

The nature of changes in endocrine and immune factors at the initial stage of the formation of chronic wounds

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Aim. The work aimed to study the nature of changes in endocrine signals (insulin and cortisol) and bioactive molecules that control inflammation and activation of process synthesis (interleukin-2 (IL-2), interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), amino-terminal propeptide of type III procollagen (PIIINP)), at the initial stage of the formation of chronic wounds in the experiment.

Materials and methods. The study was conducted in 2 groups of Wistar rats. The first group is represented by intact animals. Animals of the second group were modeled chronic wounds. On the 3rd day of the experiment, all animals were euthanized. The concentration of studied bioactive molecules was measured in blood serum by enzyme immunoassay. Selected areas of the wound were subjected to histological examination.

Results. The levels of cortisol, insulin, VEGF, IL-2, and PIIINP in animals with chronic wounds at the stage of inflammation were significantly higher compared to intact animals. A tendency towards a decrease in the expression of IL-8 was shown. Relationships between the levels of bioactive molecules in animals of different groups were studied. According to the results of studies a correlation between the concentrations of IL-8 and cortisol in rats of the intact group was established. In the group of rats on the 3rd day after wound induction, a positive correlation was noted between the levels of IL-8 and IL-2. Wound defects healed under the scab. Microscopic analysis showed that the histoarchitectonics of tissues in the area of damage on the 3rd day corresponded to the classic manifestations of the process of healing wounds at this stage. The main part of the wound was filled with fibrin strands with a predominantly inflammatory cellular component. In some areas, signs of growth and development of young granulation tissue were noted. In the marginal sections, signs of re-epithelialization of the wound defect were observed.

Conclusions. Even though in our study, the histoarchitectonics of tissues in the area of damage corresponded to the classical manifestations of the wound healing process at the stage of inflammation, the obtained changes and the relationship between endocrine and immune factors may be harbingers of the formation of the chronic wound in the future.

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Характер змін ендокринних та імунних факторів на початковому етапі формування хронічних ран

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Мета роботи – вивчення характеру змін ендокринних сигналів (інсуліну і кортизолу) та біоактивних молекул, що контролюють запалення та активацію процесів синтезу (інтерлейкіну-2 (IL-2), інтерлейкіну-8 (IL-8), фактора росту ендотелію судин (VEGF), амінокінцевого пропептида проколагену III типу (PIIINP)), на початковій стадії формування хронічних ран в експерименті.

Матеріали та методи. Дослідження здійснили в 2 групах щурів Вістар: перша – інтактна; у тварин другої групи моделювали хронічні рани. На 3 день експерименту всіх тварин вивели з експерименту шляхом евтаназії. У сироватці крові концентрували біоактивних молекул, що вивчали, вимірювали імуноферментним методом. Виділені ділянки рани дослідили гістологічно.

Результати. Рівні кортизолу, інсуліну, VEGF, IL-2 та PIIINP у тварин із хронічними ранами на стадії запалення достовірно вищі порівняно з інтактними щурами. Виявили тенденцію до зниження експресії IL-8. Вивчили зв'язки між рівнями біоактивних молекул у тварин різних груп. За результатами досліджень, у щурів інтактної групи встановлено кореляцію між концентраціями IL-8 і кортизолу. У групі щурів на 3 добу після індукції рани виявили позитивну кореляцію між рівнями IL-8 і IL-2. Загоєння ранових дефектів відбувалося під струпом. Мікроскопічне дослідження показало, що гістоархітектоніка тканин у ділянці пошкодження на 3 добу відповідала класичним уявленням щодо процесу загоєння ран на цьому етапі. Основна частина рани заповнена нитками фібрину з переважно запальним клітинним компонентом. На окремих ділянках виявили ознаки росту й розвитку молодшої грануляційної тканини; у крайових відділах – ознаки реепітелізації ранового дефекту.

Висновки. Незважаючи на те, що в нашому дослідженні гістоархітекtonіка тканин у ділянці ушкодження відповідала класичним проявам процесу загоєння рани на стадії запалення, виявлені зміни та взаємозв'язок ендокринних та імунних факторів можуть бути передвісниками формування хронічної рани в майбутньому.

