

# Addition of nitrous oxide to the carbon dioxide pneumoperitoneum strongly decreases adhesion formation and the dose-dependent adhesiogenic effect of blood in a laparoscopic mouse model

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**Objective:** To evaluate the effect of addition of nitrous oxide (N<sub>2</sub>O) to the carbon dioxide (CO<sub>2</sub>) pneumoperitoneum (PP) and the effect of blood, plasma, or red blood cells (RBCs) on postoperative adhesions in a laparoscopic mouse model.

**Design:** Prospective randomized controlled trial.

**Setting:** University laboratory research center.

**Animal(s):** BALB/c female mice.

**Intervention(s):** The effect of adding to the 60-minute CO<sub>2</sub> PP 5%, 10%, 25%, 50%, or 100% N<sub>2</sub>O on adhesion formation was evaluated. Subsequently the effect of adding 1 mL blood, or RBCs, or plasma and the effect of adding different concentrations of blood were studied. Finally, the effect of adding 10% N<sub>2</sub>O, 4% O<sub>2</sub>, or both to the CO<sub>2</sub> was evaluated in a control group and after addition of blood.

**Main Outcome Measure(s):** Postoperative adhesions after 7 days.

**Result(s):** N<sub>2</sub>O strongly reduces adhesion formation with a full effect at a concentration of 5% or 10%. Adhesions increase linearly with 0.125 mL to 1 mL blood. In both the control group and after adding blood, 10% N<sub>2</sub>O is the most effective factor in prevention of adhesions.

**Conclusion(s):** N<sub>2</sub>O, from concentrations of 5% upward, strongly prevents adhesion formation. Blood, mainly the plasma, increases adhesion formation. These data extend the concept of the role of acute inflammation and support the importance of good surgical practice with little bleeding and peritoneal cavity conditioning in adhesion prevention. (Fertil Steril® 2013;100:1777–83. ©2013 by American Society for Reproductive Medicine.)

**Key Words:** Pneumoperitoneum, adhesions, N<sub>2</sub>O, O<sub>2</sub>, peritoneal conditioning, laparoscopy, bleeding

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Abdominal surgery is associated with postoperative adhesions in 60%–90% of patients (1), being a major cause of infertility, chronic pelvic pain, and bowel obstructions. Although laparoscopy is thought to be less adhesiogenic than laparotomy, this remains unclear, and

the risk of adhesion-related complications seems to be similar (2).

The pathophysiology of adhesion formation was traditionally viewed as a local phenomenon resulting from the surgical trauma to the peritoneal surfaces as well as involving the mesothelial cells, basal membrane, and subendothelial connective tissue. This leads to a local inflammatory reaction and a cascade of events, such as exudation (3), fibrin deposition, and capillary growth at the site of injury (4). The extent of adhesions depends on the balance among the rates of fibrinolysis, peritoneal repair, and fibroblast proliferation (5).

These events at the trauma site are modulated by factors from the entire peritoneal cavity and the degree of acute inflammation (6). This acute inflammation increases with mechanical trauma as evidenced by manipulation of bowels in the upper abdomen (7) or by learning curves (8). It increases with desiccation and with the duration and pressure of carbon dioxide (CO<sub>2</sub>) pneumoperitoneum (PP) through a subnormal mesothelial partial O<sub>2</sub> pressure. It increases if the PP contains more than 10% O<sub>2</sub> through a supernormal mesothelial partial O<sub>2</sub> pressure and reactive O<sub>2</sub> species (ROS) (9). Factors that decrease acute inflammation are the prevention of desiccation by humidified gas (10), gentle tissue handling as evidenced by the decreasing adhesions during the learning curve (8), and a physiologic mesothelial partial O<sub>2</sub> pressure around 30 mm Hg by adding 4% O<sub>2</sub> to the CO<sub>2</sub> PP. In addition, acute inflammation and adhesion formation are lower when the mesothelial temperature is lower (11) and after the administration of dexamethasone (9).

Nitrous oxide (N<sub>2</sub>O), well known as an anesthetic gas, has been used for the PP instead of CO<sub>2</sub> because of the irritative effect of CO<sub>2</sub> thought to cause postoperative shoulder pain and because of the metabolic side effects of CO<sub>2</sub> following resorption (12, 13). N<sub>2</sub>O is safe gas (14) with a high solubility in water (1.5 mg/L) and a lung exchange similar to CO<sub>2</sub>. N<sub>2</sub>O in addition is less irritating with less postoperative pain compared with CO<sub>2</sub> (15). N<sub>2</sub>O has anesthetic and analgesic properties without the metabolic and cardiopulmonary side effects of CO<sub>2</sub> (16, 17). N<sub>2</sub>O, however, was never widely used owing to its explosion risks at concentrations >29% (18, 19).

Blood is considered to be adhesiogenic because bleeding enhances adhesion formation (20, 21) whereas a blood stopper decreases adhesions (22, 23). Moreover, in a cecal abrasion animal model, trauma with bleeding has been used widely as a method to induce adhesions (24). Given the importance of blood and fibrin it seemed logical to add heparin to the rinsing solution (25–29), but the clinical advantage of using heparin in rinsing solutions in the human is still unclear.

