Early Changes in the Tensile Strength and Morphology of Primary Sutured Skin Wounds in Rats

(wound healing / tensile strength measurement / tissue repair and regeneration)

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Abstract. The specific aim of this study was to measure the TS of rat skin wounds during the first week following surgical injury. Biomechanical and histological data were collected daily (days 1 to 7 following surgery) from separate groups of Sprague-Dawley rats (N = 12) each with two 3 cm long parallel skin incisions on the back. The wounds were immediately closed by four simple sutures. A control group (N = 15) was used to obtain TS measurements of unwounded skin. TS was measured by applying a ramp load until wound separation and estimated by dividing the yield strength by the wound area. The time course of biomechanical recovery followed a step-plateau pattern with the largest increase in TS observed one day after surgery $(0 - 1.60$ g/cm^2). The plateau stage extended from day 1 to 5 $(1.60 - 3.88$ g/cm^2). The final step (day 5–7) indicated a period of rapid rise in wound TS $(3.88 - 11.57$ g/cm^2). Since even on day 7 the mean TS was only 4% of unwounded skin, the wound had to be protected from tensile loads. Histological analysis confirmed that the early changes in TS (day 1) correlated with the fibrin accumulation of the wound edges followed by a plateau stage caused by the tissue proliferation. The rapid increase in wound TS was characterized by cross-linking the incisions with collagen fibres with escalating organization. We conclude that from a biomechanical perspective, sutures can be removed during the "plateau phase", but the wound must be protected from tensile loads.

Introduction

It is estimated that the morbidity associated with delayed wound healing increases, which costs the health services over $9 billion per year (Ashcroft at al., 2003). Many factors such as diabetes mellitus, AIDS, bad nutrition as well as chronic application of corticoids may negatively affect the process of wound healing in patients. Therefore, a number of experimental studies deal with new approaches improving the reparative and regenerative processes of tissues (Paul et al., 1997; Menetrey el al., 2000; Medrado et al., 2003; Milgram et al., 2004; Gál et al., 2005). Skin sutures can be removed as soon as the wound is fixed enough to no longer need mechanical support (sutures). Early sutures resection assures a uniquely better cosmetic result (Burkitt et al., 1990). Therefore, tensile strength (TS) of wounds is objective and the preferred method for wound healing evaluation and is often used in numerous experimental studies (Davidson, 1998; Dorsett-Martin, 2004). Nevertheless, no detailed biomechanical study was performed during the fist week of healing. Published studies evaluated biomechanically only certain time intervals or they observed, compared to controls, the influence of various factors on the healing process on selected days (7th and 14th day by Allendorf et al. (1997), 7th, 10th and 20th day by Andreassen and Oxlund (1987), 7th, 10th 14th, 19th 22nd, 28th 29th 84th by Paul et al. (1997) in his biomechanical and biochemical study of a standardized wound healing model, etc.). However, the most significant changes occur during the first week of wound healing and this contributes to the importance of our study (Medrado et al., 2003).

As an experimental animal model we decided to use the rat, since rat skin represents one of the most common models used in experimental studies concerning the skin wound healing. It is a useful model because we can study the healing of three different tissue types (epidermis, dermis and striated muscle) (Menetrey et al., 2000; Vidinský et al., 2006). Only the epidermis has the

capability to regenerate. The repair of injured dermis occurs in three phases: inflammation, proliferation and maturation (Barbul and Regan, 1993). The phases are not strictly separated from each other, their processes freely blend together. The healing process of injured striated muscle includes three phases: the destruction phase, the repair phase and the remodelling phase (Järvinen et al., 2005).

The aim of our study was to establish a detailed biomechanical model of skin wound healing in rats, to find the relationship of TS and morphology, and so chronologically describe this process during the first week. The use of a model is necessary to obtain information on the multifactorial nature of the wound healing process, which may be influenced by externally introduced factors (Gottrup, 2000).

