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DOSE-DEPENDENT EFFECTS OF MITOMYCIN C IN NON-HEALING WOUND MODELING

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Non-healing or chronic wounds are widely distributed complications of several pathologic states. A study of healing mechanisms requires an adequate animal model of such wounds. Use of rodents, one of the most available laboratory animals, is linked with some problems: wound edge contraction that precedes re-epithelialization. Mitomycin C (MMC), as a pharmacological inhibitor of cell proliferation, can be used in chronic wound modelling.
The aim. *The objective of the research was to create a model of non-healing (chronic) wound by surgically limiting its contraction and inhibiting recovery rate with the pharmaceutical agent mitomycin C (MMC).*
Materials and methods. *Male Balb/c mice were used. Two layers of skin were pierced through, resulting in the simultaneous formation of two wounds (~0.6 cm^3), whose edges were sutured surgically to hinder their contraction. Wounds were additionally treated with 0.5, 1, 2, and 3 mg/ml MMC. The delay of healing was assessed by measuring wound area and by morphological and histological examination.*
Results. *The application of 2 and 3 mg/ml MMC for surgically fortified excision wounds resulted in a significantly increased area by day 21 and 28 compared with groups treated with lower doses. Also, wounds had loci of necrosis and infiltration. Delayed re-epithelialization and irregular collagen fibres were observed histologically after treatment with 2 and 3 mg/ml. Considering the absence of differences between wounds treated with 2 and 3 mg/ml MMC and its potential toxic effects, 2 mg/ml was recommended for non-healing wound modelling.*
Conclusions. *An optimal model of non-healing (chronic) wound was created. The main aspects of the murine model can be outlined as follows: the use of surgical fixation of wound edges to a dense polymer base and treatment with 2 mg/ml MMC*
Keywords: *non-healing wound, chronic wound, mitomycin C, re-epithelialization, scar, fibrous tissue, keratinocytes, endothelial cells, fibroblasts*

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1. Introduction

In accordance with the guidance of the Food and Drug Administration (FDA) and the Wound Healing Society, a chronic wound is defined as a wound which does not have a normal healing sequence or progress through the wound healing without restoration of proper anatomical and functional integrity for more than 1–3 months [1]. Modern clinical classification of chronic wounds includes 4 main types: pressure ulcers, diabetic ulcers, venous ulcers and arterial insufficiency ulcers [2].

Current estimates suggest that up to 2% of developed countries population suffer from chronic wounds [3, 4]. This indicator is much higher for developing countries. Complicated, non-healing, or chronic wounds are widely distributed in the regions and states where adequate healthcare is unavailable. There may be locations with active combat operations, refugee camps, and regions torn by disasters (forest fire, flooding, tsunami). Some physical factors cause such wounds: shrapnel and bullet injuries, tissue crushing, cuts, and tissue compression with impaired blood flow. Chemical factors may include the impact of chemical weapons, contact of primary injuries with irritating agents. Also, primary wounds can be subject to bacterial contamination or be further affected by diseases such as cancer, inherent and autoimmune diseases, diabetes, and a wide spectrum of circulatory disorders.

The process of wound healing involves many types of cells: fibroblasts, immune cells, keratinocytes, and endothelial cells. Recovery of the blood supply is also a requirement. At the beginning, recovery starts from acute injury, triggering inflammation, which may persist steadily, turning an acute wound into a chronic (non-healing) one. Such wounds typically do not improve for one month or more [5–7] and require timely diagnostics and knowledge on how to treat them. There are some classical methods for chronic wound treatment and novel approaches based on stem cell use [8, 9].

Mitomycin C (MMC) is an antineoplastic cytostatic antibiotic first isolated from *Streptomyces caespitosus* broth that is used as a chemotherapeutic agent [10]. It can introduce covalent bonds in a double-stranded DNA molecules, thus hindering replication, DNA repair, transcription, and cell proliferation. MMC is widely used in medicine. It can be applied topically, administered intravenously, or orally. When MMC is applied topically on damaged skin, it may act as an inhibitor of fibroblast proliferation, suppressing gene expression [11–13]. It may

also affect other cell types: keratinocytes and endothelial cells. In this case, the wound will not proceed through the healing process. In the clinic, MMC is used as a reducer of fibroblast-dependent granulation and an inhibitor of scar formation [14].

