Impact of intraperitoneal pressure of a CO₂ pneumoperitoneum on the surgical peritoneal environment†

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BACKGROUND: Animal experiments have suggested that a high intraperitoneal pressure (IPP) might adversely affect the surgical peritoneal environment. The present experimental study investigates the impact of IPP of a CO₂ pneumoperitoneum on human peritoneum.

METHODS: Patients undergoing laparoscopic surgery were subjected to either low (8 mmHg) or standard (12 mmHg) IPP. Normal peritoneum was collected from the parietal wall at the beginning of surgery and every 60 min thereafter. Expression levels of 168 genes that encode extracellular matrix proteins, adhesion molecules or inflammatory cytokine signaling molecules were measured in peritoneal tissues using real-time polymerase chain reaction (PCR)-based assay panels. Human peritoneal mesothelial cells (HPMCs) and human peritoneal fibroblasts (HPFBs) were incubated in a CO₂ insufflation chamber for 1 h at 12 or 8 mmHg. Hyaluronan (HA) synthesis and mRNA expression levels of hyaluronic acid synthases (HAS) and hyaluronidases (Hyal) in HPMCs and HPFBs were measured at 0, 4, 8, 12, 24 and 48 h after CO₂ gas exposure by ELISA and real-time PCR, respectively.

RESULTS: Expression levels of connective tissue growth factor (CTGF), matrix metalloproteinase-9, E-selectin, chemokine (CX-C motif) ligand 2 (CXCL-2), Hyal-1 and Hyal-2 were significantly higher and those of HAS-1, HAS-3, thrombospondin-2 (TSP-2) and interleukin-10 were significantly lower in the 12 mmHg group compared with the 8 mmHg group. HA synthesis was significantly lower in the 12 mmHg group compared with the 8 mmHg group in HPMCs and HPFBs throughout the time course.

CONCLUSIONS: A low IPP (8 mmHg) may be better than the standard IPP (12 mmHg) to minimize the adverse impact on the surgical peritoneal environment during a CO₂ pneumoperitoneum.

Key words: CO₂ pneumoperitoneum / intraperitoneal pressure / laparoscopy / peritoneum

Introduction

Laparoscopic, or minimally invasive, surgery has revolutionized the peritoneal surgery field. It is not, however, without limitations. Postoperative adhesion formation and peritoneal dissemination remain as major clinical problems (Ellis et al., 1999; Canis et al., 2001). Laparoscopic surgery technology has evolved dramatically over the past two decades, and continues to advance. However, much less attention has been focused on how altering the laparoscopic surgical environment might improve clinical outcomes.

Today, in the clinical setting, 12–15 mmHg of intraperitoneal pressure (IPP) is generally applied during laparoscopic surgery. A pneumoperitoneum is required to perform laparoscopic surgery; however, animal experiments, including those performed in our laboratory, have suggested that a high IPP might adversely affect the surgical peritoneal environment at a tissue, cellular and molecular level, resulting in higher rates of peritoneal dissemination and peritoneal tissue hypoxia (Wittich et al., 2000; Bourdel et al., 2007; Matsuzaki et al., 2009, 2010a). In addition, our previous clinical study demonstrated that the tissue plasminogen activator (tPA)/plasminogen activator
inhibitor-1 (PAI-1) mRNA ratio and PAI-1 mRNA expression in peritoneal tissues were significantly lower in the 12 mmHg group compared with the 8 mmHg group during a CO2 pneumoperitoneum (Matsuzaki et al., 2011). These findings suggested that a low IPP (8 mmHg) may be better than the standard IPP (12 mmHg) for minimizing adverse effects on the peritoneal fibrinolytic system during a CO2 pneumoperitoneum (Matsuzaki et al., 2011). However, in addition to tPA and PAI-1, many other molecules in the surgical peritoneal environment might be affected by a high IPP during a CO2 pneumoperitoneum. Further evaluation is required to understand the potential adverse effects of a high IPP during a CO2 pneumoperitoneum on the surgical peritoneal environment.

We hypothesized that a high IPP during a CO2 pneumoperitoneum might adversely affect gene expression of extracellular matrix, adhesion and inflammatory cytokine signaling molecules in peritoneal tissues (Novitsky et al., 2004; Jayne, 2007), if the high IPP caused higher rates of peritoneal dissemination and peritoneal tissue hypoxia, as demonstrated in previous studies in animal models. In the present study, we investigated the impact of IPP during a CO2 pneumoperitoneum on expression levels of 84 genes known to encode extracellular matrix and adhesion molecules and 84 genes that encode inflammatory cytokine signaling molecules in peritoneal tissues using two real-time polymerase chain reaction (PCR)-based assay panels.

