ORIGINAL ARTICLE

# Protamine Impact on Viability of Bacterial and Eukaryotic Cell Cultures

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## Abstract

**Objective:** At present time, the substitution of the natural ingredients for various chemical additives used in food industry is an important problem. The perspective of this direction is protamine - small nuclear protein germ cells number of organisms. The article reveals the influence of protamine from salmon fish on the typical strains growth of pathogenic microorganisms and the viability of eukaryotic cell cultures. **Materials and Methods:** Expressed bactericidal activity of protein in concentration 4000 mcg/mL in regard to microbial strains *Escherichia coli* K-12 C 600 and *Salmonella typhimurium* LT2 was set up. **Results:** Significant antiproliferative effect on tumor cell lines Hep-2, RD, A-549, and T-98 G was marked, and less effect was found on line of human fetal lung fibroblasts FLECH 900/14. **Conclusion:** This makes possible the use of protamine as a promising antimicrobial and antitumor agent, for example, food additives with functional orientation.

**Key words:** Antibacterial activity, cell proliferation, cytostatic properties, protamine, viability evaluation

## INTRODUCTION

At present obtaining biologically active substances for medical and food purposes from low-value raw materials is becoming increasingly important, seeing the tendency of population growth, increasing consumption of drugs and food additives, and with it the reality of limited natural resources. In particular, in fish processing industry such raw material of low grade is milt, which is the source of DNA and protamines. As regards to some valuable commercial species of fish the milt is the waste. Protamines are small (molecular weight from 3500 to 6000 Da) nuclear proteins of a number of species. This protein plays a key role in the process of storage and transfer of genetic information, which allows for obtaining the DNA molecule a compact and biochemically inert form.

Characteristic feature of protamine is high basicity, which is caused by arginine content - up to 70% of the total amino acid composition. Moreover, arginine residues are located, usually in blocks of 2-7 residues along the polypeptide chain. The commonly occurring neutral amino acids in the molecules of protamine are alanine, glycine, and proline. Acidic amino acids are usually absent. Depending on the number of basic amino acids which contain protamine, they are classified into mono protamine (contain only arginine) diprotamine (contain lysine or arginine, and histidine), triprotamine (contain arginine, lysine, and histidine). Furthermore, it is the well-known protamine nomenclature, which is based on the generic names of the sources of their allocation, for example, protamine of herring *Clupea pallasii* - clupein, sturgeon *Acipenser stellatus* - stellin. In recent years, protamines from many sources have been isolated and characterized. The most studied of this class of proteins is fish protamine.

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There are several reasons for the interest in protamine. First of all, the interest in protamine is caused by the fact that this protein plays a key role in the storage and transfer of genetic information. Protamine is the only one to date preparation used in medical practice to neutralize heparin, which is a very important requirement for the success of extracorporal hemoperfusion (heart-lung machine, hemosorption, hemodialysis). These proteins were also widely used as prolongators of action of certain preparations, such as insulin.

It is well known that protamine has appreciable antimicrobial activity against a number of Gram-positive and Gram-negative bacteria. It was shown that salmine (protamine from salmon milk) has a bactericidal effect against Gram-positive bacteria growing *Brochothrix thermosphacta, Listeria monocytogenes,* and *Staphylococcus aureus*.[1] In this paper, the bactericidal effect of salmine on Gram-negative bacteria *Shewanella putrefaciens* was also observed. It was found that the protamine exerts its antibacterial action without causing cell lysis, and this operation is under the influence of the electrostatic membrane potential: The higher the value, the stronger the antagonist activity of protamine relative to the pathogenic microflora. Protamine violates the transport of energy and nutrients, and the target of its action becomes cytoplasmic membrane.

In the studies of Korean scientists, the antibacterial effect of protamine was shown against to 12 strains of bacteria, occurring in the oral cavity such as *Actinomyces naeslundii, Actinomyces odontolyticus, Enterococcus faecalis, Lactobacillus acidophilus, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Porphyromonas gingivalis*, and *Candida albicans*.[2] Effect of protamine depends on concentration and incubation time. It was shown that protamine can be used as a dental material.

