

BIOACTIVE CURDLAN/ AGAROSE DRESSING ENRICHED WITH GENTAMICIN FOR INFECTED WOUNDS – PILOT STUDIES

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Abstract

*The problem of treating chronic wounds is widespread throughout the world and carries a huge cost. Biomaterials engineering tries to solve this problem by creating innovative bioactive dressings dedicated to specific types of wounds. Both synthetic and natural polymers are the main base to produce wound dressings. Biopolymers have the advantage over synthetic polymers by being more biocompatible, non-toxic, biodegradable, and eco-friendly. The aim of this work was to produce a bioactive biomaterial based on natural polymers with potential applications to manage chronic highly exuding and infected wounds. A newly developed method for chemical synthesis of the curdlan/agarose biomaterial at high temperature combined with freeze-drying process resulted in a superabsorbent dressing material with antibiotic immobilized. The obtained biomaterial was subjected to basic microbiological in vitro tests and a cytotoxicity assay according to ISO 10993-5. Moreover, the experimental treatment of the infected wound in a veterinary patient was performed using the developed material. Based on the conducted research, it was proved that the produced dressing is not toxic to normal human skin fibroblasts. An additional advantage of the biomaterial is its ability to inhibit the growth of harmful microorganisms, such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Furthermore, the experimental treatment confirmed the validity of using the produced biomaterial as a dressing dedicated to the treatment of difficult-to-heal infected wounds. To summarize, the produced biomaterial possesses great potential to be used as a wound dressing for infected wounds.*

Keywords: biopolymers, wound dressing, cytotoxicity, antibacterial properties, wound healing

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Introduction

Wound healing is a multistep process that can be divided into four distinct phases. In the case of skin damage, hemostasis occurs up to several hours after the injury. Then an inflammatory phase is activated and it lasts 1-3 days. The next stages are proliferation and repair which can last up to 4-21 days. The final step is remodelling taking up to a year [1,2]. Disruptions at any of these stages may delay wound healing and cause excessive scarring or chronic wound formation [2]. The main problem in the treatment of chronic wounds is an excess of exudate whose level does not decrease over time, comparing to wounds amenable to treatment [3]. In the case of chronic wounds, excessive exudate secretion is observed due to the active inflammatory process [4]. To limit the amount of exudate in the wound bed, an appropriate absorbent wound dressing is desired [5]. Dressings based on both synthetic and natural polymers are commonly used to treat chronic wounds. Synthetic polymers that are primarily used to produce wound dressings include poly(ethylene oxide) (PEO), polyglycolic acid (PGA), poly(vinyl pyrrolidone) (PVP), poly(ethylene glycol) (PEG), polylactide (PLA), polyurethane (PU), and poly(vinyl alcohol) (PVA). Biopolymers widely used for dressing materials involve alginate, agarose, cellulose, chitosan, collagen, dextran, and pectin [6]. Some natural polymers possess key features that have a positive effect on the healing process. For example, due to its natural antibacterial and pro-healing properties, chitosan is often used to accelerate wound healing and prevent infections in the wound bed [7].

It is well known, that chronic wounds provide the perfect environment to develop persistent infections. *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* are the most common pathogens that hinder the healing process and cause significant tissue losses [8]. An additional problem is the ability of pathogenic bacteria (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) to form a biofilm where the cells exhibit higher resistance to antibiotics than planktonic cells [9,10]. Incorporating antibiotics into the wound dressing structure is not recommended for the topical/local treatment of bacterial colonization in chronic wounds (bacteria are present but they do not elicit an immune response). The local antibiotic treatment may result in resistant bacterial strains, limited healing, or in extreme cases, a delayed hypersensitivity reaction. However, topical antibiotic therapy is required in the case of critical colonization or a serious bacterial infection [11]. Therefore, dressings containing embedded antibiotics are only used to treat infected and difficult-to-heal wounds.

