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# Oxidative stress in lung tissue induced by $CO_2$ pneumoperitoneum in the rat

**M. Pross,[1] H. U. Schulz,[1] A. Flechsig,[2] T. Manger,[1] W. Halangk,[1] W. Augustin,[2] H. Lippert,[1] T. Reinheckel[1]**

[1] Department of Surgery, Otto von Guericke University, Leipziger Strasse 44, 39120 Magdeburg, Germany
[2] Department of Pathological Biochemistry, Otto von Guericke University, Leipziger Strasse 44, 39120 Magdeburg, Germany

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**Abstract**
*Background:* Clinical trials have found that the pneumoperitoneum has potentially hazardous side effects. The biochemical basis of organ injury induced by pneumoperitoneum is, however, not well defined. Since oxidative stress is believed to play an important role in many pathological conditions, we set out to examine oxidative stress markers in the lung, liver, kidney, and pancreas by using a rat model of laparoscopy with $CO_2$ pneumoperitoneum and comparing it to a group with gasless laparoscopy.
*Methods:* Malondialdehyde (for lipid peroxidation), protein-bound carbonyls (for protein oxidation), reduced and oxidized glutathione, and the neutrophil marker myeloperoxidase were evaluated in tissue homogenates at 2 h, 6 h, and 18 h after laparoscopy. Immunoblotting was used to analyze the modification of lung proteins by 4-hydroxynonenal at 6 h.
*Results:* Significant lipid peroxidation was found selectively in lungs at 2 h and 6 h after $CO_2$ pneumoperitoneum. This was accompanied by a loss of glutathione but only minor protein oxidation. Further, lung proteins were clearly modified by the aldehydic product of lipid peroxidation 4-hydroxynonenal. Myeloperoxidase in lungs increased continuously up to 18 h in both experimental groups, but there were higher levels in the group with pneumoperitoneum.
*Conclusion:* Oxidative stress is likely to contribute to the impairment of pulmonary function after laparoscopic operations using a $CO_2$ pneumoperitoneum.

**Key words:** Pneumoperitoneum — Carbon dioxide — Laparoscopic surgery — Lung — Oxidative stress — Lipid peroxidation

Laparoscopic techniques are commonly applied in everyday surgical practice. In contrast to the patients treated with minimally invasive surgery in its early years, the patients who now undergo laparoscopy frequently exhibit comorbid diseases, are elderly, or are even pregnant. In light of this trend, the risks of minimally invasive procedures need to be explored carefully.

From a physiological point of view, the insufflation of a gas—typically carbon dioxide ($CO_2$)—into the peritoneal cavity appears to be the most important new aspect of laparoscopy as compared to conventional surgery. Over the past several years, an increasing number of publications have reported that the $CO_2$ pneumoperitoneum has potentially hazardous effects on acid-base status, pulmonary function, cardiovascular hemodynamics, and hepatic perfusion [6, 14, 16]. Therefore, procedures using gasless abdominal wall lifting instead of pneumoperitoneum have been considered as alternative approaches [3, 10]. Because most of these studies were conducted in patients undergoing laparoscopy, the pathophysiological and biochemical mechanisms of organ impairment could not be determined in these investigations.

Oxidative stress induced by reactive oxygen species (ROS) is believed to mediate tissue injury in a large number of pathological conditions [4]. ROS are able to oxidize all macromolecular constituents of cells, such as (phospho-)lipids of membranes, proteins, and DNA. The most likely causes of ROS production as a consequence of $CO_2$ pneumoperitoneum are an inflammatory reaction due to tissue trauma, ischemia/reperfusion due to changes of abdominal pressure, and diaphragmatic dysfunction. Hence, oxidative stress may represent an important link between laparoscopy and clinically important side effects of this procedure.

The purpose of the present study was to evaluate markers of oxidative stress in the lung, kidney, liver, and pancreas of animals who underwent $CO_2$ pneumoperitoneum and compare the results to a group that underwent gasless laparoscopy with mechanical lifting of the abdominal wall. For practical reasons, the use of a laparoscopic rat model seemed especially suitable to address these problems [17].

*Correspondence to:* T. Reinheckel, Institut für Molekulare Medizin und Zellforschung, Tumorbiologie / Labor Nothnagel, BreisacherStrasse 117, D-79106 Freiburg, Germany

## Materials and methods

### *Animals*

Male Wistar rats (Harlan, Borchen, Germany) weighing 290–330 g were used in the experiments. The experimental protocol was approved by the committee responsible for animal use and care (Regierungspräsidium Sachsen-Anhalt, Dessau).