Alternatively, MMC can be used for the investigation of wound healing when applied topically to the skin of experimental animals [13, 15]. Biological effects of MMC depend on concentration and exposure time: the higher the concentration and the longer the duration of exposure, the stronger the inhibitory effect. However, there are some problems connected with animal modelling of chronic wounds, especially with rodents, because of a number of anatomical and physiological differences between human and rodent derma [16]. Firstly, rodents have thin dermal layers and a high density of hair follicles. Secondly, the skin of mice or rats has a developed layer of striated muscles (*panniculus carnosus*), which is practically absent in humans. This layer is largely responsible for the rapid closure of injured skin [17]. This brings the edges of the wound closer together, promoting their connection. Then re-epithelialization starts. Conversely, the human skin restores mainly via re-epithelialization when keratinocytes are accumulated in the granulation tissue covering the injury site [16, 18].

In order to form an adequate model for chronic wound studies using mice as one of the most available and easy-to-handle laboratory animals, it is necessary to prevent the contraction of wound edges and to hinder the proliferative stage of reparation. Thus, the objective of research is to create a murine model of non-healing wound by limiting contraction surgically and altering recovery with the pharmaceutical agent MMC.

2. Planning (methodology) of research

The study protocol describing the stages of the work is represented in the flow chart below (Fig. 1).

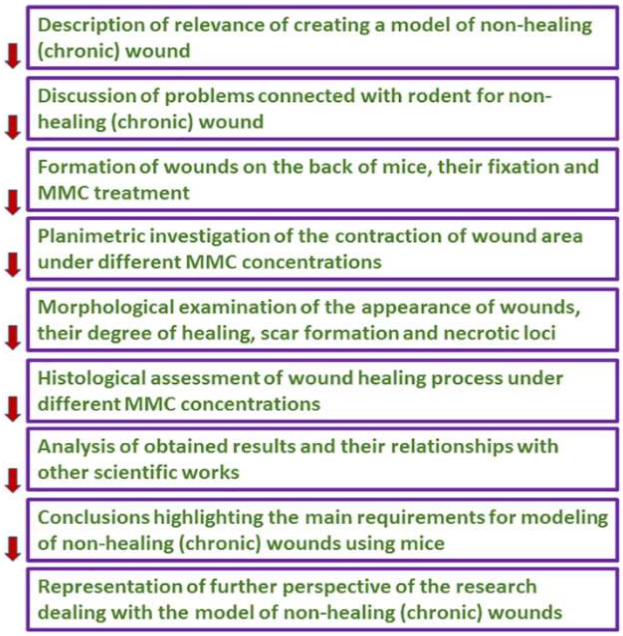

Fig. 1. Methodology of the research

3. Materials and methods

The studies were conducted on male Balb/c mice (n = 94) aged 5–6 months (body weight: 25–30 g). The work was carried out in the period from 2023 to 2025. The experiments were approved by the Bioethics Committee of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine (15.03.2023, Protocol 3). They were aligned with the provisions of the "4th European Convention for the Protection of Vertebrate Animals" (ETS 123, Strasbourg, France, 1986) and complied with the "General Principles of Experimentation adopted by the 5th National Congress on Bioethics (Kyiv, 2013).

Animals were placed six per cage before surgery, after which they were separated and maintained individually. Animals received regular food and water. All manipulations with animals were carried out in compliance with the rules of aseptics and antiseptics. Manipulations with animals were performed using general anesthesia.

Wound formation.