A dressing for the treatment of chronic wounds should absorb and remove excessive exudate from the wound bed while providing an optimal moist environment. It should also be flexible and easily adaptable to the shape of the injury. At the same time it ought to cover the wound bed, providing mechanical protection against transmission of microorganisms. A wound dressing should tightly adhere to the wound bed but resist the skin cells adhesion so that changing the applied dressing will not destroy the newly formed tissue [11,12]. Hydrogel and hydrocolloid dressing materials appear to be the ideal candidates to manage chronic wounds as they address all of these criteria. Recently, the incorporation of bioactive compounds into the biomaterial structure has become a promising trend in regenerative medicine. Modern nanocomposite polymer-based hydrogel dressings for infected wounds are often enhanced with antibacterial nanoparticles and are widely studied nowadays [13,14].

The latest scientific reports describe successful new hydrogels loaded with gentamicin [15-17]. Gentamicin is an aminoglycoside antibiotic with outstanding thermal stability [16] which is commonly used to treat chronic and post-operational infections [18,19]. It is widely applied against infections caused by strains like aerobic Gram-negative bacteria and some aerobic Gram-positive bacteria [17]. The antibiotic exhibits a therapeutic serum level of 4-8 µg/ml and a toxic level of 12 µg/ml [19]. Rs indicate that the topical gentamicin treatment significantly increases clinical efficacy and reduces the wound healing time [20].

The aim of this study was to incorporate gentamicin within the structure of the previously developed foam-like curdlan/agarose biomaterial [21] to obtain a wound dressing for highly exuding and infected wounds. The developed curdlan/agarose wound dressing demonstrated to be non-toxic, biodegradable, and endowed with a high exudate absorption capacity. Moreover, the biomaterial did not allow for fibroblast adhesion, which is a desired phenomenon enabling the painless dressing removal after the healing process [21]. In this study, the gentamicin-enriched biomaterial was subjected to the cytotoxicity test using human skin fibroblasts and the antimicrobial activity evaluation against common bacteria causing wound infections. Moreover, the experimental treatment of an infected chronic wound in a veterinary patient was performed to confirm the clinical usefulness of the tested dressing.

Materials and Methods

Materials

The curdlan powder was obtained from Wako Pure Chemicals Industries. The agarose powder (gel point $36 \pm 1.5^\circ\text{C}$), sodium dodecyl sulphate (SDS), gentamicin sulphate, phosphate-buffered saline (PBS), Eagle's Minimum Essential Medium (EMEM), penicillin, streptomycin, trypsin-EDTA, Thiazolyl Blue Tetrazolium Bromide (MTT) were purchased from Merck. The Mueller Hinton broth (MH) was obtained from Thermo Scientific. The fetal bovine serum was supplied from Pan-Biotech. Normal human skin fibroblast cell line (BJ), *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853 were purchased from American Type Culture Collections (ATCC).

Preparation of the biomaterial

The biomaterial was prepared based on the method described in Polish Patent no. 236367 (2021). A foam-like biomaterial was prepared by mixing a predetermined amount of curdlan (2% w/v), agarose (2% w/v), and gentamicin sulphate (0.2% w/v) in deionized water. Thus, the concentration of gentamicin sulphate in the polymer suspension before gelation equalled 2 mg/ml. The combined suspension of polymers and antibiotic was preheated to 50°C on a magnetic stirrer. After a homogeneous mass was obtained, it was transferred to a mould and incubated in a water bath (20 min, 95°C). The produced samples were subjected to cooling, freezing, and finally lyophilization process. As a result, the foam-like biomaterial was obtained.

In vitro cell experiments

The *in vitro* cell viability experiment was carried out using human normal skin fibroblasts (BJ cell line). The cytotoxicity test and preparation of the extract were performed according to ISO 10993-5 standard [22]. BJ cells were seeded into a 96-multiwell plate using EMEM medium at the density of 2×10^4 cells/well and cultured for 24 h.

