COLLAGEN-BASED GEL WITH ANTIMICROBIAL ACTIVITY FROM LEATHER WASTE

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The work is focused on obtaining collagen-based gel from leather waste with antimicrobial activity for further use with biomedical application and as dressings for wounds. Two antibiotics - macrolide azithromycin and phenicol chloramphenicol were tested using both laboratory strain and clinical isolates of Staphylococcus species from patients with wounds in Kyiv region. Collagen matrix was obtained from delimed pelt leather waste by acetic acid extraction. The biofilm assay and MTT assay were applied to study the effect of antimicrobial component in collagen-based gel. The reduced growth rate, cells attachment level and cells metabolic activity were the evidences of strains inhibition and biofilm development prevention.

Keywords: collagen, antimicrobial, wounds

INTRODUCTION

The skin is the largest organ of the body and the main barrier that protects the us from the external environment (Landén *et al.*, 2016). It is characterized by complexity due to the combination of sebaceous glands, blood vessels, sweat glands, sensory cells, nerves, epidermal cells and, finally, hair. The skin consists of two layers - epidermis and dermis (Benson, 2012). Incompletely permeable wounds are formed within the upper part of the dermis (epidermis) and tighten due to the regeneration process. Full-thickness wounds are associated with damage to the epidermis, dermis, and underlying fatty tissue. Such wounds are prolonged due to the processes of granulation, contraction and epithelization (reconstruction of the wound surface) (Dalton *et al.*, 2007). Effective tissue and skin surface repair requires interaction between different types of cells. This process is precisely organized and regulated at several levels - biochemical, molecular, cellular (Reinke and Sorg, 2012).

Collagen, as the main component of the skin, plays an important role in the healing process of wounds, and also affects the course of the inflammatory process. The most promising collagen for the creation of wound surface treatment agents is type I collagen. Further development of agents based on collagen and its derivatives requires solving the problems of improving the structure and maximum therapeutic effect against pathological processes (Velnar *et al.*, 2009). The pathogenesis of many orthopedic infections is associated with the presence of microorganisms in the form of a biofilm. By definition, a biofilm is a growth strategy of microorganisms in which cells attach to a surface or interphase and synthesize a polymer matrix covering intact cells (Flemming and Wingender, 2010). In biofilms, microorganisms function as clusters with structural and functional heterogeneity similar to multicellularity. Microorganisms in the form of a biofilm show less sensitivity to the action of antimicrobial substances. Biofilm tolerance to antibiotics is multifactorial, including physical, physiological, and genetic

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determinants, while antibiotic resistance of biofilm bacteria is mutational and due to repeated exposure to high-concentration antibiotics.

The aim of the work was to develop an antimicrobial component for collagen-based gel for further biomedical application.

EXPERIMENTAL

Materials

Collagen-based acid extracts from leather waste were obtained as it was described previously (Maistrenko *et al.*, 2020). Delimed pelt was used as a source of collagen type I. Two types of antibiotics – phenicol antibiotic chloramphenicol (CAP) and macrolide antibiotic azithromycin (AZM) – in collagen matrix from delimed pelt – against opportunistic pathogens were studied.

Pathogenic opportunistic infectious agents were used as test cultures - laboratory strain *Staphylococcus aureus* ATCC 25923 and two clinical isolates from wounds - *S. aureus* 1536 and *S. epidermidis* 190.

Methods

Isolation, identification and antibiotic resistance estimation were performed in Kyiv Regional Clinical Hospital (Kyiv) according to generally accepted methods and international recommendations of EUCAST with use of VITEK 2 compact 15 (France).

For biofilm studies 96-well microtiter plate assay was used. The overnight cultures (NB, HiMedia Ltd.) of studied strains were inoculated (1:10 ratio) in plates contained collagen matrices with concentration range of CAP and AZM. There was also a positive control for CAP test – 96% ethanol and a negative control - collagen without antibiotics. The strains were cultivated 24 hours at 37°C. The OD600 was measured and the crystal violet assay was applied (Kragh *et al*., 2019).

