# AMINOGUANIDINE PREVENTS OXIDATIVE STRESS INSULT FOLLOWING TRANSIENT FOREBRAIN ISCHEMIA IN THE RAT HIPPOCAMPUS

**Abdulhakeem A. Al-Majed**

لقد كان يعتقد بأن للإجهاد التأكسدي دوراً في الإصابة الدماغية الإقفارية. وقد ثبت أن للعديد من مضادات الأكسدة تأثير عصبي واق تجاه تلك الإصابة وذلك في حيوانات التجارب. تم تصميم هذه الدراسة للتحقق من التأثير الواقي المحتمل لمادة أمينو جوانيدين تجاه العطب الناتج عن الإجهاد التأكسدي في قرن آمون بدماغ الجرذ الحي بواسطة نموذج إقفاري لمقدمة الدماغ. حيث تم تعريض الجرذان إلى 5 دقائق من غلق الشريان السباتي الرئيسي من الجانبين متبوعاً بسبعة أيام من إعادة سريان الدم. ثم تم دراسة تأثير مادة أمينوجوانيدين ( 200 مغ/كغ ، بريتونياً ) بعد إحداث الإقفار الدماغي مباشرة والذي استمر لمدة مقدارها 7 أيام ، وذلك بتقييم المؤشرات البيوكيميائية المتعلقة بالإجهاد في قرن آمون في الجرذان. وبالمقارنة مع السواغ ، تسببت مادة أمينو جوانيدين في التقليل من انخفاض مستوى جلوتاثيون في قرن آمون وخفض المستويات العالية من فوق أكاسيد (بيروكسيدات) الشحوم ، وقد تبين ذلك بتقييم مستويات مادة مالونالدهيد. كما نتج عن مادة أمينوجوانيدين زيادة معنوية ($p<0.05$) في نشاط الإنزيمات المضادة للتأكسد الداخلية المنشأ وهما سوبرأوكسيد دسميوتاز والكاتلاز. إن اختزال واسمات الإجهاد التأكسدي بواسطة مادة أمينوجوانيدين كان مصحوباً باختزال معنوي في نشاط إنزيم لاكتات ديهيدروجيناز المستحث بالإقفار الدموي. تدل الدراسة الحالية على قدرة مادة أمينوجوانيدين على خفض الإجهاد التأكسدي المستحث بإقفار مقدمة الدماغ وتدل على التأثير العصبي الواقي المحتمل لمادة أمينوجوانيدين وذلك بواسطة التخفيف من إصابة الدماغ نتيجة للإقفار.

Oxidative stress has been implicated in the pathogenesis of ischemic cerebral injury. Several antioxidants have been shown to have a neuroprotective effect in experimental models of cerebral ischemia. The present investigation was designed to investigate the potential protective effect of aminoguanidine (AG) against oxidative stress damage in the rat hippocampus in an *in vivo* forebrain ischemia model. Rats were subjected to 5 min of bilateral common carotid artery occlusion followed by 7 days of reperfusion. The effect of AG (200 mg/kg, i.p), administered immediately after the induction of forebrain ischemia and continued for 7 consecutive days, was investigated by assessing oxidative stress-related biochemical parameters in rat hippocampus. Compared with vehicle, AG significantly ($P < 0.05$) reduced hippocampal glutathione depletion and decreased the elevated levels of lipid peroxides, as assessed by the levels of malondialdehyde (MDA). AG also resulted in a significant ($P < 0.05$) increase in the activity of the endogenous antioxidant enzymes, superoxide dismutase (SOD) and catalase. The reduction of oxidative stress markers by AG was associated with significant ($P < 0.05$) reduction in activity of lactate dehydrogenase (LDH; an index of lactacidosis) induced by ischemia. The present study indicates the ability of AG to reduce oxidative stress induced by transient forebrain ischemia and suggests a potential neuroprotective effect of AG by attenuating brain injury following the ischemic insult.

***Kewords:*** Forebrain ischemia, hippocampus, oxidative stress, aminoguanidine, rat

## Introduction

In cerebral ischemia, cerebral blood flow is reduced in the regions that are supplied with oxygen by the occluded vessels. The decline in cerebral blood flow and the accompanying loss of oxygen supply results in a cascade of events leading to a number of important cellular changes. These include impaired energy metabolism and a measurable decrease in adenosine triphosphate (ATP), calcium release from intracellular stores, loss of ion homeostasis, excitotoxicity, activation of enzymes

Department of Pharmacology, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Kingdom of Saudi Arabia

E-mail: majed107@hotmail.com

such as nitric oxide synthase and cyclooxygenase-2 (COX-2), arachidonic acid release and metabolism, acidosis and edema (1,2). Most of these changes are associated with massive production of reactive oxygen species (ROS) that causes severe oxidative injury to the tissue (3). Indeed, generation of ROS has been correlated with cell damage in cerebral ischemia (4).

