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Original article

Study of the effect of the combined drug PN and HA on the regeneration of collagen fibers of the skin in an experiment

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Abstract. *Relevance:* the scientifically justified use of drugs containing polydeoxyribonucleotide (PN /PDRN) to stimulate various regeneration links has been confirmed by repeated publications in medical literature. The pressing need for therapeutic medications across various medical domains underscores the urgency of exploring novel and effective drug formulations, as well as their synergistic combinations, to enhance the process of regeneration. The pharmacological properties of PN and hyaluronic acid (HA) make it clear that, when combined, these drugs will synergistically stimulate skin healing. However, there is no information about the study of the therapeutic effect of the PN/HA combination on the healing of skin wounds yet. *Aim:* to study the therapeutic effect of the PN/HA combination on the healing of skin wounds in laboratory animals. *Materials and methods:* in this work, the process of collagen fiber (CF) regeneration under the influence of this combination in the preparation **TwAc 2.0** was studied on an experimental model of a scalped wound in 17 white mongrel rats. When analyzing the results obtained: macro and microscopic parameters, morphometric parameters of collagen fibers in the field of regeneration, the dynamics of the restoration of the CF of the skin was evaluated and compared in two groups: the study group, where the combined drug PN/HA was used, and in the comparison group, where a 0.9% NaCl solution was used. *Results:* in an experiment using a combined polynucleotide preparation with HA, it was established: 1. In PN/HA group, the dynamics of maturation of CF was ahead of that of the comparison group by an average of 7 days. 1. In PN/HA group, restoration of skin appendages was noted in the regeneration zone by the 21rst day, a week earlier than the comparison group. 2. During all periods of observation, CF in the area of the scalped wound were formed with an advantage in diameter and accelerated maturation, ahead of the comparison group by 7 days. 3. Scar hypertrophy was not detected in the PN/HA group, which indicates the absence of uncontrolled proliferation of CF and the skin epithelium. 4. During all periods of observation, the experimental group had no CF homogenates at the edges of the wound, unlike the comparison group, which may indicate the protective effect of the PN/HA complex on the preservation of the CF structure, however, this observation requires additional research. *Conclusions:* earlier epithelialization of the wound and maturation of the CF, the normotrophic nature of scarring in the PN/HA group indicates more favorable conditions for regeneration under the influence of the combined drug PN/HA.

Keywords: Polynucleotide, PN, PDRN, TwAc, regeneration, collagen

Introduction

In the medical community, there is an active interest in drugs obtained from the extraction of sperm in rainbow trouts[1-3], the so-called polydeoxyribonucleotides (PN, polynucleotide), whose regenerative properties are opening wider and deeper as research progresses. By composition, PN is a mixture of deoxyribonucleotide polymers with a chain length from 50bp to 2000bp in the form of a sodium salt.

Although they have been studied since the end of the 20th century throughout Europe, to date, the mechanism of action of PN has not been fully studied and there are no final conclusions on how to implement its effects. It is known that the polynucleotide enters the cells by endocytosis (pinocytosis), due to which the cells receive the basis for the synthesis of their own nucleic acids (purine and pyrimidine bases,) and related biologically active molecules in the **damage zone**[3-5]. The activation or inhibition of purinergic receptors (A1, A2A, A2B and A3) also plays a **significant role in the implementation of** biochemical effects[6-8]. In response to the stimulation of these receptors, the humoral regulation of local homeostasis occurs, aimed at the restoration, regulation of inflammatory responses, renewal of damaged and worn-out structural elements of body tissues, including the regeneration of the skin and connective tissue matrix[9-16].

In addition, the authors of scientific papers indicate the absence of side effects or complications due to the use of PN drugs. Studies of acute and chronic toxicity of drugs during stability testing have shown that PN does not have a toxic effect on liver, lung, brain, skeletal muscles, and heart cells, which was established by the results of observations and a histological analysis[4].

However, at the moment there are not a lot of reports of studies on the effect of the currently popular combination drugs consisting of PN and hyaluronic acid (HA) on skin regeneration.

Known works are by Stefano Guizzardi[6], but they mainly concern studies of the potentiation of PN by hyaluronic acid in vitro.

The therapeutic activity of hyaluronic acid[17,18] gives reason to expect synergy in combination with PN to stimulate regeneration. Therefore, there is a question: how does the use of the combination of PN/HA affect the regeneration of collagen fibers and whether the excessive stimulation requires further scientific research.

