

Prevention of adhesion formation in a laparoscopic mouse model should combine local treatment with peritoneal cavity conditioning[†]

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BACKGROUND: Adhesion formation results from a series of local events at the trauma site. This process can be enhanced by factors derived from the peritoneal cavity such as mesothelial cell hypoxia (pneumoperitoneum with pure CO₂), reactive oxygen species (pneumoperitoneum with more than 4% oxygen), desiccation and mesothelial trauma produced through manipulation. Adhesion prevention, therefore, should combine local treatment while minimizing adverse peritoneal factors through conditioning of the pneumoperitoneum.

METHODS: In a laparoscopic mouse model, adhesion induction comprised a mechanical lesion together with a humidified pneumoperitoneum for 60 min with pure CO₂ at 37°C. Adhesion prevention consisted of a combination of treatments known to reduce adhesions, i.e. pneumoperitoneum with CO₂ with the addition of 3–4% O₂, reduction of body temperature (BT) to 32°C and application of antiadhesion products such as anti-inflammatory drugs (dexamethasone, nimesulide), calcium-channel blockers (diltiem), surfactants (phospholipids), barriers (Hyalobarrier gel), reactive oxygen species scavengers (superoxide dismutase and ascorbic acid) and recombinant plasminogen activator.

RESULTS: The addition of 3% O₂ to the pneumoperitoneum or a lower BT decreased adhesions by 32% or 48%, respectively ($P < 0.05$, Wilcoxon), but were without additional effects when combined. In addition, if dexamethasone or Hyalobarrier[®] gel were administered, the total reduction was 76% ($P = 0.04$) or 85% ($P < 0.02$), respectively.

CONCLUSIONS: Combining pneumoperitoneum conditioning together with dexamethasone or a barrier resulted in significant adhesion reduction in a laparoscopic mouse model.

Key words: post-operative adhesions / laparoscopy / humidified gas / pneumoperitoneum conditioning / mouse model

Introduction

Adhesion formation consists of a series of local events at the trauma site. Peritoneal injury caused by surgery, infection or irritation initiates an inflammatory reaction with fibrin exudate and deposition into which white blood cells, macrophages, fibroblasts and mesothelial cells migrate, proliferate and/or differentiate. A key factor in adhesion prevention is fibrinolysis, which is regulated by the plasminogen system. The inactive proenzyme plasminogen is converted into plasmin by tissue-type plasminogen activator (tPA) and/or urokinase-type plasminogen activator. The fibrin matrix serves as a scaffold for fibroblasts and capillary ingrowth and for extracellular matrix (ECM)

deposition. During normal healing, the fibrin matrix is rapidly removed and the ECM will be degraded by metalloproteinases (MMPs). On the contrary, if the fibrin matrix persists too long, or when the ECM degradation is inhibited, peritoneal adhesions will be formed (Holmdahl, 1997; diZerega, 2000).

These local events are modulated by factors derived from the peritoneal cavity such as mesothelial cell hypoxia (pneumoperitoneum with pure CO₂), reactive oxygen species (pneumoperitoneum with more than 4% oxygen), desiccation and mesothelial trauma, which have been recognized as cofactors in adhesion formation. Today, the adhesiogenic factors recognized acting upon the entire peritoneal cavity are mesothelial hypoxia (Molinas *et al.*, 2001), mesothelial

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hyperoxia (Elkelani *et al.*, 2004) and reactive oxygen species (ROS) (Binda *et al.*, 2003), desiccation (Binda *et al.*, 2006) and mesothelial mechanical trauma (Schonman *et al.*, in press). Indeed, adhesions increase with the duration of pneumoperitoneum and the insufflation pressure, and this effect is prevented by the addition of 3% oxygen but not by using helium instead of CO₂ for the pneumoperitoneum (Molinas and Koninckx, 2000; Molinas *et al.*, 2001). Moreover, with the addition of more than 3–4% oxygen to CO₂, the pneumoperitoneum is deleterious probably due to ROS production (Elkelani *et al.*, 2004). To understand the importance of oxygen concentration in the pneumoperitoneum, we should remember that the normal partial pressure of oxygen (ppO₂) of mesothelial cells is between 5 and 40 mmHg (Guyton and Hall, 2000), which is similar to the addition of 3–4% oxygen at a pressure of 770 mmHg (atmospheric pressure of 760 mmHg plus insufflation pressure of 10 mmHg). The addition of 12% oxygen at 770 mmHg thus results in a ppO₂ of 92 mm Hg, which is much higher than the normal intracellular ppO₂ and, therefore, hyperoxic. In addition, desiccation is clearly adhesiogenic and this can be prevented by using humidified gas (Binda *et al.*, 2006; Peng *et al.*, 2008). Manipulation of omentum and bowels in the upper abdomen also increase adhesion formation at the trauma site in the lower abdomen (Schonman *et al.*, in press), confirming unequivocally that factors derived from the peritoneal cavity modulate adhesion formation at the trauma site. Another argument to conclude that CO₂ pneumoperitoneum and desiccation constitute an injury to the mesothelial cells is derived from scanning electron microscopy describing bulging up of the mesothelial cells and exposure of the extracellular matrix (Volz *et al.*, 1999). Not surprisingly, hypothermia attenuates hypoxia-, hyperoxia- or desiccation-enhanced adhesion formation (Binda *et al.*, 2004, 2006).