Сучасні медичні технології. 2023. № 2(57). С. 34-39

The skin is a complex organ that has many strategies to protect the body from external influences. It contains a highly specialized network of immune cells critical for defense and repair and maintaining tissue homeostasis [1]. Trauma sets off a cascade of events designed to rapidly restore skin integrity. Inflammation, a typical pathological process, is necessary to combat the attack of invading pathogens and to remove dead tissue from the site of damage. The initial stage is the same for both acute and chronic inflammation. But if in the case of acute inflammation, there is a completion of the stages, which pass one into another, then in the case of chronic inflammation due to, for example, the impossibility of removing the altering factor, the process becomes chronic in the future.

The dynamics, quantitative and qualitative characteristics of reparative processes are strictly coordinated and regulated by many factors, among which inflammation regulators (interleukins) and growth factors are of great importance [2]. Growth factors and cytokines allow the formation of positive and negative feedback loops to control the ability to inhibit or initiate recovery processes [3]. Clinically, chronic wounds are those wounds that cannot be healed through orderly healing phases but linger in a stage of self-perpetuating inflammation [1]. The other extreme in inflammatory processes is associated with fibrosing activity, as measured by collagen synthesis and degradation [4]. Understanding the mechanisms governing the inflammatory response and its resolution is an important task.

Research is currently mainly focused on elucidating wound healing factors, gene expression networks, and signaling pathways directly involved in tissue repair. Less studied, but important factors are endocrine signals, whose role in the formation of chronic wounds is of particular importance. Through the activation of their respective receptors, they can exert pleiotropic effects on many processes by altering the signaling of localized cellular processes that determine the rate or quality of tissue regeneration [5].

Aim

The work aimed to study the nature of changes in endocrine signals (insulin and cortisol) and bioactive molecules that control inflammation and activation of process synthesis (interleukin-2, interleukin-8, vascular endothelial growth factor, amino-terminal propeptide of type III procollagen), at the initial stage of the formation of chronic wounds in the experiment.

Materials and methods

The study was carried out on 12 Wistar rats, weighing 220–250 g. The experiments were carried out by the Principles of the European Convention for the Protection of Verte-

brate Animals (Strasbourg, 1986), the EU Council Directive of September 22, 2010 “On the protection of animals used in scientific purposes” and were approved by the Bioethics Committee of Kharkiv National Medical University.

Animals were divided into 2 groups. The first control group (Int) is represented by 6 intact animals. Animals of the second experimental group (Exp) were modeled chronic wounds [6]. In our study, a model of a chronic wound was chosen with the reproduction of conditions of local hypoxia and microcirculation disorders, since it is known that ischemia and circulatory disorders are the main causes of delayed healing of trophic wounds [7]. For anesthesia, an intramuscular injection of zoletil (tiletamine hydrochloride and zolazepam hydrochloride) (Virbac, France) at a concentration of 10 mg/kg body weight was used. After 3 days, the animals were euthanized by inhalation of chloroform in a confined space. Blood was obtained by open heart puncture. The concentrations of the studied bioactive molecules in the blood serum were determined by enzyme immunoassay in accordance with the manufacturer's instructions for the reagent kits. Vector-Best kits (Ukraine) were used to determine interleukin-2 (IL-2), interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), insulin, and cortisol levels. Amino-terminal propeptide of type III procollagen (PIIINP) concentrations were obtained using the eBioscience kit (USA).

A section of the wound for histological examination, including all its departments (central, main, edge) was cut out after euthanasia. The samples were fixed in 10 % neutral formalin solution and dehydrated in alcohols of increasing concentration (50°, 70°, twice 96°). The material was passed through alcohol-chloroform and chloroform and embedded in paraffin. The prepared sections, 5–7 μm thick, were stained with hematoxylin and eosin, as well as picrofuchsin according to van Gieson following the standard method. The preparations were analyzed and photographed using a PrimoStar microscope (Zeiss) and a Microocular digital camera.

