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Combined Effect of Diluted Betadine and *Serratia marcescens* Nuclease on Viability of Rat Hepatoma Cells.

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ABSTRACT

In order to enhance the tumoricidal efficacy of the diluted Betadine® its water solutions were combined with the buffer solutions of *Serratia marcescens* nuclease. Examination of the combined effect on viability of cancer cells of rat hepatoma (H4-II-E-C3) in vitro showed the enhancement of a tumoricidal effectiveness of the prepared composition in comparison with the diluted Betadine® or the nuclease solutions. The Betadine® water solutions containing up to 5% of povidone-iodine were found to be compatible with *Serratia marcescens* nuclease solutions.

Keywords: *Serratia marcescens* nuclease, Sma nuc, Betadine, tumoricidal effect, cancer cells, rat hepatoma.

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INTRODUCTION

Betadine®, also familiar as Povidone-iodine (PVP-I), is well known antiseptic. It is very effective against a broad range of gram-negative, gram-positive and acid- tolerant bacteria as well as fungi, mold, yeast, protozoa and viruses. It is widely used in human medicine. An active component of Betadine® is activated iodine that gradually and evenly releases from the complex with PVP and provides Betadine® with prolonged high antiseptic properties even in the presence of blood and plasma. The microbicidal activity of Betadine® is due to the strong oxidizing effects of free iodine on functional groups of amino acids, nucleotides and double bonds of unsaturated fatty acids [1- 11]. On the basis of results of electron microscopy and biochemical analysis's carried out with PVP-I on models gram-positive, gram-negative and eukaryotic micro-organisms pore formation or generating solid-liquid interfaces at the lipid membrane level leading to loss of cytosol material and additionally enzymes denaturation was concluded [12].

Additionally Betadine® displayed the tumoricidal effectiveness as a disinfectant when performing abdominal operations [13-15]. Abdominal irrigation with 0.25 % PVP-I solution significantly reduced the number of animals with port-tumor recurrences in a splenic tumor model [16]. Study of prevention with PVP-I of intra- and extraperitoneal metastases by intraperitoneal instillation of the cells of human colon adenocarcinoma as well as gallbladder carcinoma revealed a significant decrease in tumor cell growth in vitro as well as intraperitoneal tumor growth in vivo [17]. The whole-colon washout using 5 % PVP-I was found to be clinically feasible in substitution of preoperative bowel preparation and helpful in prevention of recurrent cancer due to implantation of viable exfoliated tumor cells [18, 19]. A peritoneal washing in vivo in a model of peritoneal carcinomatosis in the rat with 1% diluted PVP-I has also good but an incomplete effect in the prevention of peritoneal carcinomatosis [20]. The examination of diluted PVP-I cytotoxicity in vitro on rat colon cancer cells (DHD/K12/PROb) and human colon cancer cells (HT29) displayed a high efficacy. The effectiveness of PVP-I solutions depended on both its concentration and the time of application. Thus 0.01% water solution of PVP-I was found to be not tumoricidal, neither in vitro nor in vivo towards human colonic carcinoma cells SW620. PVP-I of 0.05 and 0.1% solutions were found to be less effective in vitro than 5%, but could prevent in vivo proliferation of the same model unless an adjustment of the residual number of viable tumor cells was performed. 5 % water solution of PVP-I killed all or almost all of SW620 human colonic carcinoma cells in vitro and prevented their growth in vivo [21, 22]. Similar good results were achieved upon investigating the efficacy of intra-operative whole-colon washout using PVP-I in an experimental model of anastomotic tumor growth in the colonic lumen of Fischer rats. The anastomotic tumor growth was significantly reduced after tumor cell inoculation followed by whole-colon lavage and luminal incubation for 20 min with 5% PVP-I. After 30 min incubation with 5% PVP-I several animals died, that was obviously due to the prolonged exposition [14]. Thus although actually good tumoricidal efficacy of PVP-I solutions was demonstrated a trend to enhance its tumoricidal action to complete cell death is obvious.

In order to enhance the tumoricidal efficacy of PVP-I, that was a goal of the current study, we decided to combine the Betadine® solutions with another compound, Sma nuc nuclease.

