

## **Preliminary experiment in the healing acceleration and tissue recovery in animals (rabbits) with the application in the plague of an extract of *Benincasa hyspida***

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### **Abstract**

*We used in our experiment an extract of Benincasa hyspida (Cerifera savi) applied on wounds to demonstrate the healing of skin more rapidly in comparison with the evolution of a classically treated wound.*

*The experiment was performed on rabbits with 2 paramedian laparotomies; a plague treated in the classic way and the other treated with our extract.*

*5 days after the incision with serial histopathological examinations there was a much faster healing of the plague treated with the extract compared to our healing classical witness plague.*

Keywords: healing, regeneration, cytokine, collagen, mythogenic.

### **Introduction**

Capacity for regeneration phenomena and the local repair are ubiquitous. Sequence and their essence is the same in any type of tissue repair. But there are features of evolution, depending on many factors (1).

The first factor is the ratio of the magnitude of damage and the repair capacity;

Second group of factors consists of various types of development of local inflammation, each corresponding to a type of local healing;

Third group of factors consists of the relationship between the amount of damaged epithelium and epithelial remaining resources as well as the topographic of the epithelial remnants found in the plague area;

The fourth group of factors consisting of the specific reactivity of the tissue concerned;

Fifth group of factors consists of the specific agent vulnerable;

The sixth group of factors relates to architectural condition created by trauma and inflammatory phenomena;

The seventh group of factors relates to the functional reserve of the area affected.

These factors may be modulated by other exogenous factors and pharmaceutical products (active principles) which changes the rate and duration of healing.

Incised plague is a plague that leaves scars.

The healing of the scars and skin are directly subject to the section depth (if plague is penetrating in derma or deeply until lax subcutaneous tissue).

If the plague is intradermic, the granulation is unobtrusive and the repair is regenerative with "restitutio ad integrum", but with a delay of healing.

If plague enter the lax tissue conjunctive, within the profound plans, granular invade plague in depth, entailing a rapid healing and apparition of scars (fibroblasts of conjunctive lax tissue are more reactive and initiates the process faster).

Plague contains devitalized tissue, damaged extracellular structures, broken capillary with bleeding, bound by viable tissue.

Healing a wound is a complex and prolonged process of tissue repair and recast in response to injury. The whole process of healing is a complex series of events which run from time of injury and lasts for weeks, months or years.

In wound healing, this view includes several phases and stages, as follows (3, 14):

I. Inflammatory Phase (immediate to 2-5 days) includes:

- Hemostasis stage: Vasoconstriction
- Platelet aggregation
- Thromboplastin makes clot
- Inflammatory stage: Vasodilatation
- Phagocytosis

II. Proliferative Phase (2 days to 3 weeks) includes:

- Granulation stage: Fibroblasts lay bed of collagen
- Fills defect and produces new capillaries
- Contraction stage: Wound edges pull together to reduce defect
- Epithelialization stage: Crosses moist surface
- Cell travel about 3 cm from point of origin in all directions

III. Remodeling Phase (3 weeks to 2 years)

- New collagen forms which increases tensile strength to wounds
- Scar tissue is only 80 percent as strong as original tissue

The processes involve the coordinated cell activation, cell division (see also: Cell cycle), chemotaxis and migration (see also: Mutagenic cytokines), and differentiation of many different cell types. Dysfunctions of the different physiological phases of wound healing may be responsible for disorders of normal wound healing and may be the underlying basis for many fibrotic disorders.

In order to balance degradation and regenerative processes these events require a finely tuned control of various biochemical, cellular, and immunological reaction cascades. They are mediated by locally released growth factors and cytokines, which may act in an autocrine or paracrine manner. All phases of wound healing are either directly or indirectly controlled by cytokines. It appears that it is the balance of these cytokines and other mediators rather than the mere presence or absence of one or more cytokines that plays a decisive role in regulating the initiation, progression and resolution of wounds. In addition, cell-cell and cell-matrix interactions, mediated, for example by various cell surface adhesion molecules, play an important role in wound healing.

The first step in the wound healing process involves the activation of the intrinsic part of the blood coagulation cascade. This event is triggered by the rupture of blood vessels and initiated by the contact of plasma with tissue and basal membranes of cells and the exposure of subendothelial collagen to platelets. Some of the components of the coagulation cascade

may be involved also in other reactions such as myogenesis (see, for example: Factor X, Factor Xa, Protein S).

