

# Glibenclamide ameliorates the expression of neurotrophic factors in sevoflurane anaesthesia-induced oxidative stress and cognitive impairment in hippocampal neurons of old rats

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## Abstract

**Introduction:** Several antidiabetic medications have been proposed as prospective treatments for cognitive impairments in type 2 diabetes patients, glibenclamide (GBC) among them. Our research aimed to evaluate the impact of GBC on hippocampal learning memory and inflammation due to enhanced neurotrophic signals induced by inhalation of sevoflurane. **Material and Methods:** Rats (Sprague Dawley, both sexes) were assigned to four groups: a control (vehicle, p.o.), GBC (10 mg/kg b.w.; p.o.), low-dose sevoflurane and low-dose sevoflurane + GBC (10 mg/kg b.w.; p.o.) for 23 days. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining was performed to analyse the count of apoptotic cells and ELISA was conducted to assess the protein signals. A Western blot, a Y-maze test, and a Morris maze test were performed, and the results analysed. Blood and tissues were collected, and isolation of RNA was performed with qRT-PCR. **Results:** The Morris maze test results revealed an improvement in the length of the escape latency on days 1 ( $P < 0.05$ ), 2 ( $P < 0.01$ ), 3, and 4 in the low-dose Sevo group. Time spent in the quadrant and crossing axis and the percentage of spontaneous alterations showed a substantial decrease in the low-dose Sevo group which received GBC at 10 mg/kg b.w. Significant increases were shown in IL-6 and TNF- $\alpha$  levels in the low-dose Sevo group, whereas a decrease was evident in the GBC group. **Conclusion:** Our results indicate that glibenclamide may be a novel drug to prevent sevoflurane inhalation-induced impaired learning and reduce brain-derived neurotrophic factor release, which may be a vital target for the development of potential therapies for cognitive deficits and neurodegeneration.

**Keywords:** glibenclamide, sevoflurane, neurotoxicity, Morris maze test, neurotrophic factor.

## Introduction

Over the past few years, clinical and preclinical studies have reported that the threat of dementia and memory impairment is increasing due to type 2 diabetes (T2D), which affects more than 400 million patients worldwide (8, 37) and could affect 640 million by 2040 (45). Type 2 diabetes is a chronic metabolic disorder with a negative impact mainly on insulin-producing regions. In the brain, insulin resistance is extreme in areas that are responsible for cognition, namely the hippocampal region, which contains greater insulin receptor concentrations (5). Neuroimaging showed that an attribute of T2D is reduced hippocampal volume (31). The hippocampus, as the region carrying out such functions as thinking and memory, is the region where the impairment associated with sporadic Alzheimer's

disease (AD) has its cause. This impairment is in cognition and occurs (as does sporadic AD in general) most frequently in geriatric populations (7, 14), in whom T2D is also more common. Emerging studies have reported pre- or fully developed T2D in patients with Alzheimer's (5), supporting the involvement of brain insulin resistance in sporadic AD pathophysiology. The dysregulation of the insulin-signalling pathway leads to compromised cognition (16).

Sevoflurane is a surgical anaesthetic inhalant (36) which caused cognitive dysfunction in various experiments on animals (41). Long-term exposure of rat models to a volatile anaesthetic resulted in symptoms similar to those present in autism spectrum disorders. Our research was conducted on senile rat models to determine the molecular mechanisms that induced neurotoxicity due to sevoflurane inhalation and whether

modulating these mechanisms may limit neurotoxicity along with cognitive learning impediment and memory impairment in rat models. Several studies show that exposure to sevoflurane induces oxidative stress, increases neuronal apoptosis in the hippocampus, decreases learning and memory, and substantially attenuates brain-derived neurotrophic factor (BDNF) signals. As a result, they demonstrate that BDNF has a neuroprotective effect in senile rat models that have suffered neurotoxicity produced by sevoflurane and have impaired cognition.

Evidence of diminished expression of BDNF implied its significant contribution to the progression of different neurological disorders, including Parkinson's disease and AD (28). This warrants the prescription of anti-diabetics to treat impaired cognition in patients with AD. BDNF serum levels in a mouse model increased significantly after the T2D drug metformin was administered in Parkinson's studies (34). In addition, BDNF expression was significantly restored in a high-fat-diet rat model following treatment with metformin alone or in combination with another T2D drug, glimepiride, BDNF transcription nevertheless reducing in elderly C57BL/6J mice (2). The protective effect was manifested strongly after administration of vildagliptin, another anti-diabetic drug, with an increase in brain BDNF expression improving memory impairment in diabetic rats (17). Similarly, intragastric alogliptin administration once a day for three weeks has shown increases in BDNF levels in the brain cortex and hippocampal regions in C57BL/6J mice. No studies have yet been performed to assess the effects of glibenclamide (GBC) on neurotrophic expression or specifically on BDNF levels in older rat models with impaired cognition due to sevoflurane-induced dysfunction. Our research therefore aimed primarily at evaluating the impact of GBC on hippocampal learning, memory, and inflammation by assessing the two inflammatory cytokines tumour necrosis factor alpha (TNF- $\alpha$ ) and interleukin 6 (IL-6). This experiment sought to recognise and understand the pathways involved in neurotoxicity due to enhanced neurotrophic signals induced by inhalation of sevoflurane, which contributed to increased oxidative stress and diminished cognition in neurons.

## Material and Methods

### Calculation of the sample size and justification.

The sample size was determined using power analysis, the standard deviation was utilised to ascertain the variability of the sample with type 1 error at  $P < 0.05$ , and two-tailed tests were administered with an 80% power analysis. The sample size was calculated using the G power software (19).