The enzyme Sma nuc nuclease originated from Gram negative bacterium *S. marcescens* demonstrated a very potent digestive activity towards DNA and RNA [23, 24]. It heads a broad range of homological non-specific nucleases, which widely spread in the world. It is one of the most studied bacterial nuclease. Its structure, mechanisms of action and regulation, as well as physical, chemical and biochemical properties are well known [25-34]. A broad specificity of Sma nuc nuclease [23-24] is applied for degradation of nucleic acids [35]. It was used in development of antiviral and antiphagel activities of Sma nuc nuclease [36-37]. Earlier broad specificity of Sma nuc nuclease was explored for development of anticancer activity [38].

Thus, searching a combined tumoricidal effect in vitro towards hepatoma cells (H4-II-E-C3) taking as a model, we decided to cooperate actions of Betadine® and Sma nuc nuclease, substances belonging to different chemical groups, a iodophor representing a complex of synthetic polymer polyvinylpyrolidone with iodine, and a catalyst of polypeptide nature.

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EXPERIMENTAL

Materials

We used preparations of yeast DNA (Sigma, USA); Betadine®, a commercial preparation for external use containing 10% povidone-iodine, PVP-I (Egis Pharmaceutical, Hungary); Live/Dead Cell Viability Kit (Fluka, Switzerland) containing Calcein-AM and Propidium iodide, PI; Dulbecco's modified Eagle's medium with high glucose amount, DMEM (Sigma-Aldrich, USA) additionally containing 10% of Fetal bovine serum, FBS (Sigma, USA); 2 мM L-glutamine, and a mixture of penicillin and streptomycin (Sigma-Aldrich, USA); a cell line of rat hepatoma (H4-II-E-C3) from the American type culture Collection (ATCC, USA); human embryonic kidney cell culture HEK293T (ATCC Number: CRL-11268; Manassas, VA, USA).

Isolation of *Serratia marcescens* nuclease

Sma nuc nuclease was isolated and characterized as previously noted [30, 39]. After that 1 mg of the lyophilized preparation was dissolved with 1 ml of the equilibrium buffer, 0.1 M Tris-HCl-, pH 8.5, and subjected to a column chromatography (0.8 x 16.0 cm) on previously equilibrated Sephadex G-100 (fine). Elution was carried out with the equilibrium buffer. The fractions of highest absorption at 280 nm and enzymatic activity were selected to study the biological effects.

Concentration of Sma nuc solution was calculated based on the absorption of protein solution at 280 nm and molar extinction coefficient of 47.292 $M^{-1} \cdot cm^{-1}$ [40].

Nuclease assay

The nuclease activity was assayed by the previously described method [23, 24]. After addition of the nuclease aliquot to 9-fold volume of assay mixture containing 50 mM Tris-HCl buffer, pH 8.5, 0.3 mM DNA, and 6 mM $MgSO_4$ the incubation was performed at 37°C for 15 min so that about 15-50% of the substrate was converted to acid-soluble products. The hydrolysis was stopped with an addition of chilled 4% perchloric acid. The precipitate was removed by centrifugation. The absorption of supernatant was monitored at 260 nm. Each experiment was repeated not less than 6 times.

Cytotoxicity

Cytotoxicity of Betadine® or Sma nuc nuclease solutions was studied using CellTiter 96®A Queous Non-Radioactive Cell Proliferation Assay (MTS assay, Promega, USA) which is widely used to study cytotoxicity of compounds in vitro [41, 42]. Initially the human embryonic kidney cell culture, $5x10^3$ cells/well in 96-well plate, was incubated in DMEM, additionally containing 10% FBS, 2 мM L-glutamine and a mixture of penicillin and streptomycin under humid atmosphere containing 5% CO_2 at 37°C. After 24 h the culture medium was gently removed and changed with the fresh medium additionally containing Sma nuc nuclease with final activity at 30 U/ml, 120 U/ml or 600 U/ml corresponding respectively to 20, 80 or 400 ng/ml of the protein or the diluted Betadine® with final PVP-I amount of 0.00003 or 0.003%. After 30 min incubation, a mixture of MTS and PMS (9:1 ratio) was added by 10 µl/well. After 30 min incubation, increased absorption was measured at 490 nm with the multimode reader Infinite M200Pro (Tekan, USA). Each experiment was performed in 4 replicates.

