

## Research report

# Transgenic rat with overproduction of ubiquitous angiotensin-(1-7) presents neuroprotection in a model of ischemia and reperfusion

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## ABSTRACT

Recent studies showed that angiotensin-(1-7) has cerebroprotective actions in stroke. In the present study, we aim to test whether tissue overexpression of Angiotensin-(1-7), mainly in the brain provides neuroprotection in a model of ischemia/reperfusion by bilateral common carotid arteries occlusion/reperfusion (BCCAO/R). Evaluation of neurological deficit scores and bilateral asymmetry test (BAT) were performed seven days after transient BCCAO/R in transgenic rats (TG-7371) overexpressing Angiotensin-(1-7) and Sprague-Dawley (SD) rats. To assess blood-brain barrier (BBB) permeability Evans blue dye (EB) was intravenously injected. Cytokine levels were quantified in the whole brain through Elisa assay and oxidative stress was measured 7 days after ischemia. The expression of AT<sub>1</sub> and Mas receptors and inducible nitric oxide synthase (iNOS) was evaluated by RT-PCR. Neurological deficits were observed in both SD-BCCAO/R and TG-BCCAO/R, contrasting to sham-operated groups. However, TG-BCCAO/R showed a significant lower neurological score and latency in BAT when compared with SD-BCCAO/R. BBB integrity in TG-BCCAO/R was improved, since these animals showed lower extravasation of EB than SD-BCCAO/R. Interestingly, TG-BCCAO/R presented lower levels of pro-inflammatory cytokines when compared to SD-BCCAO/R. Levels of IL-10 were higher in SD-BCCAO/R than in SD control and even higher in TG-BCCAO/R. TG-BCCAO/R animals presented decreased levels of TBARS and increase in SOD activity and GSH levels when compared to SD sham rats. RT-PCR results showed higher levels of AT<sub>1</sub> receptor and iNOS in SD-BCCAO/R compared to TG-BCCAO/R, but no difference was observed for Mas receptor. The present study shows that lifetime increase in cerebral expression of an Ang-(1-7)-producing fusion protein induces neuroprotection in experimental global cerebral ischemia and reperfusion, reassuring that, pharmacological strategies leading to increase in Ang-(1-7) can be an additional tool for stroke therapy.

## 1. Introduction

Recent studies showed that Angiotensin converting enzyme 2 (ACE2)/Angiotensin-(1-7) [Ang-(1-7)]/Mas receptor pathway has cerebroprotective actions in stroke (Bennion et al., 2015a; Jiang et al.,

2013a; Peña Silva and Heistad, 2014; Regenhardt et al., 2014a; Sumners et al., 2013). Zang et al. (2008) suggested for the first time that Ang-(1-7) might play a potential beneficial role in the treatment of acute stroke. Using focal cerebral ischemia/reperfusion in rats they showed that medium or high-dose of Ang-(1-7) stimulates NO release and

**Abbreviations:** Ang II, Angiotensin II; AT<sub>1</sub>, Angiotensin II receptor type I; Ang-1–7, Angiotensin-(1-7); ACE, Angiotensin-converting enzyme; ACE2, Angiotensin-converting enzyme 2; BAT, Bilateral Asymmetry test; BBB, Blood-brain barrier; CAT, Catalase; ET-1, Endothelin-1; ELISA, Enzyme linked immunosorbent assay; EB, Evans-Blue; GSH, Glutathione; iNOS, Inducible nitric oxide synthase; RAS, Renin-Angiotensin System; SD, Sprague-Dawley; SOD, Superoxide Dismutase; TBARS, Thiobarbituric acid-reactive substances; TG, Transgenic; TG – 7371, Transgenic rats which overexpress Ang-(1-7).

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upregulates eNOS mRNA and protein expression in ischemic tissues (Zhang et al., 2008). Three years later, Mecca et al. (2011) showed that intracerebroventricular (ICV) infusion of Ang-(1-7) prior to and during ischemic stroke elicited by Endothelin-1-induced (ET-1) middle cerebral artery occlusion (MCAO) produced a significant reduction in the resulting intracerebral infarct volume (Mecca et al., 2011). The decrease in intracerebral infarct size was associated with increased neuron survival in the cortex and striatum, as well as decreases in behavioral deficits caused by MCAO, as evidenced by neurological tests.

Furthermore, activation of the neuroprotective ACE2 in a rat ischemic stroke model under similar conditions as those used for Ang-(1-7) effectively decreased the intracerebral infarct volume and behavioural deficits resulting from ET-1-induced MCAO (Bennion et al., 2015b). These protective actions of Ang-(1-7) and ACE2 activation were abolished by previous administration of the receptor Mas antagonist A779 (Bennion et al., 2015b; Mecca et al., 2011). In addition, a more recent study from Bennion and colleagues showed that oral Ang-(1-7), attached to hydroxypropyl- $\beta$ -cyclodextrin [HP $\beta$ CD-Ang-(1-7)] given post-stroke, decreased infarct volume and enabled improvement of neurological scores in rats, without affecting their blood pressure, heart rate and cerebral blood flow (Bennion et al., 2018).

