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Hyperoxia and prevention of adhesion formation: a laparoscopic mouse model for open surgery

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Objective CO_2 pneumoperitoneum with more than 10% oxygen enhances adhesions. As during open surgery the peritoneum is exposed to air (20% oxygen), in this hyperoxia-enhanced adhesion model we evaluated the effect of hypothermia and products with known effectiveness in hypoxia (pure CO_2 pneumoperitoneum) and normoxia (CO_2 pneumoperitoneum plus 3–4% oxygen) models. Results were expected to be important for adhesion prevention in open surgery, and, moreover, similarities and differences between the three models would be important to identify differences in pathways of adhesion formation between laparoscopy and laparotomy.

Design Two experiments were performed in which the effect of hypothermia (32°C), a surfactant (phospholipids), a barrier (Hyalobarrier® gel), reactive oxygen species scavengers (superoxide dismutase, SOD, and ascorbic acid, AA), anti-inflammatory agents (dexamethasone and nimesulide), a calcium channel blocker (diltiazem) and recombinant plasminogen activator (r-PA) were evaluated upon adhesions.

Setting University Hospital.

Population BALB/c mice.

Methods Hyperoxia-enhanced adhesions were induced by performing laparoscopically bipolar lesions during 60 minutes of CO_2 pneumoperitoneum plus 12% oxygen at 37°C body temperature.

Main outcome measures Adhesions were scored after 7 days.

Results In this model, adhesions were reduced by hypothermia ($P < 0.02$; Wilcoxon), phospholipids ($P = 0.03$), Hyalobarrier® gel ($P < 0.004$), dexamethasone ($P < 0.005$) and diltiazem ($P < 0.01$). A significant but quantitatively borderline effect was seen for AA ($P < 0.002$) and r-PA ($P = 0.0005$), whereas SOD and nimesulide did not have any effect.

Conclusions Hyperoxia-enhanced adhesions were prevented by hypothermia, dexamethasone, phospholipids, Hyalobarrier® gel, diltiazem, r-PA and AA. All effects were similar to those in the hypoxia-enhanced adhesion model, suggesting that the underlying mechanisms are similar.

Keywords Adhesion formation, hyperoxia, laparoscopy, mouse model, open surgery, prevention.

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Introduction

Adhesions are clinically important because they can cause intestinal obstruction,[1,2] female infertility,[3] chronic pain,[4] and also difficulties at the time of re-operation. It has been claimed that laparoscopy is less adhesiogenic than laparotomy, but it was difficult to substantiate this claim in both human and animal models.[5–7] This is not surprising considering that factors derived from the peritoneal cavity can enhance post-traumatic adhesions.[8]

We developed three models in which different types of insufflation gas are used: the hypoxic model, in which pure CO_2 is used, corresponds to a traditional laparoscopy; the normoxia model, in which 3% oxygen is added to the pneumoperitoneum; and the hyperoxia model, in which 12% oxygen is added to the pneumoperitoneum, and which mimics open surgery because tissues are in contact with the high oxygen concentration of air. During these studies, we demonstrated that adhesion formation is increased using pure CO_2 pneumoperitoneum and when

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more than 10% oxygen is added to the pneumoperitoneum, in comparison with a CO_2 pneumoperitoneum with 3–4% of oxygen.[9,10] The beneficial effect of the addition of 3–4% oxygen could be explained by the fact that 3–4% oxygen at 770 mmHg (atmospheric pressure of 760 mmHg plus insufflation pressure of 10 mmHg) results in a pO_2 of 23 mmHg, which is remarkably similar to the normal intracellular pO_2.[11] The addition of 12% oxygen at 770 mmHg results in a pO_2 of 92 mmHg, which is higher than the normal intracellular pO_2. We, therefore, can talk about mesothelial hypoxia, normoxia and hyperoxia for the respective models. Preliminary studies show positive staining for hypoxia at the mesothelial layer during pure CO_2 pneumoperitoneum, and no staining, meaning no hypoxia, when 3–4% oxygen is added to the pneumoperitoneum (PR Koninckx, unpubl. obs.).

