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Hypoxaemia induced by CO_2 or helium pneumoperitoneum is a co-factor in adhesion formation in rabbits

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A prospective randomized trial in a rabbit model was performed to test the hypothesis that the increase in adhesion formation following prolonged pneumoperitoneum is mediated by peritoneal hypoxaemia. Laparoscopic standardized opposing lesions were performed in uterine horns and pelvic sidewalls by bipolar coagulation and CO_2 laser in six groups of eight animals. Pure CO_2 or helium pneumoperitoneum was used for 10 (groups I and IV) or 45 min (groups II and V) to confirm the effect of duration of pneumoperitoneum and 96% of CO_2 or helium with 4% of oxygen (group III and VI) for 45 min to assess the effect of the addition of oxygen. After 7 days, adhesion formation was scored by laparoscopy. By two-way analysis of variance, total, extent, type and tenacity of adhesion scores increased ($P = 0.0003$, $P = 0.0004$, $P = 0.0004$ and $P = 0.004$) with increasing duration of pneumoperitoneum and decreased ($P = 0.02$, $P = 0.03$, $P = 0.01$ and $P = 0.05$) with the addition of oxygen. No differences were found between CO_2 and helium. In conclusion these data confirm the effect of pneumoperitoneum upon adhesions and demonstrate its reduction by oxygen, strongly suggesting that the main cause of adhesion formation is the relatively superficial hypoxaemia produced by the pneumoperitoneum.

Key words: adhesions/helium/hypoxaemia/laparoscopy/pneumoperitoneum

Introduction

Intraperitoneal adhesions are clinically important because of the associated complaints and complications. They are a major cause of intestinal obstruction, chronic pelvic pain and infertility (Rapkin, 1986; Ellis, 1997). Adhesions may be classified as congenital or acquired which can be post-inflammatory or post-operative. Some 10% of patients without previous surgery have adhesions, 9% post-inflammatory and 1% congenital. In contrast, adhesions are found in 94% of patients with at least one experience of previous surgery, 92% post-operative, 1% post-inflammatory and 1% congenital (Ellis, 1997). The peritoneal injury during either a laparotomy or a laparoscopy activates the cascade which leads to adhesion formation (Chegini, 1997; diZerega, 1997; Holmdahl, 1997).

In humans, laparoscopy was claimed to be less adhesiogenic than laparotomy (Lundorff *et al.*, 1991). More and large clinical studies have not been performed because of the obvious difficulties and ethical concerns for follow-up and scoring of adhesions. In animals, some studies failed to show differences between laparoscopy and laparotomy (Filmar *et al.*, 1987; Marana *et al.*, 1994; Jorgensen *et al.*, 1995) whereas others did show significantly less adhesions after laparoscopy in rats (Schäfer *et al.*, 1998), dogs (Schippers *et al.*, 1998), pigs (Fowler *et al.*, 1994) and rabbits (Luciano *et al.*, 1989).

CO_2 pneumoperitoneum was recently shown to play a role in peritoneal adhesion formation in rabbits (Ordoñez *et al.*, 1997) and mice (Yesildaglar *et al.*, 1999). This could be important since CO_2 is generally used for pneumoperitoneum for safety reasons because of its high solubility in water and exchange rate in the lungs. CO_2 induces local changes such as intraperitoneal acidosis (Volz *et al.*, 1996, 1997; West *et al.*, 1997). In the absence of moistening, desiccation of mesothelial layers will occur (Ryan *et al.*, 1973), whereas the intraperitoneal pressure will induce adverse effects upon peritoneal microcirculation (Taskin *et al.*, 1998, 1999) possibly inducing hypoxaemia. The hypothesis of hypoxaemia in the peritoneal superficial layers was moreover suggested by the observations that adhesions decrease when oxygen is added to the CO_2 during pneumoperitoneum (P.R. Koninckx *et al.*, unpublished).

In order to confirm that superficial mesothelial hypoxaemia is a key adhesiogenic factor, this prospective, randomized trial in a rabbit model was designed using helium instead of CO_2. This experiment should confirm the effect of duration of pneumoperitoneum and evaluate the relative importance of acidosis and hypoxaemia upon adhesion formation.

Materials and methods

Subjects and surgical procedures

Laparoscopies were performed in 48 adult, female, New Zealand white rabbits weighing between 2.5 and 3 kg. The study was approved by the Institutional Review Animal Care Committee and the animals were kept under standard laboratory conditions at the Centre for Laboratory Animal Care of the Catholic University of Leuven.

