

## Neuroprotective effects of albumin, pioglitazone and insulin against ischemic brain damage in mice

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### ABSTRACT

Stroke is a leading cause of death and permanent disability in adults worldwide. Intravenous recombinant tissue plasminogen activator (rt-PA) is the only FDA-approved treatment for administration within 3 hours of onset of acute ischemic stroke. The current study has investigated neuroprotective effects of human serum albumin (20% solution) (HSA) in a vehicle (sodium chloride 0.09%) (0.04ml/ 20g) was administrated intravenous at a constant rate over 3 min 2 h after the onset of ischemia. Pioglitazone (40 mg/kg/day, as a suspension in 0.5% carboxymethylcellulose) was orally administrated for three days before ischemia and for two days after ischemia. Insulin was administrated 0.3 IU/kg (via tail vein) at the beginning of MCAO and after 6 h of MCAO. Human serum albumin (HAS), pioglitazone and insulin demonstrated neuroprotective effects against brain damage induced by permanent MCAO in mice as evidenced by the reduction in initiation of walking time and prevention of leukocyte infiltration and brain edema.

**Key words:** Albumin-pioglitazone-insulin-stroke-ischemia-tissue plasminogen activator

### INTRODUCTION

Stroke is the second leading cause of death in industrialized countries and the most frequent cause of permanent disability in adults worldwide (Lipton, 1999; Woodruff *et al.*, 2011). It is a multifactorial disease in which neuronal cell death proceeds through a mixture of mechanisms including oxidative stress, excitotoxicity, apoptosis, and inflammation (Van Leyen *et al.*, 2006). Human serum albumin (20% solution) (HSA) in a vehicle (sodium chloride 0.09%) was administration intravenous (1% body weight) at a constant rate over 3 min immediately 2 h after onset of ischemia (Huh *et al.*, 1998). This dose for rat, so convert it to dose of mice is (0.04 ml/ 20g). Human serum albumin (HAS) has been studied as a hemodiluent in several previous reports (Aronowski *et al.*, 1996; Cole *et al.*, 1996). HAS inhibits platelet aggregation (Jorgensen and Stoffersen, 1980) and influences erythrocyte aggregation, increasing low-shear viscosity (Reinhart and Nagy, 1995). Several reports strongly support a

physiological role for human serum albumin as a scavenger of oxygen free radicals (Wasil *et al.*, 1987; Halliwell and Gutteridge, 1990). Pioglitazone (40 mg/ kg/ day), as a suspension in 0.5% carboxymethylcellulose was administrated orally for three days before ischemia and two days after ischemia. Carboxymethylcellulose solution was administered to mice as a vehicle according to the same volume and time schedule of pioglitazone (Lee *et al.*, 2009). Pioglitazone is a synthetic PPAR- $\gamma$  agonist, acts as an insulin sensitizer and is used in the treatment of type 2 diabetes. Pioglitazone was previously reported to mediate intracerebral activation of PPAR- $\gamma$  and inhibition of inflammatory responses at early time points after stroke (Zhao *et al.*, 2005). In addition, pioglitazone was shown to down regulate tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-6 (Wang *et al.*, 1996; Jiang *et al.*, 1998; Streit *et al.*, 1998; Lee *et al.*, 2000; Niino *et al.*, 2001; Pineau and Lacroix, 2007). PPAR- $\gamma$  agonists attenuate

the expression of ICAM-1, matrix metalloproteinase (MMP)-9 and many other inflammatory cytokines following brain ischemia (Pereira *et al.*, 2005; Luo *et al.*, 2006; Culman *et al.*, 2007). Insulin dose 0.3 IU/ kg via tail vein at the beginning of MCAO and 6 h after MCAO. Sodium chloride 0.09% was administrated intravenous via tail vein to mice as a vehicle according to the same volume and time schedule of insulin. This dose was selected based on a previous study in rats (Collino *et al.*, 2009). This dose for rat, so convert it to dose of mice ( $2 \text{ IU} \times 0.15 = 0.3 \text{ IU}$ ). Animal and human studies demonstrate that insulin reduces brain damage evoked by ischemia/reperfusion (I/R) injury (Hui *et al.*, 2005; Rizk, *et al.*, 2006). Insulin is a well-characterized inhibitor of the activation of the glycogen synthase kinase-3 beta (GSK-3 $\beta$ ) (Cohen and Goedert, 2004). GSK-3 has emerged as a key regulatory switch in the modulation of neurodegeneration and inflammation (Martinez *et al.*, 2002; Dugo *et al.*, 2007).

