Edema quantification by computerized morphometry as an evaluation parameter for the resistance of colon anastomoses[1]

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ABSTRACT – Purpose: This work had the objective of verifying the validity of using computerized morphometry as a method of quantitative analysis of the interference of edema in scar formation following colon anastomosis. **Methods:** Forty-five adult female Wistar rats were utilized, divided into three groups of 15 animals according to whether sacrifice was performed on the first, second or seventh postoperative day. Each group was subdivided into a main group consisting of 10 animals, and a control group consisting of five animals. In the main group, in addition to the quantitative computerized morphometric analysis of the edema in the submucosal layer, the resistance of the colon anastomosis to bursting strength was verified. In the control group, edema quantification was studied alone. **Results:** The results found via the computerized morphometry method showed that there is a 7% decrease in the presence of edema during the first postoperative week. They confirmed that there is an inverse statistically significant relationship (p<0.001) between edema presence and the resistance of the anastomosis to bursting strength. **Conclusion:** The use of computerized morphometry is a reliable, fast, objective and low-cost methodology for the quantification of edema in colon anastomoses.

KEY WORDS – Wound healing. Edema. Collagen. Anastomosis, surgical. Colon.

Introduction

There is a need to study the factors involved in scar formation following colon anastomoses, in order to define mechanisms and pathways that would improve the results from such interventions. This is particularly so in situations of interference with the normal scar-formation process. Such situations are customarily found among nephropathic and diabetic patients and those undergoing hormonal or non-hormonal anti-inflammatory therapy.

Qualitative analyses have already demonstrated that edema is one of the components responsible for reducing the resistance of anastomoses[1-4]. In this way, the employment of the basic principles of good surgical techniques (regular margins, adequate hemostasis, colligation without tension) will avoid a severe

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inflammatory process and, ultimately, edema. In consequence, this may avoid increasing the incidence of dehiscence in colon anastomoses.

The possibility of quantitatively evaluating edema, by means of a fast, objective, low-cost and easily accessible method would contribute towards better understanding of the scar-formation characteristics of colon anastomoses.

The objective of the present study was to evaluate edema quantitatively using a computerized method and also its interference in the resistance of colon anastomoses.

Methods

This study was undertaken in accordance with Federal Law no. 6638 and followed the guidelines of the Brazilian College for Animal Experimentation (COBEA).

Forty-five female Wistar rats (_Rattus norvegicus Barkenhout_) were utilized. They had an average age of 90 days, weighed between 250 and 350 g, and came from the vivarium of the University of Mogi das Cruzes.

The animals were housed in individual cages with a semicontrolled macroenvironment in relation to light, noise, humidity and ambient temperature. The rats received potable water and standard feed for the species _ad libitum_ until the eve of the operation, when they began a 12-hour fast.

On the day of the operation, they were submitted to inhalatory anesthesia using 98% ethyl ether via an open mask. After they no longer reacted to painful stimuli they were restrained on an appropriate surgical table, in horizontal dorsal decubitus. Depilation of the anterior abdominal region was performed, from the costal margin to the radix of the thighs.

The 45 animals were randomly divided into three groups of 15 animals, to be sacrificed on the first, second or seventh day after the surgical procedure. Each group was divided into two subgroups, with ten animals in the main group and five in the control group, for each of the sacrifice days.

The animals were submitted to asepsis and antisepsis using polyvinyl pyrrolidone-iodine (Povidone) and caudal longitudinal median celiotomy of 2 cm in length. After opening up the peritoneal cavity layer by layer, the rectosigmoidal portion of the colon was identified, which was invariably located behind the uterus. Peyer's patch[5] was located and from this, a distance of 3 cm was measured in the cranial direction.

At this point, after kneading the colon contents, transverse sectioning was performed with the preservation of the vascularization.

End-to-end anastomosis was performed on a single extramucosal layer using 6-0 monofilament thread and a 1.5-cm gastrointestinal needle. Separate stitches were used, always applied at the four cardinal points. Between these, always starting with the mesenteric margin, the segments were brought together with the precaution of inverting the mucosa of the anastomotic openings. The threads were always tied off using three knots. After inspecting the cavity, the abdominal wall was closed along two layers using separate 2-0 cotton stitches, involving the aponeurosis and skin, for all the animals in all the experimental groups.

