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TITLE: WOUND HEALING: DEVELOPMENT OF TENSILE STRENGTH VS. TIME FOR WOUNDS CLOSED UNDER TENSION IN RATS

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Would Healing: Development of Tensile Strength vs. Time for Wounds Closed under Tension in Rats

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FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council (DHHS, PHS, NIH Publication No. 86-23, Revised 1985).

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WOUND HEALING: DEVELOPMENT OF TENSILE STRENGTH VS. TIME FOR WOUNDS CLOSED UNDER TENSION IN RATS

ABSTRACT:

Previous studies have shown wound tensile strength to be greater in wounds closed under tension. The purpose of this study was to delineate a post-operative time interval when this difference becomes evident. Ninety-four Sprague-Dawley rats were divided into five groups- 5, 7, 10, 14 and 21 days. Transverse incisions on the backs of control rats were closed with minimal tension. Experimental animals had skin excised to create closing tensions in excess of 70 grams. Sutures in all of the rats were removed at five days and animals were sacrificed at the designated healing intervals. Tensile strength was not significantly different at five days. However, wounds closed under tension showed significantly higher tensile strength at 7, 10, 14 and 21 days. Polynomial regression reveals a cubic relationship between healing time and tensile strength where there are two periods of rapid increase wound strength. These results suggest that tensile strength of wounds closed under tension exceeds that of tensionless wounds as early as seven days following surgery.

INTRODUCTION:

In the surgical literature, a great deal of attention is dedicated to the effects of tension on wound healing. Some wounds, where extensive amounts of tissue are missing, must be closed under tension. It is the surgeon's duty to identify ways of preserving wound strength while affording the best cosmetic result. Not only is a wound's ultimate strength and scar width of interest, but the rate at which these characteristics develop is also important. For example, defining the time period when tensile strength of wounds under tension exceeds that of wounds under no tension, gives the surgeon directives about the length of time that suture support will be required. In addition, information can be gained about the expected interval when full function can be resumed safely.

Most of the surgical literature supports the notion that wounds subject to dynamic or static separating forces ultimately assume greater strength, a desired quality of a wound. Utilizing sensitive measurements of closing tension, resultant breaking load and wound thickness, Morin\(^1\) showed significant increases in wound strength at 28 days of healing when closing tensions exceeded 84 grams. The question that remains is at what period in the healing process do wounds under tension become stronger than wounds closed with no tension? And, is there a linear or exponential equation that describes the relationship between time and tensile strength of wounds closed with or without tension.

The following study uses the same methodology as that used by Morin to study the time course of healing in tension and tensionless wounds. Studying wounds at specified healing times, we hypothesize that wounds closed under no tension will initially have higher tensile strength. At some time interval however, wounds under tension become as strong and subsequently stronger. This study attempts to estimate that interval. The implications for wound closure and further study are discussed.

DEFINITION OF TERMS:

Closing Tension (grams as weight) is equal to the sum of the forces required to bring each wound edge to the closing point.
Tensile Strength (MPa) is equal to the breaking load divided by the cross-sectional area of the scar.

MATERIALS AND METHODS

One hundred rats were chosen based on levels of significance reported in Morin's study. Sprague-Dawley rats of the male sex weighing approximately 250-350gm received a pellet diet and water ad libitum. The rats were kept in metal cages allowing motion without running. After acclimation to the laboratory environment for one week animals were divided into five groups with healing times of 5, 7, 10, 14, and 21 days. In each time period there were ten experimental and ten control animals. Weight at the time of sacrifice was controlled between tension and control groups since this has been shown to be a factor in wound tensile strength. All surgical procedures and post-operative care were conducted in the Department of Clinical Investigations at WRAMC by the principal and associate investigators with the help of technicians.

