Research Paper: Effects of Different Pressures of CO₂ on P33 Tumor Inhibitor Gene in Liver and Spleen Tissues During CO₂ Pneumoperitoneum in Adult Rats

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ABSTRACT

Introduction: We aimed to study the effects of different CO₂ pressures on expression of P33 gene and apoptosis in liver and spleen cells during CO₂ pneumoperitoneum.

Methods: This study was performed on 30 male Sprague-Dawley rats, weighing between 280 and 340 g (procured from Tehran Pasteur Institute’s animal house). They were randomly divided into 3 equal groups. Groups 1 and 2 received 10 and 20 mm Hg CO₂ pressures during pneumoperitoneum, respectively, and group 3 was the control group. CO₂ gas was insufflated through a cannula into abdominal cavity of rats in groups 1 and 2 for one hour; then perfusion was performed for half an hour. In group 3, cannula was put into the rats’ abdominal cavities without releasing any gas. Then the rats were killed, and their livers and spleens were removed after laparotomy to study expression of gene P33 and apoptosis using RT-PCR and TUNEL techniques.

Results: The TUNEL technique revealed a significant rise in apoptosis in liver cells of rats that received 20 mm Hg pressure of gas compared to rats that received 10 mm Hg pressure of gas and the control group (P<0.001). Similarly, the increase in apoptosis in spleen cells was also significant in rats that received 20 mmHg gas pressure compared to rats in 10 mmHg gas pressure and control groups (P<0.006). Furthermore, RT-PCR revealed a significant decrease in P33 gene mRNA in liver and spleen cells in 20 mmHg group compared to other two groups (P<0.001).

Conclusion: Pressure level and duration of CO₂ gas administration affect viability of liver and spleen cells. Too high a pressure or too long a duration may release cytokines and free radicals from cells of these organs, which can lead to transient or serious dysfunction.

Key Words:
CO₂ pneumoperitoneum, Apoptosis, P33, Liver, Spleen
1. Introduction

Laparoscopic surgery was first described in 1987 by Mouret in Paris [1], and gradually became the gold standard for diagnosis and effective treatment of various diseases in children and adults [2]. Benefits of less invasive surgery in appropriate procedures are well-accepted today. Less postoperative pain, rapid return to normal activities, reduced postoperative ileus, decreased immune response, and considerable economic savings are among benefits of laparoscopic surgery [3]. In this method, gases like helium, normal air, or CO₂ are insufflated into abdominal cavity for better surgical access and view. Because of its availability, chemical stability and non-explosive/non-inflammable properties, CO₂ is the most common gas used in this method. Moreover, this gas is easily eliminated from blood through increased ventilation [4].

Yet, in recent years, many studies have shown that laparoscopic surgery may have transient or permanent side effects on patient’s internal or external abdominal organs, depending on temperature and pressure of gas used during surgery [4-7]. In most cases, complications occur due to production of free radicals, stimulated immune response, and production of cytokines and ischemia [8, 9]. Considering that most surgeries are performed at pressures above port pressure (10-15 mmHg), portal blood flow is temporarily reduced. However, if CO₂ pressure is far greater than port system pressure, or if the surgery takes too long, ischemia may become permanent and disrupt function of intra-abdominal organs [9].

If a cell is exposed to trauma such as irradiation, heat, or chemical compounds, it quickly produces a variety of tumor inhibitor genes to maintain genomic stability [10]. Tumor inhibitor genes are vitally important because they control cell cycle regulation, apoptosis, senescence, and repair of the injured DNA due to UV radiation [11]. Inhibitor Gene Growth (ING) family belongs to tumor inhibitor genes with 5 members [12]. ING1 is located on the long arm of chromosome (ING) family belongs to tumor inhibitor genes with 5 members [12]. ING1 is located on the long arm of chromosome 13 [13, 14], and encodes a protein family that includes P24 (ING1c), P33 (ING1b), P47 (ING1a), and P27 (ING1d) [12], with P33 having the highest expression [15-17]. P33 protein has a role in control and regulation of cell growth, apoptosis, maintaining genome stability, regulation of cell cycle, and especially in cell aging process [18].

Overexpression of protein P33 inhibits cell growth through cell cycle arrest in G1 phase [19], and induces apoptosis [20, 21]. Reduction or inhibition of protein P33 expression leads to loss of cellular growth control and immortality. On the other hand, complete absence of P33 function reduces sensitivity of cell to apoptosis, which can facilitate cancer formation process [19].

The majority of studies on laparoscopic surgery mainly emphasize organs and their physiopathology, while, only very few studies have been conducted on expression of different genes of intra-abdominal organs. Considering the importance of protein P33 in regulating apoptosis, the effect of CO₂ with pressures of 10 and 20 mmHg on liver and spleen cells in laparoscopic surgery were studied to investigate apoptosis using TUNEL technique, and expression of P33 gene using RT-PCR technique.

