Effects of methylene blue, indigo carmine solution and autologous erythrocyte suspension on formation of adhesions after injection into rats

A. Gül¹, Ç. Kotan², İ. Dilek³, T. Gül⁴, A. Taş⁵ and M. Berktaş⁶

Departments of ¹Obstetrics and Gynecology, ²Surgery, ³Hematology and ⁶Microbiology, Faculty of Medicine, and ⁵Department of Surgery, Faculty of Veterinary Medicine, Yüzüncü Yıl University, 65200, Van, Turkey; ⁴Department of Obstetrics and Gynecology, Faculty of Medicine, Dicle University, Diyarbakir, Turkey

The aim of this study was to determine whether autologous erythrocyte suspension can be used as a dye for evaluation of tubal patency and whether it has any advantages over methylene blue or indigo carmine solutions. Reproductively healthy female nulliparous Wistar Albino rats ($n = 30$), aged 6 months, mass 165–195 g, were assigned randomly to three groups. Rats received a 1 ml i.p. injection of 5% (w/v) methylene blue solution (methylene blue group: $n = 10$), 5% (w/v) indigo carmine solution (indigo carmine group: $n = 10$) or 5% (v/v) fresh autologous erythrocyte suspension (autologous erythrocyte group: $n = 10$). At 4 weeks after injection, a small sterile opening was made in the peritoneal cavity of each rat. The cavity was rinsed once with TCM-199 to collect macrophages. The rinsed peritoneal contents were cultured overnight to evaluate macrophage activation. The peritoneal opening was expanded for evaluation of adhesion formation. Only one rat from the autologous erythrocyte group had intra-peritoneal adhesions (score 2), whereas all rats in the methylene blue group (score 1: $n = 1$; score 2: $n = 4$; score 3: $n = 4$; and score 4: $n = 1$) and seven rats in the indigo carmine group (score 1: $n = 1$; score 2: $n = 2$; score 3: $n = 3$; and score 4: $n = 1$) had intra-abdominal adhesions. Macrophage activity was observed in the cultured peritoneal contents collected from the methylene blue and indigo carmine groups but not from the autologous erythrocyte group. Adhesion formation could be due to macrophage activation caused by methylene blue and indigo carmine solutions. These results indicate that tubal patency can be observed by laparoscopy using autologous erythrocyte suspension. The results of this study are believed to be the first to indicate that a patient's own erythrocyte suspension could be used during observation of tubal patency by laparoscopy. However, further studies are required.

Introduction

Surgery in the abdominal or pelvic cavity can lead to the formation of adhesions (Frantzen *et al.*, 1982; Puri *et al.*, 1984; Diamond *et al.*, 1987), which can lead to infertility (Trimbos *et al.*, 1982; Gomel, 1983). A number of techniques have been developed to reduce or prevent the formation of adhesions as a result of surgery (Halme *et al.*, 1982; Adhesion Study Group, 1983; De Cherney *et al.*, 1984; Rosenburg *et al.*, 1984; Diamond *et al.*, 1991). Adhesion formation may result from peritoneal trauma or infection (Gomel *et al.*, 1996), as well as the introduction of foreign bodies.

In the case of peritoneal injury, blood present in the peritoneal cavity adheres to the injury site and stimulates adhesion formation. This effect may be the result of the excessive amounts of fibrin contributed by the blood.

Furthermore, in the event of peritoneal injury, heparinized blood fails to stimulate adhesion formation (Ryan *et al.*, 1971). Mature erythrocytes in peripheral blood do not have adhesion molecules (Hoffbrand and Pettit, 1993).

Gynaecologists frequently use methylene blue and indigo carmine during laparoscopy or tuboplasty to demonstrate tubal patency in women (Ansari, 1968; Speroff *et al.*, 1999a). Clinically this practice appears to be devoid of toxicity but it has not been tested thoroughly for safety (McFadyen, 1992). Both methylene blue and indigo carmine appear to induce adhesion formation as well as macrophage activation *in vitro*. In addition, observations of macrophage activation have been made after exposure to methylene blue and indigo carmine solution *in vivo* (Prien *et al.*, 1995).

The aim of the present study was to assess the effects of methylene blue, indigo carmine solution and fresh autologous erythrocyte suspension on adhesion formation in a rat model. Observations of adhesion formation were made

Received 7 January 2000.

in vivo after injection of methylene blue, indigo carmine or heparinized fresh autologous erythrocyte suspension into the peritoneal cavity of rats.

