A Research Model of Measuring the Tensile Strength of Colonic Anastomosis in Wistar Rats

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Summary

The present experimental study investigates the mechanical behavior of intestinal anastomoses in Wistar rats. More specifically the response of the anastomoses to a properly applied uniaxial direct tensile load is studied and the respective tensile strength is determined. The surgical procedure of large bowel anastomoses is described in detail. In addition the authors provide a thorough description of the experimental apparatus, designed especially for measuring the tensile strength of the specimens under study, with special consideration in gripping of the intestine, the load application and the data acquisition and storage systems. This experimental model provides an excellent method for measuring the anastomotic strength and therefore a flexible tool for the comparative evaluation of various anastomotic techniques.

Introduction

Intestinal wound healing is an essential process for surgical reconstruction of the digestive tract. Compromised healing is considered a life-threatening complication, leading to prolonged hospitalization, decreased quality of life and increased medical costs (*Fielding et al., 1980).*

Investigating wound healing and attempting to improve its outcome necessitates process quantification (*Zografos et al., 1992*). Parameters for anastomotic repair and adhesion formation (*Zografos et al., 2002)* may be mechanical, biochemical, or histological (*Hendriks et al., 1990).* Histology is one of the tools for quantification,

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when comparing various series of experimental anastomoses. Certainly, it is very useful to describe the course and eventual result of the healing sequence at the tissue level. Also, the successive infiltration of various cells into the wound area may be followed, and obvious differences between anastomoses (e.g., ileal and colonic) will certainly be demonstrated this way. However, the measurement of choice to evaluate anastomotic repair and the effects of variations in surgical techniques, administration of drugs, or of any other modification to establish procedures, will mostly be either mechanical or biochemical or both.

The developing mechanical strength is, without doubt, a meaningful parameter to follow while investigating anastomotic healing. For this purpose, two fundamentally different approaches can be chosen. Bursting strength, expressed either as bursting pressure or bursting wall tension, which is the measure of resistance of the intestinal wall

to increasing intraluminal pressure or breaking strength, reflects resistance of the intestinal wall to forces exerted in a longitudinal direction (*Hendriks et al., 1990; Ikeuchi et al., 1999)*.

The basic purpose of the present experimental protocol is the study of the mechanical behavior of intestinal anastomoses and more specifically the study of their response to a direct uniaxial tensile load and the determination of the respective tensile strength, with the authors describing in detail the process itself and analyzing the parameters taken into consideration.

Materials and Methods
Animals
Twenty five male sibling Wistar rats, aged 8 weeks, all coming from the National Research Center of Natural Sciences "Dimokritos" (Athens, Greece), with an average weight of 200-250 g, were studied. All animals were supplied by van in filter boxes and quarantined for 2 weeks in the Central Animal Laboratory of the Department of Experimental Surgery and Surgical Research of the University of Athens.

The experimental protocol was approved by the General Directorate of Veterinary Services of the Prefecture of Athens, Attica, Greece (permit no. K/355/27-1-2005), according to Greek legislation regarding ethical and experimental procedures (Presidential Decree 160/1991, in compliance with EEC Directive 86/609, and Law 2015/1992, and in conformance with the European Convention "for the protection of vertebrate animals used for experimental or other scientific purposes, 123/1986").

Husbandry during experiment
All animals were housed, in an open system, two per cage. The polycarbonate cages used had the following dimensions: 480 x 265 x 210 mm, floor area: 940 cm^2 (2154F, Tecniplast, Italy). Wooden, dust-free, litter was used for bedding, with no pretreatment (Scobis-Uno, Italy). The conditions in the animal house were 15 air changes/hour, with regulated environmental temperature at 22±2 ºC, regulated relative humidity 55±10% and artificial

light/dark at 06.00/18.00, using fluorescent lighting c.300 lux. All animals were acclimatized to the laboratory conditions for a period of one week prior to the experiment.

Feeding
The animals were fed *ad libitum* a commercial pelleted food (510K, Greek Animal Food Industry, Greece), with no pretreatment, the nutrient contents of which are described in Table 1, and had free access to mains water.

Table 1. Nutrient contents of the commercial food used in the laboratory.

