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EXPERIMENTAL STUDY

Effect of sub-chronic intraperitoneal administration of aminoguanidine on the memory and hippocampal apoptosis--related genes in diabetic rats

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ABSTRACT

Memory impairment is a common disorder in diabetes mellitus which is associated with hippocampal neuronal apoptosis. The present study was conducted to examine the effect of one-week intraperitoneal (ip), administration of aminoguanidine (AG) on passive avoidance learning (PAL) and Bcl-2 family gene expression in the hippocampus of rats. Sixty male rats were divided into ten groups: non-diabetic/diabetic animals with/without AG (50, 100, 200 and 400 mg/kg, ip) treatment for one week. PAL and Bcl-2 family genes were examined. AG (100 and 200 mg/kg) improved both memory and Bax, Bak, Bcl–2 and Bcl-xl deficiency significantly in diabetic rats. AG treatment also ameliorated the diabetes-induced changes in (Bcl–2+Bcl-xl)/(Bak+Bax) ratios considerably. These results propose that one-week ip administration of AG may recover the deficit cognition in diabetic rats via enhancing (Bcl–2+Bcl-xl)/(Bak+Bax) proportions *(Tab. 2, Fig. 4, Ref. 55)*. Text in PDF *www.elis.sk.*

KEY WORDS: rats, diabetes mellitus, aminoguanidine, passive avoidance learning, Bcl-2 family genes.

Introduction

Diabetes mellitus (DM) is described by continuous carbohydrate metabolism disorders (1). Neuropathy is one of the DM complications which is created by nerve regeneration capacity failure (2, 3). The most important DM difficulties in CNS are the cognitive impairments such as general intelligence, speed of information processing and learning (4). The pathogenesis of diabetic neuropathy is connected to several factors (5). The major source of cognitive deficits which has been suggested is the imbalance between free radical production and antioxidant defense systems (6). Oxidation of glucose and/or decreased levels of endogenous antioxidants and antioxidant enzymes result in oxidative stress in DM. Hence, antioxidants might have a beneficial effect in DM-induced cognitive deficits (7).

Hippocampal cell death in DM results from apoptosis triggered by oxidative stress (8). End products of Bcl-2 family genes are the major factor which control apoptosis (9). Bcl-2, Bcl-xl, Bax and Bak proteins, etc. are the major Bcl-2 family genes. The induction of apoptosis by Bax and Bak is neutralized by Bcl-2 and Bcl-xl products. Aminoguanidine (AG), also known as pimagedine, is an amino derivate of guanidine and a nucleophilic hydrazine compound with antioxidant properties which have several biological effects, including inhibition of amine oxidase, inducible nitric oxide synthase (iNos) and developing advanced glycation end products (AGEs) (10). Protective effects of AG have been shown in peripheral nerves' functional and structural deficits induced by DM (11) and transient focal cerebral ischemia (12) and stroke (13). Paradoxical results are available relating to the effect of AG on memory of animals including positive (14–18) and negative effects (19–22). To our knowledge, few studies have been published concerning the effect of ip administration of AG on memory and anti/apoptotic genes in diabetic animals. Recently we have demonstrated that a single intra-hippocampal AG injection (30 μg/rat) recovered the memory retrieval in step-through passive avoidance task in diabetic rats, which is associated with reduced apoptotic gene expressions (23). Because peripheral administration of drugs is used in DM treatment, it is preferable to find the best dose for the ip route. However, there is no study regarding the effects of one-week ip injections of AG on learning and anti/apoptotic gene expressions in diabetic rats.

The present study was conducted to examine the effect of one-week ip AG (50, 100, 200 and 400 mg/kg) administrations on step-through passive avoidance memory impairment induced by DM as well as its role in the apoptosis by assessing the expression of Bcl-2 family genes.

Materials and methods

Chemicals

Streptozotocin (STZ) and AG were purchased from Merck, Germany. Pure RNA™ kit was obtained from TaKaRa (Japan).

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Reverse Transcription Polymerase Chain Reaction (RT-PCR) kit was purchase from Qiagen (Germany).