Oxidative stress is one of the most important factors that exacerbates damage by ischemia. A large body of experimental research clearly shows that ischemic injury involves oxidatively damaging events (5). The brain is particularly vulnerable to oxidative injury because of its high rate of oxidative metabolic activity, intense production of ROS metabolites, high content of polyunsaturated fatty acids, relatively low antioxidant capacity, low repair mechanism activity and non-replicating nature of its neuronal cells (6).

Forebrain ischemia in rats is a model for transient human cerebral ischemia resulting from transient cardiac arrest (7). Transient forebrain ischemia in humans and animals induces selective death in vulnerable neurons, namely hippocampal neurons (7-9). Forebrain ischemic insults result in significant brain injury that matures over hours to days after the insult. Hippocampal neuronal injury is observed several days after untreated forebrain ischemia in the rat, gerbil, and human (7,8,10). Several lines of evidence indicate that oxidative stress contributes to delayed neuronal death after global cerebral ischemia (11,12) suggesting that ROS formation may cooperate in a series of molecular events that link ischemic injury to neuronal cell death.

Since global cerebral ischemia injury is associated with an imbalance of oxidative stress and antioxidant defense systems, it would be theoretically possible to limit oxidative damage and disease progression by supplementing antioxidants. Aminoguanidine (AG) is an antioxidant drug (13,14). It decreases ROS (namely, superoxide anion and hydrogen peroxide) generation (15,16). The protective effect of AG has been addressed previously in other various models of oxidative stress-induced cell damage. For instance, AG has been shown to be beneficial in ameliorating symptoms of diabetes (16,17). In addition, AG has shown to be protective against oxidative stress-induced lung and liver injuries (14,18). The present work was conducted to investigate the potential protective effect of AG against forebrain ischemia-induced oxidative damage.

## Materials and Methods

The experiments were approved by the Research Ethics Committee of College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

*Animals:*

A total of 50 male Wistar albino rats obtained from Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia were used. The animals weighed between 230-250 g and were housed in metabolic cages under controlled environmental conditions (25$^{o}$C and a 12 h light/dark cycle). Animals had free access to pulverized standard rat pellet food and tap water unless otherwise indicated.

*Drugs and chemicals:*

Aminoguanidine and thiobarbituric acid were purchased from Sigma Chemical Co. (St. Louis, MO. USA), while Ellman's reagent (5-5'-dithiobis-(2-nitrobenzoic acid; DTNB) was purchased from Fluka Chemical Company (Switzerland). Superoxide dismutase analysis kit was purchased from Randox Laboratories Ltd., UK. All other chemicals were of the highest analytical grades commercially available.

## Experimental

Rats were randomly divided into 5 groups of 10 each. Two of the groups were control groups receiving drinking water or AG (200 mg/kg, i.p) respectively. The third group was subjected to sham-operated ischemia. Rats of the fourth group were subjected to 5 min forebrain ischemia, while rats in the fifth group were subjected to 5 min forebrain ischemia and received AG (200 mg/kg, i.p). The dose of AG (200 mg/kg, i.p) was administered immediately after induction of forebrain ischemia and continued for 7 consecutive days.

*Transient forebrain ischemia model:*

Transient forebrain ischemia was induced in the rats under general anaesthesia (sodium pentobarbital; 30 mg/kg, i.p.) with 2-vessel occlusion model (19). The common carotid arteries were exposed by means of a ventral midline neck incision. Both common carotid arteries were exposed, separated from the

vagus nerve and occluded for 5 minutes with microaneurysmal clips. At the end of the occlusion period, the clamps were released, allowing for restoration of carotid blood flow, and the incision was sutured with 2-0 silk sutures. In the sham-operated groups, the arteries were freed from the surrounding connective tissue but were not occluded.