Rats were anesthetized with ketamine (44mg/kg IM) and xylazine (2.5mg/kg IM) prior to the procedure. Following the induction of anesthesia, the back of each rat was shaved, prepped with Betadine solution, and washed with alcohol. A single 4cm transverse skin incision through the panniculus carnosus was made midway between the sacrum and occiput. Previous reports show that this transverse incision runs parallel to relaxed skin tension lines on the backs of rats and other small rodents. The ends of this incision were connected to a more caudal second transverse incision. Closing tensions in excess of 80 grams are required before wound tensile strengths are statistically greater than controls. Thus, widths of 50-60mm of skin between incisions were excised on experimental rats to produce closing tensions of 70-120 grams. Control rats receive a single transverse incision without skin excision.

Force required to move each skin edge to the closing line was measured with strain gauges. The gauges were calibrated with measured weights on a Metier balance. The strain gauge sensitivity (half the scale) is 1-5 grams. All measurements were repeated three times and the means from each side were added together to arrive at the closing tension.

Wounds were closed with a running-locking 5-0 Nylon suture, cleansed with hydrogen peroxide, and dressed with Bacitracin ointment. At 5 days the skin sutures were removed while securing the rat with a gloved hand. At the specified time intervals, rats were euthanized prior to scar removal and the scars were pinned on a cork board like their in vivo dimensions.

Three-five specimens were excised from each intact wound using an acrylic block with razor blades spaced at a fixed distance of 5mm. After excising the scar, the skin was wrapped in foil, frozen in liquid nitrogen (-224°C), stored in a carbon dioxide cold storage chamber (-92.8°C), and tested within 10 days of freezing. Prior to testing, specimens were placed in a saline bath at room temperature for two minutes. This method of freezing has been shown to maintain the tensile strength of specimens without deterioration. Scar thickness (vertical height) was determined by a pressure sensitive caliper as previously described and the Instron tensiometer measured specimen breaking load. Tensile strength was calculated by dividing the breaking strength by the scar's surface area (5mm X scar thickness) and reported in MPa. These measurements were performed by J. Vossoughi, MS, at Catholic University. The average of tensile strength for each wound was used for statistical analysis. Scar thickness and breaking load determinations were not subject to observer bias, as readings are determined mechanically.

Results were analyzed using the non-paired student t-test, to compare experimental and control groups at the various time periods. In addition, a curve was generated using a least squares linear regression and polynomial regression analysis, plotting time against resulting tensile strength for the two groups. Multiple regression was used to examine the influences of other variables on the final results.

RESULTS:

Average values for initial weight, sacrifice weight and closing tension for experimental and control animals are presented in table I. Even though strain gauges used for control animals were accurate to 1 gram, closing tensions in these animals were almost immeasurable. Some tension could be measured however, so closing tension was record-
ed in ten control animals in the 5 day group. The average value, 4.40 grams (sd=1.80), was used as the closing tension in control groups at 7, 10, 14 and 21 days. Closing tensions in experimental groups ranged from 68.2 to 126.8 grams. In rats where excision of skin resulted in closing tensions greater than 130 grams, the animals were excluded from the experiment. Three rats in the experimental group were excluded when wound dehiscence occurred prior to day 5. These wounds dehisced when animals removed their own sutures on the second and third post-operative day. Otherwise, when sutures were removed at day 5, all wounds remained closed until the sacrifice date. Another three rats died from over sedation in the first postoperative day. There were no wound infections and small hematomas were noted at wound harvest in three animals (a 5 day control, a 7 day tension and a 10 day tension). These were felt to have no appreciable effect on wound healing. All wound samples remained stable throughout the harvesting process. During break load testing, all wounds ruptured at the incision line.

Tensile strength of control and experimental wounds are presented in Table I along with probabilities for unpaired t-values. Bonferroni adjustment for type I error (a) was used to determine a level of significance of p=0.01. No significant difference in tensile strength was noted at 5 days (p=0.12). After day 5 however, all experimental group wounds had significantly greater tensile strength (p< 0.01 at day 7, p=0.0001 at day 10, p=0.01 at day 14 and p=0.0001 at day 21).

To verify that both differences between experimental and control groups and the trends of increasing tensile strength were not the result of variations in rat sacrifice weight or closing tension, multiple regression analysis of tensile strength was carried out. Variables included healing time, sacrifice weight and closing tension. In this study, tensile strength was influenced by healing time, with variations in closing tension in experimental groups and sacrifice weight of both groups making insignificant contributions.