Experimental animals

We used male Wistar rats weighing 202–247 g. The animals were housed in Plexiglas cages with a 12-h light/12-h dark cycle and controlled temperature (22 ± 2 °C). They were given food and water *ad libitum*. Each animal was used only once per experiment and all procedures were carried out in accordance with Institutional Animal Ethical Committee guidelines for animal care and use.

Animal grouping

The animals were divided in ten experimental groups as shown in Table 1 (n = 6).

Diabetes induction

The diabetes was induced by an injection of streptozotocin (STZ, 50 mg/kg, ip) (24). Fasting blood sugar levels (FBS) were measured after 3 days. The animals were considered diabetic when blood glucose levels were more than 250 mg/dL.

After diabetes had been labeled, either saline or AG (50, 100, 200 and 400 mg/kg, ip) was injected daily for one week. Body weights were re-checked weekly and the diabetic status was re-confirmed. Memory and gene expressions were evaluated 7 weeks after AG treatment.

Passive avoidance learning (PAL) step-through test

The PAL was determined by a shuttle box device (25). In brief, the device consisted of two compartments: a lit compartment (20 × 20 × 30 cm) made of transparent plastic and a dark compartment, the walls of which were made of dark opaque plastic (20 × 20 × 30 cm). The ground floor of chambers was built of stainless steel rods (3 mm in diameter) spaced 1 cm apart. The floor of the dark chamber could be electrified via a shock generator. A guillotine door was placed in the connection of the two compartments.

Training session

At the start, the rats were familiarized with the apparatus in two sessions. The animals were put in the lit compartment of the apparatus and the guillotine door was opened after 10 seconds. Since normal animals prefer darkness, they went into the dark compartment and the door was closed. After 30 seconds the rats were returned to their home cage. The process was repeated after 30 min using an interval equal to that in the first acquisition trial. The entrance latency to the dark compartment, STLa, was recorded when the animal laid all its paws on the floor of the dark compartment.

For a number of acquisition trials (TA), the animals went right away into the dark compartment, the guillotine door was closed and a mild electrical shock (0.5 mA) was given for 3 seconds. After 30 seconds, the rats were returned to their home cage. After 2 min, the above process was repeated. When the rats reentered the dark compartment, they received a foot-shock. Training was terminated once the rat stayed in the lit chamber for 120 consecutive seconds.

Tab. 1. Experimental details: groups, STZ-induced diabetes and the AG doses which were administered. (+) shows diabetes.

Groups	Diabetes	AG dose (one week)
C	–	–
AG50	–	50 mg/kg
AG100	–	100 mg/kg
AG200	–	200 mg/kg
AG400	–	400 mg/kg
D	+	–
DAG50	+	50 mg/kg
DAG100	+	100 mg/kg
DAG200	+	200 mg/kg
DAG400	+	400 mg/kg

Tab. 2. Primers which used to RT-PCR: Bcl-2, Bcl-xl, Bax, Bak and GAPDH measurement.

Primers	Product size	Gene
F: 5'-CTG GTG GAC AAC ATC GCT CTG-3' R: 5'-GGT CTG CTG ACC TCA CTT GTG-3'	228 bp	Bcl-2
F:5'-AGG CTG GCG ATG AGT TTG AA-3' R: 5'-TGA AAC GCT CCT GGC CTT TC-3'	357 bp	Bcl-xl
F:5'- TGC AGA GGA TGA TTG CTG AC -3 R: 5'- GAT CAG CTC GGG CAC TTT AG-3'	173 bp	Bax
F: GTCCATCAAGGCCCTCCAAACC R: AACAGGAGCATGGGTGAAGGTGG	259 bp	Bak
F: 5-'GGC CAA GAT CAT CCA TGA CAA CT-3' R: 5'-ACC AGG ACA TGA GCT TGA CAA AGT-3'	461 bp	GAPDH

Testing session

Twenty-four hours subsequent to the PAL acquisition trial, long-term memory retrieval was assessed by placing the animals in the lit compartment. After 10 seconds the door was opened, and then both the step-through latency of retention (STLr) and time spent in the dark compartment (TDC) were recorded for up to 600 seconds. During the test session, the electric shocks were not induced by the floor grid (26).