The hair was removed from the back of animals. A skin fold on the back was retracted, and two layers of skin were pierced through with a metal punch with a 7 mm diameter, resulting in two full-layer wounds simultaneously formed with a depth to the fascia. The bleeding was stopped. The injury surface area immediately after the puncture was about 0.6 cm^3 (diameter 8.5–9 mm). 75 µl of preheated (35–40°C) MMC solution with concentrations of 0.5, 1, 2, and 3 mg/ml was applied to the surface of each wound for 10 min. After that, the MMC solution was removed using a gauze swab. To prevent contraction of edges, they were fixed using polymer medical plaster (Dr. House, China) and BF-6 glue (Lubnypharm, Ukraine). Additionally, plaster was fixed with 8 surgical stitches on each wound. Then, wounds were covered with a perforated medical film. An elastic bandage was also applied. Sham-operated animals were used as controls. They received saline solution instead of MMC. Evaluation of the healing rate in mice was carried out based on visual, planimetric, morphological, and histological studies.

Visual observation.

The wounds were examined on the 3rd, 7th, 14th, 21st, and 28th days. Mice were sedated, the dressing was removed, and the wound condition was macroscopically assessed. Simultaneously with the visual assessment, photographs were taken.

Planimetric measurements.

Photographs were taken using a 10 × 10 mm stencil. Planimetric studies included measuring the wound area from images obtained with a Digital IXUS 80 IS camera («Canon», Japan) using ImageJ v. 1.5b (National Institutes of Health, USA). The stencil was used to set the scale. When measuring, just the site, which was not covered by epithelium, was taken into account.

The percentage of the wound surface closure was calculated using the formula

$$((So - St) / So) \times 100\%.$$

Morphological assessments.

Semi-quantitative assessment of the degree of wound healing was additionally assessed on a 6-point scale as described in Ariawan, 2018 [19], with some modifications:

1) red, moist;

2) pink, moist;

3) light brown, generally dry, but with moist foci;

4) dark brown, dry with scab;

5) pink, soft, dry, sometimes dry remnants of scab;

6) pale, soft skin.

In each group, these scores were summed, and the average for the group was found.

The presence of necrosis and scar tissue formation was also described. If foci of necrosis were observed, the wound was assigned the index + or ++, if not – NA:

+ – some necrotic foci at the edge of the wound;

++ – significant foci in the middle of the wound;

NA – no foci of necrosis.

If scar formation was observed, the wound was assigned the index +, ++, or +++, if not – NA:

+ – formation of scar tissue at the edge of the wound;

++ – scar tissue occupied about half of the wound;

+++ – If scar tissue occupied almost the entire wound area;

NA – no scar formation was observed.

The number of NA-, +, ++-, and +++-wounds was represented as a percentage for each group.

Histological studies.

For histological studies, animals were taken out of the experiment on days 7, 14, 21, and 28 by cervical dislocation after narcotization. Skin fragments with wound defects were excised. Then, they were fixed in a 10% formalin solution. According to the standard method, 7-μm-thick paraffin sections were prepared and stained with hematoxylin and eosin.

Histology was visually assessed with a light microscope, AmScope XYL-403, and analysed using AxioVision Rel. 4.8 software. For the semi-quantitative analysis, histological characteristics were assigned numbers from I to IV and a score of +, ++, or +++ depending on the intensity of histological characteristics.

Histological signs that were taken into account:

I – epithelialization;

II – infiltration;

III – granulation tissue;

IV – capillary density in granulation tissue.

Statistical analysis.

The data were processed statistically using "Origin 9.1" software and represented as a dependence of the period of observation versus wound area contraction on the graph. The points on the curve were expressed as a mean ± standard deviation. Results were considered significantly different at $p < 0.05$.

4. Results

Morphological analysis.

In order to select the optimal concentration of MMC that would guarantee slowing of wound healing, several concentrations of MMC were selected: 0.5 and 1, 2, and 3 mg/ml. To prevent edge contractions, the fixation with a polymer medical plaster and BF-6 glue was used as described previously [8, 9]. Additionally, we added 8 surgical stitches for each wound to fix the patch.

