The Effect of TISSEEL® on Colorectal Anastomosis Healing Process in a Diabetic Animal Experimental Model

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Abstract. *Background/Aim: Diabetes mellitus is an established risk factor of colorectal anastomosis failure. The purpose of the present study was to evaluate the effect of TISSEEL® in anastomotic healing. Materials and Methods: Forty male, Sprague-Dawley rats were used. Diabetes was induced in half of them by intraperitoneal injection of Streptozotocin, 60 mg/kg. One week after the injection, animals were operated and a 1 cm segment was removed and an end-to-end hand sewn anastomosis was performed. TISSEEL® was applied in each group (diabetic, non-diabetic) following randomization. Results: The pathology analysis revealed improved tissue remodeling in the TISSEEL® group, both for the normoglycemic and the diabetic group. Specifically, the extent of inflammation was decreased ($p<0.001$), whereas fibroblast and collagen formation were improved ($p=0.040$ and $p=0.008$). Neovascularization was also improved ($p=0.047$). Conclusion: Application of TISSEEL® on colorectal anastomoses improves healing in rats that suffer from severe hyperglycemia.*

Surgical operation is currently the definitive treatment option both for colorectal cancer and a variety of benign colorectal modalities, such as inflammatory bowel disease (IBD), inflammatory strictures, diverticulosis-diverticulitis, obstructive defecation, volvulus, fistulae *etc.* (1). Despite the fact that

colorectal surgeons undergo a high-level training and specialization in advanced techniques, colorectal operations still demonstrate serious complications (2). The most frightening and dramatic complication of colorectal resections with restorative anastomoses is anastomotic dehiscence (3). Anastomotic leakage remains a relatively frequent complication of colorectal operations even in high volume specialized centers, with reported rates of 2.5%-15% for rectal cancer resections and 3%-7% after colon cancer surgery (4). The consequences of this complication are devastating as it has been related to higher recurrence rates, increased morbidity and mortality rates, elevated treatment costs and negative influence in the quality of life of patients (5, 6).

Despite technological advances, including transanal total mesorectal excision (TME), laparoscopic and robotic approach, and the new trends followed during post-operative management of patients that undergo colorectal resection, such as enhanced recovery after surgery (ERAS), there are several risk factors that still exert a significant impact on the outcome of colorectal anastomoses (7, 8). So far, patient-related factors (male sex, ASA score>II, radiotherapy, immunosuppressive therapy, bevacizumab intake), tumor-related factors (distal size>3 cm, advanced stage, emergency surgery, metastatic disease), comorbidities (diabetes, cardiovascular disease, poor nutrition) and operation-related factors (operative time, blood loss, need for transfusion) have been considered as determinants of failed anastomotic healing and leakage (9). Diabetes mellitus has a special position among the previously mentioned risk factors, as it is a well-established independent factor for anastomotic leakage, that has been associated to increased mortality after anastomotic dehiscence as well (10).

Previous studies have suggested that the negative impact of diabetes mellitus on tissue healing is based on the exacerbated inflammatory processes (11, 12). Diabetes also dysregulates the transformation of macrophages from their

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Figure 1. Animal randomization flow diagram.

inflammatory-state into their healing-state (13). Furthermore, the disease results in the formation of extracellular neutrophilic traps that contain chromatin and cytotoxic proteins, a process called NETosis, that damages healthy tissues (14). Peripheral angiopathy is another factor that is frequently encountered in diabetic patients which per se results in impairment of the integrity of colorectal anastomoses (15). Diabetes affects a large number of physiological factors involved in the healing process, such as growth factor production, angiogenic response, collagen concentration, tissue granulation and extracellular matrix accumulation and remodeling by matrix metalloproteinases (16). However, fibrin sealants have been used as hemostatic factors and enhancers of the wound healing process in various surgical procedures including gastrointestinal anastomoses, plastic surgery, gynecologic and urologic procedures (17, 18).

The aim of the present study was to investigate the effect of TISSEEL®, which is a fibrin sealant that simulates the final stages of the coagulation cascade, in tissue healing of colorectal anastomoses in diabetic rats.

Materials and Methods

Animals. Forty male, Sprague-Dawley rats (Hellenic Pasteur Institute, Department of Animal Models for Biomedical Research, Greece) were transferred in the Laboratory of Experimental Surgery “NS Christeas” of the National and Kapodistrian University of Athens. The animals remained in atmosphere-controlled chambers (temperature $20\pm1^{\circ}\text{C}$, humidity $55\pm5\%$) and under controlled light (12 h per day) for 7 days in order to adapt to their new environment. Full nutrient supplementation was provided ad libitum using ELVIZ

510 food pellets. Animal care, surgical operations and postoperative care were approved by the Athens University Medical School Ethics Committee and by the Veterinary Directorate of the Ministry of Agriculture in agreement with the EU Directive 2010/63/EU for animal experiments. The animals were not given food the night before the surgery.

