PERITONEAL FIBRINOLYTIC ACTIVITY AND ADHESIOGENESIS

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Keywords: *adhesions, fibrinolysis, tPA, PAI-1*

Abstract: *The triggering of the formation of adhesions is caused by the organization of the fibrin matrix, an organization that takes place during the coagulation processes when there is suppression of fibrinolysis. In this study two groups of patients are analyzed. First group was used for extract the tPA and PAI-1 from the peritoneal tissue. The second group is the control group in whose cases none of the aggression factors (traumatic, chemical, bacterial) noticed in the patients of the first group was proven to exist. The aim of this study was to evaluate the role of PAI-1 and tPA genes at peritoneal tissue level. Peritoneal tissue was obtained during surgery and we have quantified the variation of expression of PAI-1 and tPA genes. The obtained results highlight an increase of expression in PAI-1 gene and decrease of expression in tPA gene, which indicated an decrease in the fibrynolitic potential.*

Cuvinte cheie: *aderenţă, fibrinoliză, t-PA, PAI-I*

Rezumat: *Formarea aderenţelor se datorează organizării matricei de fibrină ce apare în procesul de coagulare când fibrinoliza este redusă. În acest studiu au fost analizate două grupuri de pacienţi. Primul grup a fost folosit pentru extragerea de t-PA şi PAI-I din ţesutul peritoneal. Al doilea grup a fost grupul de control la care nu s-a putut demonstra existenţa niciunuia din factorii de agresiune (traumatic, chimic şi bacterian) observaţi la primul grup. Scopul studiului este evaluarea rolului genelor t-PA şi PAI-I la nivelul ţesutului peritoneal. Ţesutul peritoneal a fost obţinut prin intervenţie chirurgicală şi s-a cuantificat variaţia expresiei genelor t-PA şi PAI-I. Rezultatele obţinute subliniază expresia crescută a genei PAI-I şi expresia redusă a genei t-PA, ceea ce indică o scădere în potenţialul fibrinolitic.*

INTRODUCTION

The formation of adherences following abdominal and pelvic operations remains still frequent enough and is an important source of morbidity. The incidence of intraperitoneal adhesions ranges from 67% to 93% after general abdominal operations and up to 97% after gynecological operations in the pelvic region. The factors associated with the formation of postsurgical adhesions include: trauma, thermal injuries, infection, ischaemia, and alien bodies.[157] Many other factors, including very tight sutures, when the tension in the sutured peritoneum brings about ischemia, abrasion, exposing to aggression factors such as talcum powder in the gloves, reactive sutures materials, contents of bowels, superheating due to the lamps or to the irrigation fluid, can all contribute to the formation of postsurgical adhesions.

The formation of adherences occurs, typically, in the place, where two peritoneal surfaces come in contact. The triggering of the formation of adhesions is caused by the organization of the fibrin matrix, an organization that takes place during the coagulation processes when there is suppression of fibrinolysis. The tissular surgical lesions diminish or eliminate the blood flow, giving rise to ischaemia the effect of which being the local persistence of the fibrous matrix, gradually replaced vascular tissue of granulation, which contains macrophages, fibroblasts, and giant cells. The adhesions can subsequently maturate in the shape of fibrous bands, which often contain small calcified nodules and are frequently covered with mesothelium, blood vessels and fibres of conjunctive tissue, inclusively elastin.

MATERIALS AND METHODS

Structure of patient groups. In this study two groups of patients are analyzed:

The **first group** is composed of twelve patients operated on in the clinic for surgical acute abdomen, in whose case the presence of a factor of peritoneal aggression (FPA) susceptible to bring about the formation of postsurgical peritoneal adhesions was particularly noticeable during the operation. The factors of peritoneal aggression have been divided in three categories: traumatic, chemical, and bacterial. This group was used, both for evidencing the peritoneal **fibrinolytic activity** by determining the fibrinogen and the fibrine degradation products (FDP) in the peritoneal liquid and for extract the tPA and PAI-1 from the peritoneal tissue.

The **second group** is the control group made up of six patients operated on in the clinic for defects of the abdominal wall (three patients) and for acute catarrhal appendicitis with peritoneal serous reaction (three patients), respectively, taking into consideration the fact that – in these cases – none of the aggression factors noticed in the patients of the first group was proven to exist. The patients have consented to be included in this study, which was approved by the ethical committee of the

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hospital.

