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Influence of CO_2 pneumoperitoneum on intracellular pH and signal transduction in cancer cells*

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Abstract: Object: The authors studied the influence of CO_2 pneumoperitoneum on intracellular pH and signal transduction arising from cancer cell multiplication in laparoscopic tumor operation. Method: They set up a simulation of pneumoperitoneum under different CO_2 pressure, and then measured the variation of intracellular pH (pHi) at different time and the activity of protein kinase C (PKC) and protein phosphatase 2a (PP2a) at the end of the pneumoperitoneum. After 1 week, the concentration of cancer cells in the culture medium was calculated. Result: When the pressure of CO_2 pneumoperitoneum was 0, 10, 20, 30 mmHg respectively, the average pHi was 7.273, 7.075, 6.783, 6.693 at the end of the pneumoperitoneum; PKC activity was 159.4, 168.5, 178.0, 181.6 nmol/(g·min) and PP2a was 4158.3, 4066.9, 3984.0, 3878.5 nmol/(g·min) respectively. After 1 week, the cancer cells concentration was 2.15×10^5, 2.03×10^5, 2.20×10^5, 2.18×10^5 L^{-1}. Conclusion: CO_2 pneumoperitoneum could promote acidosis in cancer cells, inducing the activation of protein kinase C and deactivation of protein phosphatase 2a, but it could not accelerate the mitosis rate of the cancer cells.

Key words: Cancer, CO_2 pneumoperitoneum, Intracellular pH, Signal transduction
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INTRODUCTION

Since the first laparoscopic cholecystectomy was performed successfully in France, minimally invasive surgical techniques continued to advance in capability and popularity. Shorter hospital stays, decreased postoperative pain, more rapid return to preoperative activity, and decreased postoperative ileus demonstrated that laparoscopic surgery has distinct advantages over conventional surgery for a number of operative procedures. But there is still much debate on whether CO_2 pneumoperitoneum in laparoscopic tumor operation will hinder trocar implantation and extensive abdominal metastasis. Compared to conventional operation, more extensive metastasis occurs after laparoscopic colectomy (Paik *et al*., 1998). Many surgeons think that physical factors (such as floatage of cancer cells, conglutination on the incision, impact of CO_2 gas column, etc.) could explain the widespread metastasis following laparoscopic colectomy (Cataldo, 1998; Ziprin *et al*., 2003). We also found some cases that the cancer was widely spread after laparoscopic exploration. That cannot be explained by physical factors.

The effects of CO_2 pneumoperitoneum on the intracellular environment and signal transduction are still unknown. The purpose of this study was to analyze the chemical influence of CO_2 pneumoperitoneum on intracellular pH and activities of PKC and PP2a.

MATERIALS AND METHODS

Materials

Walker-256 tumor cells (China Center for Type

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Culture Collection, Wuhan University, CCTCC), Biscarboxyethyl carboxyfluoresein tetraacetoxymethyl ester (BCECF/AM, Sigma, USA), Nigericin (Sigma, USA), PSS solution (KCl 4 mmol/L, NaCl 140 mmol/L, $CaCl_2$ 1.8 mmol/L, $MgCl_2$ 1.0 mmol/L, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 20 mmol/L, glucose 10 mmol/L, 37 °C, pH 7.4), protein phosphatase test kit (Promega, USA), [r^{32}P]dATP (Yahui Biological Medical Corporation, Beijing, China), PepTag assay (Promega, Madison WI), Fluorescence microscope (Nikon UK), LS-50B Fluorescence spectrophotometer (PE, USA).

Preparation of walker-256 tumor cells

Walker-256 tumor cells, taken from breast cancer in rats, were immersed in absolute culture. After 2~3 lifetimes (about 1 week), the cell number was counted under fluorescence microscope after the cell had undergone coloration by 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT). Then the cells concentration was adjusted to 5×10^5 L^{-1}.

Setup simulating CO_2 pneumoperitoneum

Twenty ml 5×10^5 L^{-1} tumor cells culture was introduced into four medical plastic bags with valves, thus dividing cells into four groups into each bag, each was then added 80 ml absolute culture in bags. So that the tumor cells in each plastic bag was 1×10^5 L^{-1}. An automatic pneumatic apparatus was used to infuse CO_2 gas into the plastic bags with the pressure in the bags being kept to 0, 10, 20 and 30 mmHg respectively for 3 h (the average time of laparoscopic tumor operation).