In comparison with a CO_2 pneumoperitoneum with 3–4% of oxygen (normoxia model), the enhancing factors identified today are the CO_2 pneumoperitoneum (hypoxia)[9] and the CO_2 pneumoperitoneum with more than 10% oxygen (hyperoxia).[10] As both mesothelial hypoxia (a model for laparoscopy) and hyperoxia (a model for open surgery) enhance adhesion formation it is not surprising that comparative results were not consistent.

Other enhancing factors identified as derived from the peritoneal cavity are desiccation,[12,13] insufflation pressure[9] and manipulation.[14] In the hypoxia model, adhesions are further enhanced by desiccation and by tissue manipulation, and are reduced by cooling.[12,15] All three effects are dose dependent, and in this model adhesions also increase with duration of pneumoperitoneum.[9] Prevention of adhesion formation could thus assist in reducing the local effects at the level of surgical trauma. In addition, prevention should aim at minimising the adhesion-enhancing effects mediated by the pneumoperitoneum. Barriers and fibrinolytic drugs are obviously based upon the first effect. For many other factors and drugs, such as cooling, dexamethasone, hypoxia-inducible factors (HIF) inhibitors, calcium channel blockers and reactive oxygen species (ROS) scavengers, it is less clear whether the effect is transmitted through an effect upon the peritoneal cavity only, or whether these factors/products are effective at the local level and at the peritoneal cavity level. Only prevention of manipulation, i.e. gentle tissue handling, was unequivocally effective, by reducing the adhesion-enhancing effects of the peritoneal cavity.[14] In the hypoxia model, the adhesion-reducing effects of known factors such as barriers, phospholipids, HIF inhibitors, non-steroidal anti-inflammatory drugs (NSAIDs), corticoids, anti-tumour necrosis factor α (anti-TNFα) antibodies, calcium channel blockers, ROS scavengers, etc. were screened for their adhesion-reducing properties, in order to obtain a comprehensive view of efficacy when screened in one model.[16–18] Subsequently, these factors were screened in the normoxia model.[8] As cooling and normoxia had similar effects, we suggested that cooling made the mesothelial cells more resistant to the deleterious effects of hypoxia. Other factors such as dexamethasone and Hyalobarrier® gel were effective in both models.

In the hypoxia model, the adhesion-enhancing effects are thought to be generated by the effect of hypoxia[9] and the HIF cascade,[19] ultimately leading to angiogenic factors.[20] In the hyperoxia model, adhesion enhancement was thought to be mediated through an increase in ROS.[21] However, the hypoxia can also increase ROS activity by decreasing the scavengers. As in open surgery cells are exposed to air with high oxygen concentration, we wanted to evaluate in detail the hyperoxia model, in which mesothelial cells are exposed to more than 10% of oxygen. Indeed it was unclear whether adhesion prevention in open and laparoscopic surgery would be similar clinically. We thus wanted to know the quantitative effect of all factors in the hyperoxia model in order to permit comparison with the normoxia and hypoxia models. We wanted to know more specifically whether hypothermia can prevent adhesions by making cells more resistant to the toxic effects of the hyperoxia. In addition, comparing differences in relative effectiveness of the different products with their know mechanisms of actions in the three models should give indications of the underlying mechanisms.

Materials and methods

The laparoscopic mouse model for adhesion formation

Experimental set-up, i.e. animals, anaesthesia and ventilation, laparoscopic surgery, induction and scoring of intraperitoneal adhesions, has been described in detail previously.[8–10,12,14–20,22–26]

Briefly, adhesions were induced during laparoscopy by creating a mechanical lesion. Pneumoperitoneum was maintained for 60 minutes using humidified CO_2 with the addition of 12% of oxygen at 15 mmHg insufflation pressure, a model that henceforth we will call the hyperoxia model or hyperoxia-enhanced adhesions. Gas and body temperatures were strictly maintained at 37°C using a heated chamber.

Animals

Eighty-eight 9- to 10-week-old female BALB/c mice weighing 20 g were used. Animals were kept under standard laboratory conditions, and were fed with a standard laboratory diet with free access to food and water at any time.