Materials and Methods

Patients

Patients undergoing laparoscopic hysterectomy with or without premenopausal hysterectomy for benign gynecological disease were recruited. The following general criteria were also required for the enrollment: (i) no previous history of pelvic surgery, endometriosis and/or infection and (ii) BMI <30 kg/m². In the present study, we included only patients who had a macroscopically normal peritoneum. Insufflation of CO2 gas was performed using a Storz electronic endoflator (Karl Storz Endoscopy & GmbH, Tuttlingen, Germany). When the IPP reached 15 mmHg, four trocars were inserted, immediately after which the IPP was decreased to 12 or 8 mmHg and then maintained at these levels throughout surgery. The duration between insufflation of CO2 gas and insertion of the four trocars was <5 min. In our clinical setting, the minimum IPP required to perform major laparoscopic surgical procedures is 8 mmHg; 12 mmHg was applied to patients who underwent surgery performed by M.C., whereas 8 mmHg was applied to patients who underwent surgery performed by R.B. and K.J. The three surgeons (M.C., R.B. and K.J.) used the same surgical technique previously described by our group (Rivoire et al., 2007). Macroscopically normal peritoneum was collected from the anterior parietal wall at the beginning of surgery and every 60 min thereafter. Because the vagina is opened at the end of the total hysterectomy procedure, all of the samples in the present study were collected before opening the vagina for evaluation of the impact of a CO2 pneumoperitoneum at a constant IPP. The area from which the peritoneal biopsy was acquired was intact and located at a constant distance from the port through which the CO2 gas was insufflated. In view of the fragility of the mesothelial layer, the peritoneal biopsies were performed meticulously to minimize the possibility of trauma to the specimens. In addition, a study demonstrated the influence of the sampling method on the peritoneal fibrinolytic activity (Neudecker et al., 2002). The full thickness of the peritoneum was excised using only a pair of scissors, and peritoneal tissues were then collected by atraumatic forceps in all cases.

None of the patients had intra- or post-operative complications. In addition, no cases were converted to laparotomy. Finally, peritoneal samples were collected from a total of 36 patients during CO2 pneumoperitoneum at 12 mmHg and 32 patients at 8 mmHg. Clinical characteristics of the patients are shown in Table I. As this is a pilot study, a power calculation was not performed. Peritoneal samples were collected at 0 and 1 h from a total of 20 patients at 12 mmHg and 17 patients at 8 mmHg, and at 0, 1 and 2 h from a total of 16 patients at 12 mmHg and 15 patients at 8 mmHg. Peritoneal samples were immediately collected in RNAlater (Ambion, Cambridgeshire, UK) and stored at −20°C until further analysis.

For in vitro analysis, macroscopically normal peritoneum was collected from the anterior parietal wall at the beginning of surgery at 12 mmHg of IPP from a total of 10 patients (age range, 42–49 years). Peritoneal samples were immediately collected in Hank’s balanced salts solution (HBSS) and transferred to the laboratory for in vitro analysis.

All tissue samples were obtained with full and informed patient consent. The research protocol was approved by the Consultative Committee for Protection of Persons in Biomedical Research of the Auvergne region.

RNA extraction

Total RNA was extracted using the Qiagen RNeasy Mini Kit according to the manufacturer’s instructions and stored at −80°C until use. To eliminate potential genomic DNA contamination, RNA samples were treated with DNasel (15 U; DNasel, Courtaboeuf, Qiagen, France) at room temperature for 15 min.

Examination of RNA yield and integrity

RNA yield and integrity were analyzed using the RNA 6000 Pico kit and the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) (Matsuzaki et al., 2010b). The RNA 6000 Pico kit allows determination of the integrity of very small amounts of RNA as well as estimation of the quantity of the isolated RNA, which has a linear range of 200–5000 pg/µl. The RIN (RNA integrity number) value was >8.0 in all cases.

**Table I** Clinical characteristics of patients.

<table>
<thead>
<tr>
<th>Group</th>
<th>12 mmHg</th>
<th>8 mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cases</td>
<td>36</td>
<td>32</td>
</tr>
<tr>
<td>Age</td>
<td>48.5 (42–69)</td>
<td>48.0 (42–63)</td>
</tr>
<tr>
<td>Parity</td>
<td>3 (0–6)</td>
<td>3 (0–5)</td>
</tr>
<tr>
<td>Menstrual cycle (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferative</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Secretory</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Anovulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>With GnRH agonist</td>
<td>8 (22.2)</td>
<td>6 (18.8)</td>
</tr>
<tr>
<td>With oral progesterone</td>
<td>5 (13.9)</td>
<td>4 (12.5)</td>
</tr>
<tr>
<td>Post-menopausal</td>
<td>23 (63.9)</td>
<td>22 (68.8)</td>
</tr>
<tr>
<td>Indication (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uterine fibroma</td>
<td>12 (33.3)</td>
<td>10 (31.3)</td>
</tr>
<tr>
<td>Uterin fibroma + abnormal bleeding</td>
<td>8 (22.2)</td>
<td>7 (21.9)</td>
</tr>
<tr>
<td>Genital prolapse</td>
<td>16 (44.4)</td>
<td>15 (46.9)</td>
</tr>
</tbody>
</table>