Use of medical plaster fragments that were not fixed surgically resulted in their displacement by day 14 and 21 in most of the animals. MMC dose-dependently slowed down the wound healing process (Fig. 2, 3). MMC in a concentration of 0.5 mg/ml did not significantly affect reparative processes. 1, 2, and 3 mg MMC mg/ml significantly slowed down the recovery rate. The differences between these groups, control (w/o MMC) and 0.5 mg/ml were noticeable starting from day 7 after surgery. Wound area in the group with 1 mg/ml MMC differed from the control by 1.5 times, whereas that of having 2 or 3 mg MMC/ml by 2.1 times.

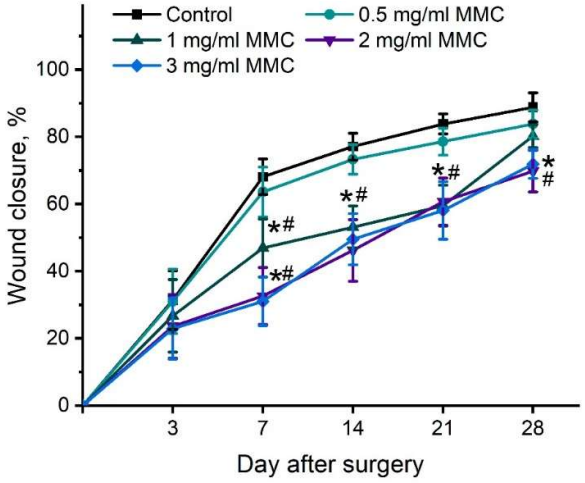

Fig. 2. The effect of MMC concentration on wound closure rate: # – $p < 0.05$ as compared to control; * – $p < 0.05$ as compared to the 0.5 mg MMS group

Wounds treated with higher concentrations of MMC (1–3 mg/ml) showed slower healing. For example, by day 14, the area decreased only by 1.5 times compared to the previous observation period (day 7), and only 20% by day 21 (Fig. 3).

In the final week, wound closure in groups with 2 and 3 mg /ml MMC reached about 70%, while in the control group it was 90%. (In the groups with 0.5 and 1 mg/ml MMC, the effect of delayed healing disappeared by day 28 (Fig. 3).

In groups treated with 2 and 3 mg/ml of MMC, a lower degree of scar formation was observed (Table 1). The majority of animals had indices + and ++ by day 21 and 28, demonstrating reduced cell proliferation and recovery. Conversely, in the control group and after treatment with 1 mg/ml MMC, the majority of animals had indices ++ and +++, pointing to the formation of scar tissue. There were no significant differences between 2 and 3 mg/ml of MMC.

It can be seen that necrotic changes in control and group with 1 mg/ml MMC were absent in the majority of animals at week 3, and at week 4 – in almost all animals (Table 2). In groups with 2 and 3 mg/ml MMC, about half of the wounds had minor foci of necrosis, which also indicates a violation of reparative processes in tissues.

