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_2O) to the carbon dioxide (CO_2) 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_2 PP 5%, 10%, 25%, 50%, or 100% N_2O 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_2O, 4% O_2, or both to the CO_2 was evaluated in a control group and after addition of blood.
Main Outcome Measure(s): Postoperative adhesions after 7 days.
Result(s): N_2O 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_2O is the most effective factor in prevention of adhesions.
Conclusion(s): N_2O, 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_2O, O_2, 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_2) pneumoperitoneum (PP) through a subnormal mesothelial partial O_2 pressure. It increases if the PP contains more than 10% O_2 through a supernormal mesothelial partial O_2 pressure and reactive O_2 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_2 pressure around 30 mm Hg by adding 4% O_2 to the CO_2 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_2O), well known as an anesthetic gas, has been used for the PP instead of CO_2 because of the irritative effect of CO_2 thought to cause postoperative shoulder pain and because of the metabolic side effects of CO_2 following resorption (12, 13). N_2O is safe gas (14) with a high solubility in water (1.5 mg/L) and a lung exchange similar to CO_2. N_2O in addition is less irritating with less postoperative pain compared with CO_2 (15). N_2O has anesthetic and analgesic properties without the metabolic and cardiopulmonary side effects of CO_2 (16, 17). N_2O, 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_2O 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_2O to the CO_2 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_2O in CO_2, two thermoflators, one delivering CO_2 and the other N_2O, 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) $\times$ 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_2 compared with 100% N_2O 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_2O group).

Experiment I. Experiment I (n = 30) was a dose-response experiment following the unexpectedly strong antiadhesiogenic effect of 100% N_2O in the pilot experiment. This experiment was designed to evaluate adhesion formation following a 60-minute PP with 100% CO_2 (control group) and with the addition of 5%, 10%, 25%, 50% ,and 100% N_2O to the CO_2 (n = 5/group). To obtain a mixture of the various concentrations of CO_2 and N_2O, 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_2O, 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 senus 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, 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_2 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_2O and of 4% O_2, 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.

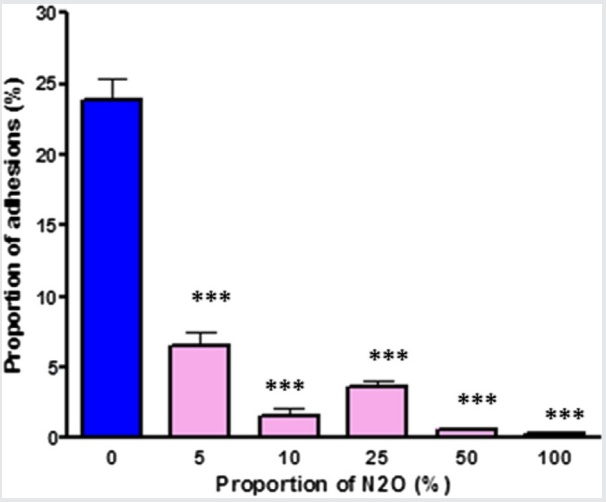
Pilot Experiment

The use of a PP for 60 minutes with 100% N_2O instead of 100% CO_2 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 ± 0.3 to 1.6 ± 0.4 (P=.009), from 10.8 ± to 5.5 ± 0.6 (P=.023), from 10.8 ± 1.2 to 5.6 ± 0.4 (P=.025), and from 11.3 ± 1.3 to 4.9 ± 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_2 the addition of 5%, 10%, 25%, or 50% of N_2O or the use of 100% N_2O 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_2O (P=.0001 for all groups). Adhesions formation between any of the groups receiving 5%, 10%, 25%, 50%, or 100% N_2O was not significantly different. The effect of as little as 5% N_2O 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_2, with the addition of 5%, 10%, 25%, or 50% N_2O to the CO_2 PP, or with 100% N_2O 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_2: ***P<.0001.

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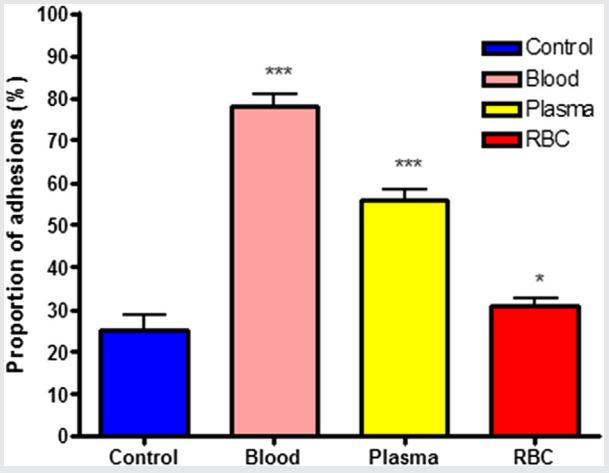
FIGURE 2



One milliliter of blood, plasma, or resuspended pellet of red blood cells was injected i.p. following 60 minutes of pneumoperitoneum with humidified CO_2. 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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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 ± 2.74 to 78.5 ± 2.20 (P<.0001), 55.5 ± 1.84 (P<.0001), and 30.5 ± 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_2, CO_2 with 4% O_2, with 10% N_2O, or with both 10% N_2O + 4% of O_2 (P=.009, P<.0001, and P<.0001, respectively). Also, after the addition of 0.5 mL blood, the use of these three gas mixtures decreased adhesions compared with the group with 100% CO_2 (P<.0001 for all groups). The addition of 10% N_2O was more effective than 4% O_2 (P<.0001) with little additive effect of adding 4% O_2 to the 10% N_2O (NS; Fig. 3). Qualitative scores showed similar results for all groups.