In the current study, brain ischemia was induced by permanent middle cerebral artery occlusion (MCAO) to estimate the possible protective role of human serum albumin, pioglitazone and insulin against brain injury induced in mice.

## 1. Material and methods

### Animals

Adult male balb-c mice aged 8-12 weeks weighing 20-25 gm were purchased from the Theodore Bilharz Research Institute, Cairo, Egypt. The mice were kept under standard environmental and nutritional conditions throughout the investigation. All experiments were carried out in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes and were approved by the Ethical Committee for Animal Handling at Zagazig University (ECAHZU)

## Experimental design

108 adult male balb-c mice aged 8-12 weeks weighing 20-25 gm were randomly distributed into 3 major groups as follows:

Albumin group (A) which was subdivided into: Sham group (A Sham) treated with vehicle (intravenous 0.9% saline), MCAO group (A MCAO) treated with vehicle (intravenous 0.9% saline) at a constant rate over 3 min immediately 2 h after onset of ischemia and Albumin group (Albumin) treated with human serum albumin (20% solution) (HSA) in a vehicle (saline 0.9%) was administration intravenous (0.04 ml/ 20g) at a constant rate over 3 min immediately 2 h after onset of ischemia.

Pioglitazone group (P) which was subdivided into: Sham group (P Sham) treated with the vehicle (oral 0.5% carboxymethylcellulose), MCAO group (P MCAO) treated with the vehicle (oral 0.5% carboxymethylcellulose) and pioglitazone group (pioglitazone) treated with pioglitazone (40 mg/ kg/ day) as a suspension in 0.5% carboxymethylcellulose was administration orally in divided dose daily for three days before ischemia and two days after ischemia.

Insulin group (I) which was subdivided into: Sham group (I Sham) treated with the vehicle (intravenous 0.9% saline), MCAO group (I MCAO) treated with the vehicle (intravenous 0.9% saline) and insulin group (insulin) treated with 0.3 IU/ kg in 0.9% saline via tail vein at the beginning of MCAO and 6 h after MCAO

## Induction of ischemia

Surgery was performed under anesthesia using tribromoethanol (15 ml/ kg of 2%, intraperitoneal, Sigma Aldrich, Germany) (Muhammad *et al.*, 2008; Barakat *et al.*, 2009) and the animal body temperature was adjusted to  $37 \pm 1^\circ\text{C}$  during and after the operation until they regained consciousness (Barakat *et al.*, 2009). MCAO was induced

by using bipolar coagulator (ERPE, Electromedizin-GMPH, Germany) to occlude the MCA. Sham-operated mice were exposed to the same anesthesia and surgical procedure without electrocoagulation of MCA (Muhammad *et al.*, 2008; Colak *et al.*, 2011; Barakat *et al.*, 2014). The mortality rate in the present study was less than 20%, and less than 10% of the animals were devoid of ischemia (were excluded from the data analysis).

### **Perfusion and sampling**

After 48 hours, the animals were anesthetized and the thoracic cavity was opened to expose the heart and visualize the ascending aorta. The animal was perfused using 18-gauge butterfly with 50 ml Ringer's solution while a cut was made into the right atrium. The whole brain was cut from olfactory bulb to the cerebellum and preserved in formalin, washed by alcohol then xylene and mounted in wax. Then, it was cooled and cut by microtome at thickness 3  $\mu$ m on slides and stained by Hematoxlin and Eosin (H&E) staining.

### **Behavioural test to detect sensorimotor recovery**

The performance in the initiation-of-walking test (latency to move) was used to assess the sensorimotor deficit based on the time needed for a mouse to move one body length (7 cm). This test was performed 3 times to calculate the mean-time one day before and two days after ischemia (Hattori *et al.*, 2000).

