Surgical Manipulation of the Small Intestine and Its Effect on the Lung1

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Background. Surgical manipulation of the intestine results in generation of oxygen free radicals leading to mucosal damage as evidenced by ultrastructural and biochemical changes. It is likely that the gut-derived mediators can bring about damage to distant organs such as the lung.

Methods. Surgical manipulation of the gut was performed by opening the abdominal wall and handling the intestine. Lung damage was assessed by histology, markers of oxidative stress, and protein content in bronchoalveolar lavage fluid. Protection offered by pretreatment with various compounds such as allopurinol, L-arginine, quinacrine, and indomethacin was also studied.

Results. Gut manipulation resulted in neutrophil infiltration, oxidative stress, and permeability changes in the lung and these changes were maximum 30 and 60 min following surgical manipulation, which recovered with time and reversed to normal by 24 h. Prior treatment with inhibitors of xanthine oxidase, phospholipase A2, or cyclooxygenase showed a protective effect against lung damage.

Conclusion. This study has shown that laparotomy and intestinal handling result in distant organ (lung) damage which is probably brought about by neutrophil infiltration and oxidative stress on the lung. This is likely mediated by compounds generated in the intestine and transported into the systemic circulation since inhibition of generation of chemical mediators in the intestine offers protection against lung damage.

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INTRODUCTION

Acute respiratory distress syndrome (ARDS) and multiple organ failure (MOF) are the most common causes of death in surgical intensive care units. A variety of stimuli such as surgery, trauma, shock, burn injury, acute pancreatitis, and ischemia–reperfusion injury initiate a systemic inflammatory response that contributes to the development of these complications [1]. Surgery on any part of the body results in a wide spectrum of alterations in normal body homeostasis and the gastrointestinal tract is now recognized to play an important role in the etiology of these complications [2]. Loss of gut barrier function together with bacterial and endotoxin translocation has been suggested as the major cause for these complications [3]. Reactive oxygen species (ROS), such as the superoxide anion liberated by neutrophils recruited to sites of inflammation, are proposed to be a major cause of the cell and tissue damage. The proteases and oxidants released by the neutrophils can also induce activation of complement and coagulation cascades, which along with the endothelial cell can affect microcirculation. This results in a decrease in oxygen delivery to the gut, leading to hypoperfusion, which might aggravate the intestinal damage. The basic pathophysiology in sepsis, shock, and MOF is a diffuse impairment of oxygen consumption and tissue perfusion, focused mostly on microcirculatory and capillary dysfunction [4].

Acute lung injury characterized by increased microvascular permeability is one feature of multiple-organ failure and adult respiratory distress syndrome (ARDS). Intestinal ischemia–reperfusion has been linked to this type of acute lung injury [5]. In animal models, intestinal ischemia–reperfusion produces distant organ injury by various mechanisms such as neutrophils, reactive oxygen metabolites, and cytokines [6, 7]. Recently it has been demonstrated that hemorrhagic shock-induced lung injury was completely pre-
vented by the division of the mesenteric lymphatics, indicating that the lung injury is possibly produced by hypoperfusion of the intestine followed by generation of some mediators, which are transported through the lymphatics into the systemic circulation [8]. The circulating levels of cytokines, eicosanoids, and platelet-activating factor have been related to remote organ injury in the lung and liver and this injury is associated with sequestration of polymorphonuclear neutrophils (PMNs) in these organs [9]. Oxidants are thought to be responsible for much of the cell and tissue damage that occurs in the injured lung [10].

Our earlier work using a rat model showed that laparotomy with mild intestinal handling, which could occur during any abdominal surgery, can result in increased intestinal permeability and oxidative stress in the enterocytes and this damage is reversible with time [11]. Surgical manipulation also resulted in structural and functional alterations in the brush border membranes through oxidative stress, which include phospholipid degradation by activation of phospholipase A$_2$ and arachidonic acid generation [12]. The small intestine is increasingly recognized as a primary effector of distant organ injury [13] and it is likely that the gut-derived chemical mediators are transferred to distant organs through the lymph. Since lung represents the first vascular bed exposed to mesenteric lymph, this study looked at the extent of lung injury after various periods following surgical manipulation of the small intestine. The study was also extended to study the protection offered by various compounds that can prevent the biochemical events taking place in the small intestine following surgical manipulation of the small intestine.

