem brain samples of individuals diagnosed with AD[6]. Aluminium is also capable of inducing neurofibrillary degeneration (NFD)[7].

The brain regions particularly affected by Al neurotoxicity include those involved in memory and learning. This may be due to the specific distribution of transferrin receptors (TfRs) and neuroanatomical connections between brain regions important for cognitive processes[8,9]. TfRs are important for toxic effects of Al in the brain. Al is transported by the iron-carrier protein transferrin (Tf) that enables its entry into the cell by binding to TfRs on the cell surface[8]. Some brain regions, especially the fronto-temporo-parietal cortex and hippocampus, express a high density of TfRs[8]. Therefore, the neurotoxic effects of Al predominate in the cortex and the hippocampus and can provide a neuroanatomical basis for Alzheimer-type dementia that develops as a consequence of Al intoxication[10].

One of the first steps in the chain of Al toxicity is the impairment of the pentose phosphate pathway of glucose metabolism that is catalyzed, in addition to other enzymes, by glucose-6-phosphate dehydrogenase (G6PDH). G6PDH activity is the main source of the reduced form of nicotinic adenine dinucleotide phosphate (NADPH) that is an essential supplier of reducing equivalents. Reducing equivalents are necessary for the adequate function of oxidative phoshorylation and antioxidative defense, including the conversion of glutathione from its oxidized form to its reduced form (GSH)[11]. Previous research showed decreased activity of G6PDH in the brain upon A1 exposure, which is achieved through G6PDH activity inhibition[12].

Both AD brains and brains exposed to Al, particularly basal forebrain, are characterised by the loss of cholinergic innervation, the decreased activities of acetylcholinesterase (AChE) and choline acetyltransferase, the markers of cholinergic system function and memory deficits[13,14]. Anatomical and neurochemical connections between brain structures involved in memory processes are of great importance to understand the involvement of cholinergic system in AD and Al-induced neuropathology. This accords with the suggestion of Dani and coworkers[15] that stimulated ACh release from cholinergic neurons would help AD patients. Deficits in cholinergic innervation of the FBCx and limbic structures, such as the hippocampus and the basal forebrain (BF), are very important for cognitive function.

Serious cognitive deficits result from the BF injury, including the aspects of attention concerning memory and learning. This could be attributed to the loss of both corticopetal cholinergic projections and hippocampal cholinergic inputs from the BF[16,17]. Some studies have indicated a discrepancy between the degree of cholinergic damage and cognitive impairment, *i.e.* more extensive loss of cholinergic neurons was followed by milder learning and memory impairment and *vice versa*[14]. However, the damage to the nucleus basalis of Meynert within the BF, which contains 90 per cent of the cholinergic neurons, has consistently been associated with impaired attention. Glutamatergic systems have also been shown to be important for learning and memory, *e.g.* in the perforant path and its terminals in the dentate gyrus[18]. The retrograde damage to the entorhinal cortex could also be produced by Al injection into the hippocampal CA1 field[18].

In the study, the active avoidance behaviour of rats was investigate after intrahippocampal injection of Al, as well as biochemical and immunohistochemical changes in brain regions involved in attention, learning and memory processes. Experiments were also performed using exposure to G6PDH, which was applied prior to Al application to study whether G6PDH pre-treatment could influence brain Al toxicity.

Material & Methods

This study was conducted in the Institute of Medical Research and National Poison Control Centre, Military Medical Academy, Belgrade, Republic of Serbia. The research protocol was approved by the

Ethical Committee of the Military Medical Academy, Belgrade, Republic of Serbia.

Animals: The experiments were performed on 12 week-old adult male Wistar rats. The animals were housed in polypropylene cages put in an air-conditioned room (temperature: 23±2ºC, dark/light cycle: 13/11 h). The animals were allowed free access to commercial rat food and tap water.

Experimental design: Before treatment, the rats were randomly assigned to three groups: A control group injected with 0.9 per cent w/v NaCl; a group pre-treated with G6PDH immediately before $AlCl_3$ injection; and a group injected only with $AlCl_3$. Each group consisted of 12 rats.