Double staining with Live/Dead Cell Viability Kit assay

The effectiveness of tested substances towards the rat hepatoma cell culture (H4-II-E-C3) in vitro was studied using double staining with Live/Dead Cell Viability Kit assay on the basis of standard protocol provided from the company [43]. 10 µl of Calcein-AM solution was added to 5 µl of PI solution and mixed with 5 ml of sodium phosphate buffer (PBS), pH 7.0. Resulting staining solution was applied in the ratio of 1:2 on cell culture grown at coverslips and pre-treated with the test compounds. After staining, cells were fixed for 20 min with 4% paraformaldehyde in PBS, washed once with PBS, pH 7.0, and embedded in Moviol mounting medium. Quantitative assessment of the living, dead and damaged cells was performed by direct counting using fluorescent microscope AxioVision (Carl Zeiss, Germany) with an excitation wavelength of 490 nm in at least 10 fields of view (approx. 300 cells).

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Preparation the rat hepatoma (H4-II-E-C3) cell culture

Rat hepatoma cell culture (H4-II-E-C3) initially was seeded ($5x10^5$ cells/well) on 4-well plate with round cover slips and incubated in DMEM additionally containing 10% FBS, a mixture of penicillin G (10 000 U/ml) and streptomycin sulfate (10 mg/ml) under humid atmosphere containing 5% CO_2 at 37°C. In accordance with recommendations (Serva, USA) the surface of the wells with cover glasses was covered with a 0.04% solution of collagen R in 0.1% acetic acid after initial mixing with sterile PBS solution, pH 7.0 [44]. After three days the culture medium was gently removed, the culture was washed with PBS solution and evaluated on viability (the intact cell culture) by the assay of double staining with Live/Dead Cell Viability Kit.

Examination of the effect on viability of the hepatoma cell culture

To study the substance effect on viability of the hepatoma cell culture in vitro the removed PBS solution was changed with the tested solutions added by 0.2 ml/well. To examine the Betadine® effect it was diluted with distilled water to PVP-I amount of 1- or 0.005% and incubated with the cell culture for 6 min. Studying Sma nuc nuclease effect the buffer solutions, containing 0.1 M Tris-HCl, pH 8.5, 0.01 M Mg^{2+} and the nuclease with activity at 12.88-, 1288 - or 6440 U/ml corresponding respectively to 8.6-, 858- or 4293 ng/ml of the protein, were incubated with the cell culture for 15 min. To evaluate the self-effect of 0.1 M Tris-HCl buffer, pH 8.5, containing 0.01 M Mg^{2+}, used as a control, it was incubated for 15 min.

To study a combined action of Sma nuc nuclease and Betadine® and its dependence on PVP-I amount or the enzymatic activity, first, water diluted Betadine, containing 1- or 0.005% PVP-I, was incubated with the cell culture for 6 min. Second, the nuclease previously diluted with 0.2 M Tris-HCl buffer, pH 8.5, containing 0.02 M Mg^{2+}, to the activity at 25.76 -, 2576 - or 12880 U/ml was equally added into the same wells and then incubated with the culture for 15 min. After incubation with the cell culture the tested substances were gently removed, the culture was washed with PBS solution and evaluated on viability by the assay of double staining with Live/Dead Cell Viability Kit.

Examination of Betadine® effect on the nuclease activity

To test the Betadine® effect on the nuclease activity water or buffer solutions of the nuclease mixed with the equal volume of Betadine® and incubated for appropriate time in the dark at 4-8° C. Then the enzymatic activity was assayed as described above taking the initial activity (prior to incubation) as 100%.
Each experiment was repeated not less than 6 times.

RESULTS AND DISCUSSION

Results of Sma nuc nuclease preparation by the gel filtration is shown in Table 1. Fractions # 4, most concentrated, was selected for further experiments.