Statistical analysis was performed using Statistica software v. 12.0 (StatSoft, USA). The significance of the differences between groups was evaluated using the non-parametric Kruskal–Wallis test for independent samples ($p < 0.05$). The results are expressed as the means \pm standard error of the mean (SE). The relationship between the levels of the studied bioactive molecules was evaluated according to Spearman's rank correlation coefficient (R). GraphPad Prism 7 package (GraphPad Software, USA) was used for plotting.

Results

The concentrations of insulin, cortisol, interleukins, VEGF, and PIIINP in the blood serum of animals are presented in *Fig. 1*.

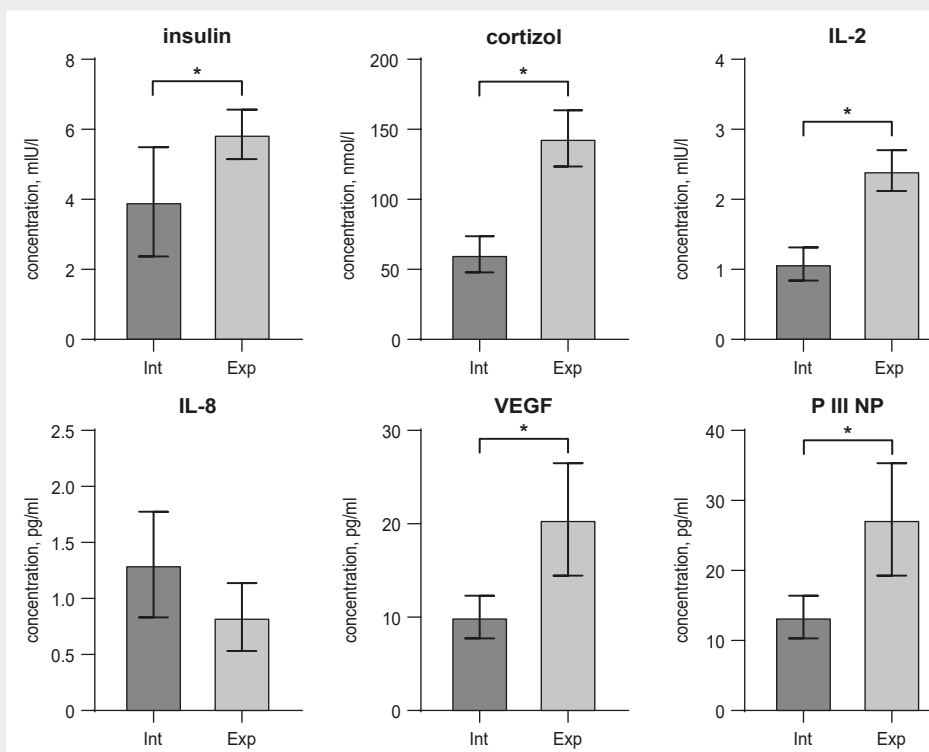


Fig. 1. Changing the levels of the studied indicators in the blood serum of animals (* $p < 0.05$). The error bars represent the standard error of the arithmetic mean for each indicator ($n = 6$).