Processing of the peritoneal tissue with the view to determining tPA and PAI-1. In order to extract the tPA and PAI-1 from the peritoneal tissue, fragments of peritoneal tissues freshly collected during the operation from the patients operated on for surgical acute abdomen were used, in which the presence of one of the peritoneal aggression factors indicated in the table below had been established. For comparison purposes, the witness group was made up of patients with uncomplicated hernias of the abdominal wall, considering that – at the level of the sacular peritoneum – none of the studied aggression factors act, and the collection of peritoneal tissue does not involve any supplementary surgical gestures since the resection of the hernia sac or the eventration represent a usual stage within the framework of the surgical treatment of the abdominal pariental defects.

Diagnosis	Traumatic FPA	Chemical FPA	Bacterial FPA
Hemoperitoneum by spleen rupture	blood	0	0
Hemoperitoneum by entero-mesenteric wound	blood	0	0
Perforated duodenal ulcer	0	bile+gastric juice	0
Perforated gastric ulcer	0	gastric juice	0
Perforated gangrenous calculous cholecystitis. Biliary septic peritonitis	0	infected bile	E.coli
Perforated gangrenous acalculous cholecystitis. Biliary chemical peritonitis	0	uninfected bile	0
Perforated gangrenous appendicitis Generalized peritonitis.	0	0	E.coli
Diastatic caecum perforation due to colon cancer. Generalized peritonitis.	0	0	E.coli
Uroperitoneum by traumatic rupture of bladder.	0	urine+blood	0
Uroperitoneum by PNL perforation of renal pelvis.	0	infected urine	E.coli
Ruptured ovarian endometriosic cyst. Endometriosic peritonitis.	0	endometriosic liquid	0
Ruptured tuboovarian abscess. Pelviperitonitis.	0	0	E.coli

Biopsy procedure. The biopsy was conducted on the parietal peritoneum (punch for biopsy with a 6-mm diameter, available, Stiefel Lab., Woobum Green, UK) as quickly as possible after the opening of the peritoneal cavity. The tissue disk was dissected from the adjacent subperitoneal tissue and minced in fragments with dimensions smaller than 1 mm, which were introduced in recipients covered with RNA stabilizer and kept in a refrigerator at a temperature of 4°C until they were processed.

Extraction of RNA from the tissue using the RNeasy® LIPID TISSUE KIT

Principle of method. The RNeasy Lipid Tissue Kit combines the method of lysis of the cells on the basis of phenol-guanidine with purification of the total RNA by means of of minicolumns of silicon dioxide. The lysis reactive QIAzol is a monophase solution of phenol and guanidine thiocyanate, ideal for the lysis of the various tissues (mainly in the case of tissues containing much fat) and for the inhibition of RNase. In a first stage, the following operations are conducted: homogenization of the tissue in the lysis reactive QIAzol, addition of chloroform and separation – by centrifuging – of the homogenate in the aqueous phase and the organic phase. In the following stage, the superior phase, which is aqueous (and contains the total RNA) is collected and then mixed with ethanol and the mixture is passed through an RNeasy minicolumn. The total RNA is then tied to the little column, and the phenol – as well as other contaminants – are eliminated. After successive washing by means of various washing swabs, the total RNA is eluted in water without Rnaze.

Statistical evaluation of results. The interpretation of results was effected by utilizing the statistical analysis conducted by means of the Excel and Epi Info computer programs. The homogeneity of data distribution was determined by the Bartlett test. A value of p lower than 0.05 of the Bartlett test is normally considered to be significant and it shows a non-homogenous distribution of the data to be analyzed, while a value of p equal to or higher than 0.05 indicates a similar dispersion of the subsamples. The significance of the differences between the patient groups with peritoneal risk factors and without peritoneal risk factors was determined by utilizing the parametric tests Student for two groups and ANOVA for more than two groups, as well as the non-parametric tests Mann-Whitney for two groups and Kruskal-Walliy for more than two groups. The difference is statistically significant if the probability p of these tests is lower than 0.05.

The Student and ANOVA tests are applicable only in the case of a homogenous data distribution (the Bartlett test has a $p \geq 0.05$). In the case of a non-homogenous dispersion of subsamples (the Bartlett test has a $p < 0,05$), the Bartlett test is not appropriate and a non-parametric statistics (the Mann-Whitney and Kruskal-Wallis tests) is to be used.