The anaesthesia was induced with i.m. ketamine (50 mg/kg, Ketalin®; Apharmo, Duiven, The Netherlands) and xylazin (6 mg/kg, XYL-M 2%®; VMD, Arendonk, Belgium) and maintained with inhalational halothane (2%, Fluothane®; Zeneca, Destelbergen, Belgium) and oxygen (1.5 l/min).

The surgery was performed under strict aseptic condition and no antibiotics were administered. The rabbits were placed in supine

position and the abdomen was shaved and disinfected with polyvidone iodine (iso-Betadine®; Asta Medica, Brussels, Belgium). A 12 mm trocar (Apple®; Medical Corporation, Bolton, MA, USA) was introduced by open laparoscopy through a 1 cm incision caudal to the sternum. The pneumoperitoneum was created using 100% of CO_2 or helium or a mixture of 96% of CO_2 or helium with 4% of oxygen. This was achieved using two insufflators (Thermoflator®; Karl Storz, Tuttlingen, Germany), one for CO_2 or helium and one for oxygen. To obtain a homogeneous mixture, the output of both insufflators was mixed in a mixing chamber which was connected to a water valve to limit the insufflation pressure at 10 cm of water (Koninckx and Vandermeersch, 1991). Therefore a slightly higher insufflation pressure was used for both insufflators (8 mm Hg), whereas 4% of oxygen was achieved using 24 l/min of CO_2 or helium and 1 l/min of oxygen. For reasons of standardization 25 l/min was used for pure CO_2 or helium, knowing that all excess gas would escape from the water valve. A 12 mm 0° endoscope (Karl Storz), connected to a single chip video camera (Karl Storz) and light source (Karl Storz), was used. After the establishment of the pneumoperitoneum a 5 mm trocar (Apple®; Medical Corporation) was introduced, under direct laparoscopic vision, at the level of the umbilicus to allow the introduction of the necessary instruments. Taking into account the high exchange capacity of the peritoneum and to maintain the concentration of the gases used, a continuous flow rate through the abdominal cavity of 1 l/min was used to remove constantly any oxygen which could be diffused from the circulation. To achieve this an 18 gauge catheter (Insyte-W®, Vialon®; Becton Dickinson, Madrid, Spain) was inserted in between the first and second trocars.

The rabbits were placed in 45° Trendelenburg position. Standardized opposing lesions of 2 cm^2 were performed randomly on the uterine horns and in the pelvic side walls by bipolar coagulation in one side, using a 5 mm forceps (Ethicon Endo-Surgery, Cincinnati, OH, USA) with a power of 10 watts (Force 30®; Valley Lab, Longbow Drive Boulder, CO, USA) and in the other side by CO_2 laser (Sharplan 1040, Tel Aviv, Israel) with a spot diameter of 1 mm and a power of 10 watts in the continuous super-pulse mode. Laser and bipolar lesions were used since the former was a lesion leaving a layer of some 100 μm of damaged cells only, whereas a bipolar lesion would induce necrosis up to at least a few mm. Since differences in healing between these lesions cannot be ruled out, the effect of pneumoperitoneum was investigated using both. The procedures took some 5–6 min and the pneumoperitoneum was maintained subsequently up to 10 or 45 min. At the end of the surgery the abdominal incisions were sutured with polyglactine 3–0 (Vicryl®, Ethicon®; Johnson and Johnson, Brussels, Belgium). Adhesion formation was scored after 7 days by second look laparoscopy since it was assumed that the laparoscopic evaluation might be more precise than a post-mortem evaluation by laparotomy because of the magnification and because of the distended abdomen by the pneumoperitoneum. This, however, will have to be validated since it cannot be excluded that adhesions might be separated by the pneumoperitoneum.

Experimental design

Six groups of eight animals were used. In group I pneumoperitoneum was maintained for 10 min and in group II for 45 min using 100% of CO_2 and in group III for 45 min using 96% of CO_2 with 4% of oxygen. In group IV pneumoperitoneum was maintained for 10 min and in group V for 45 min using 100% of helium and in group VI for 45 min using 96% of helium with 4% of oxygen.

