The Feasibility of Studying Neodymium Laser Effects on Wound Healing in Mice. Design of a Preclinical Experiment

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Introduction

Scars often cause a complex of symptoms in the patient associated with aesthetic and functional disorders, and they are socially significant pathological conditions, reducing the result of operations on open areas of the body, especially when performing operations for aesthetic purposes. The problem of treating patients with keloid and hypertrophic scars has not been completely solved [1]. This is due to the fact that there is no single effective method of managing such patients. Rational tactics for dealing with pathological scars include therapeutic, physiotherapeutic, radiological, surgical and cosmetic methods [2]. Unfortunately, among all these methods, there is not one that gives a 100% result - the scars do not completely disappear, but only become less noticeable or recur over time. For the prevention of scar formation, it is necessary to represent the entire pathological process of wound regeneration and the morphological process of scar formation.

Wound Process

In the process of wound healing, several phases are conditionally distinguished, replacing and often overlapping each other: the phase of exudation and inflammation, the proliferation phase, the reorganization phase, and some authors distinguish more phases: hemostasis, inflammation, angiogenesis, granulation, re-epithelialization and remodeling [3, 4]. In the 1st phase (exudation and inflammation), the blood coagulation system is activated and a platelet-fibrin clot is formed at the site of injury, which leads to bleeding arrest and the creation of a temporary matrix consisting of glycoproteins (collagen), proteoglycans and hyaluronic acid, which serves as the basis for connective tissue synthesis. Subsequently, against the background of fibrinolysis and destruction of platelets, numerous growth factors (transforming growth factor β, epidermal growth factor, insulin-like growth factor, platelet growth factor, etc.) are released and neutrophils are attracted, phagocytizing foreign particles and activating keratinocytes and macrophages. Proven link between inflammatory infiltrate cells and fibrosis formation via signaling, as evidenced by independent studies. For example, it was found that neutrophiles are the first to migrate to the site of inflammation, and their representation in the wound is maximum in the first 2 days after injury. The role of these cells is to cleanse the wound of bacteria and dead cells through phagocytosis. However, in the scar granulation tissue of long-term non-healing wounds, a large number of neutrophils is observed, which emphasizes their role in stimulating fibrosis [5]. In a study in neutrophil-depleted mice, wound closure was faster [6]. Interestingly, scarless healing in the fetus is characterized by a mild or no inflammatory reaction due to the immaturity of the immune system, as well as a different representation of specific fibroblasts in the fetal wound compared to adult wounds. Engrailed-1 fibroblast line as the main effector cells in production of ECM during wound healing was identified by a cell surface marker and accounted for 1% in the skin of fetuses during early pregnancy, and its proportion increased to >75% in the skin of newborns [7]. Later, this lineage of fibroblasts was found to be responsible for the formation of scars during the healing process [8]. Thus, the activity or severity of the inflammatory response is one of the key factors in the fibrotic transformation of the wound. Monocytic chemotactic protein-1, fragments of extracellular matrix (ECM) proteins, transforming growth factor beta (TGF-β) attract monocytes to the wound on days 2-3, which turn into macrophages in it, phagocytizing the previously arrived apoptotic neutrophils and promoting not only healing wounds, but also scar formation [9]. Macrophages have not only the function of cleaning the wound, but also a synthetic role, producing vascular endothelial growth factor (VEGF), FGFs, PDGF, TGF-β, necessary for re-epithelialization, growth of granulation tissue and scar formation [10]. However, the absence of macrophages does not interfere with repair. In response to signals emanating from necrotized wound areas, granulation tissue is formed, the basis of which is newly formed capillaries. Budding capillaries contribute to the release of plasma proteins, activation of blood circulation and edema, and the high proteolytic activity of leukocytes promotes proteolysis of necrotic tissues. Walraven et al. performed immunohistochemical staining of second trimester adult and fetal skin tissues and lymph nodes and reported lower numbers of macrophages, dendritic and T cells, mast cells, and Langerhans cells in fetal tissues compared with adults [11]. They found, using the leukocyte marker CD45,
that there were fewer white blood cells in the fetal skin but more cells in the lymph nodes, and found no significant difference in vascular density between fetal and adult skin. Based on the results, the authors concluded that less the number of immune cells in fetal skin is not associated with lower vascular density or immaturity of the immune system.