*Median (range).*
PCR array analysis

For screening, gene expression levels were compared between peritoneal biopsies at 2 h and initial biopsies within the same patient in five patients in each group (12 and 8 mmHg IPP). Total RNA (200 ng) was subjected to an RT reaction using the RT² First Strand kit (SuperArray, Frederick, MD, USA) following the manufacturer’s instructions. PCR array analysis of a subset of the 84 genes related to extracellular matrix and adhesion molecules (Human extracellular matrix & adhesion molecules RT² Profiler™ PCR Array, SuperArray) (Supplementary data, Table S1) or (Human inflammatory cytokines & receptors RT² Profiler™ PCR Array, SuperArray) (Supplementary data, Table S2) was performed using the ABI-Prism 7300 Fast Real-time PCR system (Applied Biosystems, Courtaboeuf, France).

Quantitative real-time RT–PCR

Quantitative real-time RT–PCR with a Light Cycler was performed as previously described (Matsuzaki et al., 2010b, 2011). Total RNA (100 ng) was subjected to an RT reaction using Superscript II Reverse Transcriptase (Invitrogen, Cergy Pontoise, France). Quantitative real-time PCR was performed in a LightCycler System using the FastStart DNA Master SYBR Green I kit as recommended by the manufacturer (Roche, Mannheim, Germany). In a total volume of 20 μl, each reaction contained 2 μl SYBR green I reaction mix (consisting of Taq DNA-polymerase reaction buffer, dNTP mix, SYBR green I, MgCl₂ and Taq DNA polymerase), 0.3–0.5 μM of each primer, 4 mM MgCl₂, 2 μl cDNA and standard or nuclease-free water as a negative control. Primer sets are shown in Table II. Quantification of the targets in the unknown samples was performed using a relative quantification method with external standards.

Table II. Sequences of the primers used for mRNA quantification by real-time RT–PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense primers</th>
<th>Antisense primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9</td>
<td>5′-AGAGATGCCTGGAGATCTGAAA-3′</td>
<td>5′-AAGGTTTGGAATCTGCACCAGT-3′</td>
</tr>
<tr>
<td>E-selectin</td>
<td>5′-GTAGGAGGAAGCTGTTGCA-3′</td>
<td>5′-ACAATTCTGAGCTTCGCTG-3′</td>
</tr>
<tr>
<td>CTGF</td>
<td>5′-GGCCAGACCCCAACTATGATTA-3′</td>
<td>5′-AGGGGCCTGTTGATGTGAAC-3′</td>
</tr>
<tr>
<td>TSP-2</td>
<td>5′-TCACCTTGGTCTTCCATGCTCTG-3′</td>
<td>5′-CAGAGCGAGGATGCAGTGTAT-3</td>
</tr>
<tr>
<td>CXCL-2</td>
<td>5′-CGCCCAAACCGGAGCTCATAG-3′</td>
<td>5′-AGCAAGCTTCTTGCCATCTT-3</td>
</tr>
<tr>
<td>IL-10</td>
<td>5′-GCCTAACATGCTTCGAGATC-3′</td>
<td>5′-TGTATGCTGGTCTTGCCTG-3′</td>
</tr>
<tr>
<td>HAS-1</td>
<td>5′-TCCACTGGTATCCTCGATCAG-3′</td>
<td>5′-GCCCTAAGAAATCGCTGAAA-3′</td>
</tr>
<tr>
<td>HAS-2</td>
<td>5′-AGGCAGCTAGTCCAGGAGAAG-3′</td>
<td>5′-AGAACCCAGGAGCCAGAAT-3</td>
</tr>
<tr>
<td>HAS-3</td>
<td>5′-CTCTGGTTGTCCTCAAGACACCA-3′</td>
<td>5′-AGGCGTAGTGGTCCTGGAT-3</td>
</tr>
<tr>
<td>Hyal-1</td>
<td>5′-CTGGGTCGTCGAGTGGAAAATA-3′</td>
<td>5′-GGCAGGTTAAGGGAGAGAG-3</td>
</tr>
<tr>
<td>Hyal-2</td>
<td>5′-TGTCAGCTCTCTGTCAGTAC-3′</td>
<td>5′-GTCCTGAGTGTCCTGGTGA-3</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-TGCCACCCAATCTGCATTAG-3′</td>
<td>5′-CCTTGCCACCATGTTCA-3′</td>
</tr>
</tbody>
</table>