2. Materials and Methods

This was an experimental study, conducted on a sample of Sprague-Dawley male rats. A total of 30 rats, weighing between 280 and 340 g and aged approximately 8 to 10 weeks were randomly divided into 3 equal groups. Group 1 (n=10): CO₂ was blown into abdominal cavity at 10 mm Hg pressure; Group 2 (n=10): CO₂ was blown into abdominal cavity at 20 mm Hg pressure; and Group 3 (control group) (n=10): Cannaula was inserted into the abdominal cavity, but no gas was blown in. During the study period, the animals had free access to food and water and the animal house was maintained on an inverted 12:12 h light-dark cycle at the University of Medical Sciences. All experiments were conducted according to the Guide for the Care and Use of Laboratory Animals.

Rats were anesthetized with 80 mg/kg of ketamine and 10 mg/kg of xylazine. Then, an IV tube set was used to simultaneously blow CO₂ into the abdominal cavity. Abdominal wall was incised and IV tubes were inserted into the cavity, with one end inside the abdominal cavity and the other side attached to CO₂ gas inhaler. Before commencement, each tube connection was examined and CO₂ was blown into the abdomen in accordance as planned. Sixty minutes later, the procedure was stopped, and vessels were perfused. For reperfusion, rats were killed after waiting for 30 minutes. Laparotomy was performed immediately after rats’ death; their livers and spleens were removed, and parts of liver and spleen tissues were placed in formalin 10% for fixing. The other parts were transferred to nitrogen tank, and stored at -70°C for RNA extraction and Reverse Transcriptase Polymerase chain Reaction (RT-PCR) test.

Reverse transcriptase polymerase chain reaction

Total RNA from rats’ livers and spleens was extracted using RNA extraction kit (Roche, Germany) according to manufacturer’s instructions. cDNA was prepared from total RNA, using cDNA synthesis kit (Fermentas, Lithuania) according to manufacturer’s instructions, and stored at -70°C for later use.
To determine mRNA expression of p33 gene, semi-quantitative RT-PCR reaction was performed. RT-PCR solution (final volume of 20 µL) contained 2 µL of cDNA, 10 µL of main PCR buffer mixture 2 ×“Qiagen Multiplex” (Qiagen, Germany). About 10 pmol of each P33 specific primer and also 10 pmol of GAPDH gen primer, as internal control were prepared. Then, 5 mL of the final product obtained from proliferation of each sample was electrophoresed on a 3% agarose gel containing ethidium bromide. Finally, bands were observed and photographed using a Bio-Imaging System.

**TUNEL staining**

TUNEL kit was procured from Roche Company, and samples were stained according to relevant protocol as follows: drying sections in an oven (at 60°C for 1-2 hours), dewaxing/deparaffinizing sections in two stages using xylene (5 minutes each stage), hydration with descending concentrations of ethanol, rinsing with Phosphate-Buffered Saline (PBS) (in three stages of 2 minutes each), blocking peroxidase in tissue with 3% H₂O₂ in methanol (for 10 minutes), rinsing with PBS, incubation of samples with proteinase K (at room temperature for 15 to 30 minutes), 3 times rinsing with PBS, incubation with TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and labeled nucleotides in a humid and dark environment at 37°C (for 1 hour), rinsing 3 times with PBS, incubation with Converter-POD (containing conjugated anti-fluorescein antibody with Horse Radish Peroxidase (HRP) in a humid environment at 37°C for 30 minutes), rinsing with PBS 3 times, incubation with diaminobenzidine (DAB) substrate at room temperature for 10 minutes, rinsing with PBS 3 times, incubation with hematoxylin at room temperature for 1 minute, rinsing with distilled water for 5 minutes, placing slides in PBS until they turn blue (nearly 30 seconds), rinsing with distilled water, dehydration with ascending concentrations of ethanol, clarification with xylene in two stages and mounting with a special adhesive.

**Statistical analysis**

Data were analyzed by performing ANOVA and post hoc tests using SPSS15.

### 3. Results

The effects of CO₂ pressures of 10 and 20 mmHg on liver and spleen cells in laparoscopic surgery were studied to investigate apoptosis using TUNEL technique, and

**Figure 1.** Semi-quantitative RT-PCR analysis of expressions of P33 gene in liver tissue of 20 mmHg CO₂ pressure group, control group, and 10 mmHg CO₂ pressure group of rats. Amplification of the P33 gene (263 bp) compared with GAPDH gene (461 bp). The RT-PCR revealed a decrease in P33 gene mRNA in rats’ liver cells in 20 mmHg group compared to the 10 mmHg pressure and control groups.

**Liver cells RT-PCR results**

Semi-quantitative RT-PCR revealed a decrease in P33 gene mRNA in rats liver cells in 20 mmHg group compared to 10 mmHg pressure and control groups (Figures 1 and 2).