A 2×2 factorial design (groups I, II, IV and VI) was used to evaluate the effect of duration of pneumoperitoneum (10 and 45 min) and the effect of insufflation gas (CO_2 and helium) upon adhesion formation. Similarly a 2×2 factorial design (groups II, III, V and VI) was used to evaluate the effect of the addition of oxygen (100% of CO_2 or helium or 96% of CO_2 or helium with 4% of oxygen) and the effect of the insufflation gas (CO_2 or helium). Groups I, II and III moreover should confirm the previous observation of an increase in adhesion formation with the duration of CO_2 pneumoperitoneum (I versus II) and a decrease with the addition of oxygen (II versus III).

Block randomization by days was used. Each block of six animals thus was operated on during the same day. All surgery was performed by the same surgeon during 8 consecutive days for the first and the second look respectively.

All second look laparoscopies were video-recorded and subsequently adhesion formation was scored blindly by two independent observers taking into account extent (0: no adhesions, 1: 1–25%, 2: 26–50%, 3: 51–75%, 4: 76–100%), type (0: no adhesions, 1: filmy avascular, 2: dense avascular, 3: dense with capillaries, 4: dense with larger vessels) and tenacity (0: no adhesions, 1: essentially fall apart, 2: required traction, 3: required sharp dissection).

Since the lesions inflicted in either the right or the left side were performed in the same way (laser or bipolar), scoring was done separately for right and left side, thus obtaining separate laser (L) and a bipolar (B) lesions adhesion scores.

Laser total (L-total) adhesion score was obtained adding laser extent (L-extent), laser type (L-type) and laser tenacity (L-tenacity) adhesion scores. Bipolar total (B-total) adhesion score was obtained adding bipolar extent (B-extent), bipolar type (B-type) and bipolar tenacity (B-tenacity) adhesion scores. Extent, type, tenacity and total adhesion scores were defined as the sum of L-extent and B-extent, L-type and B-type, L-tenacity and B-tenacity and L-total and B-total, respectively.

Statistics

Statistical analysis was performed with the SAS system (SAS Release 6.12, 1998) using Wilcoxon analysis and two-way analysis of variance. Since data were not normally distributed because of 0 scores, the general linear model (Proc GLM) was used instead of analysis of variance. All data are presented as mean ± SEM. The advantage of the 2×2 factorial design was that to achieve the same statistical precision as with a one at a time approach, twice as many observations would have been needed. The power of the observed effect of duration of CO_2 and helium, and the effect of adding oxygen thus is comparable to experiments with 16 animals in each group. This increase in power of the factorial design was only valid when the effects of the two factors were additive, i.e. when no interaction between the two factors was present. The possibility of detecting an interaction, i.e. a different effect of one factor at different levels of the other factor, could however also be considered an advantage of the factorial design, since this effect could otherwise easily be missed. When the number of observations is low, one should be aware that a positive interaction (with subsequent reduction of power to demonstrate the effect of the two factors) could be missed, especially when the between subject variability is high (Armitage and Berry, 1987).

Results

By increasing the duration of pure CO_2 or helium pneumoperitoneum from 10 to 45 min the total adhesion score increased (Wilcoxon) from 4.6 ± 1.3 to 10.5 ± 1.8 for CO_2 ($P = 0.04$) and from 3.1 ± 1.2 to 9.7 ± 1.8 for helium ($P = 0.02$) confirming the effect of duration of the pneumoperitoneum. For CO_2, extent increased from 1.7 ± 0.5 to 4.5 ± 0.9 ($P = 0.03$), type from 1.3 ± 0.3 to 3.1 ± 0.5 ($P = 0.01$) and tenacity from 1.6 ± 0.4 to 2.9 ± 0.6 (not significant, NS).