Research involving human participants and/or animals. The present study was conducted in accordance to the Declaration of Helsinki concerning animal and Human Rights and approved by the Athens University Medical School Ethics Committee and by the Veterinary Directorate of the Ministry of Agriculture in agreement with the European Union directive 86/609.

Diabetes induction. Type I diabetes was induced by intraperitoneal injection of Streptozotocin, 60mg/kg of body weight, one week following the admission of animals. Streptozotocin or Streptozocin is a naturally occurring alkylating antineoplastic agent, which is especially toxic to the insulin-producing pancreatic β -cells in mammals. Verification of the increased blood glucose levels was performed during the 3rd and 7th day post injection by collecting blood samples from the vascular plexus of the outer canthus. Animals having blood glucose levels above 200 mg/dl were considered diabetic. Following computer generated randomization, animals in each group were sub-grouped to receive TISSEEL® or not. Four groups were, therefore, formed as depicted in Figure 1.

Experimental protocol and surgical procedures. Anesthesia was performed using Xylazine (7 mg/kg) and Ketamine (70 mg/kg). Xylazine is an analogue of clonidine and an agonist at the α -2 adrenergic receptors. Ketamine is a drug mainly used for inducing and maintaining anesthesia. It induces a trance-like state while providing analgesia, sedation, and memory loss. The anterior abdominal surface was prepared and draped, using povidone iodine solution, alcohol and single-use sterile drapes. A 4 cm midline

Table I. Baseline and Timepoint 1 weight and glucose values of enrolled animals.

Studies parameter	Group 1	Group 2	Group 3	Group 4	p-Value
Baseline weight (g)	211±17 212 (191-233)	216±16 222 (183-228)	207±16 206 (186-237)	208±18 202 (180-232)	0.631
Baseline glucose (mg/dl)	104±17 100 (85-129)	98±15 103 (82-134)	103±17 98 (84-122)	108±19 106 (78-118)	0.836
Timepoint 1 weight (g)	240±19 242 (228-264)	248±16 239 (219-273)	239±23 244 (232-252)	245±12 248 (222-256)	0.239
Timepoint 1 glucose (mg/dl)	321±59 343 (213-384)	309±66 293 (227-414)	96±12 96 (80-112)	121±74 97 (84-133)	<0.001

incision was performed, starting 1 cm above the external genitalia. After entering the peritoneal cavity, the left colon was recognized and a 1 cm segment, 3 cm above the peritoneal reflection, was removed. An end-to-end hand sewn anastomosis, with interrupted 5-0 monofilament absorbable (Poly-L-lactide-co-caprolactone) sutures was performed. The groups DM+S+ TISSEEL® and S+ TISSEEL® received 1 ml/cm² dose of TISSEEL® on the anastomosis site. Closure of the abdomen was performed using a mass closure 4-0 multifilament (90% glycolide, 10% L-lactide) Vicryl continuous suture and the skin was closed with 4-0 monofilament non-absorbable, nylon continuous suture. All animals, following the operation received analgesia with subcutaneous Tramadol, at a dose of 0.01 mg/kg. Post-operatively the animals had free access to food and water, as per ERAS, and were euthanized on postoperative day 10 to minimize the possibility of false positive results concerning the integrity of anastomoses. At that time, an 1cm segment of the colon, 0.5 cm above and 0.5 cm below the anastomosis was removed and histopathologically analyzed. We considered not to include the tensile strength nor the bursting pressure measures in our experiment, since we needed intact perianastomotic segments for healing evaluation and since the clinical result was already evaluated prior to biopsy.

Fibrin sealant. The fibrin sealant (TISSEEL®; Baxter Healthcare Corporation, Deerfield, IL, USA) is a biocompatible agent that contains a mixture of coagulation factors, the active ingredients of which include two sterile, deep-frozen solutions: the sealer protein solution and thrombin solution, each in a separate preloaded double-chamber syringe in a mixture of coagulation factors. The sealer protein solution consists of synthetic aprotinin, factor XIII, and fibrinogen while the thrombin solution contains human thrombin and calcium chloride as active ingredients, fractionated from pooled human plasma. After thawing and warming to 37°C, the two solutions were mixed and approximately 0.4 ml was applied to the colorectal anastomosis.

Pathology analysis. Tissue specimens were processed using standard procedures. Diagnosis was based on 4 µm thick formalin fixed, paraffin embedded hematoxylin and eosin stained sections and in Masson's trichrome staining. The latter staining helps determine the extent of collagenous connective tissue fibers. The Ehrlich-Hunt model was used to evaluate tissue healing by assessing inflammation, fibroblastic activity, neovascularization and collagen formation in a 5-point scale (0: no evidence, 1+: occasional evidence, 2+: light scattering, 3+: abundant evidence, 4: confluent cells or fibers) (19).