Our group has recently characterized the cardiovascular phenotype of a new transgenic rat line (TG-7371) that expresses an Ang-(1-7)-producing fusion protein driven by the GFAP promoter mainly in vasculature and brain. The results showed that overexpression of Ang-(1-7) induced a hypotensive phenotype due to vasodilatation in several vascular beds leading to a fall in the peripheral resistance (Alves et al., 2021). TG-7371 can be therefore considered a new tool to investigate Ang-(1-7) effects in different pathophysiological conditions, such as ischemic stroke. Considering that Ang-(1-7) induces neuroprotection in models of ischemic and hemorrhagic stroke (Bennion et al., 2015b, 2015a; Mecca et al., 2011; Regenhardt et al., 2014a, 2014b; Sumners et al., 2013), we hypothesized that transgenic rats overexpressing Ang-(1-7) in the brain (TG-7371) could be protected against brain injury in global cerebral ischemia and reperfusion. To address this hypothesis, we evaluated neurological deficits, inflammation, oxidative stress, and gene expression in TG-7371 rats subjected to bilateral common carotid artery occlusion/reperfusion (BCCAO/R).

## 2. Material and methods

### 2.1. Animals

Adult male Sprague-Dawley (SD) and transgenic rats overexpressing Ang-(1-7) in the brain (TG-7371), 3–4 months old, were obtained from the animal facility of the Laboratory of Hypertension, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Brazil. Animals were housed in the animal facility, kept in controlled room temperature (22–24 °C) at a 12/12 h light/dark cycle. The characterization of TG-7371 transgenic rat model has been previously described elsewhere (Alves et al., 2021). All experimental protocols were approved by the Institutional Ethics Committee on the Use of Animals at the Universidade Federal de Minas Gerais (CEUA/UFGM) (no. 49/2013) and conducted in accordance with the guidelines from the National Council for Animal Experimentation Control (CONCEA-BRAZIL).

### 2.2. Bilateral common carotid artery occlusion/reperfusion

Rats were anesthetized with intraperitoneal injection of ketamine hydrochloride (100 mg/kg) and xylazine (10 mg/kg). Surgical procedure was performed on a heating pad with all animals spontaneously breathing. Transient global cerebral ischemia was induced by BCCAO/R as previously described (Kumar et al., 2016; Gonçalves et al., 2022). Briefly, an incision was made in the ventral neck to expose the common carotid arteries. Brain ischemia was induced for 25 min by bilateral occlusion of the common carotid arteries using non-traumatic clips.

After this period, clips were removed and the cerebral flow was definitively re-established. The incision was then closed with sutures. Rats were placed in a warm environment for approximately 1 h, and then returned to their home cages and housed individually with free access to water and soft food.

### 2.3. Assessment of neurological deficit

The neurological deficit scores were obtained 24 h after transient BCCAO/R for each animal. Deficit scores were carried out according to a five-point scale from a previous publication (Toscano et al., 2016) where: 0, no neurological deficit; 1, ptosis and/or ataxia; 2, animal persistently walks in circles; 3, ptosis, ataxia and animal persistently walks in circles; 4, animal was no longer able to walk spontaneously; 5, animal dies after recovery from the anesthesia.

### 2.4. Bilateral asymmetry test (BAT)

BAT is now commonly used to investigate stroke related impairments of tactile extinction and was performed accordingly to Veizovic et al. (2001). Briefly, strips of tape (1 × 5 cm) were wound around each forepaw in random order. Animals were placed in an observation cage and timed for latency to remove each tape. Rats were tested 7 days after surgery.

### 2.5. Assessment of blood-brain barrier integrity

The integrity of the blood-brain barrier (BBB) was investigated using Evans-Blue (EB) dye as previously reported (Saria and Lundberg, 1983). Rats were intravenously injected with 0.2 mL of 1 % EB solution (Sigma-Aldrich, St. Louis, MO), 7 days after the occlusion/reperfusion procedure. One hour later, animals were perfused intracardially with 5 mL of PBS. Brains were removed, samples weighed, and EB extravasation evaluated by formamide incubation (1 mL) for 24 h. The amount of EB in tissue extracts was measured by absorbance at 610 nm as an index of increased capillary permeability. Results are shown as the amount of EB ( $\mu$ g) present in 100 mg of brain tissue.

### 2.6. Enzyme linked immunosorbent assay (ELISA) of cytokines in cerebral tissue

Seven days after BCCAO/R, animals of all groups were deep anesthetized and the whole brain, excluding lower brainstem and cerebellum, was removed and rapidly frozen on dry ice, and stored at –80 °C until use. For the assay, brain was homogenized (100 mg/mL of extraction solution). The homogenate was centrifuged at 3000 g for 10 min at 4 °C, and the supernatant was collected and stored at –20 °C. Concentrations of interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-10 (IL-10) were measured using commercially available antibodies and according to the procedures supplied by the manufacturer (R&D Systems, Minneapolis, MN, USA). Results are expressed as pg/100 mg of tissue.

### 2.7. Quantification of oxidative stress markers

To indirectly assess the presence of oxidative stress in the brain of rats submitted to brain ischemia and reperfusion, lipid peroxidation and antioxidant enzymes activity were measured 7 days after ischemia.

Superoxide Dismutase (SOD) activity was measured according to Gao et al. (1998) based on the capacity of SOD to inhibit pyrogallol autoxidation using a spectrophotometric assay at 420 nm. The amount of SOD that inhibited the oxidation of pyrogallol by 50 % (relative to the control) was defined as a unit of SOD activity (Gao et al., 1998). Catalase (CAT) activity was measured in the brain tissues by the method of Nelson and Kiesow (1972) in which the decomposition of peroxide is followed spectrophotometrically at 240 nm. The consumption of

peroxide was accompanied by a decrease in absorbance for 1 min and catalase was defined as the amount of enzyme that decomposed one mol of peroxide (expressed as nmol/min/mg of protein) (Nelson and Kiesow, 1972).

