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Anthony Senagore

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I. H. CHAUDRY

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A SERIAL EVALUATION OF ANASTOMOTIC HEALING WITH COMPARISON OF CZERNY-LEMBERT AND CIRCULAR-STAPLED TECHNIQUES

By

Anthony Senagore

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ABSTRACT

A SERIAL EVALUATION OF ANASTOMOTIC HEALING WITH COMPARISON OF CZERNY-LEMBERT AND CIRCULAR-STAPLED TECHNIQUES
By

Anthony Senagore

This project was performed in four sections. Section I was a serial evaluation of the healing process of Czerny-Lembert (2-layer handsewn, inverted) and circular stapled (double staple row, inverted) anastomoses, representing the first direct comparison. Both anastomoses healed via second intention with no significant differences in anastomotic blood flow, bursting pressure, hydroxyproline content, gross or inflammatory scoring, or the incidence of anastomotic healing complications.

Section II assessed anastomotic healing in non-diverted animals and those with proximal diverting colostomy which has been associated with increased anastomotic stenosis. No significant differences were identified for any of the healing parameters. There was no predilection for diverted anastomoses to stenose.

Section III allowed development of a reproducible model of anastomotic ischemia and prospective

assessment of laser doppler velocimetry (LDV) and tissue pH (pHi) measurements in predicting subsequent tissue viability. It was apparent that pHi was superior to LDV in identifying critical ischemia and predicting anastomotic outcome. There was no significant difference in anastomotic healing or complications for CL or CS techniques.

Section IV assessed the serial effects of preoperative radiation therapy on anastomotic healing. We confirmed that preoperative radiation therapy (4500cGy) induces an early and persistent ischemic insult in the colorectum and increased the incidence of healing complications. 60μmol/kg ATP Magnesium Chloride (60μmol/kg) during each radiation treatment session resulted in a marked improvement in early anastomotic blood flow, increased Hydroxyproline content and decreased gross inflammatory scoring. Potential mechanisms of action of the ATP-$MgCl_2$ in this model include a decrease in the ischemia resulting from the endarteritis or direct protection from cytotoxic effect of radiation exposure.

This thesis is dedicated to my wife, Patricia, and my children, Antonio and Christina, who encouraged me during the difficult times of this project, and understood the absences required by this work. It is also dedicated to my parents, Antonio and Kathleen, who instilled in me the importance of the pursuit of knowledge.

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LIST OF ABBREVIATIONS

The following abbreviations have been employed:

CL	:	Czerny-Lembert two layer handsewn anastomosis
CS	:	EEA® circular stapled anastomosis
LDV	:	Laser doppler velocimetry
pHi	:	Intramural pH reading
ATP-$MgCl_2$	:	Adenosine Triphosphate-Magnesium Chloride
cGy	:	Centigrays (unit of radiation dose)

IA. GENERAL INTRODUCTION

Benjamin Travers, an English surgeon trained by Sir Astley Cooper, ushered in the modern age of intestinal suturing, with his report on anastomotic healing in 1812, stressing the need for accurate, circumferential serosal apposition for healing of enteric anastomoses.[1] Antoine Lembert, working in France in 1827, described a two layer inverting anastomotic technique based on two tenets 1) sutures should be placed in the submucosa because of its considerable strength; 2) the serosal surface should be accurately apposed circumferentially.[2] It is this technique, later modified by Czerny and Connell, which remains the most widely used today.[3]

Healing of enteric hand-sewn anastomoses has been widely studied, with comparisons made between single and double layer techniques, inverting and everting anastomoses, and between different suture materials. Over the last two decades enteric stapling has become more popular, especially when used to create end to end low rectal anastomoses with the circular EEA® instrument. However, little is known regarding the differences in healing between two layer handsewn and circular stapled anastomoses. In fact, there has been only one direct comparison between those two techniques in either clinical or basic research projects.

IB. SURVEY OF PUBLISHED WORK

1) Review of Intestinal Wound Healing

Wound healing in the gastrointestinal tract is understood to follow the same natural history as wounds elsewhere in the body. In general, wound healing occurs in three stages: 1) lag phase (0-5 days); 2) period of rapid collagen synthesis (5-17 days); 3) remodeling phase (17 days-2 years).[4] The lag phase implies a time period of minimal activity when in reality this is a very active phase dominated by the inflammatory response. Immediately after wounding there is platelet activation and activation of Hageman factor which results in a cascade of humoral responses including the complement system, coagulation cascade kinin activation, and plasmin generation.[5] The net result of these responses is hemostasis and attraction of a number of cell lines important in wound healing and repair.[5] Platelets release a number of growth factors which attract and stimulate fibroblast ingrowth as well as to stimulate neovascularization. In the first 24 hours, polymorphonuclear phagocytes and monocytes enter the wound area to begin the period of inflammation with degradation and removal of necrotic tissue.[6] The amount of necrotic tissue present in the wound can greatly affect the degree and rapidity with which successful wound healing can proceed by altering the intensity of this inflammation.

At approximately day 5, the rate of collagen synthesis begins to increase dramatically and this continues at a high rate to approximately day 17.[7] During this period, tensile strength increases correspondingly. However, the ability of the fibroblast to produce collagen is dependent on delivery of nutrients, especially oxygen. Initially, oxygen is delivered by diffusion but soon neovascular in-growth allows return to more normal levels of microvascular blood flow and nutrient delivery.

The period of collagen remodeling begins at approximately day 17 with collagen cross linking accounting for any further increases in wound tensile strength. During this time period, an equilibrium is reached between collagen production and collagen degradation so that no net increase in collagen content occurs after this time.

Inflammation without infection is a significant early determinant of the success of gastrointestinal wound healing. However, it should be noted that the mere act of performing an anastomosis results in significant local inflammation at the anastomotic line. First, tissue disruption by bowel division sets into action the previously discussed humoral cascade systems. Additionally, suture material represents a foreign body in the wound. Shambaugh and Dumphy, in 1937, found that silk (less inflammatory material) decreased the incidence of wound infection when compared to catgut.[8] Madsen studying 12 different suture materials described a marked exudative tissue reaction and delayed collagen formation in association with absorbable suture material.[9] Lord et al, identified marked injury to the submucosa, associated with braided versus monofilament suture material.[10] Similarly, Herrmann et al, identified histologic changes of sloughing and necrosis in inverted handsewn colonic anastomoses with marked inflammation.[11]

2) Review of Enteric Suturing

Suture material is a foreign body, and as such produces a local tissue inflammatory reaction which impedes anastomotic healing. In the 1940's, it was found that silk produced less inflammatory response and therefore shortened the lag phase of healing and increased early bursting strength.[12] In comparison, plain cat gut and chromic cat gut incited a significant inflammatory response which resulted in sloughing of that portion of the anastomosis with healing by second intention.[13] More recently, less inflammatory absorbable suture materials such as polyglycolic acid have been evaluated and found to be comparable to silk in anastomotic strength and degree of inflammatory response.[14] Therefore, the majority of studies have concentrated on chromic catgut, silk, or polyglycolic acid suture material.

In comparing single versus double layer handsewn anastomoses, Sako and Wangensteen in Minneapolis in 1951, found significant reduction in stomal size and increased inflammation in association with a double layer inverted suture technique.[15] Hamilton, in 1967, confirmed the disadvantages associated with a double layer handsewn anastomosis by identifying increased edema of the inverted cuff and a correspondingly higher risk of postoperative obstruction at the anastomosis.[16] Letwin and Williams found that single layer anastomoses exhibited greater bursting strength, improved anastomotic blood flow (using alphazurine dye) and less tissue necrosis at the anastomotic line. In addition, they identified an earlier rise in collagen content anastomotically with a single layer technique.[17] Thus, studies have uniformly supported single layer suture technique over double layer for optimal anastomotic healing. Despite these experimental data, a two layer handsewn technique is still widely employed in clinical surgery.

In comparing inverting and everting techniques the differences have not been as clear cut. It has been found, however, that everting anastomoses tend to be more dependent on adhesions for viability and anastomotic integrity, and may be somewhat weaker in the early postoperative period.[18-22]

Mechanical instruments for performing intestinal anastomoses originated in the work of Humor Hultel of Budapest, Hungary. In 1908, he demonstrated a stapling device to the Second Congress of the Hungarian Surgical Society, designed for use in distal gastrectomies. He had performed 21 of these procedures in the previous year without complications using this stapler.[23] Alador Von Petz subsequently improved on Hultel's instrument in 1924, producing a lighter, more easily usable instrument.[24]

Significant advancement in surgical stapling occurred in the 1950's at the Scientific Research Institute of Experimental Surgical Apparatus and Instruments in Moscow. They developed staplers for vascular anastomoses, terminal staplers for the GI tract, and a circular stapler for rectal and esophageal anastomoses.[25-27] These instruments provided accurate reproducible staple lines which were readily applied to virtually any clinical situation.

Stapling techniques for creating gastrointestinal anastomoses were introduced in this country in the late 1960's by Ravitch. Since that time staplers, have gained widespread popularity in gastrointestinal surgery, and especially in colorectal procedures as they allow an anastomosis to be performed lower in the pelvis than previously technically feasible. As a result, a surgeon is more likely to be able to preserve anal sphincter function in patients with rectal tumors or inflammatory disease, thus avoiding a permanent colostomy. Clinical experience with stapling techniques has generally been very good, however, some concerns have been raised regarding anastomotic integrity with leakage rates ranging from 2 to 68%.[28-31] In addition, it is felt that low anterior stapled colorectal anastomoses have a higher risk of stenosis based on retrospective uncontrolled clinical studies reporting incidences ranging from 13 to 35%.[28,32-34] Therefore, concern remains over the integrity of these stapled anastomoses. Despite a significant clinical experience with anastomotic stapling there have been few experimental evaluations of anastomotic healing, and in particular, there has been no comparison between the standard Czerny-Lembert (2 layer) sutured anastomosis and any of the stapling techniques.