Adhesion prevention can be achieved by using barriers that separates traumatized areas for at least 5 days. This has been translated clinically using flotation agents or barriers such as SprayGel, Intercoat or Hyalobarrier[®] gel, all achieving an adhesion reduction of 40–50%. Besides this, many other products have been described in a great variety of animal models to prevent adhesion formation. In order to obtain comprehensive comparative data, these products have been evaluated in one model, i.e. our laparoscopic mouse model comprising 60 min of pure CO₂ pneumoperitoneum. In brief, our results confirmed that barriers such as SprayGel and Hyalobarrier[®] gel were effective in reducing adhesion, by 58% and 90%, respectively and a surfactant such as phospholipids was also effective giving a 35% reduction (Binda *et al.*, 2007a). They also confirmed that adhesions were reduced, by around 20% and 30%, when using ROS scavengers, i.e. ascorbic acid and superoxide dismutase (SOD), respectively, by 32% when using dexamethasone, by 36% when using a calcium-channel blocker as diltiem (Binda *et al.*, 2007b) and by 40% when using r-tPA (Binda *et al.*, 2009). Moreover, no reduction or a marginal reduction was observed using non-steroidal anti-inflammatory drugs (NSAIDs) (ibuprofen, tenoxicam, nimesulide, parecoxib) and anti-TNF-alpha antibodies (Binda *et al.*, 2007b). To summarize, besides local factors such as barriers and fibrinolysis, we identified several mechanisms, all of which could be addressed to reduce adhesions. We, therefore, confirmed several adhesion prevention mechanisms, such as avoiding mesothelial hypoxia through the addition of 3–4% of oxygen (Molinas and Koninckx, 2000; Molinas *et al.*, 2001), applying wortmanin, an HIF inhibitor (Binda *et al.*,

2007a), neutralizing the effect of ROS using ROS scavengers and avoiding inflammation using dexamethasone (Binda *et al.*, 2007b). Several anti-inflammatory agents were tested but only dexamethasone was effective, and this suggests the involvement of other mechanisms, i.e. glucocorticoids that indeed can inhibit fibroblast proliferation and can have immunosuppressive effects on the production and release of cytokines (Brunton *et al.*, 2006). Diltiazem, a calcium-channel blocker, reduced adhesion formation in our laparoscopic model (Binda *et al.*, 2007b). The suggested cause of this effect involves mechanisms such as interference with the inflammatory response (Szabo *et al.*, 1997), protection against the toxic effect of the ischemic–reperfusion cell injury (Wang *et al.*, 2002) or activation of cellular processes (Elmslie, 2004).

After having identified different mechanisms affecting adhesion formation, we evaluate which therapies have additive effects, i.e. which therapies could be used simultaneously to minimize adhesion formation. Therefore, besides preventing tissue trauma and desiccation, we first investigated whether the addition of 3–4% oxygen and slight cooling, factors that could be used routinely during surgery, have additive effects. Subsequently, we wanted to know which other factors could advantageously be used in a model without desiccation and hypoxia.

Materials and Methods

The laparoscopic mouse model for adhesion formation

Each aspect of the experimental setup, i.e. strain of mice used (Molinas *et al.*, 2005), anaesthesia and ventilation (Molinas *et al.*, 2004), laparoscopic surgery for induction of adhesions, duration and pressure of the pneumoperitoneum, type of gas used (Molinas *et al.*, 2001; Elkelani *et al.*, 2004), humidification and temperature (Binda *et al.*, 2004, 2006), has been described in detail previously.