Once the surgical procedure had been terminated, the animals were suitably identified using a standardized tag and were returned to the individual cages. During the postoperative period, water ingestion was permitted 12 hours and feed 24 hours after the surgery, _ad libitum_.

A second surgical procedure was performed in the same way as the first, on the dates scheduled for each group. After opening up the abdominal wall at the same place as for the previous incision, an inspection was made of the peritoneal cavity. The suture line was located and a colon segment containing the anastomosis at its midpoint was removed. The animals, still anesthetized, were submitted to the inhalation of a toxic dose of ethyl ether with a lethal outcome.

Measurement of the bursting strength

The measurement of the bursting strength was performed using the same methodology for all the animals of the main subgroups. A metal pachymeter was utilized to measure a distance of 1.5 cm in the cranial and caudal directions from the colon anastomosis. This segment was dried for measuring the bursting strength. The distal extremity of this loop was occluded by means of a ligature using 2-0 cotton threads. A polyethylene tube was introduced through the proximal extremity, coming from a bursting strength measuring device that was developed specifically for this purpose[6]. This tube was fixed using 2-0 cotton thread that was tied off carefully so as not to alter the diameter of the lumen. Thus prepared, the intestinal loop was submerged in water contained in a Becker tube. The anastomosis was always kept at the same depth in the water, in relation to the surface, thereby eliminating interference from the external hydrostatic pressure exerted on the anastomosis[6] (Figure 1).
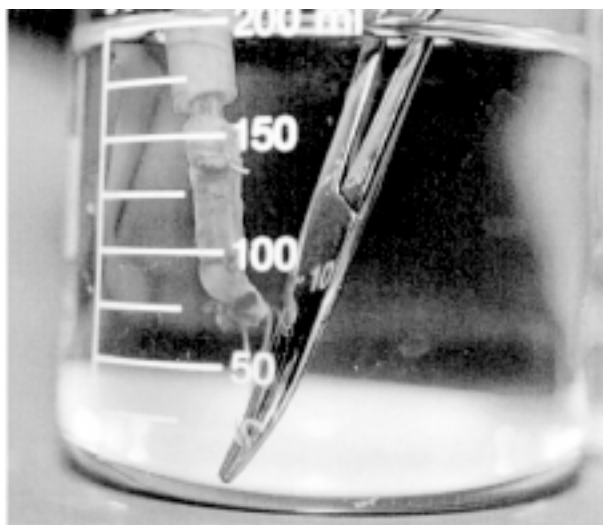
FIGURE 1 – Photograph of the anatomical specimen containing the anastomosis, submerged in water to facilitate the demonstration of the exact moment when the colon loop ruptured.

The intraluminal pressure was increased by means of the insufflation of air, until the loop ruptured. This moment was observed when the water in the Becker tube started to bubble, and thus the bursting strength reached at the exact moment of the rupture could be determined (Figure 2).
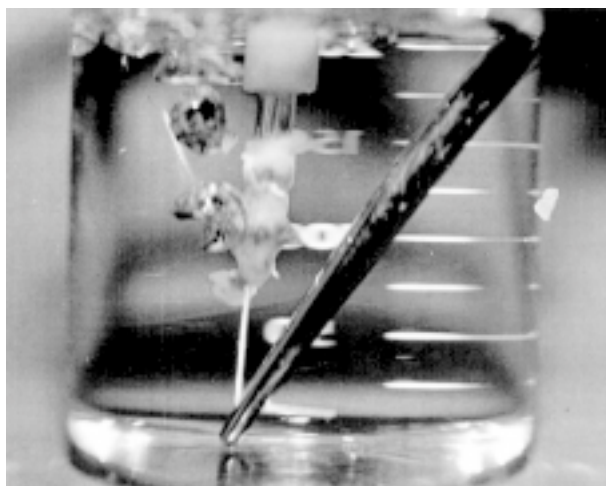

FIGURE 2 – Photograph of the anatomical specimen showing the moment when the colon loop ruptured.