Material and Methods

Animal model

Female Sprague-Dawley rats (N = 99) 8–9 months of age were used in this study. The 84 animals were randomly divided into seven groups of 12 animals per group and used for wound healing evaluation. A separate group of 15 animals was used for the TS measurement of unwounded skin.

A combination of ketamine (Calypsol, Richter Gedeon, Budapest, Hungary), xylazine (Rometar a. u. v., Spofa, Prague, Czech Republic) and tramadol (Tramadol-K, Krka, Novo Mesto, Slovenia) was intramuscularly injected to anaesthetize 84 rats in doses of 40 mg/kg ketamine, 15 mg/kg xylazine and 5 mg/kg tramadol. Atropin was administered subcutaneously as premedication (Atropin, Hoechst-Biotika, Martin, Slovakia) in a dose of 0.05 mg/kg.

Two 4-cm long parallel full-thickness skin incisions were performed under aseptic conditions on the left and right sides of each experimental rat dorsum and immediately sutured by four simple sutures (Chiraflon 3/0, Chirmax, Prague, Czech Republic). Animals were caged individually after undergoing wounding to avoid damage to the wound.

The experimental protocol and animal care were in compliance with the requirements of the Ethics Committee of the Faculty of Medicine of Pavol Jozef Šafárik University in Košice and approved by the State Veterinary Administration of the Slovak Republic.

Wound TS measurement

The device for measuring wound-breaking strength was assembled in our laboratory (Gál et al., 2005). The main component of the device is a stand with a moving arm, which transfers force from the sample to a piezoelectric sensor FSG15N1A (Honeywell International, Minneapolis, MN) (Fig. 1). As the sensor-computer interface we used an intelligent module ADAM 4011 (Advantech Co., Cincinnati, OH). To achieve vertical tensile force, we used a servomechanism with a power supply ± 3V and the range of output force from 0 to 30 N, compatible with the range of the piezoelectric sensor and covering continual tensile force increase to the breaking point of the sample. Software for data processing, recording and analysis was implemented in MatLab.

On days 1 to 7 after the surgery, six animals in each group were sacrificed by ether inhalation and the skin wounds were carefully excised immediately after euthanasia to prevent post mortem transformation. The sutures were removed and using a template the skin area with wound was adjusted to an optimal 3 x 2 cm (length of incision = 2 cm) strip to obtain uniform samples. The samples were placed between the two clamps of the tensiometer. Testing force was applied perpendicular to the direction of the incisions. The maximal breaking strengths (MBS) were registered for each sample.

Data of TS of unwounded skin were obtained with the FP100/1 device (Heckert, Chemnitz, Germany). Fifteen rats from a separate group were sacrificed by ether inhalation. The skin samples were carefully excised and adjusted using a template (Fig. 2) to a 5 x 3 cm strip with 2-cm wide measured skin area. The MBS was registered for each sample.

1. piezoelectric sensor
2. clamps
3. sample
4. servomechanism
5. counterweight
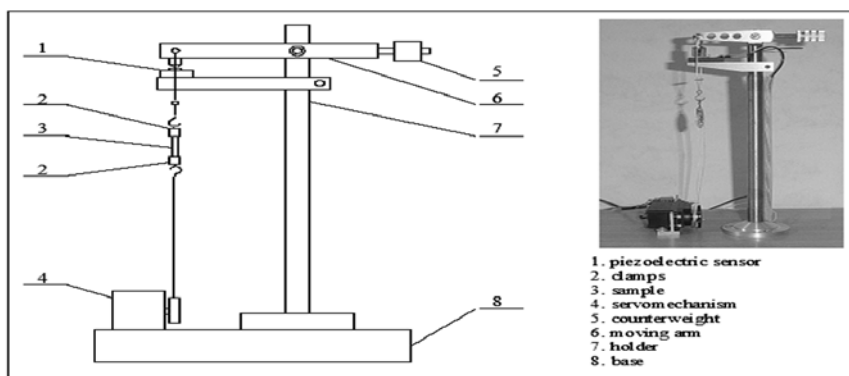6. moving arm
7. holder
8. base

Fig. 1. Mechanical parts of the tensile strength testing device.

