Study and Analysis of the Effects of Psychological Stress, Mechanical Stresses and Wound Shape in Wound Healing Process both *in vivo* and *in silico*

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Abstract

Wound healing is a specific biological process related to the general phenomenon of tissue growth and regeneration, which involves the combination of cells, scaffolds, and bioactive agents to fabricate functional new tissue to replace damaged tissue. In previous research, it has been investigated that not only psychological stress can delay wound healing process but also the mechanical stresses that are produced due to the forces generated by wounding affect this process. By this way, it can be confirmed that there is a relationship between wound shape and wound healing. In our current study, we found that stresses formed due to the forces were placed mainly at wound corners. In addition, the experiments performed at Biodonostia Health Research Institute, showed that the effect of stresses formed in wound borders tend to form a new wound shape that can make it heal much faster. For instance, rectangular and triangular wounds tend to become a circular form because of the tension in the edges, meanwhile circular shape converts into a more flattened circular profile.

1. Introduction

Human skin is the outer covering of the body and plays an important role in protecting the body against pathogens and external insults. It is a living complex material composed by several heterogeneous layers. Firstly, the epidermis, is the outermost layer of the skin which consists on a network of collagen with interspersed elastic fibers (the basement membrane) covered by an epidermal keratinocyte layer. Secondly, dermis, is the layer beneath the epidermis and it provides tensile strength and elasticity to the skin. And finally, the hypodermis that is located between dermis and underlying tissues and organs. It consists of mostly adipose tissue and is the storage site of most body fat [1]. Moreover, the thickness of each skin layer varies as a function of age, body zone or hydration [2].

The human skin is exposed to frequent external injury. Therefore, different wound closure techniques have evolved from the earliest development of suturing materials to comprise resources that include synthetic sutures, absorbable, staples, tapes, and adhesive compounds [3].

Wound healing is a complex biological process related to the general phenomenon of growth and tissue regeneration in which mechanical forces regulate the quality and speed of healing. They influence the gene expression, the synthesis of growth factors and the inflammatory mediators and cellular processes such as proliferation of many cells. The wound process can be divided into four consecutive phases: hemostasis, inflammation, proliferation and remodelling with scar formation.

1. Hemostasis: it plays a protective role in which vascular constrictions and fibrin clot formation control the bleeding.
2. Inflammation: it almost occurs simultaneously with hemostasis. It is a beneficial body's immune response in which damaged cells, irritants or pathogens are removed and the healing begins.
3. Proliferation: it happens simultaneously or after the migration phase, which involves the movement of epithelial cells and fibroblasts to the injured placed in order to replace damage tissue. During the proliferation stage, different processes happen: angiogenesis, granulation tissue formation, collagen deposition, etc.
4. Remodelling with scar formation: involves the formation of cellular connective tissue and strengthening of the new epithelium which determines the nature of the final scar.

All these phases are influenced by mechanical forces, since they directly affect cellular functions. During the wound healing process, the mechanical forces are sensed, transduced and transformed into intracellular biochemical cues. Also, mechanical forces modulate integrin-mediated processes and other mechanosensors such as gap junctions, hemichannels, transient receptor potential channels (TRP channels) and intracellular mechanosignalling pathways [4].

The application of mechanical forces may be a useful method for acceleration of cutaneous wound healing. If an external mechanical force is applied, it can be useful to reduce collagen deposition during tissue repair and scar formation. The most important forces that affect the cellular functions are tension, shear force, osmotic pressure and mechanical stimuli between cells and chemical signals [5].

The impact of stress on cutaneous wound healing process was assessed in a murine model [6]. For that, female, hairless SKH-1 mice, 6-8 weeks of age were subjected to restraint stress (RST) 3 days before and for 5 days following dorsal application of a 3.5 mm diameter full thickness punch wound, while control mice were only wounded. During this research, it was seen that a higher percent of control mice fully healed wound as compared to stressed mice. Therefore, it can be stated that the wound healing process is slower in stressed mice than in control mice. In addition, this statement can also be supported by another research [7], in which some of the virus-antibody free SKH-1 female mice, 6-8 weeks of age, were subjected to restraint stress (RST).

On the other hand, wound shape can affect the wound healing process [8]. Concave edges (positively curved edges pointing towards the removed skin) clearly advanced at a slower pace compared with convex edges (negatively curved edges pointing towards the remaining skin). This has a relationship with the mechanical forces that are mostly applied in the edges of the wound. Moreover, during wound closure [9] it was showed that actin protrusions generated traction forces and the wound closure was initially driven by cell crawling. The orientation of these forces depended on the curvature of the wound edge. It was demonstrated that the leading edge was exhibited a convex contour with traction forces pointed away from the wound. In contrast, regions exhibiting a concave contour showed that the traction forces pointed towards the wound.