Canadian Scientists Hansen LT. and Gill TA. evaluated protamine antimicrobial activity against *L. monocytogenes* and *Escherichia coli* in concentrations of 50-10,000 pg/mL at pH from 5.5 to 8.0.[3] It is revealed that the protamine inhibited *E. coli* at all these pH values, while the inhibition of *L. monocytogenes* occurred only at pH 6.5 and above. Increased antimicrobial activity of protamine at a pH close to alkaline explains likely to increase the affinity of the electrostatic surface of target cells.

There are well-known studies of the effect of chemical modification of protamine (used clupein) on its antimicrobial activity.[4] The chemical modification was conducted to block 10-71% of the guanidine group of arginine residues, which reduced the molecular charge protamine. It has been found that a modified protamine inhibited growth of *L. monocytogenes* in milk and Gram-negative bacteria (Enterobacteriaceae) in minced meat much better than native protamine. In tryptone soy broth, antibacterial activity of modified and native protamine was similar.

Protamine shows good activity (native or in combination with ethylenediaminetetraacetic acid) in inhibiting the growth of trophozoites *Acanthamoeba*, and therefore it can be a very promising solution for disinfection component contact lens.[5]

Protamine, as well as the polycationic peptides, exhibits some anticancer activity. In recent paper, we studied the effect of protamine on the proliferation of bacterial and eukaryotic cells.[6] It demonstrated that protamine causes cessation of cell proliferation as an *E. coli*, and Hela, making further studies protamine promising as an effective and specific anticancer drug.

## MATERIALS AND METHODS

The 1st step was to determine the operation of protamine antibacterial activity against pathogenic microorganisms *E. coli* K-12 C 600, *Salmonella typhimurium* LT2, *Proteus vulgaris* ATCC 13315, *S. aureus* subsp. *aureus* 209P. To determine the inhibitory action of protamine the method of double serial dilutions in physiological solution was used. The protamine concentration of 500, 1000, 2000, and 4000 mcg/mL was used for studies.

Each microbial strain was subcultured from the nutrient medium LB-agar LB liquid medium with the following composition, g/l: Peptone - 10, yeast extract 5, sodium chloride - 10. From the fresh suspension culture after 24 h of incubation, 1 mL was taken and was transferred to the test tube with 9 mL saline solution. Then, alternately transferred to 1 mL of suspension from each tube in the subsequent so that the concentration of bacterial cells *in vitro* was ninth $1 \times 10^9$ colony-forming units/mL. Thereafter, 1 mL of suspension was collected from the 6th, 7th, 8th, and 9th tubes and dispensed to sterile Petri dishes followed by LB agar filling and incubating in thermostat for 24 h at 37°C. After incubation each plate colony count was determined.

Then, the inoculum was prepared with the addition of various concentrations of protamine. Protamine sample introduced into a liquid LB medium before sowing, planting made of culture and incubated for 24 h in an incubator at 37°C. This was followed by 10-fold dilutions in saline in the same manner as in the case with the control. Interpretation of results was also carried out after 24 h of incubation.

The 2nd step was to study the effect of protamine on the viability of eukaryotic cells. As the test cultures, following continuous cell lines of human were chosen: Oral epidermoid carcinoma HEP-2, embryonic rhabdomyosarcoma RD, lung fibroblasts of normal embryo FLECH 900/14, lung carcinoma A-549, and glioblastoma T-98 G ("BioloT" St. Petersburg).

