Protective effect of aminoguanidine against oxidative stress in an experimental peritoneal adhesion model in rats

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Postoperative intraperitoneal adhesion formation is a major cause of intestinal obstruction, pain and infertility. This experimental study was designed to evaluate the degree of adhesion formation and peritoneal tissue levels of malondialdehyde (MDA), reduced glutathione (GSH) and total nitrite and nitrate (NO) and the effect of aminoguanidine (AG) on these metabolite values after postoperative intraperitoneal adhesion formation in rats. A total of 21 adult male Wistar albino rats were randomly divided into three groups. Control rats were untreated; the AG group received AG 200 mg kg^{-1} i.p. for 10 consecutive days intraperitoneally after surgery. The sham group was given 0.9% NaCl. The rats were killed on postoperative day 10. The peritoneal tissues were harvested to determine the tissue levels of MDA, GSH, and NO activity. For light microscopic evaluation, the cecum was removed. Adhesion formation scores in the AG group were significantly lower than those of the control and sham groups ($p < 0.017$, $p < 0.026$ respectively). In the AG-treated rats, tissue levels of MDA and NO were significantly lower than in the control group ($p < 0.017$). The levels of GSH in aminoguanidine-treated rats were significantly higher than those of the control group ($p < 0.01$). The severity of the inflammation was more prominent in the control group compared with the AG-injected rats. The results demonstrate that in this experimental model, intraperitoneal administration of aminoguanidine decreases the incidence and extent of peritoneal adhesions and causes a decrease in MDA and NO and an increase in GSH values. Copyright © 2005 John Wiley & Sons, Ltd.

KEY WORDS — aminoguanidine; malondialdehyde; nitric oxide; reduced glutathione; adhesion

INTRODUCTION

Postoperative abdominal adhesion formation is an important clinical problem and a common cause of complications such as intestinal obstruction, infertility and chronic pain, often requiring additional surgery.[1,2] Although the peritoneum normally possesses some preventative physiological properties for adhesion formation, mechanical trauma, tissue ischemia, thermal injury, foreign materials and peritonitis of infectious origin are the most important causes of adhesion formation.[3,4] Numerous mediators of inflammation, such as arachidonic acid, cytokines, nitric oxide (NO), and oxygen-derived free radicals may participate in postoperative formation of adhesions.[5]

To date, there is no study in the literature regarding the protective effect of AG on peritoneal wall adhesion injury. Aminoguanidine (AG), a compound structurally similar to L-arginine, which inhibits inducible nitric oxide synthase (iNOS) in a selective and competive manner, leading to decreased NO production.[6] Also, AG has antioxidant[7] and free radical scavenger properties especially on peroxynitrite (ONOO$^-$) production.[8] Additionally, the beneficial effects of AG in various experimental models of inflammation have been reported.[9] The aim of this study was to evaluate whether AG administration would protect against adhesion formation in an experimental peritoneal adhesion model in rats. To asses the ability of AG to function as an antioxidant in rats with peritoneal adhesion, we measured the levels of reduced glutathione (GSH), malondialdehyde (MDA) and total nitrite

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and nitrate (NO). We also examined histopathological changes.

METHODS

Experimental conditions

A total of 21 3-month-old Wistar-albino rats weighing 300–350 g were included in the study. Food and water were available *ad libitum*. Rats were obtained from Firat University, Animal Laboratory, Elazig, Turkey. Experiments were done at the Inonu University Experimental Research Center. 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 the Inonu University, Malatya, Turkey. Rats were kept in stainless steel cages, given food and water *ad libitum* and quarantined 7 days before surgery. Food was withheld 8 h prior to surgery, but they had free access to water. A total of 21 laparatomized rats were divided into three equal groups. A control group (traumatized only); a second group that was treated with AG (200 mg kg^{-1} intraperitoneally) both before closure and for 10 consecutive days after surgery and a sham group; that was injected i.p. with saline for 10 consecutive days after surgery to monitor the effects, if any of IP injection of the saline vehicle on inducing oxidative stress.

Operation technique

Before incision, the abdomen was shaved and prepared with betadine (Poviiodeks, Kim-Pa Corporation, Istanbul, Turkey).

Surgical procedures were performed while the rats were under intraperitoneal ketamine (50 mg kg^{-1}) and xylazine HCl (10 mg kg^{-1}) anesthesia.

