Randomized clinical trial

BJS Open Open Access

Randomized clinical trial of the effect of intraoperative humidified carbon dioxide insufflation in open laparotomy for colorectal resection

J. Y. Cheong[1,2], B. Chami[2], G. M. Fong[2], X. S. Wang[2], A. Keshava[1], C. J. Young[1] and P. Witting[2]

[1]Colorectal Unit, Concord Repatriation General Hospital, Concord Clinical School, Concord, and [2]Department of Pathology, Sydney Medical School, University of Sydney, Sydney, New South Wales, Australia

Correspondence to: Dr J. Y. Cheong, Concord Repatriation General Hospital, Hospital Road, Concord, New South Wales 2139, Australia (e-mail: juyong.cheong@gmail.com)

Background: Animal studies have shown that peritoneal injury can be minimized by insufflating the abdominal cavity with warm humidified carbon dioxide gas.

Methods: A single-blind RCT was performed at a tertiary colorectal unit. Inclusion criteria were patient aged 18 years and over undergoing open elective surgery. The intervention group received warmed (37°C), humidified (98 per cent relative humidity) carbon dioxide ($WHCO_2$ group). Multiple markers of peritoneal inflammation and oxidative damage were used to compare groups, including cytokines and chemokines, apoptosis, the 3-chlorotyrosine/native tyrosine ratio, and light microscopy on peritoneal biopsies at the start (T_0) and end (T_{end}) of the operation. Postoperative clinical outcomes were compared between the groups.

Results: Of 40 patients enrolled, 20 in the $WHCO_2$ group and 19 in the control group were available for analysis. A significant $\log(T_{end}/T_0)$ difference between control and $WHCO_2$ groups was documented for interleukin (IL) 2 (5·3 *versus* 2·8 respectively; $P = 0{\cdot}028$) and IL-4 (3·5 *versus* 2·0; $P = 0{\cdot}041$), whereas apoptosis assays documented no significant change in caspase activity, and similar apoptosis rates were documented along the peritoneal edge in both groups. The 3-chlorotyrosine/tyrosine ratio had increased at T_{end} by 1·1-fold in the $WHCO_2$ group and by 3·1-fold in the control group. Under light microscopy, peritoneum was visible in 11 of 19 samples from the control group and in 19 of 20 samples from the $WHCO_2$ group ($P = 0{\cdot}006$). The only difference in clinical outcomes between intervention and control groups was the number of days to passage of flatus (2·5 *versus* 5·0 days respectively; $P = 0{\cdot}008$).

Conclusion: The use of warmed, humidified carbon dioxide appears to reduce some markers related to peritoneal oxidative damage during laparotomy. No difference was observed in clinical outcomes, but the study was underpowered for analysis of surgical results. Registration number: NCT02975947 (www.ClinicalTrials.gov/).

Funding information
Research Foundation Grant, Colorectal Surgical Society of Australia and New Zealand
Sir Roy McCaughey Surgical Research Scholarship, Royal Australasian College of Surgeons

Paper accepted 28 August 2019

Published online 17 November 2019 in Wiley Online Library (www.bjsopen.com). **DOI:** 10.1002/bjs5.50227

Introduction

Open abdominal surgery is performed routinely for a number of diseases; however, it can be complicated by postoperative ileus, infection, anastomotic leak and, in the long term, bowel obstruction. Open surgery exposes the intestine to ambient air (20°C, 0–5 per cent relative humidity), which, combined with operating theatre negative air ventilation, has the potential to cause serosal/peritoneal desiccation[1]. Peritoneal desiccation leads to peritoneal inflammation and loss of barrier function, and increases the risk of infection[1–4]. In addition, peritoneal inflammation can lead to adhesion formation and subsequent bowel obstruction[1,2]. Bowel desiccation may also be a factor in delaying the return of bowel function after surgery. Moreover, exposure can lead to vasoconstriction of splanchnic blood flow to the intestine. Previous studies[2–4] have indicated that desiccation and cooling of

© 2019 The Authors. *BJS Open* published by John Wiley & Sons Ltd on behalf of BJS Society Ltd
This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

the peritoneum from open surgical wounds or the use of cold, non-humidified carbon dioxide insufflation may stimulate oxidative stress in peritoneal mesothelial cells. Thus, desiccation of the peritoneum may lead to peritoneal inflammation, which may manifest as oxidative damage and reduced splanchnic blood flow, with associated long-term consequences.

One pathway for mitigating bowel desiccation is the use of humidified, warmed carbon dioxide gas. Carbon dioxide is heavier (44 g/mol) and denser (1·97 kg/m^3) than the other components of atmospheric air[5] at standard temperature and pressure. Insufflated carbon dioxide therefore tends to sink to the base of the abdominal wound. Furthermore, carbon dioxide assists in maintaining heat by creating a localized greenhouse effect within the abdominal cavity, and is readily saturated to 100 per cent with sterile water, thereby acting to inhibit bowel desiccation[1,2,4,6,7]. A recently published study[8] that examined the effect of warmed, humidified carbon dioxide in simulated open abdominal surgery in mice found that peritoneal tissue damage, as determined by cellular retraction, bulging and microvillus damage, was significantly reduced in animals receiving warmed, humidified carbon dioxide compared with that in the standard laparotomy group.

The research question of the present study was whether peritoneal damage and inflammation are elicited during open abdominal operation, and whether the use of warmed, humidified carbon dioxide inhibits peritoneal damage. The primary aim was to investigate several markers of peritoneal inflammation and oxidative damage at the beginning and end of the surgical procedures in patients treated with warmed, humidified carbon dioxide ($WHCO_2$ group) and in controls. A secondary aim was to evaluate the perioperative clinical outcomes in both groups.

Methods

A single-blind RCT was performed at a tertiary colorectal unit in Sydney (Concord Repatriation General Hospital, University of Sydney). The study group received warmed (37°C), humidified (98 per cent relative humidity) carbon dioxide gas. The control group received current standard practice, and no gas was insufflated into the open laparotomy wound.

Inclusion criteria

Patients recruited into the study were aged more than 18 years and scheduled for elective resection via a midline laparotomy. Surgical indications included: potentially curable colorectal carcinoma, polyposis syndrome, diverticular disease, rectal prolapse and inflammatory bowel disease. Patients were excluded if they had emergency surgery, laparoscopic surgery and/or presented with chronic obstructive pulmonary disease (COPD) requiring home oxygen, were carbon dioxide retainers, or if it was determined that their forced expiratory volume in 1 s (FEV1) was less than 1 litre, or predicted FEV1/forced vital capacity ratio was below 50 per cent.

Primary and secondary outcomes

The primary outcome measure was an increased degree of peritoneal inflammation and damage from the beginning to the end of operation. This included changes in levels of inflammatory cytokines, measurement of peritoneal tissue apoptosis and oxidative damage, visualization of the injury to peritoneum via light microscopy and immunofluorescence. A secondary outcome measure was the perioperative clinical result.

Patient cohort and randomization

Patients undergoing elective surgery at Concord Hospital were eligible for the study. Ethics board approval was obtained and the trial was registered in ClinicalTrials.org (NCT02975947). All patients were screened before surgery, and the trial rationale and procedure were explained carefully. Patients were then given an 'opt in' participant information sheet, after which written informed consent was obtained before final enrolment. Patients were randomized to the intervention and control arm by random sequence, generated using an online tool (www.random.org)[9]. Patient allocation to specific groups was concealed in opaque numbered envelopes and kept in a central location to be opened at the time of surgery. Both patients and investigators performing the analyses were blinded to the allocation.

The humidification system

HumiGard™ (Fisher & Paykel Healthcare, Auckland, New Zealand) was used to deliver warmed, humidified carbon dioxide with the gas diffuser positioned at the upper end of the laparotomy wound at a depth of approximately 4 cm from skin after the wound retractors had been placed. Insufflation of carbon dioxide was continued until the laparotomy wound had been closed. The carbon dioxide was delivered at a rate of 10 l/min, at a pressure of 4·5 bar from pressurized gas cylinders, and then passed through the HumiGard™ system.