In these experiments, we used a laparoscopic model with Balb/c mice as a reference, a bipolar lesion and 60 min of pure CO₂ pneumoperitoneum. Insufflation gas and body temperatures (BTs) were strictly kept at 37°C using a heated chamber (Binda *et al.*, 2004), and peritoneal trauma by manipulation of peritoneal organs or desiccation was kept to a minimum by the experience of the investigator and full humidification, respectively.

Animals

In this study, one hundred twelve 9- to 10-week-old female BALB/c mice weighing 20 g were used. Mice were kept under standard laboratory conditions and were fed with a standard laboratory diet with free access to food and water at anytime. The study was approved by the Institutional Review Animal Care Committee.

Anaesthesia and ventilation

Mice were anaesthetized with i.p. 0.08 mg/g pentobarbital, intubated with a 20-gauge catheter and mechanically ventilated (Mouse Ventilator MiniVent, Type 845, Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany). The ventilation was done at a tidal volume of 250 µl at 160 strokes/min as this condition prevents hypercarbia/acidosis enhanced by the pneumoperitoneum (Molinas *et al.*, 2004) and humidified room air was used to prevent cooling (Binda *et al.*, 2004).

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 created with pure CO₂ or CO₂ with the addition of 3% oxygen at 15 mmHg insufflation pressure using the Thermoflator Plus (Karl Storz) and a water valve to damper pressure changes. The gas was humidified (Storz Humidifier 204320 33, Karl Storz) and the whole set up was kept in a chamber at 37°C in order to obtain the insufflation gas at 37°C and with 100% relative humidity. We used, as described previously, a controlled flow of the insufflation gas through the abdominal cavity of 23 ml/min using a 26-gauge needle, in order to ascertain a continuously pure CO₂ or 3% O₂ environment by removing constantly 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 min 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, standard coagulation mode).

Control of temperature

Since anaesthesia and ventilation can influence BT, the timing was strictly controlled. The time of anaesthesia injection was considered time zero (T₀). The animal preparation and ventilation started after exactly 10 min (T₁₀). The pneumoperitoneum started at 20 min (T₂₀) and was maintained for 60 min till T₈₀. BTs were strictly controlled at 37°C using a heated chamber. In some groups, cooling was induced and BT was reduced to 32°C as explained previously (Binda et al., 2004).

Scoring of adhesions

Adhesions were qualitatively and quantitatively scored. Scoring was done blindly (the investigator was not informed of the group being evaluated) after 7 days during laparotomy using a stereomicroscope (Wild Heerbrugg M7A, Gais, Switzerland) and it was standardized for all groups. 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) × 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 used

Anti-inflammatory drugs. Dexamethasone (Aacidexam 5 mg for injection, Organon, Bruxelles, Belgium) was prepared the day of the experiment as indicated by manufacturer and diluted to 80 µg/ml in saline (NaCl 0.9%) and kept at 4°C. Nimesulide (Sigma, Bornem, Belgium) was dissolved in DMSO (30 mg/ml) and kept at –20°C; on the day of the experiment, it was diluted to 0.2 mg/ml in PBS.

ROS scavengers. Superoxide dismutase from bovine erythrocytes (Sigma, Bornem, Belgium) was dissolved in saline to 3000 U/ml and kept at

–20°C till used. Ascorbic acid (AA) (Sigma) was dissolved to 20 mg/ml in saline before using.

Calcium-channel blocker. Diltiazem hydrochloridum (Tildiem i.v. 25 mg, Sanofi-Synthelabo S.A.N.V., Bruxelles, Belgium) was prepared on the day of the experiment as indicated by manufacturer, diluted to 0.2 mg/ml in saline and kept at 4°C.

Barriers. Hyalobarrier® gel is a sterile, transparent and highly viscous gel, obtained by condensation of hyaluronic acid through an auto-cross-linking 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 before use.

Recombinant human PA. Reteplase (Rapilysin® 10 U, Roche) was prepared as indicated by manufacturer and diluted to 2 µg/ml and kept at –20°C.

All the dosages used in these experiments were shown to be effective to prevent adhesions in our model as published (Binda et al., 2007a, b, 2009).

Experimental design

As a comparison, adhesions caused by a mechanical lesion enhanced by 60 min CO₂ pneumoperitoneum strictly at 37°C but without desiccation was used. Addition of 3–4% of oxygen to the pneumoperitoneum, cooling and all products had been demonstrated to be effective in reducing adhesions previously (Molinas et al., 2001; Binda et al., 2004, 2006, 2007a, b, 2009; Elkelani et al., 2004).

