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doi: 10.14735/amcsnn2017584

Sub-chronic Intra-hippocampal Aminoguanidine Improves Passive Avoidance Task and Expression of Bcl-2 Family Genes in Diabetic Rats

Aminoguanidin podávaný subchronicky intrahipokampálně zlepšuje u diabetických potkanů plnění úkolů pasivního vyhýbání a expresi genů z rodiny Bcl-2

Abstract

Aims: The beneficial effect of intra-hippocampal single dose of Aminoguanidine (AG) on deficit in experimental animal model of diabetes has been reported. This study was conducted to investigate the effects of seven-day intra-hippocampal AG injections on memory impairment induced by diabetes mellitus and its role in the apoptosis. *Materials and methodology:* 72 male rats were divided into 9 groups: control, control treated with normal saline, control treated with AG 10, 30 and 90 µg/rat, diabetics and diabetics treated with AG 10, 30 and 90 µg/rat. Then the passive avoidance learning and the Bcl-2 family genes was measured by RT-PCR. *Results:* AG significantly ameliorated the cognitive deficits (the number of trials to acquisition, step-through latency of retention and the time spent in the dark compartment) in diabetic rats. Moreover, AG treatment significantly modified the diabetes induced changes in Bax, Bcl–2 and Bcl-xl expression. *Conclusions:* Seven-day intra-hippocampal injections of AG, may improve the impaired cognitive tasks in diabetic rats by increasing either Bcl-2 or Bcl-xl and decreasing Bax ratios.

Souhrn

Cíle: Pozitivní vliv jednotlivých dávek aminoguanidinu (AG) podaných intrahipokampálně na postižení způsobená diabetem u experimentálního zvířecího modelu diabetu již byl popsán. Cílem této studie bylo zjistit účinky 7denního intrahipokampálního podávání injekcí AG na postižení paměti vyvolané diabetem a jeho roli v apoptóze. *Materiály a metodologie:* 72 samců potkanů bylo rozděleno do 9 skupin: kontrolní, kontrolní léčená fyziologickým roztokem, kontrolní léčená AG 10, 30 a 90 µg/potkana, diabetici a diabetici léčení AG 10, 30 a 90 µg/potkana. Následně bylo měřeno zvládání pasivního vyhýbání a pomocí RT-PCR geny z rodiny Bcl-2. *Výsledky:* AG významně snížil kognitivní postižení (počet opakování do osvojení si, latence udržení u step-through testu a doba strávená v zatmaveném oddíle) u diabetických potkanů. Léčba AG navíc významně ovlivnila diabetem vyvolané změny v expresi Bax, Bcl-2 a Bcl-xl. *Závěry:* Sedmidenní intrahipokampální injekční aplikace AG může zlepšit zhoršený kognitivní výkon u diabetických potkanů zvýšením Bcl-2 nebo Bcl-xl a snížením poměrů Bax.

This study was supported by a research grant from Zanjān University of Medical Sciences, Zanjān, Iran.

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.
Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.
The Editorial Board declares that the manuscript met the ICMJE "uniform requirements" for biomedical papers.
Redakční rada potvrzuje, že rukopis práce splnil ICMJE kritéria pro publikace zasílané do biomedicínských časopisů.

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Accepted for review: 2. 5. 2017
Accepted for print: 27. 7. 2017

Key words

diabetes mellitus – aminoguanidine – hippocampus – passive avoidance learning – Bcl-2 family genes

Klíčová slova

diabetes mellitus – aminoguanidin – hipokampus – osvojení pasivního vyhýbání – rodina genů Bcl-2

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Introduction

Diabetes mellitus (DM) is a common metabolic disorder characterized by chronic hyperglycemia and disturbances in the metabolism of carbohydrates [1]. Neuropathy is one of the DM complications with nerve regeneration capacity defect being the main factor involved [2,3]. Central complications include impairment of cognitive functions, such as general intelligence, speed of information processing and learning [4]. Pathogenesis of central neuropathy associated with DM is multifactorial [5]. The decrease in brain glutathione levels (antioxidant mechanisms within the cell) and oxidative stress have been suggested as the major factors involved in cognitive deficits [6]. Common mechanisms leading to oxidative stress in DM include either induction of free radical production followed by spontaneous oxidation of glucose or decreased levels of endogenous antioxidant such as A, E, C vitamins and reduction of antioxidant enzyme activities. Therefore, antioxidant treatments may be a suitable therapeutic approach in DM-induced cognitive dysfunctions [7].