The rats were intraperitoneally anaesthetised using 0.04 g/kg body weight (bw) sodium thiopental. A single dose of $AlCl_3$ (Sigma, USA) (3.7×10^{-4} g/kg bw dissolved in deionized water) was injected into the left side of the CA1 sector of the hippocampus using a stereotaxic instrument for small animals. The position for injection into the CA1 sector of the hippocampus was determined from the skull surface, relative to the lambda suture defined from its centre: 3.1 mm dorsally, 4.3 mm laterally and 2.5 mm ventrally[19]. Immediately before $AlCl_3$ application, the second study group of rats received 2500 U/ml of a G6PDH suspension (Sigma) into the CA1 hippocampal sector. The control group of animals received saline in the same manner (also injected into the CA1).

Seven days after the treatment, the rats were subjected to a two-way active avoidance (AA) task over five consecutive days. Twelve days after treatment, the rats were sacrificed by decapitation and the brains of eight animals in each group were removed, flash-frozen in liquid nitrogen and used for biochemical analyses.

The brains from the remaining four animals of each group were used for immunohistochemical analysis, removed and fixed for 24 h in 4 per cent paraformadehyde (TAAB Laboratory Equipment, Aldermaston, UK), cryoprotected in graded sucrose at 4ºC. Thereafter, these were frozen in methylbutane and stored at -70º C until cryosectioning (Reichert Jung Cryocut-E, Leica Microsystems, Cambridge UK).

Biochemical analyses: Biochemical analyses were done in crude mitochondrial fractions kept on ice dissected from frozen ipsi- and contralateral FBCx, hippocampus and BF[20].

Activity of acetylcholinesterase: The acetylcholinesterase (AChE) activity determination was based on the degradation of acetyl thiocholine iodide into a product that binds to 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and turns yellow. The kinetics of the reaction was followed spectrophotometrically over 3-5 min at 412 nm. AChE activity was expressed as mM acetylthiocholine/min/g protein[21].

Activity of glucose-6-phosphate dehydrogenase: Activity of G6PDH was measured *via* the absorbance of NADPH generated in the reaction between G6PDH and its substrates glucose-6-phosphate (5 mM) and NADP (1 mM). The reaction mixture included Tris-Hepes 200 mM, *p*H 7.4 and 0.1 mM Na_2EDTA. Absorbance was measured at 340 nm. G6PDH activity was expressed in nM NADPH/h/mg protein[22].

Test of active avoidance: The acquisition of two-way active avoidance (AA) task was studied in a series of automatically-operated commercial shuttle-boxes and programming-recording units (Automatic Reflex Conditioner 7501, Ugo Basile, Milan, Italy). The acquisition of AA responses was based upon 50 attempts daily over five consecutive days. A conventional two-way AA attempt schedule was separated with intervals of 30 sec. Each attempt began with a conditioned stimulus (CS) (broad-band noise of 68 dB, lasting seven seconds) followed by an unconditioned stimulus (US) (foot shock of 3 mA, three seconds duration) which was delivered through the grid floor. Crossing responses during the CS (AA response) terminated the CS and prevented the onset of the US. The response after the onset of US (escape response) terminated both conditioned and unconditioned stimuli. Inter-trial crossings were not punished.

Immunohistochemistry: Frozen 8 μm thick sections of three bilateral brain structures (FBCx, hippocampus and BF) from each study group were mounted on poly-L-lysine coated slides and allowed to air dry. A DakoCytomation EnVision + System-HRP kit, Denmark was used for a two-step immunohistochemical staining technique. Cryostat sections were fixed in acetone. Endogenous peroxidase activity was blocked for 15 min by 0.03 per cent w/v hydrogen peroxide containing sodium azide (DakoCytomation). Slides were incubated with an appropriate dilution of monoclonal mouse antibody anti-CD71 (TfR) 1:400 (Abcam, Cambridge, UK), as well as rabbit polyclonal antibodies: anti-Abeta 1:50 (Abcam) and anti-Tau 1:50 (Abcam) for 60 min. Thereafter, slides were incubated with labelled polymer (DakoCytomation) conjugated to goat anti-rabbit or goat anti-mouse immunoglobulins in Tris-HCl buffer containing stabilizing protein, an antimicrobial agent and 5 per cent normal rat serum

for 30 min. Staining was completed by a 5-10 min incubation with 3,3'-diaminobenzidine (DAB) + substrate-chromogen (DakoCytomation), resulting in a brown coloured precipitate at the antigen site. Finally, slides were counterstained with hematoxylin and mounted with Kaiser gel (Merck, Germany). Control slides were incubated in the same way using mouse isotype-matched irrelevant antibody (Military Medical Academy, Serbia).

