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Original Article

Spiroconjugated 1,2,3-triazolo[5,1-*b*]1,3,4-thiadiazine stimulates functional activity of fibroblasts under skin injury regeneration

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Abstract

Background and purpose: One of the most important mechanisms of tissue regeneration is the high functional activity of cells, including proliferation. Currently, there are practically no effective skin cell activators on the pharmaceutical market. The purpose of this work was to demonstrate the stimulating effect of spiroconjugated 1,2,3-triazolo[5,1-*b*]1,3,4-thiadiazine (STT) on the functional activity of fibroblasts.
Experimental approach: STT containing ointment for dermal application was made. To assess *in vivo* effect of the STT a linear wound model in rats was tested. A combination of histological techniques and mechanical testing was employed to estimate the stimulating effect of STT on the functional activity of fibroblasts.
Findings/Results: The STT significantly increased the number of fibroblasts as well as the density and order of produced collagen fibers in the dermis during the wound healing process. As a result, a tissue was formed at the site of damage with the structure corresponding to normal skin. In addition, skin functions were restored, in particular mechanically.
Conclusion and implications: The results suggested the stimulating effect of the STT on fibroblast activity and demonstrated its potential for skin regeneration.

Keywords*:* Collagen; Fibroblasts, Ki-67; Mast cells; Skin wound; 1,2,3-Triazolo-1,3,4-thiadiazine; Vimentin.

INTRODUCTION

Skin injury is an acute issue in modern medicine. Damage skin is characterized by changes in morphology and loss of functionality. Successful cutaneous wound repair requires a series of tightly coordinated steps including inflammation, tissue formation, and remodeling (1-3). Increasingly, some studies reveal the role of different tissue elements' interaction in regeneration. It is known that fibroblasts are the most important element of granulation tissue. These cells determine the processes of formation and remodeling of new tissue. Fibroblasts are also significant because they coordinate the activities of other cells in different phases of regeneration. The fibroblasts' involvement in recovery processes is determined by the level of their proliferative and synthetic activity (1,2,4). Restoration of the skin fibrous component is also crucial because it provides the mechanical characteristics of the skin and maintains dermis architectonics (5).

Because of the regeneration complexity a failure of wound healing takes place. The repair process mainly becomes recurrently deficient due to extended trauma, infections, and inflammation (6). Despite advances in skin wound treatment, a therapeutic effect is not always achieved. In this line, extensive research is underway, focused on searching for regeneration stimulators, so far, the number of effective substances is limited to a few examples. Standard wound healing therapy relies mainly on proliferation activators of natural origin. For example, to accelerate the regeneration Solcoseryl® is widely used (7).

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Chitosan-based products showed reduced inflammation, improved angiogenesis, and accelerated epithelialization (8). The popular substance methyluracil also has a stimulating effect on cell proliferation and microcirculation (9).

Previously, compounds that selectively accelerate the proliferation of human skin fibroblasts *in vitro* were discovered (10). It was found that ethyl 5-(4-ethoxybenzoyl)-5,7- dihydrospiro[[1,2,3] triazolo[5,1-*b*][1,3,4] thiadiazine-6, 1'-cyclopentane]-3-carboxylate (STT) stimulates skin regeneration (5,11). However, the way to achieve this effect is still unclear. In this paper, we evaluated regenerative process parameters that can indicate a possible mechanism of STT action and help determine further approaches to its study.

MATERIALS AND METHODS

Animals

The experiment utilized 63 male outbred laboratory rats (434.7 ± 8.8 g) and complied with the Principles of Laboratory Animal Care. All the experiments were permitted by the Ethics Committee of the Institute of Natural Sciences and Mathematics, UrFU, Russia (Ethic No. 2/2022).

Animals were randomly divided into 4 groups, including 5 (morphological studies) or 4 (mechanical studies) individuals: (1) intact rats (INT); (2) untreated rats as the control group (CONTR); (3) rats exposed to vaseline (VAS); and (4) rats exposed to STT (STT).

Chemicals

Vaseline and Analgin were obtained from Samaramedprom JSC (Russia) and Pharmstandard-UfaVITA JSC (Russia), respectively. The synthesis of STT was carried out at the Institute of Chemical Engineering (UrFU, Russia) (12).