Preparation of thin sections

The portion of the colon loop between the mesenteric and anti-mesenteric margins, chosen in an alternating manner, was stretched over a cork and fixed in 10% formol for four days, changing the solution every 24 hours. After concluding this stage, the specimens were washed in uninterrupted running water for 24 hours and then set in paraffin blocks. After this solidification, six sections were cut by means of a microtome calibrated to make sections of 6 microns in thickness. The first three cut sections were discarded, such that each thin section was made up of three tissue samples from the median portion of the section made between the mesenteric and anti-mesenteric margins.

The thin sections were stained using hematoxylin and eosin, and were always observed in a predetermined region, the submucosa of the area around the anastomosis, using a pre-established magnification (400x).

Quantification of the edema via computerized morphometry

The histopathological analysis was processed by utilizing computer-assisted image morphometry, with the objective of discriminating quantitative differences in inflammatory edema between the thin sections[7].

Image capture system

The image capture system consisted of a video camera (CCD/RGB) coupled to a microscope (Litz Fotolux) and connected to an auxiliary monitor (Sony Trinitron) and a personal computer with a 486 processor (Figure 3).

FIGURE 3 – Photograph of the equipment needed for accomplishing the computerized morphometry.

The image of the thin section observed on the monitor screen was focused and then captured by an acquisition board (Iris 16), by means of commands pre-established by the program that were put into action via the computer keyboard.

Image processing

The program displayed two side-by-side images on the screen: one formed by color levels and the other by colored dots and distinct tones. This allowed structures to be visualized that could not otherwise have been differentiated.

Element subtraction

Upon selecting a certain structure, in this case edema, the program allowed it to be seen alone in color, on a gray background, highlighting the structures of the image by means of the specific staining, in this case Hematoxylin-Eosin.

Final analysis of the image

The program associated a table of colors to the numerical values of the dots that made up the image.

Only the structure made up by the number selected was shown in color. Next, by means of startup commands, the video camera was calibrated and the image was manipulated, acquired, filtered and subtracted, guided by the program itself, such that the edema was picked out from the other structures. The final analysis of the image was then processed, with the quantification of the edema in the tissue in absolute or percentage numerical values (Figure 4).
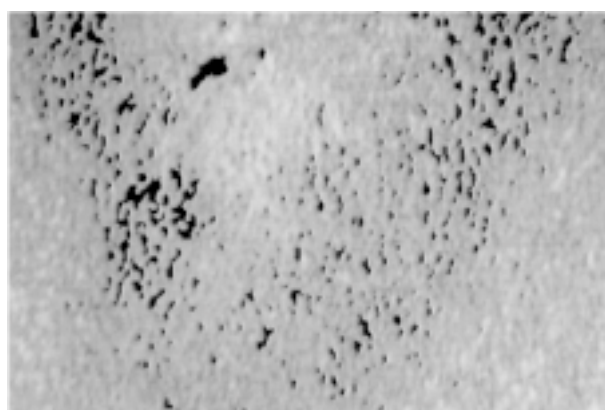

FIGURE 4 – Photograph of the thin section at the end of the quantification process, showing the edema stained in black.

Statistical analysis

For the statistical analysis of the results obtained, a significance level of 5% (p£0.05) was adopted. The following models were utilized in this analysis: arithmetic average, standard deviation, equality test on the averages (Student's t test), variance analysis, Student-Newman-Keuls test, and Pearson's parametric correlation. Whenever the results allowed a statistically significant difference to be identified (level of 5%), the result was accompanied by an asterisk (*), representing the rejection of the H_0 hypothesis.

Results

The classical relationship defined for the resistance of the anastomosis is presented as a graph of average bursting strength against the number of postoperative days (Figure 5).
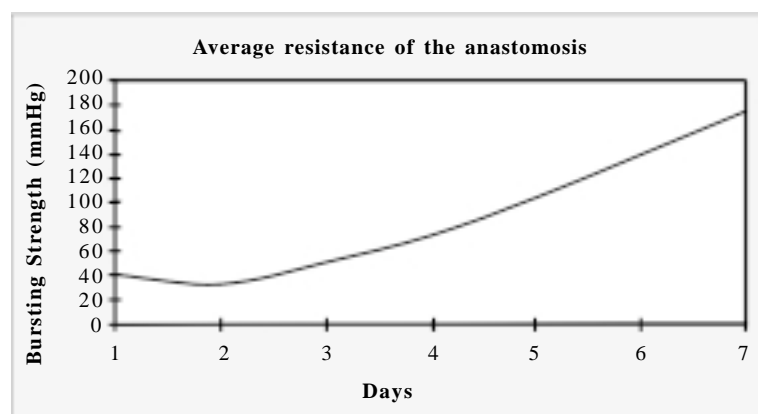

FIGURE 5 – Graph of average resistance of the anastomoses during the postoperative period.

