Histol Histopathol (2018) 33: 299-306

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Histopathological changes associated to an absorbable fibrin patch (Tachosil®) covering in an experimental model of high-risk colonic anastomoses

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Summary. Background. TachoSil® is a fibrin sponge that contains fibrinogen and thrombin and is a useful adjuvant to enhance control of air leaks in thoracic surgery and to control bleeding in vascular and general surgery. Its use in intestinal surgery to prevent suture dehiscence is currently under investigation.

Material and Methods. We report the results of a prospective randomized experimental study on 33 large white pigs in which a high-risk suture was created by induction of ischemia. We randomly employed TachoSil® to cover the anastomosis in half of the animals compared to a control group of uncovered anastomosis. After euthanasia, postmortem analysis was performed describing the findings related to anastomotic leakage, peritonitis and grade of adhesions. The entire anastomosis was resected in bloc and sent for histopathological analysis. A single blinded-pathologist evaluated the histopathological features of the specimens.

Results. We found statistically significant differences favouring the patch in decreasing leakage in the covered group. The healing process did not show significant differences between groups, although a higher rate of microscopic abscess was observed in the covered group.

Conclusion. The use of fibrin sealants covering high-risk intestinal sutures has a positive effect in avoiding macroscopic anastomotic leakage. The patch did not have any influence in the anastomotic healing process, however, as a result of the effect in containing the inflammatory response, it may increase the rate of abscess.

Key words: Colon, Anastomoses, Fibrin sealant, Ischemia, Leakage

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DOI: 10.14670/HH-11-930

Introduction

Suture dehiscence traditionally represents a major surgical complication, with a negative influence on patient prognosis (Walker et al., 2004; Branagan and Finnis, 2005). It is known that the need for reoperation is associated to a significant increase in mortality, reaching almost 30% in some series (Buchs et al., 2008). The use of fibrin sealants (FS), which are hemostatic and adhesive surgical products mainly derived from human plasma, has been shown to improve haemostasis in hepatic and pancreatic surgery (Padillo et al., 2010), is safe to use on gastric sutures of acute perforations (Di Carlos et al., 2009) and also may help to reduce air leaks in thoracic surgery (Lang et al., 2004). TachoSil® is one of the widely used FS forms approved by the European Medicines Agency (EMA) (EPAR - EMA reports on TachoSil®, 2009). Because of their characteristics, there is reasoning behind the idea that FS may be useful as a reinforcement or replacement of sutures on intestinal anastomosis. Nevertheless, a new indication for FS

would be to provide a support mechanism for intestinal sutures with the objective to reduce the possibilities of leakage especially in high-risk anastomosis.

The aim of the present study is to determine whether the use of an absorbable fibrin sealant patch with the goal to protect the anastomosis would have an effect on preventing leakages. For this reason, we designed a reproducible experimental model of delayed leakages by performing intestinal sutures with hypovascularized tissues. In addition, a secondary objective is to analyze the histopathological tissue changes associated with the patch placement.

Material and methods

Phase I. Validation of an ischemic model

We performed a preliminary study on 10 male, "Large White" pigs between 27 and 32Kg with normal anatomy to assess the feasibility of our experimental model. Following premedication with Midazolam, the animals were induced to general anesthesia using weight-adjusted dosing of Ketamine, Fentanyl and Sevoflorane. A prophylactic dose of Cefotaxime 2g was administrated in each case at 30 minutes prior to skin incision. By medial laparotomy an initial exploration of the abdominal cavity was performed. The terminal ileum and caecum were identified and exposed. The next step was to perform a devascularization of the last 15cm of the caecum with a vascular ligature along the marginal artery and its small branches (Fig. 1A). Then, the surgeons performed a hand-sewn ileo-colic anastomosis done the same way as in regular practice. The anastomoses were done between the middle third of the caecum devascularized zone on one side and the terminal ileum on the other side, in a latero-lateral fashion (Fig. 1B), with a 2cm opening, using an absorbable suture (3/0 Biosyn, Covidien®) being careful to not leave tension between the ends of the anastomoses (Fig. 2).