Materials and Methods

This prospective experimental study received institutional approval. Reproductively healthy nulliparous, non-pregnant cyclic female Wistar albino rats ($n = 30$), aged 6 months, mass 165–195 g were assigned randomly to three groups: methylene blue group: 5% (w/v) methylene blue solution (5 mg methylene blue + 95 ml saline); indigo carmine group: 5% (w/v) indigo carmine solution (5 mg indigo carmine + 95 ml saline); and autologous erythrocyte group: 5% (v/v) fresh autologous erythrocyte suspension (0.2 ml autologous erythrocyte + 3.8 ml saline). Autologous erythrocyte suspension was prepared as follows: 1.5–2.0 ml blood was collected from the tail vessel of each rat. The blood collected was centrifuged with a JUAN GR 422 centrifuge at 6500 g for 10 min. The supernatant was removed and 0.2 ml of the remaining fraction was suspended with 3.8 ml saline containing 5 iu heparin. Each rat received a 1 ml i.p. injection of methylene blue, indigo carmine or autologous erythrocyte suspension. After injection the animals were returned to standard vivarium care.

After 4 weeks the animals were killed by administration of a high dose of ether. A small sterile opening was made in the peritoneal cavity of each rat. The cavity was rinsed once with TCM-199 (Irvine Scientific, Irvine, CA) to collect macrophages. The rinsed contents of the peritoneal cavity were cultured overnight at 37°C, 5% CO₂ and 95% relative humidity to allow attachment of the cells to the bottom of the plates. After the cells were attached they were assessed visually for activation using an Olympus BX50F-3 microscope equipped with phase optics (Olympus Optical Co Ltd, Tokyo). After aspiration of the peritoneal contents, the cavity was opened completely and examined for adhesion formation by an observer with no prior knowledge about each rat's treatment. Adhesion formation was scored as: score 0; no macroscopic adhesions observed; score 1: one filmy macroscopic fibrin adhesion; score 2: multiple macroscopic filmy fibrous adhesions; score 3: one dense macroscopic fibrous adhesion with or without additional macroscopic filmy adhesions; and score 4: multiple dense macroscopic fibrous adhesions with or without additional filmy adhesions (Table 1). The data were analysed by chi-squared test.

Results

All the rats remained healthy during the 4 weeks after injection. Only one rat in the autologous erythrocyte suspension group (score 2) developed intra-abdominal adhesion, whereas all rats in the methylene blue solution group (score 1: $n = 1$; score 2: $n = 4$; score 3: $n = 4$; and score 4: $n = 1$) and seven rats in the indigo carmine group (score 1: $n = 1$; score 2: $n = 2$; score 3: $n = 3$ rats; and score 4: $n = 1$ rat) had intra-abdominal adhesions. Score 3 adhesion between intestinal loops was observed in a rat in the indigo carmine

Table 1. Adhesion score categories

Score	Adhesion	Criteria
0	No adhesion	No macroscopic adhesions observed
1	Mild	One filmy macroscopic fibrin adhesion
2	Moderate	Multiple macroscopic filmy fibrous adhesions
3	Severe	One dense macroscopic fibrous adhesion with or without additional macroscopic filmy adhesion
4	More severe	Multiple dense macroscopic fibrous adhesions with or without additional filmy adhesion

solution group (Fig. 1a). Score 4 adhesion between intestinal loops and abdominal wall was observed in a rat in the methylene blue group (Fig. 1b). Adhesion numbers and scores in each group were compared (Fig. 2a,b). The number, mean and variations of adhesion scores for each group are shown (Table 2). The adhesion numbers and scores were significantly lower in the autologous erythrocyte suspension group than in the methylene blue and indigo carmine groups ($P < 0.05$).

Qualitative morphological examination of macrophages recovered from the peritoneal cavities was performed. Activated cells had fewer points of attachment with the dish and appeared rounded, whereas non-activated cells attached fully to the plates and appeared flattened in culture (Prien *et al.*, 1995). Macrophage activation was observed in the cultured peritoneal contents collected from the methylene blue and indigo carmine solution groups, but not in the peritoneal contents collected from the autologous erythrocyte suspension group.