Finally, after anesthetizing the animals with chloroform, the skull was opened along the midline and the brain was taken out and put on an ice-cooled cutting board. Hippocampi were dissected from the hemispheres after removing the meninges, snapped frozen in liquid nitrogen and stored at –70 °C for extraction of RNA.

Preparing RNA and Semi-quantitative RT-PCR

According to the manufacture booklet, Trizol Reagent (Invitrogen) was used for total RNA extraction from hippocampi. RT was done by 1000 ng of total RNA into cDNA with RevertAid (Thermo, Scientific RevertAid cDNA synthesis Kit) and cDNA samples were stored at –70 °C. To determine the levels of Bcl-2, Bcl-xl, Bax and Bak mRNA expressions, RT-PCR was done. The RT-PCR mixture for Bcl-2, Bcl-xl, Bax and Bak genes (final volume of 25 µL) included 2 µL of cDNA, 12.5 µL of Thermo Scientific PCR Master Mix 2x and 10 pmols of each matching primer specific for Bcl-2, Bcl-xl, Bax and Bak sequences as well as Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene sequence as an internal control (Tab. 2). The details of processes were shown in our former study (23).

Statistical analyses

Data were analyzed using either two-way ANOVA, followed by Tukeys' post hoc statistical test for normal data or Kruskall-Wallis tests followed by Mann-Whitney's U test for non-parametric data. Following Mann–Whitney U test, the Holms Bonferoni's correction was used. Probability values less than 0.05 were considered significant.

Results

Behavioral results: PAL step-through test

Effects of AG administration on STLa

No significant differences in STLa were observed [Factor A × B, two-way ANOVA's $F(9, 40) = 0.756$, $p > 0.05$] which indicates an absence of sensory and motor impairments in both diabetic and non-diabetic rats (Fig. 1A).

Effects of AG administration on TA

Two-way ANOVA of mean ± SE of the TAs, indicated that one-week ip injections of AG has a positive effect on diabetes-induced impairment of TA [$F(9, 40) = 20.27$, $p < 0.0001$, Tukey's post hoc, $p < 0.05$, 0.001 and 0.001 for 50, 100 and 200 mg/kg AG, respectively versus diabetic control animals]. As shown in Figure 1B, except for the dose of 400 mg/kg, the diabetes-induced impairment of latency of retention was reversed by doses of 50, 100 and 200 mg/kg of the drug.

Effects of AG treatment on STLr

Based on non-parametric Kruskall-Wallis test of median ± quartile of the step-through latency time, there were no significant differences in STLr in non-diabetic animals (left columns of Figure 1C) which indicates that STLr was not affected in non-diabetic animals by AG ($H(4) = 0.898$, $p = 0.93$). On the other