Measurement of pHi

The CO_2 pneumoperitoneum setup was used to measure the pHi at 0, 3, 6, 9 h. Each group was measured thrice. The pHi was measured by using pH sensitive fluorescent dye Biscarboxyethyl carboxyfluoresein in its esterified form, acetoxymethyl ester (BCECF/AM), whose fluorescence intensity was measured by using a microspectrofluoremetric imaging technique (Kanaya *et al*., 2001).

Fifty μl sample was poured into 3 ml PSS solution for 5 min, 2 ml PSS solution with BCECF/AM was added and the mixture was incubated for 20 min at 37 °C in a 2.5% CO_2 incubator. The chamber was then placed on the stage of an inverted fluorescence microscope, and fluorescence emission intensity at the wavelength of 530 nm was measured after alternated excitation at 440 nm (pH insensitive) and 490 nm (pH sensitive) by using a 100 W xenon light source. The rapid shift from one wavelength to the other was obtained with a filter wheel and a shutter, the movement of which was monitored by a computer with a 50 image analysis system. The fluorescence images were captured by a charge coupled device camera and a photomultiplier, and the 490/440 ratios were transmitted directly to the personal computer. The fluorescence images were analyzed by using Hamamatsu Argus software for defining regions of interest in the fluorescence field (the selected cells). The system was calibrated at the end of each series of experiments by measuring the ratio of the fluorescence intensity of cells incubated in medium containing 140 mmol KCl, 10 mmol 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 10 mmol nigericin at pH 6.4, 6.8, 7.2, and 7.8. Because it was not possible to do the calibration on the same cells used to test the effect of sodium bicarbonate, only the changes in pHi were taken into consideration.

Measurement of the protein phosphatase 2a activity

At the end of the pneumoperitoneum where pHi was minimal, PP2a activity was measured by using PP2a catalytic subunit thrice in each group.

Five ml sample was taken from each bags and crushed by ultrasound. The cytosol and membrane complex with the protein G beads were initially washed twice with PBS buffer and finally washed with PP2a assay buffer. Immunoprecipitates with mouse IgG served as negative control. The complex was subjected to PP2a assay with a nonradioactive assay kit. In brief, the complexes containing PP2a were incubated for 20 min at 30 °C in 30 ml of assay buffer containing 200 mmol/L of phosphopeptide (KRpTIRR). The reactions were terminated by putting the tubes on ice and immediately centrifuging them at 8000 rpm for 1 min. The supernatants (25 μl/well) were pipetted into 96-well microtiter plates containing malachite green solution (100 μl/well). The absorbance at 630 nm was measured after 15 min of development time. PP2a specific activity was expressed as nmol/(g·min).

Measurement the protein kinase C activity

At the end of the pneumoperitoneum where pHi was minimal, PKC activity was measured by using PKC catalytic subunit 3 times in each group. PKC activity was determined using PepTag assay (Promega, Madison WI) as described previously. In brief, a PKC-specific fluorescent peptide was phosphorylated by the active PKCs of the cell lysate.

Twenty μl sample was introduced into a reaction solution with total volume of 25 μl and consisting of 1 μl cell lysate, 0.4 mg/ml peptide, 100 mmol HEPES (pH 7.4), 6.5 mmol $CaCl_2$, 5 mmol dithiothreitol, 50 mmol $MgCl_2$, 5 mmol adenosine triphosphate, 10 μmol leupeptin and 1 mg/ml phosphatidylserine. Subsequently, phosphorylated and dephosphorylated peptide molecules were separated by 0.8% (50 mmol Tris-HCl, pH 8.0) agarose gel electrophoresis for 15 min at 100 V. Resulting bands were visualized under UV light and documented quantitatively by densitometry (CPM)

Activity (PKC)=[CPM (sample)−CPM (contrast)]/total radioactivity×[concentration (dATP)/(7×protein content)]

PKC specific activity was expressed as nmol/(g·min).

Calculating the concentration of tumor cells

After 1 week, 10 ml sample was taken for calculating the concentration of tumor cells by using flow cytometry after coloration by MTT in each group.

Statistical analysis

The data were expressed as mean±*SEM*. Comparison within groups was made by analysis of variance.