Clinical records

Details of patient demographics (age, sex, co-morbidity, level of mobility, BMI and ASA grade) and intraoperative

© 2019 The Authors.
BJS Open published by John Wiley & Sons Ltd on behalf of BJS Society Ltd

information including the procedure performed, duration of surgery, number of assistants, duration and volume of carbon dioxide administered, intraoperative blood transfusion, intraoperative complications, allocation to an enhanced recovery after surgery (ERAS) protocol, and stoma formation were collected and used for statistical analysis. Core body temperature was measured throughout the operation using a transoesophageal probe.

Specimen collection and treatment

Two peritoneal biopsies (1 cm^2) were taken at the beginning (T_0) and end of the operation before wound closure (T_{end}) from the hepatorenal angle. All specimens were stored in 1·5-ml capped tubes (Eppendorf, Hamburg, Germany), snap-frozen in liquid nitrogen and stored at −80°C until required for biochemical or histological assessment. One of the two T_0 and T_{end} specimens was homogenized. Samples were thawed at 20°C and the tissue was transferred into a glass tube containing 1 ml of buffer A, which consisted of: phosphate-buffered saline (Sigma-Aldrich, Sydney, Australia), cOmplete™ EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich), EDTA (Sigma-Aldrich) and the water-miscible phenolic antioxidant butylated hydroxyl toluene (Acros Organics, Morris Plains, New Jersey, USA) to inhibit the artificial oxidative tissue processes. Preparations of buffer A were used for a maximum of 3 months, and subsequently replaced with freshly prepared solutions to ensure optimal antioxidant activity of constituents.

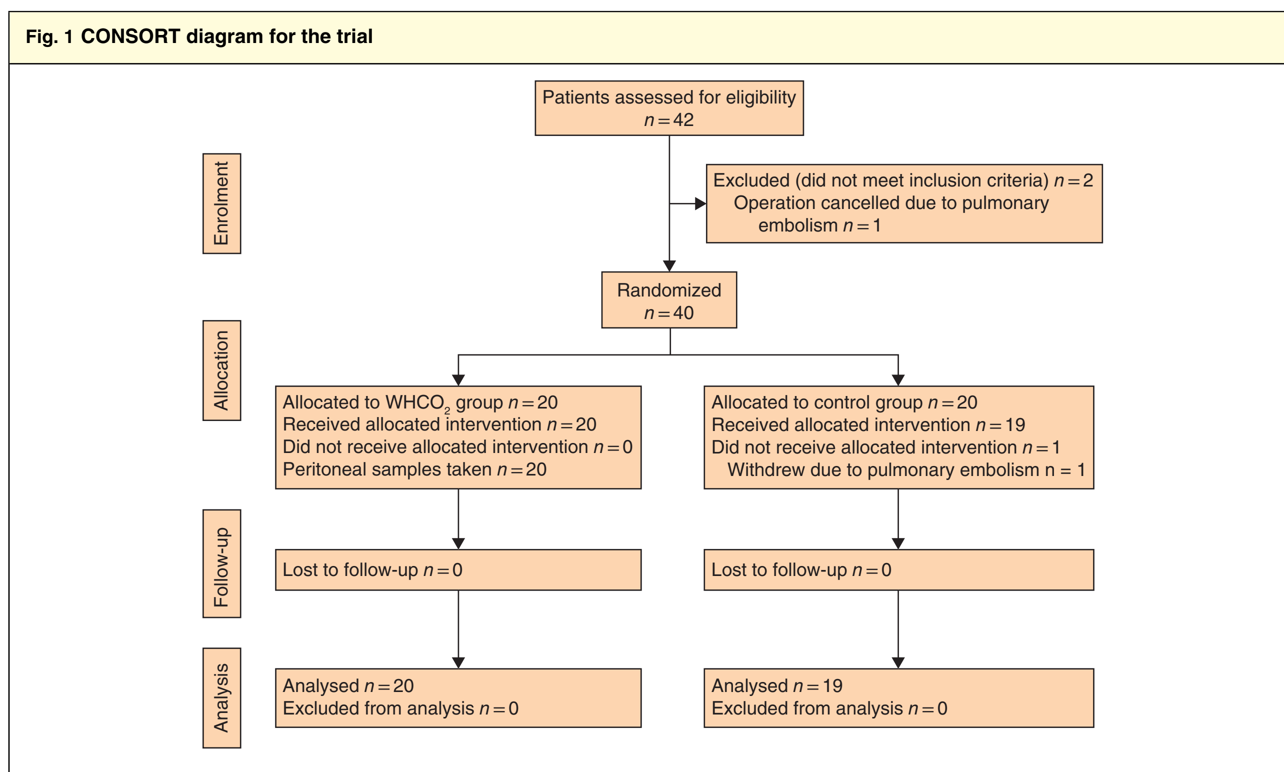
The tissue was homogenized using a matched rotating piston (set to operate at 500 r.p.m.) and matching Teflon (DuPont, Stevenage, UK)-coated glass tube, as described previously for human[10] and animal[11] tissues. The specimen was ground by the rotating piston initially for 1 min, cooled on ice (4°C) and then homogenized for a further 1 min. Finally, the tissue homogenates were aliquoted into 1·5-ml capped tubes and stored at −80°C until required for biochemical analysis.

Markers of peritoneal inflammation and oxidative damage

Tissue cytokines and chemokines

Levels of inflammatory cytokines and chemokines were measured using a commercial enzyme-linked immunosorbent assay kit (Human cytokine (16 plex)/chemokine (9 plex) – Stripwell Chemiluminescent kit; Quansys Biosciences, Logan, Utah, USA) according to the manufacturer recommendations[12,13]; each peritoneal

Fig. 1 CONSORT diagram for the trial



$WHCO_2$, warmed, humidified carbon dioxide.

© 2019 The Authors.
BJS Open published by John Wiley & Sons Ltd on behalf of BJS Society Ltd

Table 1 Demographics of patients in the carbon dioxide and control groups

	Control group (n = 19)	Carbon dioxide group (n = 20)	P*
Age (years)			
Mean	60·5	61·4	0·863†
Median (i.q.r.)	55·0 (48·0–77·5)	61·5 (56·5–70·7)	
Sex ratio (M : F)	(12 : 7)	(12 : 8)	0·839
BMI (kg/m^2)			
Mean	27·7	25·4	0·334†
Median (i.q.r.)	27·6 (21·1–34·4)	23·0 (21·0–26·6)	
BMI > 25 kg/m^2	10	7	0·130
IHD	2	4	0·339
COPD	0	1	0·299
Mean eGFR (ml per min per 1·73 m^2)	73·4	81·9	0·124†
Diabetes mellitus	4	5	0·640
Hypertension	7	10	0·267
Any co-morbidity	8	12	0·148
No. of co-morbidities			
Mean	0·7	1·1	0·332†
Median (i.q.r.)	0 (0–1·5)	1 (0–2)	
ASA grade			0·600
I–II	7	9	
III–IV	12	11	
Preoperative haemoglobin level (g/dl)			
Mean	130·7	125·1	0·485†
Median (i.q.r.)	137 (116·5–151·0)	128 (117·5–136·5)	
Preoperative INR			
Mean	1·0	1·1	0·562†
Median (i.q.r.)	1 (1·0–1·2)	1 (1·0–1·1)	
Anticoagulants	4	5	0·770
Smoker	6	3	0·219
Mobility (4 flights of stairs)	14	12	0·365
Pathology/indication for surgery			
Malignant disease	6	12	0·075
Benign disease	13	8	
ECF/parastomal hernia	3	1	
Reversal stoma	3	5	
IBD	4	2	
Diverticular disease	1	0	
Bowel fistula	2	0	

IHD, ischaemic heart disease; COPD, chronic obstructive pulmonary disease; eGFR, estimated glomerular filtration rate; INR, international normalized ratio; ECF, enterocutaneous fistula; IBD, inflammatory bowel disease. *Pearson's χ^2 test, except †Student's t test.

homogenate was assayed in duplicate. The analysis required a chemiluminescent imaging platform (ChemiDoc™ XRS; Life Science Research, Bio-Rad Laboratories, Hercules, California, USA) to image the multiplex assay plates. Quantitative analysis was performed using Q-View™ Software (Quansys Biosciences). Measurements were made of the mean fold increase in cytokine/chemokines from the samples taken at T_0 and T_{end}. The total protein concentration in each homogenate was determined using bicinchoninic acid protein analysis, and used to normalize all biochemical parameters in the corresponding homogenate. The mean T_0 value in the control group was used as a reference point to calculate the fold increase in other groups (control group T_{end}, $WHCO_2$ group T_0 and T_{end}). In addition, to compare the change in level of cytokines between $WHCO_2$ and control groups, the log of T_{end}/T_0 was used. T_{end} values were divided by T_0 values, then log (base 2) was applied and the values for the two groups were compared.