All animals were kept in our experimental institute, supervised and in climate-controlled cubicles. Oral intake was resumed 24 hours postoperatively, with free access to water and standard commercial fodder mixed with 600mg of Ibuprofen every12 hours. Basal temperature and physical exploration was performed daily to assure the welfare and clinical evolution of the animals. On the 5th postoperative day all subjects were sacrificed. A reoperation was then performed by exploring the abdominal cavity searching for adhesions, signs of peritonitis, abscess or fecal contamination. Afterwards an entire resection in bloc of the anastomosis was performed. To assess the grade of adhesions, we used a modification of Kamil's visual scale ranging from 0 to 3 (0 no adhesions, 1 loose adhesions, 2 peri-anastomotic firm adhesions, 3 anastomotic to abdominal wall adhesions) (Kamil et al., 2014).

All specimens were sent to the pathology department and a microscopic analysis of the anastomosis was performed, reporting any signs of microabscess, microscopic leakage, neovascularization and cellularity.

Phase II. FS covering vs. non covering method

After the preliminary study, a second prospective randomized phase was performed. A new set of animals underwent surgery using the previously validated model.

Following the formation of ischemic anastomosis, cases were randomized by a computer into two groups: The covered group (CG) in which the entire anastomotic line was overwrapped with a collagen patch coated with fibrin glue components (Tachosil®) versus the none-covered group (NCG) in which the anastomoses were left untouched. After surgery, pigs were continuously monitored for a period of 5 postoperative days and euthanized on completion of the observation period.

Surgical technique

After performing a surgical approach as described before and following the formation of the anastomoses, cases were randomized to the CG or NCG. In the

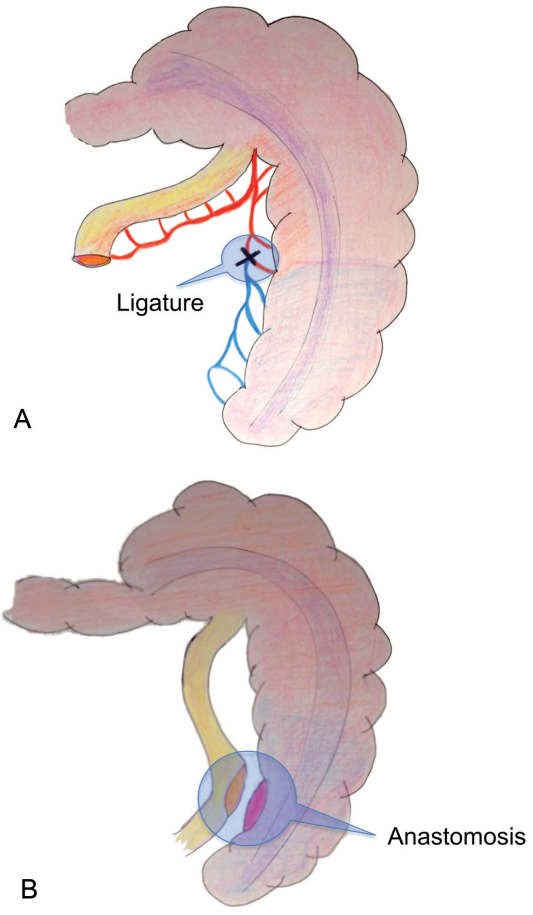

Fig. 1. Colonic devascularisation **(A)** and anastomotic conformation **(B)**.

covered group, we over-wrapped the entire anastomotic line with a collagen patch coated with fibrin glue components (Tachosil®-Takeda). The application method (shown in Fig. 3) of the patch covering the intestinal suture was carried out as described previously in the literature (Suarez-Grau et al., 2016). In short we

Fig. 2. Anastomosis between terminal ileum and a segment of ischemic colonic tissue.

