The Protective Effect of Aminoguanidine on Random Pattern Skin Flap Survival: An Experimental Study in Rats

Abstract

Objective: Distal flap necrosis resulting from ischemia is a serious problem, and increases the cost of treatment. Reactive oxygen radicals (ROS) play an important role in tissue injury and ischemia, and may lead to partial or complete flap necrosis. Aminoguanidine (AG), a potent antioxidant, prevents ROS formation and lipid peroxidation. Besides, AG inhibits inducible nitric oxide synthase (iNOS) leading to decreased generation of nitric oxide (NO).

Material and Methods: Rats were randomly divided into three groups: Control, flap elevated saline group, and AG treated group. A caudally based rectangular flap, 3 x 10-cm was elevated on the back of the rats. Flap viability was evaluated 7 days after the initial operation, measuring necrotic areas and total flap areas by computer-assisted planimetry. Malondialdehyde (MDA), NO, glutathione (GSH), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) levels were measured in flap skin tissue to observe the effects of AG.

Results: Rate of flap necrosis and MDA, NO levels were higher in the saline group compared with the control group, while G SH, GSH-Px, and SOD enzyme activities were reduced. AG administration reduced lipid peroxidation, NO generation and increased GSH, GSH-Px, SOD enzyme activities. Furthermore, it significantly reduced the rate of flap necrosis when compared with the saline group.

Conclusion: We believe that AG, a potent antioxidant and iNOS inhibitor, has beneficial effects to improve skin flap viability when distal flap necrosis is a potential complication in longer flaps.

Key Words: Aminoguanidine; surgical flaps; reactive oxygen species; malondialdehyde

Skin flap necrosis is still a controversy in the field of plastic and reconstructive surgery, although mechanisms of flap necrosis were investigated. Skin flap necrosis results from alterations in the hemodynamics of the flap.
Insufficient arterial flow, inadequate venous drainage, or combination of the two. An adequate blood supply is crucial for survival of these flaps, and any injury to flap vascularity or a too-risky flap design causes ischemia and may lead to partial or complete flap necrosis. To increase skin flap viability, many pharmacologic agents, such as sympatholytics, vasodilators, calcium channel blockers, hemorheological agents, prostaglandin inhibitors, anticoagulants, glucocorticoids, and free oxygen radical scavengers have been utilized.

One leading mechanism of skin flap necrosis is believed to involve reactive oxygen radical species (ROS) generation related to tissue ischemia-reperfusion (I/R) injury. Also, excessive iNOS production plays an important role in flap injury. NO is generated in biological systems from L-arginine, O₂, and nicotinamide adenine dinucleotide phosphate (NADPH) by a group of enzyme-denominated nitric oxide synthases (NOSs) and an iNOS originally described in macrophages. Local oxidative stress induces excessive production of NO. Based on this relationship, treatment of the flaps with exogenous SOD, catalase, allopurinol, vitamin C, glucocorticoids, anti-inflammatory agents (etodolac and etofenamate), heparin, and deferoxamine significantly increase survival.

To date, there is no study that investigates the effect of AG on flap survival. AG, a selective inhibitor of iNOS is structurally similar to L-arginine (the substrate for NO). It inhibits NO formation, and interferes with peroxynitrite (ONOO⁻) generation. Previous studies pointed to the beneficial antioxidant effects of AG and scavenger effects of ONOO⁻, which is a reactive oxidant produced from NO and superoxide (O²⁻) in various forms of tissue injury. It was also shown to possess antioxidant potential and decrease the severity of pathophysiological changes attributed to excess NO production. Additionally, Courderot-Masuyer et al. demonstrated that AG was a free radical scavenger against O²⁻ and hydroxyl radicals (OH). They revealed it to be an effective OH scavenger, by using electron paramagnetic resonance.

Since iNOS production and oxyradicals, especially ONOO⁻ generation are the main causes of skin flap I/R injury, this experimental study was designed to investigate the effects of AG, a highly effective antioxidant and free radical scavenger, on the survival of random pattern skin flap in a rat model.