Ointment preparing

Ointment for skin application was prepared by dissolving 208 mg of the 1,2,3-triazolo-1,3,4-thiadiazine in 100 µL of dimethyl sulfoxide, adding this solution to 50 g of Vaseline, and stirring to a homogeneous consistency. To avoid the effects of ointment base, a group of animals (VAS) were exposed to it. Vaseline was chosen due to its beneficial properties for wound healing (13,14).

Experimental procedures

The study included linear wound modeling. The skin from the back was prepared by hair plucking and rinsed with ethanol 96%. A linear wound of 50 ± 2 mm long was created using a surgical scalpel. Before the wound was applied, rats were injected intramuscularly with 0.2 mL Analgin 50%.

After wound modeling, animals were exposed to an experimental ointment (0.4% of STT) or Vaseline. Ointments were applied epicutaneously at the dose of 0.2 g once a day for 14 days. Animals were taken out of the experiment on the 14th and 21st days by an overdose of diethyl ether.

Histological and immunohistochemical analysis

A strip of skin tissue was obtained and fixed in 10% neutral formalin. Tissue specimens were embedded in paraffin wax, sectioned (4 µm), and stained with hematoxylin-eosin (H&E), picrofuchsin (Van Gieson's), and Azure B, to visualize the neotissue formation, collagen deposition, and mast cells number, respectively.

To estimate the cell proliferation intensity and fibroblast synthetic activity, immunohistochemical staining with antibodies against Ki-67 (SP6, rabbit monoclonal antibody, Cell Marque, USA) and vimentin (V9, mouse monoclonal antibody, Cell Marque, USA) was performed according to manufacturer's protocols. Briefly, 4-µm-thick sections were placed on poly-L-lysine-coated adhesive microscope slides (Thermo Scientific-Menzel, Germany) and then dewaxed in xylene and rehydrated in graded alcohols. Sections were incubated in Trilogy™ (Cell Marque, USA) to antigens unmasking for 20 min at 92 °C and in H_2O_2 solution for 10 min at room temperature. After that, sections were washed in tris buffered saline (TBS) immunohistochemistry wash buffer + Tween® 20 (Cell Marque, USA) for 1 min and incubated with primary antibodies for 60 min at 30 °C.

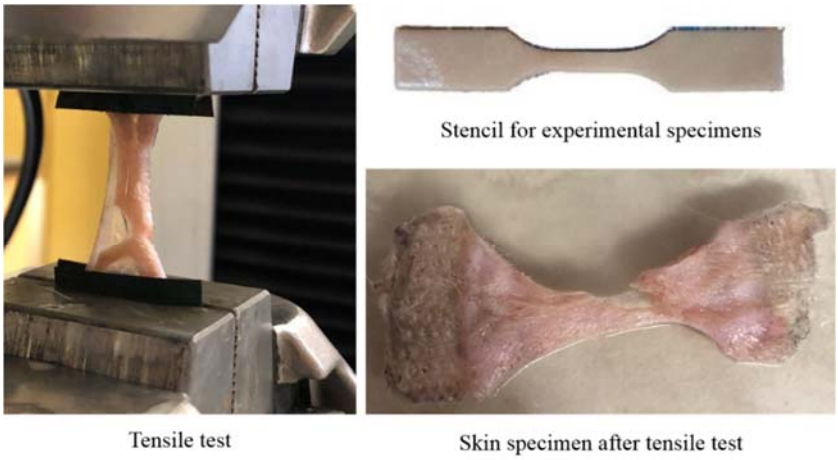

Fig. 1. Mechanical test.

Sections were washed three times with TBS before incubation with secondary antibodies (N-Histofine® Simple Stain MAX PO (MULTI), Nichirei Biosciences INC, Japan) for 30 min at 30 °C. Sections were washed three extra times in TBS and coated with DAB chromogen (DAB Substrate Kit, Cell Marque, USA), and incubated for approximately 30-60 s. After that sections were washed in distilled water and stained with hematoxylin.

Zeiss Primostar provided histological analysis. The average vessels and cell number (fibroblasts, mast cells, Ki-67- and vimentin-positive cells), collagen fibers thickness, and vessel area were analyzed by TopView software according to 10 randomly selected fields with conversion to 1 mm^2. Mast cells were defined as dark purple cells with granular cytoplasm (stained with Azur B). Fibroblasts were defined as cells with a branched basophilic cytoplasm surrounding an elliptical nucleus (stained with H&E). Cells with a brown-stained nucleus and yellow-stained cytoplasm were considered Ki-67 positive and vimentin-positive, respectively.