Fig. 2. Template for the preparation of skin samples (dimensions in centimeters).

The TS of wounds as well as of unwounded skin was calculated by using the following formula: TS = MBS/A (TS = tensile strength [g/mm^2], MBS = maximal breaking strength [g], A = wound area/skin cross section area [mm^2]). Each measurement was performed in a blind form coded for each animal sample.

Histopathological evaluation

On days 1 to 7 after the surgery, six animals in each group were sacrificed by ether inhalation and the skin wounds removed for histopathological examination. The tissue specimens were processed routinely for light microscopy (fixation, dehydration, embedding, cutting, and staining with haematoxylin-eosin (HE), van Gieson, Mallory´s phosphotungstic haematoxylin). The level of keratinocyte differentiation in the process of skin wound healing was estimated by detection of keratin type 10 using mouse monoclonal antibody (DAKO, Brno, Czech Republic). Swine-anti mouse immunoglobulin labelled by FITC (AlSeVa, Prague, Czech Republic) was used as a second step antibody. Control of the specificity was performed by replacement of the first step antibody by a monoclonal antibody of the same isotype directed against antigen not occurring in epidermis.

We were interested in the following histological structures and changes in: the epidermis (re-epithelization and keratinization), the dermis, and the striated muscle (creation of fibrin network, presence of polymorphonuclear leukocytes – PMNL, tissue macrophages, migration, proliferation and orientation of fibroblasts, creation of new extracellular matrix (ECM) – especially new collagen fibres, neoangiogenesis, and the muscle layer alone – presence of centronucleated cells). The histological structures and processes (epithelization, PMNL, tissue macrophages, fibroblasts, new collagen and neoangiogenesis) were semi-quantitatively evaluated in coded slides according to the following scale: 0, 1, 2, 3 (Table 1) as described previously (Vidinský et al., 2006).

During the post-surgery period, the animals remained healthy, without clinical evidence of infection. The values from the semi-quantitative evaluation are summarized in Table 3.

Statistical evaluation

The data obtained from TS measurements as well as the data obtained from the semi-quantitative evaluation of histological changes are presented as mean ± standard deviation (SD). The wound TS 1, 2, 3, 4, 5, 6 and 7 days after surgery were compared using one way ANOVA followed by Tukey-Kramer multiple comparison test. The significance was accepted at $P < 0.05$.

Results

Wound TS measurement

We evaluated the increase of the TS of skin wounds in rats during the first seven days after wounding (Fig. 3). During the first five days of healing the largest increase of the TS of wounds was observed one day after wounding (from 0 to 1.60 ± 0.43 g/cm^2) (Table 2).

During the evaluated time intervals of one – five days after surgery only minimal TS increases were recorded. Among the evaluated groups (1 vs. 2, 2 vs. 3, 3 vs. 4, 4 vs. 5) no statistically significant differences in the increase of wound TS were found and the increase between one and five days of healing was lower than the increase between five and six days (2.28 g/mm^2 between one and five days and 2.57 g/mm^2 between five and six days) (Table 2). Therefore, we denoted the mentioned period as the "plateau phase".

The rapid increase of TS between five and six days of wound healing (from 3.88 ± 0.68 g/cm^2 at five days to 6.45 ± 1.16 g/cm^2 at six days, $P < 0.05$) ended the "plateau phase" with a following increase in TS between six and seven days (from 6.45 ± 1.16 g/cm^2 at six days to 11.57 ± 1.77 g/cm^2 at seven days, $P < 0.05$) (Table 2).