**Experimental animals.** Sprague Dawley rats aged eighteen months and of  $585 \pm 30$  g weight were obtained

from Beijing Animal Laboratory (China). They were kept at controlled room temperature and provided with a standard animal diet. These rats were chosen based on previously available data that indicated spatial memory loss due to exposure to sevoflurane. Approval for performing the procedures was received from the Animal Ethical Committee of the Department of Medical Sciences (under approval no. BHU/011/Jilin\_67/19) at BeiHua University. Fifteen rats were assigned to each of four groups: a control group administered only the vehicle for GBC (water), a group administered GBC at 10 mg/kg b.w. (GBC), a group administered sevoflurane (Sevo) in a low dose and a group administered sevoflurane in a low dose and GBC at 10 mg/kg b.w. Control group rats were subjected for 3 h to 50% moist oxygen balanced with nitrogen in an acrylic anaesthetising chamber. The second group was administered only GBC (10 mg/kg b.w.) by oral gavage. The third group was exposed to a 3% concentration and the fourth and final group was exposed to a 7% concentration of sevoflurane in a chamber with a vaporiser for 3 h (10). The inhaled concentration of sevoflurane (0.5–3%) was in compliance with the US Food and Drug Administration recommendations for the concentration needed for surgical purposes. Our preliminary findings revealed that diminution of perception was comparable at both the low and high sevoflurane doses; therefore, for our research the low dose of sevoflurane was selected based on US FDA recommendations. Each animal was enclosed in a large plastic cage, with enough space to move and oxygen to breathe. A catheter was inserted in the tail artery every hour after inducing sevoflurane anaesthesia to draw a sample with which to examine blood gas and pH, and to measure arterial CO<sub>2</sub> and blood O<sub>2</sub> content. These parameters were measured using a device which analyses oxygen saturation. The findings revealed normal respiration rates, blood pressure, body temperature and heart rate. The study was performed according to the experimental protocol described in Fig. 1.

**Drug administration.** Four groups of rats ( $n = 15$ ), namely the control group (receiving only the vehicle), the group receiving GBC alone at 10mg/kg b.w., the low-dose sevoflurane group, and the group receiving low-dose sevoflurane and GBC at 10 mg/kg b.w. were planned following 48 h of sevoflurane inhalation. The rat models were continuously administered GBC or the vehicle (water) by oral gavage every day for 23 days. Our pilot studies established the basis for calculating the length of treatment with GBC. Throughout the investigation, a study performed to understand the improvement in damaged memory in male genetically T2D dB/dB mice related to GBC administration was referred to in order to select the dose of GBC (3). Further information on animal models and the use of medications is provided below.

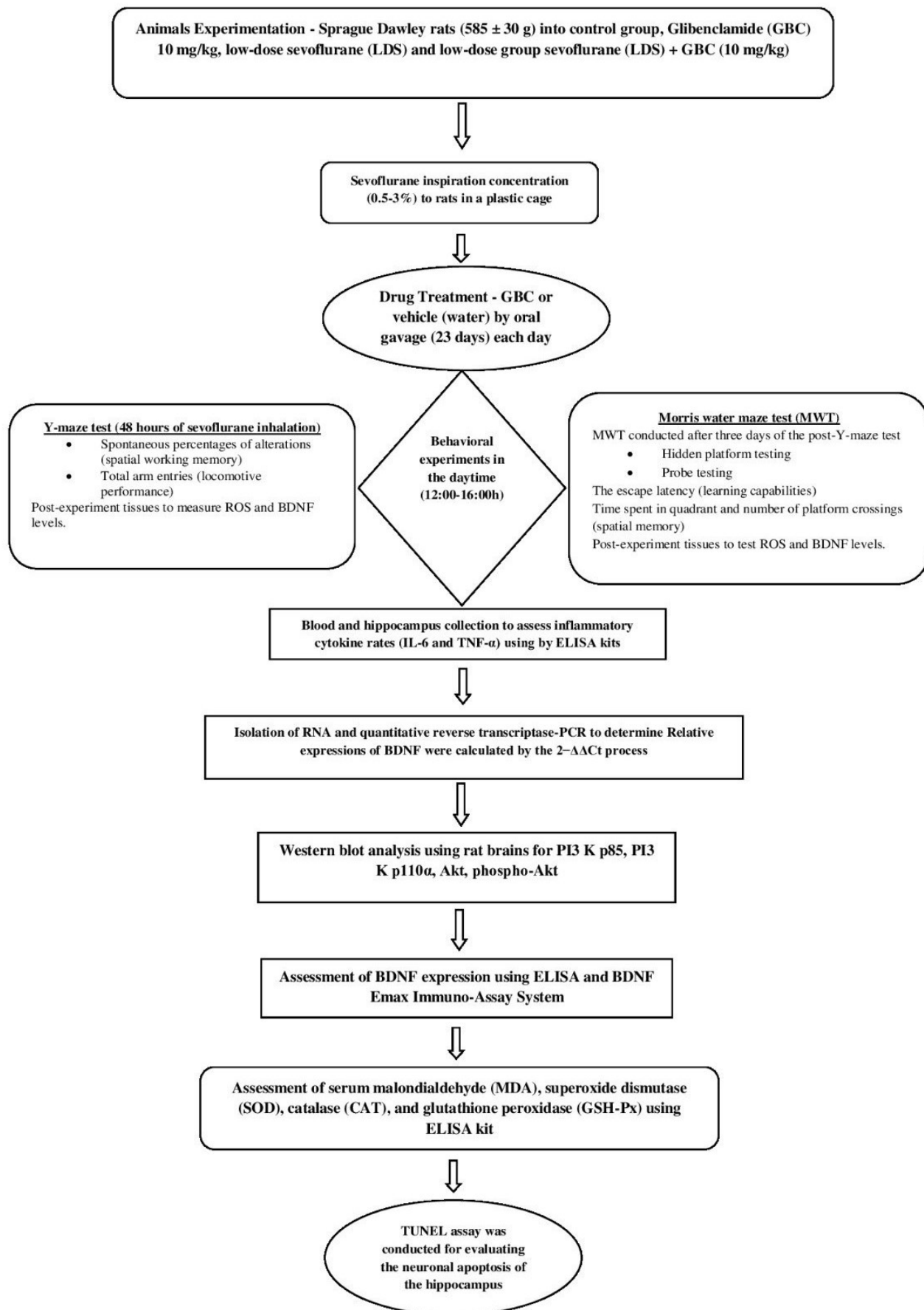


Fig. 1. Experimental protocol

**Behavioural apparatus.** Rats were chosen at random for all procedures and arbitrarily tested by a professional observer. A silent space was used for performing behavioural experiments in the daytime (12:00–16:00). These rats were allowed to stay in the room for an hour until assessment.

**Y-maze test.** A Y-maze analysis was conducted and the test was evaluated as described in a previous study (28). The percentages of spontaneous alternations revealed spatial working memory, while locomotive performance was demonstrated by total arm entries. After the experiment, tissues were collected immediately to measure reactive oxygen species (ROS) and BDNF levels.