Mechanical tests

Mechanical tests were conducted on a Shimadzu AGX 50kN tensile testing machine (Japan). The analysis of the sample's mechanical strength was carried out using the method for assessing the deformation behavior of the material under uniaxial compression (15,16). According to a special cardboard stencil, skin samples were cut out. The sample edges were rigidly fixed in the grips of the testing machine (7). After that, a uniaxial tensile load was applied to the sample (Fig. 1). The time of one test was on average 71.342 ± 4.211 s. Upon reaching the point of maximum mechanical stress and the onset of irreversible deformation (ruptures), the tests were stopped.

Statistical analysis

All data were expressed as the mean ± SEM. Statistical analysis was performed using Statistica 12.0 software by Kruskal-Wallis test with Dunn's post-hoc analysis. *P*-values < 0.05 were considered statistically significant. Mathematica software (Wolfram language) was used to visualize the received data.

RESULTS

H&E and Azure B staining

The study aimed to evaluate the effect of the chemical compound STT on the fibroblast's functional activity under linear skin wound regeneration. The points of the experiment were chosen to assess the skin condition at the final stages of regeneration.

On the 14th day, intense fibroblast proliferation was observed in the STT group. Acanthoses were detected, but on the 21st day, the epidermis acquired a uniform thickness. The dermis was divided into papillary and reticular layers. Numerous hair follicles as well as glandular structures were found. The scar was characterized by maturity with a fibrous component predominance (Fig. 2A).

