

PHARMACOLOGY

THE INFLUENCE OF STAPHYLOCOCCIN T (StT) ON THE MOUSE FIBROBLASTS (3T3) VIABILITY, AN *IN VITRO* EXPERIMENTTOMASZ DREWA¹, AGNIESZKA KRAWCZYK¹, EUGENIA GOSPODAREK²,
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Abstract: Staphylococin T (StT) is a bacteriocin, which can serve as an antibiotic. The influence of StT on the mammalian cells viability should be examined before its possible applications. The aim of this work was to study the influence of StT on mouse fibroblasts viability *in vitro*. StT was delivered as a deposit in agar. 3T3 fibroblasts were used in viability tests. Cells were incubated with StT for 24 and 48 h. In the first controls, cells grew without the agar block, in the second controls, the agar block contained no StT. Trypan blue exclusion test was performed. Growth inhibition of sensitive strain SAU-209P was observed after 24 h incubation with cell culture medium containing StT. Antimicrobial activity of StT in culture medium was confirmed even after 2 months. The viability of 3T3 cells in the second control group was 50% lower than in the first and 25% higher than in StT treated group during the first day. It was noticed mechanical damage of growing cells caused by agar blocks. The number of cells cultured with StT was similar to that of second control group after 2 days. There were no changes in morphology of cells in all groups. It follows that StT had unimportant influence on the fibroblasts (3T3) viability in culture. StT may be considered for future animal trails.

Keywords: bacteriocins, Staphylococin T, cell culture, 3T3 fibroblasts, *in vitro* toxicology

There is a rising interest in methicillin resistant *Staphylococcus aureus* (MRSA) that has dominated headlines in medical journals for the last three decades. MRSA infections cause great problems for institutions delivering health care. MRSA infections usually require treatment with parenteral second-line antimicrobials, possibly more expensive and toxic, necessitating prolonged hospital stay with many side effects (1).

Bacteriocins are antimicrobial proteins produced by bacteria that are active against closely related species. They are produced by almost all major lineages of *Eubacteria* and *Archaeobacteria* and secreted by medium (2, 3). Bacteriocins have received increasing attention for their potential use as antibiotics in animal trails and for the future clinical purposes (4).

Staphylococin T (StT) is an antibacterial agent produced by a *Staphylococcus cohnii* strain. StT was purified to homogeneity by the ammonium sulfate precipitation, gel filtration; cation exchange and fast performance liquid chromatography

(FPLC), as previously described (5). The final yield was about 20%, and over a 1000-fold increase in the specific activity was obtained. Mass determination (2166 Da), amino acid sequencing and DNA sequencing demonstrated that StT is identical to galldermin, a lanthionine-containing antimicrobial peptide. StT easily dissolves in water, 0.85% NaCl, 20%, 40%, 60% and 80% ethanol, acetone, ether and chloroform. StT is active after more than 50-fold freezing and thawing. It is also resistant to treatment with α -chymotrypsin, subtilisin, pronase E, proteinase K and pepsin. StT is susceptible to trypsin. StT is also resistant to drying. In a purified state it acts in the pH range 1-12 (5). Staphylococin T remains active at both high and low temperatures and has isoantagonistic and heteroantagonistic activity. It is active against MRSA, *Streptococcus sp.*, *Micrococcus sp.*, *Corynebacterium sp.*, *Haemophilus sp.*, *Bacillus sp.* and *Clostridium sp.* Staphylococin T can be potentially used in pseudo membranous enteritis, antibiotic induced diarrhoea and MRSA infections (5).

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The influence of StT on the mammalian cells viability should be examined before its possible clinical use. The aim of this work was to study the influence of staphylococcin T in concentration responsible for antimicrobial activity on 3T3 mouse fibroblasts viability *in vitro*.

MATERIALS AND METHODS

Viability of 3T3 mouse fibroblasts exposed to staphylococcin T

Isolation, purification and antimicrobial activity of staphylococcin T were previously described (5). Staphylococcin T used to test the cell viability was delivered for the experiment in agar. *Staphylococcus cohnii* was seeded on Mueller Hinton Agar. After 24 h of incubation, colonies were removed from agar while staphylococcin T was left as a deposit in agar. Diffusion and antimicrobial activity of StT in cell culture medium had been checked before cells were seeded. 100 μ L of cell culture medium with StT was added to a plate with culture of sensitive strain SAU-209P. After 24 h of incubation the results were obtained and staphylococcin T antimicrobial activity against SAU-209P was confirmed. It was additionally proved that the StT deposit in Dulbecco's Modified Eagle's Medium/Ham's F-12 (DMEM/Ham-F12) cell culture medium was active against sensitive strain SAU-209P even after two months. This test had confirmed the StT stability in the medium for 3T3 cells culture.