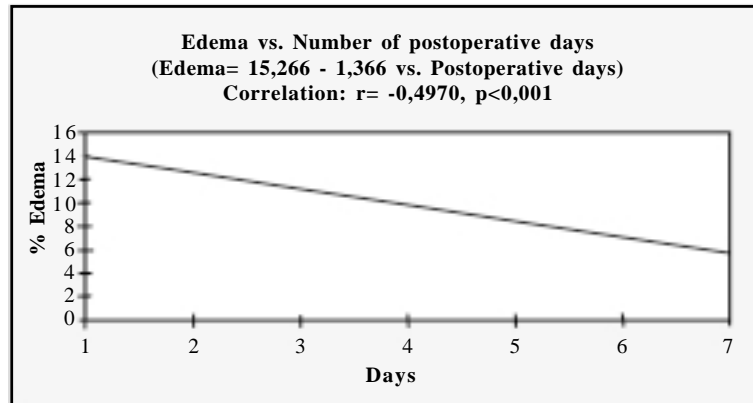The correlations between edema and postoperative days are presented in Figure 6.

FIGURE 6 – Graph of the relationship between edema and postoperative progression.

The various data correlations on the postoperative days are shown on Table 1 (as a single group), on Table 2 (main group) and on Table 3 (control group).

TABLE 1 – Edema percentage (computer-assisted image analysis), obtained using the control groups (without bursting strength) and main groups (with bursting strength).

Groups	n	Average	Standard Deviation	Standard Error
1 day	15	14.20	8.43	2.18
2 days	15	12.17	6.01	1.55
7 days	13	12.76	2.89	0.80

The variance analysis revealed: F = 0.41 and p = 0.669

TABLE 2 – Edema determination (computer-assisted image analysis), in which the bursting strength was applied (main groups).

Main Groups	n	Average	Standard Deviation	Standard Error
1 day	10	14.91	5.33	1.63
2 days	10	9.90	5.17	1.69
7 days	10	5.44	2.59	0.82

F = 10.89; p < 0.0001*

TABLE 3 – Edema determination for the control groups (without bursting strength).

Control Groups	n	Average	Standard Deviation	Standard Error
1 day	5	22.81	7.06	3.16
2 days	5	6.69	2.49	1.11
7 days	4	6.58	3.47	1.74

F = 17.76 and p = 0.0001*

In the edema comparison applied to the groups, the Student-Newman-Keuls test between the groups (1 day vs. 2 days; 1 day vs. 7 days; 2 days vs. 7 days) was not significant for p < 0.05.

Tables 4 and 5 demonstrate the data validity for the various groups without and with the use of bursting strength respectively.

TABLE 4 – Student-Newman-Keuls test for edema.

Main Groups	p < 0.05
1 day vs. 2 days	Yes*
1 day vs. 7 days	Yes*
2 days vs. 7 days	Yes*

TABLE 5 – Student-Newman-Keuls test for edema (control groups).

Control Groups	p < 0.05
1 day vs. 2 days	Yes*
1 day vs. 7 days	Yes*
2 days vs. 7 days	No

Table 6 demonstrates the data validity amongst different groups within the same day.

TABLE 6 – Student's t test for edema between the main and control groups.

Groups	Averages			Standard Deviation		GL	"t"	p
	C/PE	S/PE						
			C/PE	S/PE				
1 day	9.89	22.81	5.17	7.06	13	3.63	< 0.01*	
2 days	14.91	6.69	5.33	2.49	13	4.07	< 0.01*	
7 days	5.44	6.91	2.59	4.11	12	0.6	———	

Table 7 demonstrates that all considered variables are correlated.