*Tissue sampling:*

Seven days after ischemia, rats were decapitated, the brains were quickly removed and the hippocampus was harvested on a cold stage. The hippocampi were washed with saline, blotted dry on filter paper, weighed and then homogenate (10% (w/v)) in ice-cold saline using Branson Sonifier (VWR Scientific, Danburg, Com., USA). The supernatant was collected and processed for assessment of hippocampal tissue contents of reduced glutathione (GSH) and malondialdehyde (MDA), activities of tissue antioxidants superoxide dismutase (SOD) and catalase as well as lactate dehydrogenase (LDH) activity. The average (±S.D.) levels of GSH, MDA, and the average (±S.D.) activities of SOD, catalase and LDH for each group are reported. Protein concentration was determined parallel to any estimation.

*Lipid peroxidation and GSH assays:*

The tissue levels of acid soluble thiols, mainly GSH, were assayed colorimetrically at 412 nm (20) by using a Shimadzu (Tokyo, Japan) spectrophotometer. The contents of GSH were expressed as nmol $g^{-1}$ wet tissue. The degree of lipid peroxidation in the hippocampal neuronal tissue was determined by measuring thiobarbituric acid reactive substance (TBARS) in the supernatant tissue from homogenate (21). The homogenates were centrifuged at 3500 rpm and supernatant was collected and used for the estimation of MDA. The absorbance was measured spectrophotometrically at 532 nm and the concentrations were expressed as nmol $mg^{-1}$ protein.

*Assay procedure for SOD activity:*

The method used for the SOD assay was a slight modification of the indirect inhibition assay developed by McCord and Fridovich (22). In this method, xanthine- oxidase (XOD) was utilized to generate a superoxide flux, which reacts with 2-(4-iodophenyl)-3(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T) to form a red formazan dye. The absorbance obtained from I.N.T reduction to red formazon by superoxide was determined at 505 nm spectrophotometrically.

*Assay procedure for catalase activity*:

The catalase activity was determined spectrophotometrically by the method of Higgins et al (23), which is the assay of hydrogen peroxide ($H_2O_2$). The activity was expressed as $\mu mol^{-1}$ $min^{-1}$ $mg^{-1}$ protein using the extension coefficient of 0.0436 $mM^{-1}$ $cm^{-1}$.

*Measurement of hippocampal LDH:*

Lactate dehydrogenase (E C: 1.1.1.27) (LDH) activity was assayed according to the method of Buhl and Jackson (24) using commercially available kits (Bio-Mèrieux- RCS, Lyon, France).

*Statistical analysis:*

Data are expressed as means (±S.D.). Statistical comparison between different groups was assessed by a one-way analysis of variance (ANOVA). Post-hoc analysis, when applicable, for multiple comparisons were performed using the Tukey-Kramer multiple comparisons test. The significance level was set at $P < 0.05$.

## Results

Seven days after the induction of forebrain ischemia, there was a ~ 33% decrease in hippocampal GSH levels in comparison to control and sham-operated groups. Treatment with AG (200 mg/kg, i.p) significantly and dramatically increased GSH levels of ischemic rats ($P < 0.05$; Fig. 1).

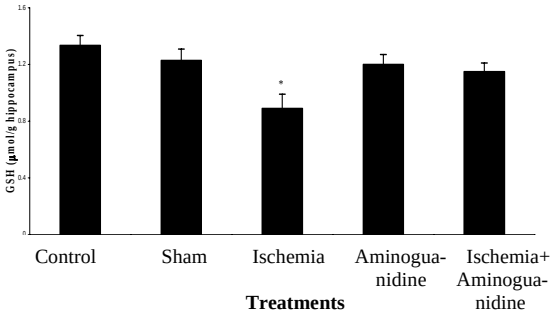


**Figure 1.** Effects of Aminoguanidine on ischemia-induced glutathione depletion in the hippocampus after 7 days of reperfusion following 5 min of forebrain ischemia. Data are expressed as means ± S.D. for all groups; n= 10 in each group. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test. *, Significantly different from control and sham-operated groups ($P < 0.05$).

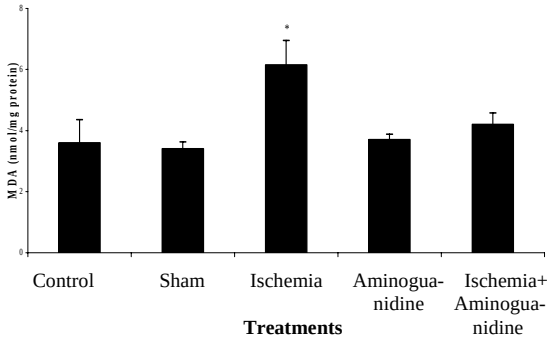


**Figure 2.** Effects of forebrain ischemia and Aminoguanidine (200 mg $kg^{-1}$, i.p) on the level of malonaldehyde (MDA) in the rat hippocampus. *, Significantly different from control and sham-operated groups ($P < 0.05$).