RESULTS

Analysis of peritoneal-tissue samples.

Relative quantification of the expression of PAI-1 gene. In order to study the expression of PAI-1 gene, the Real-Time PCR procedure was used, going through the following stages: reverse transcription of total RNA into cDNA, optimization of amplification conditions (PAI-1 gene and reference gene), achievement of the standard curve and calculation of the efficiency of PCR reaction. Finally, all the samples were run and normalized as a function of the actina reference gene, and in the end the relative expression of PAI-1 was compared between various patients.

The PCR conditions were optimized for the primer by varying the annealing temperature between 50-60°C on a gradient thermocycler IQCycler (BioRad). The PCR products were directly analyzed by electrophoresis in 2% agarose gels in 1X TAE buffer, stained with ethidium bromide, and visualized under UV light (Figs. 1 and 2). The optimum annealing temperature for all genes was 54°C.

Figure 3 shows the amplification curves which represent the fluorescent signals obtained during the reaction. In fact the graph represents the intensity of the fluorochrome interpolated at the level of the PCR products (thus measuring, in the case of the same gene, the quantity of PCR product formed in each cycle) *vs* the amplification cycle.

Figure no. 1. Temperature gradient for optimizing the amplification conditions of a fragment of PAI-1 gene. 1:

51°C; 2: 51,8°C; 3: 53,1°C; 4: 54,9°C; 5: 57,4°C; 6: 59,3°C; 7: 60°C; 8: 50bp molecular weight marker

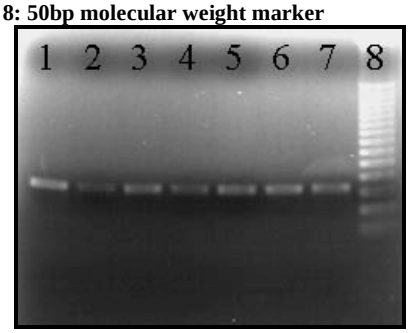


Figure no. 2. Temperature gradient for optimizing the amplification conditions of a fragment of actin gene. 1: 51°C; 2: 51,8°C; 3: 53,1°C; 4: 54,9°C; 5: 57,4°C; 6: 59,3°C; 7: 60°C; 8: molecular-mass marker 50bp

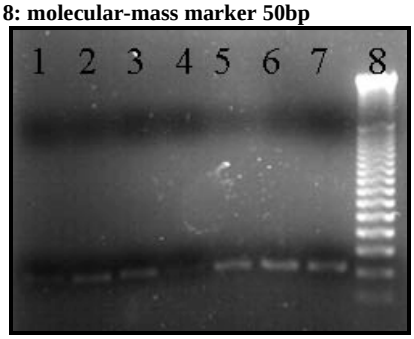
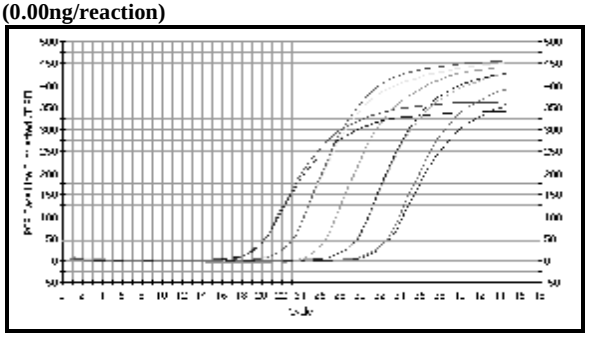


Figure no. 3. Standard curve was obtained using 5 serial dilutions of a cDNA sample: 10× dilution (10ng/reaction), 10^2 × dilution (1ng/reaction), 10^3 × dilution (0.1ng/reaction), 10^4 × dilution (0.01ng/reaction), 10^5 × dilution (0.00ng/reaction)



To calculate the efficiency of a PCR reaction, a logarithmic graph is obtained in which the values of C_t are represented *vs* the dilutions of the sample. In Figs. 4 and 5, the efficiencies of the PCR reaction are represented for the case of amplification of fragments of PAI-1 and actin genes.

The efficiency of the PCR reaction is considered to be good if it is 90-110%, which corresponds to a slope value between -3.58 and -3.10. As it can be noticed in Figs. 4 and 5 as well, the efficiencies obtained in the case of amplification of fragments of PAI-1 genes and actin were 106.6% (slope -3.173; R^2=1) and 105% (slope -3.208; R^2= o.998).