**Morris water maze test (MWT).** An MWT was conducted after the Y-maze test for three days as described in a previous study (28). The maze consisted of a large rectangular plastic tank 180 cm in diameter and 50 cm in height with a bluish coloured base placed in a room that was closed off from external stimuli by a black curtain. It was filled to a depth of 15 cm with water at 25°C and a transparent plastic spherical platform 12 cm in diameter was placed 2 cm underneath the surface of the water, onto which white talcum powder was dusted to make it opaque. Different objects or geometrical images were hung on the wall of the test room, such as circles, squares and triangles of different colours, as visual spatial clues. Each rat's swimming activity was monitored using a camera coupled to a computer monitoring system, from which the latency to locate the platform, total distance travelled, and time and distance spent in each quadrant were measured. Following the 48 h of sevoflurane inhalation, all animals received the intended treatment every day from 8 a.m. to 10 a.m., except for behavioural testing days, when such treatments were given 3 h before the testing. Ten days after the indicated treatment the animals were subjected to the first Morris water maze test, which lasted four days and consisted of four trials each day. For all trials, the platform was positioned in the NE quadrant. Thirteen days after the initial Morris water maze test, the animals received the intended treatment and were retested for two days of probe testing in the Morris water maze. The platform was removed from each trial to assess reference memory following long intervals. Learning capabilities were measured using the escape latency in seconds. Spatial memory was measured by the period spent in the quadrant of the platform and the number of crossings. After the experiment, tissues were collected immediately to test ROS and BDNF levels.

**Hidden platform testing.** Each day, the rats were subjected to four trials. At each trial, the rats were permitted 60 s to swim to the hidden platform. If the rat was successful, it was allowed to remain on the platform for 15 s. If the rat was unable to complete the task within 60 s, it was given a score of 60 s and then placed on the platform for 15 s. Trials were separated by a 4–6-min interval. The time required to locate the platform and the distance to the platform were recorded to compare groups' spatial memory and learning abilities.

**Probe testing.** Each day, three trials of 60 s each were conducted 5 min apart without the presence of a platform. The time spent by the animal in the currently "correct" quadrant was used to generate a reference memory index.

**Camera system.** The camera mounted on top of the apparatus captured all swimming activity. Wintrack software (40) was then utilised to evaluate the video document.

**Blood and tissue collection for RNA isolation.** After 48 h had elapsed since exposure to sevoflurane (for the proper elimination of anaesthetic agents from the brain), a lethal dose of pentobarbital was administered intraperitoneally. Blood was then collected, and the hippocampus was removed and maintained at –80°C as described in previous studies (28). Hippocampal tissues were homogenised in an ice-cold lysis buffer with protease inhibitor mixture. Homogenate fractions of tissues were subjected to centrifugation (4°C) at 15,000 × g for 15 minutes, and the supernatants and blood serum were stored at –80°C in sterile vials until they were processed. Hippocampal inflammatory cytokine (IL-6 and TNF- $\alpha$ ) presence was detected by ELISA kits (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) as indicated in the manufacturer's manual.

**Isolation of RNA and quantitative reverse transcriptase PCR.** RNA extraction was performed from stored tissue samples as per the instructions given in an RNeasy kit (Qiagen, Hilden, Germany). One gram of total RNA was used for reverse transcription utilising a qRT-PCR kit (Thermo Fisher Scientific, Waltham, MA, USA) as per the manufacturer's guidance. BDNF RNA was amplified in both forward and reverse directions with the priming sequences supplied in the PCR kit. Agarose gel electrophoresis analysis corrected the height of the distorted amplicons. Relative expressions of BDNF were calculated by the 2– $\Delta\Delta C_t$  process, for which the assay was performed in triplicate. For data normalisation, glyceraldehyde-3-phosphate dehydrogenase (the *GAPDH* gene) was used as a control. After normalisation, the findings were reported as mean  $\pm$  SD showing the relative fold induction of BDNF expressions of tissue specimens from their respective group.

**Western blot analysis.** Rat brains were isolated and extracted from each group shortly after sacrifice, and the hippocampus was homogenised in protease inhibitors and lysis buffers (Roche, Mannheim, Germany). An RC-DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA) was used to test protein concentrations as instructed by the manufacturer. Gel electrophoresis with the SDS-PAGE method isolated the proteins, which were then transferred to the PVDF membrane and set for one hour at 25°C in 4.5 % non-fat milk. These membranes were later tested at 4°C using rabbit anti-phosphoinositide 3-kinase (PI3K) p85, rabbit anti-PI3K p110 $\alpha$ , rabbit anti-Akt, and rabbit anti-phospho-Akt primary antibodies diluted at 1:1000 (Cell Signaling Technology, Danvers, MA, USA). Secondary antibodies diluted at 1:500 (Cell Signaling

Technology, Danvers, MA, USA) conjugated with horseradish peroxidase were pipetted onto these membranes and incubated for 1 h. Chemiluminescence was used for protein band blot analysis by comparing with  $\beta$ -actin bands to visualise their intensities.

#### Assessment of BDNF expression using ELISA.

The BDNF levels were assessed using an ELISA kit (catalogue no. ERBDNF, Invitrogen, Carlsbad, CA, USA) and BDNF Emax Immuno Assay System (Promega, Madison, WI, USA). An ELISA (Sigma-Aldrich, Shanghai, China) was used to detect serum malondialdehyde (MDA, catalogue no. MAK085-1KT), superoxide dismutase (SOD, catalogue no.19160), catalase (CAT, catalogue no. C3556), and glutathione peroxidase (GSH-Px, catalogue no. G6137). The findings were evaluated using a microplate reader set for the 575 nm wavelength for MDA, 525 nm for SOD, 450 nm for CAT and 450 nm for GSH-Px. Modified protein extraction buffer was used to homogenise the hippocampal tissue samples extracted. Bicinchoninic acid quantification analysis of the homogenised tissue was used to determine protein concentration.

**Terminal deoxynucleotidyl transferase dUTP nick end labelling assay.** Neuronal apoptosis was evaluated in 5- $\mu$ m-thick sections of the hippocampus using a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay as per the manufacturer's procedure (R&D Systems, Minneapolis, MN, USA). The image was recorded using an optical camera and associated software and magnified at 400 $\times$  with an optical microscope (Olympus Life Sciences and Olympus cell Sens, Tokyo, Japan). Quantitative analysis was carried out on the section in which light was scattered into three representative fields. Dark-violet stained nuclei indicated TUNEL-positive cells, which were quantified using the following formula: TUNEL-positive cells = total apoptotic cells/total viable cells  $\times$  100.