We performed this study to experiment the effect of the combined drug PN/HA TwAc 2.0 on the regeneration of a skin wound. Special attention was paid to the study of morphometric parameters of collagen fibers of the skin.

Materials and methods

On the basis of the Department of Experimental Surgery of the A.A. Shalimov National Institute of Surgery and Transplantology of the National Academy of Medical Sciences of Ukraine in 2018, a study of skin regeneration processes under the influence of the drug PN/HA was performed on an experimental model of scalped wounds in 17 white mongrel rats[19]. All animals passed a veterinary examination and had a group health passport with the necessary preventive measures. The ways of acquisition, conditions of detention, methods of anesthesia corresponded to the "Rules for performing work using experimental animals and caring for the ethical and humane treatment of animals", respectively, the provisions of the Council of Europe Convention on Biomedical Ethics. Furthermore, during the experiment, we were guided by the standards of Guideforthecore and Use of Laboratory Animals (National Academy Press, Revised, 1996) and the American Heart Association's "Guidelines for the Use of Animals in Research", and fulfilled the requirements of the Law of Ukraine "On protecting animals from abuse" No. 1759-IV dated 12/15/2009 and the order of the Cabinet of Ministers of Ukraine dated 07/28/2010. No. 1585 "On approval of the list of regulatory legal acts on the protection of animals from ill-treatment" and " Scientific and practical recommendations for keeping and working with laboratory animals" GFC MZO of Ukraine (Protocol No. 8 dated 22.06.2012).

In sterile conditions under anesthesia, which was provided by the intraperitoneal administration of 0.2

ml of a 5% sodium thiopental solution and 0.4 ml of a 1% propofol solution, eight rats of the study group (PN/HA group) were intradermally injected with the PN/HA drug in the upper back until a papule with a diameter of 10-15 mm which corresponds to approximately 0.10-0.15 ml was obtained. The top layer of the skin was removed within the papule. The resulting scalped wound, which had the shape of a crater (from the papillary layer of the skin at the edges and to the hypoderm in the center), was covered with an appropriately sized flap of artificial Hartmannsyspur-derm skin after applying a PN/NANA layer to the wound surface. This operation on nine rats of the comparison group (NaCl 0.9% group) was carried out in a similar way, however a sterile physiological 0.9% NaCl solution was used to form papules, and the test drug was not applied to the wound.

After 4, 7, 14, 21, and 28 days, the animals were removed from the experiment through the introduction of 0.8 ml of a 10% sodium thiopental solution, a skin area with a scar located in the center, which formed in the area of the scalped wound, was excised for histological studies. The material was fixed in 10% neutral buffered formalin. Subsequent material processing and sealing in paraffin followed a standardized protocol widely accepted in the field. The histological preparations obtained were stained with hematoxylin and eosin, picrofuxin according to Van Gieson. To compare the dynamics of regeneration in both groups, morphometric studies of collagen fibers were carried out, the diameter of their bundles was measured, their degree of maturity was assessed by tinctorial properties (affinity for dyes) and spatial orientation in the skin's thickness. The control data were the average data obtained during morphometric studies of the intact skin of the back of all 17 white rats. The obtained results were documented and recorded in the study protocol.

Histological studies were carried out using a Leica DM500 research optical microscope with a video analyzer. The preparations were photographed using a Leica ICC50 HD camera. The morphometric processing of 10 visual fields was carried out on each histological preparation (slide) at an increase of 100 using a video analyzer and the computer program "Paradise" developed by the scientific production company "Eva".

Processing of the obtained morphometric data was carried out with the Stata 12 software, and the descriptive statistics were elaborated and presented in the form of arithmetic mean and standard (mean square) deviation. Nonparametric statistics (Mann-Whitney criterion) were used to compare the indicators between the groups with the control group. The rationale for choosing a nonparametric criterion for the comparison of groups was a small number of observations in each group and a heterogeneous variability of indicators[19].

The drug

PN/HA is a combined polynucleotide preparation **TwAc 2.0** (Certificate of conformity No. 015/17/MD), which contains 5 mg of PN and 15 mg of HA in the form of a 3.0 ml solution for injection, in a syringe.

Results

After the operation in both groups, all animals survived, were mobile and active, and their vital functions were not impaired. The intact skin areas in the studied animals, the diameter of the bundles is 12.2 (0.4) microns, are formed into a three-dimensional network, typical of the skin.

The dynamics of wound healing and restoration of the matrix of connective tissue of the skin are presented below.