Isolation of human peritoneal mesothelial cells and fibroblasts

Human peritoneal mesothelial cells (HPMCs) and human peritoneal fibroblasts (HPFBs) were isolated according to the methods of Stylianou et al. (1990) and Beavis et al. (1997), respectively. To obtain HPMCs, peritoneal tissue was washed in HBSS and incubated in a solution of 0.125% trypsin and 0.01% EDTA for 20 min at 37°C with gentle agitation. The suspension was centrifuged, and the resulting pellet, which yielded a homogenous population of mesothelial cells, was washed in serum containing culture medium and seeded into 35-mm dishes that had been coated with 10% Matrigel. The remaining tissue was transferred to place HPFBs into a new flask for the next round of digestion in a solution of 0.125% trypsin and 0.01% EDTA, and the procedure was repeated one time for 20 min and two times for 40 min each. The suspension after the third and fourth rounds was centrifuged, and the resulting pellets, which yielded a homogenous population of fibroblasts, were washed in serum containing culture medium and seeded into a 35-mm dish that had been coated with 10% Matrigel. After seeding, the cells were maintained in the same medium (Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with GlutaMAX, 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite) and incubated at 37°C under an atmosphere of 5% CO₂/95% air in a humidified incubator. The medium was replaced every 2–3 days. HPMCs from the first passage and HPFBs from the third passage were used for experiments.

Immunocytochemistry

Immunofluorescent staining was performed to determine the purity of the HPMCs and HPFBs using monoclonal antibodies for human cytokeratin 19 and vimentin (Ivarsson et al., 1998; Witowski and Jörres, 2006). Cells, seeded onto 8-well Lab-Tek slides (BD, Le Pont-DECLAix, France), were fixed with ice-cold methanol for 10 min. Next, the slides were rinsed in 0.01 mol/l phosphate-buffered saline (PBS, pH 7.2) and incubated with primary antibody diluted in PBS with 3% bovine serum albumin (anti-cytokeratin 19, 1:100, DAKO, Glostrup, Denmark; anti-vimentin, 1:100, DAKO) for 1 h at room temperature. The slides were then washed in PBS and incubated for 1 h in a 1:20 dilution of fluorescein-labeled anti-mouse antibody (Dako). The slides were subsequently washed with PBS and mounted with Fluorep (bioMerieux, Marcy l’Etoile, France). The specificity of the immunocytochemical staining was...
confirmed by omission of the primary antibody. The staining was detected on an Olympus CX51 microscope (Olympus, Rungis, France) at ×400 magnification. Immunocytochemical procedures were performed on at least three different isolated cell preparations with similar results.

In vitro CO₂ pneumoperitoneum model
At our facility, le Centre International de la Chirurgie Endoscopique (CICE), we developed a 7-l insufflation chamber to simulate a CO₂ pneumoperitoneum at a temperature of 37°C. The insufflation chamber was connected to a Storz electronic endoflator (Karl Storz Endoscopy & GmbH) to adjust to the different pressures used in this study (12 or 8 mmHg). Before starting CO₂ insufflation, the boxes were filled with 15 l pure CO₂ to remove room air. Cells were seeded into 96-well plates, which had been coated with 10% Matrigel, at a density of 10,000 cells per well in 100 μl culture media. These cells were cultured at 37°C for 3 days until confluence. Then, the complete media were removed and replaced with fresh FBS-free and growth factor-free media (DMEM) with antibiotics, and the cells were cultured for 24 h. Then, cells were incubated in the CO₂ insufflation chamber for 1 h at 12 or 8 mmHg. During this time, control group cells were kept in the CO₂ incubator. Then, cells were incubated at 37°C under an atmosphere of 5% CO₂/95% air in a humidified incubator.

Determination of hyaluronan production
Conditioned media were collected at 0, 4, 8, 12, 24 and 48 h after CO₂ gas exposure. Hyaluronan (HA) production in conditioned media was confirmed by omission of the primary antibody. The staining was detected on an Olympus CX51 microscope (Olympus, Rungis, France) at ×400 magnification. Immunocytochemical procedures were performed on at least three different isolated cell preparations with similar results.