3. Review of Anastomotic Ischemia and its Assessment

Anastomotic ischemia is another factor which can affect early wound healing. When the bowel is transected, platelet activation and coagulation causes occlusion of local blood vessels to secure hemostasis. Thus, the anastomotic line is dependent on delivery of nutrients by diffusion to survive until neovascularization can occur. The decrease in local blood flow is coupled with a local increase in metabolic activity, thus worsening the impact of the borderline delivery of nutrition at a time when healing is dependent totally on oxygen delivery. Chung et al, identified acute decreases in mucosal blood at the anastomosis with either stapling or suturing using laser doppler velocimetry (LDV).[36,37]

Although ischemia at the anastomotic line has been proposed as the predominant pathophysiologic mechanism for impaired healing, it has been difficult to quantitate clinically. Currently, there is no readily available, non-invasive, accurate method of quantifying critical levels of ischemia in the GI tract which result in irreversible tissue injury. Non-quantitative data regarding gastrointestinal blood flow may be obtained by gross inspection, fluorescein injection, or doppler ultrasound.[38,39] Although, these techniques are readily available clinically, the qualitative data supplied is inadequate to allow quantification of critical levels of tissue ischemia and thus accurate prediction of successful anastomotic healing.

There are a number of currently available methods which allow quantitation of gastrointestinal blood flow: radioactive microspheres, Hydrogen clearance, laser doppler velocimetry, and tissue oximetry. Each of these techniques has relative advantages and disadvantages. Microsphere analysis allows accurate determination of differential blood flow to the layers of the bowel wall; however, this technique requires tissue removal and therefore is not useful in the clinical setting.[38] Similarly, hydrogen clearance results in accurate data in the experimental situation, however, its accuracy can be altered by diffusional shunting of the indicator or as a result of surgical manipulation of the bowel.[38-42]

A quantitative technique for measuring bowel wall perfusion which is readily available is laser doppler velocimetry (LDV). The laser doppler emits a monochromatic light with a wave length of 632.8nm. The light penetrates to a depth of 1.5mm and is reflected back to a photodiode resulting in a doppler shift in the reflected light by moving red blood cells within surface capillaries. The shift is expressed as millivolts.

Laser doppler velocimetry offers the advantage of continuous quantitative measurement of blood flow _in vivo_ at any portion of the GI tract which can be exposed at surgery.[37,40-42] Major disadvantages in using this instrument on the gastrointestinal tract are that peristalsis may limit accurate optical coupling between the probe and bowel surface and intermittent compression of the bowel wall by excess pressure applied to the probe can drastically alter local blood flow measurements in the bowel wall.[43,44]

Measurement of intramural pH (pHi) via the Khuri pH probe offers many of the same advantages as LDV. The hydrogen ion electrode and thermistor wire can be inserted at any portion of the gastrointestinal tract and allow continuous determination of pHi in vivo. This technique has a major advantage over LDV in that it supplies quantitative data which is observer independent, thereby increasing its overall accuracy. This stability was not observed with use of LDV in our study. Although blood flow is not measured with the pH probe, the effects of ischemia on tissue metabolism are directly measured, thus allowing prediction of subsequent viability. The Khuri tissue pH probe used in this study is comprised of a silver chloride, leaded glass coated, hydrogen ion electrode and thermistor probe inserted adjacently into the seromuscular layer of the colon. A remote potassium chloride salt bridge is then placed subcutaneously as an electrical reference point. Electrical potential difference is then transmitted to a computer which calculates the local tissue pH using the Nernst equation:

$$pH = \frac{E - E_o(F)}{RT}$$

Where E = tissue electrical potential, E_o= observed potential, F=Faraday's constant, R = constant, and T = absolute temperature.

The instrument has been shown to have a 95% response time less than 15 seconds and drift less than .01 pH units over a six hour period.[47]

4) <u>Review of Effect of Colostomy on Anastomotic Healing</u>

Narrowing or stricture is a recognized complication of circular stapled large bowel anastomoses.[28] Several reports have associated a marked increase in the incidence of anastomotic stenosis with the circular staple technique following proximal diversion.[28,33-35]

It is generally felt that the absence of the fecal stream for an extended period of time plays a prominent pathophysiologic role in stenosis. This could result from the loss of normal dilating effect of the fecal bolus.[35] Others have proposed an absence of short chain fatty acids which are the primary fuel source for colonic mucosa, as a potential mechanism.[48] Additionally, Buchman demonstrated an increased amount of submucosal fibrosis in diverted stapled anastomoses when compared to a two-layer, hand sewn technique.[34]

A major flaw in many of these studies is a small number of subjects as well as a lack of uniformity for the indications for proximal diversion. The lack of uniformity with regards to indications for proximal diversion may be very important, as the reason for diversion in the clinical study (i.e. abscess, multiple trauma, or shock) may be a more important factor in inducing anastomotic stenosis than the surgical technique utilized.

5) Review of the Effects of Therapeutic Radiotherapy on Colorectal Anastomotic Healing

Preoperative adjuvant radiation therapy in the range of 4-5000 rads, has demonstrated significant beneficial effects in rectal cancer patients.[48-50] These data have demonstrated tumor shrinkage or ablation, a decrease in the incidence of metastatic local/regional lymph nodes, and a decrease in local recurrence rates. Additionally, it should be noted that current experience has shown these dosages to be well tolerated by patients. Widespread acceptance of this adjuvant treatment modality has been slow over concern of adverse affects of radiation therapy when resection and primary anastomosis is considered. A number of studies have attempted to define the untoward effects of preoperative radiation therapy on anastomotic healing. Bubrick and Shauer experienced a 20-30% leak rate for low anterior anastomoses performed in dogs following preoperative doses of 2,000 and 4,000cGy's.[51] They noted that the leak rate increased dramatically to 70-80% however for animals who received 6,000cGy preoperatively.[52] Thus, they recommended that dosages less than 4,000cGy be employed to minimize complications.

The pathophysiology of anastomotic healing complications induced by radiotherapy has not been well studied.

It is clear, however, that 5-10% of patients who receive pelvic radiation will develop significant sequelae.[56,57] One mechanism for impaired anastomotic healing, following radiation therapy, appears to be direct cellular injury and death with healing occurring by fibrosis. Crowley demonstrated a significant early inflammatory reaction with loss of epithelial cells, reduction of villous height, and submucosal edema.[59] He noted, however, that by 22 days these effects had subsided almost entirely, thus the reason for the current recommendation of a three week rest period prior to surgical intervention.

Although histologic studies have implicated endothelial and connective tissue damage as the hallmark of late radiation injury, the key factor may be a failure or depletion of stromal stem cells required for adequate healing of the involved tissue.[65] This is modeled by the subsequent bone marrow failure which occurs after repeated exposures to either radiation therapy or cyto toxic drugs in lymphoreticular disorders.

It was this hypothesis of repeated stromal stem cell injury which led us to investigate ATP-$MgCl_2$ as a cytoprotective agent in radiotherapy. ATP-$MgCl_2$ has demonstrated ability to accelerate recovery in a model of post ischemic renal failure.[62] Similarly, ATP-$MgCl_2$ has demonstrated preservation of liver and cardiac function following exposure to low flow states.[61,63,64] Finally, it has been used in an in vitro model of cyclosporine renal toxicity and demonstrated a blunting of the renal dysfunction resulting from exposure to high dosages of this drug.[64] Black evaluated histologic changes following rectal irradiation in rats and identified a chronic, progressive increase in mural fibrosis and vascular sclerosis up to one year following radiation exposure, either early or late.[57] This progressive vascular sclerosis and endarteritis has been invoked as the cause of mural ischemia, resulting in the subsequent increase in anastomotic complications. Although this progressive mural ischemia has often been mentioned in the surgical literature, there has never been an attempt to quantify the magnitude of mural ischemia induced by therapeutic radiation exposure, or quantitate interventions to reverse the damage.

IC. AIMS OF THIS PROJECT

1) In particular, to assess healing in normal colorectum and under several adverse situations encountered in clinical medicine.

2) Serially compare colorectal anastomotic healing with the standard Czerny-Lembert (CL; two layer; suture; inverting) or EEA (CS; double row staples; inverting) technique a porcine model.

3) Healing was assessed at 5, 11, 60, or 120 days postoperatively to evaluate the three phases of wound healing (lag, collagen synthesis, and collagen remodeling). Parameters assessed include: a) perianastomotic blood flow; b) bursting strength; c) gross inflammatory score; d) microscopic inflammatory score; and e) hydroxyproline content.

4) Compare colorectal anastomotic healing for CL and CS techniques with or without proximal fecal diversion (colostomy).

5) Compare colorectal anastomotic healing for CL and CS techniques in the presence of ischemia.

6) Assess the accuracy of LDV and pHi to accurately quantitate critical levels of ischemia at the colorectal anastomosis and predict subsequent tissue healing.

7) Compare colorectal anastomotic healing for CL and CS techniques following 4500 cGy of preoperative pelvis radiotherapy.

8) Assess the affects of ATP-$MgCl_2$ as a cytoprotective agent during preoperative pelvic radiotherapy.

II. MATERIALS AND METHOD

A. Animals/Anesthesia

The model selected was female mixed breed pigs, age three to five months old. This animal is readily accessible, relatively inexpensive, and the distal colon resembles the human rectosigmoid anatomically with a circumferential circular and longitudinal muscle layer. Our laboratory is well equipped and experienced in porcine anesthesia and perioperative care. Preoperative mechanical bowel preparation consisted of 8oz. of magnesium citrate in the drinking water the day prior to surgery and cleansing tap water enemas immediately prior to operation. Perioperatively they received three intramuscular doses of Penicillin G (100U/kg) and Gentamicin (1mg/kg) as prophylactic antibiotic coverage administered immediately prior to surgery, and at 6 and 12 hours postoperatively.

Preoperative sedation consisted of Ketamine (2.5mg/kg) and sodium Pentothal (30mg/kg) administered intramuscularly thirty to forty-five minutes prior to surgery. Anesthesia was induced using Pentobarbital (5mg/kg IV), and orotracheal intubation was performed allowing inhalational anesthesia with halothane. The animals were allowed to recover spontaneously from anesthesia in their pens. They were allowed food and water ad libitum postoperatively. Use of animals in this project was in accordance with the National Institute of Health Publications Principles For Use of Animals and Guide for the Care and Use of Laboratory Animals. {Office for Protection from Research Risks, National Institutes of Health, Bethesda, MD 20205}. The Animal Research Review Committee of Michigan State University monitored this project in routine fashion.