The first phase of the wound process passes into the second, the essence of which is the formation of granulation tissue based on the proliferation of the endothelium of capillaries and fibroblasts. This transition occurs due to the peak accumulation of growth factor cytokines during the inflammatory phase, which explains the direct relationship between fibrosis and prolongation of the inflammatory phase [12]. A distinctive feature of endothelial cells is the high content of enzymes and the intensity of biochemical processes occurring in them. Mast cells are concentrated around the newly formed capillaries, which stimulate the process of tissue proliferation with their enzymes. Depending on the course of this process, the newly formed connective tissue in the bottom of the wound can be transformed into scar tissue in varying degrees of density. An important factor is that fibroblasts are activated only through trauma in response to the release of cytokines, growth factors, then differentiating into myofibroblasts [13].

In addition to direct interaction with immune cells, there is a significant cross-link between dermal fibroblasts and adipocytes: human skin fibroblasts express receptors for many adipokines, including leptin and adiponectin, which act as a kind of fibrosis suppressor by inhibiting fibroblast activation [14]. The emerging elements of the microvasculature are a kind of biological matrix, from which collagen-producing fibroblasts migrate. At the same time, myofibroblasts synthesize proteins actin and desmin, which improve the convergence of wound edges and reduce the area of the wound surface. Since the synthesis processes in the focus of inflammation, in the early stages of defect recovery, require significant energy supply, it is the newly formed vessels that provide oxygen delivery to the cells, which, in turn, actively synthesize protein in the wound.

That is why it is extremely important and at the same time difficult to identify the “golden mean” in the parameters of the impact on angiogenesis in a healing wound: not to transform it into a chronic or long-term non-healing wound, on the one hand, and effectively prevent excessive collagen growth in a ripening scar, on the other. As the wound defect is filled with granulation tissue, the latter becomes more and more dense. The number of microvessels decreases, partially newly formed vessels become empty, the number of cellular elements - fibroblasts, macrophages, mast cells - decreases. A feature of this phase is the predominance of embryonic collagen (type III) in the tissues of the postoperative scar, which is thin fibers and is characterized by elasticity and good extensibility. Subsequently, type III collagen is replaced by less elastic type I collagen. The final stage of any wound process (the third phase or reorganization phase) is the scar formation [3]. In this phase of wound healing, contractile proteins and various components of the extracellular matrix are synthesized by fibroblasts. Fibroblasts, mast cells and macrophages synthesize matrix metalloproteinases, which destroy the components of the extracellular matrix, the strength of scar tissue increases. Normally, the scar matures due to the formation of cross-links and gradually reaches the ratio of collagen type I and III in it, as in normal skin. Within 3 months, the wound transforms from tissue rich in capillaries and cells into a relatively avascular scar, poor in cellular elements, consisting of strong collagen bonds. One of the key processes in the synthesis of ECM, and hence scars, is the transformation of fibroblasts in an adult wound into myofibroblasts that are sensitive to chemical signals (cytokines, chemokines, and growth factors) [15]. Mesenchymal stem cells found to regulate TGF-β release to induce α-SMA expression in myofibroblasts and, as a result, promote accelerated wound healing [16]. Wound healing in adults is associated with the initial proliferation of fibroblasts followed by the synthesis of ECM, and in the fetal wound, its fibroblasts proliferate and deposit ECM simultaneously; moreover, myofibroblasts are absent in the fetal wound. However, fetal skin fibroblasts are capable of contraction despite the lack of α-SMA expression. During scarless healing, the proportion of MMPs is higher than the activity of inhibitors, in particular tissue inhibitors of metalloproteinases (TIMPs). This enzyme-inhibitor ratio promotes ECM remodeling with less collagen [24]. Moving through the wound environment, fibroblasts and other cells synthesize matrix metalloproteinases (MMPs), clearing the path of matrix fragments and cells. In normal wound healing, most fibroblasts undergo apoptosis after the synthesis of an adequate amount of ECM. Although the synthesis of collagen and ECM is necessary for effective wound closure, it can result in the development of fibrosis and the formation of skin scars [17]. However, as observations show, in many cases, the process of scar maturation stretches for 6-12 months, which becomes a new complaint of the patient when contacting doctors involved in the treatment of scars, and the removal of the scar itself becomes a long and often ineffective process [18].