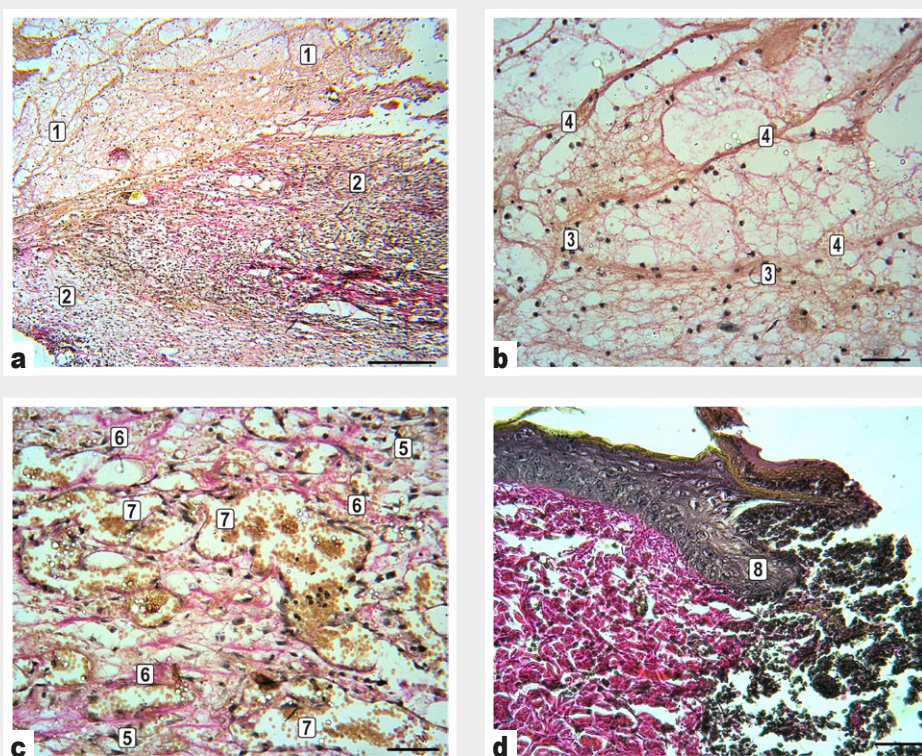


Fig. 2. Areas of the wound cavity of animals after 3 days. **A:** the upper layer of the wound (1), consisting of fibrin and inflammatory infiltrate, the lower one – young granulation tissue (2) with fibroblasts, collagen fibers, and newly formed vessels, scale bar 200 μm ; **B:** polymorphonuclear leukocytes (3) and fibrin fibers of the main part of the defect (4); **C:** young granulation tissue with fibroblasts (5), collagen fibers (6) and capillaries (7) at the bottom of the wound; **D:** proliferation and initiation of migration of cells of the integumentary epithelium at the edge of the wound (8); **B–D:** scale bar 50 μm , van Gieson's stain.

Macroscopically, the wound area in animals was covered with a dense scab 3 days after surgical modeling. Microscopic examination showed features of histoarchitectonics in different parts of the wound cavity (Fig. 2). There was a layer of leukocytes (mainly neutrophilic granulocytes) consisting of fibrin deposits and destroyed cells under the scab. The main part of the wound was filled with fibrin threads, polymorphonuclear leukocytes, and macrophages. In the areas of the bottom and along the edges of the wound, the inflammatory cellular component of the young granulation tissue was supplemented by fibroblasts, and the intercellular substance was supplemented by chaotically located single thin collagen fibers. Newly formed capillaries of various diameters were also observed here. Along the edges of the wound, signs of proliferation of the basal cells of the epidermis, and germination of the epithelial layer under the scab were noted.

Relationships between the levels of bioactive molecules in animals of different groups were studied. According to the results of studies in rats of the intact group, a negative correlation was established between the concentrations of IL-8 and cortisol ($R = -0.907$, $p < 0.05$). A positive correlation was noted between the levels of IL-8 and IL-2 ($R = 0.855$, $p < 0.05$) on day 3 after wound induction in the rat group.

Discussion

In our study, the level of serum cortisol was increased in the animals of the experimental group. Apparently, this effect was caused by the high invasiveness of surgical intervention in the formation of a chronic wound, which led to a high degree of surgical stress response, including neuroendocrine-metabolic and inflammatory-immune reactions [8]. Neuroendocrine effects are induced by changes in the hypothalamic-pituitary-adrenal and sympathetic-adrenal-brain axis. Thus, it showed that plasma cortisol levels were significantly elevated in rats subjected to chronic repetitive immobilization stress for 14 days [9].

It is known that the main glucocorticoid in primates is cortisol, while in rodents it is corticosterone due to inhibition of the CYP21 β enzyme, which is responsible for the synthesis of cortisol [10]. However, mouse serum cortisol and corticosterone have been shown to be closely correlated in dynamics under various physiological or stress conditions, with corticosterone being a more adaptation-related biomarker than cortisol under chronic stress conditions [11].

In our work, the levels of the pro-inflammatory cytokine IL-8 tended to decrease. CXCL8 is the most potent chemokine that attracts human neutrophils and plays a critical role in the response to infection and tissue damage [12]. The correlation between IL-8 and cortisol levels observed in our work in intact animals was absent after wound induction. Apparently, the activation of endocrine and immune mechanisms at the initial stage of the formation of chronic wounds was carried out by different regulatory pathways.