Chemical	analysis (%)	Additions	per kg
Protein	21	Vit. A	25.000 IU
Fat	6.2	Vit. D_3	5.000 IU
Fiber	4.5	Vit. E	100 mg
Ash	7.5	Vit. K_3	25 mg
Calcium	1.1	Vit. B_1	25 mg
Phosphorus	0.9	Vit. B_2	4 mg
Sodium	0.35	Vit. B_6	3 mg
Methionine	1.1	Vit. B_{12}	0.02 mg
		Niacin	30 mg
		Pantothenic acid	16 mg
		Choline	1000 mg
		Manganese	125 mg
		Copper	7.5 mg
		Zinc	50 mg
		Magnesium	500 mg
		Iron	40 mg
		Cobalt	8 mg
		Iodine	1.7 mg
		Salinomycin	200 mg

Experimental procedure
The experiments were carried out from September 4 to September 29, 2006 from 09.00 to 13.00, with no time interval between sampling and processing. The animals were randomized with the use of a sealed envelope in two groups. Group A (n=10) consisted of rats that were euthanized, after having undergone laparotomy, but without any colonic anastomosis. Group B (n=15) consisted of rats that were subjected to euthanasia, after having undergone colonic anastomotic surgery.

In group A, the animals, which had no preoperative preparation, were transported one by one, while in their home cage, to an adjacent room with the same ambient temperature as the animal room. Anesthesia was induced by an intraperitoneal injection of 2.5 mg/kg acepromazine maleate (Fermenta Veterinary Products, Kansas City, Mo.), 75 mg/kg ketamine (Phoenix Pharmacy Inc., St. Joseph, Mo.), and 0.02 mg/kg atropine (Phoenix Pharmacy Inc.); anesthesia was maintained with inhaled methoxyflurane (Schering-Plough, Union, New Jersey). Animals were monitored for movement and response to painful stimuli to ensure appropriate anesthesia was maintained during surgery, as previously described (*Gillingham et al., 2001*).

After thorough shaving of the abdominal area up to the middle of the anterior surface of the thorax, the area was sterilized with the use of Povidone iodine solution 10% (Lavipharm, Athens, Greece). Under aseptic conditions, a 3-cm midline ventral abdominal incision was made, allowing entry into the peritoneal cavity. The abdomen and its organs were inspected and the ileocecal valve identified.[10] The abdominal muscle wall was then closed with 5-0 Vicryl (Ethicon, Johnson & Johnson, Athens, Greece) sutures, followed by skin closure with 4-0 Silk (Medipac, Kilkis, Greece) continuous sutures. Both suture sites were sterilized with the use of Povodine iodine solution 10%. A spray film dressing (Smith & Nephew, Athens, Greece) was sprayed on

the skin closure, and the rats were put back in their cages, where they were allowed food and water *ad libitum*. On the 5th post-operational day, the animals were euthanized with the use of CO_2 as previously described (*American Veterinary Medical Association (AVMA) Panel on Euthanasia, 2001; Sharp J et al., 2006)*. The abdomen was opened with a 3-cm midline ventral incision and the ileocecal valve identified (*Komarek, 2000)*. A colonic segment, 8 cm in length, 5 cm distal to the ileocecal junction, was removed and flushed with saline to remove feces, in order to be attached to the tensile strength apparatus.

In group B, the animals were handled as animals in group A, regarding transportation and anesthesia. The surgical procedure for laparotomy was the same as in group A. However, a colonic segment, 1 cm of length, 5 cm distal to the ileocecal junction was transected and re-anastomosed end-to-end using the same 5-0 Vicryl sutures in single-layer, interrupted fashion. More specifically, two presection sutures were put in place (*Halsted, 1987)*; one on the mesenteric and the other on the anti-mesenteric border. These sutures were then used as stay sutures. The bowel was anastomosed with 8 to 10 inverting sutures to secure an inverted anastomosis without mucosal protrusion (Figure 1), which is considered to be a major cause of perianastomotic adhesions. A magnifier lamp (Magnifico, Middlesex, England) was used while

Figure 1. The anastomotic region in one of the animals from group B.

Figure 2a. The pins used to support the specimens and transfer the tensile load.

performing the anastomoses. The intestine was put back in the abdominal cavity and the abdominal muscle wall was then closed as previously described. All surgical procedures were performed by the same surgeon (PG).

