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WORKING PROTOCOL ON WOUND SPLINTING MODEL ADAPTED TO THE USE OF NANOPARTICLE-BASED OINTMENTS FOR THE EVALUATION OF SKIN REGENERATION

PROTOCOL DE LUCRU PRIVIND MODELELE EXCIZIONALE CU ATELE SILICATICE ADAPTAT LA UTILIZAREA UNGUENTELOR PE BAZĂ DE NANOPARTICULE PENTRU EVALUAREA REGENERĂRII CUTANATE

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ABSTRACT | REZUMAT

Skin is the body's largest organ in both humans and animals. The multi-layered structure is vital to the multiple functions skin performs. Wound healing pathologies may interfere with its protective and metabolic function and lead to systemic complications. Skin repair and regeneration are based on multiple and highly integrated and synchronous events. The mechanisms underpinning skin wound repair and its failure to heal are still poorly understood. Rodent animal models that can reiterate most of the human conditions were developed for use in preclinical studies exploring the wound healing process and the therapeutic options. Except for the initial contraction, the rodent wounds heal through granulation and re-epithelialization, a process similar to that in humans. The excisional splinting model is optimal for the evaluation of the bioactive glass-gold-nanoparticles-based ointments effect on the wound healing process. In laboratory animals, two or four symmetric excisional wounds are made on the upper back skin, within T1 and L1 regions, by using a biopsy punch. When rats are used as animal models, a silicon splinting ring is applied around the wound to prevent the wound closure caused by *panniculus carnosus* contraction. By using the excisional splinting model, wound closure and vascularization can be visualized, measured and assessed at different time points, and the individual factor that may bias results interpretation can be eliminated. The rat and mouse excisional wound splinting models have been used as working protocol in different studies that assessed the skin healing process and tissue regeneration following application of bioactive glass -gold nanoparticles-based ointments or stem cells transplantation. Comparison of their results could provide new insights into the healing process and could eventually be translated to viable clinical treatments in human patients with skin healing pathologies.

Keywords: skin healing, remodelling, excisional model, ointment, protocol

Pielea este cel mai mare organ al corpului, atât la om și cât și la animale. Fiind un organ multistratificat, aceasta îndeplinește multiple funcții, iar procesele patologice de la acest nivel au efect asupra întregului organism. În procesul de vindecare a pielii, corpul inițiază o cascadă complexă de procese biologice în vederea reparării și regenerării țesutului deteriorat sau pierdut. Pentru a examina și evalua procesul de vindecare și eficacitatea diferitelor formule terapeutice aplicate pe rănile cutanate au fost dezvoltate o mare varietate de modele intervenționale, majoritatea studiilor făcându-se pe șoareci și șobolani. La aceste specii procesul de regenerare al pielii se produce prin granulare și re-epitelizare, fiind similar cu cel întalnit la oameni. Deși vindecarea rănilor la animalele utilizate ca model sunt reflexia imperfectă a proceselor de vindecare a rănilor de la om, acestea continuă să fie instrumente biologice esențiale pentru elaborarea de noi strategii și abordări privind terapia vindecării rănilor. Tehnica intervențională care oferă cele mai bune rezultate în evaluarea procesului de regenerare cutanată este modelul excizional care constă în crearea de excizii dermice circulare cu ajutorul biopsy-punch. Pentru a împedica închiderea exciziei sub acțiunea puternică a mușchiului *panniculus carnosus* la șobolan, trebuie fixat în jurul acesteia un inel silicatic. Utilizarea modelelor excizionale cu 2 sau cu 4 excizii circulare de dimensiuni standard realizate în regiunea dorsală a toracelui de la T1 la L1, în condiții aseptice, pentru a evalua efectul unor unguente bioactive pe bază de nanoparticule asupra procesului de vindecare și regenerare cutanată la unele animale de laborator, oferă posibilitatea de a măsura și cuantifica cât mai exact fiecare etapă de remodelare cutanată și de formare a cicatricei. Rezultatele unor studii care au utilizat ca protocol de lucru modelul excizionl cu privire la procesul de închidere a rănii și de regenerare a pielii la șobolan și șoareci prin testarea unor unguente cu substanță bioactivă pe bază de nanoparticole de concentrație diferită sau prin utilizarea unor celule stem au permis comparații între studii, ceea ce ne determină să considerăm că aceste cunoștințe ar putea fi transferate în situații clinice întâlnite la om.