Statistical analysis. Intention-to-treat analysis was performed. Continuous variables are presented as mean and standard deviation (±SD) and as median (range) values. The normality of the distributions was assessed with Kolmogorov-Smirnov's test and graphical methods. Quantitative variables are presented with absolute and relative frequencies. Student's *t*-test or nonparametric Mann-Whitney test were used to compare means between groups. For the comparisons of proportions, we used the chi-square and Fisher's exact tests. Post-hoc analysis was performed in quantitative variables of the pathology analysis using the chi square and Fisher's exact tests. All reported *p*-values are two-tailed. Statistical significance was set at *p*<0.05. All analyses were conducted using the SPSS statistical software (SPSS Inc. Released 2009. PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc.).

Results

Overall, 40 rats were included in the present study. All animals underwent resection of a part of the colon and end-to-end anastomosis was performed. The two groups were sub-stratified to those that had TISSEEL® applied at the anastomotic site and those that did not. The basic characteristics of rats prior to the onset of the randomization (baseline) and prior to the onset of the procedure (Timepoint 1) are presented in Table I. No differences were observed at baseline in terms of animal weight and glucose values, whereas at Timepoint 1 significant differences were observed in glucose values.

The procedure was uncomplicated in all but two rats that ultimately developed significant hematochezia at post-operative day 2 (one in the normoglycemic group that did not receive TISSEEL® and another one in the diabetic group that did not receive TISSEEL®) (Figure 1). Both rats were re-operated at which point we observed that both had partial bowel necrosis. The first one had a complete recovery following the procedure; whereas the second one succumbed to the procedure.

The pathology analysis revealed improved tissue remodeling in the TISSEEL® group, both for the normoglycemic and the diabetic group as the Ehrlich-Hunt model was significantly better in the TISSEEL® group compared to the group that did

Table II. Results of the Ehrlich-Hunt model (pathology analysis). *p*-value column: The first *p*-value reflects level of significance of differences among the 4 studied groups. Post-hoc analysis is presented in the following 4 reported *p*-values that investigate differences among the mentioned groups.

	1:Diabetes – TISSEEL®	2:Diabetes – No TISSEEL®	3:Control – TISSEEL®	4:Control – No TISSEEL®	<i>p</i> -Value
Inflammation					
0	0	2	5	1	<i>p</i> <0.001
1+	1	2	4	2	Group 1 vs. 2 <i>p</i> =0.015
2+	0	4	1	4	Group 3 vs. 4 <i>p</i> =0.006
3+	1	2	0	3	Group 1 vs. 3 <i>p</i> <0.001
4+	8	0	0	0	Group 2 vs. 4 <i>p</i> =0.019
Fibroblast formation					
0	0	5	1	4	<i>p</i> =0.040
1+	2	3	1	3	Group 1 vs. 2 <i>p</i> =0.065
2+	4	2	1	3	Group 3 vs. 4 <i>p</i> =0.008
3+	2	0	3	0	Group 1 vs. 3 <i>p</i> =0.990
4+	2	0	3	0	Group 2 vs. 4 <i>p</i> >0.999
Neovascularization					
0	2	5	2	5	<i>p</i> =0.110
1+	0	4	2	3	Group 1 vs. 2 <i>p</i> =0.047
2+	3	1	1	2	Group 3 vs. 4 <i>p</i> =0.168
3+	2	0	2	0	Group 1 vs. 3 <i>p</i> =0.653
4+	3	0	3	0	Group 2 vs. 4 <i>p</i> >0.999
Collagen formation					
0	1	5	1	1	<i>p</i> =0.008
1+	1	5	1	7	Group 1 vs. 2 <i>p</i> =0.001
2+	3	0	3	2	Group 3 vs. 4 <i>p</i> =0.023
3+	4	0	3	0	Group 1 vs. 3 <i>p</i> >0.999
4+	1	0	2	0	Group 2 vs. 4 <i>p</i> =0.239

not receive TISSEEL®. Specifically, the extent of inflammation was decreased, whereas fibroblast and collagen formation were improved among animals receiving TISSEEL® (fibroblast formation was very close to statistical significance among diabetic rats with TISSEEL® compared to those without TISSEEL®) (Table II, Figure 2). Neovascularization was not different between control animals that received TISSEEL® and those that did not receive treatment; however, the effect was significant in diabetic rats, favoring the TISSEEL® group.