Table 1. Explanation of used scale in the semi-quantitative evaluation of histological sections

No.	Epithelization	PMNL	Tissue macrophages	Fibroblasts	New collagen	Neo-angiogenesis	Centro-nucleated cells
0	thickness of cut edges	minimum	absent	absent	absent	absent	absent
1	migration of epithelial cells	mild	mild	mild	mild	mild	mild
2	bridging of the incision	moderate	moderate	moderate	moderate	moderate	moderate
3	complete regeneration	marked	marked	marked	marked	marked	marked

Table 2. Comparison of tensile strength (TS) of wounded and unwounded skin

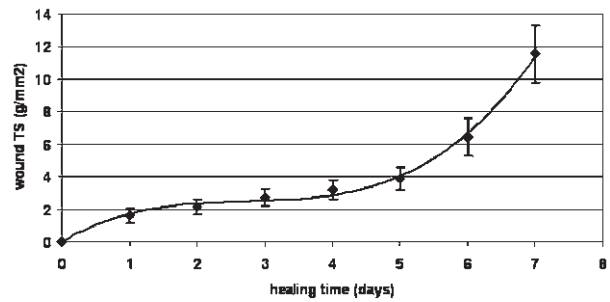| Healing time (days)	Wound TS (g/mm^2)	% of TS of unwounded skin
1	1.60 ± 0.43	0.55
2	2.14 ± 0.46	0.74
3	2.71 ± 0.53	0.93
4	3.18 ± 0.58	1.10
5	3.88 ± 0.68	1.33
6	6.45 ± 1.16	2.12
7	11.57 ± 1.77	3.98

Fig. 3. TS of wound versus time of healing. The values of TS were calculated as the average value of all samples on the day.

Table 3. Semi-quantitative evaluation of histological changes/structures during skin wound healing in rats. For each group average values with standard deviation (mean±SD) were calculated

Day	Epithelization	PMNL	Tissue macrophages	Fibroblasts	Neo-angiogenesis	New collagen	Centro-nucleated cells
1	0.00 ± 0.00	1.83 ± 0.72	1.17 ± 0.39	0.67 ± 0.65	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2	0.75 ± 0.45	2.17 ± 0.72	1.66 ± 0.49	1.75 ± 0.45	1.17 ± 0.72	0.00 ± 0.00	0.00 ± 0.00
3	1.83 ± 0.39	1.33 ± 0.65	1.92 ± 0.67	2.00 ± 0.43	1.67 ± 0.49	0.58 ± 0.52	0.00 ± 0.00
4	2.17 ± 0.39	0.42 ± 0.67	1.42 ± 0.07	2.33 ± 0.49	2.00 ± 0.74	1.58 ± 0.51	0.00 ± 0.00
5	2.58 ± 0.51	0.17 ± 0.39	1.33 ± 0.65	2.83 ± 0.49	2.58 ± 0.51	2.17 ± 0.39	0.17 ± 0.39
6	2.83 ± 0.39	0.08 ± 0.29	1.25 ± 0.62	2.92 ± 0.29	2.92 ± 0.29	2.75 ± 0.45	0.58 ± 0.51
7	3.00 ± 0.00	0.00 ± 0.00	1.08 ± 0.29	3.00 ± 0.00	2.42 ± 0.51	3.00 ± 0.00	1.17 ± 0.39

The average TS of the unwounded skin was 290.81 ± 40.42 g/mm^2.

Histopathological evaluation

Regeneration of injured epidermis

By one day after surgery the epidermis was thickened at its cut edges as a result of the mitotic activity of basal cells. After two days of healing, migration of epithelial cells beneath the scab was seen (Fig. 4). The necrotic debris on wound surfaces was almost removed and the scab was forming. The regeneration from the hair follicles was also recorded (Fig. 5). The incisions were completely bridged by three layers of new synthesized epithelial cells three days after surgery. At this time the beginning of the keratinization of the epidermis was also observed. After four days of epidermis regeneration the epithelial thickening over the incisions was evident and the surface keratinization was continued. Finally, at five days after surgery, the thickness of the keratin layer was similar to the intact epidermis, so the healing of epidermis was almost completed.