© 2019 The Authors.
BJS Open published by John Wiley & Sons Ltd on behalf of BJS Society Ltd

Apoptosis

Cell apoptosis was measured by detection of active caspase-3/7 bioluminescence, assayed in the stored tissue homogenates with Caspase-Glo® 3/7 Assay (Promega, Madison, Wisconsin, USA)[14]. The $\log(T_{end}/T_0)$ values for $WHCO_2$ and control groups were compared. A second marker of cell viability was determined using the DeadEnd™ Fluorometric TUNEL System (Promega). The extent of DNA fragmentation (a surrogate marker for apoptosis) was quantified by the measurement of green fluorescence intensity with fluorescence microscopy. A counterstain using DAPI (4′-6-diamidino-2-phenylindole) was performed and detected as blue fluorescence. Images were captured using Zeiss Axio AX10 light microscopy (Carl Zeiss, Oberkochen, Germany) with fluorescence camera AxioCam ICm 1 (Carl Zeiss). TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling)-stained images were reviewed by five scientists/pathologists independently, who were all blinded to sample identity, and the results analysed.

Estimation of peritoneal protein damage

To assess peritoneal protein damage, the level of 3-chlorotyrosine (Cl-Tyr) and total native tyrosine (Tyr) in the peritoneal homogenates was measured by high-pressure liquid chromatography with mass spectrometry (HPLC-MS). Halogenated molecules can serve as specific markers for pathological oxidation as a limited number of reactive hypohalous acids participate in protein oxidation, including hypochlorous, hypobromous and hypothiocyanous acids[15–17]. The biological oxidant and antimicrobial agent hypochlorous acid is generated by myeloperoxidase[18]. Recent studies have demonstrated that the Cl-Tyr/Tyr ratio is a specific marker for myeloperoxidase-catalysed chlorination at sites of inflammation[15]. The relative ratio of Tyr and Cl-Tyr were calculated for each patient sample at T_0 and T_{end}, and results for the control and $WHCO_2$ treatment groups were compared. Proteins in the tissue homogenates were hydrolysed to individual free amino acids[19]. Hydrolysed analytes were then purified by solid-phase extraction and analysed on an Agilent 1290 series UHPLC system tandem with 6460A triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, California, USA). Analytes within the mixture were separated using an Agilent Zorbax Eclipse XDB-C18 (4·6 × 5 mm, 1·8 μm) column fitted with a UPLC Zorbax Eclipse XDB-C18 (4·6 × 5 mm, 1·8 μm) guard column, and with a mobile phase A containing 0·1 per cent formic acid (v/v) and mobile phase B containing 0·1 per cent formic acid (v/v) in 90 per cent acetonitrile and 10 per cent water (v/v). The gradient was started at 2 per cent of mobile phase B and increased to 10 per cent at 3 min, continuing to increase to 95 per cent over 4 min and maintained for 1 min, and then returned to 2 per cent at 15 min for 1 min of equilibration. Tandem mass spectrometry was performed using electrospray ionization equipped with jet stream technology in the positive mode. The gas temperature was optimized at 350°C with a flow of 12 l/min, and the sheath gas was at 375°C with a flow of 11 l/min. Capillary voltage was 3500 MeV, and the nebulizer pressure was 25 p.s.i. All analytes were detected in multiple reaction monitoring mode with fragment voltage at 135 V, using nitrogen as the collision gas. For each analyte, one quantitative transition and one qualitative transition were monitored, including the internal standard. Data acquisition was performed using MassHunter B.07.01 (Agilent Technologies), and data analysis was

Table 2 Surgical procedures performed

	Control group	Carbon dioxide group
Rectal resection	8	4
Ileocolic resection	3	4
Small bowel resection	1	1
Total or subtotal colectomy	1	2
Other procedure	8	10

Some patients had more than one resection during the same operation.

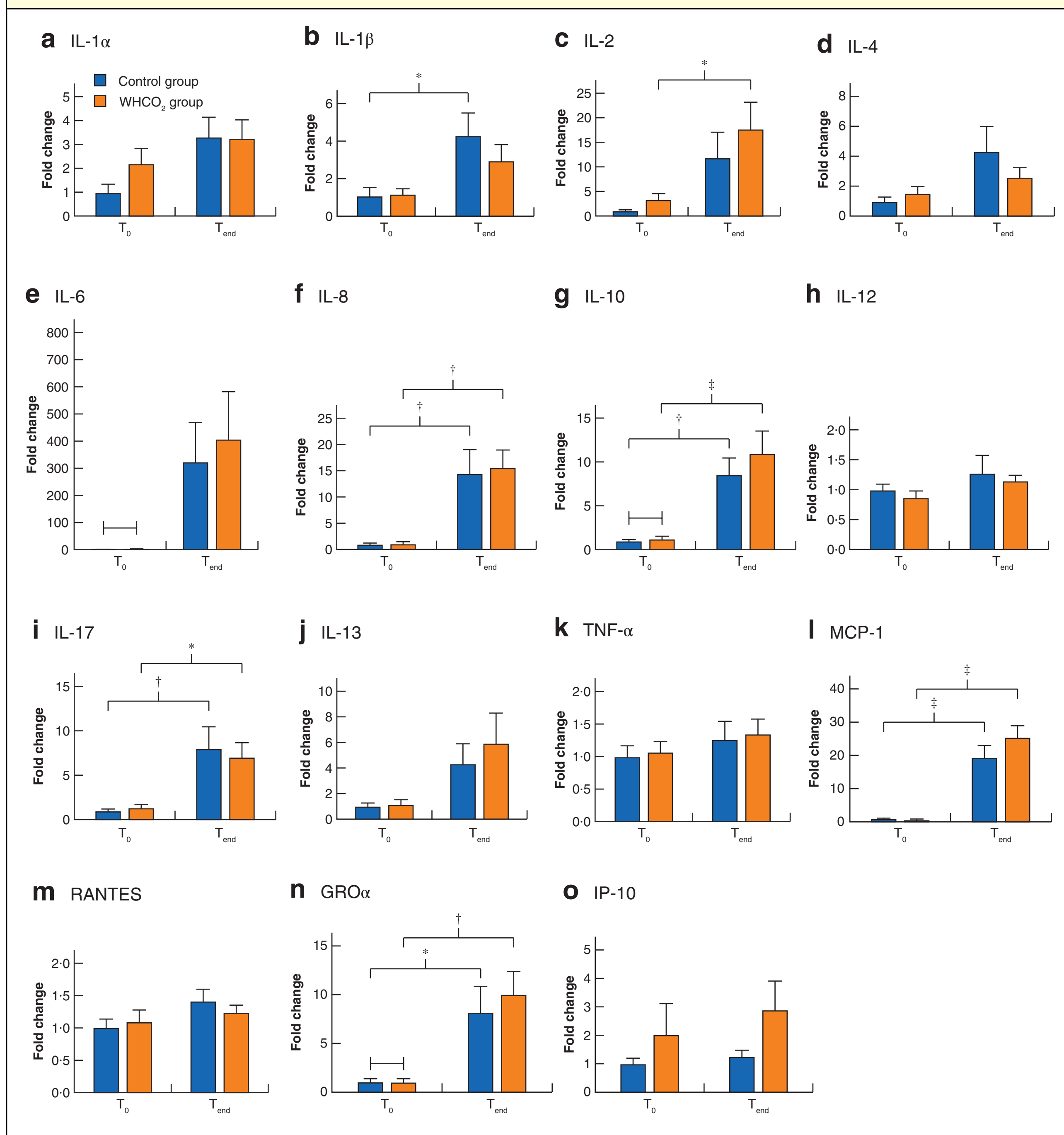
Table 3 Fold change increases in cytokine/chemokine levels

	Fold increase from T_0 to T_{end}	*P**
IL-1α	2·1	0·009
IL-1β	3·2	0·002
IL-2	6·0	< 0·001
IL-4	2·4	0·021
IL-5	3·0	0·002
IL-6	135·2	< 0·001
IL-8	13·8	< 0·001
IL-10	8·3	< 0·001
IL-12	1·3	0·120
IL-13	4·4	0·004
IL-15	4·2	0·011
IL-17	6·4	< 0·001
TNF-α	1·3	0·149
IFN-γ	1·0	0·902
MCP-1	24·2	< 0·001
RANTES	1·2	0·031
GROα	7·9	< 0·001
IP-10	1·5	0·234

IL, interleukin; TNF, tumour necrosis factor; IFN, interferon; MCP, monocyte chemotactic protein; RANTES, regulated on activation, normal T-cell expressed and secreted; GRO, growth-regulated oncogene; IP, inducible protein. *Unpaired *t* test.