Experiment I was designed to evaluate whether a lower BT and addition of 3% oxygen to the pneumoperitoneum have additive effects in reducing adhesion formation with the hypothesis that at lower temperature cells are more resistant to the deleterious effects of hypoxia and that the effects of both treatments thus should not be additive. The experiment used a factorial design, i.e. mice without (37°C) and with (32°C) cooling and mice with (pure CO₂ pneumoperitoneum) and without (CO₂+3% of oxygen) hypoxia. The four groups thus were: pure CO₂ pneumoperitoneum and mice BT at 37°C, pure CO₂ and mice at 32°C, CO₂ pneumoperitoneum + 3%O₂ and mice at 37°C and CO₂+3%O₂ and mice at 32°C (Table I). In order to permit comparison with the other groups, all mice received four doses of saline. We indeed previously demonstrated that administration of saline does not affect adhesion formation in comparison with control mice (Binda et al., 2009). Considering full humidification, the addition of 3% of oxygen and slight cooling (32°C BT) as optimal gas conditioning, we evaluated, in addition to this, whether dexamethasone, or a calcium-channel blocker (diltiazem) or a surfactant (phospholipids), could further reduce adhesion formation.

Since dexamethasone has a long half-life, i.e. 36–72 h (Nilsen, 1994; Brunton et al., 2006), treated mice received two i.p. doses of 0.5 ml immediately after performing the lesion and the day after the surgery. Since diltiazem has a shorter half-life (1.5–7 h) (Eisenberg et al., 2004), treated mice received four i.p. doses of 0.5 ml (immediately after performing the lesion, 6 h after that, 24 h after surgery and 6 h thereafter). For phospholipids-treated mice, 0.5 ml of phospholipids 3% was i.p. injected under laparoscopic view after performing the lesions (seven groups, eight mice per group).

Experiment II was designed to evaluate whether, in addition to optimal pneumoperitoneum conditioning, adhesions could be further reduced by a COX-2 selective NSAIDs (nimesulide), by a barrier (Hyalobarrier® gel),

Table I Adhesion prevention in a laparoscopic mouse model

Exp.	Treatments				Scoring (mean ± SE)					
	Gas used for the PP	BT (°C)	Products Name	# Doses	Conc.	Quantitative (proportions)	Qualitative			Total
							Extent	Type	Tenacity	
I	Pure CO ₂	37	Saline	4	—	34.4 ± 3.3	1.7 ± 0.2	1.3 ± 0.2	1.3 ± 0.1	4.3 ± 0.4
	Pure CO ₂	32	Saline	4	—	18.7 ± 3.4 ^a	1.0 ± 0.2 ^a	1.0 ± 0.2	1.1 ± 0.2	3.2 ± 0.6
	CO ₂ + 3%O ₂	37	Saline	4	—	23.4 ± 2.6 ^a	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	3.5 ± 0.2
	CO ₂ + 3%O ₂	32	Saline	4	—	19.7 ± 1.8 ^a	1.0 ± 0.1 ^a	1.0 ± 0.1	1.1 ± 0.1	3.1 ± 0.2 ^a
	CO ₂ + 3%O ₂	32	Dexamethasone	2	40 µg	8.6 ± 3.3 ^b	0.4 ± 0.2 ^b	0.4 ± 0.1 ^b	0.5 ± 0.2 ^b	1.4 ± 0.4 ^b
	CO ₂ + 3%O ₂	32	Diltiazem	4	100 µg	14.2 ± 2.8	0.8 ± 0.1	0.9 ± 0.1	1.1 ± 0.2	2.7 ± 0.4
	CO ₂ + 3%O ₂	32	Phospholipids	1	3%	14.3 ± 1.7	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	2.4 ± 0.3
II	Pure CO ₂	37	Saline	4	—	33.8 ± 2.3	1.8 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	4.2 ± 0.3
	CO ₂ + 3%O ₂	32	Saline	4	—	17.4 ± 2.5 ^a	0.8 ± 0.1 ^a	0.9 ± 0.1 ^a	1.0 ± 0.1	2.8 ± 0.3
	CO ₂ + 3%O ₂	32	Nimesulide	4	100 µg	19.6 ± 3.3	0.9 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	3.2 ± 0.2
	CO ₂ + 3%O ₂	32	SOD	1	300 U	21.3 ± 2.1	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	3.3 ± 0.3
	CO ₂ + 3%O ₂	32	AA	1	2 mg	29.5 ± 1.6 ^b	1.5 ± 0.1 ^b	1.1 ± 0.1	1.3 ± 0.1	3.9 ± 0.3 ^b
	CO ₂ + 3%O ₂	32	Hyalobarrier [®] gel	1	—	5.0 ± 1.1 ^b	0.3 ± 0.1 ^b	0.5 ± 0.1 ^b	0.6 ± 0.1 ^b	1.4 ± 0.3 ^b
	CO ₂ + 3%O ₂	32	Retepase	4	1 µg	19.7 ± 2.3	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	3.1 ± 0.2