Anesthesia and ventilation

Mice were anaesthetised with intraperitoneal 0.08 mg/g pentobarbital and intubated with a 20-gauge catheter, and

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were mechanically ventilated (Mouse Ventilator MiniVent, Type 845; Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany). Ventilation was undertaken at a tidal volume of 250 μl with 160 strokes/minute, as these conditions prevent hypercarbia/acidosis produced by the pneumoperitoneum,[25] using humidified room air to prevent cooling.[15]

Laparoscopic surgery

A midline incision was performed caudal to the xyphoides, a 2-mm endoscope with a 3.3-mm external sheath for insufflation (Karl Storz, Tuttlingen, Germany) was introduced into the abdominal cavity, and the incision was closed gas-tight around the endoscope in order to avoid leakage.

Pneumoperitoneum was produced with CO_2 plus 12% oxygen at 15 mmHg insufflation pressure using the Thermoflator Plus (Karl Storz, Tuttlingen, Germany), which permits the delivery of variable oxygen concentration between 0% and 12% in CO_2. In addition, a water valve was used to cushion pressure changes. The 12% oxygen level was chosen because the Thermoflator Plus did not have a higher range, and because previous experiments had demonstrated that adhesion formation had already clearly increased with 12% of oxygen added to the pneumoperitoneum.[10] Obviously with 20% of oxygen the increase would be more pronounced, but for evaluating the pathway of this 'hyperoxia' and its prevention, 12% seemed sufficient. The gas was humidified (Storz Humidifier 204320 33; Karl Storz, Tuttlingen, Germany) and the whole set-up was kept in a 37°C chamber in order to obtain the insufflation gas at 37°C with 100% relative humidity. We used, as described previously, a controlled flow of the insufflation gas through the abdominal cavity of 23 ml/minute, using a 26-gauge needle, in order to ascertain a continuously 12% O_2 environment by constantly removing any oxygen that might have diffused from the capillaries.

Induction of intraperitoneal adhesions

Pneumoperitoneum-enhanced adhesion formation was induced by maintaining the pneumoperitoneum for 60 minutes, and by performing standardized 10×1.6 mm lesions in the antimesenteric border of both right and left uterine horns, and pelvic sidewalls, with bipolar coagulation (BICAP™, bipolar hemostasis probe, BP-5200A, 5 Fr, 200 cm; IMMED Benelux, Linkebeek, Belgium) at 20 W (Autocon 200; Karl Storz, Tuttlingen, Germany, standard coagulation mode).

Scoring of adhesions

Adhesions were qualitatively and quantitatively scored. Scoring was performed blindly (investigator blinded to group being evaluated) after 7 days during laparotomy using a stereomicroscope.

The quantitative scoring system assessed the proportion of the lesions covered by adhesions using the following formula: adhesion (%) = (sum of the length of the individual attachments/length of the lesion) $\times$ 100. The qualitative scoring system assessed: extent (0, no adhesions; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, 76–100% of the injured surface involved, respectively), type (0, no adhesions; 1, filmy; 2, dense; 3, capillaries present), tenacity (0, no adhesions; 1, easily fall apart; 2, require traction; 3, require sharp dissection) and total (extent + type + tenacity).

The results are presented as the average of the adhesions formed at the four individual sites (right and left visceral and parietal peritoneum), which were individually scored.

Products

Mechanical barrier. Hyalobarrier® gel is a sterile, transparent and highly viscous gel, obtained by condensation of hyaluronic acid (HA) through an auto-crosslinking process, and is indicated for laparoscopic and hysteroscopic or open surgical procedures. It was kindly provided by Fidia Advanced Biopolymers SRL (Abano Terme, Padova, Italy).

Surfactant. Phospholipids solution (9%), kindly given by Dr Marc Jansen (Department of Surgery, University Clinic, RWTH Aachen, Germany) was diluted to 3% in saline (NaCl 0.9%) before use.

Recombinant human PA. Reteplase (Rapilysin® 10 U; Roche, Basel, Switzerland) was prepared as indicated in the product data sheet, and diluted to 2 μg/ml and kept at −20°C.

Anti-inflammatory drugs. Dexamethasone (Aacidexam, 5 mg for injection; Organon, Brussels, Belgium) was prepared on the day of the experiment, as indicated in the product data sheet, and was diluted to 80 μg/ml in saline and kept at 4°C. Nimesulide (Sigma-Aldrich, Bornem, Belgium) was dissolved in dimethyl sulfoxide (DMSO; 30 mg/ml) and kept at −20°C; on the day of the experiment this was diluted to 0.2 mg/ml in phosphate-buffered saline (PBS).