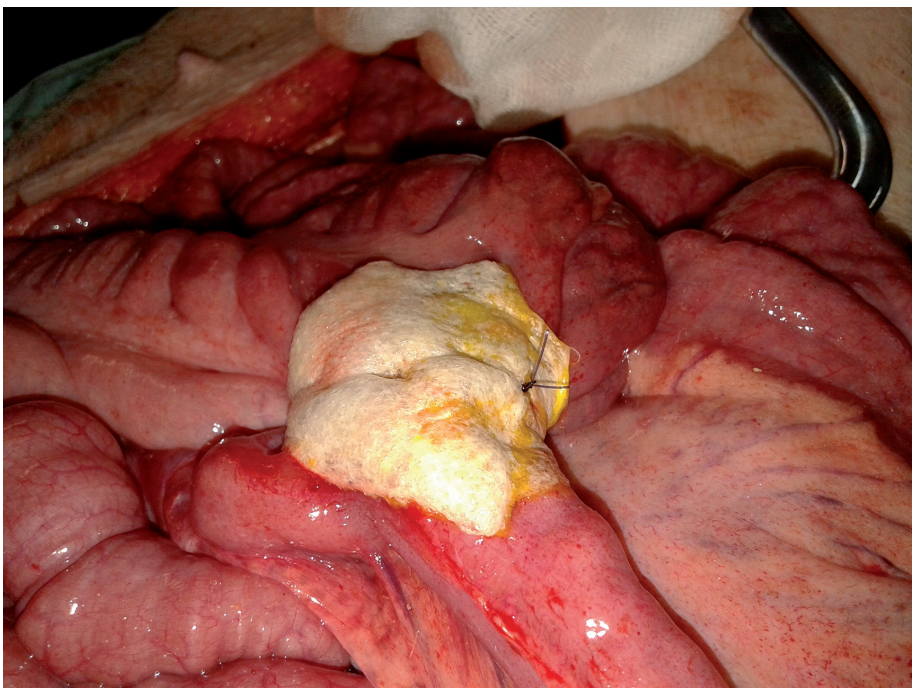

Fig. 3. Anastomosis with the Tachosil® patch overwrapping the intestinal suture.

divided the TachoSil® into two equal longitudinal parts and applied on both sides (anterior and posterior) of the anastomotic line with a light pressure using a wet gauze. In the animals randomized to the NCG, the anastomoses were left untouched. Finally, the laparotomy was closed using monofilament running sutures and the skin was approximated using staples.

After surgery, the animals were continuously monitored and fed for a period of 5 postoperative days. Animal care and observations during the postoperative period were performed by trained animal technicians supervised by the Animal Research Institute veterinary. On the 5th postoperative day (or before in the case of signs of surgical complications) the pigs were taken into the operating room to perform a new induction of general anaesthesia as described before. Then a new laparotomy was done along with evaluation of the abdominal cavity, resection of the anastomosis and fixation of the specimen and finally the subjects were euthanized with an overdose of the used anaesthetics and Pentobarbital.

Clinical variables

A first set of clinical variables were obtained at the time of surgery: animal characteristics, compliance with the surgical protocol, duration of surgery and observed intraoperative complications, and period of time to euthanasia. The surgeons described the operative findings at the time of the second operation. Abscess or leakage was defined as the presence of macroscopic defects. Abscesses were recorded as localized when pus was observed surrounding the anastomotic segment or diffused when we found free pus or faeces in the abdominal cavity. Adhesions observed between the anastomosis and the abdominal wall or between intestines were also reported using a grading scale (0 to 3) following the same scale as in Phase I (Kamil et al., 2014). Other additional findings such as the presence of perforations or other signs of necrosis over other intestinal segments, not involved in the suture, were also recorded.

Histopathological evaluation

The evaluating pathologist was blinded to the type of intervention performed on each animal., The suture segment was fixed in a formaldehyde solution to perform then a hematoxylin-eosin technique. To record the histopathological features of the anastomotic bed we used the standard criteria settled by Ehrlich and modified by Phillips et al. (1992). In short, we evaluated the kind and intensity of inflammatory reaction, the formation of microabscess (Fig. 4), the presence of fibroblasts (Fig. 5) and collagen deposit. We also measured the percentage of necrosis within the anastomosis and the number and nature of the blood vessels present in the surgical bed (Fig. 6). We performed routine 5 micron-thick haematoxylin-eosin stained slides with masson's trichrome staining for collagen evaluation.