Discussion

Methylene blue and indigo carmine solution appear to induce more development of adhesions than heparinized Ringer's lactate solution (Prien *et al.*, 1995). This finding indicates that these compounds trigger the set of biochemical events that lead to adhesion formation (Prien *et al.*, 1995). Although heparinized Ringer's lactate solution does not cause adhesion it does allow observation of tubal patency. It has been suggested that certain materials used during surgery promote the formation of adhesions. Although the biochemical mechanisms leading to adhesion remain unclear, Orita *et al.* (1986) reported that peritoneal macrophages play a crucial role in adhesion formation by secreting compounds that cause proliferation of fibroblasts. Elkins *et al.* (1987) observed a two-phase healing process after peritoneal injury. An early influx of inflammatory cells is characteristic of initial tissue repair at all sites of peritoneal injury or foreign bodies. This response includes polymorphonuclear leukocytes and surface exudate. The second phase of the healing process is observed as a response similar to the delayed inflammatory reaction, and fibroblasts, collagen, plasma cells and macrophages are observed, with formation of foreign body granulomas. In the present study, activation of macrophages

Table 2. Adhesion scores and number of rats with adhesions in each treatment group

Treatment group	Number of rats examined	Minimum adhesion score	Maximum adhesion score	Number of rats with adhesions	Total group adhesion score	Mean adhesion score
Autologous erythrocytes	10	0	2	1 ^a	2 ^a	0.2 ^a
Indigo carmine	10	0	4	7 ^b	18 ^b	1.8 ^b
Methylene blue	10	1	4	10 ^b	25 ^b	2.5 ^b

Adhesion scores: score 0: no macroscopic adhesions observed; score 1: one filmy macroscopic fibrin adhesion; score 2: multiple macroscopic filmy fibrous adhesions; score 3: one dense macroscopic fibrous adhesion with or without additional macroscopic filmy adhesions; and score 4: multiple dense macroscopic fibrous adhesions with or without additional filmy adhesions.

^{ab}Values with different superscripts within a column are significantly different ($P < 0.05$).

Fig. 1. (a) Score 3 adhesion (shown between two arrows) between intestinal loops in a rat in the indigo carmine treatment group. (b) Score 4 adhesion (shown between two arrows) between intestinal loops and abdominal wall observed in a rat in the methylene blue treatment group.

recovered from the peritoneal cavity indicates that methylene blue and indigo carmine solution stimulate macrophage activation, whereas no macrophage activation was observed when autologous erythrocyte suspension was used.

It has been reported that methylene blue significantly

reduces progesterone production by granulosa cells. Whether this effect is reversible or results ultimately in cell death needs further investigation (Maha *et al.*, 1993).

Previous studies have demonstrated a relationship between intra-abdominal adhesion formation and infertility

Fig. 2. (a) Number of rats with (□) and without (■) adhesion in the three treatment groups and (b) total adhesion score in each treatment group. Methylene blue: 5% (w/v) methylene blue solution ($n = 10$); indigo carmine: 5% (w/v) indigo carmine solution ($n = 10$); and autologous erythrocyte: 5% (v/v) fresh autologous erythrocyte suspension ($n = 10$). Adhesion scores: score 0: no macroscopic adhesions observed; score 1: one filmy macroscopic fibrin adhesion; score 2: multiple macroscopic filmy fibrous adhesions; score 3: one dense macroscopic fibrous adhesion with or without additional macroscopic filmy adhesions; and score 4: multiple dense macroscopic fibrous adhesions with or without additional filmy adhesions.

(Trimbus *et al.*, 1982; Gomel, 1983). During laparoscopy, flow of blood from the fimbrial end of the tube has been observed in almost all menstruating women (Liu and Hitchcock, 1986; Speroff *et al.*, 1999b). Although many women have reflux seeding of menstrual debris into the peritoneal cavity, none appear to develop adhesion as a result (Speroff *et al.*, 1999b). In the absence of peritoneal injury, introduction of blood into the peritoneal cavity does not usually cause adhesions

(Ryan *et al.*, 1971). The results of the present study are in agreement with this report.

Gul *et al.* (1999) investigated the possibility of using a patient's own erythrocyte suspension as a dye during observation of tubal patency by laparoscopy. Gul *et al.* (1999) assessed tubal patency by laparoscopy using Ringer's lactate solution, methylene blue solution or autologous erythrocyte suspension. Tubal patency was not observed with Ringer's lactate solution, but it was observed with autologous erythrocyte suspension and methylene blue solution.

In the present study, fresh autologous erythrocyte suspension did not cause adhesions as was observed when other dyes were used, as the autologous erythrocyte suspension required for observation of tubal patency was very dilute and the peritoneal cavity had already been washed after observation of tubal patency. The adhesion (score 2) observed in one rat in the autologous erythrocyte suspension group was probably due to contamination. It is also thought that autologous erythrocyte suspension does not have any undesirable effect on corpora lutea, as is observed after treatment with methylene blue (Maha *et al.*, 1993).