Asepsis was maintained by providing a local sterile environment. Operation gloves were washed throughly with saline before the procedure was started to remove powder particles. Using sterile techniques with both groups, a midline laparatomy was performed. On the left iliac fossa, a 1×2 cm segment of parietal peritoneum was sharply excised. In addition, the cecum was indentified and exteriorized, and an area of serosa measuring approximately 1 cm^2 on the antimesenteric surface was gently abraded along the longitudinal axis using a small brush until bleeding was observed from the serosal surface. Afterwards, the abdominal wall was closed with 2.0 silk continuous sutures.

AG (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) was dissolved in saline (0.9% NaCl w/v) to obtain a final concentration of 200 mg ml^{-1}. Because of very variable AG dosage schemes reported in the literature, we administrated AG at a dose of 200 mg kg^{-1} per day i.p., which is reported to cause marked antioxidative and iNOS inhibitor effects.[10]

All animals were maintained under the same conditions after surgery. To eliminate complications arising from diurnal effects, all rats were sacrificed on postoperative day 10 by ketamine overdose at the same time of day and peritoneal tissues and any adherent material was harvested. During re-laparatomy the presence and degree of adhesions were graded independently by two investigators who were blind to the therapy used. The extent and severity of adhesions in the operation site for each parietal peritoneum were evaluated using an established scoring system.[11] According to this system the extent of adhesions was evaluated as follows: 0, no adhesion; 1, 25% of traumatized area; 2, 50% of traumatized area; 3, total involvement. The peritoneal and cecum tissues were quickly removed. The cecum was placed in formaldehyde solution for routine histopathologic examination. The peritoneum was placed in liquid nitrogen and stored at $-85°$C until assayed for MDA, GSH and NO content.

Histological analysis

For light microscopic evaluation, tissues were fixed in 10% formalin in phosphate-buffered saline and processed with paraffin wax. Tissue sections of 6 μm were stained with hematoxylin–eosin (H–E) and were examined with a light microscope (Olympus BH-2). Five coded slides from each group were examined by a pathologist blind to all the study groups using light microscopy to evaulate for the presence and degree of neutrophil infiltration.

Biochemical analysis

Of the peritoneal tissues 100 mg were homogenized in 1.5% KCl and phosphate-buffered saline solution (1:9, w/v) using a manual glass homogenizer for approximately 5 min and flushed by centrifugation for approximately 10 s to remove large debris. The supernatant was used for analysis.

The malondialdehyde (MDA) content of homogenates was determined spectrophotometrically by measuring the presence of thiobarbituric acid reactive substances (TBARS).[12] Results are expressed as nmol g^{-1} tissue.

As tissue nitrite (NO$_2^-$) and nitrate (NO$_3^-$) levels can be used to estimate NO production, we measured

Table 1. The peritoneal tissue levels of MDA, GSH, NO and adhesion scores of each groups

Groups	n	MDA (nmol g^{-1} tissue)	GSH (nmol g^{-1} tissue)	NO (umol g^{-1} tissue)	Adhesion score
Control[a]	7	199.4 ± 25.4	1.06 ± 0.7	314.2 ± 11.7	2.5 ± 0.5
Sham[b]	7	197.1 ± 23.8	0.93 ± 0.43	308.2 ± 110.6	2.4 ± 0.5
AG[c]	7	167.8 ± 15.4	2.2 ± 0.59	210.2 ± 31.74	1.4 ± 0.7
P values					
a vs. b		0.8	0.7	0.8	0.7
a vs. c		0.017	0.01	0.017	0.017
b vs. c		0.017	0.01	0.026	0.026

GSH, reduced glutathione; NO, nitric oxide; MDA, malondialdehyde.
$p < 0.05$ was considered to be statistically significant.

the concentration of these stable NO oxidative metabolites. Quantification of NO_2^- and NO_3^- was based on the Griess reaction, in which a chromophore with a strong absorbance at 545 nm is formed by reaction of NO_2^- with a mixture of naphthylethylenediamine and sulfanilamide.[13] Results are expressed as umol g^{-1} tissue.

GSH was determined by the spectrophotometric method based on the use of Elman's reagent.[14] Results are expressed as nmol g^{-1} tissue.