Table 1: Column chromatography (0.8 x 16.0 cm) of Sma nuc nuclease solution on Sephadex G-100

Fraction #	Volume, ml	Protein, U_{A280}/ml	Activity, U/ml
1	1	0.127	8 960
2	1	0.066	9 984
3	1	0.164	138 400
4	1	0.280	257 600
5	1	0.259	436 800
6	1	0.256	228 800
7	1	0.156	49 600
8	1	0.100	3 760
9	1	0.083	3 280
10	1	0.038	191
11	1	0.030	211.8
12	1	0.028	133.4
13	1	0.023	105.6
14	1	0.022	96.6
15	1	0.020	196.8
16	1	0.017	76.8

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Cytotoxicity and tumoricidal effectiveness of Betadine® solutions

Examination of Betadine® cytotoxicity towards the human embryonic kidney cells HEK293 demonstrated its influence on the activity of mitochondrial dehydrogenases (MTS test) that was verified with variation in resulted absorption at 490 nm of the assay mixture (Figure 1). As the cells incubated in the presence of 0.00003, 0.003 % PVP-I diminished the absorption at 490 nm by about 30-70% in comparison with the control (in the absence of Betadine®), a conversion of MTS reagent to a colored formasan product was found. This conversion testified a PVP-I dependent cytotoxic effect of 0.00003 - 0.003 % Betadine® water solutions towards the human embryonic kidney cells.

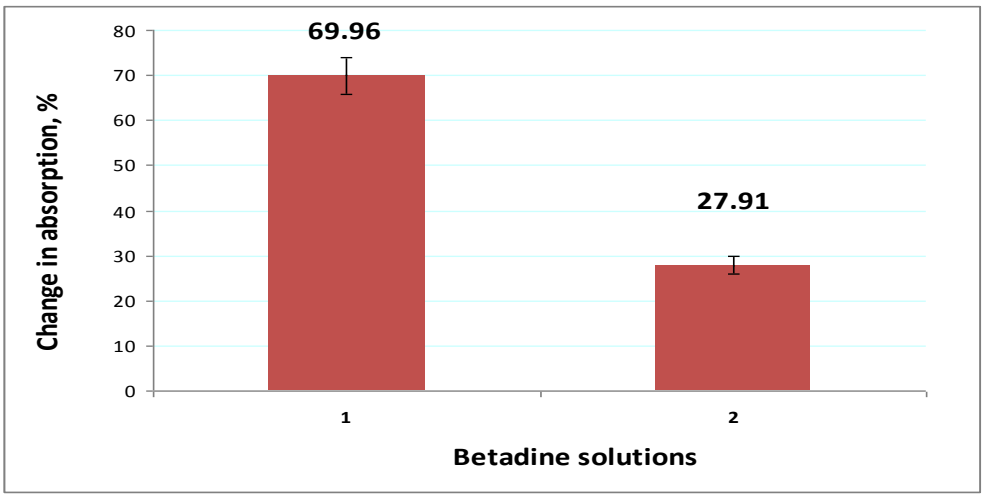

Figure 1: Cytotoxic effect of Betadine® on HEK293 cells. Variation of absorption at 490 nm characterizing the activity of mitochondrial dehydrogenases (MTS-test) upon the addition of 0.00003 or 0.003 % Betadine® water solutions (boxes 1, 2 respectively). The absorption without addition of Betadine® was taken as 100%.

Examination of Betadine® tumoricidal activity showed that although the found cytotoxic effect of 0.003% Betadine® water solution was good developed the tumoricidal activity of a similar Betadine® solution, containing 0.005% PVP-I, was not so strongly expressed. As demonstrated in Figure 2 B, 6 min incubation of the rat hepatoma cell culture with the 0.005% Betadine® solution only 1.3 - fold diminished the number of green colored cells. These cells are considered alive because of the green fluorescence of active intracellular esterases stained with Calcein-AM. At the same time the number of dead cells about 3-fold increased comparatively intact H4-II-E-C3 cell culture, used as a control and characterized in Figure 2A. The dead cells had red fluorescence because of interaction of propidium iodide with intracellular polynucleotides. The number of damaged cells, fluorescent at once with red and green as cells at the late apoptosis 10 -fold increased after 6 min the culture incubation with 0.005% PVP-I (Figure 2 B) in comparison with the control (Figure 2 A). Rising PVP-I amount to 1% significantly enhanced the tumoricidal effect of the diluted Betadine®. As shown in Figure 2 C after 6 min incubation with 1% Betadine® solution most of the cells had red fluorescence after staining with Live/Dead Cell Viability Kit. They were considered as dead. However about 3% of the cells were green or greenish colored after staining with the Kit that displayed the activity of the intracellular esterases. Thus some cells, respectively about 0.5 or 2.4 %, remained alive or damaged, but not dead, after 6 min incubation of the culture with 1% Betadine®.