Statistical analysis

The results were recorded and analyzed for statistical significance using SPSS software® (version 22.0, SPSS Inc - IBM Corp, Chicago, IL) and SOFA (version 1.4.6; 2009-15 Paton-Simpson & Associates Ltd). After general descriptive statistics with percentages, we compared the differences between groups with chi-squared test. Significant differences were defined as having a p value <0.05.

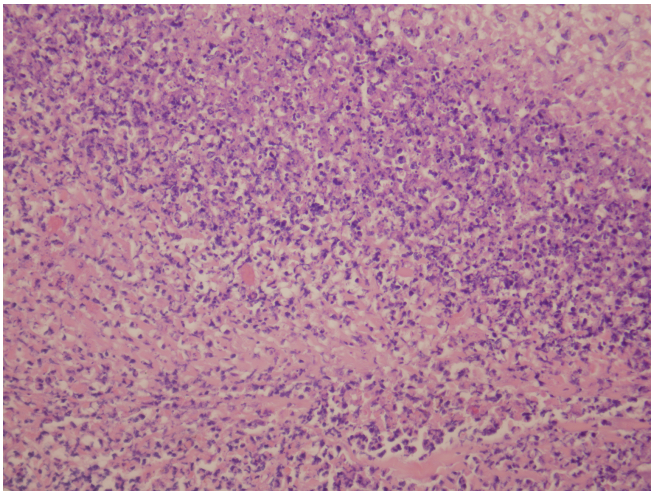

Fig. 4. Microabscess formation at the interphase between peritoneum and fibrin patch. HE, x 200.

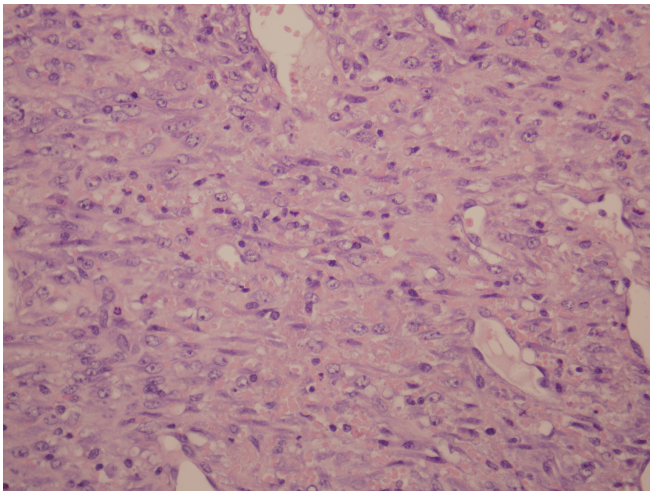

Fig. 5. Cellular fibroblastic response with little collagen deposition in the anastomotic bed. HE, x 200.

Animal safety regulations

This study is in accordance with the Animal Experiment Legislation of Madrid Community and has been registered with number 10/129734.9/14. The study has also been reviewed and approved by the Ethical Review Board of our center.

Results

First phase

All 10 procedures were performed without complications, with a median intervention time of 23 minutes. During the kept period, all animals presented with normal behaviour, obtaining the diet and medication normally, and all subjects presented with regular bowel movements. All animals were sacrificed as per protocol at the 5th postoperative day.

From a macroscopic point of view, 3 animals (30%) had a macroscopic leakage, with local abscess and major findings of faecal contamination without distant or diffuse peritonitis. The most frequent adhesion indexes were 1 and 3, without statistical significance in relation to the anastomotic leakage, possibly due to the small sample size. The microscopic study (Table 1) showed a polymorphonuclear dominance (median of 83,5%), 6 cases had important neovascularization, without relation to the presence of leakage. All the samples had signs of necrosis, with no difference of frequency between the focal or disseminate form.