Cells were grown in Dulbecco's Modified Eagle's Medium culture media (for line Hep-2, RD, FLECH 900/14) or MEM

(for the lines A-549 and T-98 G) supplemented with 10% (for Hep-2 lines, RD and FLECH 900/14) or 5% (for the lines A-549 and T-98 G) fetal bovine serum and 50 ug/mL penicillin-streptomycin antibiotic. Culture media, serum, antibiotics, and trypsin solutions version produced by "BioloT" Russia, St. Petersburg. Cultivation was carried out in polystyrene flasks (orange scientific, Belgium) with treated growth surface 25 $cm^2$ and ventilation conditions in $CO_2$-incubator (SANYO, AC-15 IVS, Japan) with a concentration of 5.0% and a gas temperature of 37°C.

After the depletion of nutrients media replacement was performed. To do this, the vials were removed from the liquid medium, the vial with the cells was washed with 2 mL of versene solution or saline solution, then the solution was removed into a vial, and fresh medium was added. The formation of a monolayer was detected by the density of cell growth in the culture vial during microscopy. After monolayer formation cells were removed and were plated in the new culture vials. Thereafter, the amount of live and dead cells was determined. Thereafter, the cell proliferation index (IPi) of and time of monolayer formation were determined. The amount of IPi was calculated as the ratio of the final concentration of cells in the original.

Detachment of cells was performed as follows: The liquid medium is removed, cells from culture flask washed with versene solution, then the solution was removed. Thereafter, 2 mL of versene solution (0.02%) and trypsin (0.25%) in the ratio of 3:1 were added into each vial. After 15 min, the flasks were placed into an incubator on a shaker at 37°C to disaggregate cells from the culture flask surface. Then, 2 mL of cell suspension and 10 mL of nutrient medium were added into a new vial and then placed in a $CO_2$ gas incubator at a concentration of 5.0% at 37°C.

All cultural experiments were carried out under aseptic conditions in a laminar flow hood II "A" 2 biological safety class (AMC MZMO, Russia).

In the experiment, after the formation of a monolayer cell suspension of each cell line were divided into five vials. One vial was used as a control sample, while the other four vials were used to assess the cytostatic activity of protamine. For this purpose, protamine in concentrations of 500, 1000, 2000, and 4000 mcg/mL and 2 mL of medium with serum were added into 4 test vials with a monolayer of cells and incubated for 1 h at 37°C in a $CO_2$ gas incubator. Thereafter, the culture medium was removed and cell monolayer was washed with 1 mL of versene solution or saline solution. Removal monolayer was performed with 1 mL 0.02% versene solution and 0.25% trypsin in a ratio of 3:1. The vials were placed into a shaker for 15 min in an incubator at 37°C and removing the washing procedure was performed for the reference bottle.

The determination of the amount of dead and live cells was carried out in the Goryaev camera. For this purpose, 1 mL of the cell suspension was collected from a control and four experimental vials and stained with 0.4% trypan blue solution. Dead cells were permeable to the dye and microscopic appearance is darker in comparison to live. A few microliters of the mixture were added under the cover Goryaev camera glass. The count was performed at a tenfold increase as follows: Under increasing 10 × were counted as follows: At 1st the cells located in the center of the square were counted on the right and lower boundaries, then 25 large squares were counted. The number of cells in the cell suspension was calculated by the formula (1):

$$N = N_{25}*2500*V_{cellsuspension} \quad (1)$$

N - cell number in the whole suspension;
$N_{25}$ - number of cells in 25 large squares;
$V_{cellsuspension}$ - total volume of cell suspension, mL.

Analysis of the kinetics of growth of the cell population was carried out by 2 parameters: Population doubling rate (X) and the population doubling time (TD). These parameters are calculated using formulas (2) and (3):

$$X = [\lg N_h - \lg N_i]/\lg 2 \quad (2)$$

$N_h$ - number of cell passages No n;
$N_i$ - number of cell passages №(n−1).

$$TD = t*\lg 2/[\lg N_h - \lg N_i] \quad (3)$$

t - cultivation time, h.

## RESULTS AND DISCUSSIONS

As a result of evaluation of antibacterial properties of protamine against selected pathogens strains the diagrams by the mean value for each concentration and control for each strain were drawn [Figures 1-4].