### *Experimental procedures*

The rats were anesthetized by intramuscular injection of 12 mg/kg xylazine (Bayer, Leverkusen, Germany) and intraperitoneal injection of 80 mg/kg ketamine hydrochloride (Parke Davis, Berlin, Germany). Laparoscopy was performed essentially as described by Sandoval et al. [17]. In brief, a trocar was introduced through a 5-mm midline incision between the xiphoid and pubis. To increase the volume of the intraperitoneal cavity, two alternative procedures were performed. In the first group, a pneumoperitoneum was produced by insufflation of $CO_2$ at a flow rate of 0.4 L/min to reach a maximal intraperitoneal pressure of 6 mmHg. For gasless laparoscopy, the abdominal wall was lifted mechanically by sutures in the four abdominal quadrants. Subsequently, in all animals a laparoscopic camera was moved forward through the trocar, and the peritoneal cavity was explored without further manipulation for 20 min. After removal of all devices, the incision was sutured.

For time-dependent investigation of organ injury, the rats were killed at 2 h, 6 h, and 18 h after the start of laparoscopy. For determination of oxidative stress markers, tissue of the lung, liver, kidney, and pancreas was kept on ice, and 500-mg samples were homogenized in 2 ml 50 mM Tris pH 7.6, 2 mM EDTA, 5 mM butylated hydroxytoluene (BHT), and 2 mM PMSF using a Potter homogenizer. The supernatant and the pellet of a 800 g centrifugation was stored in aliquots at -80°C and used for further analyses.

### *Biochemical methods*

Malondialdehyde (MDA), a marker of lipid peroxidation, was measured by derivation with thiobarbituric acid and photometric detection at 532 nm after C18-reversed phase HPLC, as described earlier [13].

The measurement of oxidative protein modification was based on the derivation of protein-bound carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) and the subsequent detection of the DNP moiety by a specific antibody (Sigma, Deisenhofen, Germany). This principle was employed in an ELISA [2] and for immunoblotting [15].

Protein modification by 4-hydroxynonenal was demonstrated by immunoblotting, as described earlier [15, 19].

Glutathione (GSH) determination was based on the reaction with 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) [1].

The assessment of myeloperoxidase (MPO) was done according to the method of Molero et al. [12]. The assay is based on the reduction of O-dianisidine in the presence of MPO and $H_2O_2$. The results were expressed in units of MPO on the basis of 1 U to oxidize 1 μmol $H_2O_2$ per min at 30°C. Protein determinations were performed using a commercially available assay (BioRad, Munich, Germany), with bovine serum albumin as standard.

### *Statistics*

Values were expressed as mean and standard deviation (SD). The group size for each time point was between three and six animals. Differences between two means were tested for statistical significance by *t*-test or, if a parameter was only investigated for three animals at a defined time point, by the nonparametric Wilcoxon rank sum test. For comparison of multiple groups, one-way analysis of variance (ANOVA) was performed. Differences found by ANOVA were confirmed by the *t*-test. In all instances, *p* values <0.05 were considered to be significant.