Oxidative stress leads to hippocampal neuronal cell death by apoptosis induction [8]. Apoptosis is regulated by two main proteins including caspase enzymes and Bcl-2 family gene products [9]. Bcl-2 family genes include Bcl-2, Bcl-xl, and Bax proteins. Bax product as an apoptotic factor neutralizes the inhibitory effects of both Bcl-2 and Bcl-xl proteins [10]. Aminoguanidine (AG), as an antioxidant, has some biological effects, including inhibition of both amine oxidase and inducible nitric oxide synthase (iNos) and formation of advanced glycation end products (AGEs) [11].

Experimental models demonstrate the protective effect of AG, such as peripheral nerves functional and structural abnormalities induced by DM [12], stroke [13] and transient focal cerebral ischemia [14]. The effects of AG on memory of animals have shown controversial, both positive [15–19] and negative [20–23], results. However, there are a few studies on the effect of AG on memory and anti-apoptotic genes in diabetic animals. We have recently showed that the single intra-hippocampal injection of AG (30 µg/rat) improved memory retrieval in step-through passive avoidance task in diabetic rats, associated with reduced apoptotic and increased anti-apoptotic gene expressions [24]. Also, one week intraperitoneally AG (100 and 200 mg/kg) improved diabetes-associated cognition insufficiency in diabetic rats by enhancing (Bcl-2 + Bcl-xl)/(Bak + Bax) proportions [25]. However, there is no study regarding sub-chronic or chronic effects of intra-hippocampal administration of AG on memory and anti-apoptotic gene expressions in diabetic animals. Our hypothesis was that repeated intra-hippocampal AG injection (10, 30 and 90 µg/rat) could also improve memory deficit induced by DM.

The present study was conducted to investigate the effect of seven-day intra-hippocampal AG injections (10, 30 and 90 µg/rat) on step-through passive avoidance memory impairment induced by DM as well as its role in apoptosis by measuring expression of Bcl-2 family genes.

Material and Methodology

Animals

The animal maintenance and procedures were carried out according to the Principles of Laboratory Animal Care (NIH publication no. 86–23 revised 1985) and were accepted by the Institutional Animal Ethical Committee (IAEC no. A-10-141-4). Healthy adult male Wistar rats weighing 232–274 g (7–9 weeks) were randomly selected and housed under controlled conditions in a 12 h light/dark cycle. The rats were fed with a standard laboratory diet and clean drinking water ad libitum.

Surgical procedures

The surgical processes were performed under ketamine-xylazine (100–25 mg/kg) anesthesia with animals being subsequently fixed with a stereotaxic device. A mid-line incision was made along the top of the head, to expose bregma; a stainless steel, 22-gauge guide cannula was inserted (bilaterally) 1 mm above the planned injection site. Stereotactic coordinates for the CA1 regions of the dorsal hippocampi were: anterior (AP) 3–3.5 mm, lateral (LAT) ± 1.8–2 mm and ventral (DV) 2.8–3 mm from the bregma, the cannula was fixed to the skull with dental acrylic [24]. Dummy guides were set into the cannula to avoid blockage by debris to enter the brain. When the experimental sessions had been finished, animals were killed with an overdose of chloroform before bilateral intra-CA1 injection of ink (1% aquatic methylene blue solution). Subsequently, the brain was extracted and fixed with 10% formalin solution for 10 days before sectioning. Sections were examined to control the location of the cannula aimed for the CA1.

Diabetes induction

Diabetes was produced by a single intraperitoneal (i.p.) injection of 50 mg/kg of streptozotocin [26]. Fasting blood glucose levels were measured 72 hours after the STZ injection. Animals were considered diabetic if their plasma glucose levels were between 13.89 to 22.22 mmol/dl and all groups were homogenous in glycemia.

Animal groups

Following surgery, the animals were allowed recovery for at least one week. The animals were divided into nine equal groups as shown in Tab. 1 (n = 8 rats per groups).

Drug treatments

When diabetes was established, they received intra-hippocampal injection of the saline, AG10, 30 or 90 µg/rat daily for 7 days, for sub-chronic evaluation of AG treatment on memory retrieval and Bcl-2 family genes in long-term STZ-induced diabetic rat outcomes. For drug administration, the animals were gently restrained by hand; the dummy guides were removed from the guide-cannula and replaced with 27-gauge injection needles (1 mm lower than the tip of guide-cannula). Each injection unit was joined by polyethylene tubing to 1 µl Hamilton syringe. The left and right CA1 regions were infused with a 0.5 µl solution on each side (1 µl/rat) over a 60-sec period. The injection needles were left in place for additional 60 sec to permit diffusion, then the dummy guides were reinserted into the guide cannula [27]. Body weights were evaluated weekly, diabetic status was reconfirmed after 7 weeks after which learning and memory were also evaluated.