ROS scavengers. Superoxide dismutase (SOD) from bovine erythrocytes (Sigma-Aldrich) was dissolved in saline to 3000 U/ml and kept at −20°C until used. Ascorbic acid (AA; Sigma-Aldrich) was dissolved to 20 mg/ml in saline before use.

Calcium channel blocker. Diltiazem hydrochloridum (Tildiem i.v. 25 mg; Sanofi-Synthelabo SA/NV, Brussels, Belgium) was prepared on the day of the experiment as indicated in the product data sheet, diluted to 0.2 mg/ml in saline and kept at 4°C.

All of the drugs were diluted in saline, except for nimesulide that was diluted in PBS for a better dissolution. All the dosages used in these experiments were shown to be effective to prevent adhesions in our hypoxia and normoxia models, as published previously.[8,16–18]

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Experimental design

As anaesthesia and ventilation can influence body temperature, the timing was strictly controlled. The time of anaesthesia injection was considered to be time 0 (T_0). The animal preparation and ventilation started after exactly 10 minutes (T_{10}). The pneumoperitoneum started at 20 minutes (T_{20}) and was maintained for 60 minutes, until T_{80}.

Two experiments were performed. Experiment I was designed to evaluate the effects of a low body temperature (32°C), a surfactant (phospholipids), a barrier (Hyalobarrier® gel) and of two ROS scavengers (SOD and AA) upon adhesion formation. Under laparoscopic view, after performing the lesions, 0.5 ml of phospholipids 3% was intraperitoneally (i.p.) injected (phospholipids treated group), or a small incision was made on the skin in order to insert the probe, and around 1 ml of Hyalobarrier® gel was applied on the lesions (Hyalobarrier® gel-treated group). As it is well know that ROS are produced during reperfusion, 0.1 ml of SOD or AA was injected i.p. at T_{75}, i.e. 5 minutes before the pneumoperitoneum ended (SOD- and AA-treated groups, respectively). Control-group mice received only saline. In this experiment, the mouse body temperature was strictly maintained at 37°C for all the groups using a 37°C chamber and a homeothermic blanket, except for the group in which the effect of low body temperature (32°C) was investigated, and this low body temperature was achieved as previously described[12,15] (six groups, with eight mice per group).

Experiment II was designed to evaluate the effect of steroidal anti-inflammatory (dexamethasone) and cyclooxygenase-2 (COX-2)-selective (nimesulide) NSAIDs, of a calcium channel blocker (diltiazem) and recombinant plasminogen activator (r-PA or Reteplase) upon adhesion formation. For the dexamethasone-treated group, mice received two i.p. doses of 0.5 ml (immediately after performing the lesion and again on the day after surgery), as dexamethasone has a very long half-life (36–72 hours and 67 hours, respectively).[27,28] For the nimesulide-treated group, mice received four i.p. doses of 0.5 ml (immediately after performing the lesion, 6-hours later, the day after surgery in the morning and 6 hours after that), as this drug has a shorter half-life (1.80–4.73 h).[27,29,30] As diltiazem has a short half-life (1.5–7 h)[31], mice received four i.p. doses of 0.5 ml (immediately after performing the lesion, 6 hours later, on the day after surgery in the morning and 6 hours after that). Four doses of 1 μg/0.5 ml r-PA was administrated i.p. in two doses on the day of surgery (immediately after performing the lesion and 6 hours after that), and two doses the day after (in the morning and 6 hours after that) (r-PA-treated group). Similarly, four doses of 0.5 ml of saline were administered in the control group. In this experiment, mouse body temperature was strictly kept at 37°C for all groups (five groups, with eight mice per group). We previously demonstrated repetitively that the administration of saline does not affect adhesion formation in comparison with a control group without saline in this laparoscopic mouse model.[18,24]

Statistics

Statistical analyses were performed with the SAS SYSTEM (SAS Institute, Cary, NC, USA). Differences in adhesion