Cytotoxicity and tumoricidal effectiveness of *Serratia marcescens* nuclease

Examination of the nuclease cytotoxicity demonstrated its weak influence on the activity of mitochondrial dehydrogenases (MTS test) of human embryonic kidney cells that was verified with slight variation in resulted absorption at 490 nm of the assay mixture (Figure 3). As the cells incubated in the presence of nuclease at the activity of 30 U/ml, 120 U/ml or 600 U/ml diminished the absorption at 490 nm by 9-14% in comparison with the control (in the absence of Sma nuc nuclease) it appeared to decelerate a conversion of MTS reagent to a colored formasan product. This suggested that Sma nuc nuclease exerted a weak cytotoxic effect at the tested amounts.

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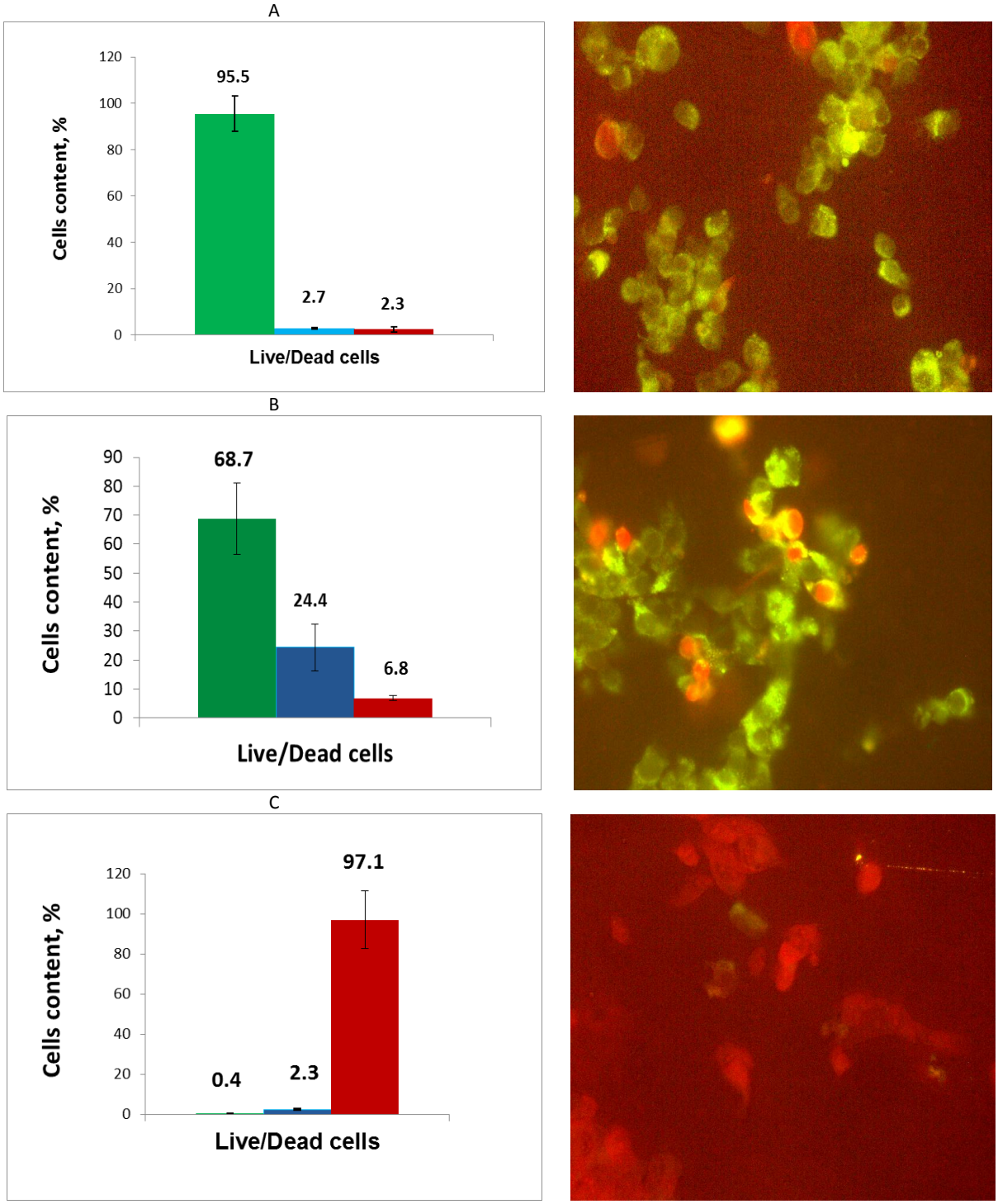