Because N<sub>2</sub>O causes less postoperative pain, possibly related to a decreased inflammatory reaction, we decided to further explore the role of acute inflammation in the entire peritoneal cavity on adhesion formation at the surgical sites by adding N<sub>2</sub>O to the CO<sub>2</sub> PP and blood in our laparoscopic mouse model. Because intraperitoneal blood is known to be associated with an inflammatory reaction, the effect of various amounts of blood and of its constituents on adhesion formation was also evaluated.

## MATERIALS AND METHODS

### Laparoscopic Mouse Model for Adhesion Formation

The model has been validated extensively and the experimental setup (animals, anesthesia, ventilation, laparoscopic surgery, induction, and adhesion scoring) previously described in detail (6–11, 30–35). Briefly, the model consisted of inducing a PP with the use of the Thermoflator (Karl Storz) and performing 2 standardized 10 mm bipolar lesions (20 W, standard coagulation mode; Autocon 350, Karl Storz) in both uterine horns and lateral side walls under laparoscopic vision (a 2 mm endoscope; Karl Storz) through two 14-gauge catheters (Insyte-W, Vialon; Becton Dickinson). All incisions were closed gas tight around the endoscope or catheter to avoid leakage, and the PP was maintained for 60 minutes at an insufflation pressure of 15 mm Hg with humidified gas (Humidifier 204320 33; Karl Storz). Because temperature is critical for adhesion formation (11), animals and equipment were placed in a closed chamber at 37°C (heated air; Warm Touch, Patient Warming System, model 5700; Mallinckrodt Medical). Because anesthesia and ventilation influence body temperature, the timing between anesthesia (T0), intubation (at 10 min [T10]), and the onset of the experiment (at 20 min [T20]) was strictly standardized.

To obtain mixtures of various concentrations of N<sub>2</sub>O in CO<sub>2</sub>, two thermoflators, one delivering CO<sub>2</sub> and the other N<sub>2</sub>O, were used. The two gases were subsequently mixed in a mixing chamber and the excess gas was permitted to escape from a water valve (experiments II and III). For the experiments, four premixed gases were used to keep flow rates of gases identical in all groups (supplied by IJsfabriek).

### Scoring of Adhesions

Adhesions were scored blindly after 7 days both quantitatively and qualitatively as reported previously. This scoring was used over the past decade with consistent results over the years. Scoring was done after 7 days for practical reasons, because scoring of adhesions on day 7, 14, or 28 gave similar results (34). The qualitative scoring comprised the extent (0: no adhesions; 1: 1%–25%; 2: 26%–50%; 3: 51%–75%; 4: 76%–100% of the injured surface involved), type (0: no adhesions; 1: filmy; 2: dense; 3: capillaries present), and tenacity (0: no adhesions; 1: easily fall apart; 2: require traction; 3: require sharp dissection) of adhesions. The quantitative scoring system assessed the proportion of the lesions covered by adhesions with the use of the following formula: (sum of the length of the individual attachments/length of the lesion) × 100. The results were presented as the average of the adhesions formed at the four individual sites (right and left visceral and parietal peritoneum), which are scored individually (36). The entire abdominal cavity is visualized by a xyphopubic midline and a bilateral subcostal incision. After the evaluation of port sites and viscera (omentum, large and small bowels) for de novo adhesions, the fat tissue surrounding the uterus was carefully removed. The length of the visceral and parietal lesions and adhesions were measured. Adhesions, when present, were lysed to evaluate their type and tenacity.

The terminology of Pouly was used (37), describing de novo adhesion formation for the adhesions formed at nonsurgical sites, adhesion formation for adhesions formed at the surgical site and adhesion reformation for adhesions formed after the lysis of previous adhesions.

## Animals

To reduce variability, inbred 9–10-week-old female BALB/c OlaHsd mice (Harlan Laboratories) weighing 18–20 g were used. Animals were kept under standard laboratory conditions and diet at the animal facilities of the Katholieke Universiteit Leuven. The study was approved by the Institutional Review Animal Care Committee (no., P040/2010).

## Experiments

All experiments were performed with the use of block randomization by day. Thus, a block of animals comprising one animal of each group was always operated on the same day, avoiding day-to-day variability. In addition, within a block, experiments were performed in random order.

**Pilot experiment.** The pilot experiment ( $n = 18$ ) was designed to evaluate the effect of PP with 100% CO<sub>2</sub> compared with 100% N<sub>2</sub>O on adhesion formation ( $n = 9$ /group). Because this experiment was the first performed by a junior investigator (K.M.) as part of her training, a higher sample size was used in each group. This also explains why two animals died during the procedure owing to intubation problems (both in the N<sub>2</sub>O group).