Then, the culture medium was replaced with 100 µl of 100% extract of the material (prepared by placing 25 mg sample in 1 ml of the culture medium, followed by 24 h incubation at 37°C) and BJ cells were exposed to the extract for 48 h and 72 h. For control samples, the fresh EMEM medium was used instead of the extract. Next, the incubation media were removed and 100 µl of 1 mg/ml MTT solution was added per well. The plate was incubated with reagent at 37°C for 180 min. Afterwards, the 0.01% (w/v) SDS solution prepared in 0.01 M hydrochloric acid was added to degrade cells, release and solve formazan crystals. The cell viability was calculated by measuring the absorbance at 570 nm and was shown as a percentage of viability compared to the control (cells incubated in the EMEM medium instead of the biomaterial extract).

Inhibition of bacterial growth test

The antibacterial activity of the tested biomaterial was evaluated using the biomaterial extract prepared according to ISO 10993-5 in Mueller-Hinton (MH) broth. After incubation at 37°C for 24 h, the biomaterial extract was transferred into a 96-multiwell plate and then bacterium suspensions of 1.5×10^5 CFU/ml of *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 were added and incubated at 37°C for 48 h and 78 h. The growth control was performed from fresh MH broth and treated as above. Afterward, the cultures absorbance was measured at 660 nm and calculated as a percent of growth control.

Experimental treatment of a veterinary patient – case presentation

The owner with a fancy 2-year-old rat (*Rattus norvegicus domestica*), an uncastrated male with an abscess on the left side of the chest came to the Division of Small Mammals at the Department of Epizootiology and Clinics of Infectious Diseases of University of Life Sciences in Lublin (Poland). The change was already large with the focus on softening. The abscess was surgically cleaned and its cavity was rinsed with a 1% povidone iodine solution (Betadine, 100 mg/ml, EGIS). The antibiotic therapy with amoxicillin with clavulanic acid (Synulox 50 mg, Zoetis) at a dose of 20 mg/kg bodyweight twice a day was also advised. After the 3-week antimicrobial therapy, a small cavity, which was the residue of an abscess, remained in the skin. Due to severe itching caused by the wound healing, the animal was treated with Hydroxyzine (Hydroxyzinum VP, 2 mg/ml, Bausch Health) at a dose of 1 mg/kg bodyweight twice a day. The wound was also washed with 0.1% Rivanol solution. However, as the performed standard treatment was unsuccessful, the owner was offered the alternative treatment with the gentamicin-enriched hydrocolloid dressing. The wound was surgically cleaned and the gentamicin-loaded curdlan/agarose dressing was sewn into the abscess cavity. The experimental treatment was performed after obtaining written consent from the pet owner.

Statistical analysis

The cytotoxicity and antibacterial activity tests were performed in at least three distinct and independent repetitions. The obtained results were presented as the mean values \pm standard deviations. The statistical analysis was performed with GraphPad Prism 8.0.1 Software with unpaired t-test ($p < 0.05$, a significance level of $\alpha = 0.05$).

Results and Discussions

The previously developed curdlan/agarose biomaterial (Polish Patent no. 236367) was modified by incorporating gentamicin during the production stage. The stiff foam-like biomaterial with a smooth surface was obtained via the high-temperature gelation of curdlan/agarose/gentamicin mixture followed by freezing and freeze-drying (FIG. 1a). The sample cross-section image shows the macroporous structure which should ensure good absorption, retention of fluids and adequate gas exchange (FIG. 1b). In the dry state, the dressing was characterized by a spongy structure. However, after its immersion into a fluid, it transformed to a soft gel able to adhere easily to the wound bed, thus acting like a typical hydrocolloid dressing. It should be mentioned that to our best knowledge there are only a few studies describing curdlan as a base for wound dressing production. The researchers created the curdlan-based hydrogel dressing by gelating the alkaline curdlan solution in a copper chloride. Nevertheless, the resultant biomaterial revealed high cytotoxicity against human skin cells [23]. In other studies, the silver-loaded nanofibrous curdlan was produced to manage difficult-to-treat wounds.

Our research team reported superabsorbent foam-like dressings made of the curdlan/agarose mixture and the curdlan/chitosan blend that proved to have great potential for the treatment of highly exuding wounds [21].