**MATERIALS AND METHODS**

Allopurinol, L-arginine hydrochloride, bovine serum albumin (BSA), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), indomethacin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), nicotinamide adenine dinucleotide (NAD) and its reduced form (NADH), nicotinamide adenine dinucleotide phosphate (NADP), oxidized glutathione (GSGS), quinacrine, reduced glutathione (GSH), 1,1'-3,3'-tetramethoxypropane, 2-thiobarbituric acid (TBA), Triton X-100, xanthine, and xanthine oxidase (XO) were obtained from Sigma Chemical Company (St Louis, MO). All other chemicals used were of analytical grade.

Animals. Adult Wistar rats of both sexes (200–250 g), exposed to a daily 12-h light–dark cycle and fed water and rat chow ad libitum, were used for the study. The study was divided into two parts. The first part consisted of five groups of rats (n = 4): Group I comprised control rats (laparotomy alone without intestinal handling). Groups II, III, IV, and V comprised rats sacrificed 30 min, 60 min, 120 min, and 24 h after surgical stress (laparotomy with intestinal handling). These periods indicate the times of sacrifice following surgical stress. In the second part, the rats were randomly divided into six groups (n = 4): Group I comprised control rats (laparotomy alone without intestinal handling); Group II, surgical stress (laparotomy and intestinal handling); Group III, allopurinol (surgical stress after allopurinol pretreatment); Group IV, arginine (surgical stress after arginine pretreatment); and Group VI, indomethacin (surgical stress after indomethacin pretreatment). In these groups, animals were sacrificed 60 min following surgical stress since our earlier work indicated maximum alterations at this period. This study was cleared by the Animal Experimentation Ethics Committee of the institution.

Surgical manipulation of the small intestine. Surgical manipulation was carried out as described [14]. Briefly, overnight fasted rats were anesthetized by ketamine injection (50 mg/kg body wt). The abdominal wall was opened by a vertical incision of approximately 4 cm. The intestine was gently moved and the ileoceleal junction identified. The intestine was then handled along its entire length from the ileoceleal junction proximally, simulating the "inspection" that occurs in a clinical setting. The intestine was then replaced in the abdominal cavity. The entire process was completed within 1 to 2 min. Afterward, the abdominal wall was sutured and the animals were killed by decapitation 30 min, 60 min, 120 min, and 24 h after the surgical procedure.

Protection studies. To study the protection offered by various compounds, animals were pretreated with allopurinol, L-arginine, indomethacin, or quinacrine prior to surgical manipulation of the intestine. For inhibition of xanthine oxidase activity, rats were given an intraperitoneal injection of allopurinol (100 mg/kg body wt), 1 h before surgical manipulation [14]. To study the role of nitric oxide, animals were injected with L-arginine (300 mg/kg body wt) intraperitoneally, 30 min before surgery [15]. For inhibition of cyclooxygenase and phospholipase A$_2$ enzyme activities, rats were fed intragastrically with indomethacin (10 mg/kg body wt) and quinacrine (20 mg/kg body wt), respectively, 30 min before surgery.

Preparation of the lung homogenate. Following surgical manipulation of the intestine, heart and lungs were removed en bloc and 20 ml sterile saline solution was infused into the right ventricle to wash the residual blood. Both lungs were then separated from the heart and the hilar structures. The lung specimens were weighed and homogenized using phosphate-buffered saline (PBS), pH 7.4 [16].

Collection of bronchoalveolar lavage fluid (BALF). The trachea was cannulated and the lungs were washed three times with 2 ml of saline to provide approximately 6 ml of BALF. Protein concentration was measured in the cell-free BALF obtained after centrifugation at 900g for 10 min.