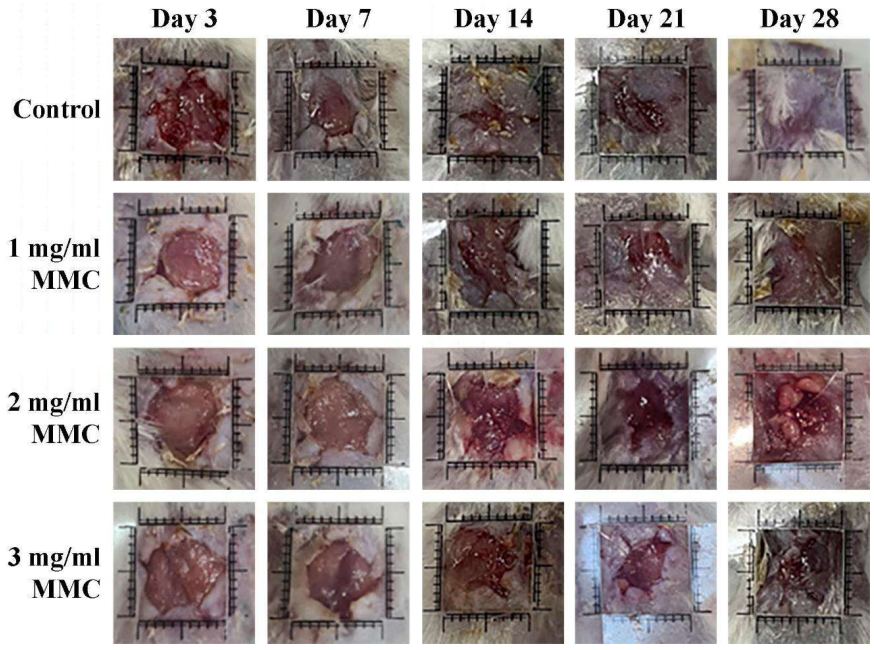

Fig. 3. Wounds after treatment with different concentrations of MMC

Table 1

Scar tissue formation at different concentrations of MMC

Experimental group	Day 21	Day 28
Control	+++ – 40%; ++ – 40%; + – 20%	+++ – 70%; ++ – 30%
1 mg/ml MMC	+++ – 50%; + – 25%; NA – 25%	+++ – 100%
2 mg/ml MMC	+ – 71%; NA – 29%	++ – 50%; + – 25%; NA – 25%
3 mg/ml MMC	++ – 83%; + – 17%	+++ – 50%; ++ – 50%

Table 2

Presence of foci of necrosis in wound beds at different concentrations of MMC

Experimental group	Day 21	Day 28
Control	++ – 20%; NA – 70%	NA – 100%
1 mg/ml MMC	++ – 13%; + – 25%; NA – 62%	NA – 100%
2 mg/ml MMC	+ – 57%; NA – 43%	++ – 25%; + – 25%; NA – 50%
3 mg/ml MMC	++ – 16%; + – 50%; NA – 34%	+ – 30%; NA – 70

By day 21, in the control and in group with 1 mg/ml MMC, most wounds were generally dry and covered with scabs. By day 28, these groups were already at the final stages of healing with scar formation on the skin. Interestingly, in groups with 2 and 3 mg/ml MMC, dry, scabbed wounds were formed only by week 4. No differences were observed between groups that received 2 and 3 mg/ml MMC (Table 3).

Table 3

Average scores for wound healing depending on the concentration of MMC

Experimental group	Day 21	Day 28
Control	3.9	5.0
1 mg/ml MMC	3.9	6.0
2 mg/ml MMC	2	3.5
3 mg/ml MMC	2.5	3.5

Histological analysis.

By day 3 in the control group, the primary changes were observed in the intact skin area surrounding wounds. Numerous foci of leukocyte infiltration were observed in the papillary layer of the dermis. Segmented neutrophils and lymphocytes were present in the infiltrate. At the edge of skin around the injury site, epidermis thickened and, in some sites, formed a migrating epithelial tongue, which was the point of epithelialization beginning (Fig. 4).

By day 7, active formation of granulation tissue occurred in wounds, partially capturing surrounding areas. Extensive sites of collagen formation and numerous cross-sectioned newly formed blood capillaries were visible. On the surface of granulation tissue, growth of epidermal projections from edges to center occurred. However, complete epithelialization had not been observed yet (Fig. 4).

By day 14, the wounds were almost closed by the newly formed epidermis, which was represented by the stratified squamous flat keratinised epithelium with the normal layer arrangement and specialisation. The remodelling process began in the granulation tissue. In some foci of the fibrous scar, the number of cellular elements was significantly reduced, and a well-developed extracellular matrix rich in collagen with fibroblasts located in some places was observed. Newly formed hair follicles were visible (Fig. 4).

By day 21, granulation tissue continued to remodel, mainly due to the formation of elastic fibres in the extracellular matrix. The formation of elastic fibres in the extracellular space and new hair follicles was seen. By day 28, granulation tissue contraction was observed. This occurred due to the contraction of newly formed connective tissue fibres and myofibroblasts. A fully formed epidermis with keratinisation and dermis with hair follicles were present at the injury site. The division of dermis into papillary and reticular layers had not yet occurred (Fig. 4).