**Experiment I.** Experiment I ( $n = 30$ ) was a dose-response experiment following the unexpectedly strong antiadhesiogenic effect of 100% N<sub>2</sub>O in the pilot experiment. This experiment was designed to evaluate adhesion formation following a 60-minute PP with 100% CO<sub>2</sub> (control group) and with the addition of 5%, 10%, 25%, 50%, and 100% N<sub>2</sub>O to the CO<sub>2</sub> ( $n = 5$ /group). To obtain a mixture of the various concentrations of CO<sub>2</sub> and N<sub>2</sub>O, two thermoflators were used with different flow rates of 2 and 2, 4 and 1, 9 and 1, and 19.5 and 0.5 L/min to obtain final concentrations of 50%, 25%, 10%, and 5% N<sub>2</sub>O, respectively.

**Experiment II.** Experiment II ( $n = 15$ ) was designed to evaluate the adhesiogenic effect of blood, plasma, and RBCs. Compared with a control group, 1 mL blood, 1 mL plasma, or 1 mL resuspended RBCs was injected intraperitoneally (i.p.) at the end of the PP ( $n = 5$ /group). Blood was obtained from the retro-orbital sinus with a Pasteur pipette or by cardiac puncture from anesthetized mice before each experiment. Blood was collected into a heparinized tube and centrifuged for 10 minutes at 1,000 rpm at 4°C to separate plasma and RBCs. The pellet containing the RBCs was resuspended to the same volume of 1 mL in an isotonic solution (145 mmol/L NaCl, 1 mmol/L CaCl<sub>2</sub>, 5 mmol/L d-glucose, 10 mmol/L MOPS; pH 7.4). Blood, plasma, or resuspended RBCs obtained from 1 mL blood was injected i.p. at the end of the PP. An eventual effect of the presence of small amounts of heparin in blood and plasma and much less in resuspended

RBC was not taken into account. Blood, RBCs, and plasma had been kept at 4°C until used.

**Experiments III and IV.** Experiments III and IV were performed in one experiment of 11 groups, which constituted the maximum number of animals that could be handled in one day by two investigators (block randomization by day;  $n = 5$ /group; total  $n = 55$ ). Experiment III ( $n = 25$ ) was designed as a dose response that evaluated in comparison with 100% CO<sub>2</sub> only (control group) the adhesiogenic effect of 0.125, 0.25, 0.5, and 1 mL blood injected i.p. at the end of the PP (5 groups). Experiment IV ( $n = 30$ ) was designed to confirm the antiadhesiogenic effect of 10% N<sub>2</sub>O and of 4% O<sub>2</sub>, to evaluate an eventual additive effect when used together in control mice, and to evaluate these effects in mice having received 0.5 mL blood at the end of the PP (twice three groups).

In both experiments III and IV, premixed gases were used to keep flow rates of gases identical in all groups.

## Statistics

Statistical significances were calculated with the SAS System (SAS Institute) using Wilcoxon/Kruskal-Wallis unpaired test for individual comparisons. Results are expressed as mean and standard deviation unless defined otherwise.

## RESULTS

In all experiments only adhesions at the surgical lesions were found. In none of the experiments adhesions at other places (de novo adhesions) were observed.

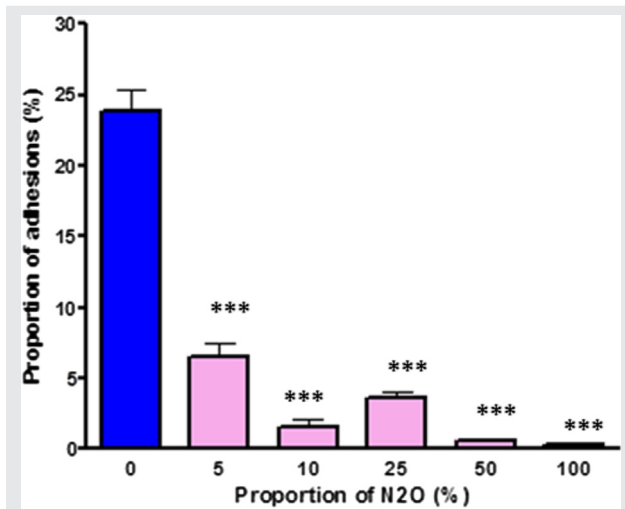
### Pilot Experiment

The use of a PP for 60 minutes with 100% N<sub>2</sub>O instead of 100% CO<sub>2</sub> strongly decreased adhesion formation whether evaluated as the quantitative score ( $P = .012$ ) or the qualitative score. Total adhesion score, type, tenacity, and extent decreased from  $3.0 \pm 0.3$  to  $1.6 \pm 0.4$  ( $P = .009$ ), from  $10.8 \pm 0.5$  to  $5.5 \pm 0.6$  ( $P = .023$ ), from  $10.8 \pm 1.2$  to  $5.6 \pm 0.4$  ( $P = .025$ ), and from  $11.3 \pm 1.3$  to  $4.9 \pm 0.8$  ( $P = .005$ ), respectively. The adhesion formation in the mouse model is known to vary with the experience of the investigator (8), which can explain the quantitative differences between the pilot experiment and the subsequent experiments.