TABLE 7 – Correlation between all the variables[#] (Correlation Test).

Variable	Edema	Number of postoperative days	Bursting strength
Edema	1.0	-0.497 (p < 0.001*)	-0.4791 (p < 0.001*)

n = 43

Discussion

The progression of scar formation in intestinal anastomoses has been studied via differing methodologies that have evaluated histological, mechanical or biochemical parameters. A methodology is still being sought today that will evaluate scar formation in a reliable, fast and objective manner that can easily be applied technically and has low cost.

It is accepted that, from a biochemical point of view, estimates of the collagen in the anastomosis constitute the best parameter for monitoring the progression of scar formation[8]. The biochemical evaluation of tissue collagen is obtained from the concentration of tissue hydroxyproline. However, it needs to be emphasized that different types of collagen present varying hydroxyproline content[9,10]. Thus, alterations in hydroxyproline concentration do not necessarily represent absolute alterations in the collagen content present in the colon wall[9]. Decreases in hydroxyproline concentration may be a consequence of a reduction in tissue collagen or the presence of non-collagen substances[11], or furthermore, a combination of these two factors.

The microscopic characteristics of scar formation in gastrointestinal wounds have patterns that are similar to scar formation models seen in all other tissues[3].

In recent anastomoses, degradation of the pre-existing collagen takes place, along with the formation of new collagen. Thus, the resistance of the anastomosis appears to also depend on the complex mechanism involving the degradation and synthesis of collagen.

In 1882, Halsted[12] was already drawing attention to the idea that the collagen content in the submucosal layer was the main factor responsible for the resistance of anastomoses. Nonetheless, the quantification of hydroxyproline is measured over the whole thickness of the intestinal wall, which makes the correlation between anastomotic resistance and the local concentration of collagen problematical.

It can be deduced from the facts presented that collagen constitutes a multifaceted component. It is thus impossible to isolate it as a single variable in evaluating a phenomenon as complex as scar formation following colon anastomosis.

Tissue collagen is not presented as an inert protein, but as a protein in a dynamic state constantly seeking equilibrium. There is no correlation between changes in the anastomotic resistance and alterations in the tissue collagen content alone. Not only the collagen concentration, but also its quality and subtypes[9,10] around the suture line are important characteristics. It needs to be considered that the strength of the anastomosis also depends on the union of the maturation process[13] with the metabolism of non-collagen substances such as iron, the contribution from tissue oxygen[14,15], and the availability of vitamin E[16], among other factors.

The possibility of performing immunohistochemical assays for measuring tissue collagen by dividing it into subtypes and thus determining the proportional responsibility of each of them for adequate scar formation has been known since the beginning of the 1990s[10]. Nevertheless, even after the passage of one decade, the method is still costly and reserved for centers doing high-complexity research. In this way, it does not appear to us that the immunohistochemical study of collagen concentrations in colon anastomoses for the evaluation of scar formation is a practical method within everyone's reach.

The measurement of the bursting strength may be a good parameter because it has been demonstrated to give the best representation of resistance physiology[1,3,17,18], as well as having low cost, ease of access and objectivity. It needs to be remembered that this method is not valid after the seventh postoperative day, since after this time the majority of ruptures occur away from the suture line[19,20,21].

The quantification of the edema in the anastomosis by means of morphometric methods has emerged as

an alternative. The edema directly reflects the degree of inflammation and presents an inverse relationship with the resistance of the anastomosis during the postoperative period, as has been demonstrated in the present work.

The methodologies employed up until now have only made it possible to have subjective verification, with non-parametric evaluations of this component that is so important in the scar-forming process. The adoption of quantification of the edema as a parameter for the progression of the formation of scar tissue has not, until now, allowed deeper studies, especially among patients with illnesses that interfere in an evident manner in the normal scar-forming process.

With the development of computerized morphometric methods, the possibility has come into existence for tissue edema to be quantified, thereby allowing a real determination of the influence of this variable on the physiological process of scar formation.

Edema is one of the factors responsible for the diminution of the resistance of anastomoses and consequent increase in the incidence of dehiscence, a complication that is greatly feared because of its high degree of mortality[22].