RESULTS

At the beginning (0 h), there were no differences of pHi between each group (7.25~7.30, $P>0.05$). After 3 h, pHi fell while CO_2 pneumoperitoneum pressure rose (7.26~6.79, $P<0.05$) (Table 1, Fig.1). At the same time, protein kinase C activity rose and PP2a fell (Table 2). There were no differences of tumor cell concentration after 1 week (2.03×10^5~2.20×10^5 L^{-1}).

DISCUSSION

Whether CO_2 pneumoperitoneum will promote trocar implantation and extensive abdominal metas-

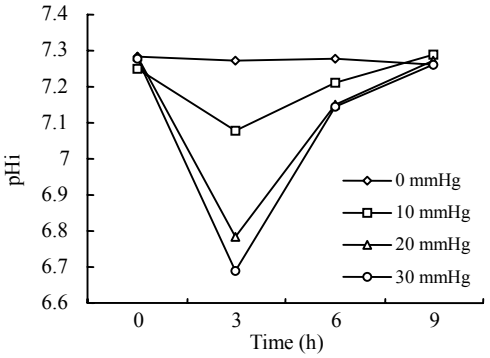


Fig.1 pHi of cancer cells at different pressure and time

Table 1 pHi of cancer cells at different pressure and time

Pressure (mmHg)	pHi			
	0 h*	3 h**	6 h***	9 h*
0	7.286±0.077	7.273±0.065	7.280±0.066	7.263±0.081
10.0	7.250±0.050	7.075±0.075	7.211±0.036	7.290±0.065
20.0	7.283±0.086	6.783±0.104	7.150±0.046	7.270±0.045
30.0	7.277±0.091	6.693±0.090	7.146±0.055	7.263±0.060

Between each groups $^*P>0.05$, $^{**}P<0.01$, $^{***}P<0.05$

Table 2 pHi, activity of PKC, PP2a and cell concentration under different pressure at 3 h

Pressure (mmHg)	pHi*	PKC** (nmol/(g·min))	PP2a* (nmol/(g·min))	Cell concentration*** (L^{-1})
0	7.273±0.065	159.4±9.7	4158.3±53.2	2.15×10^5
10.0	7.075±0.075	168.5±7.2	4066.9±48.7	2.03×10^5
20.0	6.783±0.104	178.0±10.6	3984.0±59.1	2.20×10^5
30.0	6.693±0.090	181.6±4.8	3878.5±51.0	2.18×10^5

Between each groups $^*P<0.01$, $^{**}P<0.05$, $^{***}P>0.05$

tasis after laparoscopic tumor operation was widely discussed in recent ten years, but there were still no conclusions (Zhang and Kong, 2004; Leister *et al*., 2003; Yavuz *et al*., 2003). Many surgeons found that CO_2 pneumoperitoneum would not accelerate the mitosis rate of cancer cells because blood acidosis induced by CO_2 pneumoperitoneum will be rapidly neutralized by the HCO_3^-/H_2CO_3 balance system in vivo (Tomita *et al*., 2001). Abu-Rustum *et al*.(2003) reviewed 1335 primary or metastasis malignancy cases of patients who underwent laparoscopy. Laparoscopy related subcutaneous tumor implantation is rare (0.97%) in women who underwent laparoscopy for malignant disease. Subcutaneous implantation appears to occur in patients with known metastatic disease and is detected in the setting of synchronous advanced intraabdominal or pelvic metastasis and progression of carcinomatosis (Abu-Rustum *et al*., 2004). Many surgeons think physical factors (such as floatage of cancer cells, conglutination on the incision, etc. and immaturity of laparoscopic skills) that cause trocar implantation and extensive abdominal metastasis (Hirabayashi *et al*., 2002; Lecuru *et al*., 2002). But these factors could not explain why He pneumoperitoneum would not promote trocar implantation (Neuhaus *et al*., 1998). We also found some clinical cases that cancer was widely spread after laparoscopic exploration while nothing special had been found in the laparoscopic procedure, which cannot be simply explained, by physical factors or lack of laparoscopic skills. At the same time, many researches have confirmed the high $SpCO_2$ (blood CO_2 pressure) and low pH under the gastric mucosa during laparoscopy (Celik *et al*., 2004). Blood acidosis in the portal vein system during laparoscopy may induce the extensive cancer metastasis after laparoscopy.