Passive avoidance learning (PAL) step through test

Behavioral studies were evaluated by the shuttle box apparatus. The apparatus and procedure were essentially the same as described in prior studies [28]. Briefly, the step-through passive avoidance apparatus consisted of a lighted chamber (20 × 20 × 30 cm) made of transparent plastic and a dark chamber with dark opaque plastic walls (20 × 20 × 30 cm). The floor of both chambers was made of stainless steel rods (3 mm diameter) spaced 1 cm apart. The floor of the dark chamber

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Tab. 1. Details of groups, surgery and diabetes induced by STZ and dose of AG which administered. (+) shows surgery or diabetes.

Groups	Surgery	Diabetes	AG dose (7 days)
C	–	–	–
C-S	+	–	–
AG10	+	–	10 µg/rat
AG30	+	–	30 µg/rat
AG90	+	–	90 µg/rat
D	+	+	–
DAG10	+	+	10 µg/rat
DAG30	+	+	30 µg/rat
DAG90	+	+	90 g/rat

Table 2. Primers used for RT-PCR method for Bcl-2, Bcl-xl, Bax, and GAPDH.

Gene	Product size	Primers
F: 5'-CTG GTG GAC AAC ATC GCT CTG-3'	228 bp	Bcl-2
R: 5'-GGT CTG CTG ACC TCA CTT GTG-3'		
F:5'-AGG CTG GCG ATG AGT TTG AA-3'	357 bp	Bcl-xl
R: 5'-TGA AAC GCT CCT GGC CTT TC-3'		
F:5'- TGC AGA GGA TGA TTG CTG AC -3	173 bp	Bax
R: 5'- GAT CAG CTC GGG CAC TTT AG-3'		
F: 5-'GGC CAA GAT CAT CCA TGA CAA CT-3'	461 bp	GAPDH
R: 5'-ACC AGG ACA TGA GCT TGA CAA AGT-3'		

could be electrified using a shock generator. A rectangular opening (6 × 8 cm) was placed between the two chambers and could be closed by an opaque guillotine door.

Training

Experimental groups received two trials to habituate them to the apparatus initially. For these trials, the rats were placed in light compartment of the apparatus facing away from the door and 10 sec later the guillotine door was upraised. The rats have natural preference for the dark environment. Once the rat entered the dark compartment, the door was closed and after 30 sec the rats were taken out from the dark compartment and placed in their home cage. The habituation trial was repeated after 30 min and followed after the same interval by the first acquisition trial. The entrance latency to the dark compartment (step-through latency of acquisition, STLa) was recorded when the animal had placed all four paws in the dark compartment.

For the training of the animals, once they had spontaneously entered the dark compartment, the guillotine door was lowered and a mild electrical shock (0.5 mA) was applied for 3 sec, after 30 sec, the rat was returned to its home cage. Then after 2 min, the procedure was repeated. The rat received a foot-shock each time it reentered the dark compartment and had placed all its four paws in the dark compartment but training was terminated when the rat remained in the light compartment for 120 consecutive sec. The number of trials (entries into the dark chamber, TA) was recorded.

Retention test

Long-term memory retrieval was evaluated 24 hours after the PAL acquisition trial. The rats were placed in the lighted chamber as in PAL training session and 10 sec later, the guillotine door was elevated, and the step-through latency (STLr) and the time spent in the dark compartment (TDC) were recorded for up to 600 sec. If the rat did not enter the dark compartment within 600 sec, the retention test was terminated and an upper limit of 600 sec was assigned. During this session, the electric shocks were not applied to the grid floor [29].

At the end of the experiments, weight and blood glucose level of the rats were measured. Formerly, animals were anesthetized with chloroform and the skull was opened along the midline and the brain was removed and placed on an ice-cooled cutting board. The meninges were carefully removed and hippocampus was dissected from hemispheres, snapped frozen in liquid nitrogen and stored at –70 °C for extraction of RNA.