Protamine has the greatest impact against strains of *E. coli* K-12-600°C and *S. typhimurium* LT2 since the concentration

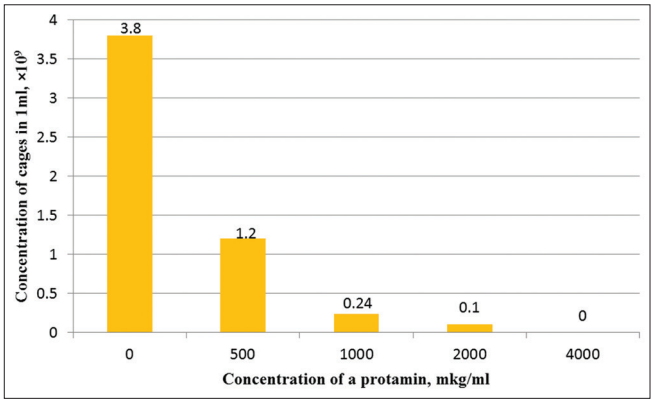


**Figure 1:** Effect of protamine per strain *Escherichia coli* K-12 C 600

of 4000 ug/mL completely inhibits growth. Protamine has a significant impact on the growth of *S. aureus* subsp. *aureus* 209P, as protamine at a concentration of 4.000 mg/mL colony growth is practically nonexistent. On strain, *P. vulgaris* ATCC 13315 protamine has little effect, as the growth of colonies is observed at 4000 mcg/mL concentration of protamine.

During the experiments with transplantable human cells, the monolayer of each cell line was obtained. Furthemore, the monolayer formation time and IPi were determined as well as analysis of growth kinetics of cell line. The following results were obtained.

The formation of a monolayer cell line FLECH 900/14 on the surface of a plastic culture flask of 25 $cm^2$ occurred for 13 days on 1 $cm^2$ monolayer proliferation area formed at 12.5 h. The IPi FLECH 900/14 was 2.36. Population doubling coefficients (X) and population doubling time (TD) for FLECH 900/14 line were amounted:

$X = [lg3,3*10^4-lg1,4*10^4]/lg2 = 1,24$

$TD = 48lg2/[lg3,3*10^4-lg1,4*10^4] = 38,73$

The formation of a monolayer line A-549 was occurred in a vial for 10 days, or the formation of a monolayer on the surface of 1 $cm^2$ was occurred for 9.6 h. The IPi of lung carcinoma cells was 3.53. Population doubling coefficients (X) and the population doubling time (TD) of the line A-549 are represented by the next equations:

$X = [lg1,2*10^5-lg3,4*10^4]/lg2 = 1,82$

$TD = 48lg2/[lg1,2*10^5-lg3,4*10^4] = 26,4$

A monolayer of Hep-2 on the surface of the bottle cell line was formed for 7 days, on 1 $cm^2$ of surface - for 6.7 h. The IPi of oral epidermoid carcinoma was 7.83. Coefficients of population doubling (X) and the population doubling time (TD) to the line Hep-2:

$X = [lg1,8*10^5-lg2,3*10^4]/lg2 = 2,97$

$TD = 48lg2/[lg1,2*10^5-lg3,4*10^4] = 16,2$

The formation of a monolayer cell line RD in a culture flask of 25 $cm^2$ occurred in 12 days, i.e., 1 $cm^2$ monolayer formed in 11.5 h. IPi was 2.4 $IP_i$. Population doubling coefficients