Biocompatibility, non-toxicity, and biodegradability of the material are some of the main requirements for modern wound dressings [24]. To verify the cytotoxicity of the produced material, the MTT assay according to ISO 10993-5 standard was performed. The test results proved that the developed biomaterial containing gentamicin was non-toxic to BJ fibroblasts. The cell viability was equal to 80.5% and 83.5% after the 48 h and 72 h exposure of the cells to the biomaterial extract, respectively (FIG. 2). It should be noted that non-toxicity of gentamicin towards fibroblasts was also confirmed by other authors. For instance, Kiliñç et al. proved non-toxicity of gentamicin at doses of 0.5-1 g/ml against L929 mouse fibroblast cells [25], whereas Hwang et al. demonstrated that the gentamicin-loaded PVA-dextran hydrogel had antibacterial properties, was non-toxic, and improved wound healing [17]. These research outcomes may indicate that the developed gentamicin-loaded curdlan/agarose biomaterial may have a beneficial effect on the healing process of infected wounds.

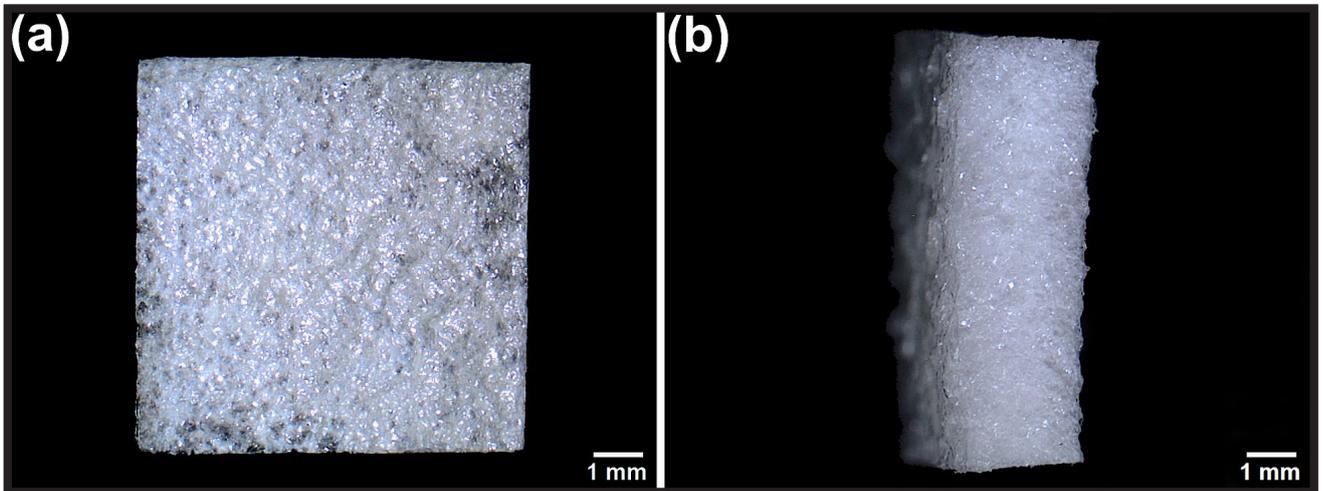


FIG. 1. Stereoscopic microscope (Olympus SZ61TR) images of the obtained biomaterial: (a) surface; (b) cross-section.