Studies of the Relationship between Angiogenesis and the Scarring Process

Particularly noteworthy are works on the study of the wound process and its relationship with angiogenesis and subsequent scarring: in the article by T. Shmakova et al. discusses the main stages of mammalian skin wound healing and their features in models of scarless or minimal healing, describes an in-depth study of the cascade of events that accompany wound healing, in particular the factors that determine the minimization of the skin scar or its absence [19]. An article by M.W. Ferguson and S.O’Kane proved the effect of growth factors (suppression of TGF-beta1 and TGF-beta2 synthesis in the wound and stimulation of TGF-beta3 production) in scar formation, and further research is related to the development of targeted topical drugs [20]. Research on laboratory animals. Studies of the healing process on laboratory models allow a deeper understanding of the essence of the pathogenesis of this process and further develop effective methods of exposure. At all stages of repair, including inflammation and maturation of scar tissue. In order to assess the changes occurring in healing wounds, it is necessary to conduct studies associated with cytological studies of wound exudate in the early stages (the first 3 days), as well as histological stud-
ies of the emerging granulation and scar tissue in the first 5-15 days after the onset of the wound. For this purpose, laboratory animals are suitable, the skin of which has the closest possible terms and stages of healing to a human. Such animals are mice [21]. Studies of the healing process on laboratory models allow a deeper understanding of the essence of the pathogenesis of this process and further develop effective methods for influencing all stages of repair, including inflammation and maturation of scar tissue. The process of wound healing in mice with a detailed histological study of sections on days 3, 7, 14 and 28 is described in great detail in the article by O. Tikhvinskaya et al. [22]. When conducting an experiment the area of injury is also important: an excisional skin wound on the abdomen of rats is characterized by faster closure and restoration of skin thickness compared to the classical model of skin damage on the back, as well as a significantly smaller scar area, moreover, it is more difficult for animals to intervene during the wound process when the presence of a different ages (8-week-old and 40-week-old males) [23]. Histological data on days 1, 3, 5 and 25 are published in the article and are approved by the authors for scientific use.

Figure 1: Comparison of wound healing slides in BALB/c and C57BL/6 mice at different stages of the process.

Based on histological data, ear structures in BALB/c mice recover more slowly than in C57BL/6, moreover, cartilage repair occurred in 8-week-old males of both strains, which was not observed in 40-wet mice, and in 8-week-old males C57BL/6 there was a complete restoration of the structures of the dermis, including hair follicles, which was not observed in BALB/c.

Conclusion
Based on the data of the works listed above, it can be concluded that for the formation of scars, along with a large number of internal and external factors, the key factors are the activity of the inflammatory response, pronounced angiogenesis, the presence of a disproportion between the synthesis of the extracellular matrix and its destruction in favor of synthesis by suppressing matrix metalloproteinases, the presence of myofibroblasts and factors regulating their activity. Middle-aged BALB/c mice can be considered an ideal model for studying wound healing in order to avoid the effects of “perfect regeneration” characteristic of young individuals, and mice of other strains.