After the calculation of efficiencies, samples from various patients were run in order to quantify the relative expression of the PAI-1 gene as a function of the reference gene – actin. In Fig. 6, the amplification curves for all samples are presented.

Figure no. 4. Efficiency of the amplification reaction of a fragment of PAI-1 gene

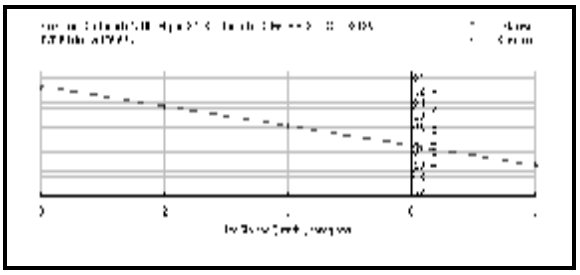


Figure no. 5. Efficiency of the amplification reaction of a fragment of actin gene

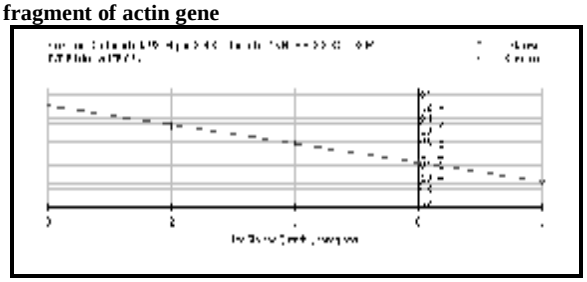
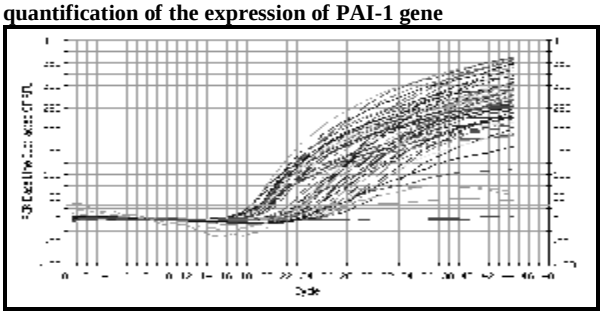


Figure no. 6. Amplification curve in the case of quantification of the expression of PAI-1 gene



The obtained melting curves have shown the existence of a single peak (89°C – PAI-1; 84°C – actin), which means that there were no non-specific amplifications, dimers of primers or contaminations with gnomic DNA.

The amplification equations were determined for both the target gene PAI-1 and the reference gene (actin); in the case of each sample we obtained the following equations:

$$N_{CtPAI-1} = N_{0PAI-1}\,(1+E)^{CtPAI-1},$$

$$N_{Ctactin} = N_{0actin}\,(1+E)^{Ctactin},$$

where

N_{Ct} = number of molecules after C_t amplification cycles,

N_0 = initial number of molecules,

E = efficiency of PCR reaction,

C_t = threshold cycle

For normalization purposes, the ratio between N_{0PAI-1} and N_0 of the reference gene (actin) was calculated with the formula:

$$N_{0PAI-1}/N_{0ref} = k\,2^{Ctref-CtPAI-1}$$

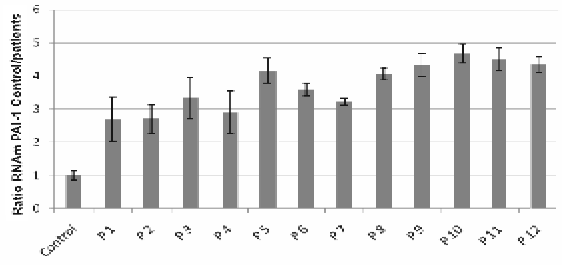
where k is a correction factor.

The above equation was used for all of the patients, including the normal patient (deemed to be the control patient). In accordance with the equation

$$\text{Control/patient} = k\,2^{Ctrefcontrol-CtPAI-1control}/k\,2^{Ctrefpatient-CtPAI-1patient}=2^{\Delta\Delta Ct},$$

all of the samples were referred to the control patient, and the diagram in Fig. 7 was obtained.