Photomicrographs were observed (magnification 400 x) by a Nikon Eclipse 50i microscope and photographed with Nikon digital camera DXM1200C (Nikon Instruments Inc., Japan).

Statistical analysis: The Student's t-test was used for comparisons between groups for biochemical data. The Mann-Whitney U test was used for comparisons of daily mean AA scores between the groups.

Results

Activity of acetylcholinesterase: AChE activity was significantly lower in both ipsi- and contralateral sides of the FBCx, the hippocampus and the BF in $AlCl_3$-treated rats compared with that in the same structures in rats injected with saline ($P<0.01$, Fig. 1). Pre-treatment with G6PDH led to the significant increase (10 to > 20 times) in AChE activity bilaterally in all three examined regions compared with those in $AlCl_3$-injected rats ($P<0.01$, Fig. 1). In addition, G6PDH induced the increment of AChE activity bilaterally over the control values in two structures (hippocampus, BF) ($P<0.01$). There were no significant differences in AChE activity between ipsi- and contralateral sides of the same brain region within all the experimental groups (Fig. 1).

Activity of glucose-6-phosphate dehydrogenase: G6PDH activity in $AlCl_3$-injected rats was significantly reduced bilaterally in all the examined brain regions compared with those in saline-injected rats (FBCx and hippocampus: $P<0.01$, BF: $P<0.05$). In rats pre-treated with G6PDH, the enzyme activity was bilaterally increased compared with control rats ($P<0.01$, except in the ipsilateral FBCx where significance was $P<0.05$) (Fig. 2).

Active avoidance test: In control and $AlCl_3$-treated rats a continuous increase in AA acquisition over five days was observed (Fig. 3). However, differences in the positive responses between these two study groups