Cuvinte cheie: piele, vindecare, remodelare, model excizional, protocol

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Skin is the body's largest organ in both humans and animals. The multi-layered structure is vital to the multiple functions skin performs. It acts as an anatomical barrier from pathogens and physical harms between the internal and external environment, it informs the body about the environment characteristics and makes exchanges with it through thermolysis, it is the major source of vitamin D and is involved in the calcium-phosphorus metabolism (3, 24).

Histologically, mammalian skin is composed of three main layers: the epidermis, the dermis and a subcutaneous fascia called the hypodermis that lies deep in the dermis. Depending on the skin region, the epidermis is composed of four to five layers: cornified, clear/translucent (only in palms and soles), granular, spinous and basal/germinal. The dermis has collagen, elastic fibres and extrafibrillar matrix as structural components and is divided into two layers, papillary and reticular dermis. The hypodermis consists of loose connective tissue and lobules of fat and contains sweat and sebaceous glands, blood vessels and nerves (11, 14).

Permanent skin exposure to the environmental harmful agents makes it vulnerable to burn injuries, ulcers, different types of wounds and other dermatological conditions. Skin wound healing is a complex process that depends on the wound type and depth, the patient's health, age, genetical factors, immune system response and injury cause. In the case of a penetrating wound that passes through all the skin layers, wound healing with scar formation occurs rather than healing with *restitutio ad integrum* in both humans and other mammals. The cascade of events involved in skin healing can sometimes be affected, resulting in chronic, non-healing wounds (8).

The mechanisms underpinning skin wound repair and its failure to heal are still poorly understood. It has been shown that the skin repair and regeneration processes are influenced by multiple factors (6). These processes are based on the interaction between several mediators, such as components of the extracellular matrix, platelets, inflammatory cells, growth factors, cytokines and chemokines, which occur in a highly integrated and synchronous manner during different phases of haemostasis, inflammation, migration, proliferation and maturation (2, 10, 24, 34). The goal of skin wound healing is regeneration without scarring. In humans, skin repair depends on its physiology, aging and the affected body region. There is an incomplete understanding of the body's natural repair process and underlying molecular basis. Controlled experiments to address this gap and provide perspectives on the treatment of non-healing wounds are limited. Even though there is wide biologic variability, animal models that properly reiterate human conditions and could be used in preclinical studies as excisional wound models are still to be identified.

Aside from appropriateness between species and transferability of information, selection of the animal model is also based on the ease of and adaptability to experimental manipulation, cost and availability, ethical implications, etc. Pigs, rabbits, mice and rats are the most frequently used animal models (8, 9). The use of the rat as excision wound animal model in the *in vivo* studies has shown the positive effects of collagen synthesis and reticulation, expression of growth factor, fibroblast proliferation, blood vessel formation, and wound contraction in the healing process (5, 29).

The aim of the current study was to evaluate and adapt the excisional wound splinting model in rats and mice, so it can be further used in studies assessing skin repair and regeneration following ointments application, and to identify the advantages and disadvantages of each model.

MATERIALS AND METHODS

Laboratory Animals

Rodent models have long been used in preclinical studies exploring wound-healing process, tissue regeneration, stem cell transplantation, tissue transplantation and host-versus-graft rejection (19, 33).

The advantages of this animal model are its frequent use and adaptability to experimental conditions and manipulation. There are, however, several limitations due to the different peculiarities of the wound healing process in rodents (8, 9, 26, 32), compared to humans (28). Rodents have loose skin and the primary wound healing mechanism is contraction, while in humans, the skin is linked to subcutaneous tissues and wounds are healed by the generation of new tissue (repair) (20). Moreover, contraction of the skin in rodent wounds is conditioned by the animal position, movements, and wound care dressing.

Another study was conducted in Wistar-Lewis rats (family *Muridae*, order *Rodentia*) (1) to assess the bioactive glass nanoparticle-based ointments effect on the wound healing process. The study comprised two experiments and 30 female rats (weight: 200 to 250 g) were included in each experiment. The excisional wound model adapted to assess the effect of bioactive glass-gold nanoparticles-based ointments on skin re-