RNA Preparation and Semi-quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from hippocampi using Trizol Reagent (invitrogen), according to the manufacturer's instructions. Reverse transcription (RT) was performed using 1000 ng of total RNA into cDNA with Revert Aid (Thermo, Scientific RevertAide cDNA synthesis Kit) according to the manufacturer's instructions, and cDNA samples were stored at –70 °C. A semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) was carried out to determine the levels of Bcl-2, Bcl-xL, and Bax mRNA expressions. The RT-PCR mixture for Bcl-2, Bcl-xL and Bax genes (final volume of 25 µl) contained 2 µl of cDNA, 12.5 µl of Thermo Scientific PCR Master Mix 2× (Qiagen, Germany) and 10 pmols of each complementary primer specific for Bcl-2, Bcl-xL, and Bax sequences as well as for GAPDH (Glyceraldehydes-3-phosphate dehydrogenase; GAPDH gene) sequence as an internal control (Tab. 2). Primers for PCR were designed by means of published cDNA sequences and analyzed with BLASTA program. The samples were denatured at 95 °C for 15 min, and amplified using 40 cycles of 95 °C for 30 sec, 60 °C for 80 sec, and 72 °C for 45 sec for Bax gene and 5 cycles of 95 °C for 30 sec, 62 °C for 80 sec, and 72 °C for 30 sec, as well as 25 cycles of 95 °C for 30 sec, 64 °C for 80 sec and 72 °C for 30 sec for Bcl-2 and Bcl-xL genes followed by a final elongation at 72 °C for 3 min on a Corbett Research thermocycler (Sydney, Australia). The best numbers of cycles were selected for amplification of all genes experimentally so that amplifications were in the exponential ranges and did not reach a plateau. All RT-PCR and PCR reactions contained the use of water in place of a template as a negative control. Eight µl of the final amplification product were run on a 2% ethidium-stained agarose gel and photographed. Finally, the densities of the bands on the agarose gels were determined by the NIH image program. Data were normalized according to the density of the GAPDH bands [24].

Statistical analyses

Data were analyzed using either two ways ANOVA, then Tukeys' posthoc statistics for parametric or Kruskall-Wallis tests fol-

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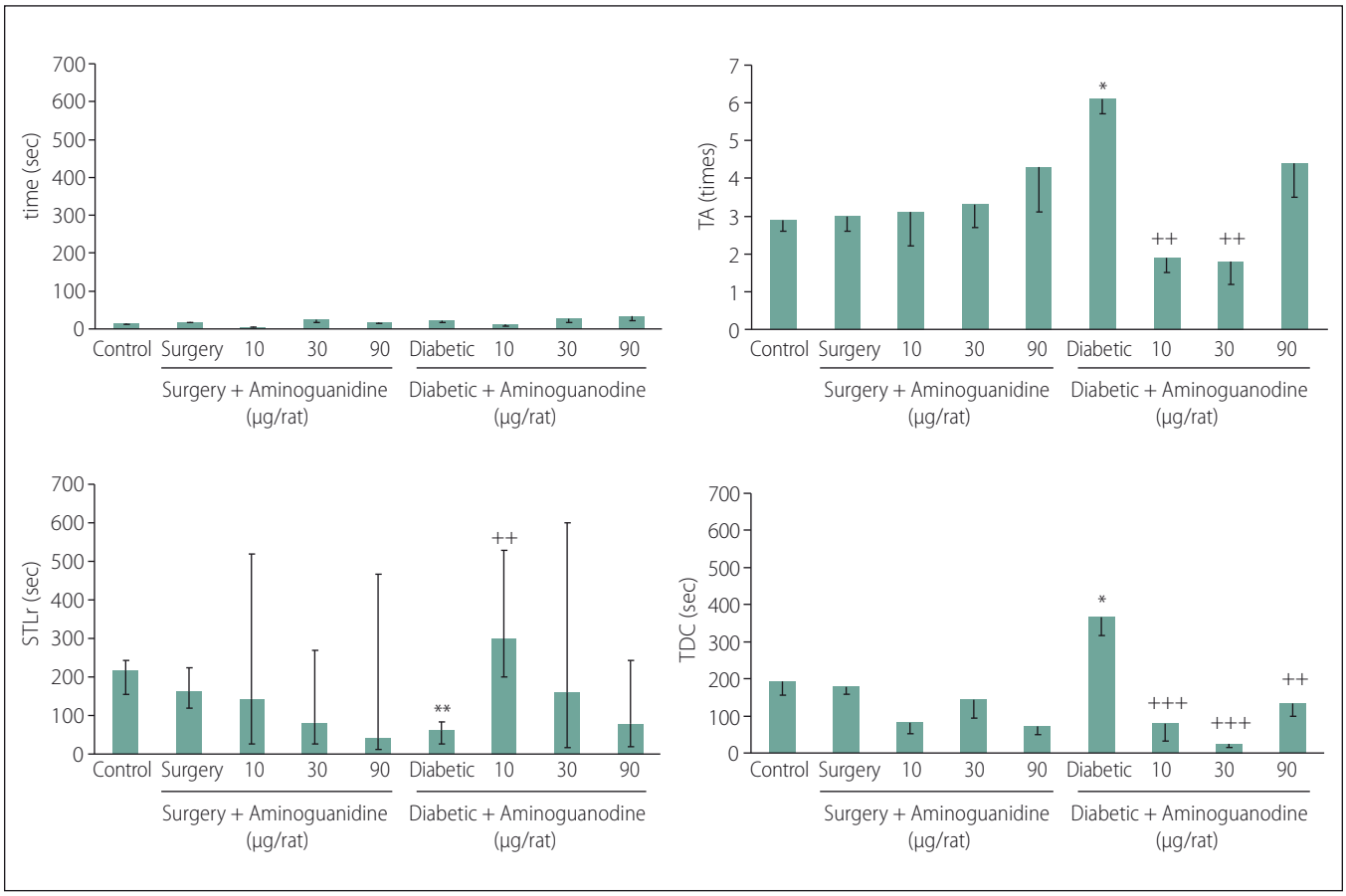