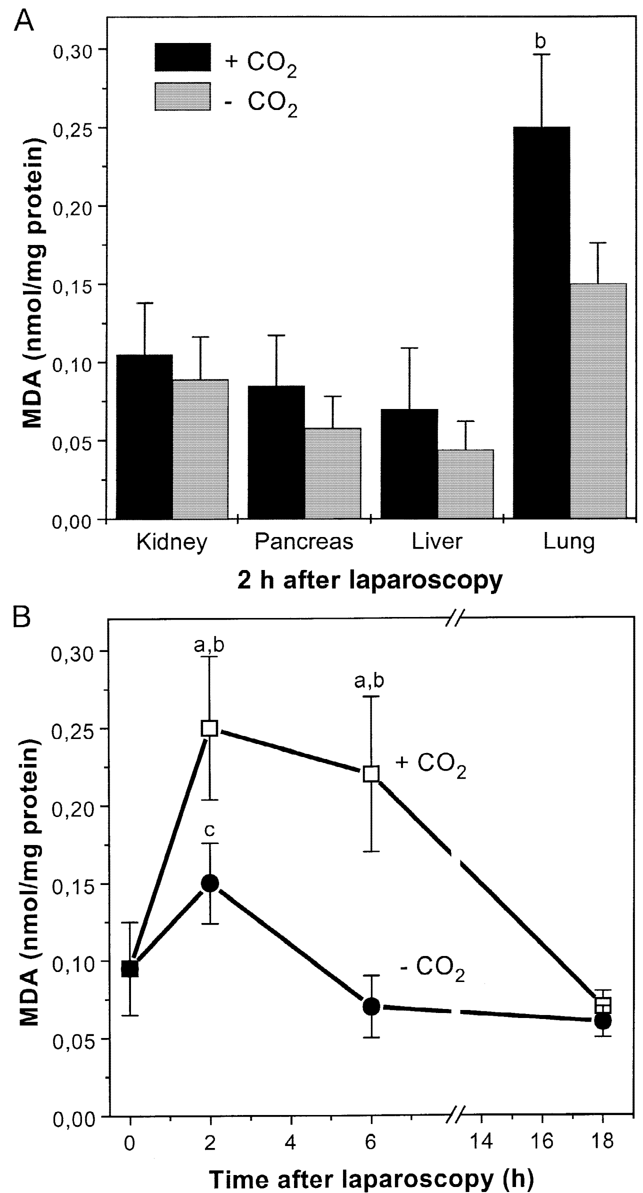


**Fig. 1.** Formation of malondialdehyde (MDA) after laparoscopy. $+CO_2$ or □ = $CO_2$ pneumoperitoneum;$-CO_2$ or ● gasless laparoscopy. **A** Malondialdehyde in kidney, pancreas, liver, and lung 2 h after laparoscopy. MDA content (nmol MDA/mg protein) at the time of laparoscopy (mean ± SD/ $n = 6$): kidney 0.108 ± 0.054, liver 0.053 ± 0.022, pancreas 0.104 ± 0.050, lung 0.095 ± 0.030. **B** Time course of MDA formation in lung tissue after laparoscopy. a) $p < 0.01$ compared to the 0 h and the 18 h time points. b) $p < 0.01$ between the experimental groups at corresponding times. c) $p < 0.05$ compared to all other time points.

## Results

Membrane lipids, mainly the polyunsaturated fatty acids, are highly susceptible to peroxidation by ROS. Lipid peroxidation results in the occurrence of relatively stable aldehydes, most notably malondialdehyde (MDA) [5]. Increased MDA was observed in the lung homogenates of rats with pneumoperitoneum, as compared to the group with gasless laparoscopy (Fig. 1). Homogenates of kidney, liver, and pancreas did not exhibit statistically significant differences 2 h after laparoscopy (Fig. 1A). The rise in lung MDA was

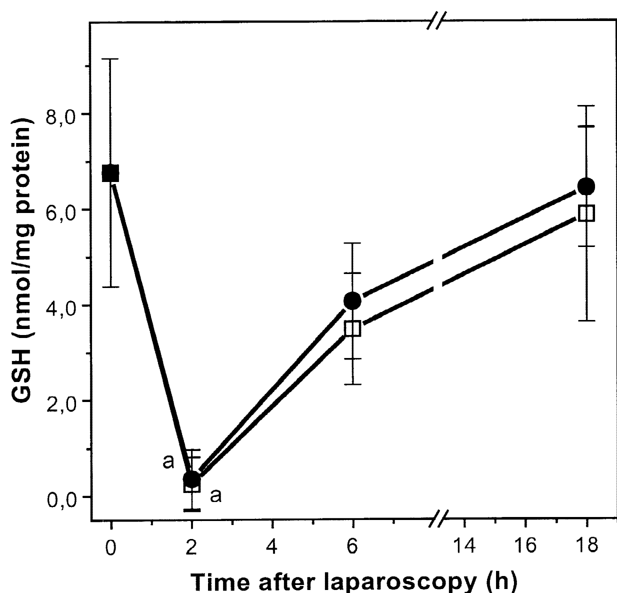


**Fig. 2.** Reduced glutathione (GSH) in lung tissue after laparoscopy. Note that oxidized glutathione (GSSG) was not increased after laparoscopy. Rather, the GSSG concentration was below the detectable level at 2 h and 6 h. □, $CO_2$ pneumoperitoneum; ●, gasless laparoscopy. a) $p < 0.01$ compared to all other time points.

transient. Control levels were detected 18 h after the procedure (Fig. 1B).

The oxidative damage of macromolecules is triggered by an impaired antioxidative defense. This is shown for lung homogenates by the decrease of reduced glutathione (GSH), a highly important water soluble antioxidant [11], at 2 h after laparoscopy, with subsequent recovery of this antioxidative compound during the next 16 h (Fig. 2). Interestingly, both experimental groups exhibited identical kinetics of GSH content. An increase in oxidized glutathione (GSSG) was not observed. In fact, GSSG was not detectable at 2 h and 6 h after laparoscopy.