### **Hematoxlin and Eosin (H&E) staining to detect the brain edema, neuronal degeneration and leukocytes infiltration:**

Brain sections were stained according to the method of Hematoxlin and Eosin (H&E) staining (Mayer, 1896; Lillie, 1965; Avwioro, 2011). The sections were

investigated under light micro scope and images were computed by using camera and the photos were analysed.

### **Statistical analysis:**

Data are expressed as mean  $\pm$  standard deviation of mean (S.D.M). Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Tukey's post Hoc test for comparisons between groups and paired *t* test for comparing changes in behavior before and after MCAO using Graph pad Prism software version 5. For all analysis, the level of statistical significance was set at  $P < 0.0001$ .

## **RESULTS**

### **Effects on behavior:**

#### **Initiation-of-walking**

A significant sensorimotor dysfunction was observed in MCAO groups when compared to before ischemia using paired *t* test as follow:

(A MCAO) group had a significant increase in the initiation of walking time when compared with the corresponding group before ischemia using paired *t* test (2.93 sec vs. 0.67 sec). (P MCAO) group had a significant increase in the initiation of walking time when compared with the corresponding group before ischemia using paired *t* test (3.01 sec vs. 0.70 sec). (I MCAO) group had a significant increase in the initiation of walking time when compared with the corresponding group before ischemia using paired *t* test (2.87 sec vs. 0.69 sec).

Furthermore, a significant sensorimotor dysfunction was observed in the MCAO groups when compared to sham group as follow:

(A MCAO) group had a significant increase in the initiation of walking time when compared with (A Sham) group (2.93 sec  $\pm$  1.08 vs. 0.65 sec  $\pm$  0.12). (P MCAO) group had a significant increase in the initiation of walking time when compared with (P Sham) group (3.01sec  $\pm$  1.08 vs.

0.61sec  $\pm$  0.14). (I MCAO) group had a significant increase in the initiation of walking time when compared with (I Sham) group (2.87 sec  $\pm$  1.07 vs. 0.66 sec  $\pm$  0.11).

(Albumin treatment) group had a significant decrease in the initiation of walking time when compared with (A MCAO) group (0.63 sec  $\pm$  0.16 vs. 2.93 sec  $\pm$  1.08). (Pioglitazone treatment) group had a significant decrease in the initiation of walking time when compared with (P MCAO) group (0.72 sec  $\pm$  0.22 vs. 3.01 sec  $\pm$  1.08). (Insulin treatment) group had a significant decrease in the initiation of walking time when compared with (I MCAO) group (0.71 sec  $\pm$  0.11 vs. 2.87 sec  $\pm$  1.07).

(Albumin treatment) improved the sensorimotor dysfunction by 78.49%. (Pioglitazone treatment) improved the sensorimotor dysfunction by 76.07%. (Insulin treatment) improved the sensorimotor dysfunction by 75.26% (fig. 1, table 1).

#### **Effects on brain edema and leukocyte**

##### **infiltration:**

**Photomicrograph (1):** (A MCAO) showed neuronal degeneration, microcytic spaces, reactive gliosis and moderate lymphocytic infiltration (bold arrow) (c and d). (P MCAO) showed moderate cerebral edema (bold arrows) (g and h). (I MCAO) group showed mild cerebral edema and leukocyte infiltration (bold arrows) (k and l). (Albumin treatment) group showed granular pink amorphous necrotic white matter (star), ruptured neurons (bold arrow), reactive gliosis, no leukocyte infiltration and no cerebral edema (a and b). (Pioglitazone treatment) group showed normal white matter (e and f). (Insulin treatment) group showed neuronal damage (star), no leukocyte infiltration and no cerebral edema (i and j). Sham groups did not show any brain edema nor leukocyte infiltration (m, n, and o).