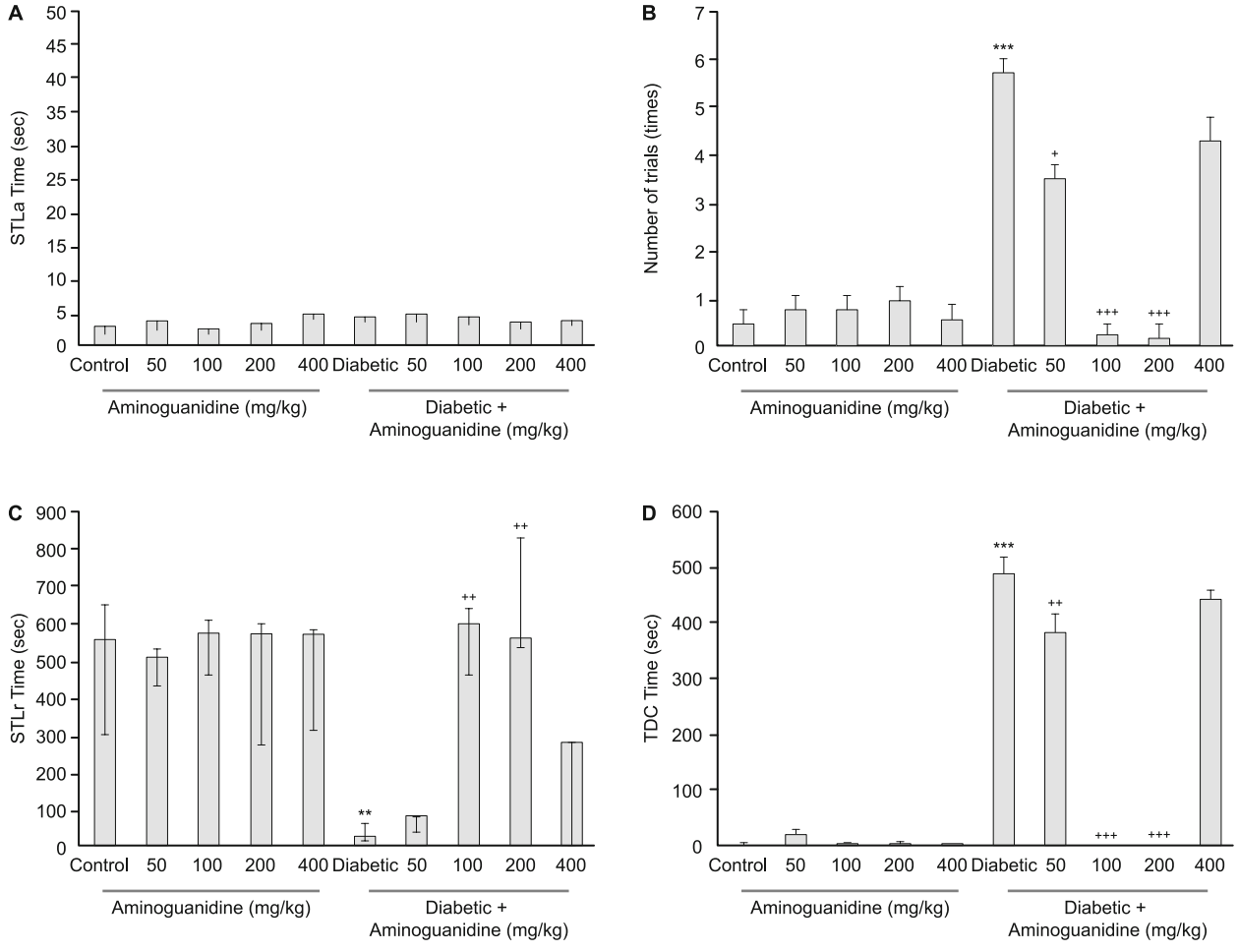

Fig. 1. Effect of one-week ip administration of different doses of aminoguanidine (AG) on the step-through latency of acquisition (STLa, A), number of trials to acquisition (TA, B), step-through latency in the retention trial (STLr, C) and time spent in the dark compartment (TDC, D) 24 h after acquisition of passive avoidance learning (PAL) task in control and diabetic animals. Each column represents mean ± SE (A, B and D) and median ± quartile (B). ** p < 0.01 and *** P < 0.001 compared with control group. + p < 0.05, ++ p < 0.01 and +++ p < 0.001 compared with diabetic group.