Materials and Methods
Based on the literature review on the study of wound healing in animals using various hardware techniques, we chose a neodymium laser for wound healing in animals. Laser sources have recently been widely used to treat a wide variety of pathologies in various fields of medicine. The mechanism of interaction of laser radiation with a biological object is complex, but in principle it can be either a thermal effect or a resonant interaction with cells. Laser radiation differs from the radiation of conventional light sources by the following characteristics:
- high temporal and spatial coherence;
- high stability of laser radiation in stationary mode;
- high spectral energy density;
- monochromaticity;
- the possibility of generating light pulses with high energy in a wide range of duration of exposure to radiation on the selected object.

The main characteristics of a laser are the wavelength, power and mode of operation, which are continuous or pulsed. For pulsed lasers, from the point of view of exposure, the pulse duration is important, which determines the time during which the laser energy acts on the selected object. Nd:Yag lasers are used to work with vascular formations. The active medium is an yttrium aluminum garnet doped with neodymium. The emitted wavelength is 1064 nm. Nd:Yag lasers have a small absorption in melanin and expressed in hemoglobin and are used for laser treatment of vascular pathologies of the skin, laser photorejuvenation, laser treatment of acne, rosacea, psoriasis and are used in laser phlebology. With all the variety of neodymium lasers on the market, we have identified a laser whose feature is the generation of laser pulses with an energy of up to 8 J and a duration of 650 μs, which allows to transfer a significant energy flow (thermal energy) to the vessel without causing it to overheat and untargeted heating of the surroundings tissues, which may be due to the use of laser pulses with a duration of exposure longer than the thermal relaxation time (TRT). Simply put, there is no risk of skin burns with effective vascular removal. The impact of laser radiation in the absorption spectrum of hemoglobin causes heating of the vessel over its entire cross section (i.e., both the walls and the center). Also for us, as practitioners, the mobility of the equipment is important - small dimensions allow doctors to move the laser between the departments of any clinic, use it in a sterile operating room and laboratory. It is also important that there are no consumables and a choice of lenses with different spot diameters. To form an optimal protocol for postoperative wound management using a neodymium laser with a wavelength...
of 1064 nm, a pulse duration of 650 μs and a maximum pulse energy of up to 8 J “Aerolase Neo Light Pod”, we developed the design of a preclinical study of this laser in the treatment of surgical wounds in animals, which, in our opinion, will help confirm or refute our hypotheses due to a series of laboratory studies of the skin, blood and wound exudate of animals at different phases of the wound process, and will also serve as a guide to conducting such studies for specialists dealing with problems of the wound process and scarring.

Experiment Design
The lines of animals are divided according to the parameters of laser exposure: three main groups. Lines K1 - control (self-healing wound without a bandage, line K2 - individuals whose skin is subjected to laser heating without causing a wound. A total of 135 individuals of 3-month-old BALB/c mice of both sexes were involved in the experiment. It was decided to take the dorsal surface of the body as the incision area, as the least favorable for healing, the incision size is 0.8 cm.

Progress of the Planned Research
1. modeling in animals of a postoperative wound;
2. visual study of the healing process (observation of animal behavior);
3. treatment of wounds with a neodymium laser, according to the design
4. studies of cells and tissues:
   • study of peripheral blood parameters: leukocyte index, catecholamines, CPK, ESR, C-RP
   • cytochemical analysis of the functional activity of neutrophilic granulocytes in peripheral blood, mast cell degranulation coefficient
   • cytological in the first 2 days
   • cytoelectrophotometry
   • histological (sections, staining for collagen, extracellular matrix, forming vessels, assessment of the cellular composition of the matrix), namely:
   1. staining of sections of healing defects with an assessment of density capillaries (CD31+ vessels without lumen)
   2. assessment on sections obtained in s.1 for the presence of small CD31+ vessels with lumen
   3. staining of sections for CD31+ and alpha-smooth muscle actin - with two goals: visualization of large vessels (with a mural GMA component) and assessment of the migration front of mature myofibroblasts [25].
   4. staining with picrosirius or masson at the end point [26].