## **DISCUSSION**

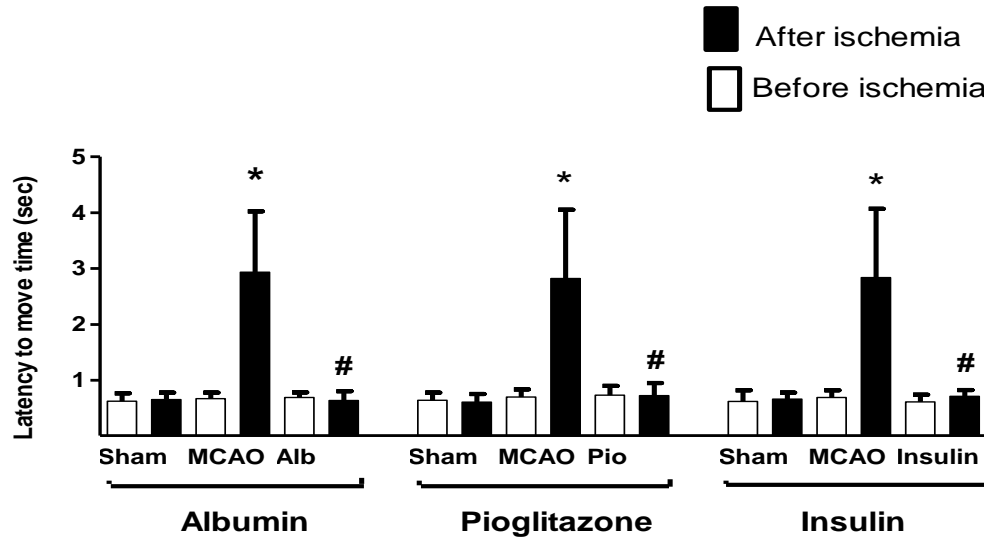
The current study attempted to investigate the possible protective effects of human serum albumin, pioglitazone and insulin against MCAO which induced brain damage in mice. In the current study, mice subjected to MCAO after 48h showed a significant increase the time of initiation of walk test (latency to move test) when compared to sham or before MCAO. Similar changes have been observed by others (Hattori *et al.*, 2000). Who used male C57/B16 mice were subjected to 60 or 90 minutes of intraluminal MCAO or sham surgery. In the first cohort of animals (n=12/group), locomotor activity, balance, and coordination were evaluated 2 weeks after surgery. In a second cohort of animals (n=10/group), the effects of 60 minutes of MCAO on subsequent learning and memory were assessed with a step-down passive avoidance task beginning 1 week after surgery. In a third cohort of animals (n=8 to 10/group), training in a passive avoidance task was completed before 60 minutes of MCAO, then retention of the task was assessed 1 week after surgery. They found that there was a significant increase in latency to move 1 body length in the 90-minute MCAO group compared with the 60-minute MCAO and sham group. Stroke followed by infiltration of leukocytes, release cytokines and chemokines and reactive oxygen species which amplify the brain-inflammatory response, eventually leading to disruption of the BBB, cerebral edema, neuronal death, and hemorrhagic transformation (Kriz, 2006; Amantea *et al.*, 2009). In the present study, mice subjected to MCAO showed significant brain edema while mice subjected to sham operation did not show significant brain edema. These results are in accordance with other researchers (Kahle *et al.*, 2009). Who found that during ischemia, cytotoxic edema occurs in all cells of the neurovascular unit, such as endothelial cell, astrocytes and

neuronal cells. Cell volume changes, due to minor changes of ion composition in the extracellular or intracellular environment, can significantly affect the function of neurons. Treatment of mice with albumin abolished MCAO induced brain edema. These results are in accordance with other researchers (Belayev *et al.*, 2001). Who reported the beneficial effects of albumin therapy against brain swelling in sprague-dawley rats subjected to brain ischemia/reperfusion. Treatment of mice with pioglitazone also prevented MCAO-induced brain edema. These results are in accordance with other researchers (Medhi *et al.*, 2010; Culman *et al.*, 2012). Treatment of mice with insulin also prevented MCAO-induced brain edema. These results are in accordance with other researchers (Kazan *et al.*, 1999; Panickar *et al.*, 2009). Leukocytes are the cells responsible for the progression from tissue ischemia to cerebral infarction (Lipton, 1999; Frijns and Kappelle, 2002; Huang *et al.*, 2006). The infiltration of leukocytes (in case of cerebral ischemia) and the activation of microglia (the resident immune cells of the brain) (Kochanek and Hallenbeck, 1992) functions to remove damaged tissue, but they have the potential to enhance infarct expansion ultimately resulting in more damage in the brain like subsequent micro vessel obstruction, edema formation, cellular necrosis and tissue infarction (Clark *et al.*, 1993). In the current study, mice subjected to MCAO by electrocoagulation for 48 h showed significant leukocyte infiltration in the ipsilateral hemisphere while sham