Reparation of injured dermis

Histological evaluation revealed the inflammatory phase during the first three days after surgery with the culmination between one and two days. After incision, blood cells and a fibrin network filled the incisional space, creating a scaffold for migrating fibroblasts (Fig. 6). On the superficial part of the dermis, necrosis was observed as a consequence of mechanical damage.
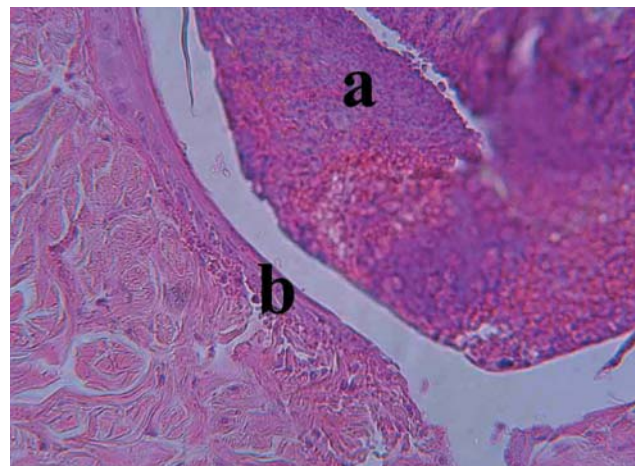

Fig. 4. Skin wound 48 hours after surgery (haematoxylin and eosin, 400x). Tissue necrosis (a) over the incision; migration of the epithelial cells beneath the scab (b).
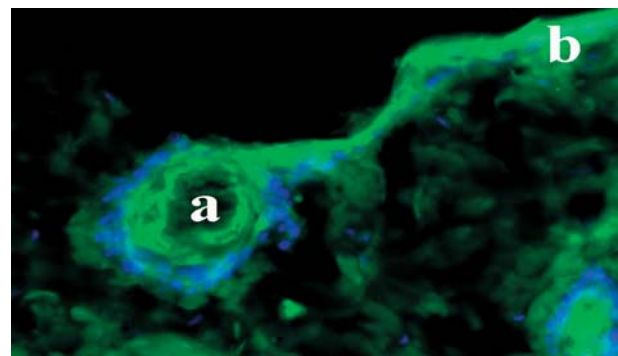

Fig. 5. Cytokeratin 10 expression in cells migrated from a hair follicle (a) into regenerated epidermis (b).

Also, at one day a demarcation line was observed under the tissue necrosis on the surface of the incisions, reflecting the initiation of the cellular reaction of the inflammatory phase. The demarcation line consisted of PMNL. Macrophages concomitantly invaded the wound area. By two days after surgery PMNL were partially replaced by tissue macrophages. However, macrophages in the tissue were almost always present during the first seven days of the healing process. By three days after surgery the inflammatory phase was almost completed.

The beginning of the proliferative phase was dated to the time period of the first day of healing with the peak between five and six days. By one day after surgery only a limited number of fibroblasts were present near the incisional space. However, two days after surgery fibroblasts were increased in number near the incisional space. Proliferation of fibroblasts and new endothelial cells with characteristic circular nuclei, which forms granulation tissue, was found in this time interval. In the wounds of animals killed three days after surgery, an increase in the number of new vessels and fibroblasts was seen. The incisional space at the layer of striated muscle and dermis contained an extracellular matrix without a significant quantity of collagen (Fig. 7). The observation of histological sections at four days of healed wounds showed vertically oriented fibroblasts. An increase in the amount of granulation tissue with new collagen was also recorded at four days. By five days after surgery the granulation tissue consisted of many new vessels and fibroblasts. The granulation tissue was most significant at the layer of the striated muscle. The assessment of histological sections after six days of healing also demonstrated vertically oriented fibroblasts. The histopathological evaluation was performed until seven days after surgery. In the time period from six to seven days after surgery more glycoproteins,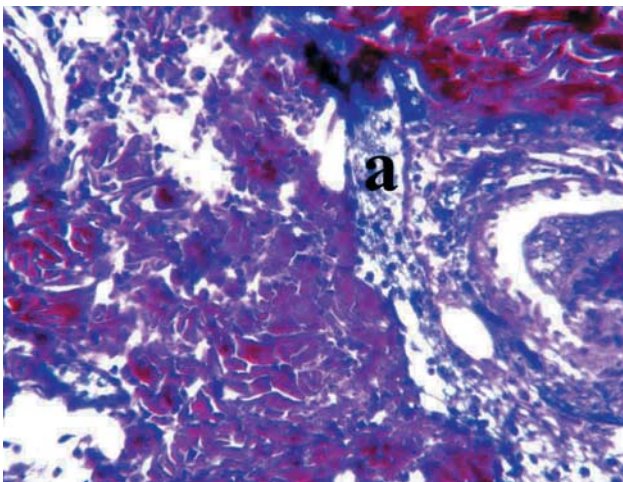 proteoglycans and collagen were synthe-sized by the fibroblasts. For the first time, at seven days, some fibroblasts were horizontally oriented, parallel to the basement membrane.