The formation of a fibrin gel serves to fix plasma proteins and blood cells and this process leads to hemostasis. Thrombin inside the plasma clot induces platelets to degranulate, i.e., to release the contents of their Alpha-granules.

Platelets are a rich source of locally active growth factors and cytokines (1). Among other things platelet-derived factors include adenosine dinucleotide (which induces platelet aggregation and also stimulates cell proliferation and migration),  $\beta$ -Thromboglobulin, bFGF, CTAP-3 Connective tissue activating protein-3), EGF, eosinophil chemotactic polypeptide-1 (i.e., RANTES), f-ECGF (Fibroblast derived endothelial cell growth factor), fibronectin (which serves as an early matrix ligand for platelet aggregation), HCl (human collagenase inhibitor), HGF (hepatocyte growth factor), HRF (histamine releasing factors), IGFBP3, NAP-2 (neutrophil-activating protein-2), NAP-4 (neutrophil-activating protein-4), PBP (platelet basic protein), PD-ECGF (platelet-derived endothelial cell growth factor), PDGF, PF4, platelet activating factor (PAF, involved also in platelet aggregation), serotonin (which induces vascular permeability and is a chemoattractant for neutrophils), somatostatin, TGF-alpha, TGF-beta, thromboxane A2 (which is involved in vasoconstriction, platelet aggregation, and chemotaxis), vitronectin (8, 9, 17).

The initial response to tissue injury subsides with the synthesis of protein-C, which inactivates the coagulation factors Factor-V and Factor VII, and the release of plasminogen activator, which initiates the dissolution of the thrombus (15).

Inadequate clot formation is associated with impaired wound healing (16). The locally formed fibrin clot effectively serves as a scaffolding matrix that can be colonized subsequently by inflammatory cells such as neutrophils, monocytes, and macrophages as well as fibroblasts and endothelial cells. The phases of normal wound healing follow an orderly sequence of events that are characterized by, and regulated by, the chronologic appearance of a number of different cell types.

Once chemotactic migration of cells into the wound milieu has been achieved this is followed by cell activation. All cells participating in wound healing must be activated, i.e., undergo phenotypic alterations of cellular, biochemical, and functional properties. Cell activation, which is initiated by the release of factors from aggregated platelets, has fundamental implications in several aspects of wound healing as this process leads, among other things, to the expression of new cell surface antigens, increased cytotoxicity, increased production and release of cytokines. Many of the factors released by activated cells present initially at the site of wound healing are responsible for mediating further phases of the wound healing response such as the activation of other cells. As a whole these mechanisms ensure a prolonged presence of cytokines in the wound milieu.

Circulating peripheral blood leukocytes migrate into the wound space. The first cells to appear in the wound area are neutrophils. Their numbers reach peak levels approximately 24 hours after injury. Their migration is stimulated by various chemotactic factors and cytokines, including complement factors, IL1, TNF-alpha, TGF-beta, and chemokines such as IL8, GRO-alpha, PF4, MCP-1, IP-10, mig, and also by bacterial polysaccharides (4). Neutrophils adhere to the endothelium by means of selectins, which function as receptors for neutrophils on the endothelial cell surface. Integrin receptors on the cell surfaces of neutrophils facilitate binding of neutrophils to the extracellular matrix. Neutrophils do not appear to play a critical role in wound healing in the absence of infections as wounds can heal in animals in which neutrophils are depleted. Neutrophils are removed by tissue macrophages when they are no longer needed (10).

Monocytes appear approximately 24 hours after injury and peak at 48 hours post-injury. Since monocytes mature into macrophages they can be considered an essential source of cytokines driving repair processes. Macrophages and monocytes are also attracted by a variety of chemokines. These chemokines contribute to the spatially and temporally different infiltration of subsets of leukocytes and thus integrate inflammatory and reparative processes during wound repair.

Tissue macrophages are the cells that essentially control and regulate the wound healing process and wounds cannot heal without the participation of these cells as shown by experiments involving depletion of wound macrophages. The differentiation of macrophages is initiated by several specific cytokines (7, 9). Many cytokines produced and secreted by activated macrophages favor further migration of inflammatory cells into the wound area (7). Macrophages also control the degradation of the extracellular matrix and regulate remodeling of the wound matrix (2, 13). Macrophages secrete cytokines and growth factors including TGF-beta, FGF, VEGF, and chemokines such as JE.