3T3 cells were cultured in 24 well plates at density of 15×10^4 cells/well. Cells were maintained in DMEM/Ham-F12 supplemented with 10% Fetal Bovine Serum (FBS) at 37°C and under 5% CO₂. Fragments of agar with StT were added to the wells with growing cells in the log phase in previously defined volume (0.4 mL). Equal volume of agar blocks warrants the similar concentration of StT in cell culture wells. Cells were incubated with agar blocks for 24 and 48 h, respectively. There were two control groups. In the first cells were incubated without agar blocks and in the second cells were incubated with the fragment of agar without staphylococcin T to exclude the impact of mechanical influence of the agar block.

The assessment of the influence of staphylococcin T on the viability of 3T3 fibroblasts was performed using trypan blue (Sigma, Germany) exclusion test. The viability of 3T3 cells was evaluated after 24 and 48 h of incubation with StT. In order to assess the viability, the fibroblasts were removed from the wells by trypsinization. Spun-down cells

were suspended in 50 μ L of culture medium. In the next step, 50 μ L of the cell suspension was mixed with 50 μ L of trypan blue. Stained cells were placed in a Neubauer chamber and counted under an inverted microscope (Nikon TMS). The cell viability was calculated from 18 separate measurements. Results were presented as mean values with standard deviations. Means were compared using Student's t-test; $p < 0.05$ was considered as significant.

RESULTS

The diffusion of StT from agar blocks to culture medium was proved. Growth inhibition of sensitive strain SAU-209P was observed after 24 h incubation with culture medium with StT. Antimicrobial activity of StT in culture medium was confirmed even after two months of incubation.

There was a statistically important difference between viability of cells in the first control group (without agar blocks) and the second control group (agar blocks without StT) after 24 h incubation (Fig. 1). There was a small but statistically significant difference between 3T3 cells viability cultured with agar blocks containing StT and control cells from the second group after 24 h incubation. The viability of 3T3 cells in the second control group was 50% lower than in the first control group and 25% higher than in StT treated group (Fig. 1). It was noticed that mechanical damage of growing cells in two control groups was caused by agar blocks and had taken place within the first day of incubation (Fig. 2). On the second day cells started to recover the growing area. Cells which were not damaged by agar blocks proliferated like cells from the first control group after 48 h test (Fig. 3). The number of cells cultured with StT was similar to second control group after 48 h incubation (Fig. 1). There were no changes in morphology of cells in all groups (Fig. 2 and 3).

DISCUSSION

Staphylococcin T appears to damage cell membrane and causes an efflux of ions and an immediate block in macromolecular synthesis within bacteria. Some bacteria demonstrated the results of several incomplete divisions, resulting in a large, misshapen morphology. The final stage of bacteria death was a process of dissolution of the entire cytoplasmic contents. This resulted in bacterial ghosts of intact bacteria walls consisting of remnants of the cytoplasmic membrane and a few dense precipitates (4, 5). The huge difference in cell viability between the first (no agar blocks) and second controls (only agar blocks

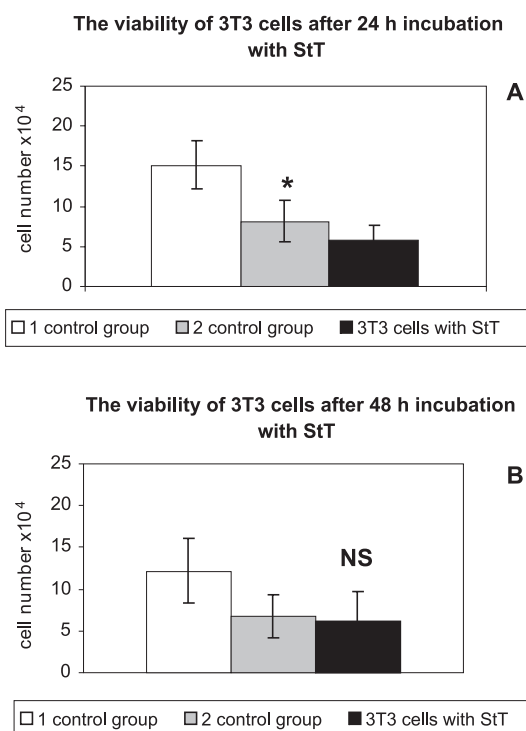


Figure 1. (A) The influence of staphylococcin T (StT) on 3T3 cells after 24 h incubation. Viability of 3T3 cells was presented as cell number $\times 10^4$. In the first control group cells were incubated without agar blocks. In the second control group cells were incubated with the fragment of agar without StT (*difference between cell number from the 1st control group, the 2nd control group and cells incubated with StT was significant, $p < 0.001$).