To estimate metabolic activity of bacterial cells MTT assay was performed as described (Wang *et al.*, 2010)

All measurements were done in 3 parallel replicas with calculation of the mean deviation. For all tests, $p < 0.05$ was considered reliable.

RESULTS AND DISCUSSION

In order to develop collagen-based gel with antimicrobial component there was a need to prepare collagen part (matrix) and the antimicrobial part. Collagen matrix was obtained from delimed pelt waste (1st extraction) by acetic acid extraction protocol. Collagen extract was washed from acetic acid by dialysis against deionized water, 18 МОм·см, pH 7 during 48 hours with changing the water every 12 hours. Strong transparent gel was obtained (Fig.1).

Figure 1. Collagen-based gel obtained from delimed pelt

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As the obtained gel was an aqueous solution the antimicrobial components should be applied as those which are dissolved in water. Both antibiotics - AZM and CAP - could be easily dissolved in water, however CAP should be dissolved in ethanol first.

During the period August 2019 —March 2021, 1560 strains of infectious agents from purulent wounds were isolated and analyzed in the Kyiv region. The most common infectious agents from wounds were representatives of the genera *Staphylococcus.* Though *Escherichia, Klebsiella*, *Pseudomonas,* and *Enterococcus* species might be presented but the number of such cases in skin wounds was much less. The most part of isolates from wound surfaces were presented by *S. epidermidis* and *S. aureus.* Among the gram-negative microflora *Enterobacteriaceae* was dominated and presented by *Klebsiella pneumoniae*, *Escherichia coli* and others. The isolates were mostly resistant to fosfomycins, penicillins, β-lactam inhibitors and monobactams. At the same time, they were highly sensitive to the last line antibiotics - teicoplanin, vancomycin and linezolid.

Monitoring of antibiotic resistance of isolates from wounds allows to assess the future prospects for antibiotic therapy and the current situation in the hospitals. At the same time, the search for promising antibiotics and antimicrobial substances for new treatment strategies and overcoming antibiotic resistance of microorganisms is continued.

Previously it was described that collagen from leather waste could be a fine matrix for bacterial biofilm formation (Iungin *et al*., 2020). Numerous studies have shown that biofilm mode of growth has greater resistance to wide range of antimicrobials compare to planktonic one (Olsen, 2015; Dier-Pereira *et al*., 2021). In the same time the generally accepted methods of antibiotic susceptibility testing are focused on planktonic mode of bacterial growth. As a result, levels of antibiotic concentrations considered to be MIC might be insufficient to suppress the development of biofilm in case of wound infection especially if the wound was already infected for a while. That is why when it comes to develop the antimicrobial component for collagen-based gel we were focused on biofilm studies only.

Within the concentration range of AZM there was definite inhibition of growth rate of all studied strains (Fig. 2).

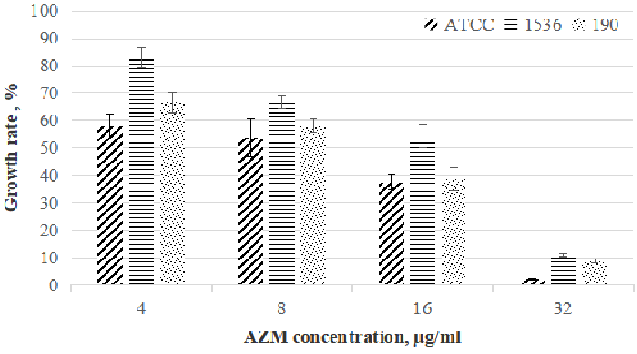

Figure 2. Staphylococcal biomass growth rate (%) in the presence of AZM

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It was shown that AZM in concentrations up to 16 µg/ml reduce the growth rate up to half whereas 32 µg/ml was enough to inhibit totally the laboratory strain. Clinical isolates were suppressed as well and the growth rate reached up 10% only.