B. Experimental Groups

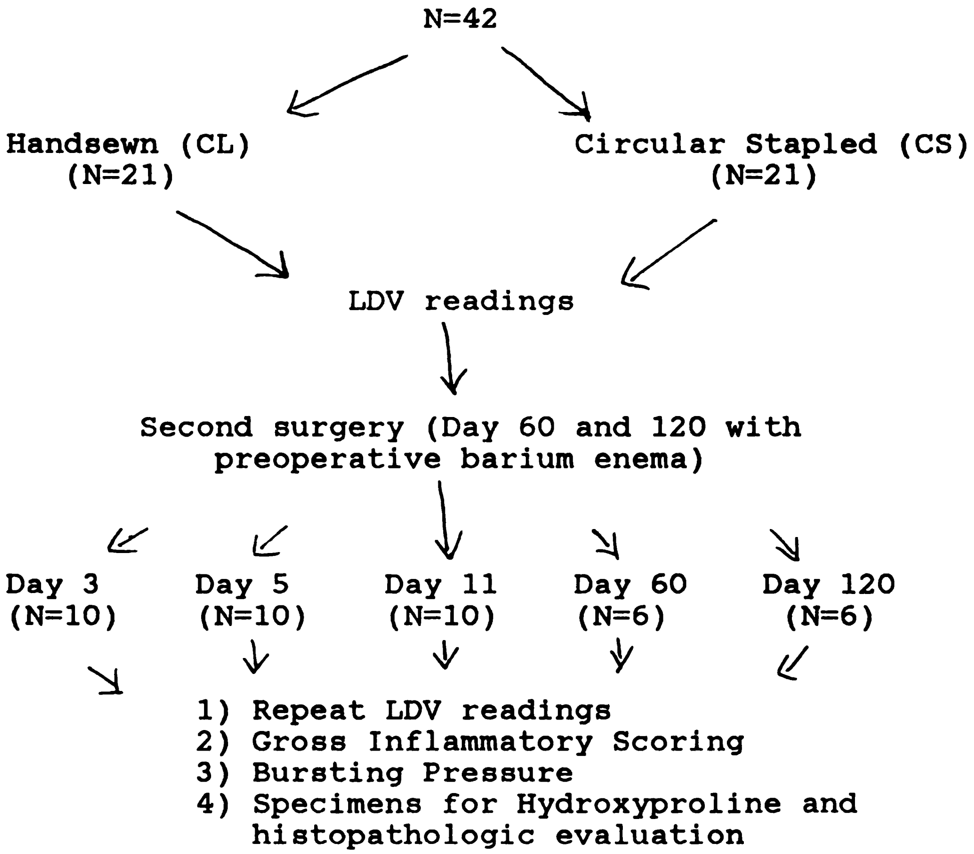
The study was performed in five sections. Section I compared the Czerny-Lembert (CL) anastomotic technique with EEA stapled technique (CS) in control animals. Section II evaluated the effects of proximal diverting colostomy (COL) to non-diversion (CON) using the same two anastomotic techniques. Section III evaluated the effects of ischemia on anastomotic healing in CL and CS anastomoses and prospectively compared the accuracy of laser doppler velocimetry (LDV) and tissue pH (pHi) in quantitating the effects of anastomotic ischemia. Section IV evaluated the effects of preoperative pelvic radiation therapy (RT) on anastomotic healing and CL or CS anastomoses. In Section V, ATP-$MgCl_2$ was infused during RT treatment sessions in two subgroups (ATP-30: 30μmol/kg; ATP-60: 60μmol/kg) in an attempt to diminish RT induced side effects.

1) Section I

In Section I (see Figure 1), the animals were randomly allocated to either CL (N=21) technique or CS (N=21) technique. The animals underwent a second surgery on postoperative day 3 (N=10), day 5 (N=10), day 11 (N=10), day 60 (N=6), or day 120 (N=6) to assess the healing process during the well documented three stages of wound healing. At the initial surgery, laser doppler velocimetry (LDV) readings were obtained 1cm proximal, at, and 1cm distal to the anastomosis. Readings were obtained at the mesenteric, anti-mesenteric and right and left aspects of the bowel at these three locations. The four values were then averaged for each level. At second surgery, assessment of the anastomosis consisted of repeat laser doppler velocimetry (same locations), bursting pressure, gross inflammatory or microscopic inflammatory, and hydroxyproline determination. In addition, the 60 and

120 day animals underwent preoperative barium enema to assess the degree of anastomotic stenosis.

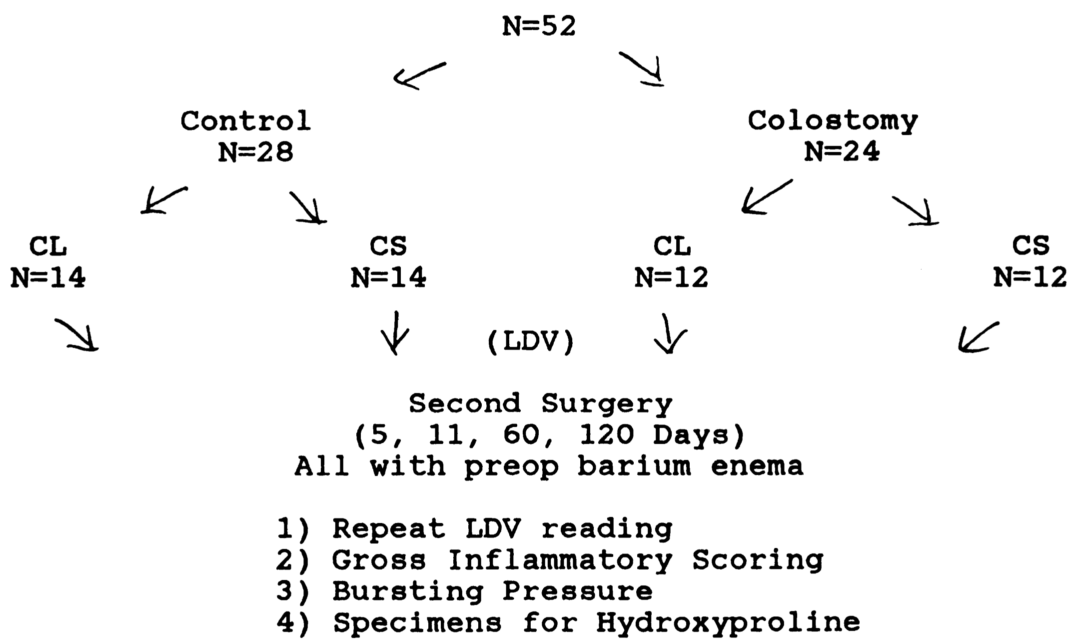
FIGURE 1: Section I, Protocol Outline



2. Section II

Section II (Figure 2) consisted of animals randomly assigned to diverting colostomy (COL, N=24) or nondiversion (CONT N=28) groups. Diverting colostomy was performed by dividing the bowel 20cm proximal to the anastomosis oversewing the distal end (leaving it intraabdominally) and bringing the proximal bowel to surface as a colostomy. Animals were also randomized to CL or CS technique with second surgery performed at 5, 11, 60 or 120 days. Similar data to Section I was collected except for microscopic inflammatory scoring.

FIGURE 2: Section II, Protocol Outline



3. <u>Section III</u>

Section III (Figure 3) was performed in two segments. First a pilot group Phase I, (N=8) in which variable portions of the mesentery were divided proximal and distal to the anastomosis to cause devascularization sufficient to induce a color change in the anastomotic segment. Subsequent anastomotic outcome was retrospectively compared to the quantitative values obtained by laser doppler velocimetry (LDV) and tissue pH (pHi) determination to allow identification of quantitative values reflecting critical levels of ischemia. The ability of these critical values to accurately predict anastomotic healing outcome (good or bad) was tested in a prospective manner (Phase II) using 24 animals allocated evenly to either CL or CS technique. Again, follow up in the prospective portion of section III was performed at 5, 11, 60 or 120 days. The purpose of this section of the study was to define the accuracy of the quantitative data in identifying critical levels of anastomotic ischemia, and to use this level of ischemia to develop a reproducible model of anastomotic stenosis.

FIGURE 3: Section III, Protocol Outline

N=32

Phase I
N=8

CL N=4 → Mesenteric devascularization
CS N=4 → Mesenteric devascularization

Mesenteric devascularization
↓
LDV Readings
pHi Readings
↓
Second Surgery
Day 5
1) Preop barium enema
2) Repeat LDV
3) Bursting pressure
4) Gross Inflammatory Scoring

(This portion allowed identification of "critical" quantitative data reflecting levels of ischemia which would impair healing.)

Phase II
N=24

CL N=12 → Mesenteric devascularization
CS N=12 → Mesenteric devascularization

Mesenteric devascularization
↓
LDV Readings
pHi Readings
↓
Second Surgery
Day 5,11,60, or 120
1) Preop barium enema
2) Repeat LDV
3) Bursting pressure
4) Gross Inflammatory Scoring

(Tested accuracy of "critical" levels to predictive good or poor anastomotic healing.)

4. Section IV

Section IV (Figure 4) consisted of animals (N=24) receiving 4500cGy of preoperative RT over five weeks followed by a three week rest period. The pelvic irradiation was administered in 425cGy fractions in a right to left then left to right fashion. A 300KV, 20mA orthovoltage x-ray unit was used with a tube to skin distance of 50cm. The radiation dose delivered was measured using a Radicon 3 with 550-5 probe which was inserted 20cm above the dentate line. The radiation was administered through a 7cm by 15cm portal. This technique assured that the total dose administered to the animals over the course of treatment was 4500cGy. This technique of radiation administration is very analogous to the treatment schedules used in clinical medicine and avoided the use of the Normal Standard Dose (NSD) formula commonly used in experimental protocols. This is especially important because recent work has demonstrated the lack of validity for the NSD formula when attempting to study chronic radiation changes.[53] The animals were then randomly allocated to either CL or CS technique. Similar data to Section I was collected except for microscopic inflammatory scoring.