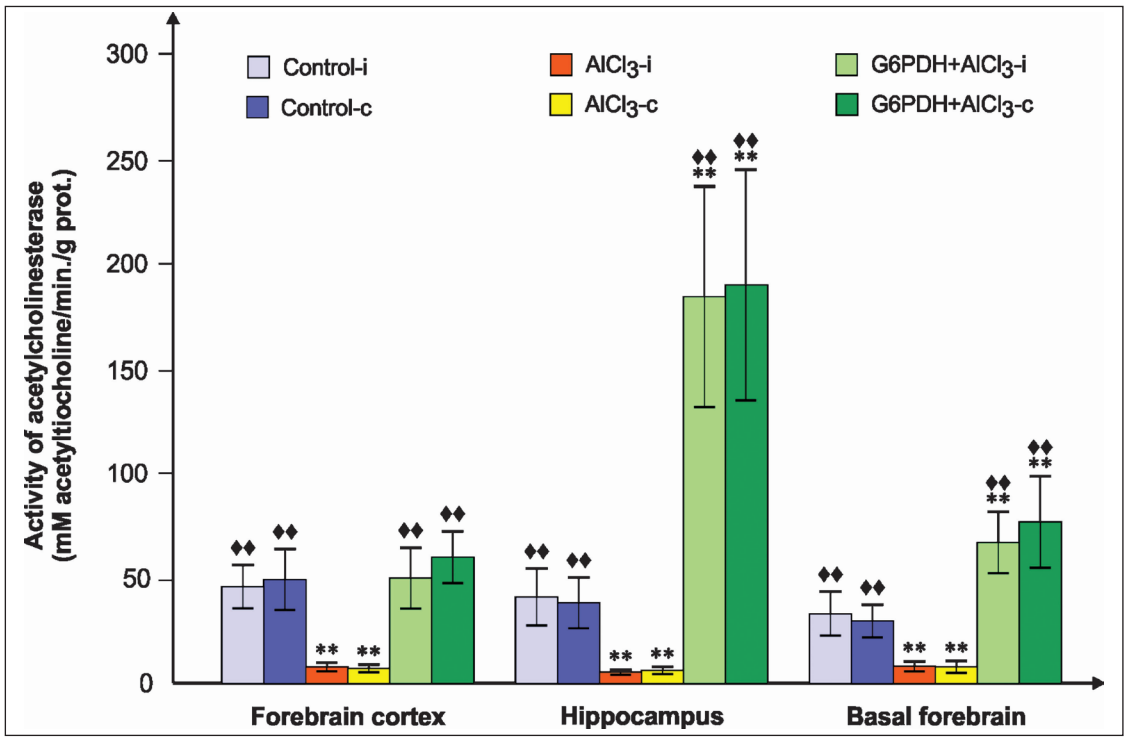

Fig. 1. Activity of acetylcholinesterase in the brain of Wistar rats (n=8 in each group) after intrahippocampal injection of either saline (control), $AlCl_3$ or glucose–6–phosphate–dehydogenase (G6PDH) applied before $AlCl_3$. Each bar represents mean±SD. ** - $P<0.01$ *vs.* corresponding ipsi– (contra–) lateral side of saline treated rats; ♦♦ - $P<0.01$ *vs.* corresponding ipsi– (contra–) lateral side of $AlCl_3$ treated rats (Student's t–test). i–ipsilateral, c–contraleteral hemisphere.

became evident for the first time after three days of testing (Fig. 3). Significant differences were achieved after the third (P<0.01), fourth (P<0.05) and fifth days (P<0.01), respectively.

In comparison with the control group, in which there were no differences connected with G6PDH, the number of correct responses was significantly increased in the G6PDH pre-treated group when compared to $AlCl_3$-treated rats (P<0.01).

Immunohistochemistry: Moderate staining for TfRs was observed in the FBCx in control, Al and G6PDH pre-treated rats, as well as in the hippocampus in the G6PDH pre-treated rats (Fig. 4, Table). Strong staining was found in the hippocampus in control and Al group. In contrast, staining was slight in the BF of all three groups.

In all three brain structures amyloid beta staining it was found to be moderate in control and G6PDH pre-treated rats, while it was very strong in Al-treated rats (Fig. 5, Table). In rats pre-treated with G6PDH before $AlCl_3$ injection the intensity of Abeta staining was moderate in all brain structures.

Slight staining for Tau protein was found in the FBCx, the hippocampus and the BF in control rats (Fig. 6, Table). In contrast, strong staining was found in the FBCx, but moderate in the hippocampus and the BF of the rats treated with Al. Moderate tau staining was found in all the examined structures of the rats pre-treated with G6PDH.

Discussion

The application of $AlCl_3$ into the hippocampal CA1 sector of rats resulted in impairment of cognitive functions accompanied by significant bilateral decreases in AChE and G6PDH activities in the FBCx, the hippocampus and the BF. The pre-treatment with G6PDH resulted in the reversion of $AlCl_3$-induced biochemical and cognitive changes.

Al salts by binding to TfR cause damage to astroglial and neuronal cells in selective brain regions of the associative Cx and the hippocampus similar to those seen in patients with AD[9]. However, the more expressive staining for TfR after Al treatment was not found in our research that could be the result of late staining (12th day) after the acute trauma induced by $AlCl_3$.

Oxidative damage to proteins and membranes is likely to be one of the mechanisms of Al's neurotoxicity. Some of these Al effects could decrease the activity of membrane-bound enzymes including Na^+/K^+-ATPase