Fig. 1. Effect of seven day intra-hippocampal injections of three different doses of aminoguanidine (AG) on the step-through latency of acquisition (STLa, A), the number of trials to acquisition (TA, B), the step-through latency in the retention trial (STLr, C) and the time spent in the dark compartment (TDC, D), 24-hour after acquisition of passive avoidance learning (PAL) task in the control and diabetic groups. Columns represent mean ± SEM of the number of trials to acquisitions (B), median ± quartile of the step-through latency times (C) and mean ± SEM of the time spent in the dark compartments (D).

* P < 0.05 and ** P < 0.01 compared with the control group. ++ P < 0.01 and +++ P < 0.001 compared with the diabetic group.

lowed by Mann Whitney's U test for non-parametric data. After Mann Whitney U test, the Holms Bonferoni's correction was used. Probability values of less than 0.05 were considered significant.

Results

Behavioral results – PAL step through test

Effects of AG treatment on STLa: There were no significant differences in the STLa, demonstrating a lack of sensory and motor disorders in animals. Two ways ANOVA's F (8.42) = 1.62, P = 0.137 (Fig. 1A).

Effects of AG treatment on the TA: Two-way ANOVA of the mean ± SE of the TAs, showed that 7-day intra-hippocampal injections of AG has an affirmative effect on diabetes-induced impaired TA (F (8.42) = = 3.59, P = 0.001, Tukey's posthoc, P = 0. 002 and 0.001 for 10 and 30 µg/rats AG, resp., in comparison to diabetic control animals) (Fig. 1B).

Effects of AG treatment on STLr: Based on non-parametric Kruskall-Wallis test of median ± quartile of the step-through latency times, there were no significant differences in the STLr in non-diabetic animals (left columns of the Fig. 1), indicating that neither surgery nor AG alone showed any effect on STLr (H (4) = 4.55, P = 0.34). On the other hand, non-parametric Kruskall-Wallis test showed that 7-day intra-hippocampal injections of AG had significant positive effect on diabetes-induced impaired STLr (7 weeks after induction of diabetes, right columns) (H (3) = 11.05, P = 0.026, Mann Whitney's U test, P = 0.006, 0.658 and 0.798 for 10, 30 and 90 µg/rats AG resp.). As a result, the 10 µg of the drug /rat could reverse the impaired latency of retention induced by diabetes (Fig. 1C).

Effects of AG treatment on the time spent in TDC: Based on two-way ANOVA of mean ± SE of the TDCs, 7-day intra-hippocampal injections of AG showed a positive effect on diabetes-induced impaired TDC within 10 min [factor A × B, F (8.42) = 7.51, P < 0.0001, Tukey's posthoc, P = 0.00002, 0.00003 and 0.001 for 10, 30 and 90 µg/rats AG resp. in comparison to diabetic control animals] (Fig. 1D).

Semi-quantitative mRNA levels of genes regulating cell death: Two-way ANOVA of the mean ± SE, showed that 7-day intra-hippocampal injections of AG has a positive effect on diabetes-induced decrease of both Bcl-2 (F (8.42) = 12.05, P < 0.001, Tukey's posthoc, P < 0.0001 for 10, 30 and 90 µg/rats AG resp. in comparison to diabetic control animals) (Fig. 3A) and Bcl-xl expressions (F (8.42) = 41.93, P < 0.001, Tukey's posthoc, P < 0.0001 for 10, 30 and 90 µg/rats

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AG resp. in comparison to diabetic control animals). As shown in Fig. 3B, 90 µg/rats AG had dual effects, it decreased the expression of Bcl-xl genes in non-diabetic but had an opposite effect in the diabetic group.