© 2019 The Authors.
BJS Open published by John Wiley & Sons Ltd on behalf of BJS Society Ltd

Fig. 2 Change in levels of cytokines/chemokines in carbon dioxide and control groups



Fold change in inflammatory cytokines and chemokines at the start (T_0) and end (T_{end}) of the operation in control and warmed, humidified carbon dioxide ($WHCO_2$) groups: **a** interleukin (IL) 1α; **b** IL-1β; **c** IL-2; **d** IL-4; **e** IL-6; **f** IL-8; **g** IL-10; **h** IL-12; **i** IL-17; **j** IL-13; **k** tumour necrosis factor (TNF) α; **l** monocyte chemotactic protein (MCP) 1; **m** RANTES (regulated on activation, normal T-cell expressed and secreted); **n** growth-regulated oncogene (GRO) α; **o** inducible protein (IP) 10. Values are mean(s.e.m.). $*P < 0{\cdot}050$, $\dagger P < 0{\cdot}010$, $\ddagger P < 0{\cdot}001$ (ANOVA using Tukey's multiple comparison test).

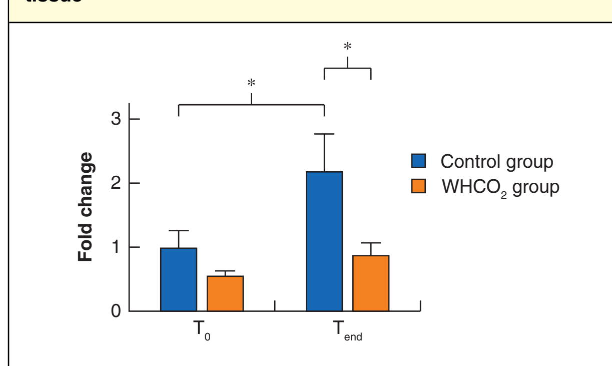
© 2019 The Authors.
BJS Open published by John Wiley & Sons Ltd on behalf of BJS Society Ltd

Table 4 Comparison of increase in cytokine/chemokines measured using $\log(T_{end}/T_0)$

	$Log(T_{end}/T_0)$		
	Control group	Carbon dioxide group	*P**
IL-1α	2·6	1·4	0·178
IL-1β	3·3	1·0	0·178
IL-2	5·3	2·8	0·028
IL-4	3·5	2·0	0·041
IL-5	2·7	3·4	0·990
IL-6	8·6	7·4	0·363
IL-8	5·0	4·1	0·805
IL-10	3·4	3·8	0·635
IL-12	0·9	1·4	0·691
IL-13	2·7	3·5	0·539
IL-15	3·7	4·3	0·675
IL-17	5·0	3·2	0·244
TNF-α	1·6	1·3	0·973
IFN-γ	0·7	2·1	0·417
MCP-1	4·0	4·8	0·112
RANTES	0·4	0·4	0·961
GROα	3·1	3·8	0·258
IP-10	0·4	0·9	0·232

IL, interleukin; TNF, tumour necrosis factor; IFN, interferon; MCP, monocyte chemotactic protein; RANTES, regulated on activation, normal T-cell expressed and secreted; GRO, growth-regulated oncogene; IP, inducible protein. *Mann–Whitney *U* test.

Fig. 3 Change in degree of apoptosis in isolated peritoneal tissue



Fold change in caspase-3/7 activity in control and warmed, humidified carbon dioxide ($WHCO_2$) groups at the start (T_0) and end (T_{end}) of the operation. Values are mean(s.e.m.). *$P \leq 0{\cdot}050$ (ANOVA using Tukey's multiple comparison test).

conducted using the accompanied MassHunter Qualitative and Quantitative B.07.00 (Agilent Technologies).

Light microscopy

Peritoneal architecture was examined by light microscopy after staining with haematoxylin and eosin. This approach has been used previously to assess peritoneal effacement[20–24]. Images were obtained using a Zeiss Axio AX10 light microscope (Carl Zeiss) with a digital camera output (Zeiss Axiocam 105 color; Carl Zeiss). The stained images were assessed independently by five pathologists at the University of Sydney Medical School, all blinded to sample assignment; their results were combined, analysed and quantified.

Postoperative outcomes

Postoperative outcomes, including postoperative pain (morphine equivalent daily dose score), duration of patient-controlled analgesia use (measured in days), duration of hospital stay (measured in days), time to return to bowel function (flatus, stool), commencement of diet, clinical complications (persistent ileus (defined as: failure of the patient to eat, pass flatus or evacuate the bowel within 5 days of laparotomy), anastomotic leak, wound infection, unexpected return to theatre, unexpected readmission, and complications, graded according to Clavien–Dindo classification[25]) were collated and analysed. Patients were followed up for 60 days from the date of discharge, by telephone interviews as well as in day clinics.

Statistical analysis

Sample size calculations were performed using the standard deviation of the measure of oxidative stress, Cl-Tyr[26–29]. For a power of 80 per cent, at 5 per cent two-sided significance, the sample size was 40 patients.