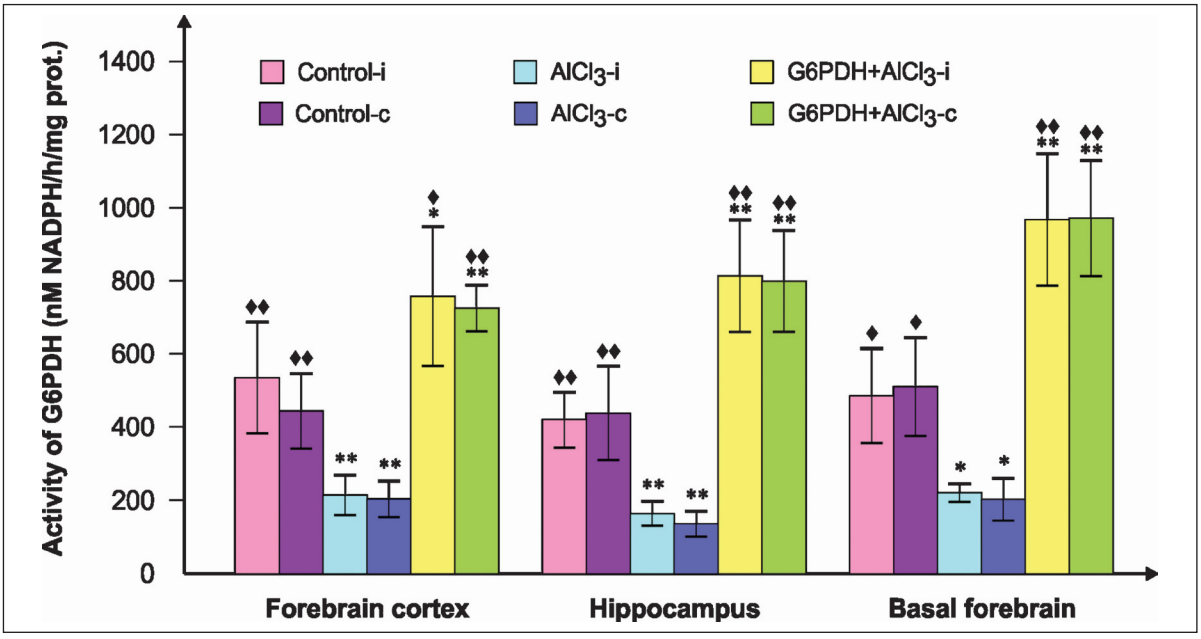

Fig. 2. Activity of glucose-6-phosphate-dehydogenase (G6PDH) in the Wistar brain of rats (n=8 in each group) after intrahippocampal injection of either saline (control), $AlCl_3$ or G6PDH applied before $AlCl_3$. Each bar represents mean ± SD. * - P<0.05 and ** - P<0.01 *vs.* corresponding ipsi– (contra–) lateral side of saline treated rats; ♦ - P<0.05 and ♦♦ - P<0.01 *vs.* corresponding ipsi– (contra–) lateral side of $AlCl_3$ treated rats (Student's t–test). i–ipsilateral, c–contraleteral hemisphere.

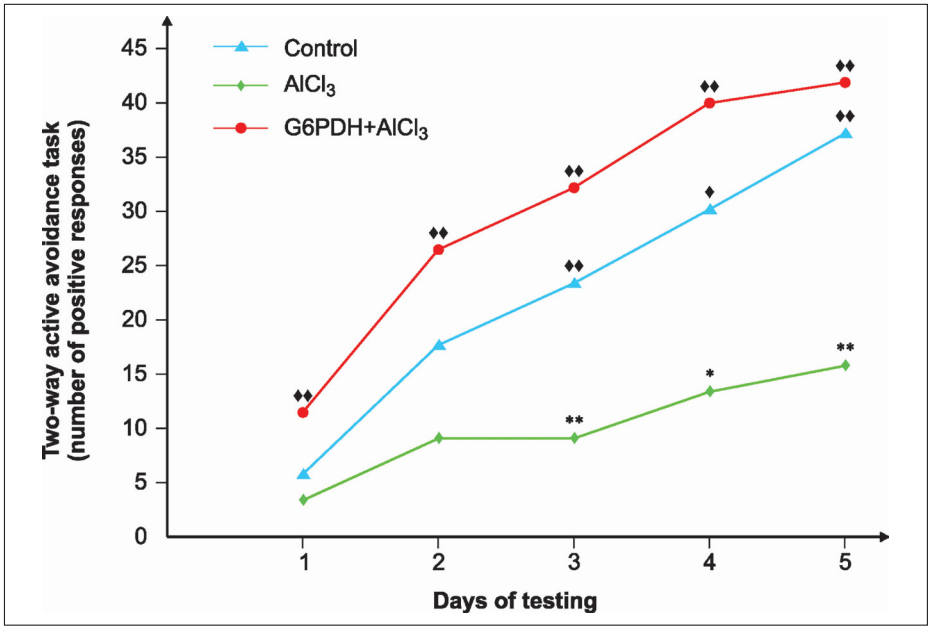

Fig. 3. The effects of intrahippocampally administered saline (control), $AlCl_3$ and glucose–6–phosphate–dehydogenase (G6PDH) administered prior to $AlCl_3$ on two-way active avoidance (AA) task in Wistar rats (n=12). Values were expressed as daily mean score for each group. * - $P < 0.05$, ** - $P < 0.01$ *vs.* corresponding value of saline treated group; ♦ - $P < 0.05$, ♦♦ - $P < 0.01$ *vs.* corresponding value of $AlCl_3$ treated group (Mann-Whitney U test for comparisons of daily mean AA scores between the groups).