To obtain the test specimen, on the 5th post-operative day, the animals were euthanized with the technique already described. The previous abdominal incision was reopened, and the anastomotic site identified and inspected for possible adhesions and leakage. An 8 cm segment of the colon with the anastomosis in the middle was resected. Care was taken not to detach adhesions from the anastomosis, but to dissect the surrounding tissues. The resected specimen was gently irrigated with saline to remove feces and was then attached to the tensile strength apparatus.

Animals in both groups received 0.5 mg/kg buprenorphine (Reckitt & Colman Products, Hull, England) subcutaneously every 12 h for 32 h after surgery, according to the Formulary for Laboratory Animals (*Hawk et al., 1995; Roughan et al., 2002)*.

The loading system

The most difficult problem to be solved in experiments with biological materials is the proper support and alignment of the specimens on the loading frame. For this purpose a special system was designed in order to achieve the fastest and safest procedure for mounting the specimens in the grips of the loading device. The system consisted of a pair of light metallic pins of cylindrical cross-section of 5 mm diameter, with a rounded head, which permitted easy entrance of the intestine in the pin, without injuring the specimen walls, thus reducing the time required for the in-situ preparation of the specimens (*Ekmektzoglou et al., 2006)*. The pins were grooved at about their mid-length and a suture which held the specimens in place was rolled up in this groove. The upper part of the pins was drilled throughout their thickness and the specimen was suspended through this hole from the upper plate of the loading frame (Figure 2a). At the same time, the second pin was fixed to the immobile plate of

the frame. The suspension and the fixing of the pins from the upper and lower traverses of the loading frame were achieved with the aid of circular, free rotating rings. In this way, the maximum possible number of degrees of freedom was given to the specimen, making possible both the self-alignment and the "un-twisting" of the intestine during tension, without external limitations and therefore without the development of parasitic tensions and disfigurations (Figure 2b). In order to minimize the accidental preloading, a spring of known elastic constant was interpolated between the ring and the lower plate.

An extremely accurate load cell of 5 N capacity and 10^{-3} N sensitivity was attached in a stiff electrical loading frame (Instron, High Wycombe, UK). This frame was selected because it provides the ability of choosing the load application speed between wide ranges (from 0.5 mm/min to 500 mm/min). This characteristic of the frame is very important where biological specimens are to be studied, since their mechanical behavior exhibits viscoelastic nature, which is strongly dependent on the strain rate induced. In the present phase of the experimental project, a relatively low-loading velocity was selected, and therefore the experiments could be considered as quasi-static. The displacement-control function mode was preferred, since, for the specific specimens, the strain was difficult to be defined and measured, rendering the strain-control function mode inapplicable. On the other hand, the load varied between relatively broad limits and therefore the load-control function mode was not advisable.

The calibration of the loads was achieved with the simplest and safest method, that of the suspension of standardized (certified) weights from the load cell. Both the absolute reading values of the load cell, as well as their linearity at the range of the expected loads were checked. The deviations detected for the absolute values of the loads did not exceed in any case the limit of 0.2% set by the "Quality Assurance System" of the Laboratory of Testing and Materials of the National Technical University of Athens

(NTUA/LTM), as described in the respective "Quality Assurance Manual" according to the ISO-9000/2000 system. On the other hand, the linearity of the values of the loading cell in relation to the respective ones of the standard weights exceeded 99,8% for the whole range of interest, as it was concluded from a linear interpolation in the experimental data, using the least square method.

The calibration of the readings of the load frame for the displacements was achieved with the aid of three Linear Voltage Displacement Transducers (RDP Electronics Ltd, Wolverhampton, UK), which have been verified with a standard micrometric vernier with an accuracy of 1 μm (Mitutoyo, Japan). Apart from the absolute values of the displacements, the parallel motion of the moving traverse of the loading frame was also checked. The deviations detected did not exceed, in any case, the limits set by "Quality Assurance System" of the NTUA/LTM. Finally, the time recording device of the data acquisition and storage system was also calibrated with the aid of a prototype chronometer (Mitutoyo, Japan).