Table 1. Quantitative and qualitative adhesion scores

Exp	Treatments				Adhesion scoring (mean ± SE)				
	BT (°C)	Product			Quantitative	Qualitative			
		Group	Concentration	Doses	Proportion (%)	Extent	Type	Tenacity	Total
I	37	Control (saline)	–	4	34.5 ± 4.1	1.8 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	4.0 ± 0.5
	32	Low temperature	–	4	18.4 ± 3.1[a,b]	1.0 ± 0.2[a,b]	1.0 ± 0.2	1.1 ± 0.2	3.3 ± 0.5
	37	Phospholipids	3%	1	22.8 ± 3.7[a,b]	1.0 ± 0.2[a,b]	1.0 ± 0.1	1.1 ± 0.1	3.1 ± 0.4
		Hyalobarrier® gel	–	1	2.8 ± 1.0[a,b]	0.2 ± 0.1[a,b]	0.3 ± 0.1[a,b]	0.4 ± 0.1[a,b]	0.9 ± 0.3[a,b]
		Ascorbic acid	2 mg	1	29.7 ± 4.4[b]	1.4 ± 0.2[b]	1.3 ± 0.2	1.3 ± 0.2	4.1 ± 0.5
		Superoxide dismutase	300 U	1	31.9 ± 3.6	1.5 ± 0.2	1.2 ± 0.1	1.3 ± 0.1	3.9 ± 0.3
II	37	Control (saline)	–	4	36.3 ± 2.8	1.6 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	4.2 ± 0.3
		Dexamethasone	40 μg	2	13.1 ± 1.8[a,b]	0.8 ± 0.1[a,b]	0.9 ± 0.1[a,b]	1.0 ± 0.1[a]	2.7 ± 0.1[a,b]
		Nimesulide	100 μg	4	30.0 ± 3.7	1.5 ± 0.2	1.3 ± 0.1	1.3 ± 0.1	4.1 ± 0.3
		Diltiazem	100 μg	4	22.5 ± 2.1[a,b]	1.2 ± 0.1[a,b]	1.1 ± 0.1	1.3 ± 0.1	3.5 ± 0.2
		Recombinant plasminogen activator	1 μg	4	29.4 ± 2.0[b]	1.4 ± 0.1[b]	1.1 ± 0.1[b]	1.1 ± 0.1	3.6 ± 0.2[b]

Wilcoxon test: Intra-experiment comparisons: [a]$P < 0.05$ versus their own control group; Inter-experiment comparisons: [b]$P < 0.05$ versus the grouped control group.

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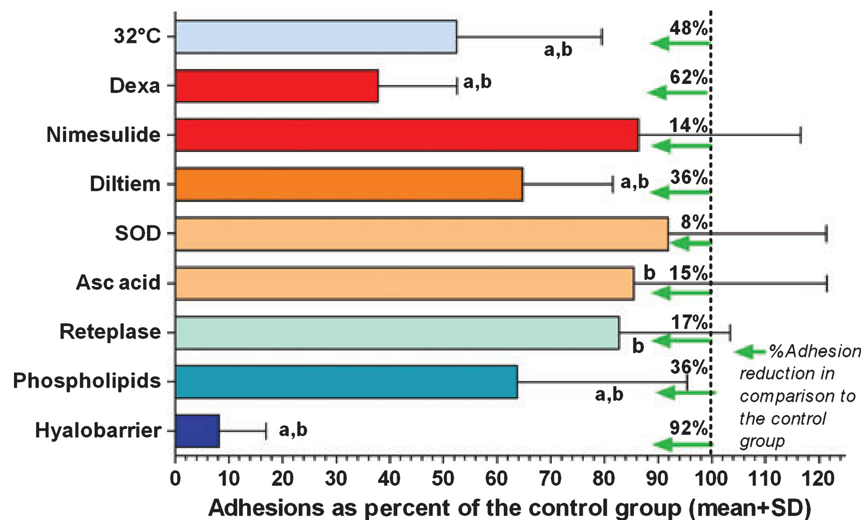

Figure 1. Prevention of hyperoxia-enhanced adhesions in a mouse model. Pneumoperitoneum was maintained for 60 minutes using humidified CO_2 with the addition of 12% oxygen at 15 mmHg insufflation pressure. Adhesions were induced during laparoscopy by making a bipolar lesion. Two experiments were performed to evaluate the effects of low temperature, surfactants (phospholipids 3%), a barrier (Hyalobarrier® gel), a fibrinolitic agent (r-PA), of anti-inflammatories (dexamethasone and nimesulide), a calcium channel blocker (diltiem) and ROS scavengers (superoxide dismutase and ascorbic acid). Adhesions were scored after 7 days during laparotomy. In order to visualise the results of the two experiments in one graph, the percentage of change in comparison with the control CO_2 pneumoperitoneum with 12% oxygen and 37°C body temperature (considered as 100%) is given for each treatment. The percentages of adhesion reduction compared with the control group were calculated, and are listed above the green arrows. To make the table clearer, only comparisons with the control group were included. Statistics: Wilcoxon test, intra-experiment comparisons, $^{a}P < 0.05$ versus their own control group; inter-experiment comparisons, $^{b}P < 0.05$ versus the grouped control group.