On the other hand, 7-day intra-hippocampal injections of AG effectively diminished the diabetes-induced increase of Bax expression ($F(8.42) = 17.19$, $P < 0.001$, Tukey's posthoc, $P < 0.0001$, $P = 0.001$ and 0.003 for 10, 30 and 90 µg/rats AG, resp., in comparison to diabetic control animals) (Fig. 3C).

Discussion

Our results showed that seven weeks after diabetes induction, STLr, TDC and TA impairment was restored by 7-day intra-hippocampal injections of AG. There are several studies indicating the effects of AG on memory. It has been shown that neuronal death and spatial memory impairments induced by lipopolysaccharides were prevented by AG [30]. Memory recovering effect of naringin in unstressed and stressed mice was improved by AG administration significantly as well [16]. Moreover, AG reverted considerably the impairment of learning and memory induced by arsenic [17]. Treatment with AG also antagonized retrograde memory impairment due to hypoxia [18]. Learning and memory deficits caused by aluminum chloride might be prevented by AG treatment [19]. On the other hand, the improving effect of atorvastatin on short-spatial recognition memory was reversed by AG [20]. In morphine-induced memory impairment, improving effect of pioglitazone in the passive avoidance task has not been modified but has reversed in Y-maze discrimination by AG [22]. Moreover, the positive effects of both atorvastatin and granisetron on memory consolidation in scopolamine-treated mice have been significantly reversed by AG [21,23].

In the present study, locomotor activity of the animals was not measured but the effect of AG on STLr can be attributed to unimpaired sensory or locomotor function since the delay in the acquisition phase (STLa) (before electrical shock) showed no significant differences between controls and the experimental group. There is little evidence regarding the effect of AG on memory deficit induced by diabetes in animals. In this study, the best STLr result was obtained with 10 µg/rat AG after 7 days of intra-hippocampal injections of the drug. This is in agreement with the results obtained with a higher single dose of AG (30 µg/rat) in our previous study [24].