The aim of this project was not only to analyze mechanical stresses distribution formed during the wound healing process in different geometries (circular, triangular and rectangular) but also to verify if applying a psychological stress could affect wound healing.

2. Materials and Methods

2.1. Animals

Animal used were C57BL/6 mice (n=18), of the 8 weeks of age, housed 1/cage on a 12 h light/dark cycle. They were obtained from Biodonostia Health Research Institute (Donostia-San Sebastián, Basque Country, Spain). Experiments were carried out after approval by the Comité de Ética de la Experimentación Animal (CEEA) of Biodonostia Institute.

2.2. Experiments fulfillment

*Some m*ice were subjected to restraint stress (RST) [6], but instead of three stress cycles, just one cycle of 2 h was performed prior to wounding. Mice were introduced in well- ventilated 50-ml centrifuge tubes.

For generating wounds, mice were anesthetized with intraperitoneal administration 0.02mg/ml Metacam. After the dorsal area was cleaned with Chlorhexidine, two symmetrical, full thickness, 0.5 cm^2 were created on the dorsum of each mouse. The shapes created were: an 8 mm diameter circle, both 1 cm base and height triangular and a 1 cm base and 0.5 cm height rectangular.

Wound areas were measured using photographs that were taken until the wound was healed around a 50%, since the beginning of the experiment (Day 0), until one week. In addition, photos were also taken at Day 1, Day 3 and Day 7. Photographs of the wound were taken always with the same camera, and then, these photos were analyzed by using ImageJ. Wound size was obtained by using the following equation:

$$Wound\ Closure\ [\%] = \frac{Area\ at\ Dx\ [cm^2]}{Area\ at\ D0\ [cm^2]} \times 100 \qquad (1)$$

Statistics were performed using MATLAB. Differences between groups over time were assessed using ANOVA in order to compare not only between the three different shapes (circular, triangular and rectangular) of the wound at a single time point but also for control and stress mice. Data is represented as mean ± SEM. Statistical significance was determined at $p \leq 0.05$ (*), $0.001 \leq p \leq 0.01$(**) and $p<0.001$ (***).

2.3. In-silico wound healing model

By using engineering software CREO PARAMETRIC, it has obtained a solid piece, that is an identical copy of the wound. The pieces were divided into three parts. On one hand, the two outer parts were equivalent to the skin and the inner part was equivalent to the scab (wound). The two outer parts were separated by stitches equivalent to the seams.

Focusing on indentation test, the Young modulus of the skin was 12.5 kPa [10]. The scab mechanical properties (Young´s modulus) was considered 10 times higher than the skin properties due to the stiffness that it presented. The contraction produced in the scab during the wound healing was simulated with a hypothetical temperature change in the scab. The thermal expansion coefficient used for this volume change was 0.001 C^{-1}.

By using ANSYS Workbench 16.2, mechanical stresses distribution was obtained. Solid meshing was performed with an element size of 0.4 mm. The analysis setting was made by inserting a thermal condition in the scab and decreasing the temperature from 22ºC (reference temperature) to 0ºC. Principal maximum stress and equivalent strain were necessary in order to analyze the obtained results.

3. Results and Discussion

3.1. Epithelization of different shapes

As it was hypothesized above, a different shape is going to have a different wound healing behavior. The initial shape area is 0.5 cm^2 and each step is equivalent to 3 days. On the one hand, in the *in vivo* results, for instance, the triangular shapes heal faster than compared to the circular and rectangular shapes as it can be seen in Figure 1. It is supposed to heal faster because the healing process takes place mostly in the very "sharp" corners, unlike the rectangular shape in which it tends to heal mostly from side to side, not from top to bottom as it can be observed in Figure 4.