Quantitative (proportions) and qualitative (extent, type, tenacity and total) scoring systems are indicated. Laparoscopy was performed using humidified pure CO₂ or CO₂ with the addition of 3–4% O₂ to the pneumoperitoneum (PP) and BT as kept at 32 or 37°C. Different products were applied. The volumes administrated were 500 µl for all the groups, except for SOD and AA that 100 µl was used and Hyalobarrier[®] gel that around 1 ml was applied. Statistic: Wilcoxon test: $P < 0.05$.

^aComparison with control pure CO₂ pneumoperitoneum, 37°C BT.

^bComparison with control CO₂+ 3% O₂ pneumoperitoneum, 32°C BT.

by ROS scavengers (SOD and AA) or by recombinant plasminogen activator (r-PA). Under laparoscopic view after performing the lesions, a small incision was done on the skin in order to enter the probe and around 1 ml of Hyalobarrier[®] gel was applied on the lesions. Since it is well known that ROS are produced during reperfusion, 0.1 ml of SOD or AA was injected i.p. at T₇₅, i.e. 5 min before the pneumoperitoneum was ended (SOD- and AA-treated groups, respectively). Since the nimesulide has a shorter half-life (1.80–4.73 h) (Bernareggi, 1998), treated mice received four i.p. doses of 0.5 ml (immediately after performing the lesion, 6 h after that, 24 h after surgery and 6 h thereafter). For the r-PA treated group, four doses of 1 µg/0.5 ml r-PA was administrated i.p. immediately after performing the lesion, 6 h after that, 24 h after surgery and 6 h thereafter. As control groups, two controls were done, the first one pure CO₂ pneumoperitoneum was induced and mice BT kept at 37°C and the second control group CO₂ pneumoperitoneum with the addition of 3% oxygen was induced and mice BT kept at 32°C (seven groups, eight mice per group).

Control group mice received only saline. In these experiments, mouse BT was strictly kept 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 BT (32°C) was investigated and this low BT was achieved as explained previously (Binda *et al.*, 2004, 2006).

Statistics

Statistical analyses were performed using SAS system (SAS Institute, Cary, NC, USA). Differences in adhesion formation were evaluated with Wilcoxon test. In Table I, the quantitative (proportions) and the qualitative (total, extent, type and tenacity) scoring values are given. All data are presented as mean ± standard error (SE) of the mean.

In order to visualize the results of the two experiments in one graph, the percentage of changes in comparison with the proportions of the control group, i.e. pure CO₂ pneumoperitoneum and 37°C BT (considered as 100%), is presented in Fig. 1 for each treatment.

Results

In Experiment I, comparing the group with 60 min of pure CO₂ pneumoperitoneum and 37°C BT, adhesions were reduced by adding 3% oxygen to the pneumoperitoneum (proportion: $P < 0.05$, total: NS, extent: NS, type: NS, tenacity: NS) and by lowering BT to 32°C (proportion: $P = 0.03$, total: NS, extent: $P = 0.05$, type: NS, tenacity: NS; Wilcoxon). Using both 3% oxygen and low temperature, adhesion formation was reduced to the same extent without an additive effect (proportion: $P < 0.03$, total: $P < 0.05$, extent: $P < 0.03$, type: NS, tenacity: NS) (Fig. 1, Table I).

In comparison with 60 min of CO₂ pneumoperitoneum with the addition of 3% of oxygen and a BT of 32°C, adhesion formation was further reduced by dexamethasone (proportion: $P = 0.04$, total: $P < 0.03$, extent: $P < 0.03$, type: $P < 0.02$, tenacity: $P < 0.02$), whereas the diltiazem and phospholipids were marginally effective (NS).