Day 4. The wound is covered with artificial skin in 100% of animals.

Group NaCl 0.9%

There is no epidermis under the artificial skin, the wound canal is still preserved, not yet completely filled with newly formed connective tissue (NST), the dermis defect begins to fill with cellular elements of the fibroblastic series, thin few collagen fibers are

formed here, the diameter of the bundles is 0.76 ± 0.04 microns, the latter are still immature, mainly picrinophilic, single large homogenized aggregates of collagen fibers are visible around the wound (highlighted by a circle) (Figure 1a).

PN/HA group

The epidermis is also absent, but there is a more pronounced increase on the edges of the wound. The amount of newly formed tissue is small, as in the previous observation. The wound canal is filled with HCT. Compared with the 0.9% NaCl group, thin bundles of CF with a diameter of 0.83±0.04 microns are observed, but fuchsinophilic collagen fibers are more mature than in the comparison group. There are no large collagen homogenates along the edge of the wound (Figure 1b).

Thus, by day 4, there are differences in the healing processes in the control and experimental groups. Animals that have been treated with PN/HA have the following advantages: the wound canal is completely made of HCT, there is an increase in the epidermis on the wound surface, fuchsinophilic, more mature CF are formed, the location of which is characteristic of healthy skin. Homogeneous seals of collagen fibers, which are formed as a result of damage and swelling of the latter in the comparison group, are absent in the experimental group. This observation may indicate the protective effect of PN/HA on the preservation of the structure of CF beams.

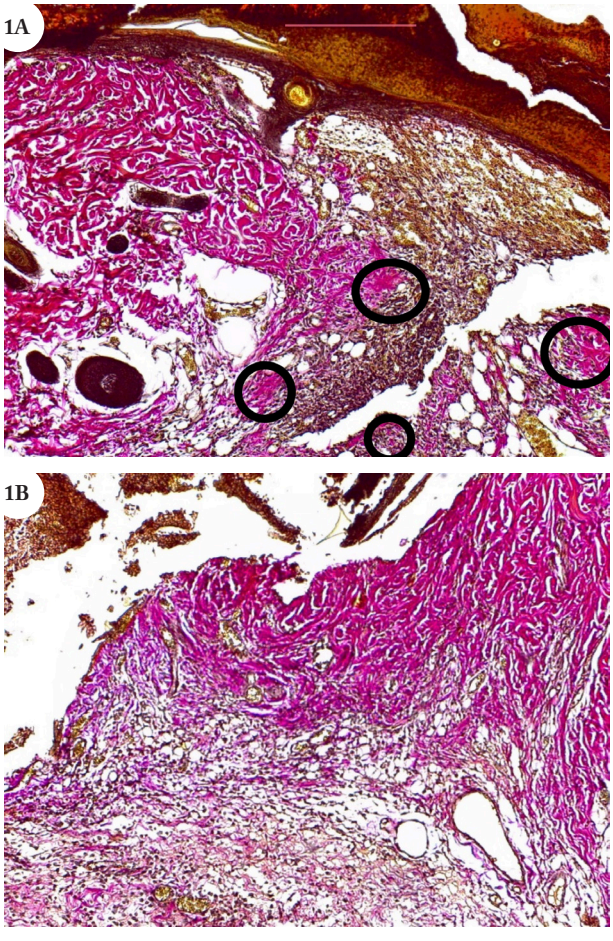


Figure 1 a-b. Scalped rat skin wound. The observation period is 4 days. Painting with picrofuxin by Van Gieson. Magnification of 100. Group I NaCl 0.9%; group II PN/HA.