Glutathione (GSH) levels were measured as described by Sedlak et al. (1968), where a colorimetric assay follows the formation of 5,5'-dithiobis-(2-nitrobenzoic acid)-sulfhydryl groups. A tissue sample (100 mg) was homogenized in 900  $\mu$ L of 0.1 M phosphate buffer at pH 6.5. The supernatant was incorporated with 5,5'-dithiobis-(2-nitrobenzoic acid) and the absorbance measured at 412 nm in a microplate reader by using reduced glutathione as external standard (Sedlak and Lindsay, 1968).

Lipid peroxidation rate was determined by the malondialdehyde content using thiobarbituric acid-reactive substances (TBARS) technique (Ohkawa et al., 1979). The protein content of tissue was determined according to Bradford (1976) using bovine serum albumin as protein standard (Bradford, 1976).

### 2.8. Measurement of AT<sub>1</sub>, Mas receptors and Inducible Nitric Oxide Synthase (iNOS) mRNA expression

Total RNA was obtained following the TRizol reagent method (Invitrogen, Life Technologies, United States) according to the manufacturer's protocol. RNA samples (2  $\mu$ g) were treated with DNase to eliminate genomic DNA present in the samples. mRNA expression was assessed by qRT-PCR after reverse transcription with MML-V (Moloney murine leukemia virus) (Invitrogen Life Technologies, United States). The cDNA for endogenous S26 ribosomal (endogenous control) and Angiotensin II receptor type I (AT<sub>1</sub>), Mas receptors and iNOS were amplified using specific primers using SYBR Green reagent (Applied Biosystems, Foster City, NY, United States). The reactions were performed using 40 cycles and annealing temperature of 60 °C (ABI Prism 7000, Applied Biosystems, Foster City, NY, United States). Gene expression was quantified using the comparative Ct (threshold cycle) method. Primers sequence of AT<sub>1</sub>: 5'-GGT GGG AAT ATT GGA AAC AG-3' (forward) and 5'-AAG AAG AAA AGC ACA ATC GCC-3' (reverse); Mas receptor: 5'-CCC ACC CAT TCC CAT AGT GC-3' (forward) and 5'-CCG AGA GGA GAG ATG CTC ATG-3' (reverse); iNOS: 5'-CCT TGT TCA GCT ACG CCT TC-3' (forward) and 5'-GGT ATG CCC GAG TTC TTT CA-3' (reverse); endogenous control S26: 5'-CGA TTC CTG ACA ACC TTG CTA TG-3' (forward) and 5'-CGT GCT TCC CAA GCT CTA TGT-3' (reverse) (Kangussu et al., 2021).

### 2.9. Statistical analysis

Differences among groups were assessed by t-student test two-way ANOVA followed by Bonferroni post-hoc test and were performed using GraphPad Prism software (version 7.0). All values were expressed as mean  $\pm$  standard error of the mean (SEM). Statistical significance was assumed for all values of  $p < 0.05$ .

### 3. Results

Both SD and TG sham-operated groups did not present neurological deficits ( $n = 10$  per group). BCCAO/R induced neurological signs in both WT ( $n = 10$ ) and TG ( $n = 10$ ) groups and were characterized by ptosis, walking in circles and/or ataxia. TG-BCCAO/R rats showed a significant lower neurological score when compared with SD-BCCAO/R rats ( $t = 8.48$ ;  $p < 0.0001$ ), Fig. 1A. Furthermore, in the bilateral asymmetry test, two-way ANOVA showed lineage effect ( $F_{(1,24)} = 17.51$ ;  $p = 0.0003$ ), ischemia ( $F_{(1,24)} = 28.19$ ;  $p < 0.0001$ ) and interaction between two ( $F_{(1,24)} = 12.41$ ;  $p = 0.0017$ ), Fig. 1B. In Bonferroni's post-hoc test, SD-BCCAO/R showed higher latency time to remove the adhesive tape ( $76.3 \pm 5$  s;  $p < 0.0001$ ) than sham group ( $39.4 \pm 4$  s;  $p < 0.0001$ ), while TG-BCCAO/R rats showed a significant reduction in latency to adhesive removal ( $44.1 \pm 3$  s;  $p < 0.0001$ ), Fig. 1B.

SD-BCCAO/R showed greater extravasation of EB ( $12.7 \pm 1$   $\mu$ g/100 mg of tissue;  $n = 7$ ) than sham group ( $2.4 \pm 0.3$   $\mu$ g/100 mg of tissue). Two-way ANOVA test showed lineage effect ( $F_{(1,21)} = 21.59$ ;  $p = 0.0001$ ), ischemia ( $F_{(1,21)} = 114.4$ ;  $p < 0.0001$ ) and interaction between two ( $F_{(1,21)} = 18.28$ ;  $p = 0.0003$ ), Fig. 2. Additionally, Bonferroni's post-hoc test, demonstrated TG-BCCAO/R had a significant reduced amount of EB ( $6.6 \pm 0.2$   $\mu$ g/100 mg of tissue;  $p < 0.0001$ ), indicating attenuation in loss of integrity of the BBB, Fig. 2.