FIGURE 4: Section IV, Protocol Outline

4500 cGy preoperative
RT over 5 weeks

Handsewn (CL)
N=24

Circular
Stapled (CS)
N=24

LDV readings

Second surgery (Day 60 and 120 with
preoperative barium enema)

Day 5 (N=12) | Day 11 (N=12) | Day 60 (N=12) | Day 120 (N=12)

1) Repeat LDV readings
2) Gross Inflammatory Scoring
3) Bursting Pressure
4) Specimens for Hydroxyproline

5. Section V

Animals (N=24) in this section (Figure 5) received 4,500cGy of preoperative RT as in Section IV. However, 12 animals (ATP-30) received intravenous infusions of 30µmol/kg ATP-$MgCl_2$ over two hours during each RT session. The other group of 12 animals (ATP-60) received 60µmol/kg of ATP-$MgCl_2$ over four hours during each RT session. Infusion rates were selected based on earlier pilot studies to identify a maximal rate of infusion which avoided hemodynamic alterations. Postoperative evaluation was limited to Day 5 and Day 11 with similar data to Section IV being collected.

FIGURE 5: Section V, Protocol Outline

4500cGy Preop RT
A(30µmoles/kg/RT treatment)
N=12

CL
N=6

CS
N=6

4500cGy Preop RT
B(60µmoles/kg/RT treatment
N=12

CL
N=6

CS
N=6

Second Surgery at Day 5 and 11

1) Repeat LDV readings
2) Gross Inflammatory Scoring
3) Bursting Pressure
4) Specimens for Hydroxyproline
5) Preoperative barium enema for stenosis or leak

RT = Radiation Therapy
CL = Czerny-Lembert (Handsewn)
CS = Circular Stapled

C. OPERATIVE TECHNIQUE

1) First Surgery

The animals were anesthetized, endotracheally intubated, placed in the supine position and surface hair was clipped. An abdominal scrub with Betadine soap followed by Betadine solution was performed. A vertical midline incision allowed entry into the abdominal cavity. The distal colon was mobilized at the pelvic brim with resection of a 4-5cm segment of colorectum. A primary anastomosis was then performed using either the Czerny-Lembert (CL; two layer hand sewn) technique or EEA (CS) stapled technique. For the CL technique, an inner layer of running 3-0 chromic was placed using full thickness bites of the bowel wall circumferentially, followed by an interrupted outer layer of 3-0 silk seromuscular Lembert sutures. The EEA stapled anastomoses (CS) were performed using either a 25 or 28mm stapler head depending on the luminal diameter of the bowel. A 2-0 polypropylene pursestring suture was placed at the opening in the proximal and distal bowel. The stapler was then inserted transanally and the anvil extended out of the distal colon. The proximal bowel was then placed over the anvil and the pursestring suture was tied securely

(Fig. 6a and b). Similarly the distal pursestring was tied securely. The stapler head was approximated and fired completing the anastomosis. The presence of a complete ring or "doughnut" of colonic wall was required from both proximal and distal colon to assure accurate firing of the stapler. Leak testing was then done following both techniques by filling the pelvis with saline and injecting air transanally while observing for bubbles as evidence of leak. If a leak was identified the location was recorded and it was repaired with interrupted 3-0 silk seromuscular sutures. Laser doppler velocimetry readings were then obtained 1cm proximal, at, and 1cm distal to the anastomosis. The abdomen was then thoroughly irrigated with saline and the fascia was approximated in a single layer using running #2 polypropylene suture. The skin was approximated using metal staples. The animals were returned to their cages and allowed to recovery spontaneously from anesthesia.

2) Second Surgery

The midline laparotomy incision was reopened following general anesthesia. The anastomotic segment was carefully exposed to allow repeat laser doppler

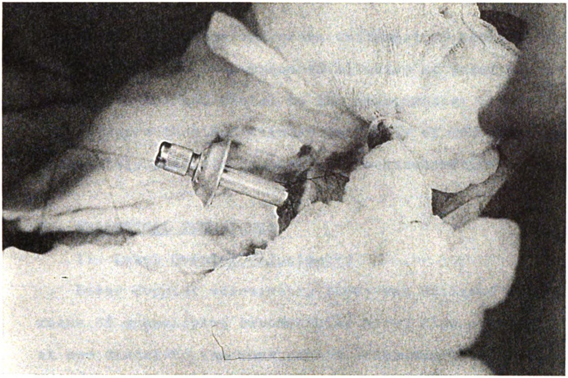
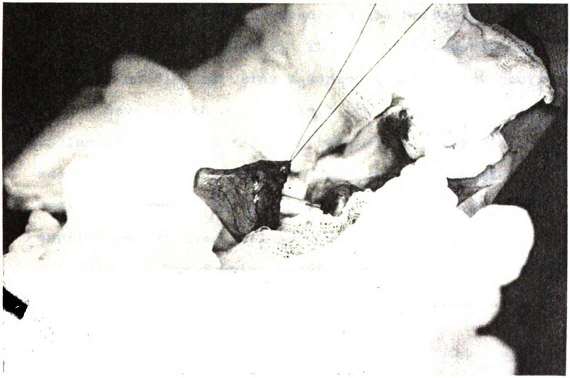


FIGURE 6A: Circular Stapling Technique

FIGURE 6B: Circular Stapling Technique



velocimetry readings and gross inflammatory scoring. The segment was then resected allowing at least 5cm of bowel proximal and distal to the anastomosis. The animal was euthanized using 0.5ml/kg IV of T61 solution immediately prior to removal of the anastomotic segment.

D. ANALYTICAL TECHNIQUES

1) Laser Doppler Velocimetry

Laser doppler velocimetry (LDV) was utilized as a means of quantifying seromuscular blood flow proximal, at and distal to the anastomosis. Seromuscular blood flow was measured because these are the layers which provide the majority of the tensile strength of the anastomosis and figure prominently in the healing process. The instrument used was the MedPacific 5000 Laser Doppler Velocimeter. This is a helium-neon Laser Doppler Velocimeter using a monochromatic light source with a wave length of 632.8mm. The light penetrates up to 1mm and is reflected back to a photo diode via a second set of fiber optics. The Doppler-shifted light, reflected from moving red blood cells within surface capillaries of the bowel is processed and expressed in millivolts. The doppler shift is proportional to the velocity of blood flow in the vessels. Readings were obtained at four points proximal (1cm proximal) at, and distal (1cm distal) to the anastomosis. The four readings at each level (proximal, at, and distal) were then used to obtain an "average" blood flow for that level. All readings are presented as millivolts (mV).

2) <u>Tissue pH</u>

The Khuri tissue pH probe was used to measure perianastomotic seromuscular colonic pH in both normal and ischemic colon. The instrument consists of a silver chloride, leaded glass, microhydrogen ion electrode which is inserted seromuscularly. A thermistor wire is inserted seromuscularly in an adjacent location. These data (hydrogen ion current and temperature) are used to calculate the tissue pH via the Nernst equation ($pH=E_o-E(F/RT)$). A reference salt (KCl) bridge is placed subcutaneously as an electrical reference point. Overall the instrument has a 95% response time of fifteen seconds with a drift of less than 0.01 pH units per six hours.[45] A pilot study was performed as part of Section III to determine normal and critical pH values induced by ischemia. These critical values were then tested prospectively in the second portion of Section III of this project. All readings are presented as pH units.

3)Bursting Pressures

Bursting pressure was determined by fixing an 18 French foley catheter in the proximal and distal end of the anastomotic segment. The catheters were secured in place by means of a pursestring suture at the ends of the bowel. One of the catheters was attached to an air inflow which allowed air to be insufflated at 1.0 liter/min. The second catheter was attached to a pressure transducer and a physiograph machine recorded pressure changes. The site and pressure (mmHg) of the burst was recorded for each anastomotic segment.

4) Hydroxyproline Assay

The assay utilized was defined by Woessner and is a colorimetric method of assessing hydroxyproline tissue content.[68] The technique is briefly described below. The tissue is hydrolyzed in 6N HCl at 60°C (1ml HCl/10mg tissue), following which the pH is raised to 6-7 using 2N NaOH. The sample is then brought to a 2ml volume by adding the appropriate amount of buffer solution as necessary. One milliliter of Chloramine T is then added. Twenty minutes later 1ml of aldehyde/perchloric acid is added to the solution which is warmed to 60°C for fifteen minutes. Absorbance is measured using a spectrophotometer set at 550nm. Standard solutions were prepared at 0.1mg/ml, 0.3mg/ml and 0.5mg/ml to allow construction of a standard curve (linear relationship) on which to plot the results of the tissue samples and allow calculation of the quantity of hydroxyproline present. Data is presented as mg/ml.

5) Gross Inflammatory Grading

A 24 point scale was used to grade the degree of serosal and mucosal inflammation at the time of second surgery. The degree of serosal inflammation, hemorrhage, edema, and adhesion formation were each graded on a three point scale. Similarly the amount of mucosal inflammation, necrosis, hemorrhage, and edema was recorded. Thus a maximum score of 24 points was available with 12 points contributed by the serosal reaction and 12 points contributed by the mucosal reaction. Grading was performed by the surgeon at the time of second laparotomy.

6) <u>Barium enema examination for stenosis or leak</u>

Barium enemas were performed immediately prior to second surgery for animals at the 60 and 120 day intervals in all groups. In the ischemic study (Section III) all animals had studies performed prior to second surgery to identify leak or stenosis. All animals receiving preoperative RT (Section IV and V) had barium studies. From the barium study, an anastomotic index was calculated by dividing the inner diameter at the anastomosis by the sum of the inner diameter of the bowel 3cm proximal and distal to the anastomotic line. A non-stenotic anastomosis would have an index of 0.5. Values less than 0.3 were considered stenotic. Leaks if present were also recorded, as well as their location.