At the same time cells attachment level has shown opposite tendency within the mentioned concentration range (Fig. 3).

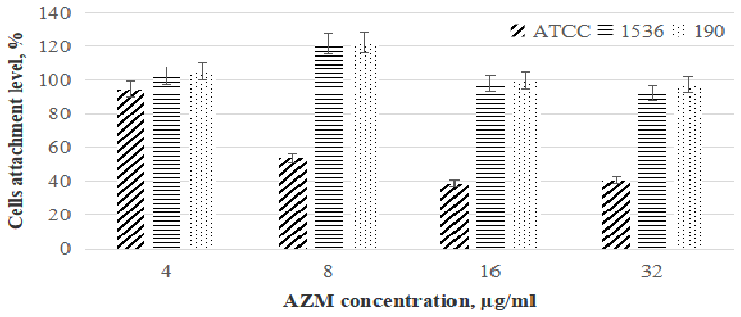

Figure 3. Staphylococcal cells attachment level (%) in the presence of AZM

Thus, the attachment level of clinical isolates was almost the same as in control. However, there was a slight raise at the point of 8 µm/ml. That could be explained by the response mechanisms to the small level stress caused by antibiotic compound. It is known that cells under small stress pressure can increase the attachment level or push planktonic cells to switch into surface-attached biofilm lifestyle (Grinberg *et al*., 2019). There is a difference between these two options. The first one is described as a shield or self-sacrifice function. In this case attached cells could act as a protector from antimicrobial surface preventing the metabolically active cells faced with the toxic molecules. It is still unknown how the part of bacterial population decide what cells should go down. The second could be connected with the presence of toxic molecule as well, however the decision of planktonic cells to attach to the surface and to change its lifestyle could be controlled by different factors including not only environmental but physiological as well (Busscher, 2012).

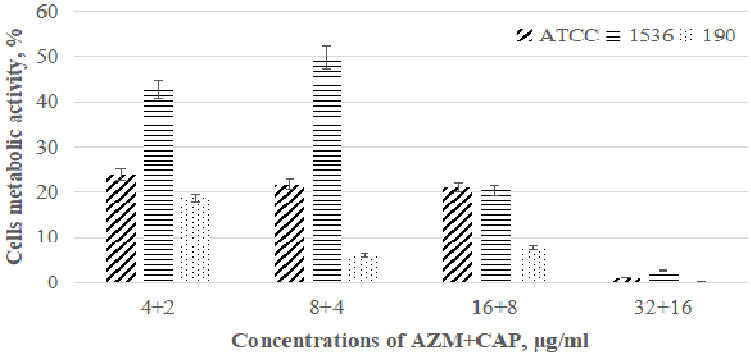

Figure 4. Staphylococcal cells metabolic activity (%) in the presence of AZM+CAP

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It was shown that combination of studied antibiotics incredibly reduces the metabolic activity of bacteria cells. The CAP itself didn't seem to reduce the growth rate, but it definitely reduces the biofilm-forming ability possibly due to the solvent ethanol presence (data is not shown). So, the role of CAP in this combination is still needed to be defined.

In general, the combination of antibiotics was more effective in fighting Staphylococcal biofilms compare to single AZM. The results also depended on the strain origin - laboratory or clinical. Clinical isolates were more adaptive to changing environments and could stand higher concentrations of antibiotics.

CONCLUSIONS

Collagen-based gel with antimicrobial component from leather waste for further biomedical application was developed. The most efficient combination of antibiotics (AZM and CAP) for treating laboratory and clinical strains was shown. However, the role of CAP is still needed to be studied. The reduced growth rate, cells attachment level and cells metabolic activity were the evidences of strains inhibition and biofilm development prevention. The slight increase in attachment level in the presence of AZM in low concentrations could be explained by small stress phenomenon.

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