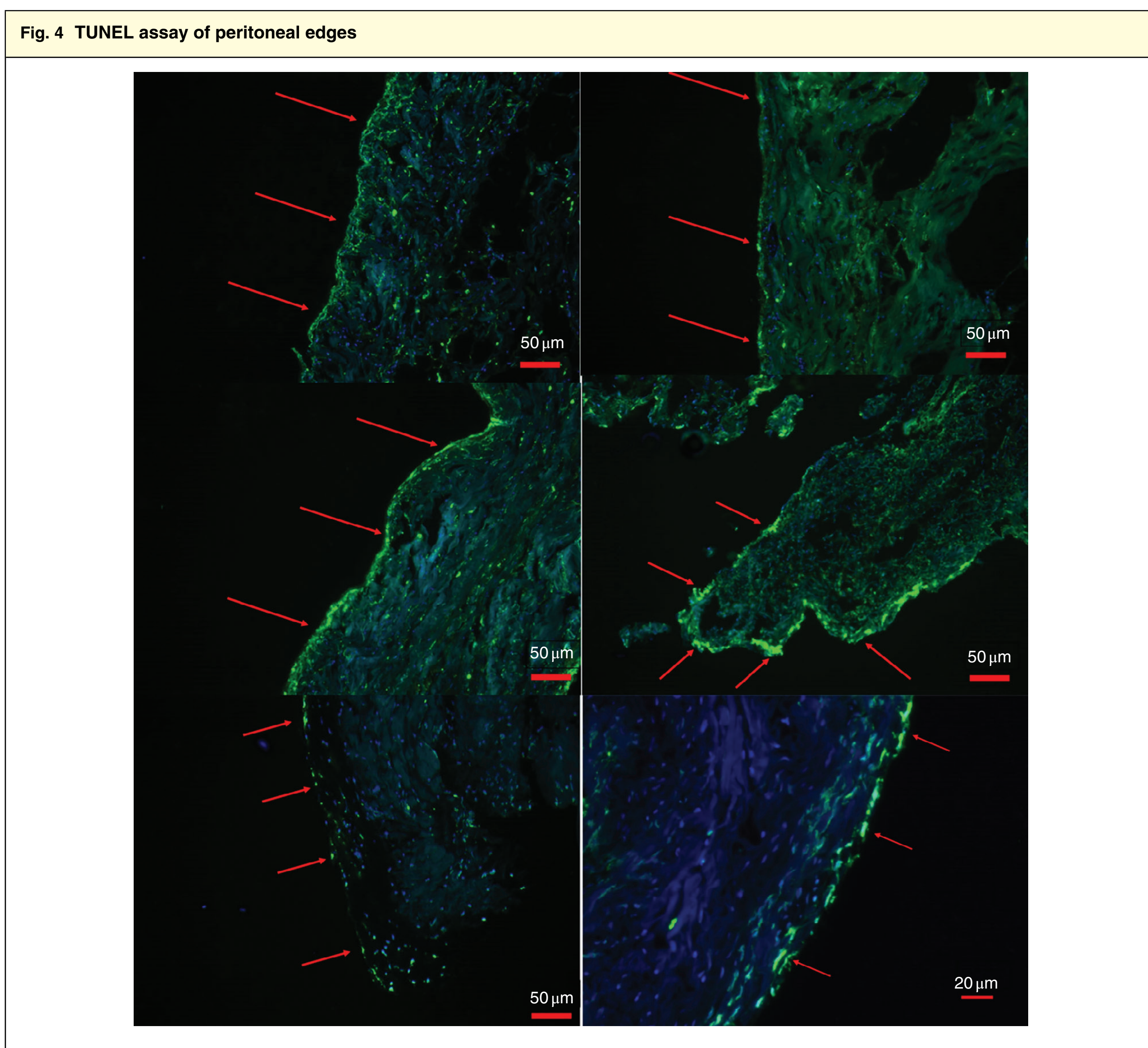
Data were analysed using IBM SPSS® version 23 (IBM, Armonk, New York, USA) and GraphPad Prism® version 7.0 (GraphPad Software, La Jolla, California, USA). Continuous variables were tested using the D'Agostino–Pearson test. Groups were compared with *t* tests or two-way ANOVA, using Tukey's multiple comparison test for parametric continuous variables and the Mann–Whitney *U* test for non-parametric continuous variables (tissue cytokines and chemokines, and apoptosis assays). The level of significance for all tests was set at $P < 0{\cdot}050$.

Results

Between February 2013 and December 2016, 40 patients were recruited for the trial and randomly allocated into two groups, each of 20 subjects. However, one patient assigned to the control group withdrew from the study due to preoperative pulmonary embolism, leaving 20

© 2019 The Authors.

Fig. 4 TUNEL assay of peritoneal edges



Red arrows indicate areas of fluorescence green (apoptosis/necrosis) along the peritoneal edge. TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling.

patients in the $WHCO_2$ group and 19 in the control group (*Fig. 1*). All data for the 39 patients were collected.

Demographics

Of the 39 patients enrolled in the study, 15 were women and the mean age was 60·9 (95 per cent c.i. 55·9 to 66·0; range 32–87) years (*Table 1*). Mean BMI was 26·5 (95 per cent c.i. 24·1 to 28·9; range 14·2–43·1) kg/m^2. Seventeen patients were overweight or obese (BMI above 25 kg/m^2). Nine patients were smokers. There were no significant differences between the groups in age, sex, obesity, background medical history (including ischaemic heart disease, diabetes, hypertension, COPD and chronic renal failure), ASA fitness grade, smoking status, mobility, preoperative haemoglobin level and coagulation level (measured as the international normalized ratio). Eighteen patients had surgery for malignancy, six in the control group and 12 in the $WHCO_2$ group, but there was no difference in the indication for surgery ($P = 0·075$).

© 2019 The Authors.
BJS Open published by John Wiley & Sons Ltd on behalf of BJS Society Ltd

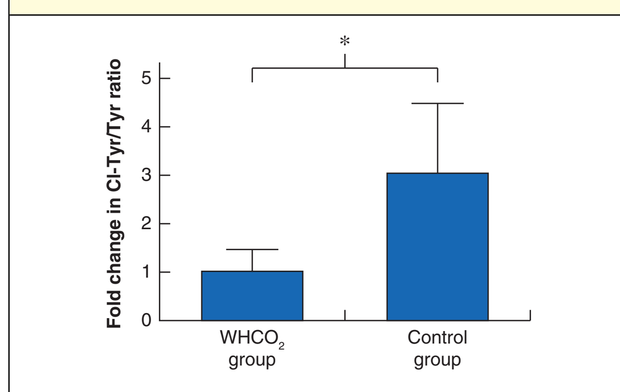
Surgical procedures

Procedures included abdominoperineal, high anterior, low anterior and ultralow anterior resections (*Table 2*). Other procedures included reversal of Hartmann's procedure, repair of enterocutaneous fistula, repair of parastomal hernia, pelvic exenteration, and resection of retroperitoneal colorectal cancer recurrence.

Inflammatory cytokines and chemokines

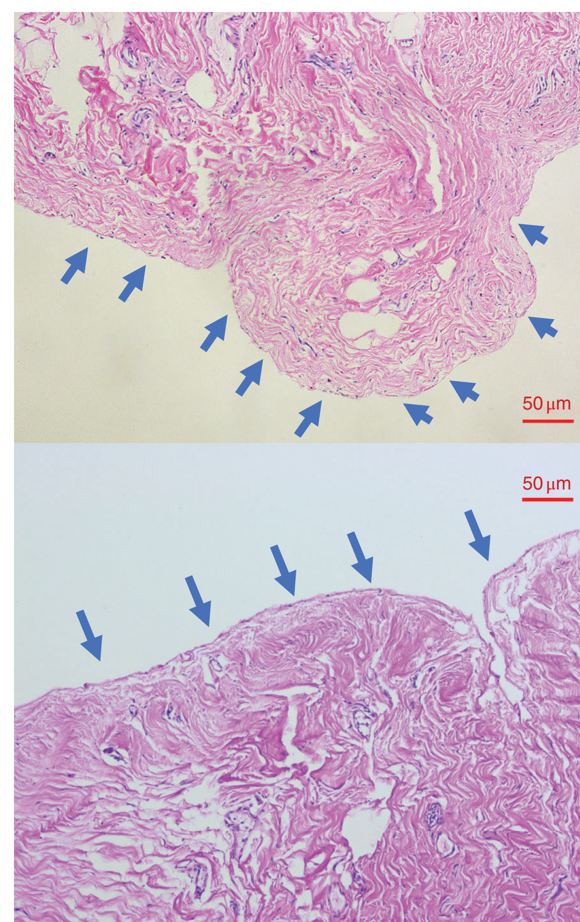
Levels of inflammatory cytokines and chemokines increased from the beginning to the end of the operation. When $WHCO_2$ and control groups were combined and analysed together, a significant increase was observed from T_0 to T_{end} for interleukin (IL) 1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-15, IL-17, monocyte chemotactic protein (MCP) 1, RANTES (regulated on activation, normal T-cell expressed and secreted) and growth-regulated oncogene (GRO) α (*Table 3*). There was no significant difference between the groups in any cytokine/chemokine level at T_0 (*Fig. 2*). In the control group, a significant increase was seen from T_0 to T_{end} in IL-1β (4·2-fold; $P = 0·026$), IL-8 (14·5-fold; $P = 0·006$), IL-10 (8·5-fold; $P = 0·006$), IL-17 (8·0-fold; $P = 0·006$), MCP-1 (19·3-fold; $P < 0·001$) and GROα (8·2-fold; $P = 0·037$). In the $WHCO_2$ group, there were significant increases for IL-2 (from 3·5- to 17·8-fold; $P = 0·047$), IL-8 (from 1·2- to 15·5-fold; $P = 0·004$), IL-10 (from 1·3- to 11-fold; $P = 0·004$), IL-17 (from 1·4- to 7·1-fold; $P = 0·045$), MCP-1 (from 0·9- to 25·4-fold; $P < 0·001$) and GROα (from 1·0- to 10·0-fold; $P = 0·003$) (*Fig. 2*). In contrast, comparison of the fold change in inflammatory cytokines and chemokines for the control and $WHCO_2$ groups using $\log(T_{end}/T_0)$ found statistically significant differences only for IL-2 (5·3 *versus* 2·8; $P = 0·028$) and IL-4 (3·5 *versus* 2·0; $P = 0·041$) (*Table 4*).