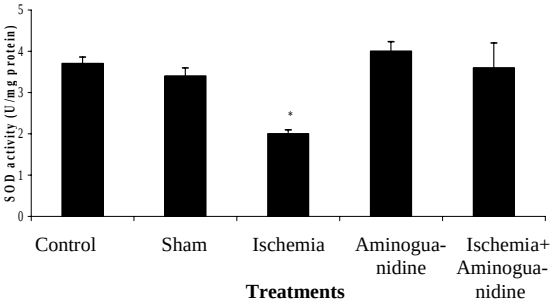


**Figure 3.** Superoxide dismutase (SOD) activity level after 5 min of forebrain ischemia and administration of Aminoguanidine (200 mg $kg^{-1}$, i.p). *, Significantly different from control and sham-operated groups ($P < 0.05$).

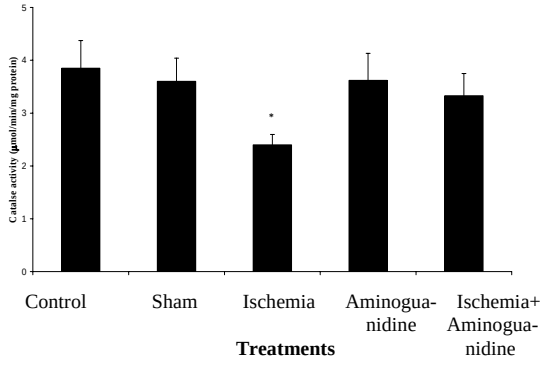


**Figure 4.** Effect of Aminoguanidine (200 mg $kg^{-1}$, i.p) on the reduced level of the catalase activity induced by 5 min forebrain ischemia. *, Significantly different from control and sham-operated groups ($P < 0.05$).

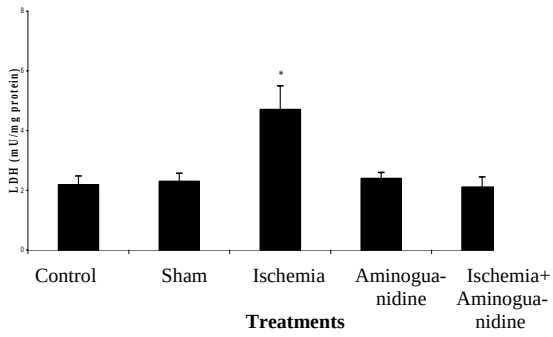


**Figure 5.** Effect of Aminoguanidine (200 mg $kg^{-1}$, i.p) on LDH activity in rat hippocampus after 5 min of forebrain ischemia. *, Significantly different from control and sham-operated groups ($P < 0.05$).

On the other hand, MDA content, an index of lipid peroxidation, was significantly elevated in ischemia-subjected rats to reach ~ 170% of control and sham-operated groups ($P < 0.05$). Ischemia-mediated lipid peroxidation was attenuated significantly in AG-administered rats as shown in Figure 2 ($P < 0.05$). The administration of AG in non-ischemic group did not alter either GSH or MDA levels.

The results of SOD and catalase activities are summarized in Figures 3 and 4. The hippocampal activity of both SOD and catalase was significantly decreased in ischemic rats as compared to the control and sham-operated groups by ~ 90 and 60 % respectively ($P < 0.05$). Treatment with AG, however, elevated the activity of the two enzymes by ~ 80 and 40%, respectively, to nearly the control value (Fig. 3,4).

Ischemic rats had a significant rise in LDH activity by 100 % compared to those in the control and sham-operated groups ($P < 0.05$). Administration of AG significantly reduced the elevation in LDH activity induced by forebrain ischemia (Fig. 5).

## Discussion

Lipid peroxidations (LPs) as well as altered levels of some endogenous scavengers are used as reliable *in vivo* indices for the contribution of ROS generation and, in turn, oxidative stress of the ischemic brain (3,6,12,19,25,26). Knowing that lipids are the most susceptible macromolecules to oxidative stress, our results showed that LPs, measured in terms of MDA level, was markedly

increased (~ 1.7 fold) after 5 minutes of ischemia and continuous reperfusion, suggesting that during forebrain ischemia there is a significant production of ROS. This finding is consistent with other findings (19,25-27) that showed that ischemia in the brain leads to lipid peroxidation.