### Experiment I

Compared with 100% CO<sub>2</sub> the addition of 5%, 10%, 25%, or 50% of N<sub>2</sub>O or the use of 100% N<sub>2</sub>O strongly decreased the quantitative scoring of adhesions in all groups ( $P = .0001$  for all groups; Fig. 1). Qualitative scores also showed a significant decrease in adhesion formation with the addition of different concentrations of N<sub>2</sub>O ( $P = .0001$  for all groups). Adhesions formation between any of the groups receiving 5%, 10%, 25%, 50%, or 100% N<sub>2</sub>O was not significantly different. The effect of as little as 5% N<sub>2</sub>O was unexpected, and the experiment was not designed to evaluate a dose response at concentrations <5%. The variability of results might be explained by the fact that in these experiments relative

FIGURE 1



The effect on adhesion formation of pneumoperitoneum with 100% CO<sub>2</sub>, with the addition of 5%, 10%, 25%, or 50% N<sub>2</sub>O to the CO<sub>2</sub> PP, or with 100% N<sub>2</sub>O was evaluated. Quantitative scoring of adhesions (proportion of adhesions) are depicted in this figure. Wilcoxon/Kruskal-Wallis unpaired test for each group compared with 100% CO<sub>2</sub>: \*\*\**P*<.0001.

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concentrations were determined by flow rates indicated on the thermoflators, which is relatively imprecise at low flow rates; in addition, flow rates of 10 L/min might induce some variability in the model by increasing pressure (Fig. 1).

### Experiment II

Experiment II demonstrated that adhesions (quantitative score) increased by the addition of blood, plasma, or RBCs compared with the control group from  $25 \pm 2.74$  to  $78.5 \pm 2.20$  (*P*<.0001),  $55.5 \pm 1.84$  (*P*<.0001), and  $30.5 \pm 1.28$  (*P*=.013), respectively. Blood is more adhesiogenic than plasma or RBCs only (*P*<.0001 for both comparisons) and plasma increases adhesions more than RBCs (*P*<.0001; Fig. 2).

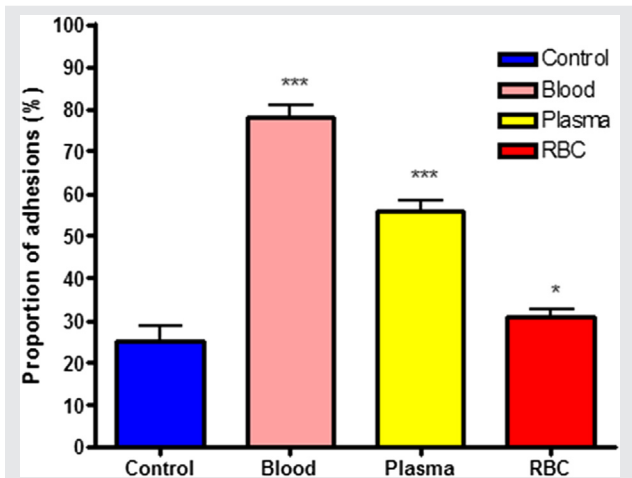
Total adhesion score, type, tenacity, and extent (qualitative score) significantly increased by the addition of blood (*P*<.0001, *P*<.0001, *P*=.0001, and *P*=.0025, respectively), plasma (*P*=.0001, *P*=.0001, *P*=.0001, and *P*=.005, respectively), or RBCs (*P*=.0001, *P*=.0025, *P*=.005, and *P*=.005, respectively) compared with the control group.

### Experiment III and IV

The dose-response curve demonstrated that as little as 0.125 mL blood already strongly increased adhesions (*P*<.0001) and that the adhesiogenic effect increased with the amount of blood up to 0.5 mL (*P*<.0001 for every amount). Adding 1 mL blood compared with 0.5 mL was not significant.

In the control group we confirmed the adhesion-reducing effect of using for the PP, instead of 100% CO<sub>2</sub>, CO<sub>2</sub> with 4% O<sub>2</sub>, with 10% N<sub>2</sub>O, or with both 10% N<sub>2</sub>O + 4% of O<sub>2</sub> (*P*=.009, *P*<.0001, and *P*<.0001, respectively). Also, after the addition

FIGURE 2



One milliliter of blood, plasma, or resuspended pellet of red blood cells was injected i.p. following 60 minutes of pneumoperitoneum with humidified CO<sub>2</sub>. Quantitative scoring system (proportion of adhesions) is indicated in the figure. Wilcoxon/Kruskal-Wallis unpaired test for each group compared with control: \*\*\**P*<.0001; \**P*=.013.

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of 0.5 mL blood, the use of these three gas mixtures decreased adhesions compared with the group with 100% CO<sub>2</sub> (*P*<.0001 for all groups). The addition of 10% N<sub>2</sub>O was more effective than 4% O<sub>2</sub> (*P*<.0001) with little additive effect of adding 4% O<sub>2</sub> to the 10% N<sub>2</sub>O (NS; Fig. 3). Qualitative scores showed similar results for all groups.