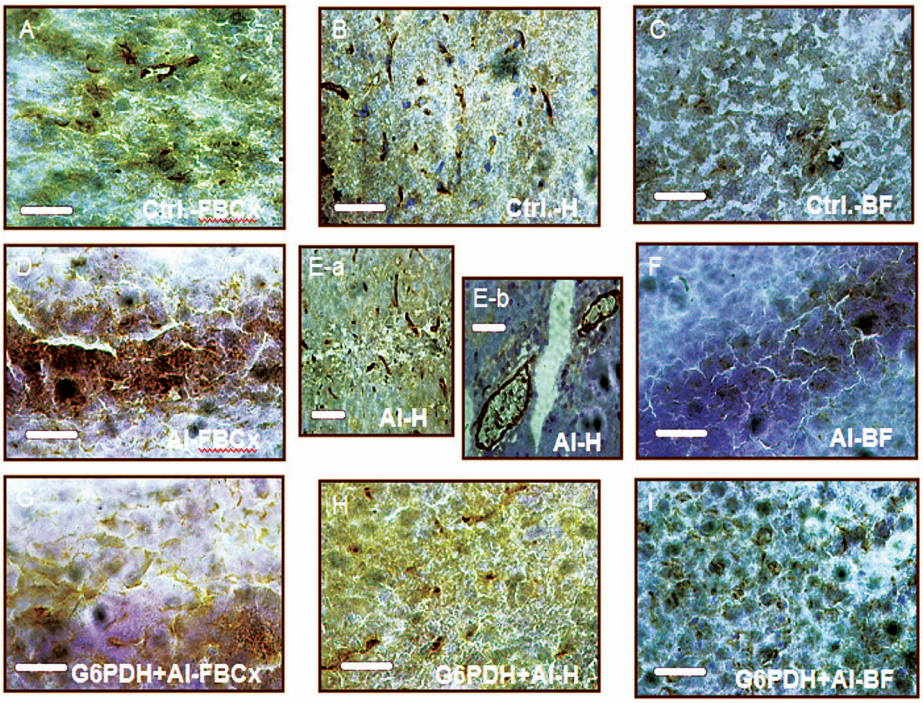

Fig. 4. Immunohistochemical staining for transferrin receptors (TfRs) in the brain of Wistar rats (n=4 in each group) after intrahippocampal injection of saline (control, Ctrl.), $AlCl_3$ (Al) or glucose-6-phosphate-dehydogenase (G6PDH) applied before $AlCl_3$ (G6PDH+Al). Moderate staining in the forebrain cortex (FBCx), strong in the hippocampus (H), and slight in the basal forebrain (BF) in the control group: micrographies **A**, **B** and **C**; moderate staining in the FBCx, strong in the H, and slight in the BF in the group treated with $AlCl_3$: micrographies **D**, **E-a** and **E-b** (perivascular staining), and **F**; moderate staining in the FBCx and the H, but slight in the BF in the G6PDH pretreated group: micrographies **G**, **H** and **I**. Magnification 400 x.

Table. Immunohistochemical staining for transferrin receptors, beta amyloid and tau protein in the brain of Wistar rats (n=4 in each group) after intrahippocampal injection of saline (control), $AlCl_3$ or glucose-6-phosphate dehydogenase (G6PDH) applied before $AlCl_3$

Antibody	Brain region/group								
	FBCx/Group			Hippocampus/Group			BF/Group		
	Control	$AlCl_3$	G6PDH/ $AlCl_3$	Control	$AlCl_3$	G6PDH/ $AlCl_3$	Control	$AlCl_3$	G6PDH/ $AlCl_3$
TfRs	++	++	++	+++	+++	++	+	+	+
Tau	+	+++	++	+	++	++	+	++	++
Abeta	++	++++	++	++	++++	++	++	++++	++

Staining intensity: very strong: ++++; strong +++; moderate: ++; slight: +
FBCx, forebrain cortex; BF, basal forebrain; G6PDH, glucose-6-phosphate dehydrogenase