**Materials and Methods**

**Experimental conditions.** Female Wistar rats weighing 150-200 g were housed in temperature (21 ± 2°C) and humidity (60 ± 5%) controlled room in which a 12:12 hr light:dark cycle was maintained. Twenty-four female Wistar rats were used, and randomly divided into three groups (n= 8 rats each group): The control group (Group 1), saline group (Group 2), and AG treated group (Group 3). Flap was elevated in the latter two groups but not in the control group to determine the effects of flap elevation. The rats were anesthetized with intraperitoneal (i.p.) injections of a mixture composed of ketamine (50 mg/kg) and xylazine (5 mg/kg). The surgeons were blinded to the treatment groups. Each animal’s back was shaved and sterile technique was employed in all surgical procedures. The animals were placed in a prone position and a caudally based rectangular flap, 3 x 10 cm was drawn onto the backs of the rats, according to the method described by Khouri and colleagues. All the rats were sacrificed with intracardiac injections of pentobarbital, after the observations were completed.

**Surgical Technique.** Asepsis was maintained by providing a local sterile environment. A dorsal random pattern skin flap with a size of 10 x 3 cm was elevated on the dorsal trunk of the rats with meticulous hemostasis; the flap was sutured back into its original place (Figure 1 a). Morbidity and mortality were not observed during our experimental study. Saline was administered i.p. 0.2 ml/day to the control and saline groups for 6 days. One hour before flap elevation, AG, a selective iNOS inhibitor with antioxidant property, was administrated 100 mg/kg/day; administration continued for 6 days. AG (Aminoguanidine Hemisulfate Salt; Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) was dissolved in saline (0.9% NaCl wt/vol) to obtain a final concentration of
100 mg/mL which was determined according to relevant literature. Intraperitoneal route was selected due to its ease of application and accuracy of dosing in rats.

Flap viability was evaluated 7 days after the initial operation, at which time a certain amount of necrosis in the distal part of all dorsal flaps was noted. The evaluators were blinded to the treatment groups. All groups were photographed (Figure 1 b, c) for demonstration and then, the necrotic skin (defined by the necrotic skin borders) and total flap (defined by the surgical borders) areas were delineated on to an acetate sheet; surface areas were calculated (in square centimeters) by using computer-assisted planimetry. The necrotic surface area was divided by the total flap area, and the results were expressed as percentages of skin necrosis. Skin biopsy was taken from an area 3-4 cm proximal of the flap to determine the levels of MDA, NO and GSH, GSH-Px, SOD enzyme activities.

All experiments in this study were performed in accordance with the guidelines for Animal Research from the National Institutes of Health and were approved by the Committee on Animal Research at İnönü University, Malatya, Turkey.

Biochemical determination. One hundred milligrams (mg) of frozen flap tissue biopsy specimens, cut into pieces with blades on dry ice, were homogenized in 1.15% KCl buffer (1:9, w/v) using a manual glass homogenizer for approximately 5 minutes and flushed by centrifugation for approximately 10 seconds to remove large debris. The supernatant was used for analysis.

The MDA content of homogenates was determined spectrophotometrically by measuring the presence of thiobarbituric acid reactive substances. Results are expressed as nmol/g tissue.

Since tissue nitrite (NO\textsubscript{2} \textsuperscript{-}) and nitrate (NO\textsubscript{3} \textsuperscript{-}) levels may be used to estimate NO production, we measured the concentration of these stable NO oxidative metabolites. Quantization of NO\textsubscript{2} and NO\textsubscript{3} was based on the Griess reaction, in which a chromophore with a strong absorbance at 545 nm is formed by reaction of NO\textsubscript{2} with a mixture of naphthylethlenediamine and sulfanilamide. Results are expressed as µmol/g tissue.

GSH was determined by the spectrophotometric method, which was based on the use of Elman’s reagent. Results were expressed as nmol/mg tissue.

GSH-Px activity was measured by the method of Paglia and Valentine. In the presence of GSH reductase and NADPH the oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP\textsuperscript{+}. The decrease in absorbance at 340 nm is measured. GSH-Px activity was expressed as U/g protein.

SOD enzyme activity determination was based on the production of H\textsubscript{2}O\textsubscript{2}, from xanthine by xanthine oxidase and reduction of nitroblue tetrazolium as previously described. The product was evaluated spectrophotometrically. Results were expressed as U/g protein.

Statistical analysis

Flap tissue MDA, NO, GSH, GSH-Px and SOD levels were analyzed by using one-way ANOVA. Post-hoc comparisons were done using Tukey’s test. Infarct size/flap size ratio was analyzed by Student’s t-test. Differences were considered significant for p< 0.05. All results were expressed as mean ± SEM.