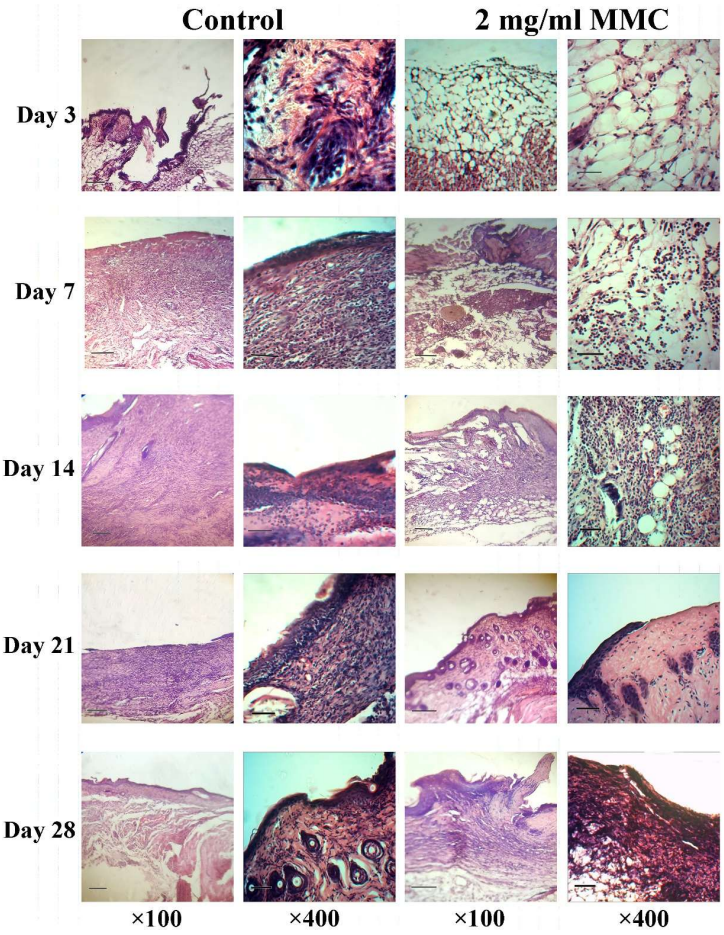

Fig. 4. Histology of mouse skin at the wound area. Hematoxylin and eosin staining

After treatment with 2 mg/ml MMC, necrotic changes were observed in adjacent skin areas and in wounds by day 3. The subcutaneous fatty tissue around injury sites consisted of a loose network of connective tissue fibres with foci of adipocytes. However, the density of fibres and cellular elements was significantly reduced. By day 7, foci of leukocyte infiltration were observed in the dermis around wounds. Granulation tissue was formed irregularly. Areas with cellular elements and newly formed collagen fibres alternated with sites depleted of collagen and cellular elements. There were some areas in which cross-sectioned blood capillaries were present. At the edges of wounds, epidermis thickened, but the formation of epithelial tongue, which is the point of epithelialization, was not observed (Fig. 4).

By day 14, the formation of epidermis was observed from the edges to the centre of the wounds. At the same time, in the peripheral areas, the epidermis was represented by multilayered flat keratinised epithelium, while in the central part, epithelialization was not observed yet. In the wound bed, active formation of the granulation tissue occurred with the formation of new capillaries, connective tissue fibres and extracellular matrix (Fig. 4).

By day 21, epithelialization was irregular. At the edges of the wounds, a full-layer epidermis with keratinisation was seen, while in the centre, a single- or double-layered epithelium was still present. Remodelling processes began in the granulation tissue. In some foci of fibrosis, the number of cells significantly decreased, and active formation of hair follicles was observed. By day 28, the histological picture was similar. The remodelling processes of granulation tissue still occurred irregularly. There were foci of infiltration. Epithelialization of the centre of wounds continued. The formed epidermis sometimes showed signs of hyperplasia (Fig. 4).