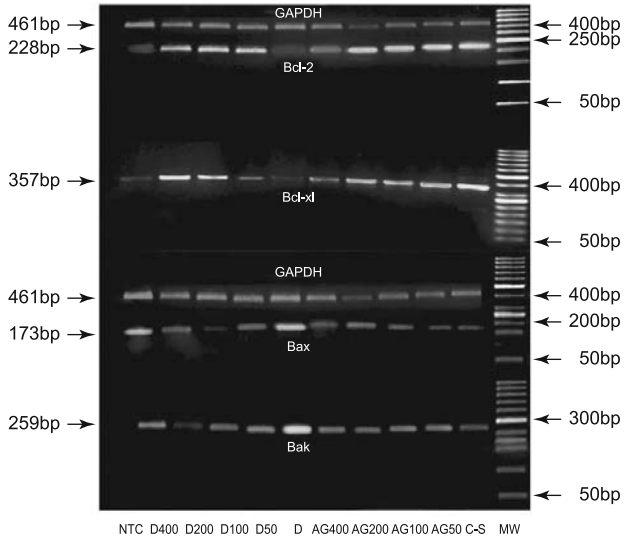

Fig. 2. Effects of Aminoguanidine (AG) on expressions of Bax, Bcl-xl, Bcl-2 and Bak mRNA in the hippocampus of rats. Expression analyses were performed by RT-PCR and the products (10 ll) were visualized following 1.5% agarose gel electrophoresis. The intensity of the bands was quantified by densitometric analyses and normalized with corresponding GAPDH. C, control; AG50, AG100, AG200 and AG400 control groups which received AG 50, 100, 200 and 400 mg/kg; D, diabetic; DAG50, DAG100, DAG200 and DAG400, diabetic treated with AG 50, 100, 200 and 400 mg/kg; MW: GeneRulerTM 50 bp DNA Ladder.

hand, non-parametric Kruskall-Wallis test showed that one-week ip injections of AG had a considerably positive effect on diabetes-induced impairment of STLr (right columns) (H (4) = 20.53, p = 0.0003, Mann-Whitney's U test, p = 0.082, 0.06, 0.06 and 0.31 for 50, 100, 200 and 400 mg/kg AG, respectively). As shown in Figure 1C, except for the doses of 50 and 400 mg/kg, the doses of 100 and 200 mg/kg of the drug could reverse the impaired latency of retention induced by diabetes.

Effects of AG administration on TDC

Based on two-way ANOVA of mean ± SE of the TDCs, one-week ip injections of AG improved diabetes-induced impairment of TDC [F (9, 40) = 161.56, p < 0.0001, Tukey's post hoc, p < 0.01, 0.001, 0.001 and 0.01 for 50, 100, 200 and 400 mg/kg AG, respectively in comparison to diabetic control animals] As indicated in Figure 1D, except for the dose of 400 mg/kg, the doses 50, 100 and 200 mg/kg of the drug could reverse the diabetes-induced impairment shown by time spent in the dark compartment.

Semi-quantitative mRNA levels of genes regulating cell death

Figure 2 shows a picture of Bcl-2, Bcl-xl, Bax and Bak gene expressions by RT-PCR with corresponding GAPDH.

Based on two-way ANOVA of the mean ± SE, one-week ip injections of AG have a positive effect on diabetes-induced decrease in both Bcl-2 [F (9, 40) = 6.53; p < 0.001, Tukey's post hoc; p < 0.01 for 50, 100 and 200 mg/kg AG, respectively in comparison to diabetic control animals] (Fig. 3A) and Bcl-xl expressions [F (9, 40) = 6.12; p < 0.001, Tukey's post hoc; p < 0.05 for 100 and 200 mg/kg AG, respectively in comparison to diabetic control animals] (Fig. 3B). As shown in figures 7 and 8, except for the doses of 400 mg/kg, the doses of 100 and 200 mg/kg of the drug could reverse the decreased Bcl-2 and Bcl-xl induced by diabetes.

On the contrary, one-week ip injections of AG efficiently reduced the diabetes-induced increase in both Bax [F (9, 40) = 11.35; p < 0.001, Tukey's post hoc; p< 0.001 for doses of 50, 100 and 200 mg/kg AG, respectively in comparison to diabetic control animals] (Fig. 3C) and Bak expression [F (9, 40) = 3.8; p < 0.01, Tukey's posthoc; p< 0.01 for doses of100 and 200 mg/kg AG, respectively in comparison to diabetic control animals] (Fig. 3D). As a result, the doses of 100 and 200 mg/kg of the drug could reverse the increase in Bax induced by diabetes. As shown in Figure 9, the dose of 400 mg/kg AG had a significant effect in comparison to non-diabetic control animals which may reveal toxic effects of the drug or other target contributions too.