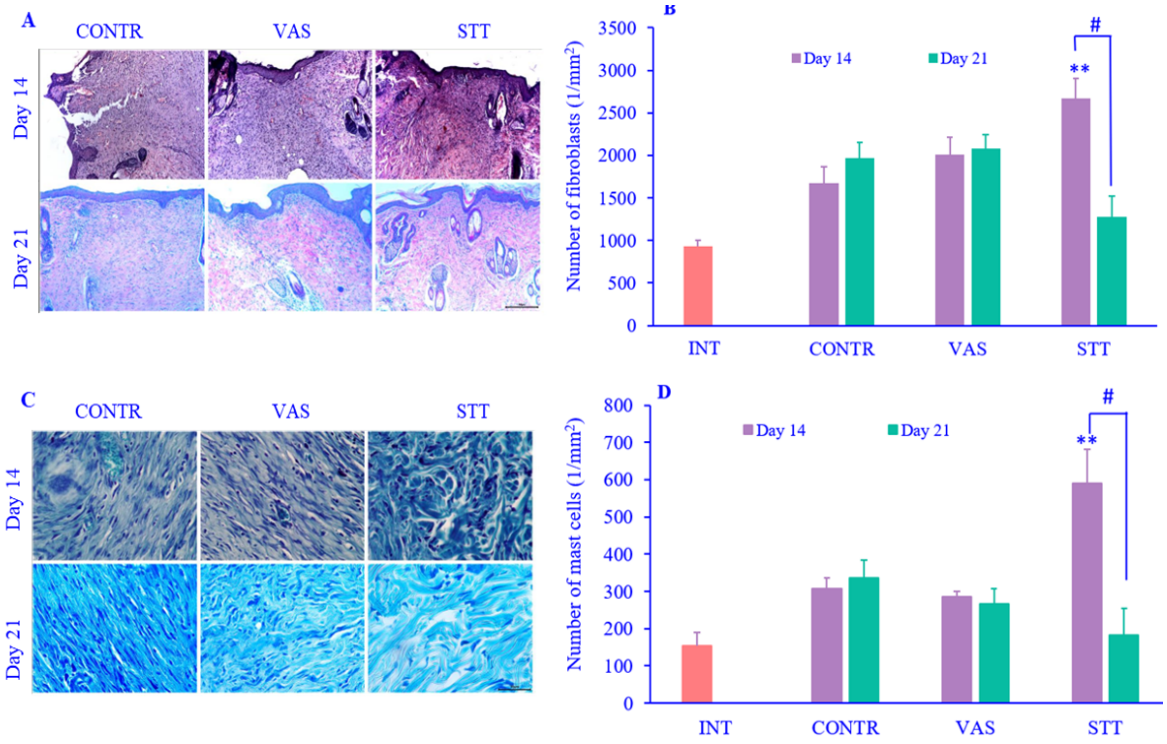

Fig. 2. (A) Hematoxylin and eosin staining, magnification: 100 ×; (B) the number of fibroblasts; (C) Azure B staining, magnification: 400 ×; (D) the number of mast cells. Data are presented mean ± SEM, n = 5. $^{**}P < 0.01$ Indicates significant differences in comparison with the INT group; and $^{\#}P < 0.05$ between the indicated groups. INT, Intact; CONTR, control; VAS, vaseline; STT, spiroconjugated 1,2,3-triazolo[5,1-b]1,3,4-thiadiazine.

The fibroblast number increased in STT groups by the 14th day relative to the CONTR and VAS groups (Fig. 2B). The high content of cells persisted by the 21st day. Changes were noted in the mast cell population (Fig. 2C). On the 14th day, the number of mast cells significantly increased in the STT group comparing other groups (Fig. 2D).

Immunohistochemical staining

The cell implication in regeneration is determined mainly by their proliferative activity (4,5). To assess the intensity of cell proliferation, the Ki-67 marker was used (17). Ki-67 positive cells were determined in the epidermis germ layer, and most of the cells were observed in the STT group on the 14th day (Fig. 3A), which was significantly higher than the values of the CONTR and VAS groups. It was typical that in the earlier period of regeneration, there were more dividing cells (Fig. 3B). However, in the STT group, the number of cells remained significantly high even by the 21st day and it also applied to the dermis (Fig. 3D).

Fibroblasts are an important link that unites various processes during regeneration (5). *Via* vimentin production, fibroblasts coordinate cell proliferation, collagen accumulation, keratinocyte transdifferentiation, and re-epithelialization (4,18-20). The number of vimentin-positive dermal cells on the 14th day of the experiment was high (Fig. 4A and B), which correlated with the fibroblasts number (Fig. 2B). By the 21st day, the number of vimentin-positive cells sharply decreased in the CONTR and VAS groups, so it was significantly less than the values of the STT group on the 14th day. The microvasculature normalization served as reliable marker of successful regeneration. Here, on the 21th day of the experiment we observe normal indicators of vessels number and area (Fig. 4C and D).