Data acquisition and storage system
The data recorded during the experiments included the values of the load as a function of the time [F=F(t)] and the values of the displacement of the moving plate of the loading frame, also as a function of time [Δs=Δs(t)]. The data acquisition system included a special multi-channel "bridge" (National Instruments, type SCXI-1000, Athens, Greece), with the ability of adjusting the sampling rate. The system includes, also, a personal computer with suitable commercial software (LabVIEW-8, National Instruments, Athens, Greece). From the F=F(t) and Δs=Δs(t) functions recorded, time was eliminated, and the applied force was obtained, as a function of the displacement induced and therefore of the overall elongation of the intestine, i.e. F=F(Δs).

A video device was added to the data acquisition system, in order to monitor the specimen during the experiments, in a mode synchronous to the recording of the values of the load and the displacement. This was considered necessary, since the records of the load versus time during the preliminary tests presented rather strong and abrupt load-drops, due to two different reasons:

· The "un-twisting" of the twisted parts or the "un-folding" of the folded parts of the intestine (due to the inevitable formation of adhesions in and around the anastomotic area), which led to a sudden length increase of the specimen, and therefore to an instantaneous unloading, that is a drop in the load recorded, as shown characteristically in the diagram of Figure 3.

· Local failures of parts of the specimen and especially in the case of anastomosed intestines,
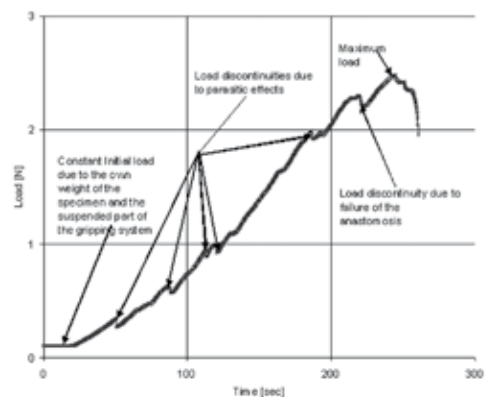

Figure 2b. An overview of the loading system.

Figure 3. A typical load-versus-time plot.

failure of the anastomotic area itself (or of directly neighboring areas due to the tearing of the material by the anastomotic suture).

Since these two discontinuities of the F=F(t) diagram cannot be easily distinguished, the synchronous video-recording of the experiment was considered necessary. In this way, it was possible to correlate the discontinuities of the plot to a specific time instant of the video recording and therefore address them to either a sudden "un-twisting" / "un-folding" of the specimen. It became possible therefore, to detect safely the crucial failure load, which did not always correspond to the maximum load recorded during the experiment.

Statistical analysis

Measurements are reported as means ± SD. Comparisons between anastomotic failure load-based measurements between rats with and without anastomosis were analyzed with the Mann-Whitney test. A p value of <0.05 was considered statistically significant.

Results

The failure load in g for group A was 205.4 ± 52.5 SD, while for group B was 114.7 ± 40.5 SD, p<0.05 (median value for group A: 214.95, median value for group B: 102.86). For group A, the load versus time plots for two characteristic experiments are shown in Figure 4. The average value for these tests was found equal to:

$$F_{cr}^{ref} = 2.03N \pm 0.52N$$

where F_{cr} is the reference critical failure force. For group B, the load versus time plots for two characteristic experiments are shown in Figure 5. The average value for the failure load of these tests was found equal to:

$$F_{cr}^{ref} = 1.13N \pm 0.40N$$

The above value is slightly lower with respect to the one mentioned by Ekmektzoglou et al., which was equal to 1.35N ± 0.42N. However, it should be mentioned that in the present study the number of tests is higher and therefore the results are more representative (*Ekmektzoglou et al., 2006*). The standard deviation (almost one third of the respective mean value) is again significant but it can be explained by taking into account the nature of the specimens and the inevitable variation of both the dimensions of the specimens as well as of the specific characteristics of the region around the anastomosis. In any case, the anastomotic operation yields failure loads decreased by about 44% in comparison to the intact specimens.
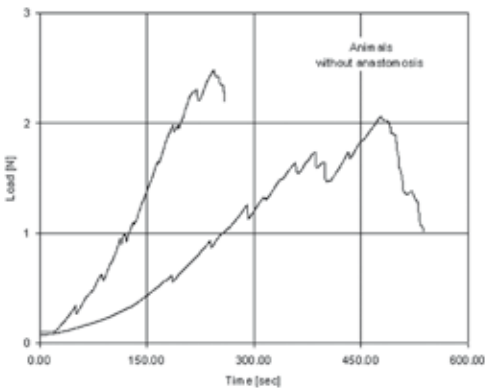