The proportion of anti-apoptotic/apoptotic mRNA levels

According to Figure 4, the drug shows a significant effect on diabetes-induced decrease in (Bcl-2+Bcl-xl)/(Bax+Bak) [F (9, 40) = 5.39; p < 0.0001, Tukey's post hoc; p < 0.05 for doses of 100 and 200 mg/kg AG, respectively in comparison to diabetic control animals]. However, as indicated in Figure 4, the dose of 400 mg/kg AG had a significant effect in comparison to non-diabetic control animals which may imply toxic effects of the drug as well as other target contributions.

Discussion

According to the results, TA, STLr and TDC impairment by diabetes was recovered by one-week ip administrations of AG. The effect of AG on memory has been evaluated by several studies. It has been demonstrated that lipopolysaccharide-induced spatial memory impairments and neuronal apoptosis are improved by AG administration (27). Also, the memory restoration with naringin in unstressed and stressed mice was advanced by AG (15). Moreover, AG administration restored significantly an arsenic-induced destruction of acquisition (16). It has been shown that AG injection reversed hypoxia-induced retrograde memory destruction (17). Moreover, the drug might protect aluminum chloride-provoked memory defect (18). In contrast, the enhancing outcome of the effect of atorvastatin on short-term spatial memory recognition has been reversed by AG administration (19). It has been indicated that AG has not changed the recovering result of pioglitazone on morphine-induced passive avoidance task deficiency but has reduced the improving effect of pioglitazone on Y-maze discrimination deficiency result from morphine (21). Also, the beneficial effects of either atorvastatin or granisetron on memory consolidation deficiency due to scopolamine have been combated by AG in mice (20, 22). However, no study has been published regarding the effect of chronic or subchronic ip administration of AG on PAL and apoptosis in the hippocampus of diabetic animals.