The overproduction of ROS can be detoxified by endogenous antioxidants, causing their cellular stores to be depleted (12,19,25-26). GSH, considered as the most prevalent and important intracellular non-protein thiol, has a crucial role as a ROS scavenger. A decline in GSH could both increase and reflect oxidative stress (28). In the current work, GSH content was significantly reduced due to ischemic insult. This could be explained by the consumption of GSH due to scavenging of the rapidly generating ROS due to ischemia. When ROS production exceeds the buffering capabilities, MDA levels increase while both non-enzymatic scavengers (GSH) and antioxidant enzymes (SOD and catalase) decrease.

In the present study, the dose of 200 mg/kg of AG ameliorated the elevated MDA level. Such a dose of AG may have reached the rat brain in a concentration sufficient to scavenge ROS and inhibit the propagating chain reaction of LPs (13). Aminoguanidine treatment was able to confer protection against hippocampal glutathione depletion (Fig. 1) and the ensuing lipid peroxidation as assessed by the increases in MDA level. No significant modification in lipid peroxidation as well as GSH level was observed in animals treated with AG without ischemia (Figs. 1, 2). This result suggests that administration of AG does not modify basal lipid peroxidation and GSH levels in the brain. The current work showed that there was a significant decline in forebrain activity of the endogenous antioxidant enzymes SOD and catalase in ischemic rats. The activities of these two antioxidant enzymes have been reported to be decreased by cerebral oxidative stress (29). The current results are in harmony with other studies (19,30).

In this model, treatment with AG was expected to protect the rat brain against oxidative damage revealed as normalization of the above-mentioned inhibited enzymatic systems. Indeed, AG (200 mg/kg, i.p) proved to be beneficial in restoring declined SOD and catalase due to ischemia insult. These findings are in accordance with several publications using the same ischemia model. Many antioxidants proved efficient as neuroprotectors, restoring deranged enzymes and parameters of oxidative damage, such as ginkgo (31), vitamin E (32), melatonin (33) currcumin (26) and nimesulide (19).

Ischemia causes lactate and $H^+$ (lactacidosis) to accumulate in the rat forebrain due to anaerobic metabolism, which lead to an increase in LDH activity to metabolize the lactate formed (32). The transport of lactate across the blood brain barrier is very limited (34). This study reported a significant increase in LDH activity after 5 minutes of forebrain ischemia, in accordance with previous results (26,35). This increase in lactate dehydrogenase activity occurs in parallel with a decline in GSH, an increase in MDA, and a decrease in SOD and catalase activities. AG (200 mg/kg, i.p) proved beneficial in restoring elevated LDH activities after ischemia, an effect similar to that observed by Mostafa et al. on the rat heart (36).

Since previous studies have shown that forebrain ischemia was associated with oxidative cell damage, therapeutic strategies are aimed at limiting free radical-mediated hippocampal injury (19,37,38). Aminoguanidine has been shown to possess antioxidant potential (13,14). Courderot-Masuyer et al. (1999) and Kędziora-Kornatowska et al. (1998) demonstrated that AG is a free radical scavenger against superoxide and hydroxyl radicals (15,16). Furthermore, Ihm et al. (1999) have demonstrated that AG was effective in inhibiting lipid peroxidation in streptozotocin-induced diabetic rats (13). This is in agreement with the similar studies which demonstrated that AG markedly diminishes tissue injury by decreasing oxidative stress and lipid peroxidation (14,18).

Aminoguanidine can also reduce ROS production indirectly. It might be possible that AG reduced ROS production by inhibiting arachidonic acid metabolism. This hypothesis is supported by recent findings that inducible nitric oxide synthase (iNOS) activates the upregulation of COX-2 after forebrain ischemia (39). The increase in COX-2 activity after cerebral ischemia has been shown to generate marked production of ROS and neuronal damage (3, 40). AG, an iNOS inhibitor, was found to suppress COX-2 activity after cerebral ischemia (39). Thus, AG could inhibit oxidative stress-induced by forebrain ischemia by reducing COX-2 activity resulting in reducing arachidonic acid metabolism and subsequently reducing ROS generation. The possible dual inhibition of ROS

obtained with AG could account for its marked anti-oxidative stress effect.

In summary, AG which was useful for amelioration of another models of oxidative stress-induced toxicities such as diabetes complication, liver and lung toxicities, can limit oxidative stress insult induced by forebrain ischemia in the rat hippocampus. Further investigations are needed to explore in more detail the protective effects of AG against forebrain ischemia-induced hippocampal oxidative damage as well as its mechanism of protection.

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