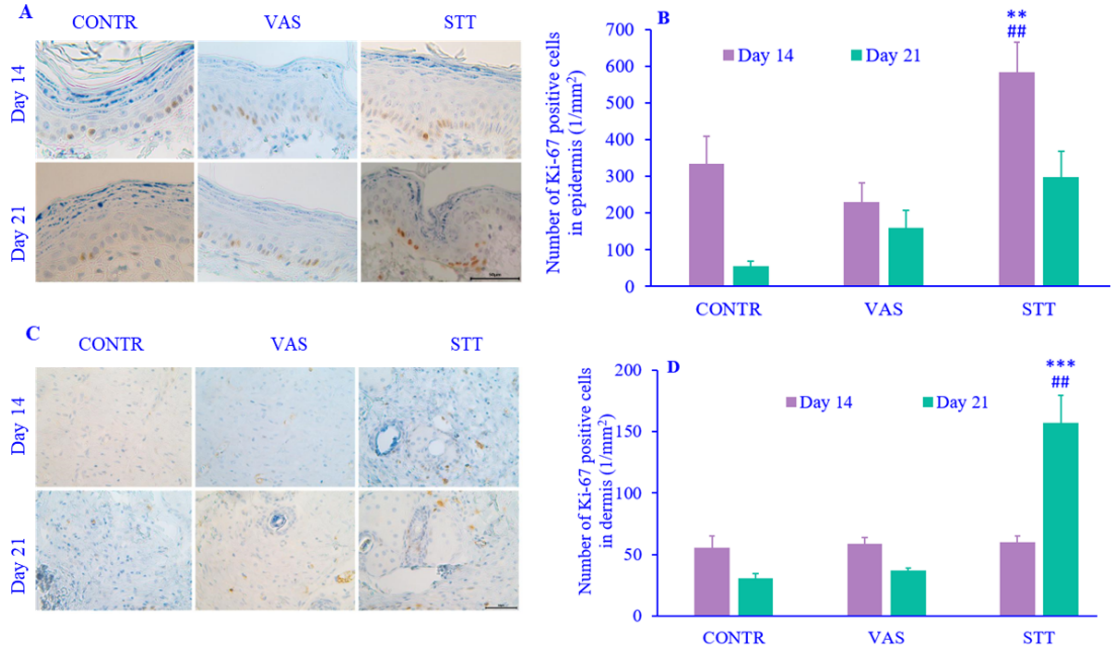

Fig. 3. (A) Immunohistochemical staining of Ki-67 in the epidermis, magnification: 400 ×; (B) number of Ki-67 positive cells in the epidermis; (C) immunohistochemical staining of Ki67 in the dermis, magnification: 400 ×; (D) number of Ki-67 positive cells in the dermis. Data are presented mean ± SEM, n = 5. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences in comparison with the control group; $^{\#\#}P < 0.01$ versus VAS group. INT, Intact; CONTR, control; VAS, vaseline; STT, spiroconjugated 1,2,3-triazolo[5,1-b]1,3,4-thiadiazine.

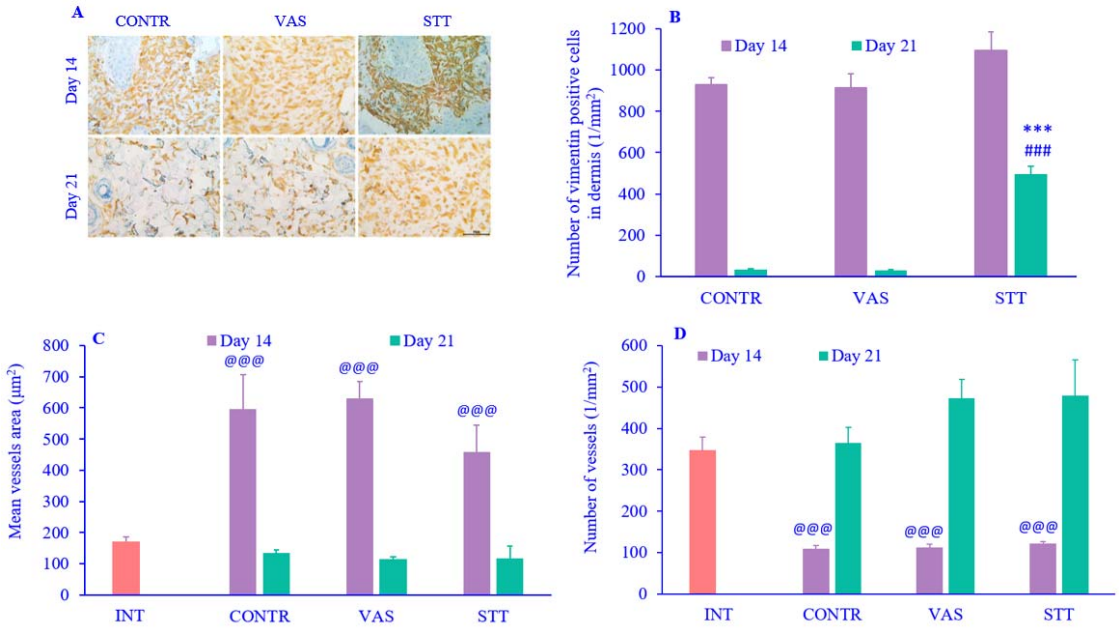

Fig. 4. (A) Immunohistochemical staining of vimentin in the dermis, magnification: 400 ×; (B) the number of vimentin-positive cells in the dermis; (C) mean vessels area; and (D) vessels number. Data are presented mean ± SEM, n = 5. $^{***}P < 0.001$ indicates a significant difference in comparison with the respective control group; $^{\#\#\#}P < 0.001$ versus VAS group; $^{@@@}P < 0.001$ against INT group. INT, Intact; CONTR, control; VAS, vaseline; STT, spiroconjugated 1,2,3-triazolo[5,1-b]1,3,4-thiadiazine.