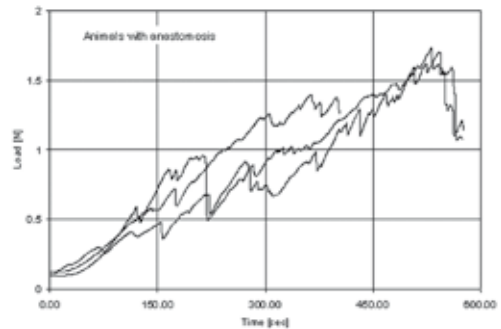**Figure 4.** Characteristic load-versus-time plots for specimens from animals without anastomosis.

Figure 5. Characteristic load-versus-time plots for specimens from animals with anastomosis.

Discussion

Anastomotic leakage is a most serious complication in gastrointestinal surgery, with concomitant high morbidity and mortality rates. Wound leakage, the major concern for every surgeon performing intestinal anastomosis, is considered a multifactorial process, upon which many factors (technical, local, systemic) accelerate or inhibit its metabolic pathway (*Ekmektzoglou et al., 2006; Norris et al., 1990*). Needless to say, that the combined presence of negative determinants may increase the risk of leakage to such an extent that the surgeon may forgo primary anastomosis.

Since anastomotic dehiscence is still considered a life-threatening situation for every patient, the experimental basis for the study of the healing of intestinal anastomoses has rested on techniques that assess its integrity and strength. Taking the 5th post-operative day, as a crucial time point upon which anastomotic failure is mostly recognized in clinical practice (*Ekmektzoglou et al., 2006)*, the authors tried to give a measure of the anastomotic strength by taking advantage of its mechanical behavior.

The values of the load as a function of the time [P=P(t)] and the values of the displacement of the moving plate of the loading frame also as a function of time [Δs=Δs(t)] were recorded, giving the load versus the displacement curve for each measurement and therefore providing the recorded discontinuities due to the anastomotic failure.

The load-versus-time plots are characterized by three distinct regions, for almost all tests carried out. The dominant one is a linear or almost linear portion interrupted by a number of abrupt load drops due to untwisting or unfolding of the specimens. This linear portion could provide an indicative modulus of elasticity, assuming that the displacement rate is properly recorded. Prior to this linear portion, an upwards curved region appears (not consistently) corresponding to a preliminary loading phase, namely the one before the complete alignment of the specimens. Finally, after the peak load, the curves start decreasing rapidly up to the complete fracture of the specimens.In general, when carrying out tests with biological tissues, it is very difficult to obtain specimens in strict conformity with any standards. The nature of these specimens dictates both their dimension and shape. This variation influences the results, since the strength of any material is in general a decreasing function of its dimensions (*Koruda et al., 1990; Kourkoulis et al., 2005*). The phenomenon, known as "size effect" in the engineering community, has been thoroughly studied many years ago for structural materials, but almost nothing is known for the case of soft biological tissues.

Meanwhile, in many cases the fracture of the anastomosis does not correspond to the maximum load recorded. Obviously, in these cases the additional load is undertaken either by the intact neighboring tissues or by the fibers used for the anastomotic operation and therefore such values should be rejected.

It should also be mentioned, that the scattering of the results appears to be rather significant. However, definite conclusions cannot be drawn, since the quantity measured is the critical load rather than the critical stress. In this context and taking into account the fact that the dimensions of the specimens influencing the critical load (namely the perimeter of the intestine and the thickness of its wall) show an inevitable scattering (due to the biological nature of the specimens), it is concluded that one should measure the active area of each specimen in order to obtain the real critical stress. Unfortunately, such a procedure is extremely difficult for biological tissues like the ones tested here.

Within the current limitations of this study, it can be concluded that the experimental apparatus and procedure described can serve as a powerful and reliable tool in measuring colonic anastomotic strength in Wistar rats.

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