Fig. 5 **Change in level of oxidative damage in control and carbon dioxide groups**



Fold change in 3-chlorotyrosine/tyrosine ratio (Cl-Tyr/Tyr) from the start (T_0) to the end (T_{end}) of the operation in warmed, humidified carbon dioxide ($WHCO_2$) and control groups. Values are mean(s.e.m.). *$P < 0·050$ (Mann–Whitney *U* test).

Fig. 6 **Visible peritoneum**



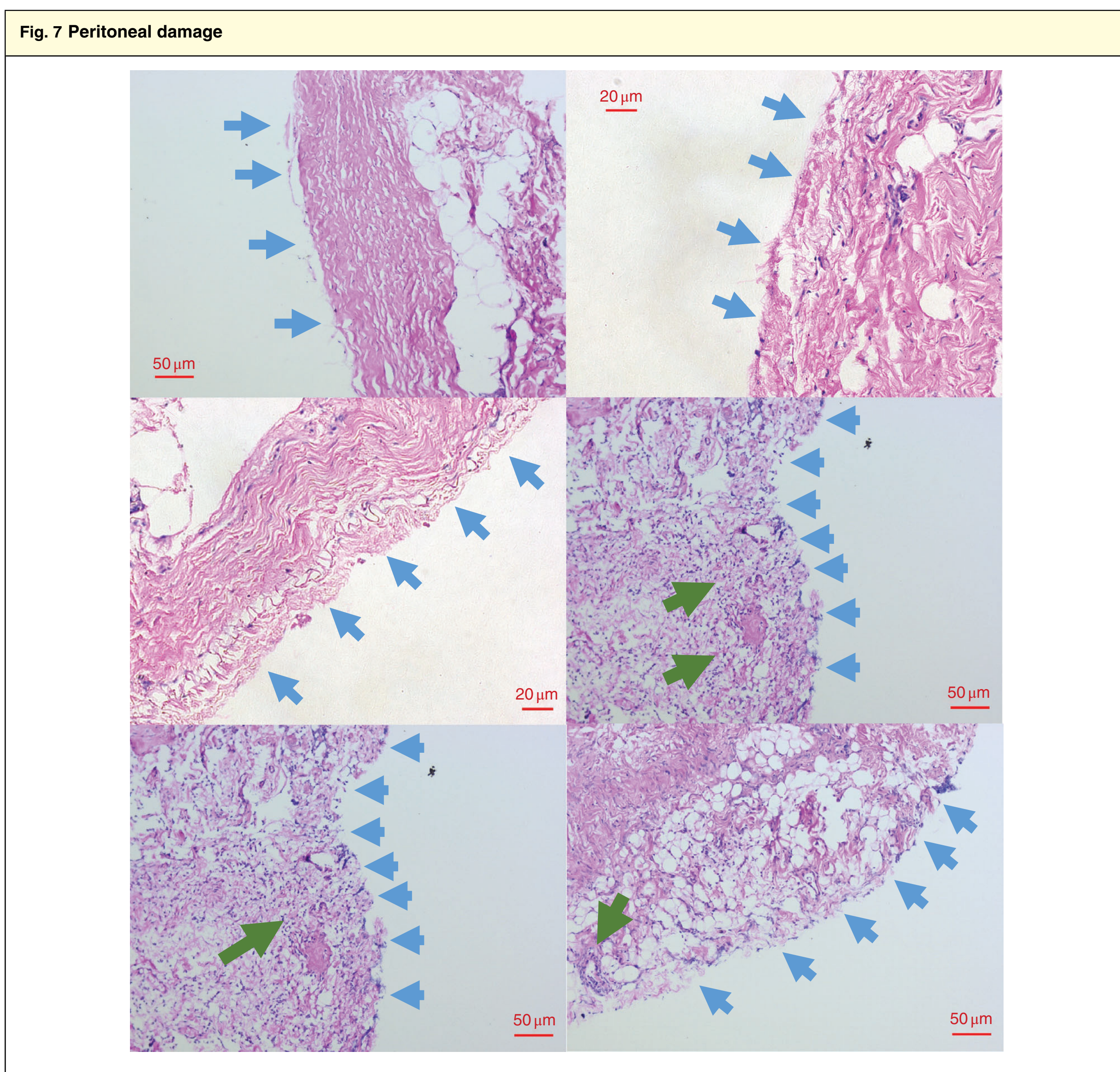
Haematoxylin and eosin-stained section showing visible peritoneal edge (blue arrows).

Apoptosis assays

Apoptosis showed a twofold increase (95 per cent c.i. 1·1 to 2·9; $P = 0·028$) in the combined data cohort from T_0 to T_{end}. When considered separately, the control group showed a borderline increase in caspase-3/7 activity ($P = 0·050$), whereas there was no significant change in caspase activity for the $WHCO_2$ group ($P = 0·883$) (*Fig. 3*). However, analysis of the change in caspase-3,7 activity by $\log(T_{end}/T_0)$ revealed no difference between control and $WHCO_2$ groups (0·5 *versus* 1·2 respectively; $P = 0·120$).

© 2019 The Authors.

Fig. 7 Peritoneal damage



Haematoxylin and eosin-stained sections showing denudation of the peritoneum (blue arrows). The last three images also show white cell infiltration of the peritoneum (green arrows).

Fig. 4 shows a diffuse green fluorescence and DAPI staining along the peritoneal edges of the section. In the combined cohort, cells comprising the peritoneal edges showed evidence of apoptosis in five of the 39 samples at T_0, increasing to 23 of all 39 samples at T_{end}. Comparison of the control and $WHCO_2$ groups at T_{end} demonstrated that green fluorescence indicative of apoptosis was visible along the peritoneal edge in nine of the 19 control samples, a proportion slightly lower than the ten of 20 seen for the $WHCO_2$ group ($P = 0{\cdot}869$).

Tissue 3-chlorotyrosine/tyrosine ratio

The mean level of chlorinated tyrosine residues (as expressed by the Cl-Tyr/Tyr ratio) was 4·3 for the $WHCO_2$ group at T_0, decreasing to 3·1 at T_{end}. The corresponding evaluation for the control group revealed an increase in the ratio, from 2·3 at T_0 to 3·6 at T_{end}.

The relative levels of Cl-Tyr/Tyr at T_{end} were re-expressed as a fraction of those at T_0: 3·1 (95 per cent c.i. 0·0 to 6·1) in the control group *versus* 1·1 (0·2 to 1·9)

© 2019 The Authors.
BJS Open published by John Wiley & Sons Ltd on behalf of BJS Society Ltd

Table 5 Summary of intraoperative and perioperative outcomes

	Total (*n* = 39)	Control group (*n* = 19)	Carbon dioxide group (*n* = 20)	*P*†
Duration of surgery (min)*	288·6	284·1	292·9	0·851‡
No. of assistants*	2·1	1·8	2·3	0·272‡
Duration of exposure to carbon dioxide (min)*		–	203·7	–
Volume of carbon dioxide (litres)*		–	2037	–
Intraoperative blood transfusion	7	1	6	0·044
Intraoperative complications	7	2	5	0·239
ERAS protocol	13	7	6	0·651
Stoma formation	14	4	10	0·060
End colostomy		1	3	
Loop ileostomy		2	4	
End ileostomy		1	3	
Duration of hospital stay (days)*	15·9	15·1	16·6	0·760‡
Time to passage of flatus (days)*	3·7	5·0	2·5	0·008‡
Time to passage of stool (days)*	4·6	5·5	3·7	0·092‡
Commenced on clear/free fluids (days)*	3·4	4·3	2·5	0·077‡
Commenced on light diet (days)*	5·9	6·9	4·9	0·125‡
Unexpected return to theatre	7	4	3	0·622
Unexpected readmission	7	3	4	0·732
Postoperative ileus	11	8	3	0·074
30-day mortality	0	0	0	–
Wound infection	9	6	3	0·219
Anastomotic leak	1	0	1	–
Clavien–Dindo complication grade				
I	21	12	9	0·256
II	8	4	4	0·935
III–IV	9	4	5	0·770

*Values are mean. ERAS, enhanced recovery after surgery. †Pearson's χ^2 test, except ‡Student's *t* test.

in the $WHCO_2$ group ($P = 0{\cdot}036$) (*Fig. 5*). Accordingly, for the $WHCO_2$ group the Cl-Tyr/Tyr ratio had increased by 1·1-fold at T_{end}, compared with the corresponding ratio measured at T_0, whereas for the control group the ratio increased by a higher 3·1-fold change from T_0 to T_{end}.