**Statistical analysis.** The data were analysed and expressed as mean  $\pm$  SD using SPSS version 16 for Windows software (SPSS Inc., Chicago, IL, USA). ANOVA (one or two-way variance analysis) was used for comparison of various means, accompanied where applicable by a Tukey's test or Student's *t*-test in case of two-group comparison. In all the comparisons,  $P < 0.05$  was deemed statistically significant.

## Results

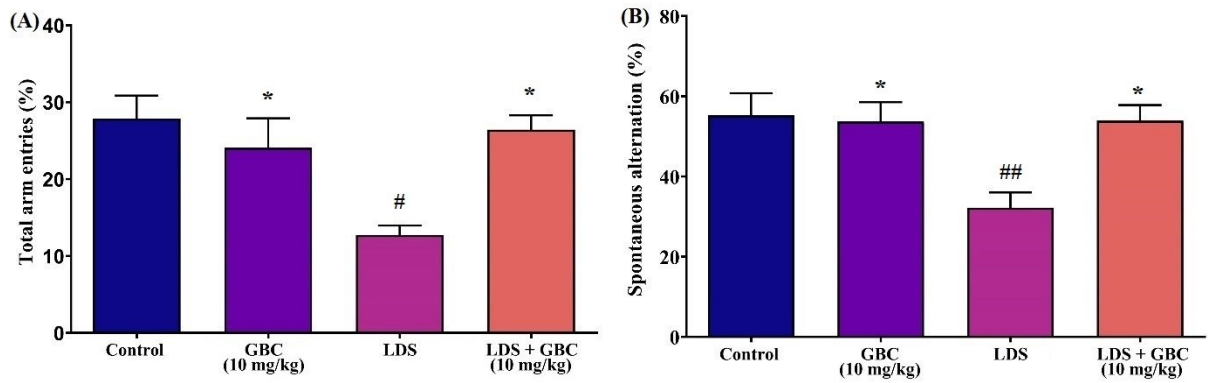
**Effects of GBC on cognitive dysfunction in elderly rats induced by sevoflurane anaesthesia: Y-maze test.** During anaesthesia induced by inhalation, the effects of glibenclamide on spatial working memory in aged rats with sevoflurane-induced cognitive dysfunction were calculated using a Y-maze study. A significant performance difference in this test was observed between groups in their percentages of spontaneous alternations (Fig. 2) ( $F(3, 26) = 15.874$ ;  $P = 0.0067$ ) and total arm entries (Fig. 2) ( $F(3, 26) = 16.623$ ;  $P = 0.0041$ ).

Figure 2 shows significant *post-hoc* comparisons of spontaneous alternations and a significant decline for sevoflurane-induced cognitive dysfunction-afflicted rats ( $P = 0.0067$ ) compared with control group rats. In addition, after GBC therapy a considerable rise was observed in the percentages of spontaneous alternations in aged rats ( $P = 0.0023$ ) compared to the animals in the low-dose sevoflurane group. Our results showed that anaesthesia induced by sevoflurane induced memory impairment in rats and that treatment with glibenclamide restored cognitive functions.

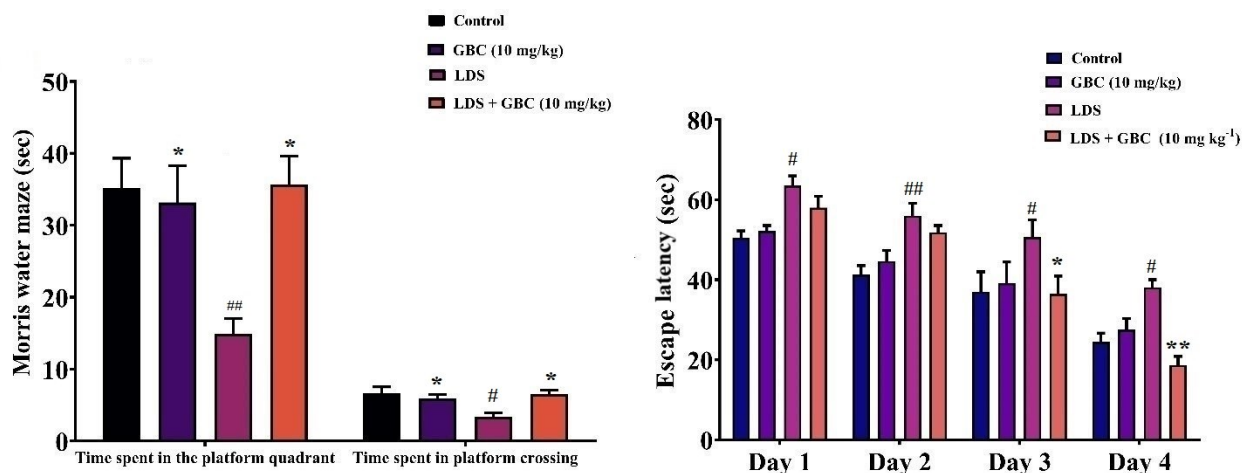
**Morris water maze test.** The Morris water maze test was used to corroborate the therapeutic effect of glibenclamide on learning and memory in rats with cognitive impairment caused by inhalation of sevoflurane. The main effects of day ( $P < 0.001$ ) and experimental group ( $P < 0.001$ ) on escape latency were analysed using two-way repeated ANOVA. No significant differences were noted between the days ( $P = 0.2055$ ) or the experimental groups. Fig. 3 shows that sevoflurane-induced anaesthesia impaired cognition ( $F(9, 48) = 1.422$ ;  $P = 0.2055$ ) such as to greatly increase the duration of escape on test days 1–4 relative to control rats. Our results demonstrated that after treatment with GBC, the escape latency in rats with sevoflurane-induced cognitive dysfunction was significantly longer on days 3 ( $F(3, 48) = 62.13$ ;  $P < 0.001$ ) and 4 ( $F(3, 48) = 15.23$ ;  $P < 0.001$ ) relative to the latency in counterpart rats administered no GBC.

In comparison, the findings of a one-way variance study showed a significant disparity between the groups over the span of time spent in the quadrants of the platform ( $F(3, 44) = 16.198$ ;  $P = 0.0087$ ) and the crossings ( $F(3, 44) = 15.631$ ;  $P = 0.0121$ ). Figure 3 shows cognitive impairment similar to Alzheimer's disease in the low-dose sevoflurane group with a significant decrease in platform quadrant time ( $P = 0.0087$ ) and platform crossing ( $P = 0.0121$ ) compared with the control group. In comparison, a significant difference was observed in the time spent in platform quadrants ( $P = 0.0077$ ) and platform crossings ( $P = 0.013$ ) between rats with low-dose sevoflurane-inflicted cognitive impairment which received GBC and rats which were administered the same low dose but no GBC. These results show that cognitive impairment in rats developed following inhalation of sevoflurane but that memory deficits improved after GBC administration to cognition-deficient rats.