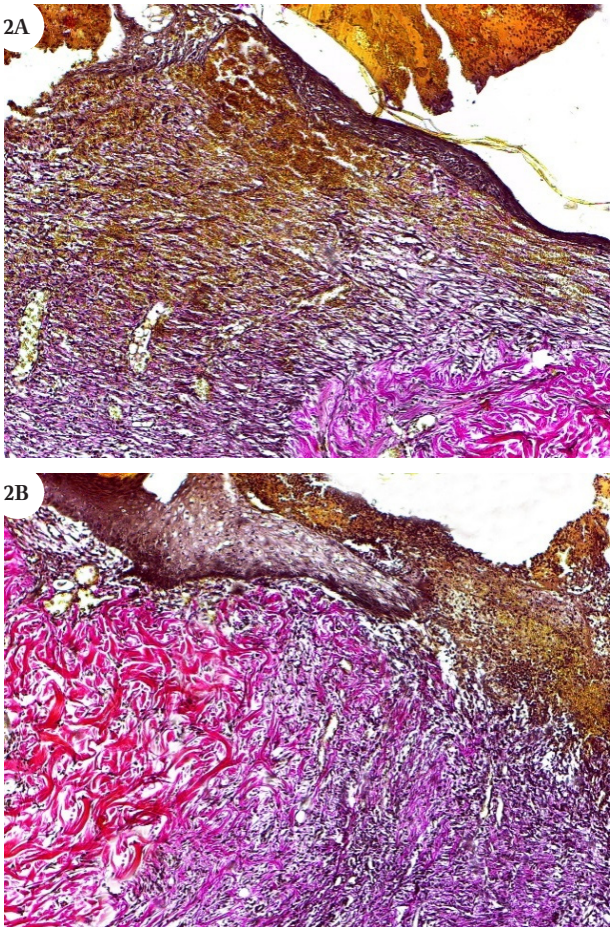


Figure 2 a-b. Scalped rat skin wound. The observation period is 7 days. Painting with picrofuxin by Van Gieson. Magnification of 100. Group I NaCl 0.9%; group II PN/HA.

Day 7

Group NaCl 0.9%.

The wound is covered with artificial skin in 28.5% of animals, and in 71.5% of operated animals it is under a scab of 3-4 mm in size. The epidermis is partially restored, crawling on the edges of the wound. The wound is filled with a vascularized granulation tissue. The bundles of collagen fibers are thin (from 0.84 to 4.48 microns), fuchsinophilic (Figure 2a).

PN/HA group.

The wound is covered with artificial skin in 66.6% of animals; in 33.3% - a wound under a scab measuring 3-4 mm. The epidermis is also partially restored. The defect zone is filled with granulation tissue with thinner (0.76-3.97 microns) bundles of fuchsinophilic CF than in the comparison group (Figure 2b).

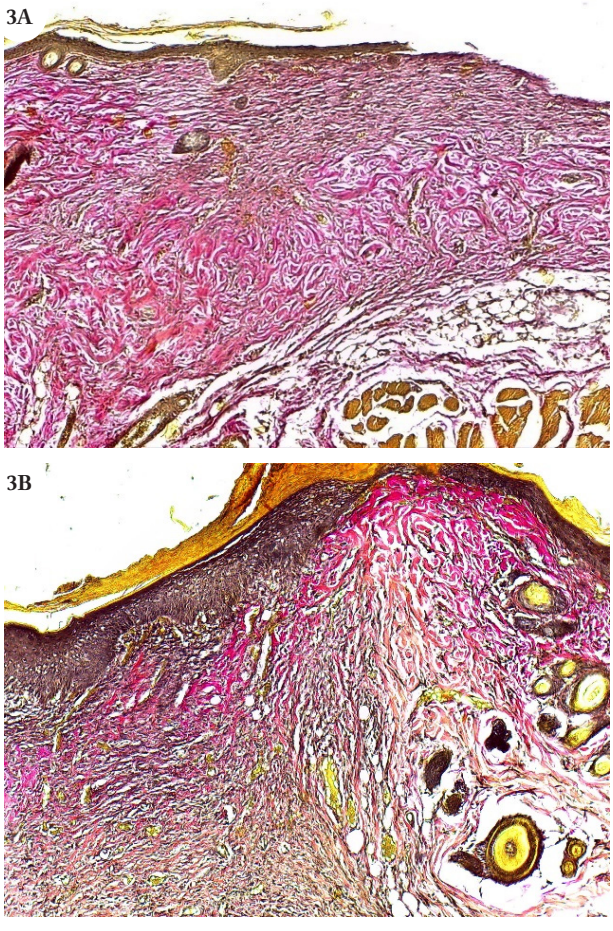


Figure 3 a-b. Scalped rat skin wound. The observation period is 14 days. Painting with picrofuxin by Van Gieson. Magnification of 100. Group I NaCl 0.9%; group II PN/HA.

Day 14.

Group NaCl 0.9%.

The scar is 3-5mm thick, hyperemic, slightly rising above the skin, the hair in the scar area does not grow. The epidermis is not completely restored, it is missing in several areas of the scar and in the suture area. The resulting scar is predominantly normotrophic, in some areas hypotrophic. The diameter of the bundles of collagen fibers has a large spread, from 1.26 to 8.22 microns, which indicates the ongoing processes of collagen formation. Collagen homogenates are also present along the edge of the wound (Figure 3a).