Regeneration processes of wounds treated with 3 mg/ml MMC were similar to wounds treated with 2 mg/ml. These groups were characterised by increased necrosis at the injury site and adjacent skin areas. Epithelial layer appeared irregular during reparation. Semi-quantitative analysis of histological sections supports the observed changes and indicates poor healing of wounds treated with 2 and 3 mg/ml MMC (Table 4). It also demonstrates that the effect of MMC was dose-dependent in the range of 0.5 to 2 mg/ml.

Table 4

Analysis of histological sections of skin

Day	CONTROL				MMC, 0.5 mg/ml				MMC, 2 mg/ml				MMC, 3 mg/ml			
	I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
3	–	++	+	–	–	+	+	–	–	–	–	–	–	–	–	–
7	++	+++	+++	+++	+	++	++	+	–	++	+	++	–	+	+	+
14	+++	+	+++	+++	+++	+	+++	+++	++	+++	+++	+++	+	+++	+++	+++
21	+++	–	++	++	+++	–	++	++	++	++	++	++	++	++	++	++
28	+++	–	+	+	+++	–	+	++	++	++	++	++	++	++	++	++

Note: I – epithelialization; II – infiltration; III – granulation tissue; IV – capillary density in granulation tissue; – absence of sign; + weakly expressed sign; ++ well expressed sign; +++ maximally expressed sign.

5. Discussion

Chronic wounds most often arise in cases of severe concomitant diseases, such as diabetes mellitus, obesity, immune system insufficiency, peripheral vascular disease, and others. The healing in acute injury normally proceeds without any hindrance and consists of four stages: hemostasis, inflammation, proliferation, and remodelling. As a result of the smooth subsequent changes of these stages, wounds heal completely. It becomes covered with a new epithelium. The scar tissue of varying density is also formed. A distinctive feature of chronic wound recovery is long-term persistent inflammation. One of the consequences of the inflammation is a risk of additional infec-

tions, which creates a vicious cycle, additionally postponing healing. The investigation of reparatory processes as well as the creation of new approaches to the treatment of such injuries is impossible without the creation of an appropriate model of chronic non-healing wound.

Skin reparative processes substantively differ in human and rodent species, and the main difference that hinders the creation of an animal model of a non-healing wound is the contraction of wound edges. There is a number of studies dealing with animal modelling using MMC, in particular with murine models [14, 20–22]. Most of these studies aim to suppress the formation of thick fibrous scar tissue by applying MMC, which shows promise in the treatment of some surgical complications [23, 24].

However, some of the murine models use MMC to decrease the formation of scar tissue but not to prevent wound contraction and, thus, are not suitable and convenient models of non-healing or chronic wounds [14]. The authors used topical applications of MMC in a concentration of 0.5–1 mg/ml, but did not prevent contractions of wound edges. Although they reported a decreased rate of injury area reduction in MMC-treated groups, qualitative analysis (histology, morphology) remained out of the scope of research, as authors focused on a plausible application of MMC to reach a "cosmetically acceptable end point" of wound healing with the reduced scar tissue.

Another murine model of intractable skin ulcer used 1 mg/ml of MMC, fixation to prevent wound edges contraction to form a model for application of mesenchymal stem cells to speed up the reparatory process and improve the outcome of healing [20]. However, the observation period did not exceed 14 days when MMC was applied, which is too short a period for chronic wound modelling.

Known methods for inducing wounds with delayed healing include the use of MMC at a concentration of 0.5 mg/ml in rats [13]. A limitation of this approach is the need for repeated MMC injections during the observation period. Furthermore, MMC is applied via gauze soaked in the specified solution, making it difficult to precisely control the amount of MMC delivered to the wound. Additionally, this method does not involve covering the wound surface, which can allow the entry of foreign substances and potentially confound the assessment of wound healing.