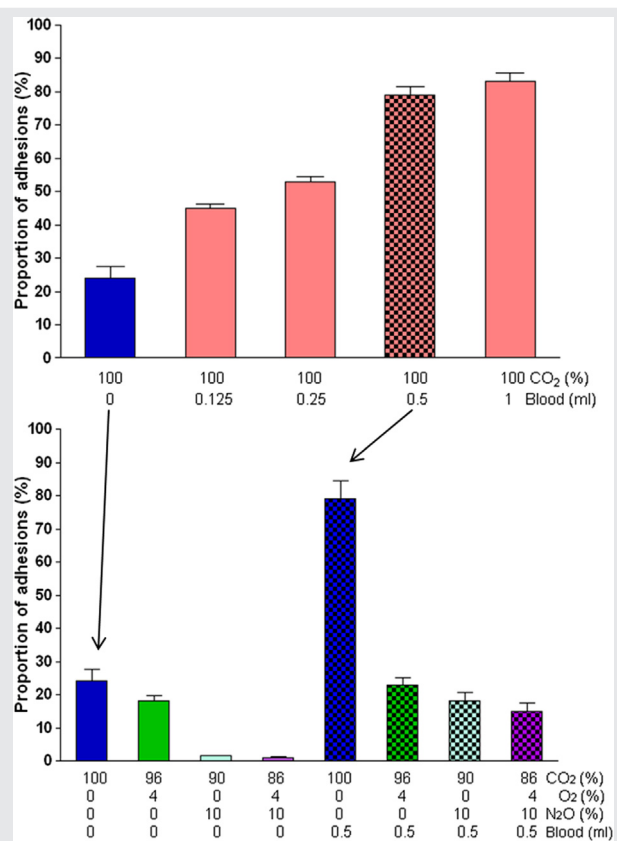
### DISCUSSION

These data demonstrate for the first time that the use of N<sub>2</sub>O instead of or in addition to CO<sub>2</sub> for the PP reduces postoperative adhesion formation in a laparoscopic mouse model. Moreover, surprisingly this effect was already obtained with a concentration of as little as 5% N<sub>2</sub>O in CO<sub>2</sub>.

The effect of N<sub>2</sub>O, especially at concentrations of 5% was unexpected and cannot be explained by current knowledge. At this moment we do not have an explanation for the effect of N<sub>2</sub>O in contrast to all previous observations in our model. The effect of lower temperatures (11) could be explained by making cells more resistant to damage, e.g., by hypoxia, and that of the addition of a physiologic concentration of O<sub>2</sub> (34) could be explained by correction of the mesothelial hypoxia during pure CO<sub>2</sub> PP or mesothelial hyperoxia when >10% O<sub>2</sub> was used (30). The effect of humidification was easily explained by preventing desiccation, whereas gentle tissue handling should decrease mechanical trauma (7, 8). The mechanism of action of N<sub>2</sub>O, however, has to be different from the effect of O<sub>2</sub>, because the effect of even 100% N<sub>2</sub>O is similar to the effect of 5%, whereas O<sub>2</sub> concentrations >10% clearly increase adhesions.

N<sub>2</sub>O, commonly known as "laughing gas," is a colorless nonflammable gas used in surgery and dentistry for its anesthetic and analgesic effects. N<sub>2</sub>O is a weak general anesthetic,

FIGURE 3



(Top) The adhesiogenic effect of adding 0, 0.125, 0.25, 0.5, or 1 mL blood following 60 minutes of pneumoperitoneum with 100% CO<sub>2</sub> is shown. (Bottom) The antiadhesiogenic effect of using CO<sub>2</sub> with 4% O<sub>2</sub>, 10% N<sub>2</sub>O, or both in the control group (left) and in the group having received 0.5 mL blood (right).

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so it is used as a carrier gas in a 2:1 ratio with O<sub>2</sub> for more powerful general anesthetic agents such as sevoflurane or desflurane. N<sub>2</sub>O is relatively nonpolar, with a molecular weight of 44.013 g/mol and a high lipid solubility. As a result, it diffuses quickly into the phospholipid cell membranes. Although N<sub>2</sub>O's exact mechanism of action is still open to some conjecture, it is known that it acts as an N-methyl-D-aspartate receptor antagonist at partial pressures similar to those used in general anesthesia. N<sub>2</sub>O also affects GABA<sub>A</sub> receptor (16), but this is still controversial. It has a lower potency by acting as a positive allosteric modulator. N<sub>2</sub>O, like other volatile anesthetics, activates twin-pore potassium channels, albeit weakly, and these channels are largely responsible for keeping neurons at the resting (unexcited) potential (17). Pure N<sub>2</sub>O has been used instead of CO<sub>2</sub> for the PP during laparoscopic surgery because it has the advantage of not inducing the metabolic side effects of CO<sub>2</sub> following resorption. It therefore was recommended to change to N<sub>2</sub>O in case of refractory hypercarbia and to use it as the primary insufflation gas in patients with little ventilatory reserve (15). In addition, N<sub>2</sub>O is a safe gas when considering the risk of gas embolism. Indeed, N<sub>2</sub>O has an even slightly better solubility in

water or blood than CO<sub>2</sub> (1.5 and 1.45 mg/L, respectively) and a slightly better lung exchange capacity. Following PP with 100% N<sub>2</sub>O, patients experience less pain (12, 13, 38, 39), on the day of surgery and following days (40). N<sub>2</sub>O, however, never became popular because of the explosion risk when used in concentrations >29%. In these concentrations, N<sub>2</sub>O might maintain combustion (18). Therefore, if gases such as methane escaped from the intestine and were ignited by electrosurgery, some explosion risk exists. This risk exists theoretically and is supported by some reported accidents (19, 41).