operation did not show leukocyte infiltration. These results are in accordance with other researchers (Tu *et al.*, 2010). Treatment of mice with albumin prevented leukocyte infiltration following MCAO as previously reported (Piryazev *et al.*, 2014). Similarly, treatment of mice with pioglitazone prevented leukocyte infiltration. This is in accordance with other researchers (Hasegawa *et al.*, 2011; and Culman *et al.*, 2012). In addition, treatment of mice with insulin had significantly decreased leukocyte infiltration. These results are in accordance with other researchers (Li *et al.*, 2009). MCAO induced cerebral edema, leukocyte infiltration and neuronal death as evidenced by H&E staining. The infiltration of leukocytes is essential to remove damaged tissue (Kochanek and Hallenbeck, 1992). However, infiltrating leukocytes release cytokines and chemokines and reactive oxygen species which amplify the brain-inflammatory response, eventually leading to disruption of the BBB, cerebral edema, neuronal death, and hemorrhagic transformation (Kriz, 2006; Amantea *et al.*, 2009).

## CONCLUSION

In conclusion, the current study has clearly demonstrated the neuroprotective effect of intravenous human serum albumin (HSA), oral pioglitazone and intravenous insulin against brain ischemia.



**Fig (1):** Effect of ischemia and treatment with albumin (0.04ml/ 3min/ 20g &i.v. infusion), pioglitazone (40mg/ kg/ day & oral) and insulin (0.3 IU/ kg &i.v.) on the sensorimotor function measured by initiation-of-walking test

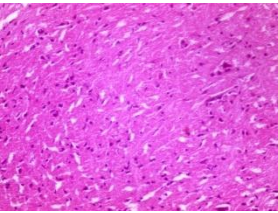
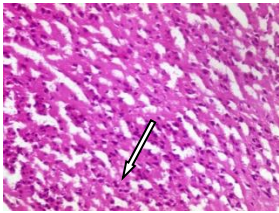
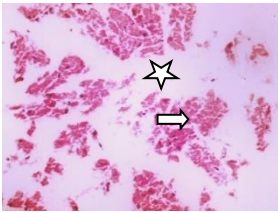
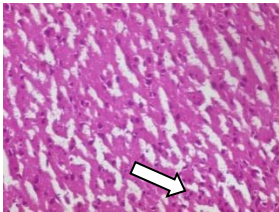
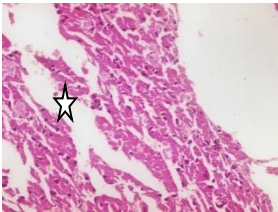
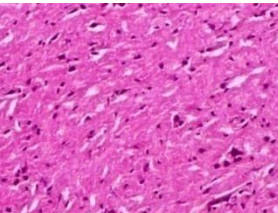
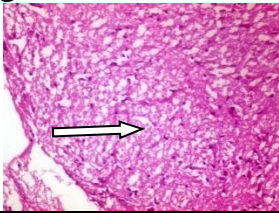
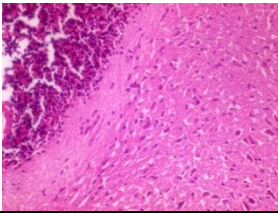
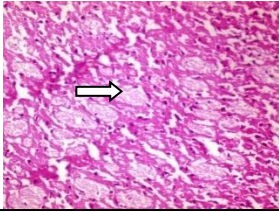
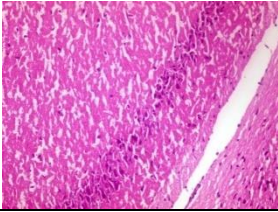
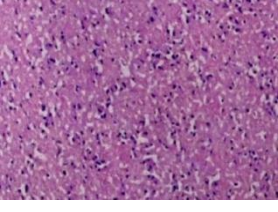
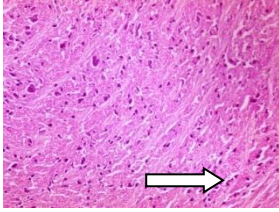