The assessment of protein-bound carbonyls by derivation with 2,4-dinitrophenylhydrazine (DNPH) is a widely used marker for oxidative protein modification [2, 15]. A small but significant increase in protein-bound carbonyls was detected only in the lungs from animals at 6 h after laparoscopy (Fig. 3). Interestingly, a decline in protein-bound carbonyls was observed in some animals after gasless laparoscopy; however, this decline did not reach statistical significance.

For closer investigation of pneumoperitoneum-induced protein modifications in lungs at 6 h, proteins from homogenates were subjected to gel electrophoresis and subsequent immunoblotting (Fig. 4). In this approach, the pattern of the total protein stain was identical. Only a few, not very distinct increases of bands were observed in the pneumoperitoneum group when the blots were tested for protein-bound carbonyls with an anti-DNP immunoglobulin. 4-hydroxynonenal (4-HNE) is an aldehydic product of lipid peroxidation that is well known for its ability to bind to proteins and affect them in their function [5, 19]. When the Western blot was tested with an antibody specific for 4-HNE–modified histidine residues in proteins, three additional protein bands appeared to be modified with 4-HNE in

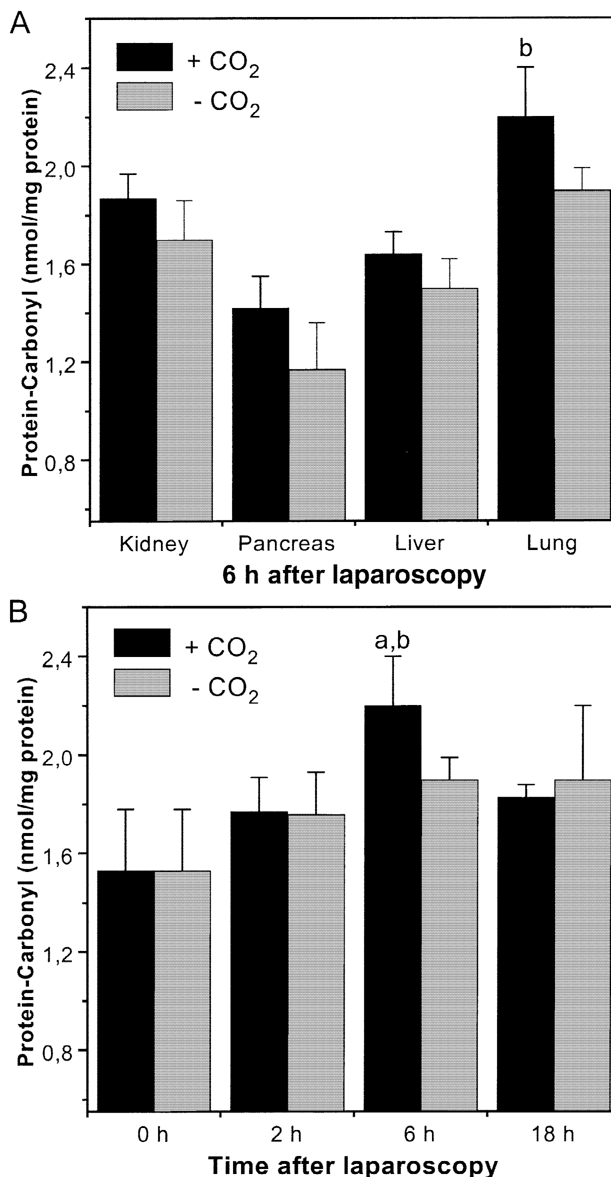


**Fig. 3.** Oxidative modification of proteins after laparoscopy. $+CO_2 = CO_2$ pneumoperitoneum;$-CO_2$ = gasless laparoscopy. **A** Oxidatively modified proteins in kidney, pancreas, liver, and lung 6 h after laparoscopy. Protein-bound carbonyls (nmol/mg protein) at the time of laparoscopy (mean ± SD/*n* = 6): kidney 2.04 ± 0.20, liver 1.63 ± 0.27, pancreas 1.60 ± 0.34, lung 1.53 ± 0.25. **B** Time course of oxidative protein modification in lung tissue after laparoscopy. a) $p < 0.01$ compared to all other time points. b) $p < 0.05$ between the experimental groups at corresponding times.

the pneumoperitoneum group. Thus, substantial lipid peroxidation with secondary modification of proteins occurs in lungs 2–6 h after $CO_2$ pneumoperitoneum.