Van Gieson's staining

Assessment of the skin's fibrous component state can serve as a marker of both the successful restoration of the skin's mechanical functions and the normal scar formation. Collagen deposition became more abundant with prolongation of the time after injury. By the 21st day in the STT group, most of the dermis was occupied by collagen fibers. The papillary region was composed of thin fibers collected in loose bundles. The reticular dermis represented by thick and crimped fibers formed certain direction bundles. The fiber dye was more intense in the CONTR and VAS groups. The morphology and distribution of collagen fibers approximated those in the normal dermis (Fig. 5A). The collagen fiber thickness showed no significant differences between the INT and STT groups on the 14th day. By the 21st day in the STT group fiber bundles were significantly thicker in comparison with the CONTR group (Fig. 5B).

Mechanical tests

Skin characteristics such as resistance to deformation or failure under applied loads can be determined from mechanical tests (16,21). Mechanical tests revealed a general trend in the dependence of the collagen fiber thickness on the skin strength properties. Therefore, on the 14th day, all samples had weak mechanical characteristics that were significantly different from the intact value (Fig. 5C). By the 21st day, the mechanical properties of the skin samples improved. STT group had the highest strength characteristics: at a maximum load of 4.903 ± 1.554 MPa, skin stretched by 34.874 ± 4.829%, which significantly differed from the values of the CONTR group (at 3.772 ± 0.640 MPa, samples stretched by 49.673 ± 14.06%) and approached the INT values (at 4.844 ± 0.206 MPa the percentage of stretch was 33.36 ± 4.92) (Fig. 5D).

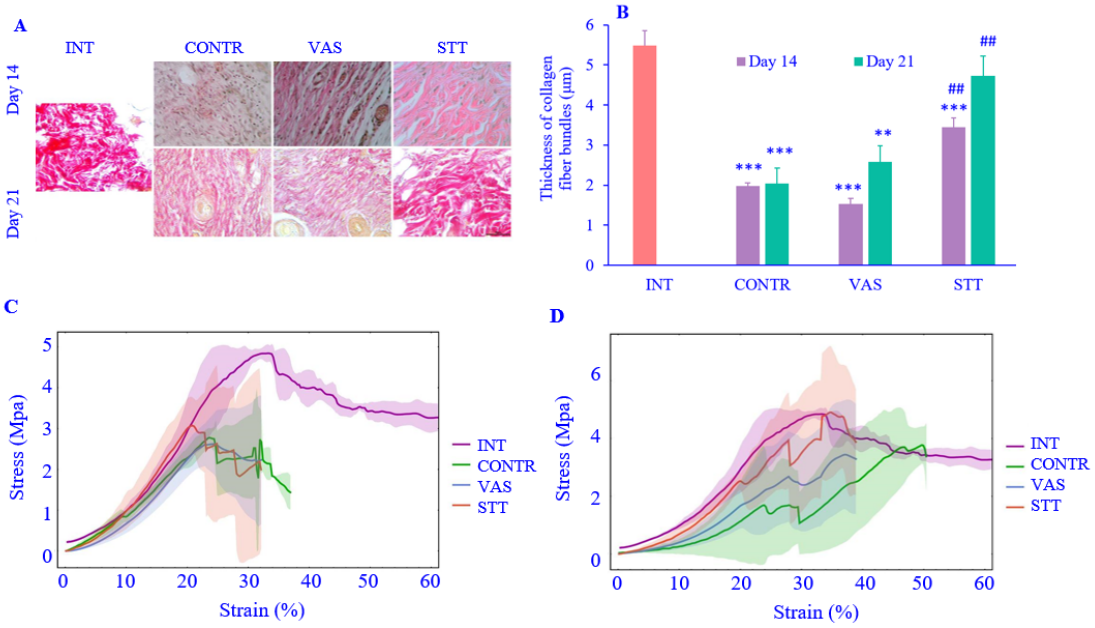

Fig. 5. (A) Van Gieson's staining, magnification: 400 ×; (B) thickness of collagen fiber bundles; (C) mechanical tests on the 14th day of the experiment; (D) mechanical tests on the 21st day of the experiment. Data are presented mean ± SEM, n = 4. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicates significant difference in comparison with the INT group; $^{\#\#}P < 0.01$ versus control group. INT, Intact; CONTR, control; VAS, vaseline; STT, spiroconjugated 1,2,3-triazolo[5,1-b]1,3,4-thiadiazine.