In the proliferative phase of wound healing fibroblasts and endothelial cells are the primary proliferating cells. Fibroplasia begins as the number of neutrophils decreases and the number of macrophages and fibroblasts in the wound area increases. Fibroblasts migrate into the wound approximately from day 3 on. They are derived from surrounding dermal elements. Fibroblasts replicate in response to cytokines and growth factors released during the earlier phases of wound healing. Some of these factors are present and stored in the fibrin clot which is invaded by these cells. Fibroblasts deposit the collagen that forms part of the substance of granulation tissue formed later during wound healing. Granulation tissue develops from the connective tissue surrounding the damaged or missing area and contains mainly small vessels, inflammatory cells, fibroblasts and myofibroblasts (2, 3).

The formation of new blood vessels is initiated by endothelial cells migrating and proliferating into the healing wound. The formation of new blood vessels within the wounded area is essential for the normal function of fibroblasts and leukocytes. The process is maintained as long as required by various angiogenesis factors (14).

TGF-beta appears to be the major factor responsible for the formation of granulation tissue and the synthesis of proteins of the extracellular matrix and thus has deserves to be called a "wound hormone". TGF-beta is a member of one of the most complex groups of cytokine superfamilies, consisting of various TGF-beta isoforms and other family members, for example, Activin A and BMP. The complexity of the wound healing process is illustrated by the observation that manipulation of the ratios of TGF-beta superfamily members, particularly the ratio of TGF-beta-1 relative to TGF-beta-3, reduces scarring and fibrosis (6, 16).

Re-epithelialization is mediated by chemotactic and mythogenic growth factors of the EGF family of growth factors. Leptin has been shown to be a potent growth factor for keratinocytes during wound healing (11, 13).

The final phase of wound healing is characterized by the gradual replacement of granulation tissues by connective tissue. This process also requires locally acting cytokines. However, little is known about the factors and mechanisms that eventually restrain tissue growth once the repair process has been completed.

The synthesis of collagen and proteinase inhibitors is stimulated, among other things, by TGF-beta and related factors. Closing of the wound and the evolution of a scar is associated with a striking decrease in cellularity, including disappearance of typical myofibroblasts. It has been suggested that cell death by apoptosis is the mechanism responsible for the evolution of granulation tissue into a scar (3, 5).

Fetal wound healing is conspicuous for its absence of scarring (12). There is some evidence that fetal and adult fibroblasts display phenotypic differences in terms of migratory activity, mythogenic response to cytokines and the synthesis of mythogenic cytokines, growth factors, and matrix macromolecules.

By manipulating the actions of growth factors and cytokines, it may accelerate or modify wound healing (1). Animal experiments and also clinical experience have demonstrated that the topical administration of various cytokines, including bFGF, EGF, KGF, PDGF, TGF-beta, either alone or in combination (see also: MBWE, mythogenic bovine whey extract), considerably accelerates wound healing by stimulating granulation tissue formation and enhancing epithelialization (see: individual factors for details; subentry Clinical use AND Significance). Some of these factors even allow a complete healing of wounds that were previously refractory to conventional treatment. Treatment of incisional wounds in a rat healing model with TGF-beta has shown that this factor enhances wound healing even if given up to 24 hours before wounding (7). The mechanism of action is unclear but it has been suggested that this effect may be due to the ability of TGF-beta to induce its own synthesis. A single dose of TGF-beta given before wounding is envisaged to prime cells throughout the body for further synthesis of TGF-beta to respond more effectively to a future injury.

The main indications for the pharmacological use of cytokines involved in wound healing processes are at present the treatment of large wound areas and burn injuries, the regeneration of the cornea following abrasion or cataract treatment, and the treatment of chronic non-healing ulcers (see also: PDWHF, platelet-derived wound healing formula).

## Material and Method

Our study shows the parallel evolution of two regeneration and incision wound healing made on the rabbit in two variants:

1<sup>st</sup> variant (witness) - incision wound with natural evolution

2<sup>nd</sup> variant (experimental sample) - incision wound on which was applied an extract of *Benincasa hispida*.