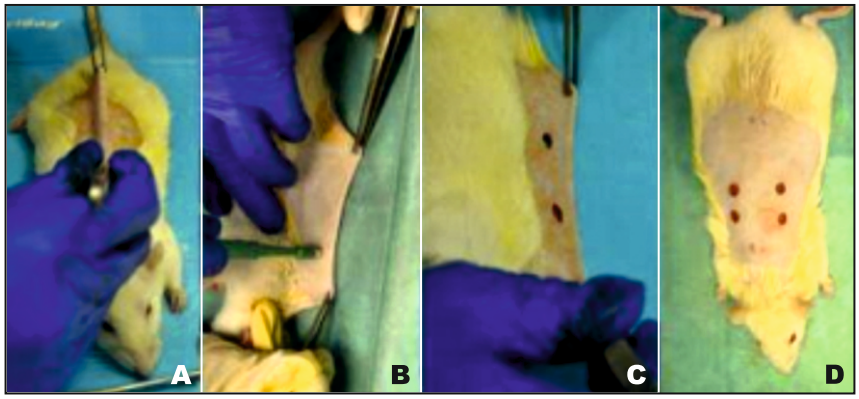

Fig. 1. Surgical steps of the excisional wound splinting model:
A. Upper back skin pinched in T1 and L1 regions; **B.** 5 mm biopsy punch instrument positioning;
C. Folded skin with skin excisions; **D.** Four symmetrical full-thickness excisional wounds

generation by application of a silicon splinting ring around the wound was used in both experiments (18). This species was selected because they are highly adaptable to experimental conditions and manipulations, have reduced sensitivity to bacterial infections and a decreased risk of spontaneous tumours development (7). The rats were supplied by the Centre for Experimental Medicine and Practical Skills of the University of Medicine and Pharmacy "Iuliu Hatieganu", Cluj-Napoca, Romania and kept in the Unit of Reproduction and Use of Laboratory Animals at the Faculty of Veterinary Medicine, Cluj-Napoca, Romania. All rats were caged in open housing conditions at a mean room temperature of 23°C with 55% humidity on a 12-hour light/dark cycle, according to the Animal welfare requirements (ISO 10993-2). All animals had water *ad libitum* and free dietary access.

The experiments were approved by the Animal Ethics Committee of the University of Veterinary Medicine and Agricultural Sciences Cluj-Napoca, Romania and by the National Sanitary Veterinary and Food Safety Authority, in accordance with the Romanian law.

Materials

All experiments were performed under sterile conditions and surgical instruments were autoclaved before use. Because it must be repeated in multiple rats, two sets of instruments should be used. Between uses, instruments should be cleaned and disinfected. The surgeon must wear clean scrubs, mask and hair cap and use fresh sterile gloves for each rat.

Materials used were: trimmer, Veet Hair Removal Cream (Health Hygiene-Home), antiseptic solutions (betadine and 70% alcohol), 5 mm sterile biopsy punch, fixed blade scalpel, surgical and haemostatic pads, scissors, sewing needle, non-resorbable suture material (Nylon 4.0), sterile wound dressing, elastic wound dressing, silicon rings and superglue.

The applied ointment

An ointment was developed by dispersion of the bioactive glass-gold nanoparticles (36) in neutral medical Vaseline (18). Vaseline was brought to 42°C, the bioactive materials were dispersed by centrifugation and the obtained product was then rapidly brought back to the room temperature.

Presurgical animal care

General anaesthesia through inhalational or parenteral anaesthetic agents is recommended for pain management. For the excisional wound models, the parenteral anaesthesia is recommended because the inhalational anaesthetic agents must be administered through a mask, making difficult the manoeuvres during the surgical procedure. After the surgery, isoflurane anesthesia is recommended for wound bandaging and healing process monitoring. In our experiments, general anaesthesia was induced with xylazine 6 mg/kg (Xylazin Bio 2%, Bioveta, Czech Republic) IP and ketamine 60 mg/kg IP (Narkamon Bio, Bioveta, Czech Republic) (35). Each rat was then positioned in ventral recumbency on a table. The dorsal surface hair was trimmed within T1 and L1 region, with a special attention being given to the withers (interscapular region) and to the vertebral and lumbar regions, including the dorsal midline sides. For complete removal of the hair, a depilatory cream is recommended to be applied for 3 minutes contact time. To remove the depilatory cream and hair, a plastic scraper should be used. Finally, the antisepsis is obtained by dorsum skin gentle cleaning with cotton pads soaked in betadine solution, followed by 70% alcohol.