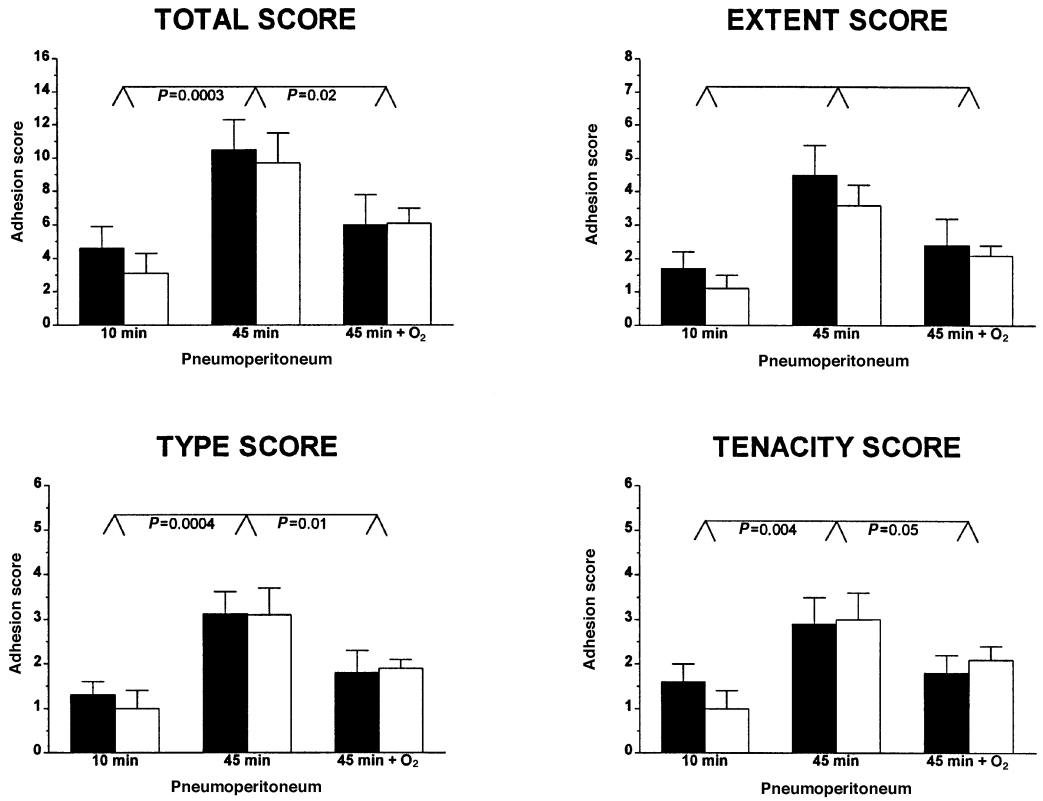

Figure 1. Effect of CO_2 (■) and helium (□) pneumoperitoneum (10 or 45 min) and of the addition of 4% of oxygen upon adhesion formation following a laser and bipolar opposing lesions. Total, extent, type and tenacity mean scores ± SEM are indicated together with *P* values (two-way analysis of variance).

For helium, extent increased from 1.1 ± 0.4 to 3.6 ± 0.6 ($P = 0.01$), type from 1 ± 0.4 to 3.1 ± 0.6 ($P = 0.02$) and tenacity from 1 ± 0.4 to 3 ± 0.6 ($P = 0.03$). By two-way analysis of variance, the effect of duration of pneumoperitoneum was highly significant for total ($P = 0.0003$), extent ($P = 0.0004$), type ($P = 0.0004$) and tenacity ($P = 0.004$) scores, whereas no differences between CO_2 and helium and no interaction between duration of pneumoperitoneum and the type of gas used were found (Figure 1).

Adding 4% of oxygen to CO_2 or helium pneumoperitoneum during 45 min, total adhesion score decreased (Wilcoxon) to 6 ± 1.8 for CO_2 (NS) and to 6.1 ± 0.9 for helium (NS). For CO_2, extent decreased to 2.4 ± 0.8 (NS), type to 1.8 ± 0.5 (NS) and tenacity to 1.8 ± 0.4 (NS). For helium, extent decreased to 2.1 ± 0.3 (NS), type to 1.9 ± 0.2 (NS) and tenacity to 2.1 ± 0.3 (NS). By two-way analysis of variance, the addition of oxygen was significant for total ($P = 0.02$), extent ($P = 0.03$), type ($P = 0.01$) and tenacity ($P = 0.05$) scores, whereas no differences between CO_2 and helium and no interaction between the addition of oxygen and the type of gas used were found (Figure 1).

Laser and bipolar lesions were evaluated separately. By two-way analysis of variance, increasing duration of the pneumoperitoneum increased L-total ($P = 0.0001$), L-extent ($P = 0.0001$), L-type ($P = 0.0001$) and L-tenacity ($P = 0.003$) adhesion scores, as well as B-total ($P = 0.05$), B-extent (NS), B-type (NS) and B-tenacity (NS) adhesion scores (Table I).

By two-way analysis of variance, the addition of oxygen decreased L-total (NS), L-extent (NS), L-type (NS) and L-tenacity (NS) adhesion scores, as well as B-total ($P = 0.02$), B-extent ($P = 0.04$), B-type ($P = 0.01$) and B-tenacity ($P = 0.05$) adhesion scores (Table I).