Samples were taken from the edge of the wound for histopathological examination:

- 3 days after incision
- 5 days after incision.

Were conducted clinical observations and the pace of recovery has been evaluated and appearance wound (cicatricial).

Please note that the wounds made with a knife usually develop after the magnitude of aggression. They cover more quickly than those made with electric knife or laser. Deep dissection and the suture made increase the amount of aggressed and devitalized tissue and also the volume of healed wound.

1<sup>st</sup> variant - incision wound (for natural healing), after 24 hours shows visible bleeding areas (the central area of black-brown color)

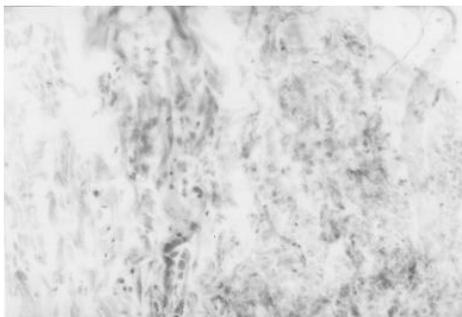
2<sup>nd</sup> variant - incision wound (using as cicatrizing extract of *Benincasa hispida*) to 24 hours to submit an area dotted with small hemorrhagic points.

## Results and Discussions

### Histopathological aspects

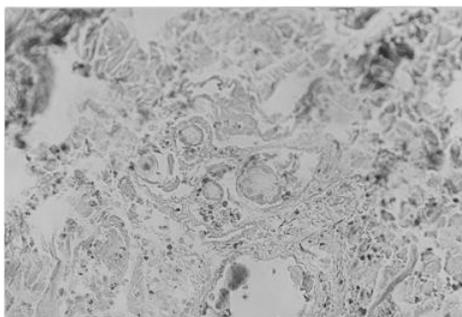
#### At 3 days after section

1<sup>st</sup> variant. In section is revealed an area of uncovered skin in surface, with vascular-enzymatic phenomena and polymorphonuclear. The aspects of morphological disorganization are high: they appear in the derm structure as a condensation of conjunctive fibers, mainly of collagen, the capillary vascularization being abundant and with many fibroblasts (see figure 1).



**Fig. 1.** Morphological disruption post-section (objective 20, HEA coloration)

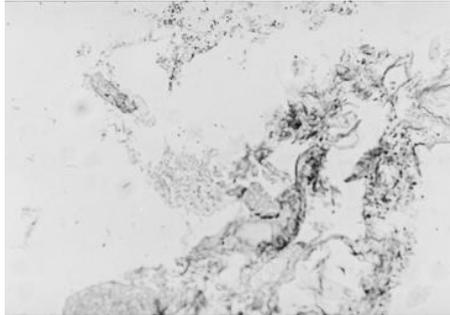
At the limit the conjunctive adipose tissue, prevailing the cytolysis phenomena and a tissue reaction in the form of a bulky escare with an abundant fibrin-leukocyte exudate. Retraction phenomena present in the edge sections: broken conjunctive fiber fascicles, embedded (see figure 2).



**Figure 2.** Tissue reaction at limit of the connective tissue (objective 20, HEA coloration)

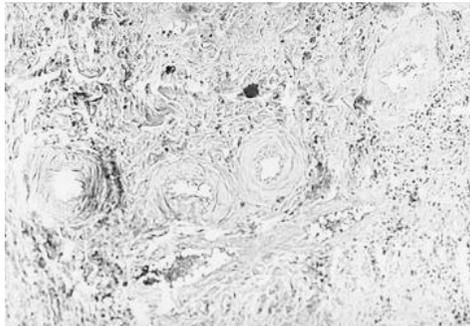
In conclusion, the overall picture prevailing congestion in the thickness of the uncovered conjunctive tissue with degeneration, cytolysis, edema; not suggestive images for granulation. 2<sup>nd</sup> variant.

In two of the sections examined – it's observed a reduced haemostatic blood clot, replaced by fibrin-leukocyte exudate (possible positive effect that reduces exposure to cell exsiccation). Cellular phenomena are more extensive in depth: between the derm and hypoderm are agglomerations of fibroblasts and fibers, 2 - 3 times wider than the 1<sup>st</sup> variant (fig.3).