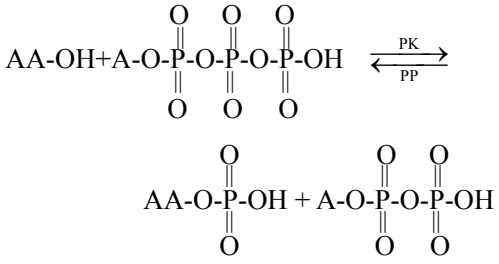
Our research found that low pHi correlated with the high pressure of simulant CO_2 pneumoperitoneum and has the ability to activate PKC and inactivate PP2a because intracellular acidosis can inhibit the phosphorylation of protease and promote the dephosphorylation of protease.

The function of protein kinase (PK) and protein phosphatase (PP) can be expressed by the equation:

$$\text{AA+ATP} \underset{\text{PP}}{\overset{\text{PK}}{\rightleftharpoons}} \text{AA-Pi+ADP}$$

AA: the protease aminophenol residue can be due to phosphorylation or dephosphorylation.

The molecular formula is



In theory, the decrease of pHi will consequentially influence the balance of PK/PP system. We think that the low pHi will activate the PK and inactivate the PP because in the acidosis environment, the organic chemical reaction with proton (H^+) participating in will react to form firmer hydrogen bond and that ATP can provide more H^+ than ADP, so the hydrogen bond of ATP is firmer than that of ADP (Ishizaka *et al*., 1999).

Protein kinase (PK) and protein phosphatase (PP) are key regulatory enzymes in the intracellular signal transduction. Almost all protease are activated or inactivated by PK/PP (Kim *et al*., 2003). PP2a is the major constituent in the signal transduction pathways for cell mitosis and has progressing amplificatory effect by activating PP family and inhibiting PK family. The biological activity of PKC is opposite that of PP2a. In addition, PKC/PP2a can regulate cell cycle, DNA copy and genes transcription (Ugi *et al*., 2003).

Oncogenes and PK perform a key function in tumor cells mitosis and mitosis-related signal transduction. Genes, such as src, ErbB, Raf, ras, etc. affect each step of the signal pathways by express growth factor, receptors, tyrosine kinase, GTP combined protein, serine/threonine kinase and transcription regulative protein, etc. In addition, PKC can influence the expression of cancer gene by phosphorylating the Ras, Raf and MAP kinase, activating transcription factors, inducing the mutation of early oncogenes such as c-fos, raf, MAP, etc.

There are close relations between PP2a and inactivation of anti-oncogene (Nazarenko *et al*., 2003). In human breast adenoma, lung cancer, rectal cancer, many researches have demonstrated the mutation of PR65/A gene, which encode PR65/A protein, a sub-

unit of PP2a and relating to cell apoptosis. PP2a can inactivate cyclin-dependent kinase (CDK) by dephosphorylation, inducing block from G_2 phase to M phase and from G_1 phase to S phase in the cell cycle (Martens *et al*., 2003). In addition, PP2a can promote cell apoptosis in coordination with apoptosis enzyme such as that of caspase-3 and adenovirus E40rf4 protein, etc.

Intracellular acidosis up regulates PKC/PP2a, but does not accelerate the mitosis rate of cancer cells, probably because intracellular acidosis promotes the apoptosis of cancer cells. The need of cancer cells large amount of energy when they are in mitosis, cannot be satisfied by cancer cell's anaerobic metabolism and acidosis makes metabolic enzyme pH far away form the optimum pHi. So many cells will go into apoptosis (Piot *et al*., 2001).

Intracellular acidosis induced by CO_2 pneumoperitoneum, on the one hand promotes cancer mitosis by up regulating PKC/PP2a in the intracellular signal transduction pathways, on the other hand promotes apoptosis of cancer cells by inhibiting energy metabolism. Though cancer cell's mitosis rate is not accelerated, the two factors may bring about a natural selective effect. That is how cancer cell subgroup came into being after long time in acidic environment, in which the group's cancer cells have more ability to resist invasive and metastasis. Intracellular acidosis causes apoptosis of cancer cells that cannot adapt to the acidic environment so the residual cancer cell's mitosis rate is accelerated. So the cancer cells after being subjected to CO_2 pneumoperitoneum have more ability against trocar implantation and abdominal metastasis.

CONCLUSION

The high pressure of CO_2 pneumoperitoneum can induce low pHi of cancer cells. And intracellular acidosis can up regulate the activity of PKC and inactive PP2a.

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