![Figure 1. a) Design of our standard rat dorsal skin flap elevation technique. b) Flap elevated saline group; notice the tissue necrosis. c) Flap elevated group was treated with i.p. AG 100 mg/kg/day; a significant enhancement of flap survival in this group.](image-url)
Results

**Evaluation of tissue enzymes.** Figure 2 summarizes the data of MDA, NO, GSH, GSH-Px and SOD levels obtained from the skin tissues of rats in all groups.

MDA, the end product of lipid peroxidation, and NO levels were higher in the saline group compared to the control group while GSH, GSH-Px, SOD enzyme activities were significantly reduced.

AG administration significantly reduced lipid peroxidation, NO generation and increased GSH, GSH-Px and SOD enzyme activities when compared with the saline group.

**Gross Observations.** No rats died during the experimental study. The regions of survival and necrosis were clearly demarcated in every flap since the flaps did not shrink on post-operative day 7. The surviving skin appeared pink-white, tender, and normal in its texture and it bled when cut, whereas the necrotic skin was black, rigid and did not bleed (Figure 1 a-c).

**Evaluation of necrotic area/total flap area ratio.** Figure 3 presents the summary of necrotic area/flap area ratio. Saline group caused a significant increase in necrotic area expressed as the percentage of risk zone (43.14 ± 4.10). AG administration significantly reduced the necrotic area (30.00 ± 3.57) (p< 0.05.).

Discussion

Random pattern flaps are widely used for the closure of skin and subcutaneous defects since flap elevation and transfer are technically simple. However, due to the unpredictable circulation of these flaps, partial flap necrosis is often encountered in the distal portion of the flap.32 In the distal region of random pattern skin flap, cutaneous blood flow decreases initially, but increases significantly 24 hours after flap elevation.33 The distal region of random pattern flap represents an incomplete ischemic state with tissue damage resulting from oxygen derived free radicals.3 An adequate blood supply is crucial for the survival of these flaps, and any injury to flap vascularity or a too-risky flap

![Figure 2](image_url)  
*Figure 2. The effects of aminoguanidine (AG) on tissue content. a: p< 0.05 vs control group, b: p< 0.05 vs saline group.*

Plastic, Reconstructive and Aesthetic Surgery

Aydoğan et al
design or I/R injury may lead to partial or complete flap necrosis.  

One leading mechanism of skin flap necrosis is believed to involve ROS generation related to tissue I/R injury. Although ROS were implicated in a wide range of biological functions, they may have both beneficial and highly toxic effects on cellular homeostasis. Several conditions are known to disturb the balance between the production of ROS and cellular defenses resulting in dysfunction and cellular destruction. An imbalance between pro and antioxidant factors plays an important role in many disease processes including I/R injury pathogenesis. The formation of oxygen free radicals begins when oxygen is reintroduced to tissues after ischemia. In other words, the fresh supply of oxygen, which accompanies reperfusion, is necessary for the production of oxygen free radicals, which are toxic to all biologic substances.

The effect of NO on the microcirculation in the periphery of a flap remains unclear; besides, its effect on flap survival is unknown because NO has a dual action. NO, on one hand, relaxes vascular tone, which is potentially protective against ischemia-induced flap necrosis and on the other hand, in the presence of O' produces a powerful oxidant, ONOO-, that may be cytotoxic to flap tissue. NO is generated in biological systems from L-arginine, O2, and NADPH by a group of enzymes denominated NOS that include two constitutive isoforms (endothelial and neuronal) and an iNOS originally described in macrophages. Excessive iNOS production plays an important role in flap injury. Plasma levels of NO are altered during experimental and clinical ischemia, hypoxia, sepsis and bacteremia, suggesting that NO may mediate important responses of the regional vascular beds in such conditions.

Previous studies have shown NO-mediated modulation of free radical generation from polymorphonuclear leukocytes (PMNs), following hypoxic-reoxygenation as well as in the normoxic cells. Also, Willemart et al. demonstrated that vasoconstriction, edema formation, accumulation and activation of leukocytes which is related to production of ROS contributed to formation of skin flap necrosis.