The rate of change in wound tensile strength over time was examined in control and experimental groups. Using linear regression, the correlation coefficients for both groups was 0.8 with the slopes of these lines being 58 MPa/day in the control group and 104MPa/day for the experimental group (figures 1 and 2). Polynomial regression however, shows a better fit to a cubic curve in both groups (figures 3 and 4). More specifically, there are two phases where there appears to be rapid increases in wound strength. The first phase occurred between day 5 and day 9, the second after day 16.

Discussion:

We have adopted a method of comparing precise measurements of wound closing tension and tensile strength. In this study we have applied these techniques to define the time period when wounds under tension have greater tensile strength than wounds closed without tension. In a previous study,1 the same techniques showed that wounds closed with greater than 84 g of tension showed greater tensile strength at 28 days. Prior studies failed to measure both closing tension and tensile strength.3, 13, 14, 25, 26, 27

The question of what biological events occur that cause wounds under tension to become stronger is best approached after a review the stages of wound healing. The first event, injury, is in this case a surgical incision. The coagulation phase of wound healing is mediated by platelets. Thrombus formation results in release of platelet factors. These factors initiate fibroblast migration to the wound site and subsequent fibroblast proliferation. Additionally these factors stimulate local vasodilatation and increase permeability which leads to the next phase of wound healing, inflammation. Here neutrophils and monocytes influx initiating a propagating inflammatory reaction. Uncertainties arise about the function of macrophages, lymphocytes and neutrophils in wound healing, but it appears that in concert, leukocytes have a role in regulating scar formation. Macrophages ingest bacteria and debree. They also release factors that stimulate collagen synthe-
Neutrophils combat bacteria, however their function may also be the release of collagenase and counterregulation of collagen proliferation. Studies involving nude mice and depletion of T cells offer conflicting information about the role of lymphocytes in wound healing. Nude mice wounds have greater breaking strength and proportionally greater hydroxyproline content than control mice 2, 3 and 4 weeks post-incision. In contrast, immunocompetent mice were treated with anti-T cell antibody to deplete animals of T lymphocytes, wounds had decreased breaking strength and hydroxyproline content when compared to controls at 2, 3 and 4 weeks. A more thorough review reveals that wound healing during the stage of inflammation is finely regulated by T cell subpopulations, macrophages and neutrophils which produce scars with maximum strength and minimum collagen content. The effects of closing tension on this inflammatory response are not well defined.

In the proliferative phase, collagen seems to be the key to wound strength. Fibroblasts synthesize and secrete procollagen. Extracellularly, end terminals of the individual chains are cleaved in a reaction catalyzed by procollagen peptidase. The resultant protein is called tropocollagen. A characteristic of these long triple helix polypeptide chains is the relatively high concentration of glycine, proline and lysine. Hydroxylation of proline and lysine amino acids on the chains creates hydroxyproline and hydroxylysine respectively. Hydroxyproline plays a role in forming hydrogen bonds to stabilize helical polypeptide chains. Aldol condensation between hydroxylysine residues leads to strong cross-linkages between aligned tropocollagen chains. Tropocollagen chains linked together in a redundant orderly fashion make-up collagen fibers and collagen formation initiates the process of scar formation. Aside from molecular stability of individual collagen fibers, tissue strength reflects the overall arrangement of these fibers. Redistribution and reorientation of collagen fibers during wound healing results in increased wound tensile strength. During this rise in wound strength when collagen degradation and synthesis is in equilibrium, haphazard collagen fibers become more compact, thicken and aligned parallel to one another. In one study, the above changes corresponding to increases in wound strength occur most rapidly between the 14th and 42nd post-operative days. Small increments in wound strength occur for up to one year with maturation of collagen bundles, depending on the species being studied. Seven types of collagen introduce another variable to scar formation, although skin contains mostly type I and III. The final phase of wound healing, contraction, is thought to result from contraction of myofibroblasts. Remodeling of collagen during this stage fixes the scar's dimensions resulting in contracture.