The observation, therefore, that as little as 5% or 10% of N<sub>2</sub>O has a strong antiadhesiogenic effect similar to that of 100% N<sub>2</sub>O brings this gas within reach for generalized use during laparoscopic surgery. Indeed at these concentrations far below the critical 29% there is no longer an explosion risk (14). Evaluation of lower concentrations of N<sub>2</sub>O was not performed in the laparoscopic mouse model for practical reasons. Indeed, 1% N<sub>2</sub>O would have required 0.5 mL/min N<sub>2</sub>O in 50 mL/min of CO<sub>2</sub>.

We recently demonstrated that in women undergoing promontofixation, postoperative pain was reduced by adding 4% O<sub>2</sub> to the CO<sub>2</sub>. Because in the mouse model, acute inflammation of the peritoneal cavity decreased by the addition of 4% O<sub>2</sub>, it is suggested that postoperative pain is at least partially mediated by acute inflammation of the entire peritoneal cavity. Because N<sub>2</sub>O was reported to decrease postoperative pain (12, 13, 38, 39, 42) and because this has been confirmed by a recent Cochrane meta-analysis (40), we expect that N<sub>2</sub>O, even at low concentrations of 5%–10%, will decrease also the acute inflammatory reaction.

To the best of our knowledge, this is the first experimental study detailing the effect of blood in the peritoneal cavity on adhesion formation. The effect was dose dependent with a sigmoid relationship between adhesion formation and amount of blood. To interpret the observation that as little as 0.125 mL already significantly increased adhesions, with a further exponential increase up to 0.5 mL blood, with little additional effect of 1 mL, it should be taken into account that 0.5 and 1 mL of blood are high volumes considering the total amount of blood in a mouse and the volume of the peritoneal cavity. The adhesiogenic effect of total blood corresponded strikingly to the sum of the adhesiogenic effect of plasma and RBCs separately, suggesting that besides the likely fibrin deposition, acute inflammation of the entire peritoneal cavity plays an important role. This is consistent with earlier observations that acute inflammation in the entire peritoneal cavity causes enhanced adhesion formation between injured areas and that this mechanism is quantitatively much more important than the adhesions resulting from peritoneal trauma only. Trauma, however, remains a prerequisite for adhesion formation, because in none of these (and earlier) experiments were de novo adhesions found, not even after the addition of 1 mL blood. We therefore suggest two separate roles of fibrin deposition in adhesion formation. After a peritoneal injury, exudation and local fibrin deposition occurs, and if this fibrin is not removed by fibrinolysis within a few days, adhesions start to form locally. Blood and/or fibrin, spilled in the peritoneal cavity, causes acute inflammation

of the entire peritoneal cavity as evidenced by the pain and the rise in C-reactive protein (CRP) following intra-abdominal bleeding (43, 44). It is unclear whether in humans fibrin deposition in the peritoneal cavity plays a role in adhesion formation only through acute inflammation, because an overload of fibrin could in addition decrease the availability of plasmin necessary for the local fibrinolysis between injured areas. These concepts that fibrin also has an effect on the entire peritoneal cavity might be useful to interpreting the unclear and conflicting results of the effect of tissue plasminogen activator (tPA) on adhesion formation (32). These data also confirm the antiadhesiogenic effect of adding 4% O<sub>2</sub> and extend the antiadhesiogenic effect of N<sub>2</sub>O and O<sub>2</sub> to adhesions enhanced by blood. Extrapolation of this effect of blood to human surgery supports the importance of meticulous hemostasis. Quantitatively, however, 1 mL blood in these small mice is probably equivalent to >1 L blood in the human.

In conclusion, our present data confirm and extend the concept (45) that acute inflammation of the entire peritoneal cavity is quantitatively the most important driving mechanism in adhesion formation, and that good surgical practice and peritoneal cavity conditioning are the cornerstones of adhesion prevention. To the already identified detrimental factors, the strong adhesiogenic effect of blood is added. The strong antiadhesiogenic effect of N<sub>2</sub>O was unexpected, especially because it is the single most important factor identified so far. That N<sub>2</sub>O is effective in low concentrations of 5%–10% indicates that it is effective not by replacing CO<sub>2</sub> but through an unknown drug-like mechanism. Most importantly, however, at these low concentrations N<sub>2</sub>O can be used without explosion risk. Human trials will have to evaluate to what extent the other beneficial effects of N<sub>2</sub>O compared with CO<sub>2</sub> will remain valid.