Laser lesions induced more adhesions than bipolar lesions as evidenced by higher total scores ($P = 0.0001$), and higher scores for extent ($P = 0.0001$), type ($P = 0.0001$) and tenacity ($P = 0.0001$).

Discussion

It appears that this is the first study of post-laparoscopic adhesion formation using helium pneumoperitoneum. Helium was chosen as an alternative for CO_2 pneumoperitoneum because it is chemically, physiologically and pharmacologically inert, non-explosive and since it does not produce hypercarbia and acidosis and the related haemodynamic and cardiopulmonary effects observed with CO_2 (Kotzampassi *et al.*, 1993; Leighton *et al.*, 1993; McMahon *et al.*, 1994; Rademaker *et al.*, 1995; Neuberger *et al.*, 1996; Declan *et al.*, 1997; Volz *et al.*, 1997). Its lower solubility in water, however, carries a higher risk of embolization and an increased lethal effect of gas embolism (Wolf *et al.*, 1994; Rudston-Brown *et al.,* 1997).

Helium pneumoperitoneum was used in this experiment to evaluate the hypothesis that the hypoxaemia, induced by compression of the capillary flow in the superficial peritoneal layers during prolonged pneumoperitoneum, is a cause of adhesion formation. It is logical to assume that increasing duration of pneumoperitoneum and higher intra-abdominal

Table I. L-extent, B-extent, L-type, B-type, L-tenacity, B-tenacity, L-total and B-total adhesion scores 7 days after the correspondent lesions using pure CO_2/helium pneumoperitoneum for 10 min (I/IV) and for 45 min (II/V) or 96% of CO_2/helium with 4% of oxygen for 45 min (III/VI). Mean ± SEM are indicated

Type of lesion	Category	Groups					
		I	II	III	IV	V	VI
Laser	L-extent	0.9 ± 0.5	2.9 ± 0.4[b]	1.8 ± 0.6	0.8 ± 0.3	2.8 ± 0.4[b]	2.0 ± 0.4
	L-type	0.5 ± 0.2	2.1 ± 0.3[b]	1.4 ± 0.4	0.9 ± 0.3	2.1 ± 0.3[b]	1.8 ± 0.3
	L-tenacity	0.8 ± 0.3	1.9 ± 0.3[b]	1.3 ± 0.3	0.9 ± 0.3	2.0 ± 0.3[b]	1.8 ± 0.2
	L-total	2.2 ± 1.1	6.9 ± 1.0[b]	4.5 ± 1.3	2.6 ± 1.0	6.9 ± 1.0[b]	5.6 ± 0.8
Bipolar	B-extent	0.8 ± 0.3	1.6 ± 0.5	0.6 ± 0.3[c]	0.3 ± 0.2	0.8 ± 0.3	0.1 ± 0.1[c]
	B-type	0.8 ± 0.3	1.0 ± 0.3	0.4 ± 0.1[d]	0.1 ± 0.1	1.0 ± 0.3	0.1 ± 0.1[d]
	B-tenacity	0.8 ± 0.3	1.0 ± 0.3	0.5 ± 0.2[c]	0.1 ± 0.1	1.0 ± 0.3	0.3 ± 0.2[c]
	B-total	2.4 ± 0.9	3.6 ± 1.0[a]	1.5 ± 0.8[c]	0.5 ± 0.5	2.8 ± 1.0[a]	0.5 ± 0.5[c]

I + IV versus II + V: [a]$P < 0.05$, [b]$P < 0.001$. II + V versus III + VI: [c]$P < 0.05$, [d]$P < 0.001$ (two-way analysis of variance).
L = laser, B = bipolar. For description of groups and categories see Materials and methods section.

pressures limit the peritoneal capillary flow, increasing hypoxaemia, and that the addition of oxygen could decrease hypoxaemia. This assumption is consistent with previous findings that duration of CO_2 pneumoperitoneum (Ordoñez *et al.*, 1997; Yesildaglar *et al.*, 1999) and that higher intra-abdominal pressures (Yesildaglar and Koninckx, 2000) increase adhesions whereas the addition of oxygen decreases adhesion formation (P.R. Koninckx *et al.*, unpublished). This experiment confirmed the observations using CO_2 pneumoperitoneum and demonstrated the same effect using helium pneumoperitoneum whereas no differences were found between CO_2 and helium. All these data together confirm the hypothesis that hypoxaemia is more important than acidosis in adhesion formation following prolonged pneumoperitoneum.