The effects of wound tension on the above stages of wound healing have not been delineated. Various studies have however, evaluated factors that may be involved in increased tensile strength in wounds closed under tension.

Numerous studies have examined the morphology of collagen bundles and fibroblasts in wounds closed under decreased, normal and increased tension. As mentioned above, there are several factors related to collagen structure that impact on wound tensile strength. Obviously abundance of collagen and fibroblasts would seem to be related to wound strength. This is the case in wounds closed under tension early on (up to 16 days), however subsequently these wounds become thinner than controls. Organization and compactness of collagen bundles is probably more important for wound strength after day 16. Studies reveal that wounds under dynamic tension show increased birefringence of collagen under polarized light when compared to wounds without tension. Birefringence suggests the degree of organization of collagen fibers. Comparison of the thickness of collagen fiber bundles in normal dermis and in skin wounds from Guinea pigs shows that fiber width (2-15 microns) in wounded dermis never reaches the width found in normal dermis (12-30 microns). The fact that wounded skin never
reaches that of normal skin implies that there is a relationship between collagen fiber width and the ultimate wound tensile strength. One could speculate that wounds under tension have greater tensile strength because of greater collagen fiber bundle diameter and more colinear, compact organization of bundles.

Composition of collagen may be an additional factor responsible for greater tensile strength of wounds under tension. Madden\(^1\) showed increased wound hydroxyproline specific activity beginning at 4 days in rats injected with radiolabeled proline 24 hours prior to sacrifice. Hydroxyproline and hydroxylysine specific activities in wounds closed under tension has not been studied specifically, but one study investigated the effects of mechanical stress on biosynthesis in aortic medial cells.\(^1\) This study showed that when cultured cells were subject to mechanical stretch, there was increased incorporation of hydroxyproline into collagen. It is reasonable to surmise that the degree of cross-linking is another factor that may result in greater tensile strength in wounds closed under tension.

The effects of leukocytes on wounds under tension is addressed in the literature. While the role of neutrophils in concert with macrophages and lymphocytes on wound healing is not well delineated, laparotomy wounds under tension in rats show increase accumulation of neutrophils.\(^9\) Conversely rat wounds under reduced tension show lesser degrees of inflammation.\(^14\) Future studies might clarify how leukocytes modulate wound healing under tension. The implication is that healing in these wounds can be altered with cyclooxygenase inhibitors and other pharmaceuticals.

Especially in the early healing period (prior to 5 days), blood supply to skin flaps in wounds closed under tension is thought to be one factor that inhibits wound healing. It was shown that closing tensions of greater than 250 grams resulted in flap necrosis in 100% of pedicle flaps in pigs.\(^20\) Diminished flap blood flow measured with laser doppler velocimetry was inversely correlated with closing tension. Wounds in our study were closed under less than 130 grams of tension and skin edges of the transverse incisions were minimally undermined. While vascular dynamics of linear incisions are quite different from those of pedicle flaps, diminished blood flow to wounds under tension must be considered with other factors that affect wound healing. Direct measurements of blood flow to wound edges in wounds closed under tension are lacking in the literature, but it is conceivable that decreased blood flow may be responsible for the attenuated tensile strength in wound under tension in the early healing period.

As discussed above, there are many aspects of wound healing that may be altered by increased closing tension. While examining the time course of healing in wounds closed under tension, this study made no attempt to identify the specific events responsible for the greater tensile strength in the experimental group. Because of the time course however, we can suggest which components may be most important.