AG acts as an antioxidant and free radical scavenger, preventing ROS formation and lipid peroxidation in cells and tissues. During the last decade, two important effects of AG were discovered, which caused this molecule to attract a great deal of interest. First, AG inhibits in vitro and in vivo formation of highly reactive advanced glycosylation end products (AGEs) associated with the pathogenesis of secondary complications of diabetes. Second, AG inhibits NOS, particularly the iNOS isoform which is associated with production of large quantities of NO, in response to various stimulants, e.g. cytokines, rendering AG an important pharmacological tool. AG is endowed with many other activities that together account for its beneficial effects. AG inhibits diamine oxidase, which catalyzes degradation of biologically active diamines such as histamine and putrescine.

However, to date there is no study that investigates any effect of AG on flap survival. The protective effects of AG were previously addressed in other models of cell damage induced by drugs. The beneficial effects of AG in various experimental models of inflammation have also been reported. Previous studies pointed to the beneficial antioxidant, and ONOO scavenger effects of AG. Recently, Al-Shabanah et al. showed that AG protected mice against hepatotoxicity induced by carbon tetrachloride and also Mansour et al. reported that AG protected against nephrotoxicity.
induced by cisplatin in rats.\textsuperscript{24,47} Mostafa et al. showed that AG prevented the cardiotoxic effects of chronic doxorubicin administration.\textsuperscript{48} Yıldız et al. also found that AG had direct scavenging activities against.\textsuperscript{49} Finally, Giardino et al. reported that AG acted as an antioxidant in vivo, preventing ROS formation and lipid peroxidation in cells and tissues preventing oxidant-induced apoptosis.\textsuperscript{50}

Based on this relationship, the present study was planned to investigate the possible protective effect of AG, a selective iNOS inhibitor and scavenger of oxyradicals, on ischemia related necrosis in rat random pattern skin flap. Since many studies are present concerning the role of ROS in the pathophysiology of flap necrosis, we decided to determine the levels of MDA, a lipid peroxidation end product, NO generation and GSH, GSH-Px and SOD enzyme activities in extracted skin tissue.\textsuperscript{3,4}

In our study, biochemical evaluation of MDA and NO levels is correlated with previous literature findings (Table I). Flap elevation significantly increased MDA and NO levels. In addition, AG administration significantly reduced lipid peroxidation and NO generation. The beneficial effects of AG probably include its iNOS inhibitor, antioxidant, free radical scavenger and protecting lipid peroxidation effects. Although this protective mechanism is not clear, AG may directly eliminate free oxygen radicals such as ONOO\textsuperscript{-} or directly increase the antioxidant enzyme activity and prevent the inhibition of these enzymes. Besides, inhibition of iNOS may contribute to this beneficial effect. The results of the present study with AG pre-treatment confirm the findings of Jakus et al. who demonstrated that AG had the potential to inhibit MDA in vitro in erythrocytes.\textsuperscript{51}

Lipid peroxidation as well as altered levels of some endogenous scavengers are taken as indirect in vivo reliable indices for the contribution of free radical generation and in turn oxidative stress.\textsuperscript{52} It was already demonstrated that depletion of GSH preceded the induction of lipid peroxidation.\textsuperscript{53} Lipid hydroperoxides may be reduced and detoxified to lipid alcohols and these reactions are catalyzed by GSH-Px oxidizing GSH to GSSG.\textsuperscript{54,55}

In the present study, flap elevation significantly reduced GSH, GSH-Px and SOD enzyme activities according to the control group. AG administration had a significantly beneficial effect on these enzymes (Figure 2).

Considering the reduced oxidative damage due to AG treatment, all investigators attributed protective actions of AG to its iNOS inhibitor, free radical scavenger and preventing ROS formation activity. This data is consistent with our results that AG administration reduced MDA and NO levels and increased GSH, GSH-Px, SOD enzyme activities. In addition, AG administration significantly reduced the ratio of skin flap necrosis, which was determined by using planimetry and supported by photography (Figure 1).

These findings strongly suggest that AG is important in preventing ischemic injury due to random pattern dorsal skin flap tissue. Based on the reduction of lipid peroxidation and NO generation by AG, which yielded parallel planimetric results, it is important to inhibit lipid peroxidation to prevent necrosis of skin flap (Figure 3).

In conclusion, we believed that the AG had beneficial effects to improve skin flap viability when distal flap necrosis is a potential complication of longer flaps. However, further investigations with larger animal models are needed to determine the optimal dose, effects of prolonged use and clarification of mechanism of action as well as new routes of administration such as topical application for the protective effect of AG on flap survival.