Assessment of cell viability
To assess the effect of a CO₂ pneumoperitoneum at different pressures on mesothelial cell and fibroblast viability, a cell cytotoxicity assay was performed using the CytoTox-Fluor Cytotoxicity Assay according to the manufacturer’s instructions (Promega, Charbonnières-les-Bains, France). After exposure to CO₂ gas in the insufflation chamber for 1 h at 12 or 8 mmHg, or in a CO₂ incubator (nontreated control cells), CytoTox-Fluor™ Cytotoxicity Assay Reagent was added in an equal volume (100 μl per well) to all wells, mixed briefly by orbital shaking, then incubated for 3 h at 37°C. Then, fluorescence was measured with the Fluoroskan Ascent Microplate Fluorometer (Thermo Scientific) at 480 nm Ex/530 nm Em. All experiments were performed in triplicate.

Statistical analysis
The Statview 4.5 program (Abacus Concepts, Inc., Berkeley, CA, USA) was used for statistical analysis. Comparisons between different groups were made using the one-way analysis of variance following Scheffe’s method or the Mann—Whitney U-test. Statistical significance was defined as P < 0.05.

To analyze PCR array data, an MS-Excel worksheet was downloaded from the manufacturer’s website (http://www.sabiosciences.com/pcrarraydataanalysis.php). For each PCR reaction, the Excel worksheet calculated two normalized average Ct values, a paired t-test P-value and a fold change. Data normalization was based on correcting all Ct values for the average Ct values of several constantly expressed housekeeping genes included in the array. The differentially expressed genes must show at least a mean 2-fold difference in expression that is statistically significant. Statistical significance was defined as P < 0.05.

Results

In vivo studies

PCR-based microarray analysis
A total of five genes [E-selectin, connective tissue growth factor (CTGF), hyaluronic acid synthase-1 (HAS-1), chemokine (C-X-C motif) ligand 2 (CXCL-2) and matrix metalloproteinase (MMP-9)] were up-regulated and one gene (thrombospondin-2, TSP-2) was down-regulated after 2 h versus at initial biopsy in the 12 mmHg group (Table II). In the 8 mmHg group, a total of five genes [E-selectin, CTGF, HAS-1, CXCL-2 and interleukin (IL)-10] were up-regulated and no gene was down-regulated after 2 h versus at initial biopsy (Table III).

Quantitative real-time PCR
Results are shown in Fig. 1. CTGF and MMP-9 mRNA expression was significantly higher in the 12 mmHg group compared with the 8 mmHg at 1 or 2 h of CO₂ pneumoperitoneum. No significant difference in

<table>
<thead>
<tr>
<th>Table III Results of PCR-based microarray analysis.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>After 2 h versus initial biopsy</strong></td>
</tr>
<tr>
<td><strong>12 mmHg (n = 5)</strong></td>
</tr>
<tr>
<td>Up-regulated genes</td>
</tr>
<tr>
<td>E-selectin (21.5)</td>
</tr>
<tr>
<td>CTGF (13.2)</td>
</tr>
<tr>
<td>HAS-1 (5.4)</td>
</tr>
<tr>
<td>MMP-9 (3.9)</td>
</tr>
<tr>
<td><strong>8 mmHg (n = 5)</strong></td>
</tr>
<tr>
<td>Up-regulated genes</td>
</tr>
<tr>
<td>E-selectin (4.1)</td>
</tr>
<tr>
<td>HAS-1 (22.3)</td>
</tr>
<tr>
<td>CXCL-2 (2.1)</td>
</tr>
</tbody>
</table>

Values in parentheses indicate the fold change.

MMP-9, CTGF, E-selectin, HAS-1, TSP-2, extracellular matrix-related genes clusters; CXCL-2, IL-10, inflammatory cytokine gene clusters.
either E-selectin or CXCL-2 mRNA expression was observed between the 12 and 8 mmHg groups at 1 h of CO₂ pneumoperitoneum, whereas E-selectin and CXCL-2 mRNA expression was significantly higher in the 12 mmHg group compared with the 8 mmHg group at 2 h of CO₂ pneumoperitoneum. No significant difference in either TSP-2 or IL-10 mRNA expression was observed between the 12 and 8 mmHg groups at 1 h of CO₂ pneumoperitoneum, whereas TSP-2 and IL-10 mRNA was significantly lower in the 12 mmHg group compared with the 8 mmHg group at 2 h of CO₂ pneumoperitoneum. The present PCR microarray analysis identified