7) <u>Microscopic Inflammatory Score</u>

A histologic grading scale was utilized by Dr. Robert Dunston of the Michigan State University, College of Veterinary Medicine, Department of Pathology, to assess the degree of inflammatory reaction at the anastomosis. Factors graded on a three point scale were: 1) erosion/ulceration; 2) necrosis; 3) congestion; 4) fibrosis; 5) inflammation; 6) PMN infiltration; 7) lymphocyte infiltration; 8) macrophage infiltration; 9) eosinophil infiltration. Thus a maximum score available was 27 points. Examination was performed separately on the mucosa, muscularis, and serosa. In addition, overall reaction to the material used in the anastomosis was graded (3 points each) by assessing: 1) fibrosis; 2) inflammation; 3) PMN infiltration; 4) lymphocyte infiltration; 5) Macrophage infiltration; 6) eosinophil infiltration. A maximum score available for this overall grade was 18 points.

Specimens were fixed in formalin for at least 48 hours and then embedded in paraffin from which three sections were cut and placed on slides. Tissue was stained with routine hematoxylin and eosin.

8)Statistical Analysis

Blood flow data was analyzed using Analysis of Variance and BonFeroni's Least Significant Difference, a posteriori test. Student's t-test was also used to compare CL and CS LDV data at each level. Bursting pressure, and tissue hydroxyproline levels were analyzed with Student's t-test. Inflammatory scores were analyzed with the rank sum test. In all cases, significance was set at $p<.05$. Results are expressed as mean ± standard deviation.

III. RESULTS

A. SECTION ONE: NORMAL COLORECTUM

1. Laser Doppler Velocimetry Results

Forty-two (42) female mixed breed pigs with an average weight of 63.7kg (58kg-69kg) were used in this study. The animals were randomized to CL (N=21) or CS (N=21) technique of colorectal anastomosis. The average perianastomotic LDV data at the initial surgery are shown in Table 1.

TABLE 1, SECTION I: LDV Readings (mV) at Initial Surgery (mV)

	Prox	At	Dist
CL (N=21)	492±154	264±121*	356±156
CS (N=21)	620±221#	329±174*	546±215#

*p<.001 ANOVA (comparing Prox, At, Dist)
#p<.05 t-test (comparing CL to CS)

For each technique there was a significant decrease in seromuscular blood flow as measured by LDV at the anastomotic line compared to flow 1cm proximal and distal. Additionally, seromuscular blood flow was significantly greater 1cm proximal and 1cm distal to the anastomotic line for the CS anastomoses compared to CL. There was no significant difference between CL or

CS techniques with respect to LDV readings at the anastomosis.

The data for perianastomotic seromuscular LDV readings demonstrates that the decrease in flow at the anastomotic line resolved by day three (Table 2). Likewise there were no significant differences between CL and CS techniques for Day 5-120 (see Table 2).

TABLE 2, SECTION I: LDV (mV) Data Day 3-120

Post-Op Day	Prox	At	Dist
Day 3CL(N=5)	475±54	353±62	421±115
Day 3CS(N=5)	338±220	230±111	408±220
Day 5CL(N=5)	684±365	707±436	682±226
Day 5CS(N=5)	533±201	315±102	448±103
Day 11CL(N=5)	640±273	541±345	561±277
Day 11CS(N=5)	462±242	407±218	342±178
Day 60CL(N=3)	356±81	213±40	249±89
Day 60CS(N=3)	331±104	228±78	344±48
Day 120CL(N=3)	290±142	224±193	320±157
Day 120CS(N=3)	240±60	144±60	280±81

CL=2 layer handsewn CS=circular stapled

2. Bursting Pressures

The bursting pressures at day three were not significantly different for the two techniques (CL:157±9mmHg; CS:133±33mmHg). The bursting pressures at day five were also not significantly different (CL:188±54; CS:231±57mmHg).

3. <u>Gross and Microscopic Inflammatory</u>

Analyzing the gross and microscopic inflammatory scores did not reveal any significant differences between the two techniques at any of the time periods (Tables 3 and 4).

TABLE 3, SECTION I: Gross Inflammatory Score Day 3-120 (max=24 point)

Post-Op Day		CL	CS
Day 3	(N=10)	4.1±1.0	3.9±1.2
Day 5	(N=10)	5.4±1.3	5.2±.9
Day 11	(N=6)	6.4±.9	5.2±.9
Day 60	(N=6)	4.2±.9	4.4±.5
Day 120	(N=6)	4.1±.8	4.6±.7

(Score is based on gross findings at the time of second surgery with a maximum of 12 points contributed by both the serosal and mucosal surfaces).

TABLE 4, SECTION I: Microscopic Inflammatory Score Day 3-120

Post-op Day	Mucosa	Muscularis	Serosa
Day 3CL(N=5)	10.2±2.9	15±1	13±2.1
Day 3CS(N=5)	13.5±4.5	14±1.6	14.25±3.3
Day 5CL(N=5)	11.6±4.6	11.0±4.4	15.2±3.7
Day 5CS(N=5)	11.4±4.2	9.2±3.8	10.8±2.4
Day 11CL(N=5)	8.2±3.1	12.6±1.9	11.0±3.7
Day 11CS(N=5)	11.25±3.0	10.5±4.5	8.25±3.2
Day 60CL(N=3)	7.7±1.5	10.0±2.6	7.0±1
Day 60CS(N=3)	10.7±2.5	7.7±2.9	4.7±2.1
Day 120CL(N=3)	6.7±1.2	6.3±1.2	3.7±2.1
Day 120CS(N=3)	9.0±3.5	4.0±2.6	5.0±2.0

CL=2 layer handsewn CS=circular stapled

4. <u>Hydroxyproline Content</u>

The anastomotic hydroxyproline content for each technique at the time interval evaluated is shown below (Table 5).

TABLE 5, SECTION I: Hydroxyproline Day 3-120 (mg/ml)

Post-op Day		CL	CS
Day 3	(N+10)	0.04±.03	0.07±.04
Day 5	(N=10)	0.14±.13	0.05±.04
Day 11	(N=6)	0.20±.08	0.14±.10
Day 60	(N=6)	0.15±.09	0.20±.1
Day 120	(N=6)	0.22±.07	0.14±.02

CL=2 layer handsewn CS=circular stapled

There was a trend for more rapid gain in hydroxyproline content for handsewn anastomoses but this did not achieve statistical significance.

5. <u>Anastomotic Diameter</u>

Anastomotic diameters and anastomotic ratios were similar for both CL and CS techniques (Table 6).

TABLE 6, SECTION I: Anastomotic Diameter and Ratios for 60 and 120 Day Groups

	Diameter (mean ± S.D.)	Ratios
60 CL	4.6±1.4	0.43
60 CS	3.4±.6	0.40
120 CL	3.6±1.4	0.37
120 CS	5.1±.5	0.42

CL=2 layer handsewn CS=circular stapled

B. SECTION TWO: COLOSTOMY (COL) VS. CONTROL (CON: NON-COLOSTOMY)

1. General Data

Fifty-two (52) mixed breed female pigs were employed in this portion of the study. The average weight was 65.3kg (57-69kg). The animals were randomly allocated to colostomy (COL, N=24) or noncolostomy control group (CON, N=28).

2. Laser Doppler Velocimetry Results

The perianastomotic blood flow as measured by LDV, at the initial surgery was similar in both techniques irrespective of the presence or absence of proximal diversion (Table 7).

TABLE 7, SECTION II: Initial Perianastomotic Blood Flow (mV)

	CL prox	CL at	CL dist
Control	627±107	330±181*	545±230
Colostomy	441±180	247±122*	522±190
	CS prox	CS at	CS dist
Control	492±153	269±121*	356±157
Colostomy	517±218	274±77*	539±170

*p<.001 ANOVA (comparing prox, at, and dist flow; no significance between flow at any level comparing CON to COL).

The early decrease in blood flow at the anastomotic line was identified in the colostomy and control animals without significant difference between the two groups. This decrease in flow resolved by day five in these animals and there was no significant difference subsequently with respect to anastomotic technique or the presence or absence of proximal diversion (Table 8).

TABLE 8, SECTION II: Perianastomotic Blood Flow (mV±SD) 5-120 day

		Prox		At	
Post-op	Day	Con(N=6)	Col(N=3)	Con(N=6)	Col(N=3)
Day 5	CL	641±344	474±63	707±436	313±69
Day 5	CS	511±206	442±143	315±102	414±171
Day 11	CL	640±273	531±204	541±345	395±53
Day 11	CS	454±243	600±62	407±218	343±46
Day 60	CL	356±81	333±17	213±40	223±55
Day 60	CS	331±104	338±70	228±78	264±114
Day 120	CL	290±142	546±124	224±193	637±402
Day 120	CS	240±60	496±127	144±60	328±195

		Distal	
		Con(N=6)	Col(N=3)
Day 5	CL	681±226	399±111
Day 5	CS	447±103	485±72
Day 11	CL	561±277	466±168
Day 11	CS	342±178	473±155
Day 60	CL	250±89	257±19
Day 60	CS	344±48	281±73
Day 120	CL	320±157	580±211
Day 120	CS	280±81	442±252

CON=control
COL=colostomy

3. Bursting Pressure

There was no significant difference in bursting pressures between diverted anastomoses and nondiverted anastomoses with respect to anastomotic technique (Table 9).

TABLE 9, SECTION II: Bursting Pressures (mmHg) Day 5

	CL	CS
Control (N=6)	188±54	231±57
Colostomy(N=6)	262±58	177±38

4. Inflammatory Scores

With respect to gross inflammatory scoring, there was no significant difference between control and proximally diverted anastomoses (Table 10).

TABLE 10, SECTION II: Gross Inflammatory Score Day 5-120
(Max=24)

Post-Op Day			Control	Colostomy
Day 5	CL	N=6	5.4±1.3	5.3±1.2
Day 5	CS	N=6	5.2±0.8	11.3±3.0
Day 11	CL	N=6	6.4±0.9	4±0.5
Day 11	CS	N=6	5.2±0.8	4±0.4
Day 60	CL	N=6	4.2±.08	5.8±3.9
Day 60	CS	N=6	4.4±0.5	4.5±3.0
Day 120	CL	N=6	4.1±0.8	6.0±1.7
Day 120	CS	N=6	4.6±0.7	6.0±1.7

CL=2 layer handsewn CS=circular stapled

5. Hydroxyproline Content

There was no significant difference in hydroxyproline content for anastomoses regardless of colostomy or anastomotic technique (Table 11).