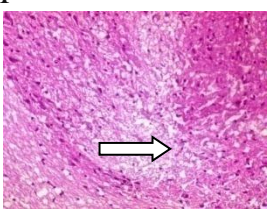
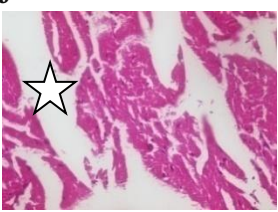
\* Significantly difference from the corresponding sham groups.  
 #Significantly difference from the corresponding MCAO groups.

**Table (1):** Effect of ischemia and treatment with albumin (0.04ml/ 3min/ 20g &i.v. infusion), pioglitazone (40mg/ kg/ day & oral) and insulin (0.3 IU/ kg &i.v.) on the sensorimotor function measured by initiation-of-walking test:

Group	Mean±SD (sec)	% Change from the corresponding MCAO group
A sham	0.65 ± 0.12	-----
A MCAO	2.93 ± 1.08*	0
Albumin treatment	0.63 ± 0.16#	78.49%
P sham	0.61 ± 0.14	-----
P MCAO	3.01 ± 1.08*	0
Pioglitazone treatment	0.72 ± 0.22#	76.07%
I sham	0.66 ± 0.11	-----
I MCAO	2.87 ± 1.07*	0
Insulin treatment	0.71 ± 0.11#	75.26%

\*Significantly different from the corresponding sham group at p<0.0001.  
 #Significantly different from the corresponding MCAO group at p<0.0001.  
 Data are expressed as mean ±SD

**Photomicrograph (1):** Effect of ischemia and treatment on leukocyte infiltration, cerebral edema and necrotic cell death detected by Hematoxlin and Eosin staining:

	Sham	MCAO	Treatment
Albumin (0.04ml/ 3min/ 20g & i.v. infusion)	m 	c 	a 
		d 	b 
Pioglitazone (40 mg/ kg/ day & oral)	n 	g 	e 
		h 	f 
Insulin (0.3 IU/ kg & i.v.)	o 	k 	i 
		l 	j 



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## التأثير العصبي لالبومين المصل البشري, البيوجليتانزون والانسولين علي التلف المخي لدى الفئران

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السكتة الدماغية هي السبب الرئيسي للوفاة والعجز الدائم لدى البالغين في جميع أنحاء العالم. الحقن الوريدي لمنشط الأنسجة البلازمينوجيني المخلق (ار تي بي اية) في غضون 3 ساعات من ظهور السكتة الدماغية الحادة هو العلاج الوحيد المعتمد من إدارة الأغذية والعقاقير (اف دي اية). في هذه الدراسة تم دراسته تأثير البومين المصل البشري (تركيز 20%) (اتش اس اية) في محلول (صديوم كلوريد 0.09%) (0.04 ملليمتر/20 جرام) عن طريق الحقن الوريدي بنسبة 1% من وزن الجسم) بمعدل ثابت على 3 دقيقة بعد ساعتان من انسداد الشريان الدماغية الاوسط (MCAO). البيوجليتانزون (40 مليجرام / كجم / يوم) في مستحلب من 0.5% كربوكسي ميثيل سلولوز) عن طريق الفم لمدة ثلاثة أيام قبل انسداد الشريان الدماغية الاوسط (MCAO) وبعد يومين من انسداد الشريان الدماغية الاوسط (MCAO). الأنسولين (2 وحدة دولية / كجم) عن طريق الحقن الوريدي مع انسداد الشريان الدماغية الاوسط (MCAO) وبعد 6 ساعات من انسداد الشريان الدماغية الاوسط (MCAO).

البومين المصل البشري, البيوجليتانزون والانسولين أظهروا تحسن العيوب العصبية الناتج عن التلف المخي المحدث تجريبيا الناجم عن انسداد الشريان الدماغية الاوسط (MCAO) الدائم في الفئران الذي يتضح من قلة الوقت الازم للشروع في التحرك وكما يتضح من انخفاض تسلسل كريات الدم البيضاء وانخفاض الأستقساء الدماغية.