Figure 1 mRNA expression of expression levels of CTGF, MMP-9, E-selectin, CXCL-2, TSP-2, IL-10, HAS-1, HAS-2, HAS-3, Hyal-1 and Hyal-2 in peritoneal tissues at 1 or 2 h of CO₂ pneumoperitoneum during laparoscopic surgery, relative to the expression levels of the reference gene, GAPDH. Results are presented as the mean (gene/GAPDH) + SEM. C, initial biopsy (12 mmHg: n = 36; 8 mmHg: n = 32); 12 mmHg, CO₂ pneumoperitoneum at 12 mmHg (1 h: n = 36, 2 h: n = 16); 8 mmHg, CO₂ pneumoperitoneum at 8 mmHg (1 h: n = 32, 2 h: n = 15). *P < 0.05 versus CO₂ pneumoperitoneum at 8 mmHg at 1 h. **P < 0.05 versus CO₂ pneumoperitoneum at 8 mmHg at 2
hyaluronan synthase-1 (HAS-1) as one of the differentially expressed genes in both groups (Table III). Thus, we further investigated expression levels of all three HAS genes, HAS-1, HAS-2 and HAS-3, as well as the HA-cleaving endoenzymes, hyaluronidases 1 and 2 (Hyal-1 and Hyal-2). HAS-1 and HAS-3 mRNA expression levels were significantly higher in the 8 mmHg group compared with the 12 mmHg group at 1 and 2 h of CO2 pneumoperitoneum. No significant difference in HAS-2 mRNA expression levels in the 8 and 12 mmHg groups was observed after 1 or 2 h compared with those of initial biopsies within the same patients. Hyal-1 and Hyal-2 mRNA expression was significantly higher in the 12 mmHg group compared with the 8 mmHg group at 1 h of CO2 pneumoperitoneum, whereas no significant difference was observed at 2 h of CO2 pneumoperitoneum.

In vitro analysis

Immunocytochemistry

HPMCs were positive for Cytokeratin 19 and vimentin. In contrast, HPFBs were positive for vimentin and negative for Cytokeratin.

HA synthesis

HA protein levels in HPMCs (Fig. 2) and HPFBs (Fig. 2) were significantly lower in the 12 mmHg group than in the 8 mmHg group throughout the time course of the study.

Quantitative real-time PCR

Mesothelial cells

Results are shown in Fig. 3. HAS-1 and HAS-2 mRNA expression was significantly higher in the 8 mmHg group compared with the 12 mmHg group from 12 h until 24 h. No significant difference in HAS-3 mRNA expression was observed between the 8 and 12 mmHg groups throughout the time course of this study. No significant difference in Hyal-1 mRNA expression was observed between the 8 and 12 mmHg groups, whereas Hyal-2 mRNA expression was significantly higher in the 12 mmHg group compared with the 8 mmHg group throughout the time course.

Fibroblasts

Results are shown in Fig. 4. HAS-1, HAS-2 and HAS-3 mRNA expression was significantly higher in HPFBs in the 8 mmHg group compared with the 12 mmHg group at 0, 24 and 48 h. No significant difference in Hyal-1 mRNA expression was observed between the 8 and 12 mmHg groups, whereas Hyal-2 mRNA expression was significantly higher in the 12 mmHg group compared with the 8 mmHg group throughout the time course.

Cytotoxicity assay

Cytotoxicity assays demonstrated that no significant differences existed among the control, high- and low-pressure groups (Fig. 5).

Discussion

HA is a major component of the glycocalyx that forms a protective barrier around mesothelial cells, and bestows upon the peritoneal membrane a slippery non-adhesive surface that prevents adhesion, infection and tumor dissemination (Yung and Chan, 2007; Jiang et al., 2011). Seprafilm, a bioresorbable membrane developed to prevent the formation of adhesions, consists of HA and carboxymethylcellulose and acts as a mechanical barrier between two damaged surfaces during the period of peritoneal regeneration. Seprafilm has been shown to reduce the severity of post-operative adhesions (Vrijland et al., 2002). An in vitro study suggested that HA synthesis is a critical event and is tightly regulated in the re-epithelialization of the peritoneal mesothelium (Yung et al., 2000). Considering the biological importance of HA in peritoneal physiology as well as its potential role in prevention of post-operative adhesion formation, we further investigated expression levels of the three HAS genes, HAS-1, HAS-2 and HAS-3, as well as those of the HA-cleaving endoenzymes Hyal-1 and Hyal-2. HAS-1 and HAS-2 encode high-molecular-weight HA, whereas HAS-3 has been shown to orchestrate the synthesis of low-molecular-weight HA (Jiang et al., 2011). In humans, the Hyal gene family includes six members (Jiang et al., 2011). The major transcript of Hyal-3 is enzymatically inactive (Jiang et al., 2011). In contrast, Hyal-1 and Hyal-2 are ubiquitous and show activity in acidic