Figure no. 7. Relative expression PAI-1 gene



In compliance with the statistical test t-test, two tails, non-paired, all of the values obtained for *p* were significant ($p<0.05$). It is apparent from the diagram in Fig. 7 that the relative expression of PAI-1 gene is significantly increased (3-4.5 times) in comparison with the case of the sample considered control sample.

Relative quantification of the expression of tPA gene. In order to study the relative expression of tPA gene, the Real-Time PCR technique was used as well. The actin gene was used as reference gene, and the stages run through were the same as in the case of the quantification of expression of PAI-1 gene: optimization of the hybridization temperature of primers, calculation of the efficiency of PCR reaction, and comparison of the expression of tPA of the patients.

The PCR conditions were optimized for the primer by varying the annealing temperature between 50-60˚C on a gradient thermocycler IQCycler (BioRad). The PCR products were directly analyzed by electrophoresis in 2% agarose gels in 1X TAE buffer, stained with ethidium bromide, and visualized under UV light (Fig. 8)

The optimum temperature of the hybridization of primers was 54°C. In Fig. 9 we have shown the obtained standard curve for tPA gene. The efficiency of the amplification reaction of a fragment of tPA gene was 99%, with a slope of -3.346 and $R^2 = 0,998$.

After the calculation of efficiency, samples from various patients were run in order to quantify the relative expression of tPA gene as a function of the actin reference gene. The samples from each patient were run in triplicate (technical replications). After the samples were run, the program of the PCR instrument established the threshold level and calculated the values of C_t for each sample. In Fig. 10 the amplification curve of all of the samples is presented.

The data obtained was processed in compliance with the equations given above, and the average values obtained – along with the standard deviation – were represented graphically in Fig. 11.

In compliance with the statistic test t-test, two tails, unpaired, all of the values obtained for *p* were significant ($p<0.05$). In the case of tPA gene, a level of the expression was obtained, which was 3 to 9 times lower than in the case of the control patients (fig. 11).

Figure no. 9. Efficiency of the amplification reaction of a fragment of tPA gene

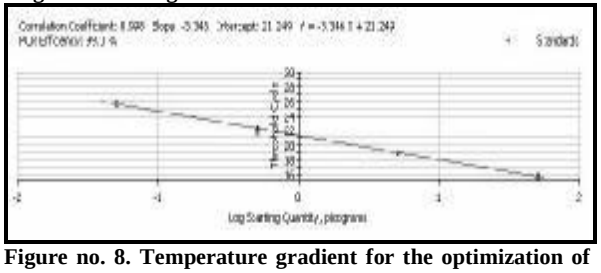


Figure no. 8. Temperature gradient for the optimization of the amplification conditions of a fragment of tPA gene. 1: 51°C; 2: 51,8°C; 3: 53,1°C; 4: 54,9°C; 5: 57,4°C; 6: 59,3°C; 7: 60°C; 8: 50 bp molecular weight marker

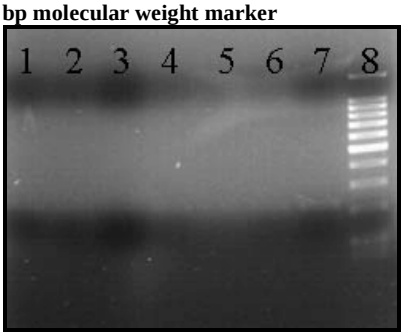


Figure no. 10. Amplification curve in the case of quantification of the expression of tPA gene