As shown in Fig. 3, levels of pro-inflammatory cytokines were increased in SD-BCCAO/R [(A) IL-1 $\beta$ :  $204 \pm 12$ ; lineage effect ( $F_{(1,18)} = 19.86$ ;  $p = 0.0003$ ), ischemia ( $F_{(1,18)} = 239.2$ ;  $p < 0.0001$ ) and interaction between two ( $F_{(1,18)} = 22.46$ ;  $p = 0.0002$ ); (B) IL-6:  $296 \pm 19$ ; lineage effect ( $F_{(1,18)} = 49.71$ ;  $p < 0.0001$ ), ischemia ( $F_{(1,18)} = 249.1$ ;  $p < 0.0001$ ) and interaction between two ( $F_{(1,18)} = 60.48$ ;  $p < 0.0001$ ); (C) TNF- $\alpha$ :  $405 \pm 17$  pg/100 mg of tissue; lineage effect ( $F_{(1,17)} = 45.91$ ;  $p < 0.0001$ ), ischemia ( $F_{(1,17)} = 351.9$ ;  $p < 0.0001$ ) and interaction between two ( $F_{(1,17)} = 44.87$ ;  $p < 0.0001$ )] when compared to SD control rats (IL-1 $\beta$ :  $29 \pm 3$ , IL-6:  $23 \pm 4$ , TNF- $\alpha$ :  $37 \pm 5$  pg/100 mg of tissue).

Interestingly, in post-hoc analysis TG-BCCAO/R presented lower levels of pro-inflammatory cytokines, Fig. 3 [(A) IL-1 $\beta$ :  $124 \pm 7$

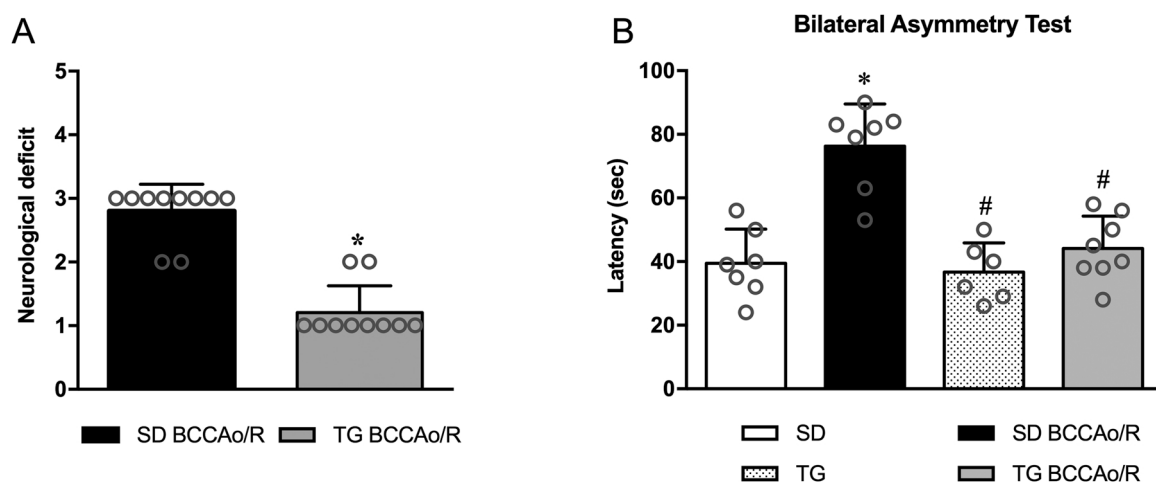
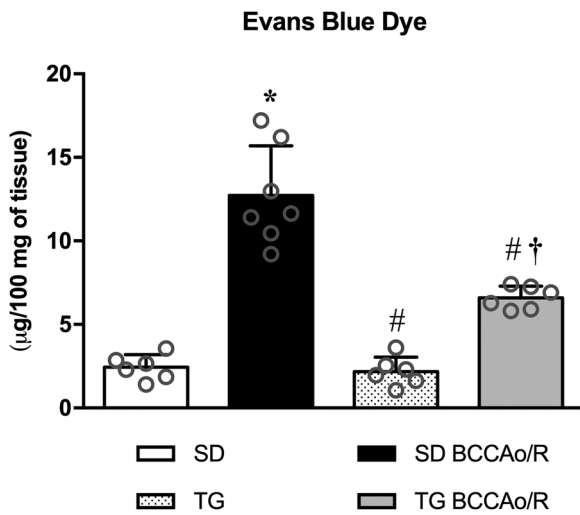


Fig. 1. Neurological score and bilateral asymmetry test were evaluated in experimental animals. For the neurological deficit scores rats were tested 24 h after global cerebral ischemia induction surgery (A) and for bilateral asymmetry test animals were tested 7 days after surgery (B). Results are expressed as mean  $\pm$  SEM.  $n = 6-7$  per group. \* $p < 0.05$  vs. SD, # $p < 0.05$  vs. SD BCCAO/R. (A) t-student test and (B) two-way ANOVA followed by Bonferroni post-hoc test.