Figure 2  HA protein levels in peritoneal mesothelial cells and fibroblasts at 0, 4, 8, 12, 24 and 48 h after CO2 gas exposure. Values are normalized to the total protein content of the cell lysate. Values are means ± SEM, n = 5. 12 mmHg, 1 h CO2 gas exposure at 12 mmHg; 8 mmHg, 1 h CO2 gas exposure at 8 mmHg; C, controls (no CO2 exposure). 1, 0 h; 2, 4 h; 3, 8 h; 4, 12 h; 5, 24 h; 6, 48 h. *p < 0.05 versus time-matched 8 mmHg group and time-matched controls. †p < 0.05 versus time-matched controls. ‡p < 0.05 versus time-matched 12 mmHg group.
environments, and Hyal-2 has very low enzymatic activity compared with Hyal-1 (Jiang et al., 2011). During a CO₂ pneumoperitoneum, the peritoneal environment is acidic (Hanly et al., 2005); therefore, Hyal-1 and Hyal-2 might be active during laparoscopic surgery. The present in vivo study demonstrated that expression levels of Hyal-1 and Hyal-2 were significantly higher and those of HAS-1 and HAS-3 levels were significantly lower in the 12 mmHg group compared with the 8 mmHg group during a CO₂ pneumoperitoneum. In addition, the present in vitro analysis demonstrated decreased expression of HASs and increased Hyal-2 mRNA expression in both mesothelial cells and fibroblasts in the 12 mmHg group compared with the 8 mmHg group during the present time course study. We also demonstrated that HA synthesis in vitro was significantly lower in both mesothelial cells and fibroblasts in the 12 mmHg group compared with those of the 8 mmHg group. These findings suggest increased HA synthesis during a CO₂ pneumoperitoneum at 8 mmHg compared with 12 mmHg during the peri-operative period. The present results also suggest that the surgical peritoneal environment of a low IPP (8 mmHg) might be better for regeneration of injured peritoneal tissues than the standard IPP (12 mmHg) during the peri-operative

Figure 3 mRNA expression of HAS-1, HAS-2, HAS-3, Hyal-1 and Hyal-2 in peritoneal mesothelial cells at 0, 4, 8, 12, 24 and 48 h after CO₂ gas exposure. Values are means ± SEM, n = 5. 12 mmHg, 1 h CO₂ gas exposure at 12 mmHg; 8 mmHg, 1 h CO₂ gas exposure at 8 mmHg; C, controls (no CO₂ exposure). aP < 0.05 versus time-matched 12 mmHg group. bP < 0.05 versus time-matched 12 mmHg group and time-matched 8 mmHg group. cP < 0.05 versus time-matched 12 mmHg group and time-matched controls.
Further studies both in vivo and in vitro are required to investigate the biological significance of altered HA synthesis during a CO₂ pneumoperitoneum at 12 mmHg. The present ELISA assay used for HA synthesis detected high-molecular-weight HA (>950 kDa), medium-molecular-weight HA (90–150 kDa) and low-molecular-weight HA (15–40 kDa).

Very importantly, HA can have very different properties depending on its molecular weight (Yung and Chan, 2007; Jiang et al., 2011). In its native state, HA possesses a high molecular weight in excess of 10⁶ kDa. High-molecular-weight HA possesses anti-inflammatory and anti-angiogenic properties, whereas low-molecular-weight HA plays pivotal roles in acute inflammation and angiogenesis (Yung and Chan, 2007; Jiang et al., 2011). The fragmentation of HA occurs through both enzymatic and non-enzymatic pathways via the

Figure 4 mRNA expression of HAS-1, HAS-2, HAS-3, Hyal-1 and Hyal-2 in peritoneal fibroblasts at 0, 4, 8, 12, 24 and 48 h after CO₂ gas exposure. Values are means + SEM, n = 5. 12 mmHg, 1 h CO₂ gas exposure at 12 mmHg; 8 mmHg, 1 h CO₂ gas exposure at 8 mmHg; C, controls (no CO₂ exposure). *P < 0.05 versus time-matched 8 mmHg group and time-matched controls. †P < 0.05 versus time-matched controls. ‡P < 0.05 versus time-matched 8 mmHg group. §§P < 0.05 versus time-matched 8 mmHg group and time-matched 12 mmHg group. ¶P < 0.05 versus time-matched 12 mmHg group and time-matched controls.
degradation of the parent molecule by Hyal or reactive species, respectively (Yung and Chan, 2007; Jiang et al., 2011). The present study demonstrated that Hyal-1 and Hyal-2 mRNA expression in vivo and Hyal-2 mRNA expression in vitro were increased in the 12 mmHg group compared with the 8 mmHg group. In addition, the generation of HA fragments occurs by reactive oxygen species (Soltés et al., 2006). A recent review also suggested an impact of IPP on oxidative stress (Sammour et al., 2009). These findings suggest that more fragmentation of HA might occur during a CO2 pneumoperitoneum at 12 mmHg compared with 8 mmHg during the peri-operative period in vivo.