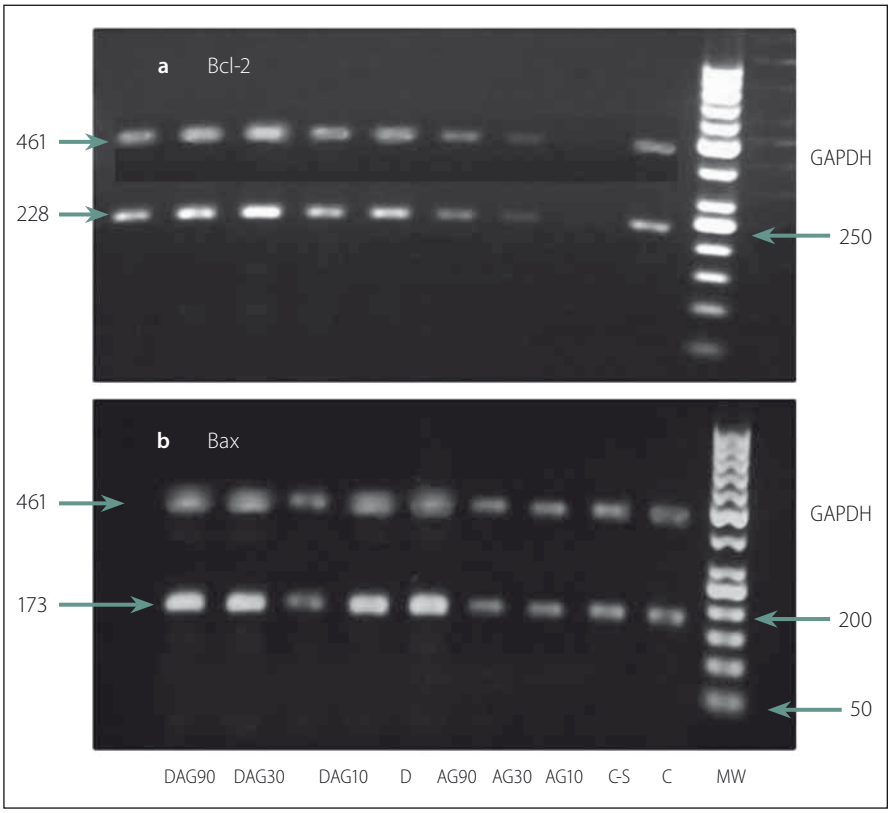

Fig. 2. Effects of aminoguanidine (AG) on expressions of Bcl-2 (a) and Bax (b). mRNA in a rat hippocampus. Expression analyses were performed by RT-PCR and the products (10 ll) were visualized following 1.5% agarose gel electrophoresis. The intensity of the bands was quantified by densitometric analyses and normalized with corresponding GAPDH. C, control; CS, control- surgery; AG10, AG30 and AG90 controls which received aminoguanidine 10, 30 and 90 µg/rats; D, diabetic; DAG10, DAG30 and DAG90, diabetic treated with aminoguanidine 10, 30 and 90 µg/rat; M: GeneRulerTM 50 bp DNA Ladder.

On the other hand, repeated AG administration (10,30 and 90 µg/rat) indicated significant positive effects on the other parameters (TDC and TA), also similar to our previous study (single dose intrahippocampal injection of the drug) [24].

Several factors are involved in the pathogenesis of diabetes-induced learning and memory deficits [5]. The beneficial effect of AG may be related to its restorative role on a variety of pathogenic processes [31]. It has been shown that AG can improve vascular and neuronal complications detected in experimentally-induced diabetes [32]. Moreover, AGEs play a principal role in learning and memory impairments induced by diabetes, AG can prevent the formation of AGEs [33–36]. Also, reactive oxygen species (ROS) and reactive nitrogen species (RNS)-induced oxidative damage are other main causes of cognitive dysfunction in diabetes [37] related to iNOS production [38]. Inhibition of iNOS by AG may be related to amelioration of diabetes-induced cognitive dysfunction [39]. Another mechanism of AG-induced neuroprotection may be attributed to its free radical scavenging properties [36, 40]. In addition, AG neuroprotective effects can be featured to a variety of other cellular metabolic pathways [41], including formation of methylglyoxal that acts as an endogenous toxic compound [42] as well as a potent source of ROS [43]. These are frequently accumulated in the hippocampal neurons under conditions of hyperglycemia and impaired glucose metabolism [44,45] and their production can be inhibited by AG [46].

In the present study, diabetes caused decrease in Bcl-2 and Bcl-xl (as anti-apoptotic genes) and increase in Bax (as an apoptotic gene) expression. Consistent with our study,

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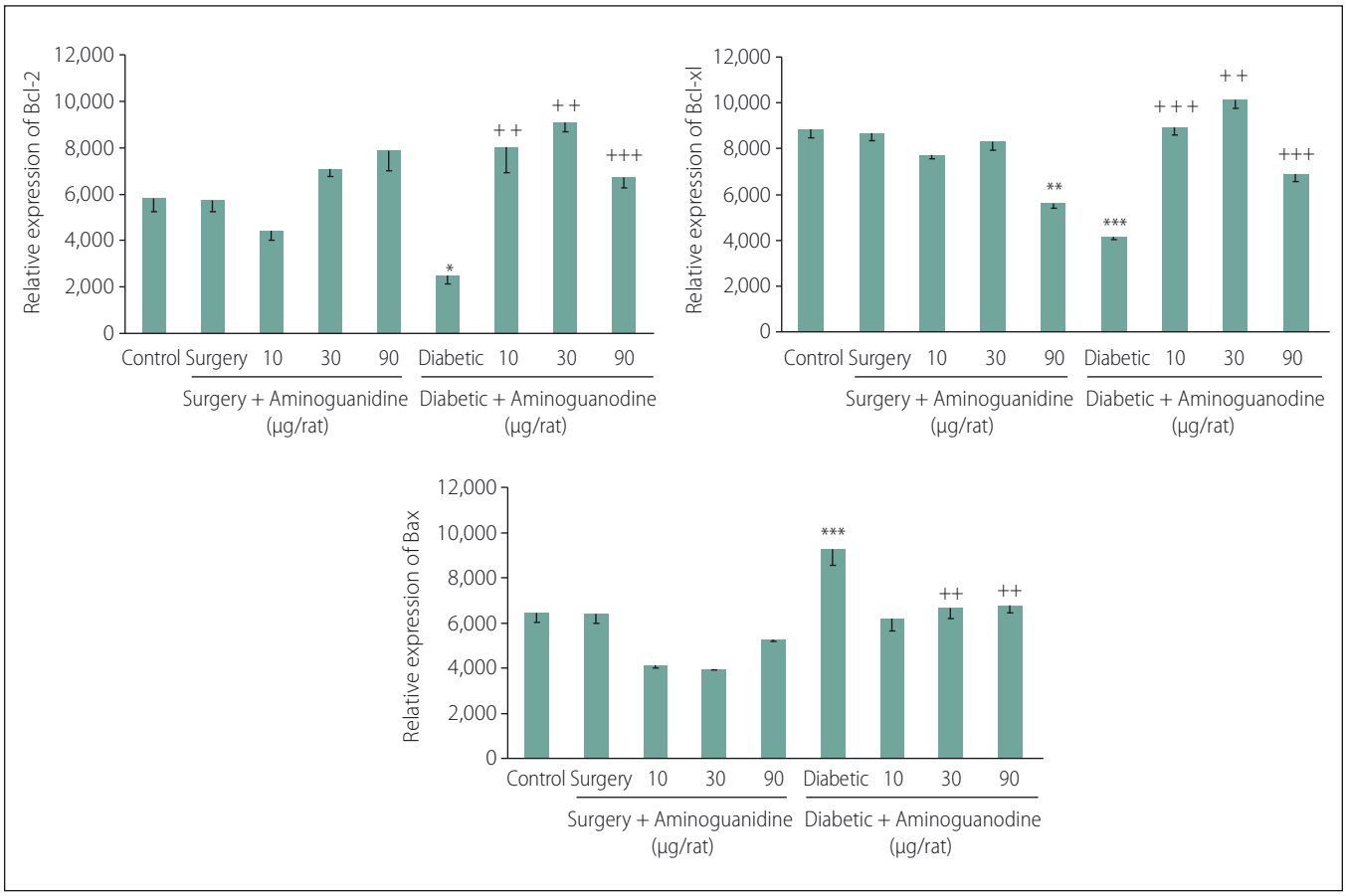