formation were evaluated with the Wilcoxon test. In Table 1, the quantitative (proportions) and the qualitative (total extent, type and tenacity) adhesion scores are presented. All data are presented as the mean ± standard error of the mean (SE).

In order to visualize the results of the two experiments in one graph (Figure 1), the changes in adhesions (quantitative score) in comparison with the control group, i.e. CO_2 pneumoperitoneum plus 12% oxygen and 37°C body temperature, are represented.

Results

In experiment I, hyperoxia-induced adhesions (12% oxygen, 37°C body temperature) were reduced by low temperature (proportion, $P < 0.02$; extent, $P < 0.02$; Wilcoxon test), phospholipids (proportion, $P = 0.03$; extent, $P < 0.02$) and Hyalobarrier® gel (proportion, $P < 0.004$; total, $P < 0.01$; extent, $P < 0.01$; type, $P < 0.01$; tenacity, $P = 0.01$). ROS scavengers such as AA and SOD did not have a significant effect. Most effective was the Hyalobarrier® gel, which was more effective than low temperature (proportion, $P < 0.01$; total, $P < 0.03$; extent, $P < 0.02$; type, $P = 0.02$; tenacity, $P = 0.03$), phospholipids (proportion, $P < 0.001$; total, $P < 0.01$; extent, $P < 0.01$; type, $P = 0.01$; tenacity, $P < 0.02$), AA (proportion, $P < 0.005$; total, $P < 0.01$; extent, $P < 0.01$; type, $P < 0.01$; tenacity, $P < 0.01$) and SOD (proportion, $P < 0.04$; total, $P < 0.03$; extent, $P < 0.02$; type, $P < 0.04$; tenacity, $P < 0.03$) (Figure 1; Table 1). Moreover, Hyalobarrier® gel was so effective that the combined effectiveness of this product when used together with the other products was technically not realistic.

In experiment II, hyperoxia-induced adhesions were reduced using dexamethasone (proportion, $P < 0.005$; total, $P < 0.005$; extent, $P < 0.005$; type, $P < 0.03$; tenacity, $P < 0.05$; Wilcoxon test) and diltiazem (proportion, $P < 0.01$; extent, $P < 0.02$). Nimesulide had no effect (not significant), and a low effect of r-PA (not significant) was observed (Figure 1; Table 1). Mice treated with dexamethasone had less adhesions than mice treated with diltiazem (proportion, $P < 0.02$; total, $P < 0.02$; extent, $P < 0.04$; type, $P = 0.05$; tenacity, $P < 0.03$), r-PA (proportion, $P < 0.01$; total, $P < 0.005$; extent, $P < 0.004$; type, $P = 0.05$) and nimesulide (proportion, $P < 0.01$; total, $P < 0.03$; extent, $P < 0.02$; type, $P < 0.04$; tenacity, $P < 0.03$).

As means and standard deviations of adhesion formation in the control groups of both experiments were similar (not significant), the results of experiments I and II were grouped as if both experiments had been performed as one. In comparison with a control group of 16 mice instead of eight, all significances were higher, as expected, i.e. adhesions decreased with dexamethasone (proportion, $P = 0.0005$; total, $P < 0.001$; extent, $P < 0.001$; type, $P < 0.02$), diltiazem (proportion, $P < 0.005$; extent, $P <$

0.008), low temperature (proportion, $P < 0.02$; extent, $P < 0.004$), phospholipids (proportion, $P < 0.01$; extent, $P < 0.01$), Hyalobarrier® gel (proportion, $P = 0.0003$; total, $P = 0.0003$; extent, $P = 0.0003$; type, $P = 0.0002$; tenacity, $P = 0.0005$). Borderline significances became significant. Adhesions were lower with AA (proportion, $P < 0.002$; extent, $P < 0.004$) and r-PA (proportion, $P = 0.0005$; total, $P < 0.001$; extent, $P < 0.001$; type, $P < 0.02$; Wilcoxon test). No effect was observed with SOD or nimesulide (not significant for all comparisons).