Light microscopy

Representative haematoxylin and eosin-stained peritoneal images are shown in *Figs 6* and *7*. *Fig. 6* shows visible peritoneum, whereas *Fig.* 7 shows denudation of the peritoneum with evidence of recruitment and infiltration of leucocytes. In the combined total cohort, peritoneum was visible in 36 of the 39 specimens collected at T_0, whereas identification of visible peritoneum decreased to 30 of 39 at T_{end}. By contrast, comparison of the control and $WHCO_2$ groups at T_{end} revealed that peritoneum was visible in only 11 of 19 samples from the control group *versus* 19 of 20 samples in the $WHCO_2$ group ($P = 0{\cdot}006$).

Surgical outcome

There were no significant differences between the control and $WHCO_2$ groups with respect to duration of surgery, number of surgical assistants, intraoperative complications, stoma formation or number of patients assigned to the ERAS protocol (*Table 5*). Intraoperative blood transfusion was significantly greater in the $WHCO_2$ group (6 of 20 patients *versus* 1 of 19 in the control group; $P = 0{\cdot}044$).

For the whole cohort, the mean duration of hospital admission was 15·9 (median 11, 95 per cent c.i. 10·6 to 21·2; range 3–77) days. Postoperative ileus occurred in 11 of the 39 patients. Wound infection developed in nine patients, and one of 30 patients with a bowel anastomosis subsequently developed an anastomotic leak. Seven patients had unexpected readmission after discharge from hospital: two for wound infection, one for a small bowel obstruction, one for perforated stomach, one for constipation due to narrowed colorectal anastomosis, one because of high-output ileostomy dysfunction and one for a pelvic

© 2019 The Authors.
BJS Open published by John Wiley & Sons Ltd on behalf of BJS Society Ltd

collection requiring drainage. No 30-day mortality was reported (*Table 5*).

There was no difference between the two groups in duration of hospital stay or postoperative complications including wound infection, anastomotic leak, unexpected return to operating theatre and unexpected hospital readmission (*Table 5*). Patients in the $WHCO_2$ group passed flatus significantly earlier than those in the control group (mean 2·5 *versus* 5·0 days respectively; $P = 0{\cdot}008$).

Discussion

Inflammation is a tightly controlled process with factors that are proinflammatory and anti-inflammatory working simultaneously[30]. Whether localized peritoneal damage will promote a cascade of inflammation to the remaining bowel peritoneum is unclear; however, studies have shown that peritoneal damage is a feature occurring in animals and humans undergoing laparoscopy/laparotomy[30].

Human and animal studies have found discrepant results regarding the effect of warmed, humidified carbon dioxide on peritoneal inflammation[30]. In laparoscopic human studies, it was reported[31] that peritoneal damage was less with the use of warmed, humidified carbon dioxide gas insufflation, but another study[32] documented no difference. Similarly, animal-based studies have shown that use of warmed, humidified carbon dioxide resulted in less peritoneal damage and desquamation[21,22,33], whereas other studies found no effect[23,34,35]. A recently published animal-based study[8] comparing the effect of warmed, humidified carbon dioxide during open abdominal surgery in mice *versus* passive airflow found significantly lower peritoneal tissue damage (as determined by cellular retraction, bulging, microvilli loss) in the $WHCO_2$ group. In addition, peritoneal hypoxia, as measured by hypoxia-inducible factor 1α concentration, was significantly lower in the $WHCO_2$ group. This study[8] also found that core body temperature was maintained better in the $WHCO_2$ group.

The present study evaluated oxidative stress on the peritoneum during laparotomy by measuring Cl-Tyr as a fraction of native tyrosine. Oxidative stress during laparoscopy has been described previously[36], and has been suggested to be due in part to the pneumoperitoneum leading to an increase in intra-abdominal pressure contributing to organ ischaemia with resultant formation of reactive oxygen species (ROS). These ROS then damage the membrane phospholipids, proteins and DNA, resulting in cellular injury. Other authors[36] have hypothesized that, although raised intra-abdominal pressure during laparoscopy (with resultant insufflation–deflation causing ischaemia–reperfusion injury) is contributory to oxidative stress, other factors could also contribute and interplay, such as anaesthesia, the surgical insult itself, patient position and the type of gas used. In addition, during laparotomy, where the peritoneum is exposed to the atmospheric conditions, factors contributing to oxidative stress could be desiccation, surgical insult and anaesthesia. Another systematic review[37] found that the increase in oxidative stress was less in the laparoscopic than in the laparotomy group, despite both groups showing a marked increase in plasma levels of markers of oxidative stress. However, most of these studies were based on plasma-based measurement of oxidative stress; only a few studies measured oxidative stress in tissue specimens (including tissue malondialdehyde concentration and gastric mucosal pH).

The reason for the greater oxidative stress observed in open surgery compared with laparoscopic procedures remains unclear. A possible explanation could be in relation to peritoneal dessication, with peritoneal dessication and activation of neutrophils leading to ROS damage to the peritoneum, which may be more marked in open surgery.

An important question derived from this study is whether the positive outcome of warmed, humidified carbon dioxide is sufficiently rigorous to demand translation into clinical practice. In this study the carbon dioxide treatment group achieved earlier passage of flatus, although the procedures were heterogeneous. Overall, the postoperative outcomes in the two groups were comparable and no other significant difference was noted. It would be interesting to note in future whether use of warmed, humidified carbon dioxide would result in a lower rate of adhesion formation. However, this study was neither designed nor powered to detect differences in adhesion formation between the intervention and control groups, nor to detect clinical difference.

Nonetheless, this trial in humans can act as a pilot for a larger study, as there were no significant technical drawbacks to the use of low-level carbon dioxide infusion. Placement of the diffuser did not interfere with the operation and therefore had no impact on duration of surgery. Future studies involving warmed, humidified carbon dioxide could investigate its effect on intraoperative splanchnic blood flow. Warmed, humidified carbon dioxide would raise local temperature, prevent heat loss through convection, and lower local pH, thereby increasing bowel perfusion. This would be especially beneficial when bowel anastomosis was being performed, as adequate perfusion is essential for anastomotic healing. Examination of the inhibition of peritoneal oxidative damage by inhibition of myeloperoxidase may be another useful study.

© 2019 The Authors.
BJS Open published by John Wiley & Sons Ltd on behalf of BJS Society Ltd

Acknowledgements

The authors thank S. Maleki and R. Cheong-soos of the Department of Pathology, University of Sydney, for helping in the analysis of this study. They also thank V. Cran of Concord Repatriation General Hospital for helping to organize the study.

This study was conducted with research funding from the Royal Australasian College of Surgeons and Colorectal Surgical Society of Australia and New Zealand.

Disclosure: The authors declare no conflict of interest.