TABLE 11: Anastomotic Hydroxyproline Content (Colostomy vs. control mg/ml).

Post-Op Day	COL	CON
Day 5 CS	.11±.04	.07±.04
Day 5 CL	.09±.04	.04±.03
Day 11 CS	.21±.05	.20±.08
Day 11 CL	.19±.04	.14±.10
Day 60 CS	.12±.05	.20±.1
Day 60 CL	.12±.02	.15±.09
Day 120 CS	.18±.9	.14±.02
Day 120 CL	.19±.10	.22±.07

6. Anastomotic Complications and Diameter

There was one anastomotic leak in the colostomy (COL) animals compared to no leaks in control (CON) animals. The anastomotic diameter was significantly more narrow for colostomy stapled anastomoses than control (non-diverted) stapled anastomoses (CON, 4.44±1cm vs 2.85±.62, $p<.001$). However, the anastomotic ratios were similar (CON, 0.41 vs COL, 0.43). The diameter for handsewn anastomoses was also more narrow for diverted animals (COL, 3.14±.31 vs CON, 4.09±1.46) which did not achieve statistical significance. The ratios were again similar (0.48 vs 0.41).

C. <u>SECTION III</u>

1. <u>Phase I</u>

a) <u>General Data on Critical Quantitative Values</u>

The criteria for impaired anastomotic healing were leak, stenosis, or bursting pressure <100mmHg. Using these criteria we retrospectively identified quantitative values associated with critical levels of ischemia, (LDV ≤200mV; pHi ≤7.0 pH units), (Table 12).

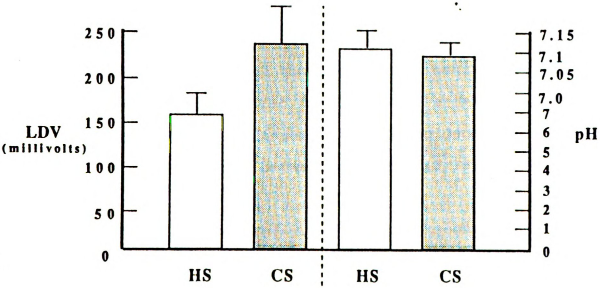
TABLE 12, SECTION III: Phase I, Comparison of Anastomotic Outcome with LDV and pHi Data in Phase I

	LDV (mv)		pHi	
	>200	<200	>7.0	<7.0
"Good" healing	5	0	5	0
Leakage or stenosis	1	2	0	3

LDV=laser doppler velocimetry
pHi=intramural pH

These critical values (<200mV, <pH7.0) were tested prospectively in phase II of the study. In phase I, there was no significant difference identified for either pHi or LDV readings when we compared HS to CS techniques (Fig. 7).

Figure 7: A comparison of laser dopper velocimetry (LDV in millivolts) and tissue pH (Khuri pH probe) determinations in the Phase I study. There were four animals in each of the hand sewn (HS) and circular stapled (CS) groups. No statistical significance existed between groups.



The average intramural pH (pHi) in these pilot animals was 7.06 (6.6-7.40) significantly less than the average arterial pH of 7.39 (7.23-7.48), ($p<0.05$, t-test).

2. Phase II

a) General Data and Complication Rates

Thirteen animals (N=13) were randomized to the HS technique and 14 to the CS technique. The standardized ischemic insult utilized in this portion of the study induced a 70% (19/27) incidence of anastomotic healing complications. At 5 and 11 day intervals, contained small leaks and decreased bursting pressures were predominantly seen (11/14). By 21 and 60 day intervals, stenosis was the predominant healing complication and occurred in 53% (7/13) animals. There was no significant difference in the incidence for healing complications between handsewn (9/13) versus CS (8/14) anastomoses.

Overall, LDV correctly predicted subsequent anastomotic healing outcome (Table 12) in 70% (19/27). This is contrasted with the significantly greater accuracy ($p<0.05$, Fishers exact t-test) of pHi at 93%

(25/27). The positive and negative predictive values were also greater for pHi (Table 13).

TABLE 13: Predictive Accuracy of LDV and pHi, Phase II

	Overall Accuracy
LDV	19/27* (70%)
pHi	25/27 (93%)

*p<.05 Fisher's exact t-test

IV. SECTION IV

1. General Data

Twenty-four (N=24) mixed breed female pigs were used in this portion of the study. All animals received 4,500cGy of preoperative radiation (RT) over four and a half weeks.

2. Laser Doppler Velocimetry Data (Controls vs RT)

The initial blood flow data for the RT animals compared to control animals demonstrated significant decrease in seromuscular anastomotic blood flow at all levels (Table 14).

TABLE 14: Initial Blood Flow (mV) Control vs. Radiation Therapy (RT)

		Prox		At	
		CL	CS	CL	CS
Control	(N=24)	644±251	627±187	345±151	329±181
RT	(N=24)	409±147*	391±103*	181±68*#	170±59*#

		DIST	
		CL	CS
Control	(N=24)	578±131	545±230
RT	(N=24)	382±77*	375±70*

*p<.05 T-test (comparing control to RT at each level)
#p<.05 ANOVA (comparing prox, at, and distal for each technique)

The blood flow data for the second surgery at 5, 11, 60 or 120 days revealed a persistent decrease in blood flow at the anastomotic line in irradiated animals when compared to controls (Table 15).

TABLE 15, SECTION IV: Blood Flow (mV) 5-120 day, Control vs. Radiation Therapy

Control

Post-Op Day	Prox	At	Dist
Day 5CL(N=3)	579±346	404±27	485±74
Day 5CS(N=3)	468±201	341±91	392±135
Day 11CL(N=3)	569±114	442±126	498±105
Day 11CS(N=3)	579±186	525±182	424±154
Day 60CL(N=3)	356±80	213±40	250±89
Day 60CS(N=3)	331±103	228±78	344±33
Day 120CL(N=3)	528±145	483±199	541±176
Day 120CS(N=3)	654±50	643±22	661±39

RT

Post-Op Day	Prox	At	Dist
Day 5CL(N=3)	304±127	165±100*	289±150
Day 5CS(N=3)	155±76*	77±27*	157±129*
Day 11CL(N=3)	360±88	197±39*	378±124
Day 11CS(N=3)	353±165	326±232*	362±260
Day 60CL(N=3)	221±120	110±79*	188±130
Day 60CS(N=3)	225±92	171±69*	221±127
Day 120CL(N=3)	290±142*	224±193*	320±157*
Day 120CS(N=3)	240±16*	144±60*	280±81*

*p<.05 t-test (comparing control to RT at each level in paired fashion)

CL=2 layer handsewn CS=circular stapled

3. Bursting Pressure

There was no significant difference between bursting pressures regardless of time or technique (Table 16).

TABLE 16: Bursting Pressure (mmHg) Radiation (RT) vs. Control, Day 5 and Day 11

Post-Op Day	Radiation	Control
CS 5	217±56	213±57
HS 5	195±48	188±54
CS 11	196±51	188±53
HS 11	201±48	191±43

4. Gross Inflammatory Scoring

Gross inflammatory scores were considerably higher in irradiated animals versus controls through Day 60 but by Day 120 there was no significant difference (Table 17).

TABLE 17: Gross Inflammatory Score (Max=24): Radiation vs. Control

Post-Op Day	Radiation	Control
Day 5 CS	10.0±4.3*	4.2±1.7
Day 5 HS	10.6±1.2*	2.6±0.9
Day 11 CS	11.0±3.6*	2.0±0
Day 11 HS	10.3±2.1*	2.6±0.5
Day 60 CS	4.6±1.2	3.6±0.6
Day 60 HS	7.0±2.0*	2.6±0.6
Day 120 CS	6.0±1.0	6.6±1.2
Day 120 HS	4.3±0.6	4.0±0

*p<.05 Rank Sum Test (comparing RT to Control)

5. Hydroxyproline Content

TABLE 18: Hydroxyproline Content

Post-Op Day	RT	Control
Day 5 CS	.13±.02	.07±.04
Day 5 CL	.11±.06	.04±.03
Day 11 CS	.11±.05	.20±.08
Day 11 CL	.13±.03	.14±.10
Day 60 CS	.10±.01	.20±.1
Day 60 CL	.11±.04	.15±.09
Day 120 CS	.18±.06	.14±.02
Day 120 CL	.21±.09	.22±.07

6. Anastomotic Complications

There was a significant increase in anastomotic leaks or stenosis in the RT group compared to normal controls (RT:7/24 vs Control:2/24, $p<.05$ Fishers exact test).

SECTION V

1. Laser Doppler Velocimetry Data (Saline, ATP-30, ATP-60)

Comparing blood flow data at the time of initial surgery for saline, ATP-30, and ATP-60 animals revealed a significant improvement in perianastomotic blood flow in the laboratory (Table 19).

TABLE 19, SECTION V: Initial Blood Flow (mV) Saline, ATP-30, ATP-60

	Prox	At	Dist
Saline(N=12)	410±147	181±68	382±77
ATP-30(N=12)	327±126	191±129	383±191
ATP-60(N=12)	586±243*	379±152*	601±175*

ATP-30=30μmol/kg/dose ATP-60=60μmol/kg/dose
p<.001 ANOVA (comparing saline, ATP-30, and ATP-60)

This difference, however, did not persist at days 5 or 11 (Table 20 and 21).