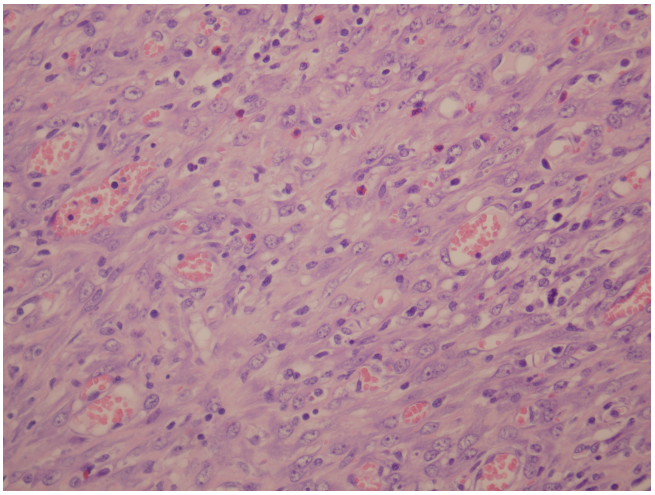

Fig. 6. Neovascularization in the anastomotic bed. HE, x 200.

Second phase

A total of 33 pigs were included in this phase of the study. Four animals died due to anesthetic complication during surgery and one died before the end of the observation period and was excluded from the pathological analysis.

Therefore, we included in the present study, data from 29 animals. The median operation time was 25 minutes, 28 animals completed the observation period until being euthanized on the 5th postoperative day. The median weight was 35Kg. We included 12 (41.3%) males and 17 (58.7%) females. A resume of animal characteristics and clinical variables is showed in Table 2.

We performed the anastomosis on each pig without intraoperative surgical complications. A total of 15 (51.7%) anastomoses were randomized to the CG using the FS patch, while the rest, 14 (48.3%) were left untouched. A total of 28 of the 29 anastomoses were resected at the end point time and underwent pathological studies. One subject of the non-covered group died on the third postoperative day due to anastomotic leak, making it impossible to perform the reoperation.

The observed results on each study variable are presented in Table 3.

Anastomotic leakage (by surgeons): Macroscopic anastomotic leakage was defined as visible anastomotic defects and faecal peritonitis and was observed in 10 cases (8 non-covered vs. 2 covered), indicating a 34.5% of macroscopic leakage. The covering of anastomoses

Table 1. Microscopic Study.

ID	Fail	PMN	Lymph	Vessels	Fibroblast	Macrophages	Necrosis	Hemorrhage
1	No	80%	10%	Neoformed	++	10%	Focal	Diffuse
2	No	95%	5%	Mature	+++	<2%	Extensive	Focal
3	No	95%	5%	Mature	++	<2%	Extensive	Focal
4	No	65%	35%	Neoformed	++	<2%	Focal	Diffuse
5	No	95%	5%	Neoformed	++	<2%	Focal	Diffuse
6	Si	90%	10%	Neoformed	++	<2%	Focal	Diffuse
7	Si	70%	30%	Mature	+++	<2%	Extensive	Limited
8	No	85%	15%	Mature	+++	<2%	Extensive	Limited
9	No	75%	25%	Neoformed	++	5%	Focal	Limited
10	Si	85%	15%	Neoformed	++	<2%	Extensive	Diffuse

Table 2. Animal characteristics and clinical variables.

Number	Gender	Weight	Cover
1	Male	35	Yes
2	Female	25	No
3	Male	30	Yes
4	Female	30	No
5	Female	30	Yes
6	Male	30	No
7	Male	32	No
8	Female	28	Yes
9	Male	27	Yes
10	Female	26	No
11	Male	27	Yes
12	Male	30	Yes
13	Male	27	Yes
14	Female	30	No
16	Male	29	No
17	Female	31	No
18	Female	26	Yes
19	Female	31	No
20	Female	26	Yes
21	Male	28	No
22	Female	27	No
23	Female	28	Yes
24	Male	26	No
25	Male	34	Yes
26	Female	24	Yes
27	Female	24	No
28	Female	30	Yes
29	Female	32	Yes
30	Female	35	No

with Tachosil® showed a significant reduction in the macroscopic leakage rate from 27.6% in the non-covered group vs. 6.9% in the covered group; p=0.013.