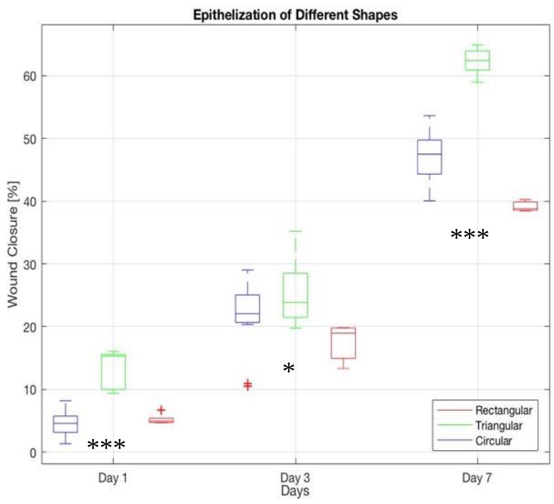

Figure 1. *Epithelization of Different Shapes. The initial area is 0.5 cm^2. Data represent mean ± SEM from three experiments during one week (Day 1, Day 3, Day 7). N=14 circular shapes/group, N=5 rectangular shapes/group and N=5 triangular shapes/group. p≤0.05 (*), 0.001≤p≤0.01(**) and p<0.001 (***). Rectangular and triangular shapes compared to circular shape (ANOVA).*

On the other hand, in the *in silico* results, it seems that the circular shape tends to heal faster than the triangular and rectangular shapes. Moreover, unlike the *in vivo* results, triangular shape lasts more time for achieving the wound closure than the other two shapes as it can be observed in Figure 2.

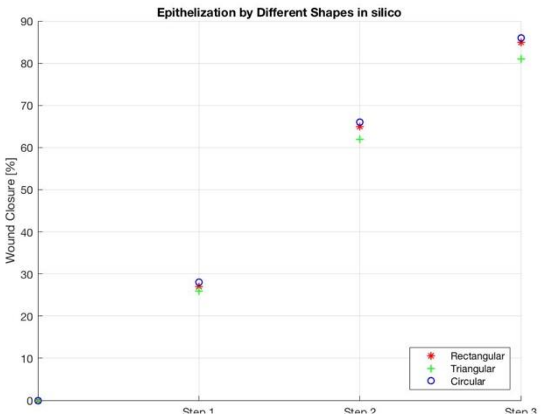

Figure 2. *Epithelization by Different Shapes in silico. Data represent mean from the different simulation during three steps. Each step is equivalent to 3 days.*

3.2. Epithelization of restraint stress

As it was supposed above, a restraint stress delays the wound healing process as it was also investigated in previous research [11]. By having a look in the Figure 3, even though it is not easy to recognize, but with stress, the wound needs more time for healing. As in the chapter above, the initial shape area is 0.5 cm^2.

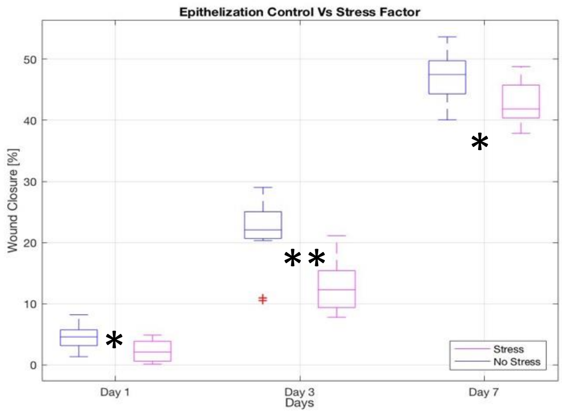

Figure 3. *Epithelization Control VS Stress Factor. Data represent mean ± SEM from two experiments during one week (Day 1, Day 3, Day 7). N=14 control/group, N=8 stress/group. p≤0.05 (*), 0.001≤p≤0.01(**) and p<0.001 (***). Stressed compared to control (ANOVA).*

3.3. Evolution

The evolution of the wound can be observed in Figure 4, from the Day 0 till Day 7. The area of each wound was assumed to be 0.5 cm^2 at Day 0.

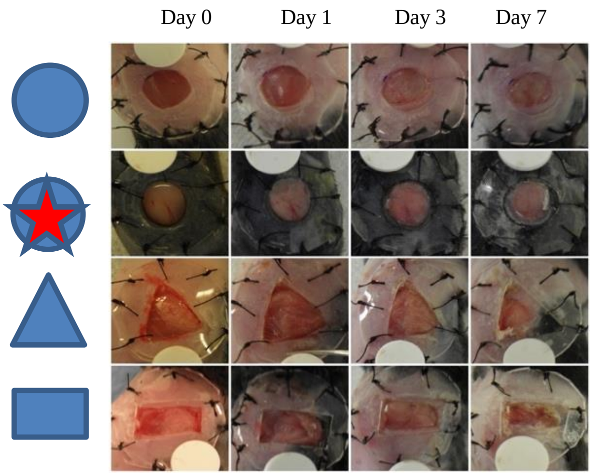

Figure 4. *Evolution of the different wound shapes and stress during one week. The circular shape with stress is represented with a star mark. Each wound had an approximate area of 0.5cm^2. In every wound, a silicone splitting ring was placed around the wound.*