The initiation of the maturation and remodelling phase of the healing process was seen six days after surgery, when a large number of organized collagen fibres were observed. By seven days after surgery a decrease in the number of new vessels was observed and collagen predominated. This reflected the scar formation (Fig. 8).

Reparation of injured striated muscle

The histological evaluation performed one day after surgery showed necrotic myofibres of injured striated muscle in the deepest part of wounds. It was the destruction phase of striated muscle healing, which continued during the next day. Concurrently, two days after surgery the repair and remodelling phase of striated muscle healing was observed. By two and three days after surgery the tissue macrophages were presented during the process of the degeneration of the necrotic
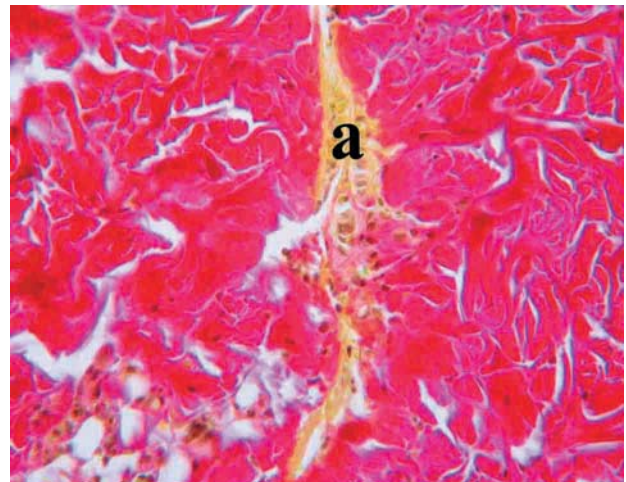

Fig. 7. Skin wound 72 hours after surgery (van Gieson, 400x). The incisional space at the layer of striated muscle and dermis without significant quantity of collagen (a).
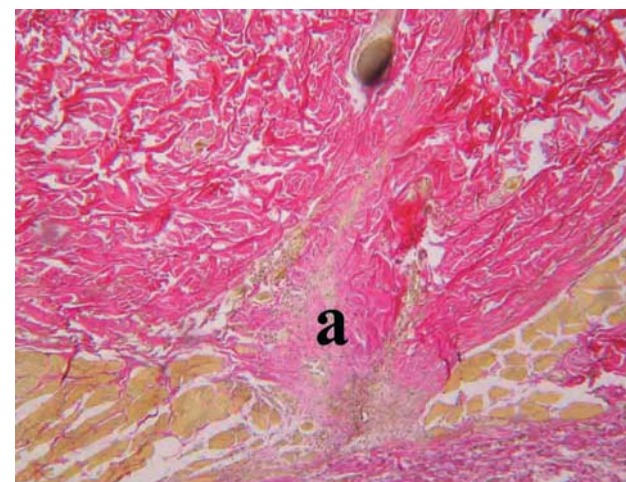

Fig. 6. Skin wound 48 hours after surgery (Mallory´s phosphotungstic haematoxylin, 400x). Fibrin network including inflammatory cells (a).