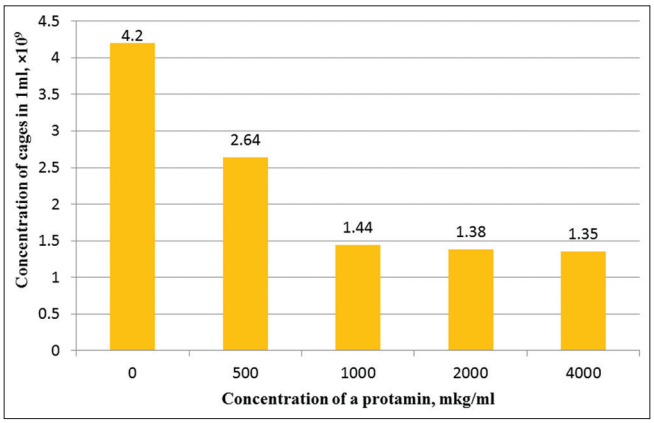


**Figure 2:** Effect of protamine per strain *Proteus vulgaris* ATCC 13315

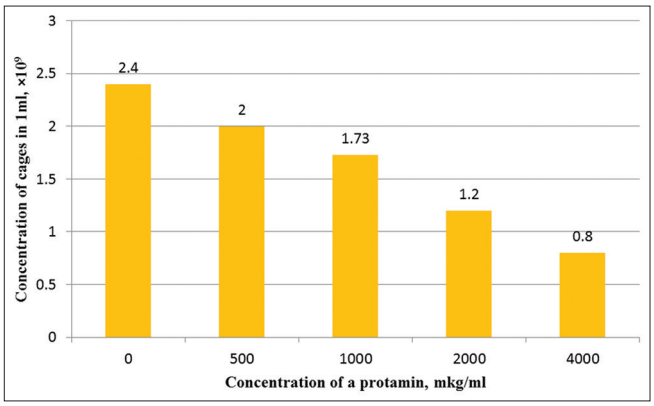


**Figure 3:** Effect of protamine per strain *Staphylococcus aureus* subsp. *aureus* 209P

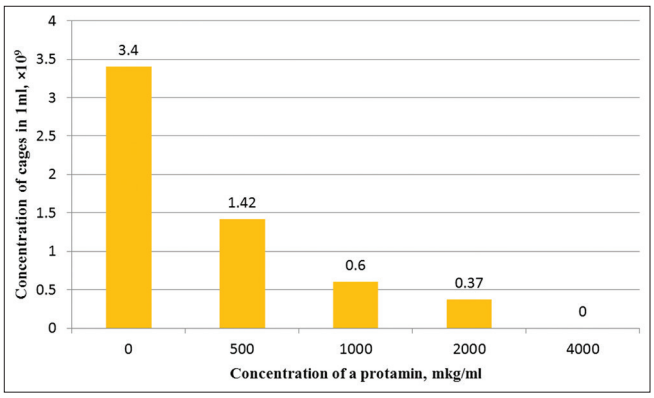


**Figure 4:** Effect of protamine per strain *Salmonella typhimurium* LT2

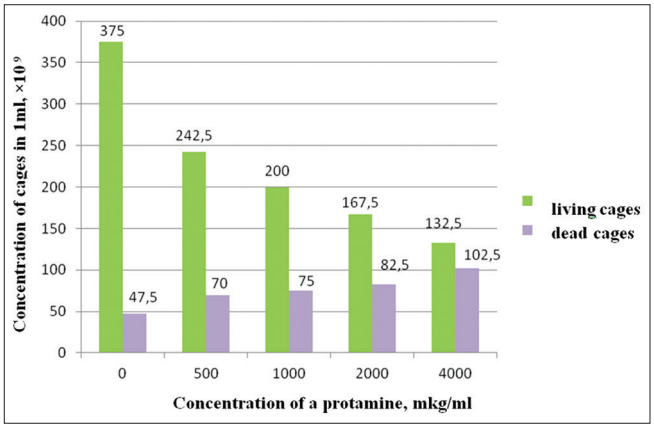


**Figure 5:** Effect on viability of protamine cell line T-98 G

(X) and the population doubling time (TD) of RD line are represented by the next equations:

$X = [lg9,1*10^4-lg3,8*10^4]/lg2 = 1,25$

$TD = 48lg2/[lg9,1*10^4-lg3,8*10^4] = 38,12$

The formation of a monolayer of T-98 G line in a culture flask of 25 $cm^2$ was occurred in 10 days, culture flask of 1 $cm^2$ - for 9.5 h. IPi was 4.33. Coefficients of population doubling (X) and the population doubling time (TD) are defined as:

$X = [lg1,3*10^5-lg3*10^4]/lg2 = 2,11$

$TD = 48lg2/[lg1,3*10^5-lg3*10^4] = 22,68$

To assess the effects of protamine on the viability of eukaryotic cell cultures, according to the results of counting the total number of cells diagrams [Figures 5-9] were plotted for the control and experimental samples.

It is found that the protamine exhibits cytostatic properties toward all studied strains of eukaryotic cells, the observed dose-dependent inhibition of viability.

The greatest inhibitory activity of protamine was observed against oral epidermoid carcinoma Hep-2. Each subsequent protamine concentration increases the amount of living cells by approximately 1.5 times in comparison with the control sample, and at concentration of 2000 mcg/mL, the number of dead cells increased 2 times as compared with the control sample. The use of protamine concentration 4000 mcg/mL leads to the predomination of dead cells in comparison with live cells.

Protamine had minimal effect against lung fibroblasts of normal embryo FLECH 900/14. Each subsequent protamine concentration increases the amount of dead cells increased slightly as compared with the control sample. Despite the reduction in the number of living cells at 500 mg/mL protamine concentration, more than twice in comparison with the control sample, further concentration increase did not lead to the decrease of live cell amount.

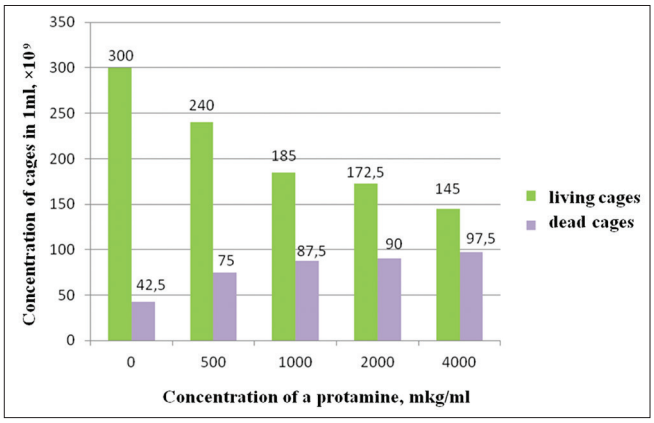


**Figure 6:** Effect on viability of protamine cell line A-549

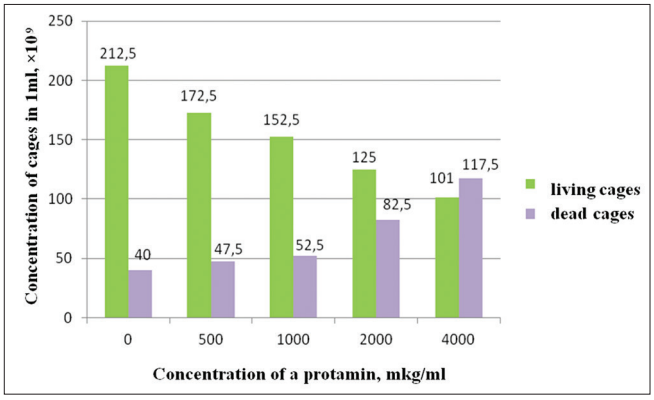


**Figure 7:** Effect on viability of protamine cell line Hep-2

## CONCLUSIONS

The article set protamine antimicrobial effect against pathogenic microorganisms of the strains *E. coli* K-12 C 600, *S. typhimurium* LT2, *P. vulgaris* ATCC 13315, *S. aureus* subsp. *aureus* 209 P. Complete inhibition of the growth of *E. coli* K-12 C 600 and *S. typhimurium* LT2 was established