Statistical analysis

The statistical package for social sciences (SPSS) version 10.0 was used for statistical analysis. Individual

Figure 2. Neutrophilic infiltration limited to the mucosa and submucosa of cecum in the AG group. Note the inflammation is less severe and the muscularis propria shows no inflammation (H & E × 100)

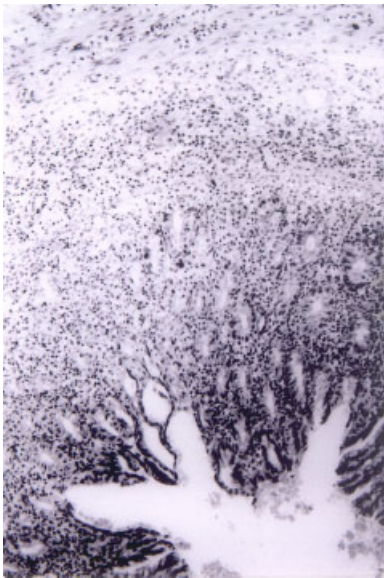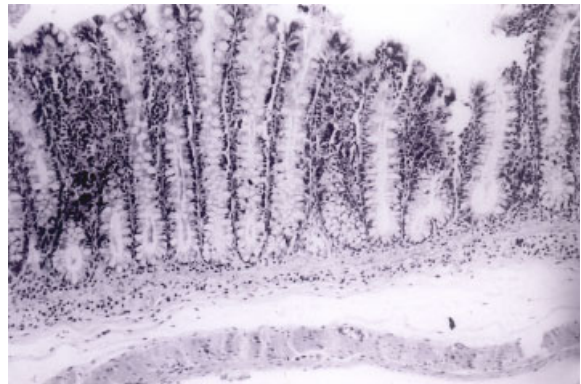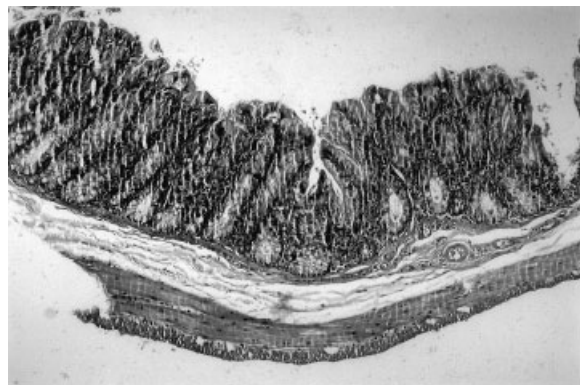Figure 3. Normal histological appearance of cecum (H & E × 100)

Figure 1. Prominent transmural neutrophilic infiltration of cecum in the control group. Severe inflammation has partially distorted the mucosal glandular architecture (H & E × 100)

group parameters were assessed with the one-sample Mann–Whitney *U* test. The results are given in the text as means ± standard deviation (SD). *P* values of < 0.05 were considered statistically significant.

RESULTS

Morbidity and mortality were not observed during our experimental study. Ascites was found in the abdomen during laparatomy in the non-AG treated control group. Also, in these rats adhesions were found between the deserosalized antimesenteric surface of the cecum and adjacent small or large bowels. The mean score of adhesions in control group was found to be 2.5 ± 0.5. In the AG-treated rats, the mean score was found as to be 1.4 ± 0.7 AG treatments significantly reduced the adhesion scores compared with the control and sham groups ($p < 0.017$, $p < 0.026$ respectively). The results for MDA, GSH and NO are shown in Table 1. In the AG-treated rats the MDA and NO levels were significantly lower than in the control ($p < 0.017$) and in the sham group ($p < 0.017$ and $p < 0.026$ respectively).

On the other hand, the levels of GSH in the AG-injected rats were significantly higher than in the control and sham groups ($p < 0.01$). Apparently, AG administration reduced lipid peroxidation, and ameliorated alterations in GSH and NO status. Histologically, a neutrophilic response was present in both groups. The severity of the inflammation was more prominent in the control group compared to the AG-injected rats. In addition, the inflammation was transmural in the control group while limited to the mucosa and submucosa in the AG group (Figures 1 and 2). Normal cecum tissue is shown in the Figure 3.

DISCUSSION

Intra-abdominal adhesions (IAA) are fibrous adhesions formed between serosal surfaces as the result of an inflammatory reaction. IAA occur after abdominal operations and bacterial infections but can also be caused by radiation, allergic reactions, chemical irritation and tissue ischemia.[15,16] Any circumstances that produce ischemia with reduction in tissue oxygenation are known to result in adhesion formation.[17] Notably, during the first 5 min of ischemia, there is already a significant production of free radicals which are able to react rapidly with oxygen exacerbating the oxygen deficit.[18] Locally generated free radicals such as superoxides, peroxides, and hydroxyl radicals are potential oxidizers of polyunsatureted fatty acids.[19] Lipid peroxidation of cellular mebranes occurs and vascular permeability increases, which leads to the formation of serosanguineous exuda, which in turn initiates adhesion formation. These adhesions are generally lysed within 72 h after formation by the endogeneous fibrinolytic activity and much of the healing is complete within 5 days.[20,21] If there is an imbalance between fibrin deposition and dissolution, deposited fibrin may persist and fibrinous adhesions may develop.[22,23]