In Experiment II, comparing the group with 3% oxygen and 32°C BT, adhesion formation were further reduced by Hyalobarrier[®] gel (proportion: $P < 0.02$, total: $P < 0.02$, extent: $P < 0.01$, type: $P < 0.04$, tenacity: $P < 0.03$), whereas Retepase, SOD or nimesulide had little effect (NS for the three comparisons). Surprisingly, an increase in adhesions formation was observed with AA (proportion: $P < 0.01$, total: $P < 0.02$, extent: $P < 0.01$, type: NS, tenacity: NS).

Discussion

These experiments confirmed that hypothermia (32°C) and the addition of 3% oxygen to the pneumoperitoneum reduce adhesions in comparison with pure CO₂ at 37°C by 48% and 32%, respectively (Binda *et al.*, 2004). Using both treatments together did not have manifested additional effects that are consistent with the hypotheses that

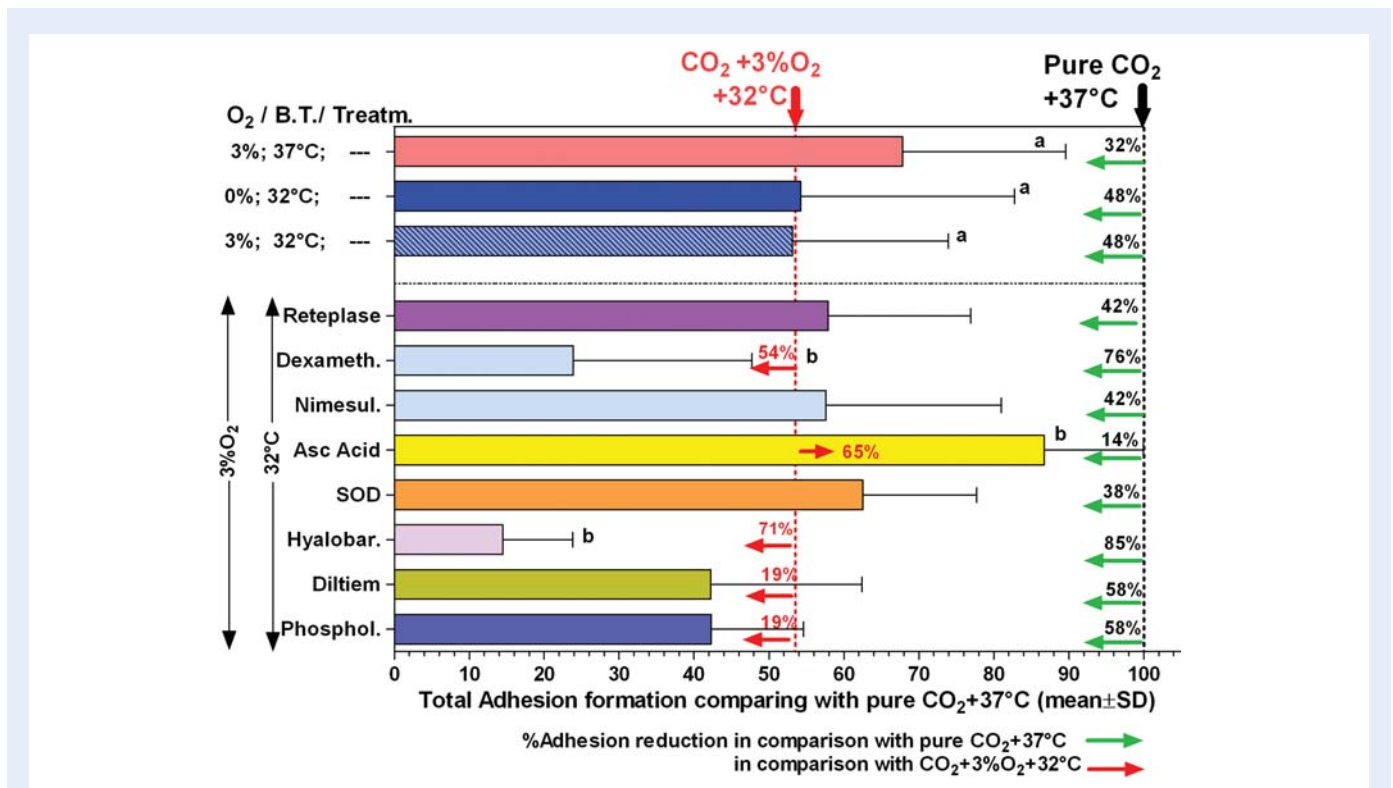


Figure 1 Adhesion prevention in a laparoscopic mouse model.