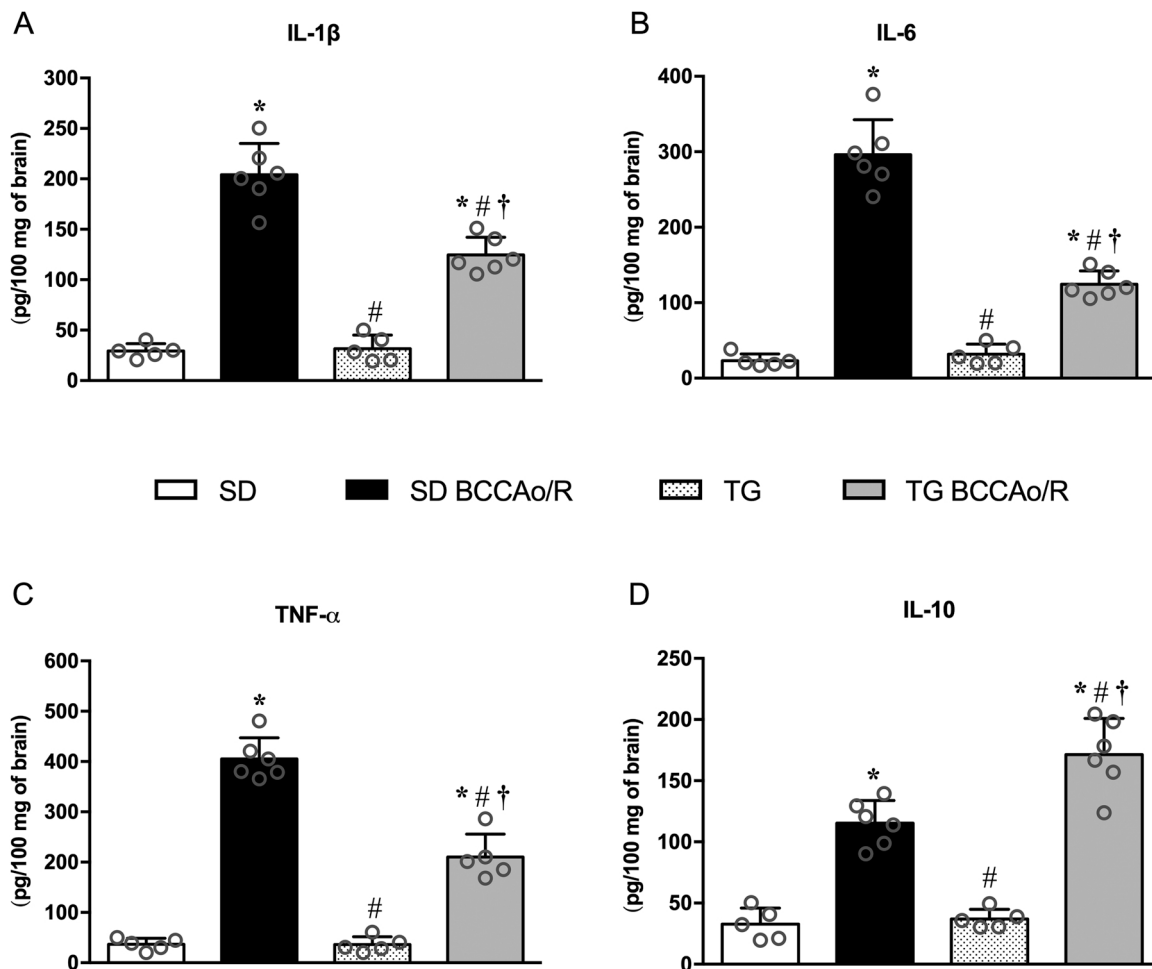


**Fig. 2.** Assessment of blood-brain barrier (BBB) permeability on experimental groups. Effects of the BCCAO/R on the changes in integrity of the BBB was assessed by the degree of extravasation of Evans blue dye (EB). Results are expressed as mean ± SEM. *n* = 6–7 per group. \**p* < 0.05 vs. SD, #*p* < 0.05 vs SD BCCAO/R, †*p* < 0.05 vs TG. Two-way ANOVA followed by Bonferroni post-hoc test.

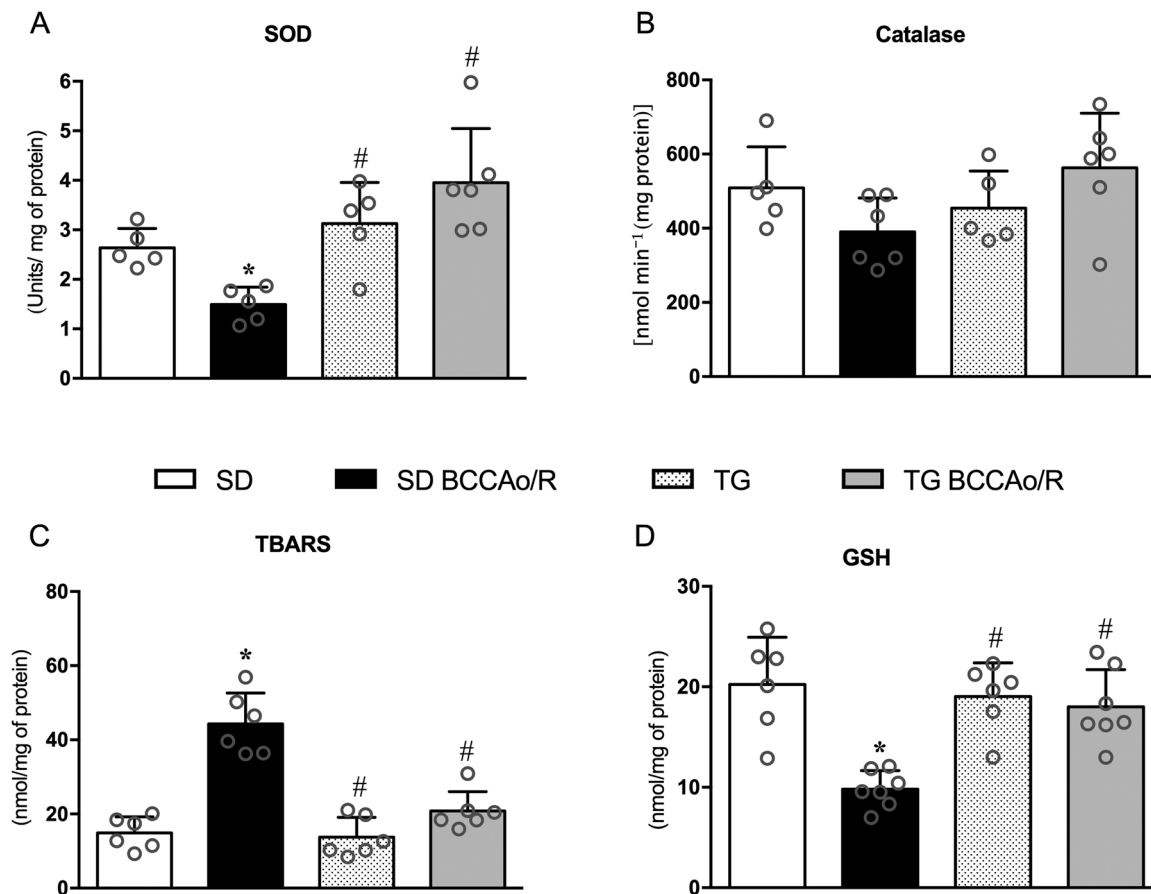
(*p* < 0.0001), (B) IL-6: 125 ± 7 (*p* < 0.0001), (C) TNF-α: 210 ± 20 pg/100 mg of tissue (*p* < 0.0001) when compared to SD-BCCAO/R. Levels of IL-10 (D) were higher in SD-BCCAO/R than in SD control 115 ± 8 vs 33 ± 6 pg/100 mg of tissue (*p* < 0.0001) and even higher in TG-BCCAO/R (171 ± 12 pg/100 mg of tissue (*p* = 0.0007).