In conclusion, the results of the present study indicate that fresh autologous erythrocyte suspension could be used safely for patients, especially those with dye allergies (Maha *et al.*, 1993), but further studies are required.

References

- Adhesion Study Group (1983) Reduction of postoperative pelvic adhesion with intraperitoneal 32% dextran 70: a prospective, randomized clinical trial *Fertility and Sterility* **40** 612–619
- Ansari AH (1968) Methylene blue test for assessment of tubal patency: a new and simple technique *Canadian Medical Association Journal* **99** 182–184
- DeCherney AH and Mezer HC (1984) The nature of post-tuboplasty pelvic adhesions as determined by early and late laparoscopy *Fertility and Sterility* **41** 643–646
- Diamond MP, McLaughlin DS, Daniel JE, Frieman S, Feste J, Vaughn WK and Martin DC (1987) Adhesion reformation and *de novo* adhesion formation after reproductive pelvic surgery *Fertility and Sterility* **47** 864–869
- Diamond MP, Linsky CB, Cunningham T, Kamp L, Pines E, DeCherney AH and Dizerega G (1991) Synergistic effects of INTECED(TC7)* and heparin in reducing adhesion formation in the rabbit uterine horn model *Fertility and Sterility* **55** 389–394
- Elkins TE, Stvall TG, Warren J, Ling FW and Meyer NL (1987) A histologic evaluation of peritoneal injury and repair: implication for adhesion formation *Obstetrics and Gynecology* **70** 225–228
- Frantzen C and Schosser HW (1982) Microsurgery and postinfectious tubal infertility *Fertility and Sterility* **38** 397–402
- Gomel V (1983) An odyssey through the oviduct *Fertility and Sterility* **39** 144–155
- Gomel V, Urman B and Gurgan T (1996) Pathophysiology of adhesion formation and strategies for prevention *Journal of Reproductive Medicine* **41** 35–41
- Gul A, Dilek I, Gul T, Sahin G and Timurkan H (1999) The evaluate of tubal patency with patient's own erythrocyte(s) suspension as an alternative method to methylene blue *Turkish Journal of Fertility* **7** 26–30
- Halme J, Becker S, Hammond MG and Raj S (1982) Pelvic macrophages in normal and infertile women: the role of patent tubes *American Journal of Obstetrics and Gynecology* **142** 890–895
- Hoffbrand AV and Pettit JE (1993) The white cells 1: granulocytes, monocytes and their benign disorders. In *Essential Haematology* pp 147–160. Blackwell Scientific Publications, London
- Liu DTY and Hitchcock A (1986) Endometriosis: its association with retrograde menstruation, dysmenorrhea, and tubal pathology *British Journal of Obstetrics and Gynaecology* **93** 210–214

- McFadyen I** (1992) The dangers of intra-amniotic methylene blue *British Journal of Obstetrics and Gynaecology* **99** 89–90
- Maha MM, Glenn AW, Scott H, Stephane B and Michael MM** (1993) Methylene blue but not indigo carmine is toxic to human luteal cells *in vitro*. *Reproductive Toxicology* **7** 631–633
- Orita H, Campeau JD, Nakamura RM and diZerega GS** (1986) Modulation of fibroblast proliferation and transformation by activated macrophages during postoperative peritoneal reepithelization *American Journal of Obstetrics and Gynecology* **155** 905–911
- Prien SD, Dunn C and Messer RH** (1995) Adhesion-promoting properties of dyes routinely used during fertility surgeries *Journal of Assisted Reproduction and Genetics* **12** 136–140
- Puri P, Guiney EJ, O'Donnell B and McGuinness EPJ** (1984) Effects of perforated appendicitis in girls on subsequent fertility *British Medical Journal* **288** 25–26
- Rosenburg SM and Board JA** (1984) High-molecular weight dextran in human infertility surgery *American Journal of Obstetrics and Gynecology* **148** 380–385
- Ryan G, Grobety J and Majino G** (1971) Postoperative peritoneal adhesions: a study of mechanism *American Journal of Pathology* **65** 117–148
- Speroff L, Glass RH and Kase NG** (1999a) Female infertility. *Clinical Gynecologic Endocrinology and Infertility* pp 1013–1042. Baltimore, Maryland
- Speroff L, Glass RH and Kase NG** (1999b) Endometriosis. In *Clinical Gynecologic Endocrinology and Infertility* pp 1058–1073. Baltimore, Maryland
- Trimbos-Kemper T, Trimbos B and Van Hall E** (1982) Etiological factors in tubal infertility *Fertility and Sterility* **37** 384–388