The effect of a low oxygen concentration in blood (hypoxaemia) or in tissues (hypoxia) differs between tissues, which could be explained by different microvascular flow reserves, oxidative capacity and metabolic need (Scannell, 1996). Hypoxia alters the metabolism of endothelial cells as shown by vascular damage and increased vascular permeability (Scannell, 1996), increased expression of adhesion molecules such as vascular cell adhesion molecule (VCAM)-1 and intracellular adhesion molecule (ICAM)-1 (Setty and Stuart, 1996) and increased polymorphonuclear (PMN) adhesion to the vascular endothelium (Kalra *et al.*, 1996). At the same time, hypoxia decreases T-lymphocyte production of interleukin (IL)-2, a key cytokine responsible for B-cell proliferation and inmunoglobulin secretion, and increases the release of tumour necrosis factor (TNF)-α, IL-1 and IL-8 by human macrophages (Scannell, 1996).

Hypoxia could modulate directly or indirectly, during pneumoperitoneum, the production of cytokines and growth factors by peritoneal mesothelial cells, macrophages and fibroblasts. Macrophages secrete cytokines and growth factors, such as IL-1, IL-4, IL-6, IL-10, TNF and transforming growth factor (TGF), which are involved in peritoneal wound healing and modulate the process that leads to adhesion formation (Chegini, 1997). TGF-β1, TNF-α and IL-1β up-regulate plasminogen activator inhibitor-1 (PAI-1) and down-regulate tissue-type plasminogen activator (t-PA), decreasing plasmin and thus inhibiting the lysis of fibrin (Tietze *et al.*, 1998). TGF-β decreases the expression of matrix metalloproteinases (MMP) and increases the expression of tissue inhibitors of metalloproteinases (TIMP), thus decreasing matrix degradation and increasing fibrous adhesions (Chegini, 1997). The effect of hypoxaemia, however, has not been investigated as was done for mesothelial cells. Recently it was shown that human peritoneal mesothelial cells cultured under hypoxic conditions (2% oxygen) increase amounts of TGF-β1 mRNA and collagen III mRNA after 6 h (Saed *et al.*, 1999a), amounts of TGF-β1 and TGF-β2 mRNA after 24 h (Saed *et al.*, 1999b) and amounts of TIMP-1 mRNA, possibly via a TGF-β1 dependent mechanism (Saed *et al.*, 1999c). To interpret these data it should be realized that in some experiments hypoxaemia varies from 0–5% of oxygen. This could be important since it should be compared to the partial oxygen pressure in the abdominal cavity.

The effect of mesothelial hypoxaemia, induced by the pneumoperitoneum during a laparoscopic surgery, upon vascular endothelial growth factor (VEGF) expression should be considered to explain the increase in adhesion formation. Indeed, hypoxia, together with other growth factors and cytokines, stimulates the production of VEGF by a variety of normal and transformed cell types (Neufeld *et al.*, 1999). Hypoxia up-regulates the production of VEGF by non-activated and by interferon-δ (IFNδ) and/or lipopolysaccharide (LPS) activated murine peritoneal macrophages (Xiong *et al.*, 1998). Increased concentrations of VEGF were detected, generally under hypoxic conditions, in ovarian hyperstimulation syndrome (Chen *et al.*, 1999), ovarian neoplasm (Yamamoto *et al.*, 1997), endometriosis (McLaren *et al.*, 1996) and ascites tumours (Luo *et al.*, 1998) and during the normal cyclic changes in the female reproductive system (Shweiki *et al.*, 1993). Furthermore, VEGF was found in peritoneal adhesions of women by immunohistochemistry (Wiczyk *et al.*, 1998) and of men and women by enzyme-linked immunosorbent assay (Diamond *et al.*, 1999) and it was shown that a polyclonal rabbit antibody to VEGF limits adhesion formation after laparotomy in a murine model (Saltzman *et al.*, 1996). These

data indicate that VEGF has a role in the development of post-operative adhesions.

It is unclear at present why laser lesions induce more adhesions than bipolar lesions. It can only be speculated that the larger denuded area might be important, but it should be investigated what is the exact role of depth and amount of tissue necrosis and whether this effect might be specific for hypoxaemia induced adhesions.

In conclusion, this experiment confirms the key role of mesothelial hypoxaemia in adhesion formation. Recent publications indicate that this could be mediated by a growth factor such as TGF-β or VEGF and more specific studies are currently being performed in order to clarify this point.

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