PN/HA group.

The scar is 3-4mm pale, below the level of healthy skin, active growth of wool in the scar area. The epidermis is completely restored, uneven in thickness, thickened closer to the edge of the wound, and thinner in the center than in intact areas. The scar is narrow, mainly normotrophic, the CF bundles are thin (0.89 - 5.28 microns), mature, thicken closer to the surface, oriented mainly tangentially. The formation of hair follicles is noted in the scar tissue (Figure 3b).

Thus, on day 14, a more pronounced, almost complete epithelialization was observed in animals of the HA-PN group, which, in combination with a mature CF, indicate a more active regeneration of the skin in the wound area. In the animals of the comparison group, areas devoid of epidermis were noted, which are heterogeneous in thickness, signs of fibrosis are noted, the skin regeneration process has not yet been completed.

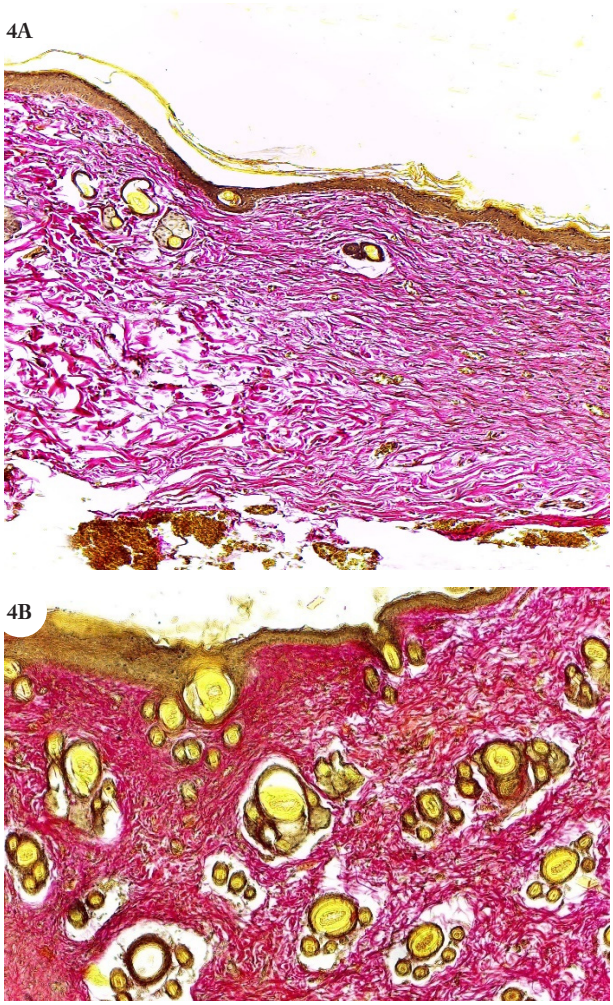


Figure 4 a-b. Scalped rat skin wound. The observation period is 21 days. Painting with picrofuxin by Van Gieson. Magnification of 100. Group I NaCl 0.9%; group II PN/HA.

21 days.

The NaCl group is 0.9%.

The wound area is overgrown with hair, a pale scar 3-4 mm thick, slightly rises above the level of intact skin, shines through from the inside with a pale blue spot, in the area of the scar there is a spike with the underlying muscles. The epidermis is restored, not thickened. The scar is formed by a mature connective tissue without pronounced signs of collagen formation, the bundles of CF with a diameter from 1.92 to 5.28 are oriented mainly tangentially. The scar is normotrophic or hypotrophic with single hair follicles (Figure 4a).

PN/HA group.

The wound area in all rats is overgrown with hair, the scar is 3-4 mm, not dense, practically invisible from the inside, there is a spike with the underlying muscles, comparable to that in the NaCl 0.9% group. The epidermis is restored, somewhat thickened in some areas. 2.94 - 6.53 microns. The scar is formed from a mature connective tissue, narrow, normotrophic, less numerous hair follicles are visible in it than in intact areas, the bulbs are not deep compared to the surrounding areas of the dermis (Figure 4b).

On the 21st day in both groups, a full restoration of the epidermis is observed. In PN/HA group, there were more numerous hair follicles in the area of the scalped wound, the CF bundles acquire the orientation characteristic of intact areas, in the NaCl 0.9% group they are tangentially oriented, and in this group, wider scars were formed.

Day 28.