Fig. 8. Skin wound 168 hours after surgery (van Gieson, 100x). Tissue scar formation (a).

myofibres. The defect in the layer of rat skin striated muscle was healed by the formation of granulation tissue, which was described in the healing of injured lower parts of the dermis. At six days after surgery, for the first time, the presence of centronucleated cells at the limbs of damaged striated muscle cells was observed with an increase in number during the following day.

Discussion

The current and previous (Vidinský et al., 2006) histological as well as current biomechanical results show that the first week of skin wound healing in rats, from the point of view of TS and histomorphology, can be divided into three basic intervals. The TS of the skin wound increases substantially during the first day and during days 6 and 7 post surgery. We interpreted the initial increase as reflective adhesion of the wound borders with fibrin (Ist Interval, 0–24 hours post surgery); a result confirmed by our and other morphological (Conolly et al., 1997) and biomechanical studies (Altmeppen et al., 2004). Concerning the mechanical properties, the healing process has a specific course during the proliferative phase in the time period between one and five days with minimal increase of the tensile strength (IInd Interval, 24–120 hours post surgery, "plateau phase"). The mechanical properties of the wound correlate with the results of our morphological study. Only a small increase in the amount of new collagen (Van Gieson staining) in the incisional gap during this time period was described, and only vertically oriented fibroblasts and no cross-linked collagen fibres were observed in the histological specimens. Therefore, we suppose that the initial increase of the TS of a wound is related to the process of the vascular reaction (by creation of fibrin network) of the inflammatory phase. A crucial effect on the increase of wound TS had the dynamic, self-remodelling, macromolecular complex – ECM – synthesized by fibroblasts (Kadler, 1995; Cotran et al., 1999; Menetrey et al., 2000). Especially the reorganization of ECM during the healing process affects the wound TS. Continuance of the "plateau phase" correlated with the proliferative phase of wound healing that started one day after surgery and persisted all seven evaluated days. According to our previous (Vidinský et al., 2006) and current results, we conclude that the synthesis of collagen prevails over its degradation just after six days, in the time period when the TS of the wound has an increasing pattern (IIIrd Interval, 120-168 hours post surgery). Cross-linking the incisional gaps with collagen fibres increases the TS in the time period between five and six days ending the "plateau phase". The increase of TS between six and seven days after wounding correlates with our parallel histological study (the greatest amount of new organized collagen, Van Gieson staining). This process also correlates with the study of the deposition of collagen in colon anastomoses and skin incisional wounds published by Oxlund and associates (1996). PMNL retarded reparative processes through enzymatic dissolution of substrates, and their long presence during wound healing decreased wound TS (Muehlberger et al., 2005). In our histological study we recorded minimum PMNL after five days of healing and the decrease in PMNL could share in causing the higher mechanical firmness of the wounds. Thus, while more fibroblasts are present within the proliferative phase, their production of collagen can diminish in the presence of an exuberant acute inflammatory response (Muehlberger et al., 2005).

In the healing of striated muscle the centronucleated cells were considered as regenerating myofibres. A significant number of these cells were present seven days after surgery. This correlates with a study researching the influence of growth factors on gastrocnemius muscle healing in rats (Menetrey et al., 2000) and also is in accordance with human muscle healing (Cotran et al., 1999; Järvinen et al., 2005).

In summary, the results from our previous (Vidinský et al., 2006) and current investigation showed that the healing of rat epidermis, dermis and striated muscle is faster than but comparable to the healing of human skin and striated muscle. In addition to the fact that the results of this work can serve as an experimental model for further research using various factors by which the process of skin wound healing can be influenced, the study also has a clinical importance. The skin sutures can be removed as soon as the wound has sufficient mechanical firmness to prevent the separation of wound borders. On the body regions of rats that have greater mechanical strain of the skin, there is the possibility to remove the suture only after the start of the maturation phase six days after surgery. After the interpretation of our results we considered that in plastic surgery, on the body region with minimal strength, there may not be any difference in removing the skin sutures two, three or four days after surgery. However, before we can apply the results from our study to humans, we will need to verify these results on other animal models.

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