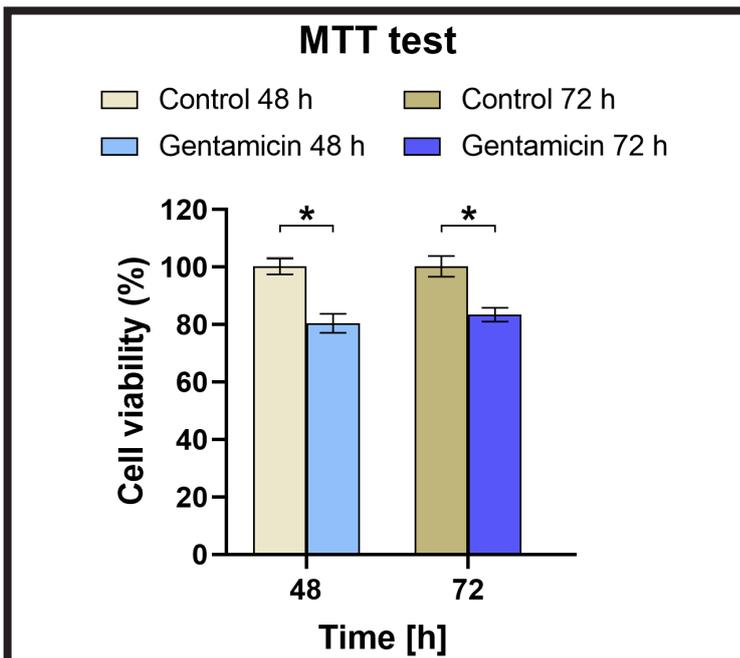


FIG. 2. Cytotoxicity of the gentamicin-enriched curdlan/agarose dressing against BJ fibroblasts evaluated by MTT assay with the 24-h extract of the biomaterial (the control – cells grown in the culture medium instead of the extract); * statistically significant results compared to the control (unpaired t-test).

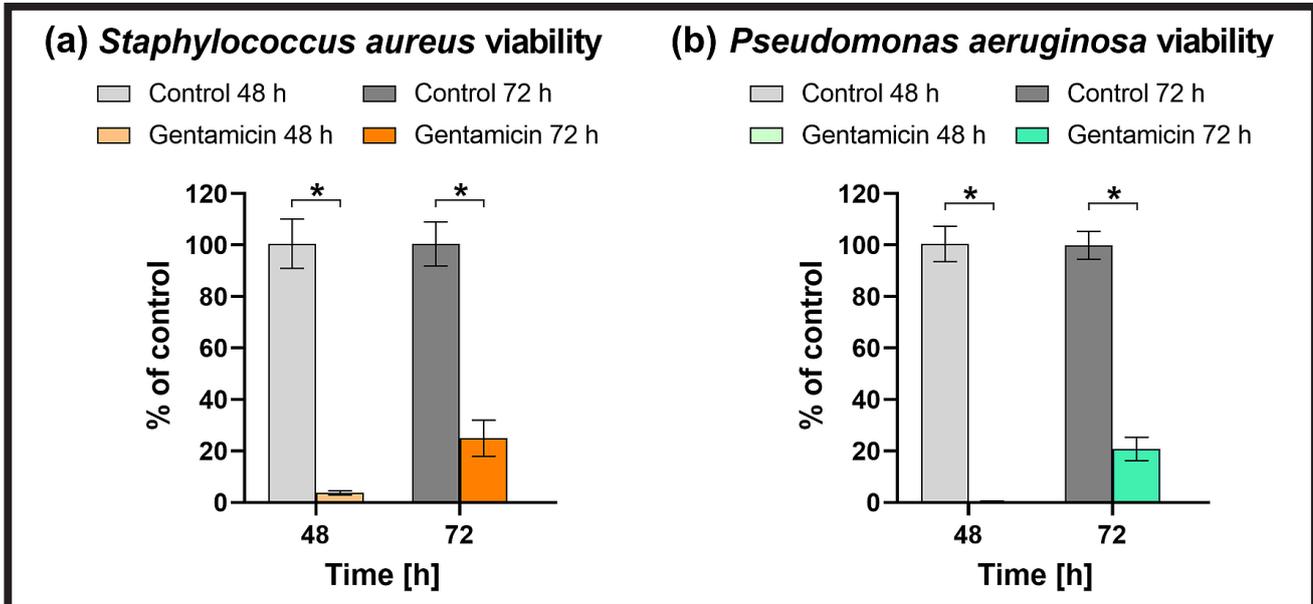


FIG. 3. Bacterial cell viability (as % of the control): (a) *Staphylococcus aureus* ATCC 25923 and (b) *Pseudomonas aeruginosa* ATCC 27853, after the 48 h and 72 h exposure to the biomaterial extract (prepared by 24 h incubation in the MH broth; the control – bacteria cultured in the MH broth instead of the extract); * statistically significant results compared to the control (unpaired t-test).