After wound induction, our work showed an increase in the expression of the pro-inflammatory cytokine IL-2 at the stage of inflammation. This appears to be because early

in the wound healing process, IL-2 signaling may play an important role in recruiting immune mediators to initiate the healing process and prevent microbial colonization of the wound site. This cytokine can also promote the proliferation of skin cells and blood vessels. Later, a decrease in IL-2 levels may help resolve inflammation by recruiting and expanding regulatory immune cells [13]. In the experimental group of rats on day 3 after wound induction, a positive correlation was noted between the levels of IL-8 and IL-2. Thus, IL-2 and IL-8 induced modulated inflammatory processes after wound induction.

It was found an increase in the level of insulin after the induction of an experimental complicated wound. There was a violation of the adaptive-compensatory metabolic reactions at the stage of inflammation after the operation. Insulin is the only hypoglycemic agent in the body. In addition, insulin also regulates the body's physiological metabolism, mainly through the receptor tyrosine kinase mechanism, to promote the body's glycogen, fat, and protein synthesis and regulate energy metabolism [14]. The results obtained in our study also are consistent with the literature data, which show an increase in insulin levels on the third day of the postoperative period [15].

Insulin has previously been shown to induce the expression of growth factors such as VEGF and promote healing primarily by increasing blood flow [16]. In our study, we also observed an increased expression of VEGF associated with the activation of this factor during tissue damage. VEGF is an important cytokine that leads to endothelial migration, production of chemotactic agents, proliferation, granulation tissue formation, and angiogenesis [17].

The secretion of pro-inflammatory cytokines, along with the above factors, affects the migration of fibroblasts that promote the deposition of collagen, epithelial and endothelial cells [18]. For example, IL-2 appears to act as a signal for fibroblast growth in a mechanism involving autophagy [19]. In our work, an increase in the concentration of PIIINP in the blood serum of animals with induced chronic wounds was observed. Serum PIIINP is associated with fibrillogenesis and thus is most commonly used as a marker of type III collagen synthesis during early wound healing [20]. Our results are consistent with the literature data, which show that an injury leads to an increase in the metabolism of type III collagen [21]. In severely injured patients, i. e., at high risk of chronic wound formation, elevated serum levels of procollagen type III propeptide were observed [22]. The histological studies carried out confirm the presence of collagen fibers in the wound samples.

Conclusions

At an early stage of chronic wound formation (inflammation stage):

1. A significant reaction of the endocrine system to damage was revealed – a statistically significant increase in the level of insulin and cortisol was found. Probably, the degree of involvement of the endocrine mechanisms of regulation

of metabolic processes is determined by the massiveness of the damage, and, along with other factors, can serve as a predictor of the chronicity of the process.

2. An increase in the level of IL-2 was found, which corresponds to the activation of inflammatory processes; the downward trend in IL-8 levels in animals with chronic wounds may seem to reflect a disruption in the mechanisms governing the inflammatory response; an increase in the levels of VEGF and PIINP indicates the simultaneous activation of synthesis processes already at this stage.

3. A change in the relationship between the regulation of the neurohumoral and immune systems was found. So, in intact animals, there was a correlation between the concentrations of IL-8 and cortisol, and after the induction of wounds, it was absent. Instead, a correlation was observed between IL-8 and IL-2 levels. That is, the activation of endocrine and immune mechanisms at the initial stage of the formation of

chronic wounds is probably carried out through different regulatory pathways.

4. Even though in our study, the histoarchitectonics of tissues in the area of damage on the 3rd day corresponded to the classical manifestations of the wound healing process at this stage, the obtained changes and the relationship between endocrine and immune factors may be precursors of the formation of the chronic wound in the future.

Prospects for further research. Currently, the formation of chronic wounds in patients with endocrine diseases is being studied. Primarily diabetes. However, in individuals without endocrine disease, wound-induced changes are possible and may be significant risk factors. The study of the relationship between endocrine and immune regulation is relevant for the development of new complex methods for the prevention and treatment of chronic wounds.

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