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## REFERENCES

1. Monk BJ, Berman ML, Montz FJ. Adhesions after extensive gynecologic surgery—clinical-significance, etiology, and prevention. *Am J Obstet Gynecol* 1994;170:1396–403.
2. Lower AM, Hawthorn RJ, Clark D, Boyd JH, Finlayson AR, Knight AD, et al. Adhesion-related readmissions following gynaecological laparoscopy or laparotomy in Scotland: an epidemiological study of 24 046 patients. *Hum Reprod* 2004;19:1877–85.
3. diZerega GS. Biochemical events in peritoneal tissue repair. *Eur J Surg* 1997; (Suppl):10–6.
4. diZerega GS. Peritoneum, peritoneal healing and adhesion formation. In: diZerega GS, editor. *Peritoneal surgery*. New York: Springer; 2000:3–38.
5. diZerega GS, Campeau JD. Peritoneal repair and post-surgical adhesion formation. *Hum Reprod Update* 2001;7:547–55.
6. Corona R, Verguts J, Schonman R, Binda MM, Mailova K, Koninckx PR. Post-operative inflammation at the abdominal cavity increases adhesion formation in a laparoscopic mouse model. *Fertil Steril* 2011;95:1224–8.
7. Schonman R, Corona R, Bastidas A, de Cicco C, Koninckx PR. Effect of upper abdomen tissue manipulation on adhesion formation between injured areas in a laparoscopic mouse model. *J Minim Invasive Gynecol* 2009;16:307–12.
8. Corona R, Verguts J, Binda MM, Molinas CR, Schonman R, Koninckx PR. The impact of the learning curve on adhesion formation in a laparoscopic mouse model. *Fertil Steril* 2011;96:193–7.
9. Binda MM, Molinas CR, Bastidas A, Koninckx PR. Effect of reactive oxygen species scavengers, anti-inflammatory drugs, and calcium-channel blockers on carbon dioxide pneumoperitoneum-enhanced adhesions in a laparoscopic mouse model. *Surg Endosc* 2007;21:1826–34.
10. Binda MM, Molinas CR, Hansen P, Koninckx PR. Effect of desiccation and temperature during laparoscopy on adhesion formation in mice. *Fertil Steril* 2006;86:166–75.
11. Binda MM, Molinas CR, Mailova K, Koninckx PR. Effect of temperature upon adhesion formation in a laparoscopic mouse model. *Hum Reprod* 2004;19:2626–32.
12. Aitola P, Airo I, Kaukinen S, Ylitalo P. Comparison of N<sub>2</sub>O and CO<sub>2</sub> PPs during laparoscopic cholecystectomy with special reference to postoperative pain. *Surg Laparosc Endosc* 1998;8:140–4.
13. Holzman M, Sharp K, Richards W. Hypercarbia during carbon dioxide gas insufflation for therapeutic laparoscopy: a note of caution. *Surg Laparosc Endosc* 1992;2:11–4.
14. Hunter JG, Staheli J, Oddsdottir M, Trus T. Nitrous oxide pneumoperitoneum revisited. Is there a risk of combustion? *Surg Endosc* 1995;9:501–4.
15. Tsereteli Z, Terry ML, Bowers SP, Spivak H, Archer SB, Galloway KD, et al. Prospective randomized clinical trial comparing N<sub>2</sub>O and carbon dioxide PP for laparoscopic surgery. *J Am Coll Surg* 2002;195:173–9.
16. Jevtovic-Todorovic V, Todorovic SM, Mennerick S, Powell S, Dikranian K, Benshoff N, et al. N<sub>2</sub>O (laughing gas) is an NMDA antagonist, neuroprotectant and neurotoxin. *Nat Med* 1998;4:460–3.
17. Gruss M, Mathie A, Lieb WR, Franks NP. The two-pore-domain K(+) channels TREK-1 and TASK-3 are differentially modulated by copper and zinc. *Mol Pharmacol* 2004;66:530–7.
18. Neuman GG, Sidebotham G, Negoianu E, Bernstein J, Kopman AF, Hicks RG, et al. Laparoscopy explosion hazards with nitrous oxide. *Anesthesiology* 1993;78:875–9.
19. Robinson JS, Thompson JM, Wood AW. Laparoscopy explosion hazards with nitrous oxide [letter]. *Br Med J* 1975;4:760–1.
20. Gadallah MF, Torres-Rivera C, Ramdeen G, Myrick S, Habashi S, Andrews G. Relationship between intraperitoneal bleeding, adhesions, and peritoneal dialysis catheter failure: a method of prevention. *Adv Perit Dial* 2001;17:127–9.
21. Gamal EM, Metzger P, Szabó G, Bráth E, Pető K, Oláh A, et al. The influence of intraoperative complications on adhesion formation during laparoscopic and conventional cholecystectomy in an animal model. *Surg Endosc* 2001;15:873–7.
22. Cömert M, Karakaya K, Barut F, Karadeniz Çakmak G, Uçan HB, Gültekin FA, et al. Does intraabdominal use of Ankaferd Blood Stopper cause increased intraperitoneal adhesions? *Ulus Travma Acil Cerrahi Derg* 2010;16:383–9.
23. Wiseman DM, Gottlick LE, Diamond MP. Effect of thrombin-induced hemostasis on the efficacy of an absorbable adhesion barrier. *J Reprod Med* 1992;37:766–70.
24. Numanoglu V, Cihan A, Salman B, Ucan BH, Cakmak GK, Cesur A, et al. Comparison between powdered gloves, powder-free gloves and hyaluronate/carboxymethylcellulose membrane on adhesion formation in a rat caecal serosal abrasion model. *Asian J Surg* 2007;30:96–101.
25. Bahadır I, Oncel M, Kement M, Sahip Y. Intra-abdominal use of taurolidine or heparin as alternative products to an antiadhesive barrier (Septrafilm) in adhesion prevention: an experimental study on mice. *Dis Colon Rectum* 2007;50:2209–14.
26. Opitz I, van der Veen H, Witte N, Braumann C, Mueller JM, Jacobi CA. Instillation of taurolidine/heparin after laparotomy reduces intraperitoneal tumour growth in a colon cancer rat model. *Eur Surg Res* 2007;39:129–35.
27. Inoh K, Muramatsu H, Ochiai K, Torii S, Muramatsu T. Midkine, a heparin-binding cytokine, plays key roles in intraperitoneal adhesions. *Biochem Biophys Res Commun* 2004;317:108–13.
28. Tayyar M, Basbug M. The effects of intraperitoneal piroxicam and low molecular weight heparin in prevention of adhesion reformation in rat uterine horn. *Res Exp Med (Berl)* 1999;198:269–75.