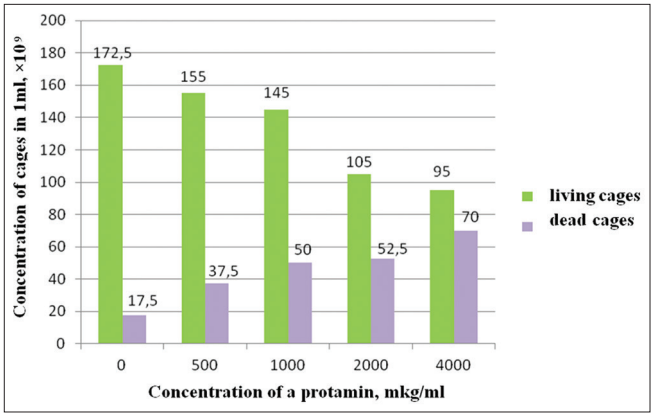


**Figure 8:** Effect on viability of protamine cell line RD

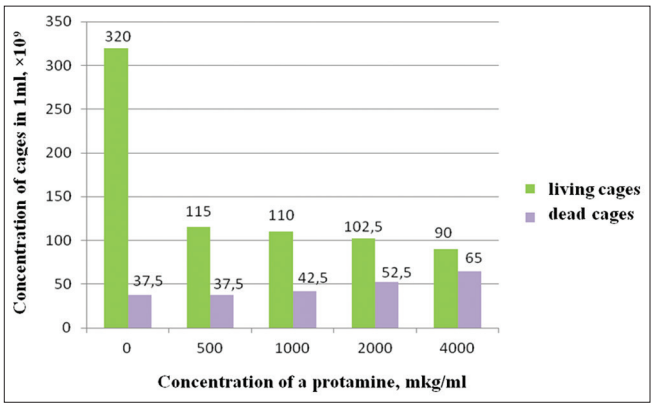


**Figure 9:** Effect on viability of protamine cell line FLECH 900/14

at 4000 mcg/mL protamine concentration, which makes possible the use of protamine as antimicrobial agent, for example, a food preservative.

The results of the study on the test cultures epidermoid carcinoma of the larynx the Hep-2, embryonal rhabdomyosarcoma RD, fibroblasts fetal human lung FLECH 900/14, lung carcinoma A-549, and human glioblastoma T-98 G show that protamine demonstrated good cytotoxic activity. It should be noted that protamine impact against noncancerous FLECH 900/14 line is expressed in least, so we can make the conclusion that protamine is very promising agent as anticancer preparation or a functional food additive.

## REFERENCES


1. Johansen C, Gill T, Gram L. Antibacterial effect of protamine assayed by impedimetry. J Appl Bacteriol 1995;78:297-303.
2. Kim YH, Kim SM, Lee SY. Antimicrobial activity of protamine against oral microorganisms. Biocontrol Sci 2015;20:275-80.
3. Hansen LT, Gill TA. Solubility and antimicrobial efficacy of protamine on *Listeria monocytogenes* and *Escherichia coli* as influenced by pH. J Appl Microbiol 2000;88:1049-55.
4. Potter R, Truelstrup Hansen L, Gill TA. Inhibition of foodborne bacteria by native and modified protamine: Importance of electrostatic interactions. Int J Food Microbiol 2005;103:23-34.
5. Vijay AK, Bandara M, Zhu H, Willcox MD. Protamine as a potential amoebicidal agent for contact lens disinfection. Optom Vis Sci 2013;90:119-24.
6. Günther K, Paradowska-Dogan A, Bärmann B, Klein H, von Eichel-Streiber C, Hartley R, *et al.* Expression of sperm-specific protamines impairs bacterial and eukaryotic cell proliferation. Histochem Cell Biol 2015;143:599-609.


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