Furthermore, de novo synthesis of low-molecular-weight HA may also occur within sites of inflammation (jiang et al., 2011). The present study demonstrated that CXCL-2 and E-selectin mRNA expression levels in the 12 mmHg group were significantly higher than those in the 8 mmHg group at 2 h of CO2 pneumoperitoneum. CXCL-2 has been shown to elicit neutrophil chemotaxis in vitro (Call et al., 2001), whereas E-selectin is critically important for neutrophil recruitment into the inflamed peritoneal cavity (Ramos et al., 1999). Although neutrophils are involved in a large number of pathological conditions, they play a particularly important role during acute inflammation in vivo. In addition, IL-10 mRNA expression levels in the 12 mmHg group were significantly lower than those in the 8 mmHg group at 2 h of CO2 pneumoperitoneum. IL-10 is an anti-inflammatory cytokine that inhibits the production of proinflammatory cytokines, including IL-1, IL-6, IL-8 and tumor necrosis factor (TGF-α) originating from monocytes (Foey et al., 1998). These findings suggest that the standard IPP (12 mmHg) might induce more inflammation in the surgical peritoneal environment compared with a low IPP (8 mmHg). More inflammation during a CO2 pneumoperitoneum at the standard IPP (12 mmHg) might result in de novo synthesis of low-molecular-weight HA. Furthermore, HA fragments themselves induce inflammatory responses in inflammatory cells (jiang et al., 2011).

In the present study, MMP-9 and CTGF mRNA expression levels were significantly higher and TSP-2 mRNA expression levels were significantly lower in the 12 mmHg group compared with the 8 mmHg group during a CO2 pneumoperitoneum. MMP-9 cleaves latent TGF-β, enabling TGF-β activation (Yu and Stamenkovic, 2000). TGF-β plays a central role in peritoneal fibrosis and is a potent inducer of CTGF (Abreu et al., 2002). TGF-β is activated by TSP-1 through a non-enzymatic mechanism, and TSP-2 inhibits activation of latent TGF-β by TSP-1 (Schultz-Cherry et al., 1995). These findings suggested that the surgical peritoneal environment under the standard IPP (12 mmHg) might increase the expression of several genes involved in peritoneal fibrosis compared with a low IPP (8 mmHg). A study demonstrated that small HA fragments enhance expression of MMP-9 in lung carcinoma cells (Fieber et al., 2004), and HA fragments augment steady-state mRNA, protein and inhibitory activity of PAI-1 (Horton et al., 2000). Our previous study demonstrated that PAI-1 mRNA expression levels in the 12 mmHg group were significantly higher than those in the 8 mmHg group during a CO2 pneumoperitoneum (Matsuzaki et al., 2011). Increased small HA fragments under the standard IPP during a CO2 pneumoperitoneum might have contributed to the increased MMP-9 and PAI-1 mRNA expression observed in the 12 mmHg group.

Although further studies both in vitro and in vivo are required to investigate whether more HA fragments might occur during a CO2 pneumoperitoneum at the standard IPP compared with a low IPP, the present findings suggest that a low IPP (8 mmHg) might minimize adverse effects of IPP on inflammation, peritoneal fibrosis and generation of HA fragments. In the present study, we used cool, dry CO2 gas, which could cause structural, morphologic and biochemical injury to peritoneal tissues (Sammour et al., 2009). Further studies are required to investigate the impact of a warm, humidified CO2 pneumoperitoneum on the surgical peritoneal environment.

However, a recent Cochrane review concluded that the safety of a low-pressure pneumoperitoneum remains to be established (Gurusamy et al., 2009). In addition, the duration of surgery was relatively short in the present study. The present study was not randomized, and different teams of surgeons operated on the 8 versus 12 mmHg groups. Further randomized controlled studies are required to confirm the present findings and to fully assess the safety and impact of IPP during a CO2 pneumoperitoneum, as well as a CO2 pneumoperitoneum longer than 2 h in duration, in patients with a pathological peritoneum (i.e. adhesion, endometriosis).

In conclusion, a low IPP (8 mmHg) may be better than the standard IPP (12 mmHg) for minimizing the adverse impact on the surgical peritoneal environment during a CO2 pneumoperitoneum. It may be better to use the lowest IPP, allowing adequate exposure of the operative field rather than using a routine pressure.
Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors’ roles

S.M. was involved in concept and design, sample collection, in vitro experiments, acquisition of data, analysis and interpretation of data; drafting the article. K.J. was involved in concept and design, and sample collection. E.M. prepared the sample and conducted PCR analysis. F.dA. was involved in in vitro experiments. M.C. played a role in concept and design, sample collection, interpretation of data, critical comments on the paper. R.B. played a role in concept and design, sample collection, interpretation of data, critical comments on the paper. All authors read and approved the final version of the paper.

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Conflict of interest

None declared.

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