29. Basbug M, Aygen E, Tayyar M, Kaya E, Narin F, Oktem O. Hyaluronic acid plus heparin for improved efficacy in prevention of adhesion formation in rat uterine horn model. *Eur J Obstet Gynecol Reprod Biol* 1998; 78:109–12.
30. Binda M, Koninckx PR. Hyperoxia and prevention of adhesion formation: a laparoscopic mouse model for open surgery. *BJOG* 2009;117: 331–9.
31. Schonman R, Corona R, Bastidas A, De Cicco C, Mailova K, Koninckx PR. Intercoat gel (oxiplex): efficacy, safety, and tissue response in a laparoscopic mouse model. *J Minim Invasive Gynecol* 2009;16:188–94.
32. Binda MM, Hellebrekers BW, Declerck PJ, Koninckx PR. Effect of Reteplase and PAI-1 antibodies on postoperative adhesion formation in a laparoscopic mouse model. *Surg Endosc* 2009;23:1018–25.
33. Molinas CR, Binda MM, Koninckx PR. Angiogenic factors in peritoneal adhesion formation. *Gynecol Surg* 2006;3:157–67.
34. Molinas CR, Mynbaev O, Pauwels A, Novak P, Koninckx PR. Peritoneal mesothelial hypoxia during pneumoperitoneum is a cofactor in adhesion formation in a laparoscopic mouse model. *Fertil Steril* 2001;76:560–7.
35. Elkelani OA, Binda MM, Molinas CR, Koninckx PR. Effect of adding more than 3% O<sub>2</sub> to carbon dioxide PP on adhesion formation in a laparoscopic mouse model. *Fertil Steril* 2004;82:1616–22.
36. Holmdahl L, al Jabreen M, Risberg B. Experimental models for quantitative studies on adhesion formation in rats and rabbits. *Eur Surg Res* 1994;26: 248–56.
37. Pouly JL, Seak-San S. Peritoneal surgery. In: diZerega GS, editor. New York: Springer; 2000:183–92.
38. Minoli G, Terruzzi V, Spinzi GC, Benvenuti C, Rossini A. The influence of carbon dioxide and nitrous oxide on pain during laparoscopy: a double-blind, controlled trial. *Gastrointest Endosc* 1982;28:173–5.
39. Sharp JR, Pierson WP, Brady CE. Comparison of CO<sub>2</sub>- and N<sub>2</sub>O-induced discomfort during peritoneoscopy under local anesthesia. *Gastroenterology* 1982;82:453–6.
40. Cheng Y, Lu J, Xiong X, Wu S, Lin Y, Wu T, et al. Gases for establishing pneumoperitoneum during laparoscopic abdominal surgery. *Cochrane Database Syst Rev* 2013:CD009569.
41. Corall IM, Elias JA, Strunin L. Laparoscopy explosion hazards with nitrous oxide [letter]. *Br Med J* 1976;1:397.
42. El-Minawi MF, Wahbi O, El-Bagouri IS, Sharawi M, El-Mallah SY. Physiologic changes during CO<sub>2</sub> and N<sub>2</sub>O pneumoperitoneum in diagnostic laparoscopy. A comparative study. *J Reprod Med* 1981;26:338–46.
43. Gupta N, Dadhwal V, Deka D, Jain SK, Mittal S. Corpus luteum hemorrhage: rare complication of congenital and acquired coagulation abnormalities. *J Obstet Gynaecol Res* 2007;33:376–80.
44. Teng SW, Tseng JY, Chang CK, Li CT, Chen YJ, Wang PH. Comparison of laparoscopy and laparotomy in managing hemodynamically stable patients with ruptured corpus luteum with hemoperitoneum. *J Am Assoc Gynecol Laparosc* 2003;10:474–7.
45. Koninckx PR, Ussia A, Adamyan L. The role of the peritoneal cavity in adhesion formation. *Fertil Steril* 2012;97:1297.