To date, although AG has antioxidant features it has not been investigated in the experimental peritoneal adhesion model in rats. The present study indicates that aminoguanidine, i.p. administration at a dose of $200 \, \text{mg} \, \text{kg}^{-1}$ on day 10, markedly reduces peritoneal adhesion formation. Our results are in agreement with previous reports of other antioxidant agents.[5,19,24] Also AG administration reduced malondialdehyde and NO production but increased GSH content. Unlike previous works, in the present study we measured the concentrations of MDA and GSH in addition to adhesion score.

A recent study using dietary vitamin E supplementation showed a significant reduction in postoperative peritoneal adhesions in rats.[25] In another study, i.p. superoxide dismutase and catalase, known to block the effect of oxygen free radicals and reactive oxygen species, have been shown to reduce the inflamatory reaction and thereby the adhesion formation in an endometriosis animal model.[26]

Measurements of the amount of MDA provides an index of oxidative stress and lipid peroxidation.[27] Locally generated free radicals such as superoxides, peroxides, and hydroxyl radicals are potential oxidizers of polyunsatured fatty acids, and therefore are postulated to induce peritoneal adhesions through damage to cellular membranes. In the present study, levels of MDA in aminoguanidine-treated rats were significantly lower than those in the control group. Although tissue MDA levels were clearly decreased by AG, its exact mechanism is not clear. Reports that AG directly scavenges hydroxyl radicals and thereby inhibits lipid peroxidation are well documented.[28,29] Reduction in MDA levels in the AG-treated rats is probably due to AG antioxidant and free radical scavenging properties. AG is readily absorbed when it is administered via any route. It seems to enter traumatized peritoneal tissues with ease where it prevents oxidative damage, preserves mitochondrial function,[9] and has low toxicity. It is possible that the interference of AG with free radical generation may be related to the decline in peritoneal adhesions formation.

Recently, Giardino *et al.*[30] reported that AG acted as an antioxidant *in vivo*, preventing free radical formation and lipid peroxidation in cells and tissues and thus preventing oxidant-induced apoptosis.

In the present study, the levels of NO in AG-treated rats, were significantly lower than in those untreated rats. Several mechanisms may contribute to a reduced

level of NO in AG-injected rats. For instance, AG, a compound structurally similar to L-arginine, inhibits inducible nitric oxide synthase (INOS) in a competitive manner, leading to decreased NO production.[6] AG has peroxynitrite (ONOO⁻) scavenger properties.[8] Possible explanations for our finding, include reduced excessive NO formation, and/or neutralization of NO once formed; this would lower oxidative damage and therefore, allow AG to reduce peritoneal adhesion formation in rats. The studies of the role of NO in adhesion formation are limited. In contrast to our study, Ozden et al.[31] showed that administration of L-arginine ($300 \, \text{mg kg}^{-1}$ i.p) increased levels of NO and reduced adhesion formation in rat uterine horn with the parietal peritoneal defect model. In another study, the NO inhibitor and free radical scavenger agent, methylene blue, was found to be effective in the prevention of adhesion formation, but this activity was attributed to inhibition of the free radical generation and not to the action of NO.[5]

In the present study, levels of GSH in aminoguanidine-treated rats were significantly higher than those in the control group. This increased GSH is consistent with the protective effects of AG against oxidative damage.

After trauma/injury to the peritoneum, there is increased vascular permeability in vessels supplying the damaged area, followed by an exudation of inflammatory cells, ultimately leading to the formation of a fibrin matrix. The fibrin matrix is gradually organized and replaced by tissue containing fibroblasts, macrophages and giant cells.[32] In our study, when AG was administered, lower clinical adhesion scores and less inflammation were detected. Intraperitonal AG enters traumatized peritoneal tissues where it may cause fibroblasts to proliferate more slowly, synthesize less collagen and reduce adhesions. Inhibition of fibroblastic proliferation, once it is established, has been described as one of the major measures in the prevention of peritoneal adhesions.

CONCLUSION

In the current study, intraperitoneal administration of AG significantly minimized adhesions in the experimental peritoneal adhesion model in rats. Considering the reduced oxidative damage resulting from AG treatment, the protective actions of AG in the current study are believed to be a consequence of direct and indirect antioxidative activities. AG could be useful in treating intra-abdominal adhesions and possibly other clinical conditions involving excess free radical production.

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