Discussion

In order to interpret the results of these experiments, the results of adhesion reduction in the hypoxia and normoxia models should be kept in mind. In brief, in our hypoxia model, CO_2 pneumoperitoneum-enhanced adhesions were reduced by low temperature, barriers, such as Spraygel and Hyalobarrier® gel, phospholipids, HIF inhibitors, such as wortmanin, a corticosteroid, such as dexamethasone, a calcium channel blocker, such as diltiazem, a fibrinolitic agent, such as r-tPA, and ROS scavengers, such as SOD.[16,17,32] A borderline effect was seen for AA. In the normoxia model, adhesions were not further reduced by low temperature, suggesting that cooling prevented the effects of hypoxia.[8] Dexamethasone and barriers remained effective.

Hyperoxia-induced adhesions were reduced by 48% by low temperature (32°C) (Figure 1; Table 1). This inhibition is quantitatively similar to the effect in the hypoxia model (51% of inhibition, with the percentage calculated from figure 2 of Binda *et al.*[15]) and in the normoxia model (48% of inhibition, with the percentage calculated from figure 1 of Binda *et al.*[8]). Lower temperature is thus equally effective in preventing both adhaesiogenic effects. Thus, low temperature also protects the mesothelial cells against the toxic effects of hyperoxia. ROS might be involved in pneumoperitoneum-enhanced adhesions because they are generated after the reperfusion of ischemic tissue,[33] and this is valid for both the hypoxia and the hyperoxia models. Consistent with this, the generation of ROS after laparoscopic surgery is well reported.[34,35] Laparoscopic surgery increases ROS availability by increasing ROS production,[35] or by decreasing ROS scavengers.[36,37] In addition, when pneumoperitoneum is induced with 12% oxygen, i.e. a level that is even less than the 20% oxygen present during open surgery, mesothelial cells would be in a hyperoxic environment, which could lead to increased ROS production or decreased ROS scavenger production in comparison with a hypoxic or normoxic environment. Hypothermia could thus prevent adhesions by reducing ROS production[38–43] and improving the recovery of energetic parameters during reperfusion.[44] It can suppress the inflammatory response after ischaemia-reperfusion.[45,46] We therefore suggest that hypothermia can protect against the toxic effect of ROS and the ischemia-reperfusion process in both the hypoxia and the hyperoxia models. However, we cannot exclude that both hypoxia- and hyperoxia-enhanced adhesions have similar underlying mechanisms. Indeed in both models ROS activity increases through ischemia-reperfusion, with, in addition, a decrease in scavengers during hypoxia and an oxygen-induced increase during hyperpoxia. As both AA and SOD have little or no effect in the hyperoxia model, the ROS pathway is probably less important.

With all of the restrictions imposed by the results obtained in different experiments, dexamethasone seemed to have a stronger inhibition in the hyperoxia model in comparison with the hypoxia model, i.e. 62% (Figure 1) versus 32% (percentage calculated from figure 2 of Binda *et al.* [16]) of inhibition, respectively. To explain this we can only speculate. It has been demonstrated that the administration of dexamethasone during hyperoxia conditions accelerated the maturation of the antioxidant enzyme system in fetal lungs, showing elevated SOD, catalase and glutathione peroxidase activities.[47,48] Dexamethasone can also reduce the hyperoxia-induced interleukin-8 (IL-8) release and mRNA synthesis by human alveolar macrophages.[49] Therefore, it might be that the high oxygen tension increases the inflammatory response by increasing cytokine production in the hyperoxia model. In order to explain the results, we should thus consider that increases in ROS and cytokine production are more important in the hyperoxia model, whereas dexamethasone inhibits inflammation and fibroblast proliferation in both the hypoxia and hyperoxia models.

In both models the anti-inflammatory drug, nimesulide, was without significant effect. The calcium channel blocker diltiazem showed a similar inhibition in both models, i.e. 36% in both hyperoxia (Figure 1) and hypoxia (percentage calculated from figure 2 of Binda *et al.*[16]) models. This suggests that the mechanisms of action of the calcium channel blocker, i.e. interference with the inflammatory response[50] and protection against the toxic effect of the ischemic-reperfusion cell injury,[51] are similar in both models.