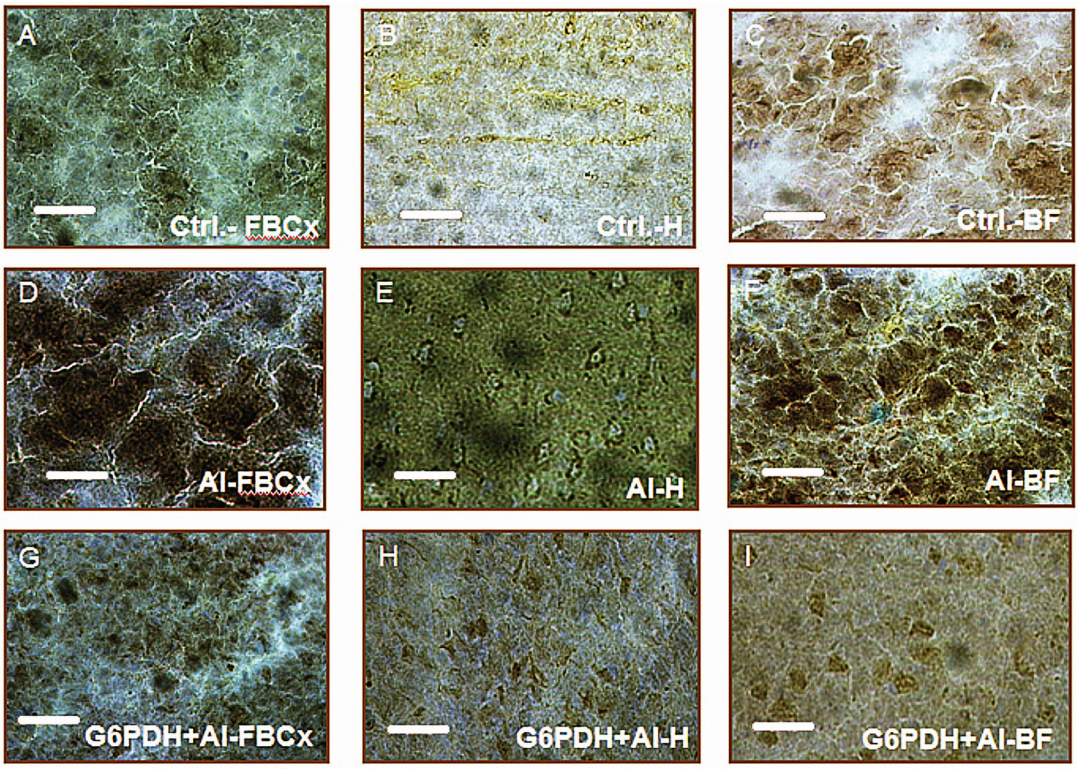

Fig. 5. Immunohistochemical staining for beta amyloid (Abeta) in the brain of Wistar rats (n=4 in each group) after intrahippocampal injection of saline (control, Ctrl.), $AlCl_3$ (Al) or glucose-6-phosphate-dehydogenase (G6PDH) applied before $AlCl_3$ (G6PDH+Al). Moderate staining in the forebrain cortex (FBCx), hippocampus (H) and basal forebrain (BF) in the control group: micrographies **A**, **B** and **C**; very strong staining in all examined structures in the group treated with $AlCl_3$: micrographies **D**, **E** and **F**; moderate staining in all examined structures in G6PDH pretreated group: micrographies **G**, **H** and **I**. Magnification 400 x.

and AChE[23]. Therfore, a significant reduction in AChE activity in the $AlCl_3$-treated rats suggests the reduced function of the cholinergic system in the BF.

Furthermore, a bilateral two-fold decrease in G6PDH activity in all the examined regions suggests the impaired pentose-phosphate metabolism. Together with the disrupted glycolysis that leads to the impaired production of reducing equivalents and consequently the redox disbalance and oxidative stress[23,24].

Bilateral reduction in both AChE and G6PDH activities in all the examined brain regions confirms the extensive spatial propagation of Al neurotoxicity and an acute dysfunction of the BF cholinergic neurons, indicating retrograde degeneration of the BF