The research carried out in our laboratory has also shown that the use of low doses of MMC (0.5 mg/ml) for the complication of a full-thickness excisional wound model resulted in the development of reparatory processes and complete re-epithelialization of wounds by week 2 [8]. This necessitates the studies aimed at improving the existing models.

In order to optimise the model, we used surgical and chemical approaches. First, in addition to adhesive material (polymeric medical plaster and BF-6 glue) applied on the excisional wound shown in the previous work [8, 9], wounds were sutured at 8 different points. This guaranteed the prevention of wound edges contractions at an extended period of observation (up to four weeks). Second, MMC was applied to suppress cellular processes that normally accompany hemostasis, inflammation, proliferation, and remodelling. We experimented with MMC concentrations ranging from 0.5 to 3 mg/ml to unveil detailed planimetric, morphological and histological differences between wounds.

Planimetric differences clearly showed the dose-dependent suppression of injury area contraction, which directed the recovery process towards re-epithelialization (the way of human wound healing). There were no differences in wound square between groups treated with 2 and 3 mg/ml. This suggests a preference for using a lower dose of 2 mg/mg MMC for non-healing wound modelling, especially when taking into account the toxic and carcinogenic effects of MMC on cells [25, 26]. The suggestion was supported by wound morphological appearance that included a lower degree of scar formation, presence of necrosis at the wound bed, and the lowest score of wound healing compared with groups treated with 0.5 and 1 mg/ml. The observations were also supported by formed irregular granulation tissue, altered alignment of collagen fibres, disrupted re-epithelization and presence of infiltration foci in the case of the application of 2 and 3 mg/ml MMC.

Practical relevance. The study of reparative processes provides a deeper understanding of the causes and mechanisms of wound healing, scar formation, and skin function restoration. This has significant practical importance in medicine and cosmetology. Developing an adequate model of chronic or non-healing wounds enables the refinement of existing treatment methods and the development of new approaches – for example, those based on the use of novel pharmaceutical agents that enhance healing, or cell therapy methods involving stem cells and their derivatives.

Research limitations. A limitation of wound models developed using rodents is the difference in healing mechanisms compared to humans: in rodents, wound closure primarily occurs through rapid contraction of the wound edges. In the present study, this issue was addressed by surgically fixing the wound edges to a polymer material. Although this surgical intervention itself does not promote healing and does not hinder the creation of the model of chronic (non-healing) wounds, it is an important factor to consider in the further application of the model – for instance, when studying the effects of pharmaceutical agents on wound healing.

Further research prospects. Attention to the benefits and potential adverse effects of different MMC concentrations must be maintained and examined, but present results clearly indicate that 2 mg/ml MMC can help extend the period of wound healing in the murine model for further use in studies aiming to understand processes of wound recovery and methods of chronic wound treatment. The adequate murine model of non-healing wounds opens new perspectives, the objective of which is the treatment of such wounds. The treatment may involve testing of new pharmaceutical chemicals or the use of novel methods of regenerative medicine based on the application of stem cells.

6. Conclusions

The obtained results indicate that MMC slows down wound healing in a dose-dependent manner. Concentration of 0.5 mg/ml was ineffective, especially by day 21 and 28. 1 mg/ml MMC was generally effective during the first two weeks. To form a wound that does not

heal for more than 3 or 4 weeks, higher doses of MMC, 2 and 3 mg/ml, are necessary. Additionally, procedures that prevent the contraction of wound edges, which include the application of surgical sutures to skin and polymer plaster with prior fixation of plaster with BF-6 glue, should be included. Since no significant differences were observed between these two MMC concentrations (2 and 3 mg/ml), 2 mg/ml MMC can be recommended for chronic wound modelling in laboratory rodents.

Conflict of interest

The authors declare that they have no conflict of interest with this research, whether financial, personal, authorship or otherwise, that could affect the research and its results presented in this article.

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Data availability

Data will be made available at a reasonable request.

Use of artificial intelligence

The authors confirm they did not use artificial intelligence technologies to create the current work.

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