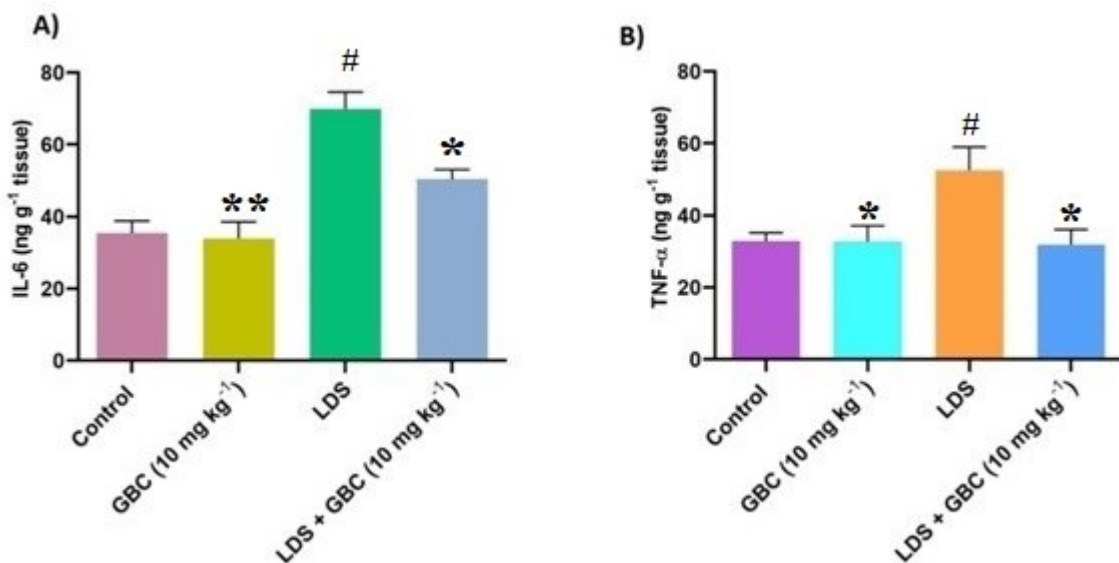
**Hippocampal inflammation.** To evaluate activation of hippocampal inflammation in rats which had been given low-dose sevoflurane anaesthesia, an assay of IL-6 (Fig. 4A) and TNF- $\alpha$  (Fig. 4B) cytokines was performed. The results revealed significant differences in rat hippocampi between IL-6 ( $F(3, 42) = 18.06$ ;  $P < 0.001$ ) and TNF- $\alpha$  ( $F(3, 52) = 4.725$ ;  $P = 0.0212$ ). Figure 4 shows a significant increase in the levels of IL-6 ( $P < 0.001$ ) and TNF- $\alpha$  ( $P = 0.041$ ) in low-dosed rats compared with the control group. In contrast, notable attenuation was observed in both IL-6 ( $P < 0.001$ ) and TNF- $\alpha$  ( $P = 0.0212$ ) rat hippocampus concentrations in the low-dose sevoflurane group which also received GBC relative to the low-dose sevoflurane group which only received the vehicle.



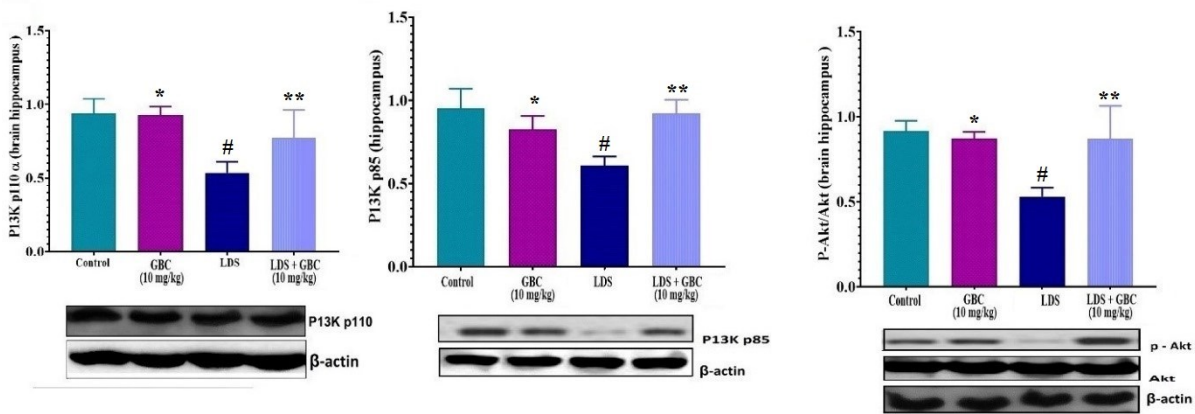
**Fig. 2.** Effects of glibenclamide on memory in rats with sevoflurane-induced cognitive impairment as demonstrated by the Y maze test. Values are presented as (A) mean ± SEM (n = 15) of total arm entries and (B) percentage of spontaneous alternations: # – p < 0.05 and ## – p < 0.01 for significant difference *versus* the control group; \* – p < 0.05 for significant difference *versus* the low-dose sevoflurane group; GBC – glibenclamide; LDS – low-dose sevoflurane