DISCUSSION

The results of this study were consistent with the previously described effect of STT associated with the activation of fibroblasts and scar formation with a developed fibrous component (11). Here, additional parameters were evaluated that can more fully describe the state of the tissue during the regeneration and indicate the possible mechanisms of STT action. On the 14th day, the epidermis in the STT group was characterized by acanthoses, which were associated with epithelium remodeling. Upon injury, keratinocytes at the wound edges form epithelial tongues that move and interact with the dermal cells and matrix to re-establish coverage of the wound bed (3,22). In the STT group, this process was probably more intense, which was confirmed by the high content of Ki-67-positive cells in the epidermis. In addition, a more intensive recovery was observed by the skin derivatives restoration, which could also be associated with increased proliferation of keratinocytes.

In comparison with the previous study, it was found that the fibroblasts number decreased only by the 21st day of the experiment, which may indicate an active restructuring of the scar. The presence of a large number of proliferating cells was correlated with the number of Ki-67-positive cells. Furthermore, a high number of vimentin-positive cells in the STT group may indicate a high functional activity of cells, in particular synthetic. Vimentin participates in numerous processes as an integrator, with functions in cell adhesion, migration, and differentiation (16-18,23-27). Thus, in the dermis, by the 21st day, an increase in the number of Ki-67 positive cells was found in the STT group. Activated fibroblasts through vimentin may coordinate the migratory and proliferative activity of other cells, for example, progenitor cells involved in scar restructuring. This correlates with studies where activated fibroblasts have been shown to facilitate cell migration and tissue reconstruction (3,22,24).

Except for vimentin, synthetic activity is certainly associated with collagens. By the 21st day, the structure of the fibrous component approached the norm. It is worth mentioning that no sclerotic changes were detected, and microcirculation indicators on the 21st day significantly corresponded to intact ones (Fig. 4C and D). Fibrosis may occur due to intense collagenases, but this did not occur in the STT group, which was probably due to the accelerated scar restructuring. Thus, it is known that rapid scar maturation and remodeling prevent the formation of hypertrophic or keloid scars.

In addition, possible interactions between fibroblasts and mast cells played a certain role here. Mast cells are known to be involved in scar remodeling processes. Thus, it is reported that mast cells are responsible for fibroblast proliferation inhibition, which prevents keloid scar formation (28). Considering our results, possibly a large number of activated-STT fibroblasts was a kind of trigger for mast cells to increase their functional activity. As a result, a better-quality scar with better mechanical characteristics was formed. As long as about 60% of the skin is collagen, it is primarily responsible for mechanical properties (29). The structure of collagen fibers in the scar differs from normal skin, which leads to functional disorders. The fibers in the scar are less differentiated, sharply thickened, and randomly located. In keloid scars, an abundance of collagen is noted not only in the reticular but also in the papillary layer (30). In our work, it was demonstrated for CONTR and VAS groups, which presented weak mechanical characteristics. However, in the STT group, the mechanical properties of the skin improved. Probably, due to the STT stimulation of fibroblasts, a mature scar with a predominance of the fibrous component was formed. Considering the described trends, it seems relevant in future studies to study key biomarkers of fibroblast to reveal the mechanisms of their activation under STT treatment. The mechanism of STT action can be clarified by gene expression analyses (such as matrix metallopeptidase 9 and matrix remodeling associated 7 genes) or protein quantification (vimentin, collagens, and fibroblast activation protein α), which play an important role in fibroblast activation during regeneration (1,31).

CONCLUSION

The results of the current study revealed the beneficial effect of STT on skin wound regeneration. Morphological studies indicated that STT was responsible for scar formation with a structure similar to normal skin. An increase in the fibroblasts number, high levels of vimentin, and an intensification of collagenogenesis point to the stimulating effect of STT on the functional activity of fibroblasts. However, detailed mechanisms remain to be explored in future studies.

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Conflict of interest statement

The authors declared no conflicts of interest in this study.

Authors' contributions

I.M. Petrova performed all histological analyses, data acquisition and analysis, statistical analysis, literature search, and wrote the first draft of the manuscript; S.I. Chebanova assisted in microscopic studies; S.L. Khatsko performed all experimental studies with the animals and provided close supervision and helpful discussions about the project direction; T.A. Kalinina synthesized STT and prepared the skin ointment; D.V. Zaitsev assisted with mechanical testing and interpretation of results; and T.V. Glukhareva participated in the concept, design, and coordination of the study and also provided proofreading of the manuscript. All authors read and approved the finalized article.

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