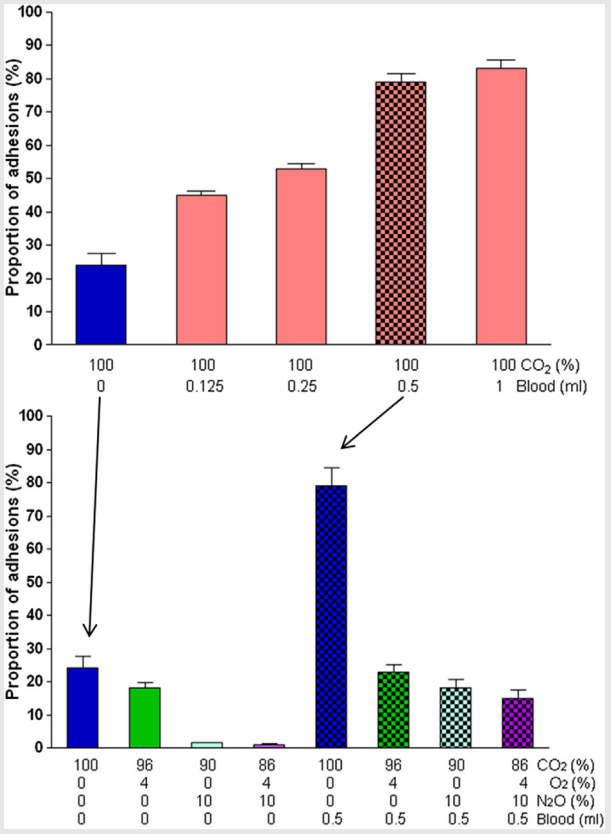
DISCUSSION

These data demonstrate for the first time that the use of N_2O instead of or in addition to CO_2 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_2O in CO_2.

The effect of N_2O, 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_2O 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_2 (34) could be explained by correction of the mesothelial hypoxia during pure CO_2 PP or mesothelial hyperoxia when >10% O_2 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_2O, however, has to be different from the effect of O_2, because the effect of even 100% N_2O is similar to the effect of 5%, whereas O_2 concentrations >10% clearly increase adhesions.

N_2O, commonly known as "laughing gas," is a colorless nonflammable gas used in surgery and dentistry for its anesthetic and analgesic effects. N_2O 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_2 is shown. (*Bottom*) The antiadhesiogenic effect of using CO_2 with 4% O_2, 10% N_2O, 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_2 for more powerful general anesthetic agents such as sevoflurane or desflurane. N_2O 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_2O'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_2O also the affects GABAA receptor (16), but this is still controversial. It has a lower potency by acting as a positive allosteric modulator. N_2O, 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_2O has been used instead of CO_2 for the PP during laparoscopic surgery because it has the advantage of not inducing the metabolic side effects of CO_2 following resorption. It therefore was recommended to change to N_2O in case of refractory hypercarbia and to use it as the primary insufflation gas in patients with little ventilatory reserve (15). In addition, N_2O is a safe gas when considering the risk of gas embolism. Indeed, N_2O has an even slightly better solubility in water or blood than CO_2 (1.5 and 1.45 mg/L, respectively) and a slightly better lung exchange capacity. Following PP with 100% N_2O, patients experience less pain (12, 13, 38, 39), on the day of surgery and following days (40). N_2O, however, never became popular because of the explosion risk when used in concentrations >29%. In these concentrations, N_2O 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_2O has a strong antiadhesiogenic effect similar to that of 100% N_2O 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_2O was not performed in the laparoscopic mouse model for practical reasons. Indeed, 1% N_2O would have required 0.5 mL/min N_2O in 50 mL/min of CO_2.

We recently demonstrated that in women undergoing promontofixation, postoperative pain was reduced by adding 4% O_2 to the CO_2. Because in the mouse model, acute inflammation of the peritoneal cavity decreased by the addition of 4% O_2, it is suggested that postoperative pain is at least partially mediated by acute inflammation of the entire peritoneal cavity. Because N_2O 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_2O, 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_2 and extend the antiadhesiogenic effect of N_2O and O_2 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_2O was unexpected, especially because it is the single most important factor identified so far. That N_2O is effective in low concentrations of 5%–10% indicates that it is effective not by replacing CO_2 but through an unknown drug-like mechanism. Most importantly, however, at these low concentrations N_2O can be used without explosion risk. Human trials will have to evaluate to what extent the other beneficial effects of N_2O compared with CO_2 will remain valid.

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