What is the cause of oxidative stress in this model? Stimulated neutrophils are known to produce an array of ROS. Therefore, the activity of the neutrophil marker enzyme myeloperoxidase (MPO) was determined in lung homogenates (Fig. 5). MPO was increased significantly at 6 h and 18 h after laparoscopy, with the highest level at 18 h. Furthermore, animals with pneumoperitoneum exhibited higher MPO activities than the group with gasless laparoscopy at 18 h. Thus, lipid peroxidation and neutrophil invasion in lungs exhibit different kinetics. This means that lipid

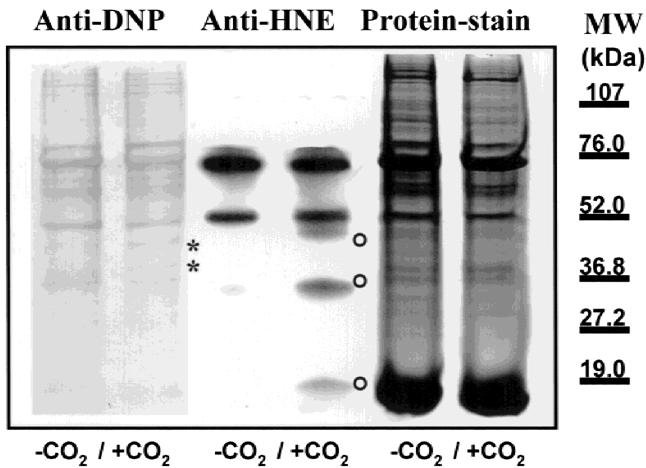


**Fig. 4.** Immunoblots for the detection of oxidatively modified proteins from lung tissue. Anti-DNP = detection of DNPH-reactive protein carbonyls with anti-DNP IgG (*selectively oxidized proteins). Anti-HNE = chemiluminescence detection of proteins with 4-HNE-modified histidine residues in the samples from the anti-DNP lanes (°selectively modified proteins). Protein stain = Coomassie stain of proteins to compare protein patterns and for "loading control" of the samples from the anti-DNP and anti-HNE lanes. $+CO_2$ = $CO_2$ pneumoperitoneum;– $CO_2$ = gasless laparoscopy.

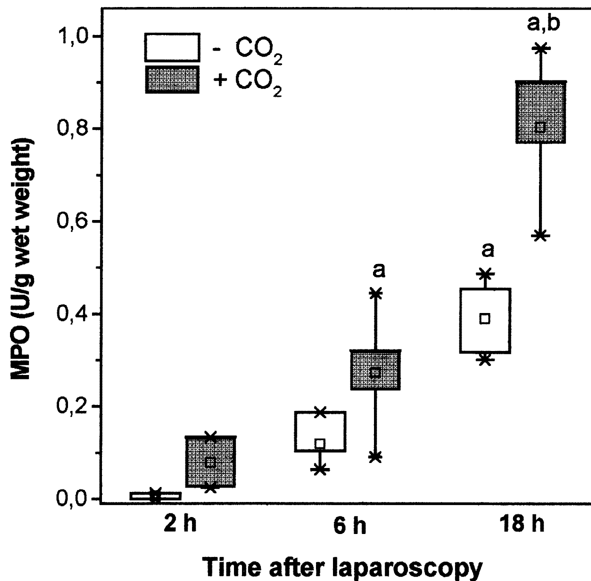


**Fig. 5.** Myeloperoxidase (MPO) in lung tissue after laparoscopy. Untreated controls exhibited MPO activities of 0.036 ± 0.041 U/g wet weight ($n$ = 3). $+CO_2$ = $CO_2$ pneumoperitoneum;$-CO_2$ = gasless laparoscopy. a) $p < 0.05$ compared to the untreated control (Wilcoxon rank sum test). b) $p < 0.05$ between the experimental groups at corresponding times (Wilcoxon rank sum test). The boxes denote values between the 25th and 75th percentile, and the square symbols represent the means of data. The error bars denote the 5th and 95th percentile values. The symbols below the 5th percentile denote the lowest value determined; the symbols above the 95th percentile denote the highest value determined.

peroxidation is unlikely to be caused by neutrophil-derived ROS.