The brain inflammation triggered by ischemia and reperfusion led us to investigate other brain aspects, such as the brain redox status. As shown in Fig. 4A, the activity of the antioxidant enzyme SOD was significantly reduced in SD-BCCAO/R rats in comparison with SD control rats [1.5 ± 0.2 vs 2.6 ± 0.2 units/ mg of protein; lineage effect ( $F_{(1,17)} = 19.56$ ; *p* = 0.0004) and interaction between two ( $F_{(1,17)} = 8.75$ ; *p* = 0.0088)]. In contrast, this alteration was normalized in TG-BCCAO/R (3.9 ± 0.4 units/ mg of protein; *n* = 6). However, there were no significant differences in catalase activity among the experimental groups [lineage effect ( $F_{(1,18)} = 1.418$ ; *p* = 0.2492), ischemia ( $F_{(1,18)} = 0.102$ ; *p* = 0.9204) and interaction between two ( $F_{(1,18)} = 5.29$ ; *p* = 0.0335)], Fig. 4B.

Levels of lipid peroxidation (TBARS) in the brain were significantly increased in SD-BCCAO/R when compared to SD control [44 ± 3.3 vs 15 ± 1.7 nmol/ mg of protein; lineage effect ( $F_{(1,17)} = 19.56$ ; *p* = 0.0004) and interaction between two ( $F_{(1,17)} = 8.75$ ; *p* = 0.0088)], TG-BCCAO/R showed significantly reduced levels of TBARS (21 ± 2.1 nmol/ mg of protein; *p* = 0.0003)], Fig. 4C. Moreover, lower levels of GSH were observed in the brain of SD-BCCAO/R (9.8 ± 0.7 nmol/ mg of protein; *n* = 7) than in SD control [20 ± 2 nmol/ mg of protein; lineage effect



**Fig. 3.** Levels of cytokines in the central nervous system in SD and TG rats sham or after transient global cerebral ischemia. All experimental groups had their brain tissue extracted for quantification of pro and anti-inflammatory cytokines 7 days after BCCAO/R. Concentrations of IL-1β (A), IL-6 (B), TNF-α (C) and IL-10 (D) were measured. Results are expressed as mean ± SEM. *n* = 5–6 per group. \**p* < 0.05 vs. SD, #*p* < 0.05 vs SD BCCAO/R, †*p* < 0.05 vs TG. Two-way ANOVA followed by Bonferroni post-hoc test.



**Fig. 4.** Levels of oxidative stress markers in the brain in SD and TG rats sham or after transient global cerebral ischemia. All experimental groups had their brain tissue extracted for quantification of SOD (A), Catalase (B), TBARS (C), and GSH (D) 7 days after BCCAo/R. Results are expressed as mean ± SEM. *n* = 5–7 per group. \**p* < 0.05 vs. SD, #*p* < 0.05 vs SD BCCAo/R. Two-way ANOVA followed by Bonferroni post-hoc test.

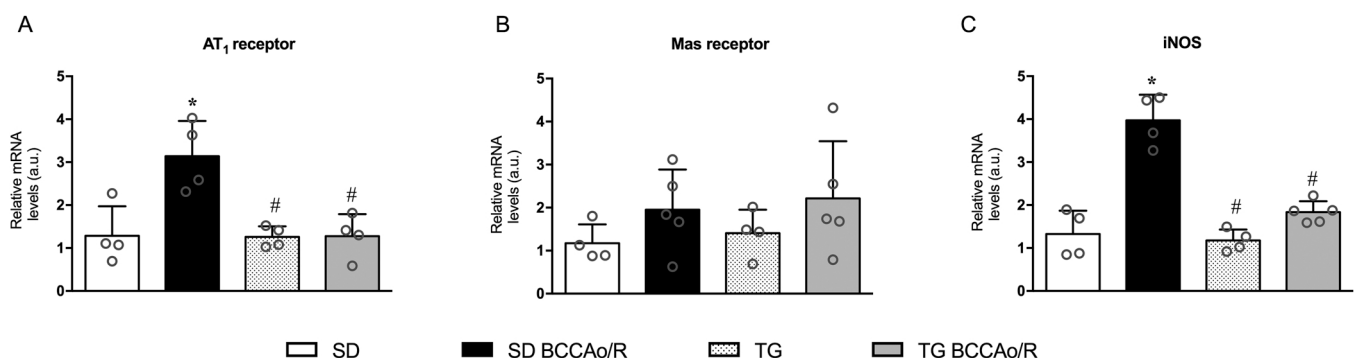
( $F_{(1,22)} = 6.43$ ;  $p = 0.0188$ ), ischemia ( $F_{(1,22)} = 17.29$ ;  $p = 0.0004$ ) and interaction between two ( $F_{(1,22)} = 11.69$ ;  $p = 0.0025$ ]. On the other hand, TG-BCCAo/R showed normal levels of GSH ( $18 \pm 1.3$  nmol/mg of protein;  $p = 0.0014$ ), Fig. 4D.