Group NaCl 0.9%.

The scar is dense, faintly noticeable, a spike with the underlying muscles has formed in the scar area, a newly formed subcutaneous vessel going to the wound site is clearly visible. The epidermis has been completely restored, without any peculiarities. Mature CF beams are thinner than intact ones, with a diameter ranging from 3.3 to 7.16 microns, located mainly tangentially. The scar tissue is mature, there are a few hair follicles in it. The scar is normotrophic (Figure 5a).

PN/HA group.

The scar is 3-4 mm, thinned noticeably below the skin level, bluish, not dense, almost invisible from the inside, the spike with the underlying muscles and the newly formed vessel are comparable to those in the comparison group. The epidermis is restored. Single hair follicles are observed in the scar tissue. The scar in some areas is hypotrophic, in some areas it is closer to normotrophic. The scar is mature, formed mainly by tangentially arranged bundles of thin (4.47 - 8.32

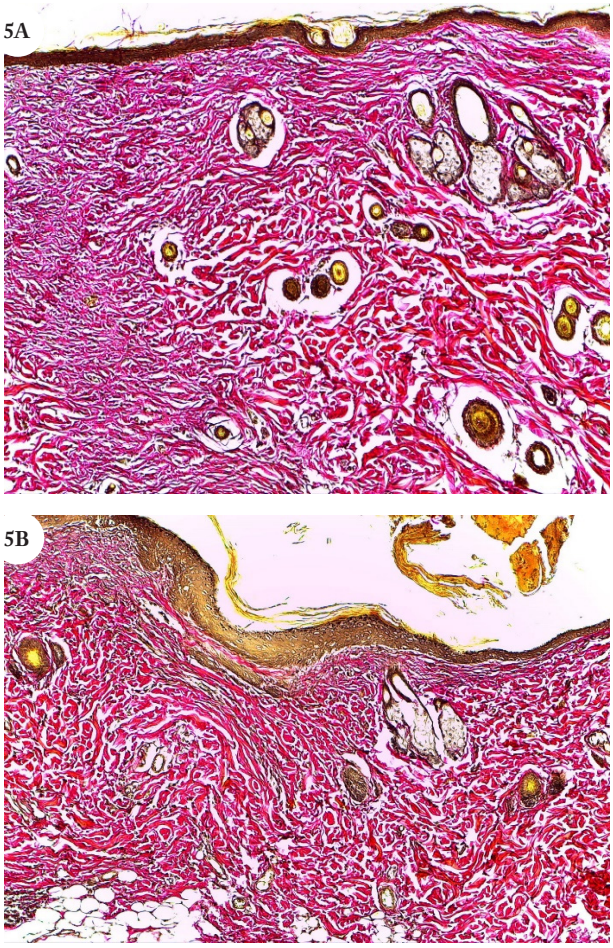


Figure 5 a-b. Scalped rat skin wound. The observation period is 28 days. Painting with picrofuxin by Van Gieson. Magnification of 100.

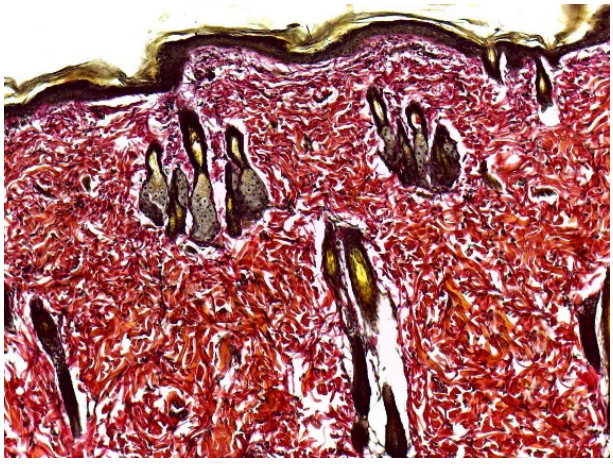


Figure 6. Intact rat skin. Painting with picrofuxin by Van Gieson. Magnification of 100.

microns) collagen fibers, but significantly larger than in the comparison group (Figure 5b).

By this time, there was a full restoration of the epidermis in both groups (Figure 6). It is noteworthy that in the group where the drug PN/HA was used, the healing dynamics is ahead of that in the **NaCl** group by 0.9% on average for 7 days, and the quality of the resulting scars is also different: normal or hypotrophic scars prevail in animals of the **HA-PN** group, and in the comparison group there is a tendency to hypertrophy.