3.4. Circumferentiality

Circular shape tends to convert into a more ellipse shape since it has less concave and convex edges. On the other hand, both triangular and rectangular shapes develop into a more circular shape in experimental analysis. Circumferentiality starts decreasing at the beginning of both analysis (*in vivo* and *in silico*). Once the stresses decreased at wound corners, the circumferentiality starts increasing. See Figure 5. This increase results on a homogenization o mechanical stresses at scab borders. This evolution is because edges of the triangular and rectangular wounds suffer higher mechanical stresses than in flat surfaces and those start healing easier.

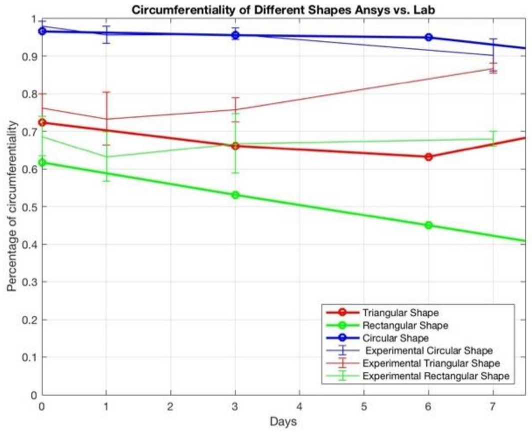

Figure 5. *Circumferentiality obtained in vivo and in silico. Experimental data represent mean ± SEM from three experiments during one week (Day 1, Day 3, Day 7). N=14 circular shapes/group, N=5 rectangular shapes/group and N=5 triangular shapes/group. Dots represent steps performed during the simulations. Each step is equivalent to 3 days.*

3.5. Principal maximum stress for growing scabs

Convex regions are where the highest value of stress is accumulated. As it was supposed, different shapes are going to have different wound healing behavior. Elements labeled with red, grow twice as fast as the blue elements, making converts it into a circular shape. Focusing on rectangular shape, the wound converges into a circular shape since the corners are the regions in where the highest value of stress is concentrated. The value of stresses formed in triangular and rectangular shape is two times higher than in circular shape due to the more pronounced and sharp corners in these geometries. The wound healing process simulated *in silico* can be seen in Figure 6.

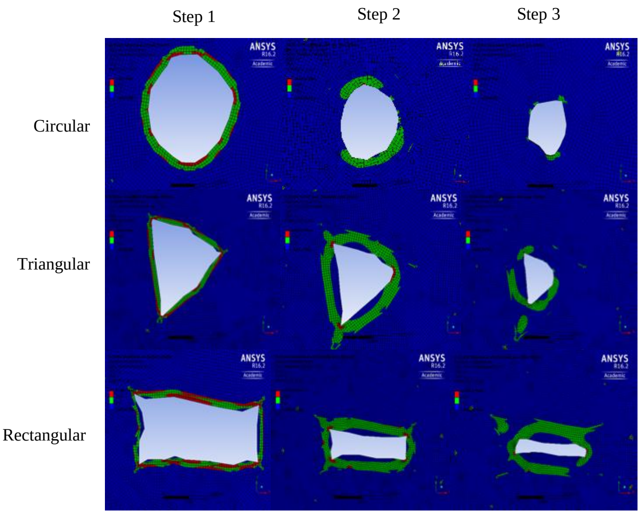

Figure 6. *Maximum principal stress for three steps for circular, triangular and rectangular shape. Step 1: Scab growth 0.5 mm from the border of the wound. Step 2: Depending on the stresses that have appeared in the Step 1 around the border of the scab of 0.5 mm, the new scab will grow: 0 Pa - 0.5 mm/step, 500 Pa - 0.75 mm/step, >1000 Pa – 1 mm/step. Step 3: Depending on the stresses that have appeared in the Step 2 around the border of the scab, the new scab formed will follow the same velocity as in the Step 2.*

4. Conclusion

The different shapes tend to close the wound as circular form, as the vertices accumulate stresses there. The way the stresses area is distributed in the wound has a relationship between wound healing and velocity. Also, psychological stress has a negative influence in wound healing, which might be related with the decreased production of three key cytokines (IL-6, IL-1β, and TNF- α).

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