In our study, wounds under tension of greater than 65 grams already had tensile strength equal to that of controls by 5 days. These results are consistent with others in the literature. Examining the effects of dynamic tension, Newberger\(^21\) demonstrated increased tensile strength of abdominal wounds by the fifth post-incision day in rats that were exercised. Rats allowed freedom of movement have greater wound tensile strength by the seventh post-surgical day.\(^22\) Cutaneous wounds on the anterior aspect of the knees of rabbits show greater tensile strength by day 21 when subjected to continuous passive motion.\(^15\) Shorter intervals were not tested. Studying the effects of reduced tension on wound healing, Brunius\(^14\) showed greater tensile strength at the earliest test period of 7 days in rats with normal tension. Sussman\(^3\) measured tensile strength after 14 days in cutaneous rats wounds under increased tissue traction. Again, values were greater in the
tension group, however shorter intervals were not tested and closing tensions were not measured. Borgstrom and Nilsson showed diminished wound tensile strength in wounds under tension at 5 days and 15 days respectively. As with most previous studies however, closing tensions were not quantitated. While the time course of healing in aponeurotic wounds does not necessarily correlate with that of cutaneous wounds, a study of abdominal aponeurotic wounds under tension in rabbits shows similar results to ours.13 Tensile strength was no different at 5 days, but significantly greater in the experimental group beginning at 7 days with a plateau at 21 days.

Wounds under tension had significantly greater tensile strength beginning at 7 days. At 10 and 21 days there appeared to be some divergence between control and experimental groups but at 14 days, wound tensile strength in the experimental group approached the limits of level of significance. In fact, when data points comparing tensile strength to healing time are analyzed, there is some suggestion that there is plateauing of the the tensile strength at 14 days. In an effort to define this relationship between tensile strength and healing time, polynomial regression was performed. This showed the best fit to a cubic curve that illustrates the two periods of rapid growth in tensile strength (figure 3 and 4). Two previous studies refer to cutaneous, fascial, muscle and gastric wounds and showed increases in tensile strength that were dependent on the phase of wound healing.23–25 They showed an early quiescent period that corresponded to the coagulative phase when tensile strength was due to sutures. The first rapid rise in tensile strength began at approximately 5–7 days and corresponded to the proliferative phase of wound healing. Reaching maximum at 14 days, there was some plateauing prior to a second rise in tensile strength at around 18–22 days. This corresponded to the phase of maturation which may continue for 60 days or longer. It is likely that our data demonstrate these two rises in tensile strength, and perhaps there is there is some decrement in the difference between control and experimental groups during the plateau period. It may also demonstrate that before 14 days, greater tensile strength in the experimental animal is mostly due to fibroblast proliferation and collagen deposition. After 14 days the difference is more likely related to reorganization and cross-linking of collagen fibers.

To examine any confounding variables, a multiple regression analysis was done. Closing tensions were within the range required to produce significantly stronger wounds according to Morin's study. However, closing tensions correlated poorly with the amount of skin excised. Even while modifying the amount of skin removed, closing tensions varied significantly. This shows how inconsistencies in the literature might exist. Weight of rats has also been found to significantly affect tensile strength. Variations in closing tension and animal sacrifice weight were shown not to be responsible for the difference in wound tensile strength between groups by multiple regression analysis. The significant variables were tension versus non-tension and healing time.

The findings in this study are remarkable in that they provide insight into the time course of wound healing in wounds under tension. Inferences are made about the possible physiologic factors that may be responsible for differences in tensile strength based on timing and pattern of increased strength. Thus, this discussion is meant to stimulate directions for further research. This investigation made no attempts to define the specific factors responsible for alteration in wound tensile strength. While the data may later be related to human skin, this study does not imply that the above time course is applicable to surgical patients. Future studies will explore different animal models such as the pig, where there is a closer relationship to human skin. Studies that use this model to investigate the effects of tissue blood flow, collagen content, collagen orientation and cross-linking, and the effects of biologically active factors will be important in the future.

**BIBLIOGRAPHY:**


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* Statistically Significant
Control Group Linear Regression

\[ y = 58x - 202.2, \quad r^2 + .8 \]
Experimental Group Linear Regression

\[ y = 104x - 399.9, \quad r^2 = 0.8 \]
Control Group Polynomial Regression

\[ y = 596.147 + 198.198x - 14.027x^2 + .397x^3 \]
Experimental Group Polynomial Regression

\[ y = 1935.559 + 621.555x - 49.667x^2 + 1.368x^3 \]