Adhesions (by surgeons): Adhesions between intestines and from the anastomosis to the abdominal wall were observed at the time of euthanasia in a high percentage of cases (19 cases; 76%). The covering of the anastomoses with Tachosil® did not have an impact in increasing these adherences; 40% of cases had adhesions in the covered group vs. 36% in the non-covered group; p=0.9.

Abscess (by pathologist): An overall of 53.5% of the animals showed abscesses in the histopathological analysis and most of these (80%) did not show macroscopic leakage. Histopathological analysis revealed a significantly higher rate of microabscesses in the animals treated with TachoSil® of 28.5% vs. 78.5%; p=0.01.

Necrosis (by pathologist): Some grade of necrosis, whether local or diffused was observed in all cases. In 11 cases (37.9%) diffused necrosis was observed, of them, 17.2% had a macroscopic leakage and 20.7% were normal; p=0.33. Necrosis was more localized in the TachoSil® group, but the difference did not reach significance (50% vs. 71.5%); p=0.19.

Table 3. Summary of histopathological features (expressed in absolute number).

Feature	Control group (14)	TachoSil (15)	P value
Anastomotic leakage	Absent: 6 Present: 8	Absent: 13 Present: 2	0.013
Adhesions	Absent: 9 Present: 5	Absent: 9 Present: 6	0.9
Abscess formation	Absent: 10 Present: 4	Absent: 4 Present: 11	0.01
Necrosis	Localized: 7 Widespread: 7	Localized: 11 Widespread: 4	0.2
Neovascularization	Absent: 8 Present: 6	Absent: 11 Present: 5	0.59

Inflammatory cell reaction (by pathologist): Inflammatory cell reaction was observed in almost 50% of the samples. To note, the reinforcement of the anastomosis with Tachosil® did not show a correlation with an increased inflammatory reaction (46.7% showed visible inflammatory reactions in the covered group vs. 42.9% in the non-covered group); p=0.83.

Neovascularization (by pathologist): Neovascularization, defined as presence of immature vessels was observed in 11(37.9%) cases. The covering of the anastomosis with Tachosil® did not have a significant impact in the generation of vessels when comparing the percentage of immature vessels in the covered group 34.5% vs. 27.6% in the non-covered group; p=0.59.

Other variables (by pathologist): Collagen deposition, inflammatory lymphocytic reaction and fibroblastic proliferation were similar between groups.

Discussion

The present randomized controlled study shows that covering the anastomosis with Tachosil® has a significant impact in decreasing macroscopic leakage in our experimental model. As we proposed in our principal hypothesis, we have validated an animal model for the study of the ischemic anastomosis, showing a 30% rate of anastomotic leakage in a species that may be considered an accurate reproduction of the human intestine (Pommergaard et al., 2011). From an ethical point of view, our model seems to be well tolerated by the subjects and generated a controlled amount of discomfort, with extensive alternatives to be reduced (analgesia, antibiotics).

Our findings are in line with what has been previously published in the literature. In a non-randomized study published (Nordentoft and Holte, 2014) using Tachosil® in pigs (to our knowledge, our study is the second one using pigs as an experimental

model), they also reported a significant decrease in leakage. This previous study has some differences compared to ours, as the author induced a standardized defect in the anastomosis, and consequently reported a higher leak rate than ours. We believe that our experimental model is a better one, as surgeons, since in a real setting a defect in the anastomosis would not be created, although, it is quite common to perform intestinal anastomoses in critical situations. Another study (Tallón-Aguilar et al., 2015) using a murine model performed a histopathological analysis of the anastomotic tissue and reported a benefit in using Tachosil® covering colonic perforations, as in our results, a high percentage of abscess.