Histological studies. Lung tissue was fixed in 10% buffered formaldehyde and then processed. Four-micrometer sections were cut and stained with hematoxylin and eosin, and quantitative morphometry was performed. Briefly, lung specimens were examined under a 40× high-power objective for the presence of PMNs and these were counted in representative sections. Data are expressed as PMNs/high-power field (HPF) [7].

Myeloperoxidase (MPO) assay. Lung myeloperoxidase was measured to quantitate the degree of pulmonary neutrophil sequestration. Lung specimens were weighed and placed in potassium phosphate buffer, pH 7.4, with 0.5% hexadeoxytrimethylammonium bromide and homogenized. The homogenate was then centrifuged at 4000 rpm for 4 min and the supernatant was used for the MPO assay. The assay procedure consists of 50 mM phosphate buffer, pH 6, 0.167 mg of o-dianisidine, 0.1 μM hydrogen peroxide, and an aliquot of the enzyme. The rate of decomposition of H$_2$O$_2$ by MPO with o-dianisidine as H$_2$O$_2$ donor is determined by measuring the rate of color development at 460 nm [17].

Enzyme assays. Lung homogenate was also assayed for various antioxidant enzymes. Homogenate was centrifuged at 4000 rpm for 4 min and the supernatant was used for the following enzyme assays. Superoxide dismutase activity was measured at 540 nm, following the reduction of the tetrazolium dye MTT by superoxide generated by the xanthine-xanthine oxidase system [18]. Total glutathione peroxidase activity was determined by the oxidation of NADPH at 340 nm using hydrogen peroxide [19]. The activity of glutathione reductase was measured at 340 nm, following hydrogen transfer.
from NADPH to GSSG [20]. The activity of glutathione S-transferase was measured spectrophotometrically at 340 nm using the substrate 1-chloro-2,4-dinitrobenzene (CDNB) [21]. Catalase activity was estimated by measuring the change in absorption at 240 nm using H₂O₂ as substrate [22].

Peroxidation parameters. Total lung homogenate was used for assessment of lipid peroxidation. Malonaldehyde (MDA) was measured using the TBA method [23]. The amount of MDA formed was calculated from the standard curve prepared using 1,1',3,3'-tetramethoxypropane and values are expressed as nanomoles per milligram of protein. For conjugated diene measurement, total lipids were extracted as described [24], dissolved in 1 ml heptane, and read at 233 nm using a Shimadzu spectrophotometer. The amount of conjugated diene formed was calculated using a molar absorption coefficient of 2.52 × 10⁴ and expressed as micromoles per milligram of protein [25]. Total thiol content was measured using DTNB as described [26] and expressed as nanomoles per milligram of protein. Tocopherol content was measured using HPLC as described for liver microsomes [27] and quantitated using a Shimadzu 6A high-performance liquid chromatograph [28].

Protein estimation: Protein was estimated by Lowry’s method using bovine serum albumin as standard [29].

![FIG. 1. Morphological studies of lungs of rats subjected to surgical manipulation revealing normal appearance in control rats (A) and in treated rats 120 min (D) and 24 h (E) after laparotomy and intestinal handling. Heavy neutrophil infiltration and disruption of the normal alveoli were observed 30 and 60 min (B and C, respectively) after surgical manipulation.](image-url)
Statistical analysis. Data are expressed as means ± SD. The Mann-Whitney and Kruskall-Wallis nonparametric tests were used to test significance of differences between groups. A probability of less than 0.05 was accepted as significant.

RESULTS

Figure 1 shows the histological evidence of neutrophil sequestration at various periods after surgical manipulation of the intestine. On histological examination, sham-operated controls showed no lung injury, with the appearance of normal alveoli and very few neutrophils (Fig. 1A). Surgical manipulation resulted in the disruption of normal alveolar architecture and heavy neutrophil infiltration at 30 and 60 min (Figs. 1B and 1C, respectively); by 120 min the control pattern was achieved (Fig. 1D) and remained the same at 24 h (Fig. 1E). These histological findings were confirmed by measurement of myeloperoxidase (MPO) activity which is a reliable and quantitative marker for neutrophil accumulation in tissues. This enzyme activity, was significantly higher 30 and 60 min following surgical manipulation and returned to normal by 24 h (Fig. 2A). Figure 2B shows the neutrophil counts per high-power field and this further confirmed the sequestration of neutrophils in the lung 30 and 60 min following surgical manipulation. Lung permeability was determined by measuring the total protein concentration in cell-free BALF and this value was found to be significantly higher 30 and 60 min following surgery. By 24 h the protein concentration returned to the control level (Fig. 2C).