In comparison with 60 min of CO₂ pneumoperitoneum, the addition of 3% of oxygen and cooling had similar but no additive effects. In order to visualize the results of the two experiments in one graph, the percentage of changes in comparison to the proportions of the control group, i.e. pure CO₂ pneumoperitoneum and 37°C BT (black vertical dashed line, considered as 100%), are represented for each treatment. The percentage of reduction of adhesion in each treatment compared with this control group was also calculated (green arrows). The similar procedure was performed for another control group, i.e. CO₂ pneumoperitoneum + 3% oxygen and 32°C BT, which is denoted as red vertical dashed line, and the percentage reduction (or increase for the AA) of each treatment compared with this control (considered as 100%) was calculated (red arrows). Statistic: Wilcoxon test: $P < 0.05$. ^aComparison with control pure CO₂ pneumoperitoneum, 37°C BT. ^bComparison with control CO₂+3% O₂ pneumoperitoneum, 32°C BT.

the addition of 3% of oxygen prevents the mesothelial hypoxic trauma caused by pure CO₂, whereas at a lower temperature cells are more resistant to hypoxia. Since our results do not permit the detection of minor differences, we cannot exclude that cooling and adding 3% oxygen might have slight additional effects. This, anyway, would not contradict the hypothesis of a common pathway. Conditioning of pneumoperitoneum, thus, should comprise today perfect humidification, the addition of a few percent of oxygen and slight cooling instead of heating as has been suggested. Considering the hypothesis that hypoxia of the superficial layer is a driving mechanism for adhesions, then delivering oxygen to the cells in order to achieve some 30–40 mmHg is crucial. Increasing the ppO₂ in the blood by ventilation will help, however, adding the oxygen directly to the pneumoperitoneum is obviously easier and more effective. Cooling together with prevention of desiccation can, however, not be achieved that easily, since it will require cooling of the peritoneal cavity by external means. Indeed, if the insufflated gas is cooled, the absolute humidity will drop, and after the gas is heated in the peritoneal cavity to the BT, desiccation is inevitable and will occur.

Many products have been described to be effective in decreasing adhesion formation both in the human and in animal models, which unfortunately have not been that strictly characterized and the experimental conditions vary widely. We, therefore, have evaluated

previously the effect of a large number of these products in our well-defined laparoscopic mouse model with 60 min of CO₂ pneumoperitoneum at 37°C, i.e. the standard conditions routinely used for laparoscopic surgery in the human all over the world. Since the addition of 3% of oxygen and slight cooling already decrease adhesion formation by some 50% in the laparoscopic mouse model, we now wanted to investigate which products are still effective in this model. Of all products investigated for further decreasing adhesion formation in addition to peritoneum conditioning, the effect of dexamethasone is remarkable. Dexamethasone reduces adhesions by 32% when pure CO₂ is used for the pneumoperitoneum (Binda *et al.*, 2007b), but resulted in a total decrease in adhesion formation of 76% in comparison with pure CO₂ at 37°C when it is combined with the peritoneum conditioning. Since nimesulide had no additive effect to adding oxygen and cooling, we suggest that the effectivity of dexamethasone is mediated through mechanisms other than reducing inflammation. Indeed, glucocorticoids can inhibit fibroblast proliferation while having an immunosuppressive effect affecting production and release of cytokines (Brunton *et al.*, 2006). Moreover, since these pathways would be different to those involved in hypothermia and the addition of 3% oxygen, the additive effect is plausible.

With peritoneum conditioning, the calcium-channel blocker diltiazem and phospholipids further decreased adhesion formation

by some 20%, resulting in a total reduction of some 60%. This additional effectivity, however, failed to reach statistical significance, which is not surprising since much larger groups would be required to demonstrate the effect unequivocally. Moreover, it would be interesting to investigate the additional effect after 3% oxygen, slight cooling and dexamethasone administration, since this would suggest a different pathway. Indeed, phospholipids are considered to lubricate the peritoneum, whereas diltiazem is considered to affect the inflammatory response while protecting cells against the toxic effect of the ischemia–reperfusion injury. Since the demonstration of the effect in a model with already 75% adhesion reduction requires such large groups, we decided to postpone these experiments until more information would have been gathered.