**Fig. 3.** Exudate at the level of adjacent derm (objective 10, HEA coloration)

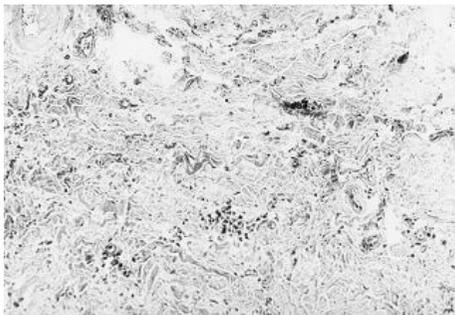
In the border area - the edge of wound (section) shows areas of capillary sinusoidal vascular proliferation that can be interpreted as initiators of granulations. It shows elements epithelial cell "submerged" edge of epithelium discontinued and the accumulation of monocytes alongside lymphocytes is also high (see figure 4).



**Fig. 4.** Vascular proliferation in the border area of the wound (objective 20, HEA coloration)

#### **At 5 days of making the incision**

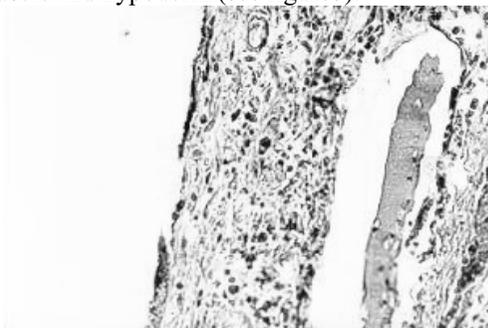
1<sup>st</sup> variant. Are caught alternating issues, areas of skin with nonepithelized area and epithelized skin area. Between "bed" and epithelium persist "clots" of fibrin and exudate of polymorphonuclear histiocitary abundant. In deep beams of derm appear dense collagen fascicles and fibroblasts arranged in concentric structures (islands) can be interpreted as fibroblastic metaplasia (see figures 5 and 6).



**Fig. 5.** Fibroblastic metaplasia area (objective 10, HEA coloration)

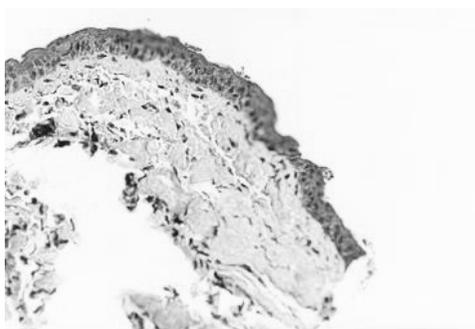
This may involve delaying a normal recovery and yields to a hypertrophic scarring that can occur in incision wound.

2<sup>nd</sup> variant. Snippets harvested highlights important areas of tissue regeneration, with the persistence of reactional phenomena strong edema in lax connective tissue and at the junction between the muscle and hypoderm (see figure7).



**Fig. 7.** Apparent tissue regeneration (objective 20, HEA coloration)

Restoration of collagen fibers is intense throughout dermal thickness, which may be related to the stimulation fibroblasts by the therapeutic agent used. Although the epithelium surface is 80% of the area provided, however, it shows slight signs of keratogenous activity and not seen signs of hair buds and hairs in the profound structure. It's observed a beginning of keratinization by the emergence of a layer of corn (see figure 8).



**Fig. 8.** Regeneration of the epithelium surface (objective 20, HE coloration)

Collagen fibers are thick, sinuous, perpendicular to the structure in the central section and less thick and sinuous at the edge of the section. Fibroblasts are globular, bulky.

## Conclusions

The pace of and recovery activity of the tissue is much increased in 2<sup>nd</sup> variant, leading to a normal healing without inflammatory phenomena and retraction. They fall within the threshold of a normal healing, reducing vascular and metabolic disorders, without the appearance of inflammatory superadded phenomena. They may be the positive result of the presence of multiple factors with anti-inflammatory effects, antibacterial, immune-modulatory and even some cytokine that may act complex throughout all structures cut that modulate the

main points of physiological regeneration. They reduce local phenomena caused by the threads of suture – they do not appear suppurative rejectee with collagenic lysis inflammatory.

Based on the results we obtained after the preliminary research on the rabbit, the *Benincasa hispida* extract can be applied successfully in accidental and surgical plague on other species of animals and humans, reducing to minimum the effects of healing pathology.

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