The experience in colonic surgery is not homogeneous and recent literature seems to indicate that the use of fibrin sealants (TachoSil) in anastomoses is a safe alternative (Nordentoft el al., 2007; Suarez-Grau et al., 2016). In this study, a systematic review shows varying results (Nordentoft et al., 2015), and we feel this fact can be explained by the lack of homogeneity of the reported studies. Most of the work is experimental and has been performed in different animal models. We have performed a conventional anastomosis on an ischemic colon segment, but we have not created any artificial defect in the anastomosis itself. It could be expected that the rate of complications in the present study mimics better the one found in usual ischemic anastomosis. For ethical and practical reasons, it would be extremely difficult to perform controlled experimental studies of the present kind in human beings and we feel pig models are the best reflection of the actual situation in humans (Pommergaard et al., 2011).

From a macroscopic point of view, TachoSil® seems to be a good candidate to control anastomotic failure due to the presence of a stable collagen base that may work as a control to the fluids with the help of the fibrin adhesive capacities. Finding a reduced rate of macroscopic failure in the CG may be in line with this capacity to contain fluids and contamination, reducing the rate of macroscopic peritonitis and thus this, reducing the clinical manifestation of said peritonitis. On the other hand, this physical characteristic of a semi-rigid patch may also justify the presence of an augmented rate of abscess in the perianastomotic area and an apparent, but non statistical significant increment on the perianastomotic adhesions.

Therefore, we consider that our experimental model shows a strong correlation with clinical situations as no controlled clinical studies with respect to TachoSil® and hypoperfunded intestinal sutures have been performed in humans to date. Another positive effect seen in the present study is that, although adhesions between intestines and to the abdominal wall were frequently seen in the study (76%), these adhesions were not increased by the use of the patch. The high percentage of adhesions may be explained by local inflammatory fibrosis due to tissue necrosis and we have not reported intestinal obstructions due to the use of the patch.

As for histopathological analysis of the anastomotic bed after fibrin sealant use, we have found two previous studies, but they chose a murine model, (Tallón-Aguilar et al., 2015; Suarez-Grau et al., 2016). These authors designed two experimental murine models to validate their clinical observations and proposed that TachoSil® behaves both as a sealing and therapeutic agent rather than being a mere reinforcement of the sutures, probably acting in the prevention of the anastomotic leak more as a physical barrier. We find it worth noting that they also found in this experimental model a higher percentage of microabscesses in the group receiving TachoSil® patches. We have confirmed their finding, but we believe the patch can act as a means of containment for inflammatory reaction. Necrosis and acute inflammation may tend to be more localized in animals treated with TachoSil® and this is associated to a lower rate of macroscopic anastomosis leakage. It might be postulated that TachoSil® contains inflammation and reduces the risk of spill over the peritoneal cavity through both a mechanical and hemostatic effect on the surgical bed. The presence of abscesses in the anastomosis could generate fear of potential infectious complications after surgery, but use of TachoSil® in kidney and pancreatic transplant surgery (Padillo et al., 2010) has been associated to a significant reduction of intraabdominal infectious complications and length of stay in hospital., During clinical follow-up we found no significant differences in the rate of fever or any other signs indicative of infectious complications, enabling us to argue microabscesses are not harbingers of more dangerous intraabdominal infectious complications. Despite this, our study was not specifically designed to test this issue and animals were euthanized too soon after surgery to draw solid conclusions regarding this issue. Future studies with longer postoperative follow-up should settle this matter.

We consider our animal model a valid imitation of the regular surgical practice. However, new promising techniques have arisen in recent years as the intraoperative indocyanine green evaluation of the anastomosis may modify the way the surgeons see anastomosis, reducing the number of ischemic anastomosis. Also, we think that a collagen analysis of the specimen could have given more information on the healing process of the anastomosed intestine. Due to the high rate of failure we do not think that a bursting pressure analysis could have given any more useful information.

Conclusions

Our porcine experimental model is a simple, effective, and feasible model for the study of anastomotic leakage after colonic surgery. The employment of a Tachosil® patch over high-risk anastomosis has a positive effect on reducing the rate and clinical consequences of anastomosis failures. However, a higher rate of local abscess is expected, as

the positive effect of the patch is due to a mechanical contention of the infection.

Acknowledgements: The authors thank Sara Rosenstone Calvo M.D. and Patricia Tejedor M.D. for editing this manuscript.

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Accepted September 7, 2017