References

1 Persson M, van Der Linden J. Intraoperative CO_2 insufflation can decrease the risk of surgical site infection. *Med Hypothesis* 2008; **71**: 8–13.
2 Binder MM. Humidification during laparoscopic surgery: overview of the clinical benefits of using humidified gas during laparoscopic surgery. *Arch Gynecol Obstet* 2015; **292**: 955–971.
3 Tsuchiya M, Sato EF, Inoue M, Asada A. Open abdominal surgery increases intraoperative oxidative stress: can it be prevented? *Anesth Analg* 2008; **107**: 1946–1952.
4 van der Linden J, Persson M. CO_2 field flooding may also reduce oxidative stress in open surgery. *Anesth Analg* 2009; **109**: 683–684.
5 Reference.com. *Is CO_2 Heavier Than Air?*; 2017. https://www.reference.com/science/co2-heavier-air-b69c3fe7a671fbad?qo=contentSimilarQuestions# [accessed 12 October 2017].
6 Persson M, Flock JI, Van der Linden J. Antiseptic wound ventilation with a gas diffuser: a new intraoperative method to prevent surgical wound infection? *J Hosp Infect* 2003; **54**: 294–299.
7 Persson M, van der Linden J. Wound ventilation with carbon dioxide: a simple method to prevent direct airborne contamination during cardiac surgery? *J Hosp Infect* 2004; **56**: 131–136.
8 Carpinteri S, Sampurno S, Malaterre J, Millen R, Dean M, Kong J *et al.* Experimental study of delivery of humidified–warm carbon dioxide during open abdominal surgery. *Br J Surg* 2018; **105**: 597–605.
9 Random.org. *Randomness and Integrity Services Ltd*. https://www.random.org [accessed 3 September 2014].
10 Raitakari OT, McCredie RJ, Witting P, Griffiths KA, Letters J, Sullivan D *et al.* Coenzyme Q improves LDL resistance to *ex vivo* oxidation but does not enhance endothelial function in hypercholesterolemic young adults. *Free Radic Biol Med* 2000; **28**: 1100–1105.
11 Witting P, Pettersson K, Ostlund-Lindqvist AM, Westerlund C, Wâgberg M, Stocker R. Dissociation of atherogenesis from aortic accumulation of lipid hydro(pero)xides in Watanabe heritable hyperlipidemic rabbits. *J Clin Investig* 1999; **104**: 213–220.
12 Trune DR, Larrain BE, Hausman FA, Kempton JB, MacArthur CJ. Simultaneous measurement of multiple mouse ear proteins with multiplex ELISA assay. *Hear Res* 2011; **275**: 1–7.
13 Quansys Biosciences. *Q-Plex™ Manuals: Human Multiplex Assays. Human Cytokine – Stripwells*. http://www.quansysbio.com/manuals [accessed 12 October 2017].
14 Promega. *Technical Bulletin. Caspase-Glo® 3/7 Assay*. https://www.promega.com/-/media/files/resources/protocols/technical-bulletins/101/caspase-glo-3-7-assay-protocol.pdf [accessed 12 October 2017].
15 Talib J, Pattison DI, Harmer JA, Celermajer DS, Davies MJ. High plasma thiocyanate levels modulate protein damage induced by myeloperoxidase and perturb measurement of 3-chlorotyrosine. *Free Radic Biol Med* 2012; **53**: 20–29.
16 Daugherty A, Dunn JL, Rateri DL, Heinecke JW. Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. *J Clin Invest* 1994; **94**: 437–444.
17 Nagra RM, Becher B, Tourtellotte WW, Antel JP, Gold D, Paladino T *et al.* Immunohistochemical and genetic evidence of myeloperoxidase involvement in multiple sclerosis. *J Neuroimmunol* 1997; **78**: 97–107.
18 Kettle AJ, Winterbourn CC. Myeloperoxidase: a key regulator of neutrophil oxidant production. *Redox Rep* 1997; **3**: 3–15.
19 Hawkins CL, Morgan PE, Davies MJ. Critical methods in free radical biology and medicine: quantification of protein modification by oxidants. *Free Radic Biol Med* 2009; **46**: 965–988.
20 Davis SS, Mikami DJ, Newlin M, Needleman BJ, Barrett MS, Fries R *et al.* Heating and humidifying of carbon dioxide during pneumoperitoneum is not indicated: a prospective randomized trial. *Surg Endosc* 2006; **20**: 153–158.
21 Erikoglu M, Yol S, Avunduk MC, Erdemli E, Can A. Electron-microscopic alterations of the peritoneum after both cold and heated carbon dioxide pneumoperitoneum. *J Surg Res* 2005; **125**: 73–77.
22 Peng Y, Zheng M, Ye Q, Chen X, Yu B, Liu B. Heated and humidified CO_2 prevents hypothermia, peritoneal injury, and intra-abdominal adhesions during prolonged laparoscopic insufflations. *J Surg Res* 2009; **151**: 40–47.
23 Sammour T, Mittal A, Delahunt B, Phillips AR, Hill AG. Warming and humidification have no effect on oxidative stress during pneumoperitoneum in rats. *Minim Invasive Ther Allied Technol* 2011; **20**: 329–337.
24 Glew PA, Campher MJ, Pearson K, Schofield JC, Davey AK. The effect of warmed, humidified CO_2 on the dissipation of residual gas following laparoscopy in piglets. *J Am Assoc Gynecol Laparosc* 2004; **11**: 204–210.
25 Dindo D, Demartines N, Clavien PA. Classification of surgical complications – a new proposal with evaluation in a cohort of 6336 patients and results of a survey. *Ann Surg* 2004; **240**: 205–213.

© 2019 The Authors.
BJS Open published by John Wiley & Sons Ltd on behalf of BJS Society Ltd

26 Buss IH, Senthilmohan R, Darlow BA, Mogridge N, Kettle AJ, Winterbourn CC. 3-chlorotyrosine as a marker of protein damage by myeloperoxidase in tracheal aspirates from preterm infants: association with adverse respiratory outcome. *Paediatr Res* 2003; **53**: 455–462.
27 Mita H, Higashi N, Taniguchi M, Higashi A, Kawagishi Y, Akiyama K. Urinary 3-bromotyrosine and 3-chlorotyrosine concentrations in asthmatic patients: lack of increase in 3-bromotyrosine concentration in urine and plasma proteins in aspirin induced asthma after intravenous aspirin challenge. *Clin Exp Allergy* 2004; **34**: 931–938.
28 Van Der Vliet A, Nguyen MN, Shigenaga MK. Myeloperoxidase and protein oxidation in cystic fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2000; **279**: 537–546.
29 Wu SM, Pizzo SV. α_2-Macroglobulin from rheumatoid arthritis synovial fluid: functional analysis defines a role for oxidation in inflammation. *Arch Biochem Biophys* 2001; **391**: 119–126.
30 Cheong JY, Keshava A, Witting P, Young CJ. Effect of intraoperative warmed, humidified CO_2 insufflation during abdominal surgery: a review. *Ann Coloproctol* 2017; **34**: 125–137.
31 Brokelman WJ, Holmdahl L, Bergström M, Falk P, Klinkenbijl JH, Reijnen MM. Heating of carbon dioxide during insufflation alters the peritoneal fibrinolytic response to laparoscopic surgery: a clinical trial. *Surg Endosc* 2008; **22**: 1232–1236.
32 Sammour T, Kahokehr A, Hayes J, Hulme-Moir M, Hill AG. Warming and humidification of insufflation carbon dioxide in laparoscopic colonic surgery: a double-blinded randomized controlled trial. *Ann Surg* 2010; **251**: 1024–1033.
33 Moehrlen U, Ziegler U, Boneberg E, Reichmann E, Gitzelmann CA, Meuli M *et al.* Impact of carbon dioxide *versus* air pneumoperitoneum on peritoneal cell migration and cell fate. *Surg Endosc* 2006; **20**: 1607–1613.
34 Hazebroek EJ, Schreve MA, Visser P, De Bruin RW, Marquet RL, Bonjer HJ. Impact of temperature and humidity of carbon dioxide pneumoperitoneum on body temperature and peritoneal Morphology. *J Laparoendosc Adv Surg Tech A* 2002; **12**: 355–364.
35 Margulis V, Matsumoto ED, Tunc L, Taylor G, Duchenne D, Cadeddu JA. Effect of warmed, humidified insufflation gas and anti-inflammatory agents on cytokine response to laparoscopic nephrectomy: porcine model. *J Urol* 2005; **174**: 1452–1456.
36 Sammour T, Mittal A, Loveday BP, Kahokehr A, Phillips AR, Windsor JA *et al.* Systematic review of oxidative stress associated with pneumoperitoneum. *Br J Surg* 2009; **96**: 836–850.
37 Arsalani-Zadeh R, Ullah S, Khan S, MacFie J. Oxidative stress in laparoscopic *versus* open abdominal surgery: a systematic review. *J Surg Res* 2011; **169**: e59–e68.

© 2019 The Authors.
BJS Open published by John Wiley & Sons Ltd on behalf of BJS Society Ltd