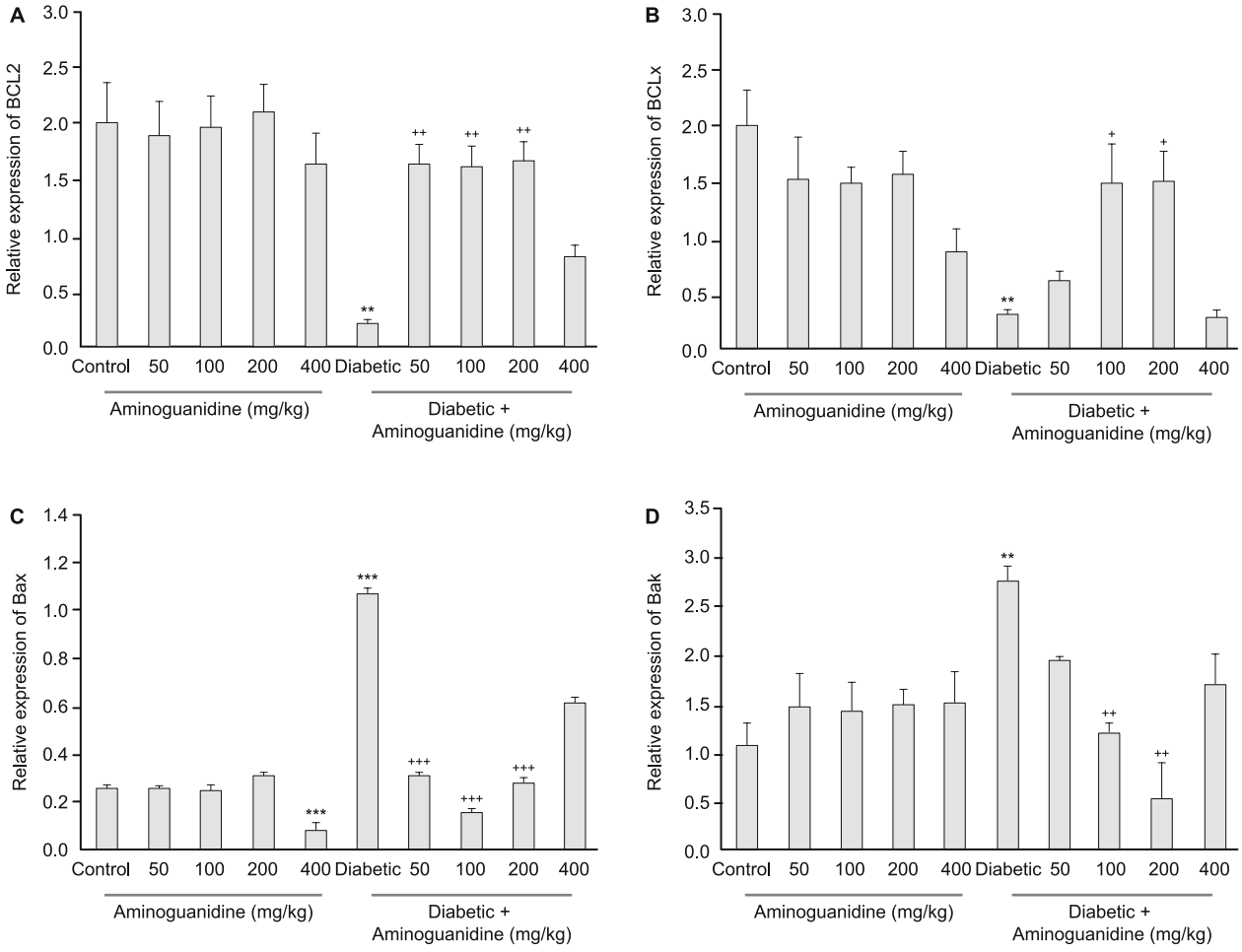

Fig. 3. Effect of one-week ip administration of different doses of aminoguanidine (AG) on the Bcl-2 (A), Bcl-xl (B), Bax (C) and Bak (D) mRNA in the hippocampus of rats. Expression analyses were carried out by RT-PCR and the products (10 ll) were visualized following 1.5% agarose gel electrophoresis (all amplifications were repeated three times). All data are represented as the mean ± SE. ** p < 0.01, *** p < 0.001 compared with control group. + p < 0.05, ++ p < 0.01 and +++ p < 0.001 compared with diabetic group.

Locomotor activities of rats were not measured in the present work. However, the useful result of lower doses of AG (100 and 200 mg/kg) on STLr might not be attributed to sensory or motor damage since the delay in the acquisition phase (STLa before electrical shock) did not demonstrate considerable differences among controls and experimental groups (diabetic and non-diabetic animals). In this work, the best results of TA, STLr and TDC were obtained with the doses of 100 and 200 mg/kg AG after one-week ip injections (peripheral administration of the drug) which is in agreement with our earlier work, i.e. single intra-hippocampal injection of AG (30 µg/rat) (23).

Several studies have demonstrated that the pathogenesis of diabetes-induced memory defects is associated with a number of factors. Several aspects including chronic hyperglycemia, microvascular and macrovascular complications, free radical production, and deficiency of antioxidant defense system have been proposed in the pathogenesis of diabetes-related cognitive deficit, but the precise mechanisms of the memory defect in diabetes is unknown and therapeutics are limited (28, 29). Several studies have indicated that administration of antioxidants reduces the hippocampal cell damage in diabetes-induced excitotoxicity (28). The positive role of AG on several pathogenic processes has been documented (30). Improvement in neuronal and vascular deficiencies in diabetic animals by AG has been reported (31). Moreover, it has been shown that AGEs play a crucial function in learning defects resulting from diabetes and that their development can be averted by AG (32–35). Besides, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are other main origins of cognitive dysfunctions due to diabetes (36) which is dependent on iNOS action (37). Inhibition of iNOS by AG may ameliorate the cognitive deficit induced by diabetes (38). Other neuroprotective mechanisms of AG may be related to its free radical scavenging properties (35, 39). Also, a number of cellular metabolic pathways about AG neuroprotective functions relate to methylglyoxal (MG) inhibition (40). MG