**Spleen cells RT-PCR results**

Semi-quantitative RT-PCR revealed a decrease in P33 gene mRNA in rats’ spleen cells in 20 mmHg group compared to 10 mmHg pressure and control groups (Figures 3 and 4).
According to data obtained from cell apoptosis in TUNEL staining technique, in rats' livers, brown apoptotic cells were distinguished from non-apoptotic cells that had turned blue in hematoxylin staining (Figure 5). Additionally, a significant increase in rats' liver cell apoptosis rate in 20 mmHg CO2 gas pressure group was observed compared to this rate in 10 mmHg and control groups (P<0.0001) (Figure 6).

**TUNEL assay of liver**

According to data obtained from cell apoptosis in TUNEL staining technique, in rats' livers, brown apoptotic cells were distinguished from non-apoptotic cells that had turned blue in hematoxylin staining (Figure 5). Additionally, a significant increase in rats' liver cell apoptosis rate in 20 mmHg CO2 gas pressure group was observed compared to this rate in 10 mmHg and control groups (P<0.0001) (Figure 6).

**TUNEL assay of spleen**

According to data obtained from cell apoptosis in TUNEL staining technique, in rats' spleens, brown apoptotic cells were distinguished from non-apoptotic cells.
ic cells that had turned blue in hematoxylin staining (Figure 7). Additionally, a significant increase in rats’ spleen cell apoptosis rate in 10 mmHg CO$_2$ pressure group was observed compared to the rate in the control group (P<0.006) (Figure 8).

4. Discussion

Study results indicate an increase in apoptotic cells proportional to an increase in CO$_2$ pressure in both liver and spleen. Furthermore, expression of P33 mRNA decreased at high CO$_2$ pressures. Various studies have shown adverse effects of CO$_2$ pressures in intra- and extra-abdominal organs [4, 8, 9, 22]. Although these effects are generally transient, some studies suggest that long duration or high pressures lead to serious impairments in organs [8, 22]. For instance, Diebel et al. reported that increased intra-abdominal pressure up to 20-25 mmHg would result in 63% reduction in mucosal blood flow in rats with normal mean blood pressure [23].

In a similar study conducted by Arikan et al. in 2007, the expression of P53 was assessed at different CO$_2$ pressures and the results showed an increase in both liver and spleen apoptotic cells, but no P53 expression in either organ was observed [1]. Moreover, Imamoglu et al. found that increased CO$_2$ pressure from 10 mmHg to 20 mmHg significantly affected testicular blood flow, oxidative stress markers, and morphology, and pneumoperitoneum caused reduction in blood flow rate [24].

In a study by Schachtrupp et al. on samples with 15 mmHg pressure, pneumoperitoneum caused 59% reduc-
tion in urine output and more than 30 mbar increase in respiratory pressure, however, coronary output did not change. Also, ami-notransferase level and serum blood alkaline phosphatase significantly increased, but creatine lactate, and lipase showed no change. In terms of tissue, liver tissue and renal proximal tubes contracted low grade necrosis and intestinal tissue was also damaged. This study showed that long term (24 hours) exposure to CO2 affects changes in body organs and their functions [22].

In 2005, Unsal et al. studied the effects of CO2 on terminal ileum tissue in 24 rats, with varying degrees of pressure from 10 to 20 mmHg. The results showed a significant increase in mucosal and sub-mucosal damage at both pressures, and ileal smooth muscle was also damaged in 20 mmHg group. In both groups, contraction due to electrical stimulation was inhibited, and acetylcholine-induced contraction in 20 mmHg group was inhibited, compared to control group. Generally, intra-abdominal pressure due to pneumoperitoneum causes inhibition of contractile response, which leads to structural changes associated with ischemic damages in terminal ileum [9].

In a study conducted by Ozmen et al. On 40 rabbits, effects of different CO2 gas pressures were examined on inflammatory cytokine levels and free radicals responses during CO2 pneumoperitoneum. It was found that CO2 gas with pressures of 10, 15, and 20 mmHg cause ischemia, liberation of free radicals and cytokines-mediated cell damage [25].

Other studies have shown that CO2 pneumoperitoneum causes increased level of prostate-specific antigen and renal damage [26, 27]. However, some studies have shown that with well-controlled gas pressure, adverse effects on hemody-namic parameters can be avoided [28, 29].

The present study showed that CO2 pressure level and its duration of use can affect liver and spleen cell viability. High pressure or prolonged use may release cytokines and free radicals from these organs, and cause transient or serious functional disorders. Moreover, a significant reduction in P33 gene is indicative of genomic damage in this process as well, which requires further investigation of results with clinical complications. In addition, study on human samples is also recommended.

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Conflict of Interest

All authors certify that this manuscript has neither been published in whole nor in part nor being considered for publication elsewhere. The authors have no conflicts of interest to declare.

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