RT-PCR was carried out to show the differences in gene expression of iNOS, AT<sub>1</sub> and Mas receptors. The results depicted in Fig. 5A showed that SD-BCCAo/R presented increased [lineage effect ( $F_{(1,12)} = 9.718$ ;  $p = 0.0089$ ), ischemia ( $F_{(1,12)} = 9.569$ ;  $p = 0.0093$ ) and interaction between two ( $F_{(1,12)} = 9.194$ ;  $p = 0.0104$ )] gene expression of AT<sub>1</sub> receptor ( $3.1 \pm 0.4$  a.u.;  $n = 4$ ) and when compared to TG-BCCAo/R animals ( $1.3 \pm 0.3$  a.u.;  $n = 4$ ),  $p = 0.0057$ . iNOS gene expression was also increased [lineage effect ( $F_{(1,13)} = 29.73$ ;  $p = 0.0001$ ), ischemia ( $F_{(1,13)} = 61.83$ ;  $p < 0.0001$ ) and interaction between two ( $F_{(1,13)} = 22.38$ ;

$p = 0.0004$ )] in SD-BCCAo/R ( $4 \pm 0.3$  a.u.;  $n = 4$ ) when compared to TG-BCCAo/R ( $1.8 \pm 0.1$  a.u.;  $n = 5$ ),  $p < 0.0001$ , Fig. 5B. On the other hand, no difference in gene expression between the groups was seen for Mas receptor [lineage effect ( $F_{(1,14)} = 0.3194$ ;  $p = 0.5809$ ), ischemia ( $F_{(1,14)} = 3.261$ ;  $p = 0.0925$ ) and interaction between two ( $F_{(1,14)} = 0.0014$ ;  $p = 0.9702$ )].

#### 4. Discussion

The main point raised by our findings in this study was that tissue overexpression of Ang-(1-7), mainly in the brain protects against ischemic injury and consequently attenuates loss in the BBB integrity resulting in mitigation of inflammatory processes and oxidative stress



**Fig. 5.** Expression of mRNA levels of AT<sub>1</sub> receptor (A), Mas receptor (B) and iNOS (C) in the brain in SD and TG rats sham or after BCCAo/R. Results are expressed as mean ± SEM. *n* = 4–5 per group. \**p* < 0.05 vs. SD, #*p* < 0.05 vs SD BCCAo/R. Two-way ANOVA followed by Bonferroni post-hoc test.

along with modifications in the gene expression of AT1 receptors and iNOS, which improves neurological and motor functions.

Brain tissue of TG-7371 rats overexpress an Ang-(1-7) producing fusion protein that induces a lifetime increase in Ang-(1-7) levels being an excellent experimental model to check the effects of chronic high levels of Ang-(1-7) in disease models (Alves et al., 2021). After being subjected to bilateral common carotid artery occlusion/reperfusion (BCCAO/R), TG-7371 rats showed decrease of neurological alterations and impairments of tactile extinction when compared to SD-BCCAO/R. In 2011, Mecca et al. showed that administration of Ang-(1-7) prior and following ET-1-induced middle cerebral artery occlusion improved animal's state (Mecca et al., 2011). Similar neuroprotective results were seen after oral administration of Ang-(1-7) post-stroke by Bennion et al. (Bennion et al., 2018). Thus, the results provided here are in agreement with the well-established neuroprotective effects of the Ang-(1-7) showed so far in the literature (Bennion et al., 2018; Mecca et al., 2011; Peña Silva and Heistad, 2014). However, most of these previous studies are related to the occlusion of the middle cerebral brain artery as a stroke model. Our results showed that Ang-(1-7) was also able to exert its neuroprotective effect in the BCCAO model. Therefore, we are expanding Ang-(1-7)'s range of action showing its efficacy in a different stroke model which supports the potential effects of this peptide against stroke.

In addition, the anti-inflammatory effects of Ang-(1-7) have been reported after ischemic stroke (Liu et al., 2016; Prestes et al., 2017; Simões e Silva et al., 2013). Our results show that overexpression of Ang-(1-7) in the brain presented attenuation in loss of integrity of the BBB. Wu et al. showed that Ang-(1-7) exerts a protective effect in BBB damage. They also showed that the protective role of Ang-(1-7) in the BBB promoted expression of zona-occludens-1 and claudin-5 (Wu et al., 2015). Thus, Ang-(1-7) actions on the BBB may be a mechanism that explains the mitigation of inflammation and oxidative stress in these animals.

In the present study, it is demonstrated that TG-BCCAO/R presented lower levels of pro-inflammatory cytokines when compared to SD-BCCAO/R. Interestingly, Jiang et al. (2012) demonstrated that Ang-(1-7) has anti-inflammatory effects and contributes to neuroprotection after permanent cerebral ischemia. The beneficial effects of Ang-(1-7) were reversed by A779, but not by PD123319. Therefore, an interaction with Mas receptor to suppress the NF- $\kappa$ B dependent pathway may represent one of the underlying mechanisms for the anti-inflammatory effects of Ang-(1-7) (Jiang et al., 2012).