Scientific reports indicate that bacterial growth is a major contributor to chronic wounds. For this reason, wound dressings should reduce the microorganisms burden without cytotoxic effect against skin cells [26]. Our microbiological study proved that the tested biomaterial extract inhibited the bacterial growth both after two and three days of the experiment. In the case of *S. aureus*, the bacteria viability was equal to 3.8% and 25% after the 48 h and 72 h exposure time, respectively (FIG. 3a). Similarly, the *P. aeruginosa* viability was only 0.4% after 48 h and it increased to 20.8% after 72 h, compared to the control (FIG. 3b). Importantly, both bacterial strains strongly inhibited bacterial growth only after 48 h, as compared to the control. Thus, it may be assumed that gentamicin lost its therapeutic effectiveness during the 72 h incubation in the bacteria suspensions. It also indicates the necessity to change the wound dressing every 2 days during the treatment. The obtained results are in agreement with the reports presented by other authors who confirmed high effectiveness of gentamicin against bacterial strains related to wound infections. Studies of Tam et al. proved different profiles of antibacterial effect of gentamicin against *S. aureus* and *P. aeruginosa* [27]. It was also proven in a porcine model that gentamicin at a concentration of 2 mg/ml reduced *S. aureus* counts in the infected wounds [28].

The obtained here results indicate the great potential of the gentamicin-loaded biomaterial for infected wounds since the developed wound dressing exhibited antibacterial properties and non-toxicity against human skin cells. Based on the promising *in vitro* cell culture and microbiological tests, a decision to implement this biomaterial in the treatment of the veterinary patient was made.

Experimental treatment of a veterinary patient

Prior to the experimental treatment with the newly developed dressing material, the fancy rat was subjected to the standard treatment. However, the wound remained open and was oozing serous-purulent discharge, indicating secondary infection (FIG. 4a). Due to the unsuccessful standard treatment, the owner was offered a topical antibiotic therapy with the gentamicin-loaded curdlan/agarose dressing. To avoid the biomaterial damage by the scratching animal, the dressing was sewn into the abscess cavity. Immediately after the procedure, the normal healing process was observed. After 7 days of treatment, the dressing fell off along with the scab. The healed skin did not reveal any signs of infection (FIG. 4b). After 10 days, the reepithelialization process was almost completed (FIG. 4c), whereas on the 14th day the remaining scab fell off and the skin healing process was fully completed (FIG. 4d).

It should be noted that effective antibiotic release into the wound area is important to treat the infection [25]. Within this study, it was demonstrated that the 24-hour extract of the biomaterial had sufficient antibacterial activity *in vitro*. The antibacterial effectiveness of the dressing was also confirmed during the treatment of the veterinary patient. The advantage of the produced biomaterial was associated with its topical application to the infected wound. During contact with the exudate, the antibiotic was released from the dressing structure, reaching a therapeutic concentration that was not achievable with the systemic treatment. It is worth mentioning that by incorporating gentamicin within the curdlan/agarose biomaterial, it is possible to achieve the optimal drug accumulation tailored to the specific type and condition of the infected wound. To produce the optimal gentamicin-loaded dressing for infected wounds, it is planned to furtherly optimize the biomaterial gentamicin content to determine the antibiotic release profile.