Discussion

The reduction of morbidity and mortality following colorectal resections due to benign and malignant modalities would be of great socioeconomic importance. Several factors seem to affect the integrity of colorectal anastomoses including smoking, excessive blood loss and need for transfusion, the use of immunosuppressive therapy as well as the site of anastomosis (20). Diabetes mellitus is one of the most widely studied risk factors for colorectal anastomoses, with a proven destructive effect on the tissue healing process (10). Our study demonstrated an improvement in tissue remodeling after colorectal anastomosis following application of TISSEEL® both for

diabetic and non-diabetic subjects. Specifically, inflammatory response and fibroblast accumulation were diminished for both groups after application of TISSEEL® on colorectal anastomoses. At the same time, an increase of neo-vascularization was observed after the application of TISSEEL® in diabetic rats, although this effect was not evident in the non-diabetic group.

Despite the proven effect of several risk factors related to colorectal anastomoses on specific molecular pathways, fibrin glue has been studied only in animal models without additional risk factors (21). On the other hand, several experimental studies have suggested that the implementation of biological sealants may be effective as an adjunct factor that could ensure the integrity of colorectal anastomoses (22). Vakalopoulos *et al.* have evaluated the efficacy of several biological fibrin sealants on sutureless repair of colonic defects. They have observed that Histoacryl Flex® exhibited significantly higher collagen formation at day 10 compared to other sealants (23). In addition, a previous systematic review produced by the same study group, has revealed that application of fibrin sealants on colonic defects is promising (24). However, the most recent systematic review relevant to this field failed to show a clear benefit from the use of fibrin glue on colorectal anastomoses (25).

Figure 2. Description of the procedure. A: Bowel exteriorization and site selection for excision; B: application of TISSEEL® in site of end to end bowel anastomosis; C: extensive inflammation and thickening of the muscularis layer (H&E×4) in a diabetic animal that did not receive TISSEEL®; D: neovascularization (H&E×20) in a diabetic animal that received TISSEEL®; E: extensive inflammation (H&E×4) in a non-diabetic animal that did not receive TISSEEL®; F: TISSEEL® fragments in serosal macrophages (H&E×20) in a non-diabetic animal that received TISSEEL®.

Moreover, van der Vijver *et al.* have argued in favor of the beneficial effect of fibrin sealants on patent anastomoses in low risk conditions (26). Nevertheless, to date, none of the aforementioned fibrin sealants has been studied in severely diabetic colorectal patients who require anastomosis.

To the best of our knowledge, this is the first study, which evaluates the effect of a fibrin sealant on the proven destructive role of diabetes mellitus during the healing

process of a colorectal anastomosis. Another major strength of the current study is the randomization of the animals during the second stage of the experiment prior to the application of TISSEEL®. Furthermore, all experiments were performed by the same operator and the pathologist that conducted the histopathologic analysis was blinded to the application or not of the agent. A potential limitation of the present study is the relatively small sample size of animals

used. However, its clinical importance is not diminished as it may serve as a pilot for future experimental and clinical studies in the field. Furthermore, bursting pressures were not applied; however, the clinical efficacy of the experiment may be proven by our decision to implement ERAS guidelines and by the number of postoperative days that exceed the time of anastomosis failure.

Experimental and clinical studies demonstrate that the application of fibrin sealants may have an effect on healing of colorectal anastomoses. In addition, the improvement of the healing process seems to be crucial for anastomotic stability in diabetic patients that undergo colorectal anastomoses. The findings of our study could be the basis for the design of further experimental studies in order to examine the effect of other agents on diabetic colorectal anastomoses, as well as on the safety of application of such agents. The next large step would be the application of fibrin sealants on colorectal anastomoses of diabetic human patients in order to identify their possible effect on diminishing morbidity and mortality following colonic resections.

In summary, application of TISSEEL® on the colorectal anastomoses improves healing in rats that suffer from severe hyperglycemia. This effect is mainly attributed to improved fibroblastic activity and collagen formation as well as a decrease in inflammation. Vascularization may also be improved, although the sample size of our study did not suffice to reach statistical significance. Therefore, our study may serve as a pilot for future research in larger animals, as well as in clinical trials to reach firm conclusions regarding the beneficial effect of TISSEEL® in diabetic patients that undergo colectomy.

Conflicts of Interest

The Authors report no conflict of interest regarding this study.

Authors' Contributions

Konstantinos Stergios and Vasilios Pergialiotis: conceived the idea, designed the protocol, performed the pilot study and revised the manuscript, Konstantinos Stergios, Maximos Frountzas, Laskarina Maria Korou, and Ioannis Thivaios performed the procedures and were responsible for animal care, Nikolaos Nikiteas, Despina N. Perrea and George Vaos wrote the manuscript. All Authors critically revised the manuscript and approved it prior to submission.

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