Although we were expecting a significant effect of ROS scavengers, SOD and AA were ineffective. AA showed a borderline effect after grouping all the control groups, demonstrating that the sample size may be too small to detect small differences. Nevertheless, the effect of SOD in the hyperoxia model was less than in the hypoxia model (8% versus 36%, respectively; Table 1, and percentage calculated from figure 2 of Binda *et al.*[16]). Thus ROS may not be such an important factor. Reteplase has a borderline effect of 17% in the hyperoxia model (Figure 1), which is probably less than the 40% effect observed in the hypoxia model (percentage calculated from figure 3 of Binda

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et al.[32]). This could be explained as follows. In cell culture, fibrinolytic activity is regulated by partial oxygen tension, i.e. urokinase Plasminogen activator (uPA) and Plasminogen activator inhibitor (PAI) levels were both very low in hypoxic conditions: uPA release peaked in normoxic conditions, whereas PAI release was highest at a hyperoxic pO_2.[52] When applied to our adhesion models, less fibrinolysis would be expected in the hyperoxic conditions in comparison with the hypoxic conditions because PAI, which inhibits PA and PAI release, is higher in hyperoxic conditions.

Phospholipids and Hyalobarrier® gel showed a similar inhibition in the hypoxic and hyperoxic models, i.e. 36% and 90% (Figure 1, and percentage calculated from figure 2 of Binda *et al.*[17]), respectively. This was as expected, as both compounds have a mechanical or lubricating effect upon the peritoneum, by keeping the two damaged layers separate.

Conclusion

We confirmed that hyperoxia-enhanced adhesions were also reduced by cooling, pointing to a protective effect upon the mesothelial cell similar to the effect in the hypoxia model. ROS scavengers, such as SOD and AA, were unexpectedly not very effective, at least not more effective than in the hypoxia model. The effectiveness of products as phospholipids, Hyalobarrier® gel and diltiazem was similar in the hyperoxia and hypoxia models. The only differences observed between the hyperoxia and hypoxia models were that dexamethasone probably had a more pronounced effect and Reteplase exhibited a lower effectiveness in the hyperoxia model. With all of the limitations imposed by results from separate experiments (although results in the inbred strains were highly reproducible), we suggest differences in the inflammatory/immune pathways, and in fibroblast proliferation and fibrinolysis.

Our previous prevention studies indicated that the combination of therapies targeting the different mechanisms involved achieved the highest adhesion prevention, to a level of 90% or more.[8,16,17,32] A key factor is the 'conditioning of the abdominal cavity': i.e. minimising tissue trauma, preventing desiccation, creating normoxic conditions by the addition of 4% oxygen, and cooling the peritoneal cavity to some 32°C. In addition to this, dexamethasone and calcium channel blockers (such as diltiazem) are effective. Finally, barriers such as Hyalobarrier® gel or a surfactant such as phospholipids are additionally effective. These considerations seem to be important for the clinical evaluation of anti-adhesion products. Indeed it would be hard to prove effectiveness in a model in which there are already 70–80% less adhesions because of conditioning the abdominal cavity, and using drugs such as dexamethasone or calcium channel blockers.

Although we suggested the use of hypothermia during laparoscopy to reduce adhesions,[8] this is unlikely to be adopted during open surgery. We therefore suggest the use of superficial hypothermia, by working in an operating theatre at room temperature, as a method of adhesion prevention. This would be as safe as and more effective than the alternatives in existance today.

We therefore consider it important that we evaluated the different pathways in the hyperoxia model, as a model for open surgery, in order to consider combination therapies with an expected efficacy of over 90% in humans in the future. Obviously the potential harmful effects of decreasing body core body temperature will have to be evaluated and monitored closely when organizing trials to translate the observations of the mice experiments into clinical medicine.

Disclosure of interests

MMB and PRK have no conflicts of interest to disclose.

Contribution to authorship

MMB designed the study, analysed the results and drafted the article. PRK supervised the research. All authors have approved the final version.

Details of ethics approval

The procedures of the study received approval from the ethical committee of the Catholic University of Leuven (reference number: P085/2004).

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