TABLE 20, SECTION V: Blood Flow (mV) Day 5, Saline, ATP-30, ATP-60

	Prox	At	Dist
Saline(N=12)	237±114	121±82	223±145
ATP-30(N=12)	186±30	111±46	226±46
ATP-60(N=12)	226±65	184±46	265±97

ATP-30=30μmol/kg/dose ATP-60=60μmol/kg/dose

TABLE 21, SECTION V: Blood Flow (mV) Day 11 Saline, ATP-30, ATP-60

	Prox	At	Dist
Saline(N=12)	403±183	262±164	367±186
ATP-30(N=12)	311±118	223±177	297±155
ATP-60(N=12)	215±82	146±79	238±66

ATP-30=30μmol/kg/dose   ATP-60=60μmol/kg/dose

2. Gross Inflammatory Scoring

Gross inflammatory scoring was also significantly less at second surgery in the ATP-60 group of animals (Table 22).

TABLE 22, SECTION IV: Gross Inflammatory Score (Max=24) Saline, ATP-30, ATP-60

	Day 5	Day 11
Saline	10.3±2.9	10.7±2.7
ATP-30	7.2±2.3	6.8±3
ATP-60	6.8±1.9*	6.0±1.9*

ATP-30=30μmol/kg/dose   ATP-60=60μmol/kg/dose
*p<.001 Rank Sum Test (comparing ATP-60 to saline)

3. Incidence of Cutaneous Lesions in Radiotherapy Portals (Saline, ATP-30, ATP-60)

Additionally, the incidence of cutaneous side sores in the radiation portals was much less in the ATP-30 and ATP-60 groups (saline: 9/12; ATP-30:0/12*; ATP-60:2/12*; p<.001 Fisher's exact test).

4. Hydroxyproline Data Saline, ATP-30, ATP-60

Comparing hydroxyproline data at days 5 and 11 revealed a significant increase in hydroxyproline content at the anastomotic line at day 5 and 11 for the ATP-2 group compared to the other two groups (Table 23).

TABLE 23, SECTION V: Hydroxyproline (mg/ml) Day 5 and 11 (Saline, ATP-30, ATP-60)

	Day 5	Day 11
Saline	0.12±.05	.12±.04
ATP-30	.06±.02	.11±.05
ATP-60	.24±.05*	.20±.03*

ATP-30=30μmol/kg/dose ATP-60=60μmol/kg/dose
*p<.05 ANOVA (comparing saline, ATP-30, and ATP-60)

5. Bursting Pressure

There was no significant difference in bursting pressures between the three groups of animals (Table 24).

TABLE 24: Bursting Pressure (mmHg) Saline, ATP-30, ATP-60

Post-Op Day	Saline	ATP-30	ATP-60
CS 5	217±56.2	198±46.3	186±47.3
HS 5	195±47.5	187±49.1	189±51.4
CS 11	196±51.3	210±50.3	200±49.1
HS 11	201±47.8	221±53.2	202±53.7

6. Incidence of Leak or Stenosis

There was no significant difference in the incidence of leak or stenosis between the three groups of animals (Saline:3/12; ATP-30:4/12; ATP-60:5/12).

IV. DISCUSSION

A. General Discussion of Colorectal Anastomotic Healing

The incidence of colorectal cancer continues to increase with over 43,000 cases predicted in 1988.[58] The majority of these patients will undergo colorectal resection of the tumor and primary anastomosis. In addition, approximately 112,000 patients underwent colorectal resection and anastomosis for inflammatory bowel disease or complications of diverticular disease in 1983 alone.[63] These facts, coupled with the high incidence (2-65%) of anastomotic complications, emphasize the need for greater understanding of wound healing in the colorectal anastomosis.[28-30,32-35] In particular, a careful study of the patterns of healing in a mechanically stapled anastomosis (CS) versus the standard two-layer hand sutured technique (CL) may allow identification of attributes of each technique for use in specific situations.

The colorectum was chosen as the site to evaluate the CL and CS techniques because of the relatively high incidence of leak and stenosis associated with this location compared to other areas of the GI tract.[32-35] The colorectum is an especially hostile environment for anastomotic wound healing because it is a watershed area for blood flow, as there is a transition from visceral arterial supply to systemic arterial supply. Another risk factor is performance of anastomoses at or below the peritoneal reflection, as the absence of the serosa may affect anastomotic healing.[29] In addition, unique clinical problems in the region greatly increase the hazards of anastomotic healing such as diverticular abscess, inflammatory bowel disease and neoplasms. Therefore, the study was designed to allow comparison between the two anastomotic techniques in normal colorectum, and in situations which are potential causes of adverse anastomotic healing.

Wound healing in the gastrointestinal tract follows the same natural history as wounds elsewhere in the body, with three stages: 1) lag phase (0-5 days); 2) period of rapid collagen synthesis (5-17 days); 3) remodeling phase (17 days-2 years).[5] The lag phase is dominated by an inflammatory response during which necrotic tissue is removed.

At approximately day 5 the rate of collagen synthesis begins to increase dramatically and this continues at a high rate for approximately 17 days.[5]

During this period, tensile strength increases correspondingly. However, the ability of the fibroblast to produce collagen is dependent on delivery of nutrients, especially oxygen. Initially, oxygen is delivered by diffusion but over a 3-5 day period, neovascular in-growth allows return to more normal levels of blood flow and nutrient delivery.

The period of collagen remodeling begins at approximately day 17 with collagen cross linking accounting for further increases in wound tensile strength.[5] During this time period, an equilibrium is reached between collagen production and collagen degradation so that no net increase in collagen content occurs after this time.

Because surgical technique impacts heavily on the degree of inflammation and subsequent pattern of anastomotic healing, it is important to elucidate any difference in healing between a two-layer handsewn and circular stapled anastomosis. A potential advantage for the stapled anastomosis is that it is formed using relatively inert metal staples which theoretically cause minimal inflammatory reaction compared to the suture material.[4]

B. Healing in the Normal Colorectum

The present study (Section I) is the first to directly compare an inverted two-layer handsewn anastomosis (CL) with an inverted circular stapled (CS) technique. We were unable to identify any significant differences in the pattern of healing for these two techniques. Unexpectedly, gross and microscopic grading of inflammation at the anastomosis was similar at the intervals assessed (days 3, 5, 11, 60, or 120, Table 3, page 40 and Table 4, page 41). When we specifically examined the degree of mucosal ulceration and subsequent submucosal fibrosis, there was no significant difference between these two techniques. Additionally, bursting strength (page 41) and hydroxyproline content (Table 5, page 41) were similar for the two techniques. Therefore, it appears that both anastomotic techniques cause similar degrees of anastomotic inflammation without significant effect on the pattern or quality of healing. In fact, based on these data the pattern of both CL and CS techniques are identical. Initially there is an inflammatory reaction and sloughing of the inverted anastomotic line, leading to ulceration, which then heals by epithelial ingrowth (second intention healing) irrespective of the technique.

These results are contrary to those reported by Ravitch et al who described primary healing (initial mucosal apposition without ulceration) along the sutured anastomotic line.[14] A significant difference exists between the stapling techniques used in Ravitch's work compared to the present study. Ravitch used a TA stapler to create an everted staple line thus providing mucosal apposition at the time the anastomoses is created and allowing healing by first intention. The inversion created by both the EEA stapler and 2 layer HS technique create identical pathophysiologic changes at the anastomotic line resulting in ulceration of the mucosa at the anastomosis requiring healing by second intention.

C. Anastomotic Ischemia

Another factor which can affect early wound healing is the relative ischemia at the anastomosis. The anastomotic line is dependent on diffusional delivery of nutrients, and in particular, oxygen, to survive until neovascularization can occur. The LDV data (Table 1, page 38) confirms the fact that microvascular blood flow is diminished at the anastomotic line and that this decrease is similar with either CL or CS techniques. This is in accordance with the findings of Chung et al who identified acute decreases in mucosal blood at the anastomosis with either stapling or suturing.[41,42] An interesting finding was the significant decrease in the blood flow 1cm proximal and 1cm distal to the anastomosis for the CL technique when compared to CS (Table 1, page 38). This may reflect a greater degree of adjacent tissue trauma due to handling during the performance of a handsewn anastomosis. In a precarious situation, stapling may provide an edge with respect to preserving blood flow locally at the anastomosis by minimizing tissue trauma due to handling during surgery. We did note, however, that by day 3 (Table 2, page 39) perianastomotic blood flow had improved in both types of anastomoses without significant differences between techniques.

Although ischemia at the anastomotic line has been proposed as the predominant pathophysiologic mechanism for impaired healing, it has been difficult to quantitate clinically. Currently, it is difficult clinically to quantify critical levels of ischemia in the GI tract which result in irreversible tissue injury. Non-quantitative data regarding gastrointestinal blood flow may be obtained by gross inspection, fluorescein injection, or doppler ultrasound.[41,42] Although, these techniques are readily available, the qualitative nature of the data is inadequate to allow identification of critical levels of tissue ischemia and thus accurate prediction of successful anastomotic healing.

Laser doppler velocimetry offers the advantage of continuous quantitative measurement of blood flow in vivo at any portion of the GI tract which can be exposed at surgery.[43,46-48] Use of this instrument on the gastrointestinal tract is hampered by peristalsis which impairs accurate optical coupling between the probe and bowel surface. Intermittent compression of the bowel wall by excess pressure applied to the probe can drastically alter local blood flow measurements in the bowel wall by inducing local ischemia followed by a reactive hyperemia.

We compared LDV with the Khuri tissue pH probe which also provides quantitative data regarding adequacy of tissue perfusion as the degree of tissue acidosis correlates well with impairment of oxygen delivery.

Using the Khuri pH probe we were able to predict subsequent impairment of anastomotic healing based on initial intraoperative tissue reading in the perianastomotic region. The predictive accuracy (Table 13, page 50) obtained with this device (93%) was significantly greater than was obtained with LDV (70%). Additionally, we did not identify any significant differences in healing of the ischemic anastomosis between handsewn or circular stapled techniques. Tissue pH measurements allowed us to reproduce a consistent ischemic insult which resulted in impaired anastomotic healing in 63% of animals without frank gangrene of the bowel segment. Therefore, this tool can be used to reproduce a model of anastomotic stenosis.