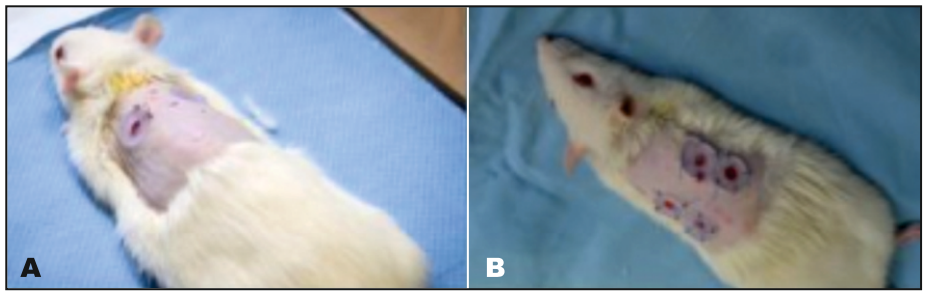

Fig. 2. Rat excisional wound splinting model with two (**A**) and four (**B**) excisions

Surgical procedure

The surgical procedure should be performed in a sanitized surgery room by using sterilized instruments. Because it must be repeated in multiple rats, two sets of instruments should be used. Between uses, instruments should be cleaned and disinfected. The surgeon must wear clean scrubs, mask and hair cap and use fresh sterile gloves for each rat. Two or four dermal excisions can be performed in the disinfected area, according to the following steps. The anesthetized rats are placed in ventral recumbency on the surgical table. The upper back skin of each rat is pinched in T1 and L1 regions with two haemostatic pads (Fig. 1A). After moving the rat in lateral recumbency position, the dorsal skin is tensed along the spine. The punch biopsy instrument of 5 mm diameter is placed 8 mm far from the dorsal skin fold (Fig. 1B). The folded skin is punched through its both layers to create two or four symmetrical full-thickness excisional wounds beneath the interscapular region and beside the dorsal midline (Fig. 1C). Measured on the relaxed skin, the distance between two symmetric wounds should be 16 mm (Fig. 1D) (32). Figures 2A and 2B display the rat excisional wound splinting model with two and four excisions, respectively.

After the dermal excisions, wound cleansing with sterile swabs is recommended. To minimize wound closure caused by *panniculus carnosus* contraction, a sterile rounded-shaped silicon splinting ring should be placed around each wound. The splinting ring is made-up by 1 mm thick semi-hard silicate that is secured to the skin with four interrupted sutures. The splinting rings are obtained using metallic transducers and cropping silicate spheres of 15 mm external diameter and 6 mm internal opening. These splinting rings are used after they have been sterilized in disinfectant solution and stored in sterile envelopes. To centre the wound within the splint and maintain this position, the instant-bonding adhesive Superglue is spread on the side of the splint, which is in direct contact with the skin. The splint is then secured to the skin with the four interrupted sutures of 4.0 nylon (Fig. 2B). The splint should be used to prevent wound closure caused by *panniculus carnosus* contraction and thus to allow the wound to heal through granulation and re-epithelialization, a process similar to that in humans. This protocol was adapted from the mouse excisional wound splinting model proposed by Wang et al. (32). The excisional wound location is chosen based on the limited movements of the rat in the respective body area.

Following the silicon splinting ring cleansing (Fig. 3A), the bioactive glass-gold nanoparticles embedded in Vaseline should be applied with a spatula on the opened wounds (Fig. 3B). The ointment should cover the entire excision without passing the silica ring edges (25). A sterile wound dressing should then be applied on the silicone rings, followed by an elastic wound dressing wrapped over the rat's body, between the forelimbs, to prevent wound contamination with foreign bodies and to keep intact the silicone rings.

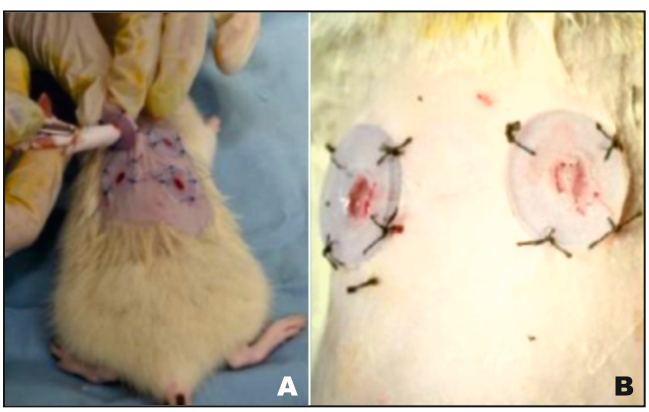

Fig. 3. **A**. Cleaning of silicon splinting ring; **B**. Open wounds with bioactive glass nanoparticles embedded in Vaseline

Post-operative care

The rats should be placed in individual cages under a warming lamp until they are fully recovered from anaesthesia. They should be checked daily to ensure

that the bandage is on. In our experiments, the rats were anesthetized with isoflurane and wound bandages were taken off to monitor the healing process at days 1, 4, 7 and 12 after the surgery. Ointment re-application should be performed at pre-specified time intervals (according to the procedure described above). If a crust is developed at the excision level, it should be removed with forceps and the ointment should be re-applied. Photographs of individual wounds should be taken with a digital camera to assess excision diameter by direct measurement.