(B) The influence of StT on 3T3 cells after 48 h incubation. The viability of 3T3 cells presented as cell number $\times 10^4$ (difference between cell number from the 2nd control group and cells incubated with StT was not significant, NS).

without StT) confirmed the mechanical damage of cell culture caused by agar blocks. There were no morphological changes in 3T3 cells incubated with StT after 24 and 48 h. Cells morphology in culture with StT resembled the normal growing fibroblasts monolayer. The characteristic “cell ghosts” were not observed. Cells divided very quickly in all groups. The mitotic activity of cells was observed (Fig. 3). All these observations confirm that the StT has not influenced the 3T3 cells growth in culture.

There was no statistical difference between cells number in culture with StT agar blocks compared with second control group after 48 h of incubation. The morphological observation revealed that lower viability of cells cultured with agar blocks could be caused by mechanical damage of cells by agar blocks within the first day. Small movements of the agar blocks were observed which could influence the cell viability *in vitro* (Fig. 2). It is supposed that the differences in cell number found after 24 h

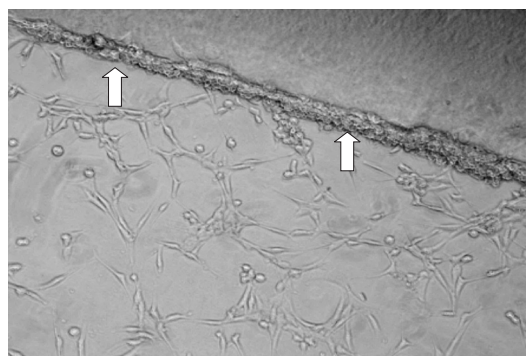


Figure 2. Mechanical damage of 3T3 cells by agar block during 24 h of incubation was noticed. Small movements of the agar block rolled the cells under the block edge (arrows, inverted microscope, and magnification 100 \times).

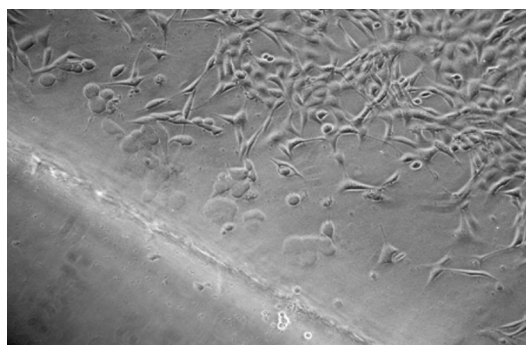


Figure 3. 3T3 cells started to proliferate under agar block with StT deposits within the second day of the experiment (inverted microscope, magnification 100 \times).

were connected with mechanical damage. After 48 h there was no difference in cell number between the second control group and StT treated group. The antimicrobial activity of StT against SAU-209P strain was observed even after 2 months and it was the proof that StT antimicrobial concentration had been maintained on the second day of the experiment. On the other hand, StT was released from agar to the medium in the concentration sufficient to inhibit the model SAU-209P strain in a very short period of time (24 h).

793 strains of *Staphylococcus* sp. (including 137 methicillin-resistant *Staph. aureus* strains) are sensitive to purified StT. All these strains are resistant to numerous antibiotics including aminoglycosides, second and third generation cephalosporins, lincomycins and rifamycines. StT is also active against strains that belong to the *Neisseriaceae* family, including four main representatives, *Acinetobacter*, *Branhamella*, *Moraxella*, and *Neisseria*. StT has no effect on any species that belong to the *Enterobacteriaceae* and *Pseudomonadaceae* families and yeast-like fungi (5).

Watanabe and Saito proved that some bacteriocins can be toxic to mammalian cells as well. Pyocin S2 inhibited the growth of not only tumor cells but also normal cells, like 3T3 mouse fibroblasts and BHK 21 cells (6). In this work, it has been proven that StT had unimportant influence on mouse 3T3 fibroblast cell line viability. There is a need to study the StT influence on the *in vivo* animal model.

CONCLUSIONS

Staphylococcin T in concentration sufficient for antimicrobial *in vitro* activity has unimportant influence on the mouse fibroblasts (3T3) in culture. StT may be considered for an animal trails.

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Received: 24.04.2006