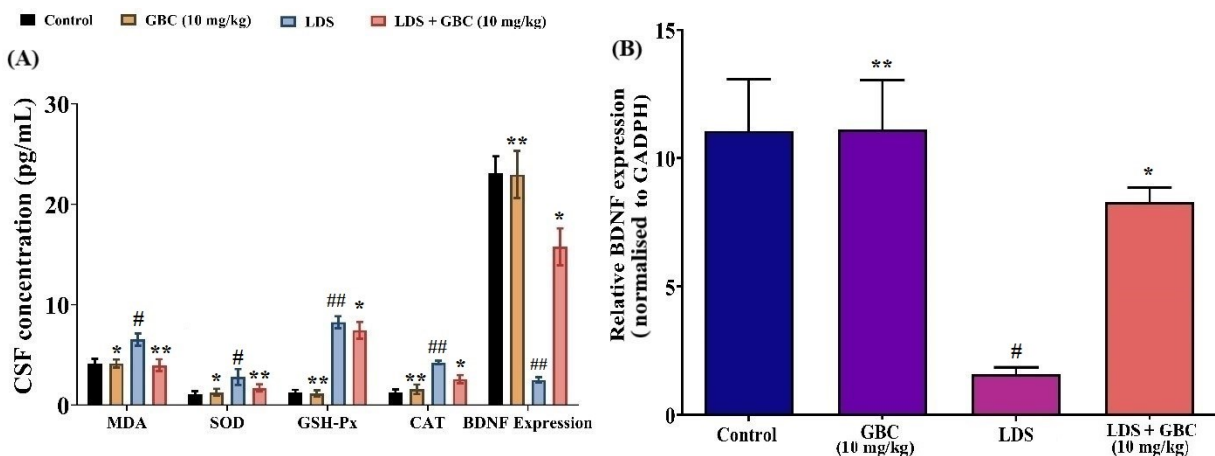
**Fig. 3.** Effects of glibenclamide treatment on learning and memory in rats with sevoflurane-induced cognitive impairment in the Morris water maze test. Values are presented as mean ± SEM (n = 15) escape latency, time spent in the platform quadrant or the platform crossings. # – p < 0.05 and ## – p < 0.01 for significant difference *versus* the control group; \* – p < 0.05 and \*\* – p < 0.01 for significant difference *versus* the low-dose sevoflurane group



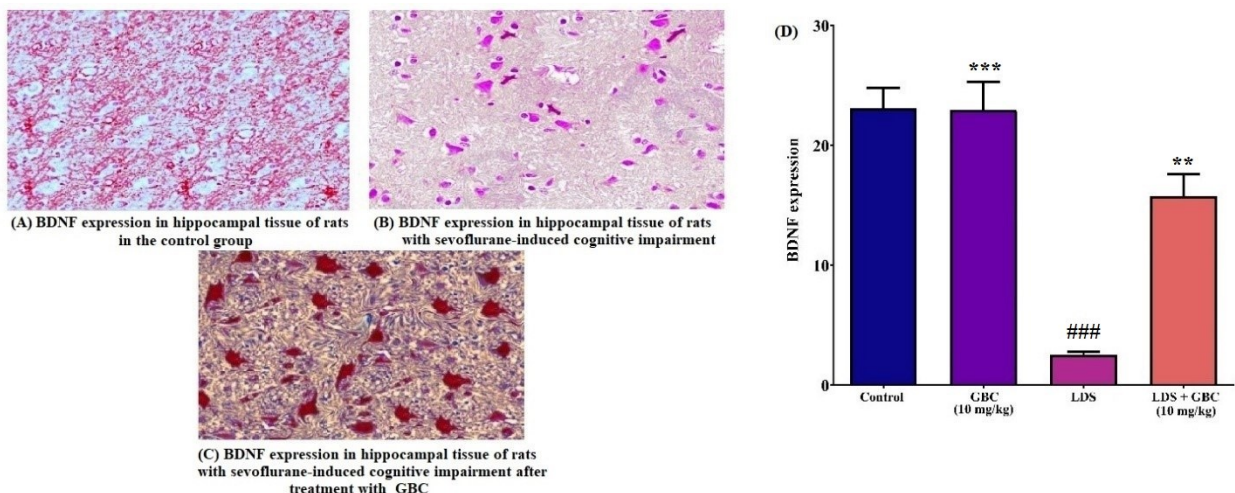
**Fig. 4.** Effects of glibenclamide treatment on interleukin 6 (IL-6) (A) and tumour necrosis factor alpha (TNF-α) (B) levels in the hippocampus of rats with sevoflurane-induced cognitive impairment. Values are presented as mean ± SEM (n = 15) of the concentrations. # – p < 0.05 for significant difference *versus* the control group; \* – p < 0.05 and \*\* – p < 0.01 for significant difference *versus* the low-dose sevoflurane group; GBC – glibenclamide; LDS – low-dose sevoflurane



**Fig. 5.** Effects of glibenclamide treatment on attenuation of phosphoinositide 3-kinase (PI3K)/Akt signalling pathways in sevoflurane-induced cognitive impairment in the hippocampal regions of aged rats. Values are presented as mean ± SEM (n = 15) of the concentrations. # – p < 0.05 for significant difference versus the control group; \* – p < 0.05 and \*\* – p < 0.01 for significant difference versus the low-dose sevoflurane group; GBC – glibenclamide; LDS – low-dose sevoflurane



**Fig. 6.** Effects of glibenclamide treatment on BDNF expression in sevoflurane-induced cognitive impairment in rats. (A) Serum levels of BDNF and indicated oxidative stress markers in rats with or without effects of sevoflurane inhalation; (B) Expression of *BDNF* mRNA in sevoflurane-induced cognitive impairment in the hippocampal regions of aged rats. Data represent mean ± SEM (n = 15). # – p < 0.05 and ## – p < 0.01 for significant difference versus the control group; \* – p < 0.05 and \*\* – p < 0.01 for significant difference versus the low-dose sevoflurane group; GBC – glibenclamide; LDS – low-dose sevoflurane



**Fig. 7.** Brain-derived neurotrophic factor (BDNF) expression in sevoflurane-induced cognitive impairment in rats. (A) Expression of BDNF protein in the control group; (B) BDNF expression in sevoflurane-induced cognitive impairment in the hippocampal regions of aged rats; (C) Expression of BDNF protein after treatment with glibenclamide in sevoflurane-induced cognitive impairment in the hippocampal regions of aged rats; (D) Relative quantification of BDNF expression in the control group, GBC-only group, low-dose sevoflurane group without GBC, and low-dose sevoflurane group with GBC (n = 5). Significant differences versus the low-dose sevoflurane group: ### – p < 0.01 for significant difference versus the control group; \*\* – p < 0.01 and \*\*\* – p < 0.001 for significant difference versus the low-dose sevoflurane group; GBC – glibenclamide; LDS – low-dose sevoflurane

**PI3K/Akt attenuation of signalling pathways in aged rat hippocampal regions following sevoflurane-induced anaesthesia.** Our analysis measured the impact of inhaling sevoflurane on PI3K/Akt activation (Fig. 5). Treatment with sevoflurane resulted in a decline in the expressions of PI3K-p85 and PI3K-p110 $\alpha$  but a slight rise in their expressions resulted from GBC treatment at 10 mg/kg b.w. ( $F(3, 38) = 19.840$ ;  $P = 0.0046$  and  $F(3, 38) = 17.935$ ;  $P = 0.0088$ , respectively). No changes in terms of PI3K-p85 and PI3K-p110 $\alpha$  were observed in the control group or GBC-administered group (10 mg/kg b.w.) without sevoflurane. Furthermore, inhalation of sevoflurane also contributed to a decrease decline in P-Akt/Akt production in the hippocampus. In comparison, treatment with glibenclamide considerably reversed the impact of sevoflurane on the activation of P-Akt/Akt. No significant changes in P-Akt/Akt expression ( $F(3, 38) = 18.589$ ;  $P = 0.0070$ ) were observed in the control group or GBC group.