Oxidation of polyunsaturated fatty acids by oxygen free radicals has been postulated as a mechanism responsible for damage and/or disruption of biological membranes. Assessment of lipid peroxidation (MDA and conjugated diene) showed significantly more peroxidation products 30 and 60 min after surgical manipulation and these parameters returned to control levels.

**FIG. 2.** (A) Myeloperoxidase activity of lung in control rats and in rats subjected to surgical manipulation. Time points on the x axis represent minutes or hours after intestinal handling. (B) Quantitative morphometric evaluation for lungs from control rats and from treated rats at various periods after surgical manipulation. PMN/HPF, neutrophils per high-power field. (C) Total BALF protein concentration of the lungs obtained from control and animals at various time periods after surgical manipulation. Each value represents the mean ± SD of four separate experiments. *P < 0.05 when compared with sham control. **P < 0.05 when compared with 60 min after surgical manipulation.
by 24 h (Figs. 3A and 3B). Total thiol content in the lung homogenate also decreased by 60 min after surgical manipulation (Fig. 3C) and returned to the control level by 24 h.

A major defense mechanism against oxygen free radicals includes both enzymatic and nonenzymatic antioxidants. The activity of some of the antioxidant enzymes was assayed in the lung homogenate various periods after intestinal handling. Superoxide dismutase and catalase, the superoxide and hydrogen peroxide scavenging enzymes, decreased significantly 30 and 60 min following surgical manipulation and with time, returned to control levels (Figs. 4A and 4B). The nonenzymatic antioxidant \( \alpha \)-tocopherol was also significantly decreased 30 and 60 min following surgical manipulation, and returned to the control level by 120 min (Fig. 4C). Significant decreases in glutathione peroxidase and glutathione S-transferase enzyme activities were observed 30 and 60 min following intestinal handling and these decreases were maintained throughout the study period studied (data not shown).

Our earlier studies showed activation of xanthine oxidase (XO), phospholipid degradation, and arachidonic acid generation in the small intestine following surgical manipulation [12, 14]. We also observed protection against mitochondrial damage by nitric oxide donor \( L \)-arginine following surgical manipulation [15]. Hence studies were carried out using allopurinol, XO inhibitor, \( L \)-arginine, nitric oxide donor, quinacrine, a phospholipase \( A_2 \) (PLA2) inhibitor, and indomethacin, a cyclooxygenase inhibitor. Since maximum damage to the lung was observed 60 min following intestinal handling, further experiments were carried out at this period. On histological examination, sham-operated controls showed no lung injury with the appearance of normal alveoli (Fig. 5A). Surgical manipulation resulted in disruption of normal alveolar architecture and heavy neutrophil infiltration (Fig. 5B). Pretreatment with allopurinol, indomethacin, or quinacrine prior to surgery had a protective effect against lung injury following intestinal handling (Figs. 5C, 5E, and 5F) as seen by considerably reduced neutrophil infiltration, and this was not observed with \( L \)-arginine (Fig. 5D). The histological finding was further confirmed by

**FIG. 3.** Oxidative stress parameters (malonaldehyde, A; conjugated diene, B; total thiols, C) in lungs isolated from control and at various periods after surgical manipulation. Each value represents the mean ± SD of five separate estimations. *P < 0.05 when compared with control. #P < 0.05 when compared with 60 min after surgical manipulation.
measurement of MPO activity and neutrophil counts, which were low in lungs pretreated with allopurinol, indomethacin, or quinacrine (Figs. 6A and 6B). Neutrophil counts and MPO activity were not altered in L-arginine-pretreated rat lungs and were similar to levels 60 min following surgical manipulation of the small intestine. Allopurinol, indomethacin, and quinacrine also reduced the total BALF protein concentration (Fig. 6C).