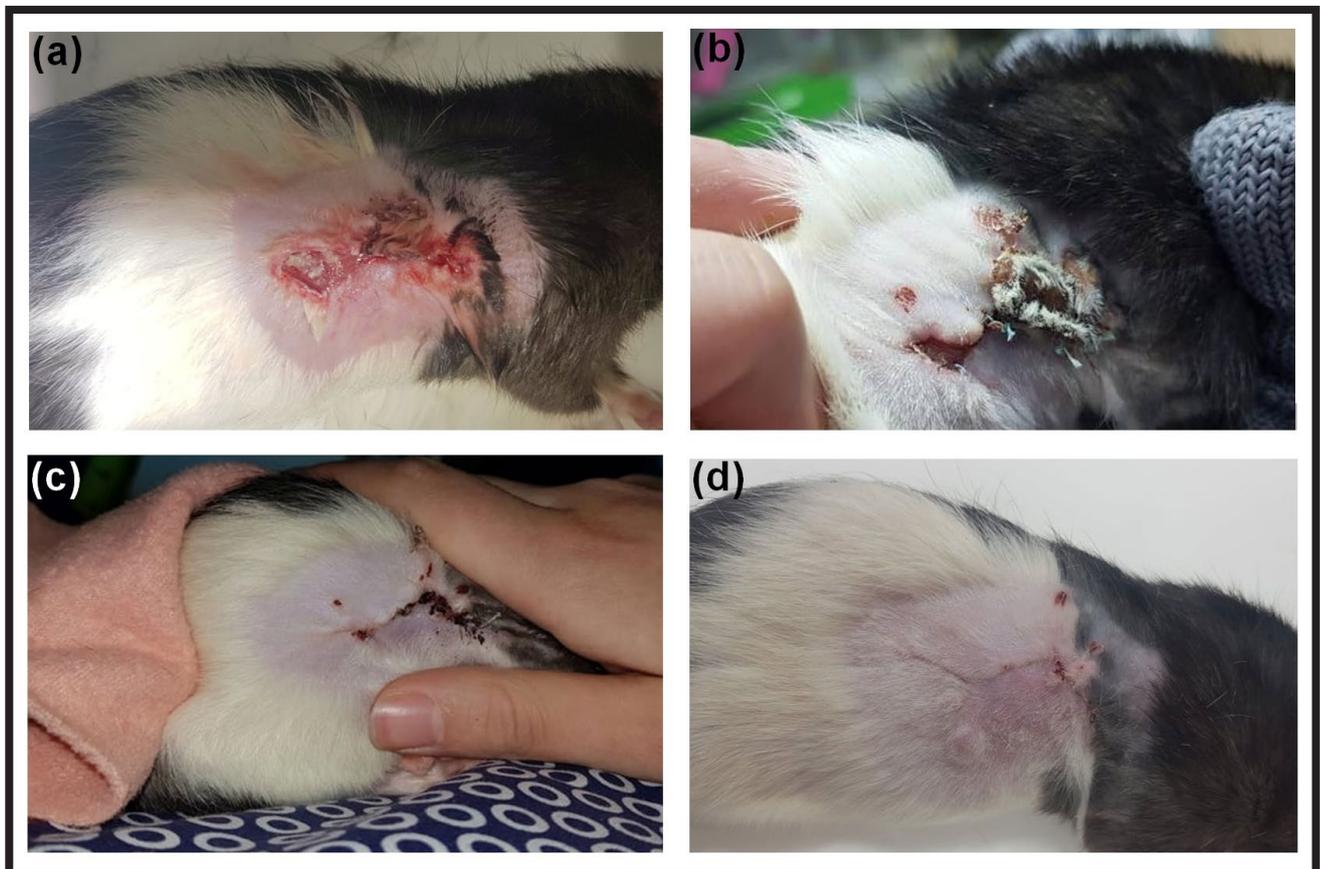


FIG. 4. Images of the wound area and its regeneration in a veterinary patient (*Rattus norvegicus domestica*): (a) before applying the dressing material, (b) the wound after 7 days, (c) the wound after 10 days, (d) the wound after 14 days.

Conclusions

The studies presented the production and basic characterization of the antibacterial hydrocolloid dressing material composed entirely of natural polymers (curdlan and agarose). The biomaterial contained a bioactive compound (i.e. gentamicin) that was loaded into its structure during the production stage. The conducted research proved that the biomaterial was not-toxic to normal human skin fibroblasts and exhibited the antibacterial activity against *S. aureus* and *P. aeruginosa* strains. Moreover, the presented clinical case proved that the gentamicin-loaded curdlan/agarose dressing applied as a drain for the local treatment of the infected wound significantly reduced the bacterial growth and accelerated the wound healing process. The biomaterial also limited the granulation tissue development and excessive scarring. The obtained results are promising, however, it is necessary to conduct further studies in order to confirm the applicability of developed biomaterial as a dressing used in the management of infected chronic wounds.

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