**Sevoflurane-induced oxidative stress injury, neuronal apoptosis and BDNF expression inhibition in aged rat hippocampal neurons.** To investigate the impact of sevoflurane-induced oxidative stress, neuronal apoptosis, ROS species production and BDNF expression inhibition, hippocampal intracellular concentrations were measured. The low dose of sevoflurane resulted in significant induction of ROS generation followed by significant apoptosis in the neurons of the hippocampal regions as determined by TUNEL assay. Treatment with GBC, however, significantly reversed ROS levels (Fig. 6) and lowered the percentage of apoptotic cells.

The above effects of ROS generation resulting in oxidative stress further motivated us to evaluate serum rates of activity of MDA ( $F(3, 52) = 15.409$ ;  $P = 0.0138$ ), CAT ( $F(3, 52) = 19.47$ ;  $P = 0.0003$ ), GSH-Px ( $F(3, 52) = 48.84$ ;  $P < 0.0001$ ), and SOD ( $F(3, 52) = 20.41$ ;  $P < 0.001$ ). Sevoflurane stimulated marked serum level rises in each of these oxidative stress markers. In comparison, treatment with GBC greatly decreased serum rates of activity of MDA, CAT, GSH-Px, and SOD (Fig. 6) in the hippocampi of the rats with low-dose sevoflurane-mediated cognitive dysfunction relative to these rates in the vehicle-treated low-sevoflurane group.

Our findings showed that low-dose sevoflurane anaesthesia altered the production of BDNF in aged rat hippocampal neurons. A significant decline in the transcription of BDNF mRNA accompanied by simultaneous down-regulation of BDNF proteins was evident, as seen in Fig. 6. A substantial decline in serum BDNF levels in aged rats after inhalation of sevoflurane is clearly seen in the findings. In comparison, treatment with GBC following low-dose sevoflurane exposure caused a dramatic increase in serum BDNF levels as well as up-regulated BDNF protein production with a substantial increase in BDNF mRNA expression ( $F(3, 32) = 19.898$ ;  $P = 0.0014$ ) in rat hippocampi relative to the low-sevoflurane group administered no GBC.

## Discussion

The specific pathophysiological mechanisms underlying cognitive dysfunction and brain damage in diabetes mellitus are unknown; however, various factors such as hyperglycaemia, vascular illness, hypoglycaemia, and insulin resistance play considerable roles. Microvascular complications and poor glycaemic control (defined as elevated glycated haemoglobin) contribute to morphological and physiological modifications in the central nervous system seen in greater cortical atrophy and micro-structural malformations which lead to cognitive dysfunction linked with intellectual disability, deficient working and verbal memory, and involuntary movements. Hypoglycaemia and hyperglycaemia have both been shown as key contributors to cognitive impairment. Diabetes mellitus (type 2) accelerates the ageing process of the brain, causing cerebral atrophy; additionally, it appears to interfere with cerebral amyloidogenesis, resulting in tau hyperphosphorylation *via* a mechanism related to insulin resistance, neuroinflammation, and synaptic plasticity disruption (38).

Sulfonylureas and metformin, alone or in combination, were found to lower the risk of dementia in a previous study (21). There have been no preclinical or molecular studies on the possible mechanisms involved in these drugs' regulation of cognitive function; however, two of the sulfonylureas, glyburide and glipizide, were shown to intervene as mammalian target of rapamycin (mTOR) inhibitors (23). Given the role of the mTOR pathway in memory and learning, as well as its suggested role in neurodegenerative disorders such as Alzheimer's disease (9, 13), such drugs may be beneficial in the treatment of cognitive impairment in diabetic patients. A recent study showed that glimepiride protects neurons from amyloid- $\beta$  induced synapse damage, and that reducing synapse damage may slow the onset of cognitive decline in Alzheimer's disease (33). Finally, glibenclamide was found to be effective in restoring memory function and normalising blood-brain-barrier modifications in db/db mice, a mouse model of spontaneous diabetes with hyperinsulinaemia, but the effects of glibenclamide on cognitive improvement involving BDNF expression have not been studied.

BDNF is a ubiquitously expressed neurotrophin which is present in the hippocampal region and contributive to neuroplasticity. It is responsible for the proper functioning of the nervous system and for the development of the embryo. Learning and memory mechanisms are regulated by BDNF signals, as was demonstrated with an improvement in hippocampal-dependent cognitive tasks (25, 27). Anxiety and other emotions may arise with the involvement of the hippocampus and the level of BDNF in this region may be influential upon origination of these feelings (35). Hence reduced signalling by BDNF owing to the intake of sevoflurane induces clustered cognitive dysfunction.



Boosting BDNF expression may contribute to mitigation of the impairment in cognitive abilities.

Studies have shown that BDNF and synaptotagmin-1 protein levels both decrease significantly after the intake of sevoflurane, which affects synapse transmission and memory development. It was also found that postsynaptic density protein 95 expression decreases cognition development with the ingestion of sevoflurane in the medial prefrontal cortex of the neonatal, adult or geriatric rat populations (43), although the precise role of these signalling pathways is uncertain.

The results showed that the pathology of impaired cognition featured decreased BDNF expression. Analysis of gene expression was not performed in our research, which is one of the drawbacks of our analysis. This study helps to explain the consequences of sevoflurane inhalation and the impact of BDNF on gene expression when GBC restores the neurotrophin to the normal level. While we did not determine if replenished BDNF could minimise or prevent hippocampal neuronal apoptosis due to sevoflurane exposure, our findings nevertheless suggest that apoptosis was inhibited by it; however experimental verification is needed.

Higher concentrations of advanced glycation end products in the brain promote glycation in the plasma membrane and raise the number of receptors, leading to an alteration in the intracellular signalling pathway, an upregulation of gene expression, the release of pro-inflammatory molecules, and the accumulation of scavengers of reactive oxygen species, all of which could result in increased oxidative stress and more prevalent microvascular pathophysiological changes. All these processes have been linked to neural ageing and hippocampal atrophy (10).