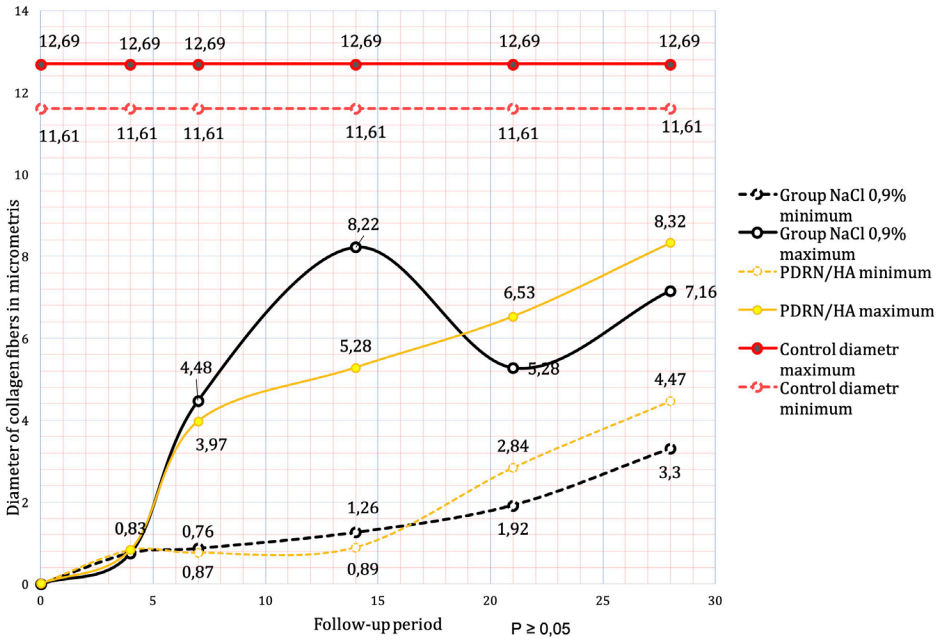
Table 1 shows the average values of the diameter of the CF beams and the presence of various signs of their maturation in both groups during all observation periods.

Discussion

When studying the results of macro-, microscopic studies in dynamics, we found the following features.

The growth of the epithelium on the edges of the wound surface with partial epithelization in the PN/HA group occurred already on day 4, and in the NaCl group 0.9% only on day 7. The **complete epithelialization** of the wound and the completion of the regeneration process in the PN/HA groups were noted already on day 14, and in the **NaCl group 0.9%** only on the 21rst day.

In animals of both groups, scar tissue has characteristic differences: in animals of PN/HA group, normal or hypotrophic scars predominate, and in the comparison group there is a tendency to hypertrophy. The increase in the diameter of the CF bundles and their maturation in animals that were treated with a combined HA polynucleotide was more pronounced at almost all follow-up periods, except for 14 days, and took place at an earlier time than in the **NaCl 0.9% group** and was significantly higher by 28 days, indicating an earlier regeneration in the PN/HA group. The fuchsinophilicity of CF in the polynucleotide group was detected by 4 days, a three-dimensional network of CF bundles was formed by 14 days, during the same period the wound regeneration process was basically completed. The same processes in the comparison group occurred 4-7 days later.



*the differences between the group and the control are true. **the differences between the PN/HA and NaCl 0.9% groups are true

Figure 7. Dynamics of the diameter of newly formed CF beams in the area of the scalped wound in both groups during all observation periods.

Both processes - the synthesis of CF and their maturation during all periods of observation in PN/HA group were ahead of those in the comparison group, due to the local stimulation of metabolism by the drug PN and HA (Figure 7).

According to the data obtained, under the influence of a combined polynucleotide preparation, the body mobilizes reserves for regeneration more effectively from the **first day**, as indicated by the larger diameter of the CF and the presence of fuchsinophilic CF already on day 4 in the PN/HA group, unlike the comparison group, where picrinophilic fibers were mainly noted during the same period. The use of the studied drug eventually reduces the healing time by 7 days[19].

Particular scientific attention is paid to the data on skin appendages in NCT in these groups. Hair follicles are significantly more common in those treated with the PN/HA drug and 7 days earlier than the comparison group.