Hyalobarrier[®] gel clearly decreased adhesion formation in addition to 3% oxygen and cooling, resulting in some 85% adhesion reduction in total, thus confirming the effect of Hyalobarrier[®] in the CO₂ pneumoperitoneum model at 37°C (Binda *et al.*, 2007a). This additional effect is not surprising since the mechanism of action of Hyalobarrier[®] is considered to be a local barrier, whereas 3% of oxygen and cooling are considered to act upon the entire peritoneal cavity. At present, we can only speculate based on their mechanisms of action that barriers will decrease adhesion formation by more than 90% when given in the 3% oxygen and cooling model along with dexamethasone treatment.

Superoxide dismutase, an ROS scavenger, did not show an additional effect to the treatments, 3% oxygen addition and cooling. Since a 38% reduction was achieved in the CO₂ model at 37°C BT, we postulate that this is due to the decrease in ROS scavengers in this model (Binda *et al.*, 2007b). To explain the increase in adhesions by AA in the 3% oxygen and cooling model, and the borderline effect in the pure CO₂ model at 37°C, we have to postulate some irritation by the acidic AA. Although Reteplase has shown a 40% effective in the pure CO₂ 37°C model (Binda *et al.*, 2009), it failed to demonstrate additional effect in the 3% oxygen–cooling model.

Adhesion prevention by conditioning the pneumoperitoneum during surgery, e.g. adding 3% of oxygen and slight cooling without desiccation, together with the administration of dexamethasone and a barrier, after surgery is theoretically and clinically attractive. Theoretically, the mechanisms of adhesion prevention are believed to be different. Adding 3% oxygen to the pneumoperitoneum and cooling prevent mesothelial cell hypoxia with induction of angiogenic factors and decrease in ROS. Both of these, as well as desiccation, have an effect upon the entire peritoneal cavity. The mechanisms of adhesion prevention by dexamethasone is less clear but given the ineffectivity of COX-2 inhibitors, it is probably not through the reduction of inflammation, and a barrier has a mechanical effect by acting locally. Clinically, this combination therapy is attractive since it combines pneumoperitoneum conditioning during surgery, with drug treatment and a barrier after surgery, with a speculated overall effect of more than 90% adhesion reduction. Obviously, this will have to be validated in clinical trials.

Although the side effects of the low temperature are well known (Insler and Sessler, 2006), induced hypothermia is one of the most promising neuroprotective therapies (Hemmen and Lyden, 2007). Application of therapeutic hypothermia after cardiac arrest could help to improve the neurological recovery (Bernard *et al.*, 2002; Hypothermia after Cardiac Arrest Study Group, 2002; Holzer *et al.*, 2005). Moreover, the hypothermia–cerebroprotection effect can be

improved by additional pharmacotherapy in rats subjected to ischemia–reperfusion (Schmid-Elsaesser *et al.*, 1999). In addition, combined therapies with mild hypothermia (33°C) were efficient for neuroprotection during cerebral ischemia in cerebrovascular surgery (Zausinger *et al.*, 2003). These examples are different from our model; however, they both have the common pathway of the ischemia–reperfusion and the protection of its toxic effects. Since hypothermia was induced for longer time and in the whole body, those would be extreme examples suggesting that hypothermia can also be used locally in humans. A recent article of Ozgonul *et al.* (2007) supports our theory of using low temperature locally. In this study, hypothermic CO₂ (21°C) used for pneumoperitoneum was compared with isothermic gas (37°C) during laparoscopic cholecystectomy in a prospective randomized study. Measurements were done before insufflation, at 30 min of pneumoperitoneum and 30 min after desufflation. No significant difference was observed in core BT and blood arterial pH, arterial carbon dioxide pressure, arterial oxygen pressure and bicarbonate values, only the mean skin BT was significantly higher in the isothermic group than the hypothermic group. We indeed should apply hypothermia carefully in women. This will be the next experiment since by preliminary data demonstrate that when cooling the abdominal cavity, the cooling is only superficial. Our hypothesis is that superficial mesothelial cooling is sufficient to prevent the hypoxic effect.

In summary, we demonstrated that conditioning the pneumoperitoneum by cooling and/or adding 3% oxygen, some 50% reduction of adhesion formation can be achieved. Adhesion formation can be further reduced to 76% by adding dexamethasone or 85% by applying Hyalobarrier[®] gel after surgery. With these data, we suggest that adhesion formation prevention should become multifactorial combining pneumoperitoneum conditioning, medical treatment and mechanical barriers. Whereas for now, dexamethasone has been identified as effective, the exact place of calcium channel blockers or phospholipids in this combination model remains unclear.

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