These research methods will give an idea of the correlation of the density of vessels of various calibers in the study groups with an outcome in the form of fibrosis. - immunohistochemical analysis of cells entering the cell cycle (visualization of endothelialocytes as the direct culprits of neoangiogenesis) - physical (Doppler blood flow, thermometry).

Results of the Study (Planned)
We currently hypothesize that postoperative wound management in BALB/c laboratory mice will lead to an acceleration of the exudation or inflammation phase and proliferation, i.e. granulation tissue will mature faster in mice whose wounds have been treated with a neodymium laser. We also assume that in this group the formation of a more elastic and full-fledged dermis, compared with rigid scar tissue in mice from the control group.

Discussion
When searching for articles on the topic of prevention of skin scars, attention was drawn mainly to the pharmacological and biomolecular focus of scarring prevention:
1. Mark W. J. Ferguson, Sharon O’Kane, 2004 [18]. The article discusses a method for suppressing the synthesis of TGF-beta1 and TGF-beta2 in the wound and stimulating the production of TGF-beta3. The study was conducted on volunteers who made linear incisions (1 cm) in the axillary regions, one incision was treated with the study drug, the second, control, was carried out in the traditional way. Thus, the influence of growth factors in the formation of scars has been proven, and further research is related to the development of targeted topical preparations.
2. Daegu Son, Aram Harijan, 2014 [27]. The article discusses general methods for the prevention of pathological scars, including the removal of tension from tissues in the early postoperative period, the use of non-absorbable inert suture material, wound management in a humid environment, the use of pressure silicone patches for the first 3-6 months after surgery.
3. Kovalevsky A.A., 2005 [28]. Proposal of a method for administering verapamil to fresh post-burn wounds using phonophoresis - the drug has shown high efficiency in early (therapy started 2 weeks after wound epithelialization) prevention of keloid and hypertrophic scars, which is a common complication of burns of 3a degree and deeper. The technique is recommended for the prevention of scars with extensive wound surfaces.
4. Luisa A. DiPietro, 2016 [29]. This study provides a detailed analysis of the process of angiogenesis as a central pathogenetic process in inflammation, wound healing and subsequent scar formation. The conclusion of the research article is the idea of selective suppression of vascular growth by acting on VEGF and pro-inflammatory cytokines.
5. T.V. Shmakova, E.Yu. Kananykhina, G.B. Bolshakova, 2019 [19]. The review discusses the main stages of skin wound healing in mammals and their features in models of scarless or minimal healing (early mammalian fetuses, Acomys spiny mice, oral mucosa, wound on the abdomen of rats). Describes a deep study of the cascade of events, that accompany wound healing, in particular the factors that cause the minimization of the skin scar or its absence. Also in their study, scientists determined that the location of the wound in a laboratory animal (rats, in particular) is of great importance. So they came to the conclusion that a wound on the back heals worse than a wound on the stomach.
Thus, most of the works devoted to the early prevention of fibrous transformation of wounds have a biochemical or pharmacological focus. In our opinion, a significant drawback of these methods is their unpredictability when they enter the systemic circulation and interact with numerous biochemical processes of an individual. Hardware techniques refer to physical methods of influence, they realize their effects locally, which reduces the risk of systemic side effects and adverse events. The economic component is also important: the presence of one or several devices in the clinic is cheaper in the long wound in areas less accessible to the paws and teeth. When searching for an ideal laboratory animal to study the wound healing process at all stages of this process, an article turned out to be practically applicable, in which the authors compared the process of regeneration of all ear structures, including muscle and cartilage, in mice of two lines BALB/c and C57BL/6 and run than the purchase of high-tech drugs, which also applies to their mass production [24].

Conclusion
If our hypothesis is confirmed in the course of this laboratory study, we will conduct a clinical study among patients surgical hospitals, the purpose of which will be the early healing of postoperative surgical wounds in order to prevent their fibrous transformation. These protocols can significantly improve the results of treatment of patients in surgical hospitals, which will affect the quality of their future life, and will also solve one of the most common medical and social problems - the problem of scars.

References

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