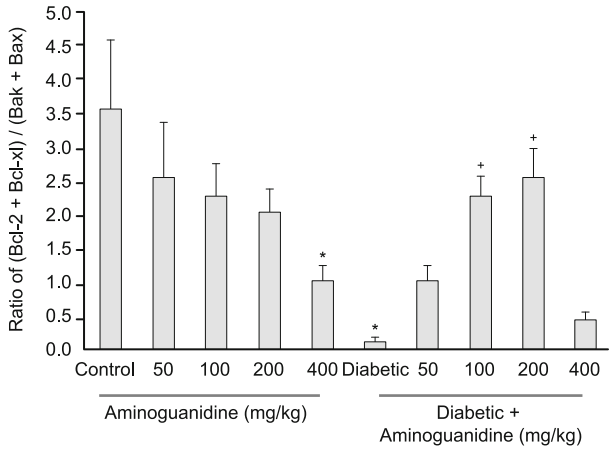

Fig. 4. Effect of oneweek ip administration of different doses of aminoguanidine (AG) on the (Bcl-2+Bcl-xl)/(Bax+Bak) ratios in the hippocampus of rats. Expression analyses were carried out by RT-PCR and the products (10 ll) were visualized following 1.5% agarose gel electrophoresis (all amplifications were repeated three times). All data are represented as the mean ± SE. * p < 0.05 compared with control group. + p < 0.05 compared with diabetic group.

acts as either an endogenous fatal substance (41) or an effective source of ROS (42), which frequently build up in the hippocampal neurons in hyperglycemia (43, 44, 45). However, the lack of MG, AGEs, ROS and iNOS measurement is a limitation in our study.

In this work, a decreased expression of Bcl-2 and Bcl-xl (as anti-apoptotic genes) and increased expression of Bax and Bak (as apoptotic genes) in diabetic rats were detected. An increase in Bax expression in the hippocampus has been reported in type-I diabetes which led to neuronal cell death (46). In the memory defect due to diabetes, apoptosis in hippocampal neurons plays a primary role (47, 48). On the other hand, it has been indicated that apoptosis in the hippocampus due to altering expression of Bcl-2 family proteins in the neurons could relate to reactive MG, ROS and RNS (44, 47, 49, 50). The imbalance of pro/anti-apoptotic signals of Bcl-2 family plays a crucial role in the release of apoptogenic mitochondrial mediators (51, 52). It has been shown that STZ-diabetes-induced hippocampal apoptosis in rats is related to the mitochondrial pathway (53, 54).

According to our results, one-week ip injection of AG demonstrated a beneficial effect on (Bcl-2+Bcl-xl)/(Bax+Bak) ratio due to diabetes. As seen in Figure 4, the dose of 400 mg/kg AG demonstrated adverse effects (in comparison to lower doses, 100 and 200 mg/kg) in diabetic and non-diabetic rats, which may be related to off-target or other pharmacologic effect which shows up at a high (toxic) dose of the drug.

To our knowledge, few reports exist regarding the effect of AG on the cited gene expressions in the hippocampus of diabetic rats. It has been indicated that AG can reduce apoptosis by its antioxidant activity and inhibition of either amine oxidase or iNos enzymes (49). Therefore, the beneficial effects of AG on memory might be dependent on altering the apoptosis in the brain regions which are associated with cognition. It has been demonstrated that the Bcl-2 or Bcl-xl to Bax expression ratios play a crucial role in determining cell life or apoptosis (13). It has been revealed that hyperglycemia due to STZ raises the ratio of Bax/Bcl-2 which results in cortical neurons apoptosis in newborn rats (55). In the present study, the beneficial effect of one-week ip administration of AG (100 and 200 mg/kg) on anti/apoptotic genes in STZ-induced diabetic rats is in favor of our earlier study (single-dose intra-hypocampal injection of the drug (23).

Our results suggest that one-week ip administration of AG (100 and 200 mg/kg), may improve the memory deficit in diabetic rats by increasing the (Bcl-2+Bcl-xl)/(Bax+Bak) proportion. However, further pathological and molecular studies are required to elucidate the exact mechanisms underlying the neuroprotective effect of AG on the memory in diabetic rats.

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