Assessment of oxidative stress in the lungs of rats pretreated with various compounds showed that the lipid peroxide levels were almost similar to those of the sham-operated controls when rats were pretreated with allopurinol, indomethacin, or quinacrine (Figs. 7A, 7B, and 7C). Here again, L-arginine did not have a protective effect. Similarly, antioxidant enzyme activities were decreased 60 min following intestinal handling, and this was prevented by pretreatment with allopurinol, indomethacin, or quinacrine. On the other hand, L-arginine pretreatment did not protect the lungs following intestinal surgical manipulation (Fig. 8).

**DISCUSSION**

It has been suggested that the gastrointestinal tract plays an important role in distant organ injury in conditions such as surgical stress, hemorrhagic shock, burn trauma, and intestinal ischemia-reperfusion. The intestine is considered the “motor” in surgical stress-induced trauma and multiple organ failure syndrome (MOFS) [30], where bacterial translocation resulting from a breakdown of intestinal barrier function is said to be an etiological factor. One of the distant organs affected in these conditions is the lung, and it has been suggested that acute lung injury is a result of factors generated in the intestine and transported through the lymph into the systemic circulation [31]. This facilitates recruitment of PMNs, and their inter-

**FIG. 4.** Superoxide dismutase (A) and catalase (B) activity and α-tocopherol content (C) of lung homogenate obtained from control rats and from rats at various periods after surgical manipulation. Each value represents the mean ± SD of four separate estimations. *P < 0.05 when compared with sham control. #P < 0.05 when compared with 60 min after surgical manipulation.
action with vascular endothelial cells results in destruction of capillary vascular patency and increase in tissue permeability. Our earlier work showed that surgical manipulation of the intestine leads to activation of superoxide-generating enzyme, xanthine oxidase (XO), in the small intestine and this results in considerable alterations of intestinal structure and function [11, 14]. In the present study it was shown that surgical manipulation of the intestine also results in oxidative stress in the lung which is probably due to sequestration of activated neutrophils by the lung as shown by histology, quantitative morphometric analysis, and elevation of lung myeloperoxidase activity. The oxidative stress-related changes in the lung were at a maximum 30 and 60 min following surgical manipulation and a return to the control pattern was observed by 24 h.

Activated neutrophils generate oxygen free radicals
and are proposed to be a major cause of cell and tissue damage, including apoptosis, associated with many chronic inflammatory diseases [33–35]. It has also been shown that lung cells are susceptible to the injurious effect of oxidants and these cells can release inflammatory mediators and cytokines such as tumor-necrosis factor α (TNFα), IL-1, and IL-8 in response to oxidative/nitrosative stress. The release of cytokines induces neutrophil recruitment and activation of key transcriptional factors such as NF-κB and activator protein 1 (AP-1), thereby augmenting the inflammatory response and tissue damage [36, 37]. The increase in protein permeability across the endothelial and epithelial barriers of the lung is an early characteristic feature of lung injury and it is thought that PMNs can create injury to lung tissue, leading to flooding of alveoli by plasma liquid and proteins. An increased BALF protein concentration 60 min following surgery indicated increased permeability of the lungs. Oxygen free radicals are known to cause lipid peroxidation in the membranes and there is abundant evidence that lipid peroxides are potentially harmful to cells and tissue [38–40]. In the lungs increased levels of MDA and conjugated dienes and a decreased total thiol content were observed following intestinal handling and the changes were at a maximum 60 min following surgical manipulation. A similar increase in lipid peroxidation products in the lungs was observed following burn trauma and this was suggested to be due to generation of ROS by the infiltrating neutrophils [41].

Antioxidant status in the lung following surgical manipulation revealed decreased superoxide dismutase and catalase activities 60 min following intestinal manipulation and these activities returned to control lev-
els by 24 h, indicating the reversibility of damage with time. Studies have shown decreased activity of superoxide dismutase in the lungs following antigen-induced asthma [42] and a therapeutic role for superoxide dismutase and catalase has been suggested in preventing the oxidant-induced damage to the lung [43, 44].