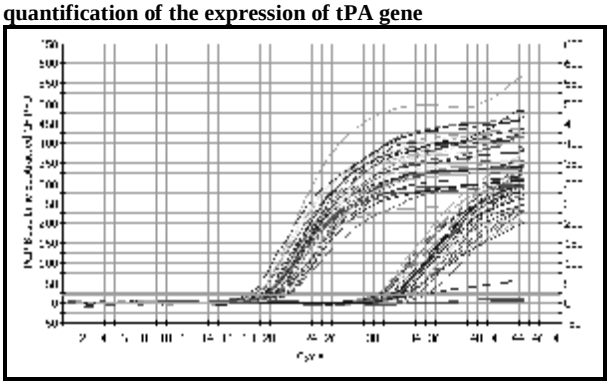
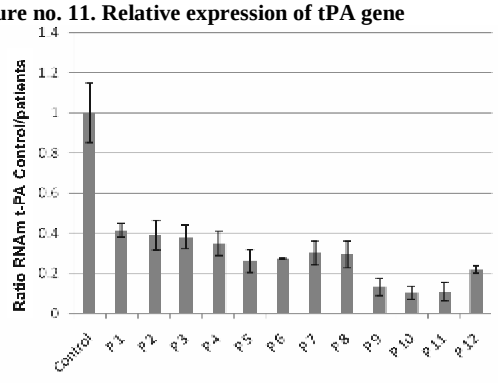
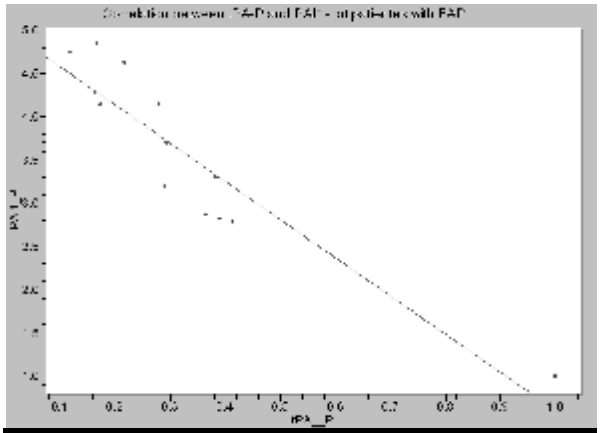


Figure no. 11. Relative expression of tPA gene





DISCUSSIONS AND CONCLUSIONS

The physiological disappearance of the provisional matrix of the clot is as important as its formation. The main proteolytic enzymes, the activators of plasminogen and the plasmin avoid the action of such inhibitors of proteases as the inhibitor of the activator of plasminogen and the α2-antiplasmine, linking themselves with the fibrine clot and the cellular surfaces. Although the activator of the plasminogen and the plasmin can degrade a wide range of proteins of the extracellular matrix, a specific inhibitor of the activator of plasminogen (PAI-1) links itself with the extracellular matrix and limits the matriceal degradation to a very small space neighbouring the cellular surfaces. The inadequate removal of fibrin can hinder the normal healing process of the wounds and may lead to the formation of fibrous adherences.

PAI-1 is a glycoprotein of 50kD produced at the level of the endothelial cells, which belongs to the superfamily of superprotease inhibitors (serpin-1) and represents the major inhibitor of the tPA activator (tissue plasminogen activator) and of the activator uPA (urokinase plasminogen activator), at the level of the plasma and of the peritoneum (Kruithof, 1988; van Hinsbergh *et al.*, 1990), determining the formation of the inactive enzimatic complexes PA-PAI. The high levels of expression of PAI-1 and tPA are associated with the coronarian risk at the level of arteries. The increased activity of tPA is an indicator of the increased fibrinolytic potential. The action of PAI-1 is controlled by various citokines and growth factors, such as TGF- β. Thus, TGF- β stimulates the secretion of PAI-1 in various cellular lines (Rondeau et al., 1995) and *in vivo* (Sawdey&Loskuttoff, 1991).

The fibrinolytic system plays an important role in the formation/reformation of the peritoneal adherence. In the first stages of the healing process, between two peritoneal surfaces wounded, from the fibrinous matrix fibrin bands develop subsequently, which can be removed in a natural way by fibrinolysis, the result being the formation of fibrin degradation products (FDP). In the pathological processes of the adherential formation, the fibrinolytic mechanisms are alterate, permitting the formation and persistence of adherences.

The continuation of research is necessary for knowing the mechanisms involved in adesiogenesis from a molecular and cellular point of view and for determining with more precision the role of fibrin, as well as the regulation of fibrinolysis and coagulation, all of these from the point of view of the involvement in the generation of adherences. Also, by understanding the biochemical and morphological phenomena of the normal peritoneal healing and of cicatrisation, it will be possible to obtain a modality of separating the peritoneal surfaces and increasing fibrinolysis. The difference between the adherences that appeared at different intra-abdominal or intrapelvic levels must be studied further. A better understanding of the adherences that predispose to complications and the knowledge of the places where these appear more frequently is necessary. Further research is required for a better knowledge of the newly discovered agents, with special emphasis on the degree of adherence prevention. It is also necessary to identify the best diagnosis procedures to permit the visualization of the adherences that appeared after the operation.

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