The local vascular response to the trauma causes edema in the intestinal wall in the area of the anastomosis, during the first week. On the first day, the submucosa is to be found congested and swollen, although as the days pass by the quantity of edema regresses until by the seventh day it is practically non-existent[10]. These observations were confirmed in the present study from an analysis of the values obtained in Table 2 and Figure 6. It needs to be emphasized that these values were statistically significant, as can be seen in Table 3. These observations were in agreement with other studies that found more severe edema on the first days after the operation that practically disappeared around the seventh day[3]. These same authors observed that the integrity of the serous layer was restored, although the repair of the intestinal mucosa was still incomplete by that time[3,10].

The quantification of the edema in the present study was obtained by means of a computer-assisted image analysis method[7]. The possibility of quantitatively evaluating the color intensity allowed for numerical translation of the quantity of edema into percentages. The quantity of edema present in the submucosa layer was thus determined for all the anastomoses performed. In this way, it could be seen that there was a reduction in the edema in the submucosa layer from 12% to 5% during the first postoperative week. In other words, over

the first seven days following the construction of the colon anastomosis, there was a 7% reduction in the quantity of edema in the submucosa layer (Figure 6).

The utilization of control groups for each sacrifice day was based on a concern about the influence of the use of bursting strength on the disjunction of the tissue fibers, which could visually simulate an area of interstitial edema. To eliminate this presumed diversion, it was decided to divide the experiment into a main group, in which in addition to the computerized morphometric quantification of the edema the explosive pressure was studied, and a control group in which the explosive pressure evaluation was not performed.

This division of the groups presented statistical significance when analyzed for the main groups (Tables 2 and 3) and control groups (Tables 4 and 5) individually over the course of the postoperative period. However, the division was not significant when the data was analyzed as a single group (Table 1). The differences were mutually annulled, except on the seventh postoperative day, when the presence of edema became so small that it did not cause differences between the groups studied (Table 6). Thus, despite the computerized analysis method being influenced by prior application of bursting strength, it is reliable when applied in isolation.

The computerized morphometric analysis utilized in the present study was able to confirm the inverse relationship between the resistance of the anastomoses and the presence of edema over the course of the first postoperative week (Figures 5 and 6 and Table 7). The data obtained were in agreement with observations by various other researchers[1-3,10], and were complemented by the quantitative evaluation. It needs to be emphasized, furthermore, that this methodology utilized in this study also makes it possible to evaluate edema in adverse situations, such as in diseases that increase the risk of complications in intestinal anastomoses.

Conclusions

It was verified that the use of computerized morphometry as an evaluation method for the presence of edema in colon anastomoses has a valid role. It not only allows for fast, objective and low-cost qualitative analysis but also, especially, quantitative evaluation of such anastomoses.

It was furthermore observed that there is a 7% reduction in the quantity of edema during the first week after the surgical intervention. An inverse relationship is presented between the quantity of edema and the resistance of the colon anastomosis.

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RESUMO – Objetivo: Verificar a validade do emprego da morfometria computadorizada, como método de análise quantitativa da interferência do edema na cicatrização das anastomoses cólicas. **Métodos:** Foram utilizados 45 ratos, Wistar, fêmeas adultas, divididas em três grupos de 15 animais segundo a data do sacrifício ter sido realizada no primeiro, segundo e sétimo pós-operatório. Cada grupo foi subdividido em grupo principal, constituído de 10 animais, onde além da análise morfométrica computadorizada quantitativa do edema na camada submucosa foi verificada a resistência da anastomose cólica à pressão de explosão e um grupo controle, constituído de cinco animais, onde se estudou isoladamente a quantificação do edema. **Resultados:** Os resultados encontrados pelo método morfométrico computadorizado constataram que existe redução de 7%, em valores percentuais, da presença do edema durante a primeira semana pós-operatória e confirmaram que existe relação inversa, estatisticamente significante (p<0,001), entre a presença de edema e a resistência a pressão de explosão da anastomose. **Conclusão:** O emprego da morfometria computadorizada é metodologia fidedigna, rápida, objetiva e de baixo custo na quantificação de edema nas anastomoses cólicas.

DESCRITORES – Cicatrização de feridas. Edema. Colágeno. Anastomose cirúrgica. Colon.

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