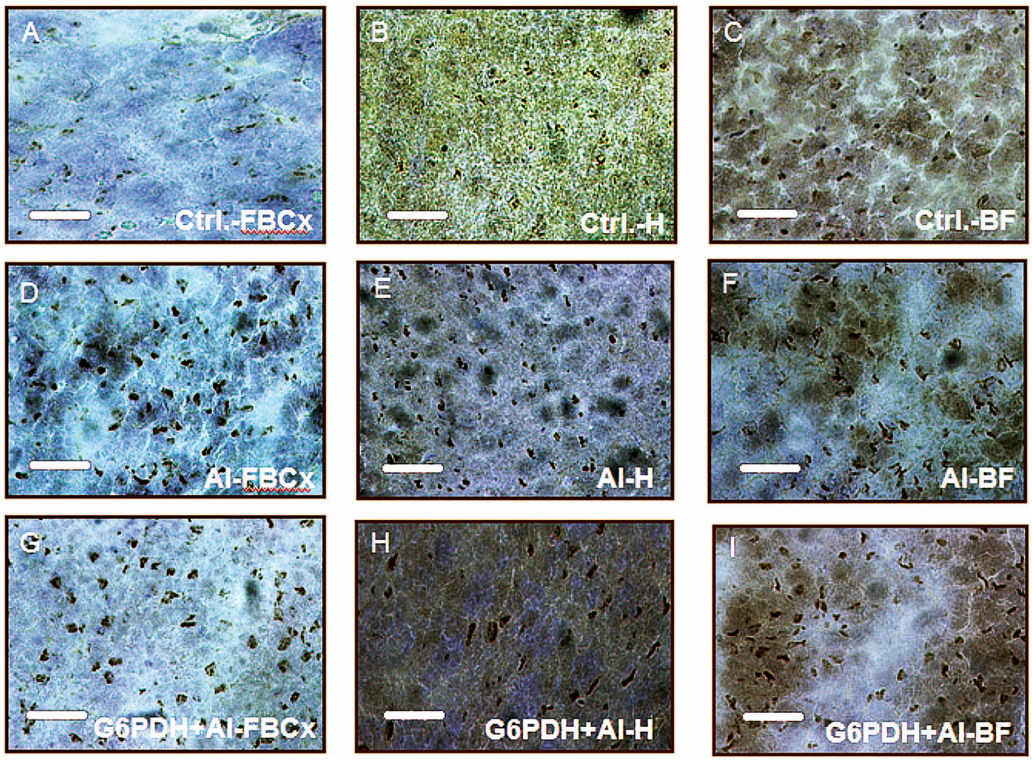

Fig. 6. Immunohistochemical staining for tau protein in the brain of Wistar rats (n=4 in each group) after intrahippocampal injection of saline (control, Ctrl.), $AlCl_3$ (Al) or glucose-6-phosphate-dehydogenase (G6PDH) applied before $AlCl_3$ (G6PDH+Al). Slight staining in the forebrain cortex (FBCx), hippocampus (H) and basal forebrain (BF) in the control group: micrographies **A**, **B** and **C**; strong staining in the FBCx, moderate in the H and the BF in the group treated with $AlCl_3$: micrographies **D**, **E** and **F**; moderate staining in all examined structures in G6PDH pretreated group: micrographies **G**, **H** and **I**. Magnification 400 x.

cholinergic neurons and impaired neuroanatomical communication between the BF and the hippocampus, as well as with the FBCx. In favour of this, there is the strong tau protein staining in the FBCx and the moderte in the hippocampus and the BF (Fig. 6, Table) reflecting the beginning of tau hyperphosphorylation, an early stage of Al-induced NFD. Consistent with our findings are the results of Ansari and Scheff who reported the decreased activity of G6PDH in brains of people who suffered from AD[25].

The decrease in the number of correct responses in AA testing was the expected consequence of Al-induced toxicity of the BF cholinergic system considering the role of ACh in learning, memory and attention. Such serious cognitive disorders are possible only if there are lesions of both the ipsi- and the contralateral hippocampus[26]. However, the progressively increased number of correct responses in rats treated with G6PDH+$AlCl_3$ showed that G6PDH prevented the effects of $AlCl_3$ and even improved the number of correct responses (Fig. 3), suggesting the involvement of G6PDH in cognitive functions.

To sum up, the experimental evidence of neurochemical, behavioural and some immunohistochemical changes (Abeta) induced by $AlCl_3$ injection into the hippocampal CA1 region and the propagation of Al toxic effects throughout three brain structures involved in cholinergic neurotransmission provides a sound basis for further work in order to thoroughly understand the pathophysiological events during experimental AD. Furthermore, the improvement of all the examined parameters in the G6PDH+$AlCl_3$-treated group suggests that G6PDH is involved in the pathogenesis of $AlCl_3$ neurotoxicity and could be effective in the protection against it under such conditions.

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Reprint requests: Dr Marina D. Jovanović, Military Medical Academy, Institute for Medical Research
Crnotravska 17, 11000, Belgrade, Serbia
e-mail: zarijasonja@gmail.com