Finally, the wounds should be completely recovered with the sterile and elastic wound dressings.

Histologic analysis

On day 15 after the surgery, when wound healing is complete, all rats can be euthanized by overdose of anaesthetic and cervical dislocation.

From each wound excision, skin tissue samples should be harvested from both scarring site and surrounding normal tissue (including a 1 cm skin border).

RESULTS AND DISCUSSIONS

Punch biopsy, also named biopsy through perforation in human medicine, is one of the most widely used technique in studies assessing skin regeneration (16).

To better understand the skin healing process and the therapeutic management of the skin wounds, animal models such as the rat and mouse excisional wound healing models have been developed (21). These species are preferred because of their availability, reduced body size, simplicity, mammalian struture, low costs, etc. The wound undergoes a relatively fast healing process that is highly influenced by physiology, age and external factors (21). To better understand wound healing in humans, animal studies are needed even though the wound healing process in animals may differ (21). The healing process involves a cascade of highly integrated and synchronous events, including coagulation, granulation, re-epithelialization, connective tissue formation and tissue regeneration (16). The process has three phases: acute inflammatory stage, collagen synthesis, and remodelling stage, when the granulation tissue is gradually remodelled forming scar tissue (22).

In our experiments conducted to assess the effect of the bioactive glass-gold nanoparticles embedded in Vaseline on the skin healing and cutaneous regeneration, the Wistar rat animal model was used (13, 30).

The excisional wound splinting model was created using a 5 mm biopsy punch (12, 23, 31). A silicon splint was applied to prevent wound closure by contraction and to allow the wound to heal through granulation and re-epithelialization, a process similar to that in humans (4). The experimental model can have either two or four symmetrical incisions on the upper back skin, based on the type and concentration of the bioactive glass-gold nanoparticles dispersed in Vaseline. If the experimental product has a single bioactive glass-gold nanoparticle, the product should be compared to a previously tested product and the healing process to the normal healing. In this case, the excisional wound splinting model with two excisions is the first choice and the investigational product should be applied on the right-side excision; standard Vaseline or other licensed product on the left-side excision, as Control. If more than one experimental product is to be assessed, the excisional model with four excisions is preferred. The standard product should be applied on the cranial left-side excision (Control) and the investigational products on the remaining excisions. In skin regeneration studies, the experimental products and control should be applied on the same animal to make sure that the individual factor that may have biased results interpretation was eliminated.

The excisional wound model with an odd number of lesions is not preferred when biopsy punch technique is used, because subdermal tissue lesions can occur during the surgical procedure and have a negative influence on dermal regeneration. Wound closure, granulation and vascularization can be visualized, measured and assessed at different time points. The visual assessment can be done by direct photographs or measurement with a transparent polyethylene paper placed on the top of the excised surface immediately after the excision at day 1, 4, 7 and 12. The wound closure percentage can be calculated throughout the healing (12, 23, 31).

Other advantages of this model are results uniformity, wound closure can be prevented and variations in application of the wound dressing are minimal. Moreover, the investigational products can be applied at any time during the healing process. The first marker of the excisional wound healing process is the wound closure speed (4). Dermis vascularization is an important step in the healing process (17, 27) and the ingrowth of capillaries can be assessed through microscopic examination. The neovascularization and angiogenesis are activated by capillary sprouting from the excision edges and can be observed in the first days after wound creation (18).

CONCLUSIONS

The rat excisional splinting model with two or four symmetric wounds on the upper back skin, in-between the T1 and L1 regions, is an optimal model to assess the bioactive materials effect on the wound healing and tissue regeneration. To prevent wound closure caused by *panniculus carnosus* contraction, a silicon splinting ring should be applied around the wound.

First application of the investigational product should be performed after silicon splinting ring positioning and wound disinfection. Subsequent applications should be done only after crust removal.

The use of laboratory animal models in wound healing studies provide major advantages: wound closure, granulation and vascularization can be visualized, measured and assessed at different time points, and the individual factor that may bias results interpretation can be eliminated by placing the investigational product and control on the same animal.

The rat and mouse excisional wound splinting model have been used as working protocol in different studies that assessed the skin healing process and tissue regeneration following application of nanoparticles-based ointments or stem cells transplantation. Comparison of their results could provide new insights into the healing process and could eventually be translated to viable clinical treatments in human patients with skin healing pathologies.

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