In agreement with earlier published results, our analysis showed that impaired memory in older rats was due to the elevated levels of the hippocampal inflammatory cytokines IL-6 and TNF- $\alpha$  following sevoflurane inhalation (5, 10, 27, 30). We found that when administered with a lower dose of sevoflurane, glibenclamide was able to reduce certain hippocampal inflammatory cytokines in rats with cognitive disability. A study reported that the hippocampal TNF- $\alpha$  levels in ischaemia-reperfusion injury rats were decreased by GBC, which was also the orientation of our results (29).

Glibenclamide was also able to reduce sevoflurane-mediated memory impairment. A clinical study also reported that memory in diabetic patients had increased with GBC therapy. Similarly, to our results, a report showed better memory in ischaemic or traumatically brain-injured rats in the subjects treated with GBC (39). Another study observed a decline in cognitive dysfunction related to a high-fat diet in mice with obesity (32). In their Y-maze and MWT studies, Chen *et al.* (11) recorded a lessening of previously experimentally induced memory impairment, regeneration of hippocampal neuroplasticity, and a decrease in amyloid- $\beta$  infiltration, which crosses the blood-brain barrier in male mice with T2D (db/db),

when subjects were treated with glibenclamide. Baraka *et al.* (6) also found related effects of decreased tau protein hyperphosphorylation in the hippocampus and increased memory and learning in an MWT when GBC was administered to rats challenged with amyloid- $\beta$  for 3 weeks. These results indicate that treatment with glibenclamide mitigates behavioural signs of diminished cognitive functioning that are consistent with those observed in AD (20).

The PI3K/Akt signalling mechanism was examined to further investigate the impact of GBC in rat models with impaired cognition due to low-dose sevoflurane inhalation. The association of neurotrophic factors with their receptors (6) resulted in the activation of the PI3K/Akt signalling pathway, contributing to morphological changes such as enhanced neuroplasticity and neuronal survival along with enhanced neuronal development, regeneration and differentiation. The PI3 kinase is a heterodimer consisting of p85 and p10 subunits which have respective regulatory and catalytic functions in the phospholipid kinase family. Glibenclamide stimulates PI3K, which transforms into phosphatidylinositol (4,5)-biphosphate and binds to Akt by an unclear mechanism. It induces Akt phosphorylation in the presence of 3-phosphoinositide-dependent protein kinase-1, leading to Akt activation (22). Phosphoinositide 3-kinase shows Akt as its essential downstream molecule, and upon activation, Akt may trigger the phosphorylation of other downstream molecules which regulate cell proliferation and existence, thus protecting the cells damaged by ischaemic injuries (44).

In histological analysis of the hippocampus, elevated levels of TUNEL-stained activated cells were found. In numerous other studies which reported apoptosis in the hippocampal region, such findings were also made, and activation of the pro-apoptotic protein signal was attributed to consistent sevoflurane ingestion. These studies also confirmed that anaesthetic agents induced pro-apoptotic effects, namely early neuronal deficiencies, which resulted in later-stage behavioural deficits (42) that were reduced when treated with GBC. Hence, the mechanism by which GBC reversed the effects of sevoflurane neuro-damage was the suppression of neuronal apoptosis.

Accumulated ROS could also result in enhanced apoptosis (26). Several reports have shown that anaesthetic agent-induced brain intoxication was characterised by elevated oxidative stress leading to escalated lipid peroxidation, cell destruction and neuronal death (24). Our results indicated increased MDA levels while sevoflurane decreased SOD and CAT levels. The fall in SOD and CAT levels owing to sevoflurane suggests a higher potential for the disruption of the pathological process. The decrease may be attributed to a particular route of absorption of sevoflurane which has a direct effect on the brain's antioxidant process (1). In numerous animal experiments, it was also found that GBC minimised

neuronal damage by modulating antioxidation and the concentration of free scavenger radicals inside the brain (18). At the same time, it was observed that GBC often decreases the oxidative stress induced by different pathways leading to renal injury or neutrophilic inflammation (46). We found that GBC decreased oxidative stress and restored the antioxidation mechanism in the hippocampus. This suggests that GBC greatly mitigated the effects of oxidative stress attributable to sustained sevoflurane exposure.

When assessed using the Morris water maze test, significantly augmented memory and spatial learning in sevoflurane-induced rats was noted following GBC (10 mg/kg b.w.) treatment. Interestingly, in the low-dose Sevo group we found longer escape latencies than in the control group. The results are shown in Fig. 6. We also observed that treatment with GBC (10 mg/kg b.w.) significantly reduced the time needed to identify the platform by rats with cognitive impairment induced by sevoflurane. Sevoflurane greatly reduced the number of times platform sites were crossed over when compared with the control group during the testing procedure. Furthermore, GBC was found to reverse the effects induced by sevoflurane. These findings suggested that GBC effected a marked improvement in memory and learning behaviour in sevoflurane-induced cognitively dysfunctional rats.

One limitation of the study is that it did not include the demonstration of how the administered GBC reaches the plasma, cerebrospinal fluid and hippocampus of the treated rats. Measuring the GBC concentration in these tissues in order to demonstrate that the observed effect is due to GBC would therefore be useful. Another limitation is that the effect of GBC treatment on blood glucose and/or insulin concentration was not analysed although it could have been to prove that the GBC application worked as an antidiabetic drug. The study also needs to be accompanied with the corresponding images of brain tissue/hippocampal histological analysis. The final possible shortcoming of the study is that it did not include immunostaining results for neuronal apoptosis.

Our study identified that the replenishment of BDNF due to the action of GBC inhibits oxidative stress and cognitive impairment mediated by sevoflurane. The PI3K/Akt signalling pathways were also triggered by GBC, resulting in decreased hippocampal neuronal death. Under GBC therapy at 10 mg/kg b.w., sevoflurane-induced neurotoxicity, spatial learning deficits, and cognitive loss were diminished as changes related to inactivation of IL-6 and TNF- $\alpha$  in the hippocampus. Our results indicate that GBC may be a novel drug for preventing sevoflurane inhalation-induced impaired learning in the hippocampus. Our study therefore suggests that BDNF may be a vital target for the development of potential therapies in cognitive deficits and neurodegeneration. Further research is still needed, however, for confirmation by molecular and genetic analyses.

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