Because of its ease of use, tissue pH determination may be very helpful in a number of clinical situations when prediction of subsequent tissue viability is difficult and highly desirable. It could be used as an indicator of critical levels of ischemia at a worrisome anastomosis as may be encountered following low anterior resection or in resections for mesenteric arterial or venous occlusion. It could also provide useful information following reduction of a strangulated obstruction or following revascularization of acute mesenteric ischemia, possibly limiting the amount of bowel resected or avoiding an unnecessary high risk second look laparotomy. A worrisome colostomy could easily be monitored at the bedside using this technique. This instrument also provides the ability to longitudinally follow tissue pH, as the probe could be implanted seromuscularly at an impaired segment of intestine with the probe wire brought out through the abdominal wall much like a pacemaker wire used in cardiac surgery, allowing one to identify progression or regression of intestinal ischemia at the bedside over hours or days.

D. Effects of Preoperative Pelvic Radiotherapy

Preoperative adjuvant radiation therapy in the range of 4-5,000 rads, as utilized in this study, has demonstrated significant beneficial effects in rectal cancer patients.[48-52] These data have demonstrated tumor shrinkage or ablation, a decrease in the incidence of metastatic disease local/regional lymph nodes, and a decrease in local recurrence rates. Additionally, it should be noted that current experience has shown these dosages to be well tolerated by patients. Widespread acceptance of this adjuvant treatment modality has been slowed over concerns of increasing anastomotic complications.

The pathophysiology of anastomotic healing complications induced by radiotherapy is not well studied. One mechanism for impaired anastomotic healing, following radiation therapy, appears to be direct cellular injury and death with healing occurring by fibrosis. Crowley demonstrated a significant early inflammatory reaction with loss of epithelial cells, reduction of villous height, and submucosal edema.[59] He noted, however, that by 22 days these effects had subsided almost entirely, thus the reason for the current recommendation of a three week rest period prior to surgical intervention. The results of this study indicated a significant increase in anastomotic inflammatory reaction at the 5 and 11 day intervals for irradiated animals (Table 17). Therefore, it is demonstrated that inflammatory reaction is increased at a time when one would assume that the majority of the effects of radiation exposure had subsided.

Although progressive mural ischemia has often been mentioned in the surgical literature, there has never been an attempt to quantify the magnitude of mural ischemia induced by therapeutic radiation exposure. Using laser doppler velocimetry we were able to confirm and quantify a significant decrease in seromuscular blood flow which occurred as early as four weeks following completion of the radiation treatment regimen (Table 14, page 51). Again, it should be recalled that this was identified at a time that the majority of the inflammatory reaction induced by radiation exposure should have subsided. We also noted that the level of anastomotic ischemia persisted as long as four months following exposure to the radiation protocol (Table 15, page 52). There did not appear to be any significant difference in perianastomotic blood flow based on the anastomotic technique used. We were also able to identify a significant increase in anastomotic complications in the irradiated group of animals (RT: 7/64; Control: 2/24 $p<.05$ Fishers Exact Test).

E. Effects of ATP-$MgCl_2$ on Adverse Effects of Radiotherapy

Although a significant decrease in anastomotic blood flow associated with preoperative RT was identified, the mechanism remained unclear. Assuming that the majority of injury was due to direct cellular injury to mesenchymal stem cells followed by healing in the form of perivascular fibrosis and an endarteritis attempts to reduce this injury with ATP-$MgCl_2$ were made. ATP-$MgCl_2$ has demonstrated beneficial cytoprotective effects during ischemic insults to the liver, kidneys and myocardium during and following low flow conditions.[61-63] Additionally, in vitro data demonstrates its ability to diminish renal toxicity from cis-platinum, a chemotherapeutic agent.[64] We found that administering 60μmol/kg of ATP-$MgCl_2$ during each treatment session of radiation therapy markedly reduced many of the measurable side effects. Anastomotic blood flow at the initial surgery was significantly improved (Table 19, page 55). The degree of gross perianastomotic inflammation was diminished (Table 22, page 56), the incidence of cutaneous lesions was reduced, and hydroxyproline content (Table 23, page 57) was significantly greater in the 60μmol/kg ATP-$MgCl_2$ group of animals. Therefore, these data indicate that ATP-$MgCl_2$ at a minimum dose of 60μmol/kg diminishes many of the adverse effects of preoperative radiation therapy in colorectal anastomoses. This potential benefit coupled with its exciting antineoplastic abilities to increase permeability in transformed cells and to halt their growth in the S-phase of the cell cycle may result in a new, less dangerous combination adjuvant treatment protocol.[66-68]

F. Effect of Proximal Colostomy

Stenosis

Stenosis or anastomotic narrowing is a well recognized complication of circular stapled colonic anastomoses. Several investigators have proposed that a proximal diverting colostomy increases the risk of anastomotic narrowing.[28-30,32-35,45] Waxman and Ramsey using an ewe model found significant narrowing in diverted stapled anastomoses independent of stapler diameter.[35] Similarly, Buchmann found that diverted stapled anastomoses had greater submucosal fibrosis than sutured anastomoses.[34]

In the current project the incidence of stenosis was similar for both diverted (1 CL; 0/CS) and colostomy animals (0/CL; 1 CS). Although the stapled colostomy animals had significantly more narrow anastomoses (2.86cm versus 4.44cm, $p<.001$) there was no significant difference between the anastomotic ratios between diverted and nondiverted animals (0.43 versus

0.48). Similarly, the handsewn anastomoses were more narrow in diverted animals (3.14cm versus 4.09cm) although this difference was not significant and again the anastomotic rations (0.48 versus 0.40) were similar. Most important, however, is the fact that there was no significant difference between handsewn or stapled anastomoses with or without fecal diversion. Proximal diversion simply narrowed the entire length of distal colon rather than any specific effect on the anastomosis. Similarly, there was no significant difference with respect to anastomotic blood flow (Table 7 & 8, page 43-44), hydroxyproline content (Table 11, page 46), or bursting pressures (Table 9, page 45) with or without diversion.

Therefore, the results indicate that proximal diverting colostomies do not inherently change the pattern of colorectal anastomotic healing. It is more likely, however, that the initial indication for diversion (ie. lack of prepared bowel, local infection, or shock state) is the etiologic factor in adverse anastomotic healing. Specifically, proximal diversion does not increase the risk of poor anastomotic outcome when the circular stapler is used to reconstitute bowel continuity, as has been purported in the literature.[34,35]

V. CONCLUSIONS

1. Circular stapled (CS) and two-layer handsewn (CL) anastomotic techniques produced similar levels of anastomotic ischemia as measured by laser doppler velocimetry.

2. There is no difference in the incidence of anastomotic healing complications (leak or stenosis) between CS and CL techniques.

3. Healing of the mucosal aspect of CS and CL anastomoses occurs by second intention. This work is contrary to data obtained by Ravitch (1967) that stapled anastomoses healed by primary intention and handsewn anastomoses healed by second intention.

4. Proximal fecal diversion did not increase the risk of anastomotic stenosis or leak regardless of anastomotic technique. In addition, bursting pressures and hydroxyproline content at the anastomosis were similar with or without fecal diversion.

5. Tissue pH determination (pHi) is superior to laser doppler velocimetry (LDV) in predicting tissue viability following an ischemic insult based on quantitative data.

6. Controlled anastomotic ischemia using pHi allows reproduction of consistent ischemic insult to produce a high incidence of anastomotic stenosis without frank gangrene of the segment.

7. There is no significant difference in the incidence of healing complications in the face of ischemia between CL and CS techniques.

8. Preoperative external beam pelvic irradiation (4500cGy) produced an early and persistent decrease in seromuscular blood flow compared to non-irradiated colon.

9. Preoperative radiotherapy significantly increases the incidence of anastomotic healing complications compared to control animals.

10. ATP-$MgCl_2$ in doses of 30µmol/kg or 60µmol/kg administered intravenously during each radiotherapy treatment session significantly decreased the incidence of cutaneous lesions in the radiation portals compared to saline treated animals.

11. ATP-$MgCl_2$ (60µmol/kg) if used during each RT treatment session significantly increased early Hydroxyproline production at the anastomosis at day 5 and 11.

12. ATP-$MgCl_2$ (60µmol/kg) infused during each RT treatment session increased anastomotic seromuscular blood flow at the time of initial surgery.

13. ATP-$MgCl_2$ (60µmol/kg) infused during each RT session significantly decreased postoperative gross inflammatory scores at day 5 and day 11.

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LIST OF REFERENCES

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APPENDICES

APPENDIX A

A. PRESENTATIONS

1. Colonic Mural Blood Flow: The Effects of Radiation and Differing Anastomotic Techniques.

   Resident Competition, 1988
   Society of University Surgeons
   San Antonio, Texas

   Annual Meeting FASEB, 1988
   Las Vegas, Nevada

2. Intramural pH: A Quantitative Measurement for Predicting Colorectal Anastomotic Healing.

   Annual Research Forum, April 1988
   Michigan State University
   East Lansing, Michigan

   GRAMEC Annual Research Day, May 1988
   Grand Rapids, Michigan

   American Society of Colon and Rectal Surgeons, Annual Meeting, June 1988
   Anaheim, California

3. Reduction of Preoperative Intestinal Radiation Injury by ATP-Magnesium Chloride.

   Residents Program, February 1988
   Society of University Surgeons

4. Does Proximal Colostomy Affect Colorectal Anastomotic Healing?

   Poster Presentation
   American Society of Colon and Rectal Surgeons, Annual Meeting, June 1989
   Birmingham, England

5. A Direct Comparison Between Czerny-Lembert and Circular Stapled Anastomotic Techniques in Colorectal Anastomoses.

   American Society of Colon and Rectal Surgeons, Annual Meeting, June 1989
   Toronto, Canada

APPENDIX B

B. PUBLICATIONS

1. Senagore A, Milsom JW, Mazier WP, Walshaw R, Chaudry IH. Colonic Mural Blood Flow: The Effects of Radiation and Differing Anastomotic Techniques. FASEB 1988; 2(4):A742.

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3. Reduction of Preoperative Intestinal Radiation Injury by ATP-$MgCl_2$ Administration. Senagore A, Milsom JW, Walshaw RK, Mostosky UV, Chaudry IH. FASEB 1989;3(3).