\textbf{REFERENCES}

5. Arima H, Nishimoto Y, Motoyama K, Hirayama F, Uekama K. Inhibitory effects of novel hydrophilic cyclo-
dextrin derivatives on nitric oxide production in macro-
phages stimulated with lipopolysaccharide. Pharm Res
Involvement of spontaneous nitric oxide production in the
diabetogenic action of streptozotocin. Pharmacology
7. Sagi A, Ferder M, Levens D, Strauch B. Improved sur-
vival of island flaps after prolonged ischemia by perfusion with
8. Hardy SC, Homer-Vanniasinkam S, Gough MJ. Effect of
free radical scavenging on skeletal muscle blood flow dur-
ing postischaemic reperfusion. Br J Surg 1992;79:1289-
92.
9. Im MJ, Manson PN, Bulkley GB, Hoopes JE. Effects of
superoxide dismutase and allopurinol on the survival of
in a rat epigastric island skin flap model. Ann Plast Surg
and systemic disposition of hexamethylene lauramide and its
penetration enhancement effect on hydrocortisone in a rat
Effects of some pharmacological agents on the survival of
unipedicle venous flaps: An experimental study. Micro-
decreases ischemia-reperfusion injury in isolated canine
changes in experimental flaps in a rabbit model. J Re-
const Microsurg 1998;14:121-6.
attenuates ischemia-induced reperfusion injury in the skin
and muscle of myocutaneous flaps in the pig. Plast Re-
16. Misko TP, Moore WM, Kasten TP, et al. Selective inhibi-
tion of the inducible nitric oxide synthase by aminogua-
17. Suzuki N, Sakamoto A, Ogawa R. Effect of L-canavanine,
an inhibitor of inducible nitric oxide synthase, on myocard-
dial dysfunction during septic shock. J Nippon Med Sch
18. Ihm SH, Yoo HJ, Park SW, Ihm J. Effect of aminogua-
nidine on lipid peroxidation in streptozotocin-induced dia-
nidine inhibitors of nitric-oxide synthase react with per-
oxynitrite and protect against peroxynitrite-induced oxida-
peroxynitrite decomposition catalyst protects against the
development of doxorubicin-induced cardiac dysfunction.
novel inhibitor of nitric oxide formation, prevents diabetic
22. Couderot-Masuyer C, Dalloz F, Maupoil V, Rochette L.
Antioxidant properties of aminoguanidine. Fundam Clin
23. Khouri RK, Angel MF, Edstrom LE. Standardizing the
24. Mansour MA, Mostafa AM, Nagi MN, Khattab MM, Al-
Shabanah OA. Protective effect of aminoguanidine against
nephrotoxicity induced by cisplatin in normal rats. Comp
25. Haener GM, Wechselberger G, Otto-Schoeller A, Gu-
rulugolugu R, Piza-Katzer H, Schoeller T. Improved dorsal
random-pattern skin flap survival in rats with a topically
applied combination of nonivamide and nicobixon. Plast
effect of melatonin on random pattern skin flap necrosis in
27. Sahna E, Paralapkiran H, Ozturk F, Cigremis Y, Acet A.
The protective effects of physiological and pharmacologi-
cal concentrations of melatonin on renal ischemia-
28. Cortas NK, Wakis NW. Determination of inorganic nitrate
29. Paralapkiran H, Ozer MK, Sahna E, Vardi N, Cigremis Y, Acet A. Aminoguanidine in experimental isch-
31. Suzuki S, Miyachi Y, Niwa Y, Ishihi K. Significance of
reactive oxygen species in distal flap necrosis and its sal-
vage with liposomal SOD. Br J Plast Surg 1989;42:559-
64.
32. Gurlek A, Schusterman MA, Evans GR, Gherardini G.
Blood flow and microcirculatory changes in an ischemia-
reperfusion injury model: Experimental study in the rabbit.
33. Halliwell B, Gutteridge JM, Cross CE. Free radicals,
antioxidants and human disease: Where are we now? J
34. Yagmurca M, Faddioglu E, Erdogan H, Ucar M, Sogut S,
Irmak MK. Erdosteine prevents doxorubicin-induced car-
35. Darley-Usmar V, Halliwell B. Blood radicals: Reactive
tion nitrogen species, reactive oxygen species, transition metal