## Discussion

Laparoscopic operations such as laparoscopic cholecystectomy are associated with a reversible impairment of pulmonary function [6, 9]. Hypothetically, this decline in lung function could be caused by three major factors—general anesthesia, the laparoscopic procedure itself, and tissue trauma due to the operation (such as the removal of the gallbladder). The present experiments were designed to investigate the influence of the laparoscopic intervention independent of the other factors and to elucidate some of the pathophysiological aspects of laparoscopy-induced organ injury. Consequently, major tissue trauma due to an operation was omitted in our rat model. Further, the inhalative anesthesia employed in patients was replaced by an intravenous protocol in the animals. Hence, we were able to study the effects of the laparoscopic procedure with $CO_2$ insufflation alone, as compared to the gasless procedure. However, in this experimental setting, we cannot differentiate the metabolic effects of $CO_2$ from the effect of increased intraabdominal pressure on the physiological or biochemical parameter of interest.

The present investigation of oxidative stress-related parameters in the liver, kidney, pancreas, and lung revealed that only the lung tissue was affected by the laparoscopic procedure. GSH is considered the most important antioxidant in lungs; it is also present in the epithelial lining fluid in concentrations ~100 times higher than that found in extracellular fluid of other tissues. We observed an almost complete disappearance of lung GSH in both experimental groups at 2 h after the procedure. Since GSSG, which represents the oxidized form of GSH, did not increase accordingly in the tissue, a loss of this tripeptide needs to be assumed [7]. Thus, a process not related to oxidative stress may cause the GSH loss early after laparoscopy. However, this does not exclude the possibility that GSSG is formed before it is lost.

The alteration of antioxidant systems, such as the GSH/GSSG system, is believed to render cells or tissues more susceptible to ROS attack [18]. Interestingly, lipid peroxidation in lung tissue occurs only after $CO_2$ pneumoperitoneum and appears to be a significant component of lung injury after $CO_2$ pneumoperitoneum, as demonstrated by the significantly higher invasion of neutrophils at 18 h as compared to the group with gasless laparoscopy.

At first sight, lipid peroxidation results in the damage of biological membranes and therefore cell damage and tissue injury. In addition, an array of secondary products is formed during membrane peroxidation. Among this products are reactive, relatively stable aldehydes, which have their own biological effects [5]. For example, the aldehyde 4-HNE is cytotoxic at high concentrations, exhibits genotoxic effects, and disturbs cell proliferation at low concentrations. 4-HNE is able to react with sulfhydryl and amino groups of proteins. This covalent modification affects the function of proteins and is a possible mechanism of the biological effects of 4-HNE.

In the present study, the immunoblot using antibodies directed against HNE-modified histidine residues of proteins clearly showed distinct 4-HNE–modified lung proteins 6 h after induction of $CO_2$ pneumoperitoneum.

The reaction with aldehydes is not the only mechanism of oxidative protein modification. Proteins are also known to be damaged by ROS directly or in the course of glycoxidation. All of these reactions can result in the occurrence of carbonyl groups in the protein molecule. Therefore, the as-

sessment of protein-bound carbonyls by derivation with DNPH is a widely used marker for oxidative protein modification [2, 15]. In the present experiments, comparatively minor increases of DNPH-reactive protein carbonyls were observed. Thus, oxidative protein damage to the lungs of the animals who underwent $CO_2$ pneumoperitoneum is probably largely due to protein modification by aldehydes.

It might be hypothesized that neutrophils are the sources of the ROS responsible for induction of the lipid peroxidation process in the lung. However, the kinetics of neutrophil invasion in this tissue do not support this assumption. Clearly, lipid peroxidation is highest at times when only minimal invasion of myeloperoxidase-containing granulocytes is observed. In contrast, the maximal occurrence of neutrophils in the lungs is accompanied by already normalized levels of MDA as well as GSH. Thus, we conclude that periods of ischemia/reperfusion due to pneumoperitoneum-caused changes of abdominal and thoracic pressure are the most likely source of ROS in this model.

In conclusion, oxidative stress was identified as a component of $CO_2$ pneumoperitoneum–induced lung injury using a small-animal model of laparoscopy. As judged by the parameters investigated by us, oxidative stress was absent in the kidneys, liver, and pancreas as well as the lungs of animals in which gasless laparoscopy was used instead of a pneumoperitoneum. It should, however, be emphasized that this "advantage" of gasless laparoscopy is only temporary and that it is a purely biochemical advantage. Rather, the complex antioxidant systems seem to be able to terminate the pro-oxidative processes in the lungs of animals who undergo pneumoperitoneum. However, in the light of the continually increasing number of indications for laparoscopy, we conclude that the nutritional status of patients should be considered carefully, with special attention to the intake of vitamins and other antioxidants.

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