Fig. 3. Effect of seven-day intra-hippocampal injections of three different doses of aminoguanidine (AG) on the Bcl-2 (A), the Bcl--xl (B) and the Bax mRNA in a rat hippocampus. Expression analyses were performed by RT-PCR and the products (10 ll) were visualized following 1.5% agarose gel electrophoresis (all amplifications were repeated three times). The data are expressed as the mean ± SEM.

* P < 0.05, ** P < 0.01 and *** P < 0.001 compared with the control group. ++ P < 0.01 and +++ P < 0.001 compared with the diabetic group.

it has been shown that increased Bax expression and caspase-3 activity in a diabetic hippocampus can cause neuronal cell death [47]. Hippocampal neuron apoptosis has an essential role in the learning and memory impairment in diabetes [48,49]. Furthermore, it has been revealed that either reactive methylglyoxal [50,51] or RNS [44,45] are the components inducing apoptosis in the hippocampus by altering expression of Bcl-2 family proteins in the neurons. The pro- and anti-apoptotic balance signals of the Bcl-2 family have a critical role in the release of apoptogenic mitochondrial mediators [52,53]. It has been demonstrated that the mitochondrial pathway plays a major role in apoptosis due to diabetes in hippocampus of STZ-induced diabetic rats [54,55].

Our results demonstrated that 7-day intra-hippocampal injections of AG had a positive effect on diabetes-induced decrease of both Bcl-2 and Bcl-xl expressions. On the other hand, the drug significantly diminished the diabetes-induced increase of Bax expression.

To our knowledge, studies regarding the effect of AG on the mentioned gene expressions in diabetic animal's hippocampus are lacking. It has been shown that AG as an antioxidant and amine oxidase and iNos inhibitor can inhibit apoptotic cell death [50]. Therefore, the benefitial effects of AG on memory might be related to alteration of apoptosis in the brain areas involved in learning and memory. It is clear that the ratio of Bcl-2 or Bcl-xl to Bax expression determines cell fate and cell leading to the death or life [13]. It has been reported that hyperglycemia induced by STZ results in apoptosis of cortical neurons of newborn rats by increasing the Bax/Bcl-2 ratio [56]. The positive effect of repeated AG administration (10, 30 and 90 μg/rat) on Bax, Bcl-2 and Bcl-xl expression in diabetic animals are in agreement with our previous studies (single dose intra-hippocampal (30 μg/rat) and intraperitoneal (100 and 200 mg/kg) injection of the drug) [24,25].

Much more reliable results could be obtained by real-time PCR and protein level measurements and this is considered as a limitation of our study.

In conclusion, 7-day intra-hippocampal injections of AG may improve impaired cognitive tasks in diabetic rats by increasing either Bcl-2 or Bcl-xl and decreasing Bax ratios. However, additional pathological and molecular investigations are required to explain the detailed mechanisms underlying the neuroprotective effect of AG on memory in diabetic rats.

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