Regenhardt et al. (2013) demonstrated that the beneficial effect of Ang-(1-7) during ischemic stroke is associated with an anti-inflammatory effect of this peptide and suggest that the cerebroprotective action of this peptide in ischemic stroke may involve effects on nitric oxide generation by microglia. Moreover, they also demonstrated in another study that central administration of Ang-(1-7) into stroke-prone spontaneously hypertensive rats, a model of hemorrhagic stroke, increases lifespan and improves the neurological status of these rats, as well as decreases microglial numbers in the striatum, implying attenuation of cerebral inflammation (Regenhardt et al., 2014b). Therefore, our results, along with what has been seen in the literature, suggest that Ang-(1-7) neuroprotective actions might be influenced by its anti-inflammatory effects.

It is well established that during brain ischemia and reperfusion induced by BCCAO/R occurs intense oxidative stress, an increase of reactive species of oxygen levels, increase of lipid peroxidation and reduction of activity of antioxidant enzymes (Homi et al., 2002; Kakkar et al., 2013; Mukherjee et al., 2007). The antioxidant effect of Ang-(1-7) is already shown in different experimental conditions (Jiang et al., 2013b; Lin et al., 2016; Rabelo et al., 2011; Zhang et al., 2015). As expected, overexpression of Ang-(1-7) in the brain is associated with reduced oxidative stress. Zheng et al. (2014) provided the first evidence that activation of the ACE2/Ang-(1-7)/Mas axis produces a direct protective effect during cerebral ischemic injury by alleviating cell swelling

and cell death via decreased Nox expression and resulting in lower ROS production. In addition, the protective effect of Ang-(1-7)/Mas activation on ischemic injury in the brain shows an age-dependent trend (Zheng et al., 2014). Taken together, these results indicated that Ang-(1-7) mediates neuroprotection in a BCCAO/R, at least in part, through inhibition of oxidative stress.

Gene expression of AT1 receptor was increased in the brains of SD-BCCAO/R animals when compared to TG-BCCAO/R. This is in line with a study from Vikam et al., which evaluated the mRNA expression of different receptors after ischemia in human tissue and found that AT1 receptors levels were increased (Vikam and Edvinsson, 2006). In addition, many studies also showed that the use of AT1 receptor antagonists before or after an ischemic event improves stroke outcome (Arroja et al., 2016; Culman et al., 2017, p. 1). Thus, our results support the negative role of AT1 receptor in the ischemic environment and show that Ang-(1-7) is able to decrease AT1R levels which might be part of its neuroprotective action in stroke.

iNOS mRNA levels were also increased in SD-BCCAO/R animals when compared to TG-BCCAO/R. One of the first studies that showed Ang-(1-7) effects on the level of iNOS post stroke was from Iadecola et al., where the activation of Ang-(1-7) axis was able to decrease the levels of iNOS (Iadecola et al., 1995). In 2013, Regenhardt et al. showed that central Ang-(1-7) treatment in ischemic stroke resulted in attenuation of the increased levels of iNOS mRNA (Regenhardt et al., 2013). Therefore, the increased levels of iNOS seen in SD-BCCAO/R and reduced levels in TG-BCCAO/R support these findings suggesting a role of Ang-(1-7) action, during stroke, through reduction of nitric oxide.

Although, it is reported in the literature that after ischemic stroke Mas receptor mRNA levels are increased in the brain tissue of rats (Lu et al., 2013), no differences in Mas receptor gene expression was seen in our results. This could be an indication that a lifetime overexpression of Ang-(1-7) may induce a homeostasis situation within the Ang-(1-7) axis that is not affected by an ischemic stroke event.

The occlusion/reperfusion method employed in the present study reveals some limitations that should be taken into account. The transient bilateral common carotid artery occlusion, due to the collateral circulation in the circle of Willis and to the anastomoses in the cerebral vessels, induces a global ischemia, especially in the forebrain and, therefore, not exactly a stroke model. Nevertheless, in this model, the reperfusion is the critical element to evaluate tissue damage, such as those evoked by inflammation and oxidative stress.

## 5. Conclusion

In conclusion, our results show that transgenic rats with increased brain Ang-(1-7) presents neuroprotection in global cerebral ischemia and reperfusion, improving neurological function, attenuation in loss of integrity of the BBB, inflammation, oxidative stress, and decreased AT<sub>1</sub> receptor and iNOS levels suggesting that pharmacological strategies that lead to increase in Ang-(1-7) may be an additional tool to treat stroke. In addition to the present findings, further studies will be needed to increase our understanding of the molecular mechanisms of cerebroprotection mediated by Ang-(1-7).

## CRedit authorship contribution statement

**Lucas Miranda Kangussu:** Conception and design of research, Performed experiments, Analyzed data, Prepared figures, Interpreted results of experiments, drafted manuscript, edited and revised manuscript. **Ana Flávia Almeida-Santos:** Performed experiments, analyzed data, **Lorena Figueiredo Fernandes:** Analyzed data, drafted manuscript. **Natalia Alenina:** interpreted results of experiments, edited and revised manuscript. **Michael Bader:** Interpreted results of experiments, edited and revised manuscript. **Robson A.S. Santos:** interpreted results of experiments, edited and revised manuscript. **André Ricardo Massensini:** analyzed data, interpreted results of experiments, drafted

manuscript, edited and revised manuscript. **Maria José Campagnole-Santos**: analyzed data, prepared figures, interpreted results of experiments, drafted manuscript, edited and revised manuscript. All authors contributed to and approved the final version of manuscript.

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## Conflict of interest

There are no conflicts of interest or disclosures.

## Data Availability

Data will be made available on request.

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