α-Tocopherol is the first line of defense against peroxidation of polyunsaturated fatty acids present in cellular and subcellular membrane phospholipids. A decrease in tocopherol level was observed 30 and 60 min following surgical manipulation and this decrease correlates with the increased lipid peroxidation seen at these periods.

Earlier it was shown that laparotomy and intestinal handling result in structural, functional, and biochemical alterations in the enterocytes, which are due to the generation of superoxide by xanthine oxidase activation [14]. It was also shown that surgical manipulation results in structural and functional alterations in the brush border membranes (BBMs) through oxidative stress, which include phospholipid degradation by activation of phospholipase A2 and arachidonic acid generation. Allopurinol, an inhibitor of xanthine oxidase, prevented PLA2 activation and arachidonic acid generation [12]. It was also observed that the nitric oxide donor L-arginine could offer protection against the mitochondrial damage observed in enterocytes following surgical manipulation of the intestine [15], but L-arginine could not prevent PLA2 activation and arachidonic acid generation [12]. To understand the biochemical mechanisms leading to lung damage, studies were initiated to inhibit various events taking place in the intestine following surgical manipulation. Animals were pretreated with allopurinol, an inhibitor of XO which generates superoxide, L-arginine, a nitric oxide donor, quinacrine, a phospholipase A2 inhibitor, and indomethacin, a cyclooxygenase inhibitor, prior to surgical manipulation of the intestine. It was observed that allopurinol almost completely prevented this surgical manipulation-induced lung injury, suggesting that xanthine oxidase-generated superoxide in the intestine probably plays a role in lung damage. It is likely

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**FIG. 7.** Oxidative stress parameters (malonaldehyde, A; conjugated diene, B; total thiols, C) in lungs isolated from control animals and animals pretreated with allopurinol, L-arginine, quinacrine, or indomethacin before surgical manipulation. Each value represents the mean ± SD of five separate estimations. *P < 0.05 when compared with control. **P < 0.05 when compared with 60 min after surgical manipulation.
that the effect of allopurinol pretreatment is due to inhibition of intestinal XO since lung has negligible XO activity. Although we had earlier observed protection of enterocyte mitochondria by the NO donor L-arginine following surgical manipulation, this compound did not prevent lung injury. It was earlier observed that arachidonic acid (AA) is the main product of PLA2 activation in the intestine following surgical manipulation, and in the present study inhibition of phospholipase A2 by quinacrine offered protection against lung injury. Phospholipase A2 comprises a superfamily of enzymes that catalyze the hydrolysis of ester bonds at the sn-2 position of membrane phospholipids. The role of PLA2 is well documented in the rate-limiting step in the production of lipid inflammatory mediators, including eicosanoids (e.g., prostaglandins and leukotrienes), lysophospholipids, and platelet-activating factor [45]. It is likely that AA or its products have a role in inducing lung injury following surgical manipulation of the intestine. Experiments with the cyclooxygenase inhibitor indomethacin showed a protective role, thereby indicating that prostaglandins may act as a downstream mediator of lung injury following intestinal manipulation. Pretreatment with either quinacrine or indomethacin did not affect XO activation or superoxide generation in the intestine following surgical manipulation (data not shown).

In conclusion this study has shown histological and biochemical alterations in the lung following laparotomy and intestinal handling; these alterations are at a maximum 30 and 60 min and the tissue recovers by 24 h after surgical manipulation. These changes are due to oxidative stress resulting from infiltration into the lung of activated PMNs, which appear to be an important effector cell in surgical manipulation-induced lung injury. This study has also shown the biochemical events occurring in the intestine that are responsible for the lung damage. Superoxide generated by XO activation leads to PLA2 activation and generation of AA, which by itself or after conversion to prostaglandins probably brings about the lung damage. This is supported by inhibition of the lung damage by
pretreatment with allopurinol, quinacrine, or indomethacin. Further studies are needed to establish the chemical nature of the mediator generated in the intestine and its mode of action resulting in lung damage.

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