It is also important that in the studied animals of the PN/HA group, a normotrophic scar is formed (*Fig. 2.II. and Fig. 3.II.)* and morphometric parameters of the diameter of the CF bundles by the end of the month are greater than in the comparison group (the diameter of the bundles is 6.5 and 5.1 microns, respectively), but as a result, in both groups the indicators did not reach the control values (12.1 microns). These facts together allow us to think about the absence of the effect of uncontrolled proliferation of collagen fibers in the combined polynucleotide TwAc 2.0[14,19].

Our data correlates with the results of other studies describing the improvement of the regeneration of ischemic wounds in the experiment, wound healing in rats and mice with diabetes, pressure sores and trophic ulcers in clinical practice and the growth of fibroblasts in culture, under the influence of the topical use of drugs containing PN (adenosine receptor A2A agonists)[8,15,16,19-21].

Conclusions

As a result of studying skin regeneration in an experiment using a combined polynucleotide preparation with HA, it was found:

1. In PN/HA group, the dynamics of maturation of CF was ahead of that of the comparison group by an average of 7 days.

Table 1. Dynamics of changes in morphometric parameters of CF maturation in the wound area during all follow-up periods in the VC/Rfbr groups NaCl 0.9%, and control data.

Terms of observation	**4 days**	**7 days**	**14 days**	**21 days**	**28 days**
Diameter of beams CF control control	12,2 (0,4)	12,2 (0,4)	12,2 (0,4)	12,2 (0,4)	12,2 (0,4)
Diameter of beams CF PN/HA microns	0,83 (0,15)	2,1 (1,3)	2,9 (2,1)	4,3 (1,5)	6,5 (1,6) *
Diameter of beams CF NaCl 0.9% microns	0,76 (0,19)	2,3 (1,6)	4,0 (2,6)	3,6 (1,5)	5,1 (1,8) *
the criterion for the reliability of Mann-Whitney differences between groups	p= 0,274	p=0,710	p= 0,212	p= 0,211	p= 0,032*
Picrinophilicity Control	absent	absent	absent	absent	absent
Picrinophilicity PN/HA	absent	absent	absent	absent	absent
Picrinophilicity **NaCl 0.9%**	present	present	absent	absent	absent
Fuchsinophil Control	present	present	present	present	present
Fuchsinophil PN/HA	present	present	present	present	present
Fuchsinophil NaCl 0.9%	absent	present	present	present	present
Spatial organization of CF beams into a three-dimensional network Control	present	present	present	present	present
Spatial organization of CF beams into a three-dimensional network **NaCl 0.9%**	absent	absent	absent	present	present
Spatial organization of CF beams into a three-dimensional network PN/HA	absent	absent	present	present	present
The presence of skin appendages (hair follicles) control	present multiply	present multiply	present multiply	present multiply	present multiply
The presence of skin appendages (hair follicles) **NaCl 0.9%**	absent	absent	absent	present single	present single
The presence of skin appendages (hair follicles), group PN/HA	absent	absent	beginning of formation	present multiply	present multiply
Mann-Whitney p-criterion; * - the differences between the compared groups are significant					

1. In PN/HA group, restoration of skin appendages was noted in the regeneration zone by the 21rst day, a week earlier than the comparison group.

2. During all periods of observation, CF in the area of the scalped wound were formed with an advantage in diameter and accelerated maturation, ahead of the comparison group by 7 days.

3. Scar hypertrophy was not detected in the PN/HA group, which indicates the absence of uncontrolled proliferation of CF and the skin epithelium.

4. During all periods of observation, the experimental group had no CF homogenates at the edges of the wound, unlike the comparison group, which may indicate the protective effect of the PN/HA complex on the preservation of the CF structure, however, this observation requires additional research.

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Conflict of interest: The author of the article, as well as the co-authors, at several times received fees from the Yuma Medical LP company and other companies related to aesthetic

medicine as consultants. Yuma Medical LP was the sponsor of this study but avoided any kind of pressure on the authors.

List of abbreviations

PN/HA group is an experimental group, a group of animals where a papule was formed during the operation and an application was applied to the wound surface with a sterile combined polynucleotide with hyaluronic acid preparation TwAc 2.0;

The NaCl 0.9% group is a comparison group, a saline solution group, a group of animals where, during surgery, a papilla was formed using an intradermal injection of a sterile 0.9% physiological NaCl solution;

CF - collagen fibers

NCT - newly formed connective tissue;

PN - polydeoxyribonucleotide;

bp is the number of DNA base pairs, which is understood as the "molecular weight" of DNA.

HA - hyaluronic acid

TwAc 2.0 is a combined polynucleotide preparation with hyaluronic acid;

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