Figure 2: Tumoricidal effect of water diluted Betadine® on H4-II-E-C3 cells. Variation of fluorescence at 490 nm characterizing the activity of intracellular esterases, stained here and further in green with Calcein-AM solution (alive cells) or cellular membrane damage stained here and further in red with PI solution (dead cells) after the addition of 0.005 % or 1% Betadine® water solutions (B and C, respectively). A - The variation of fluorescence without addition of Betadine®, intact culture. Cells stained with green and red at once considered as damaged (blue boxes at diagrams)

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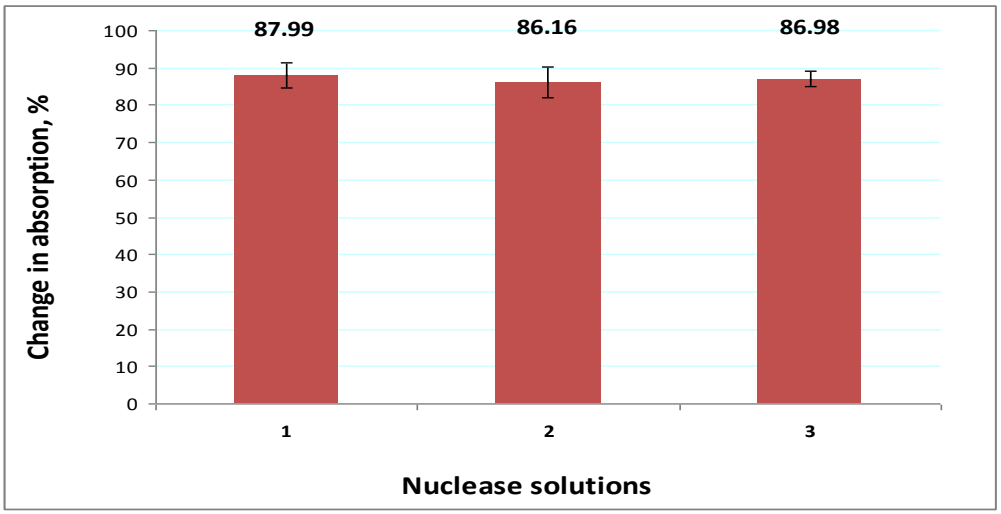

Figure 3: Cytotoxic effect of Sma nuc nuclease solutions on HEK293 cells. Variation of absorption at 490 nm characterizing the activity of mitochondrial dehydrogenases (MTS-test) upon the addition of Sma nuc nuclease solutions to final activity 30 U/ml, 120 U/ml or 600 U/ml (boxes 1,2,3 respectively). The absorption without addition of Sma nuc nuclease, taken as 100%.

Examination of tumoricidal effect in vitro of the nuclease solutions containing 0.1 M Tris-HCl buffer, pH 8.5, and the activating 0.01 M Mg^{2+} showed (Figure 4) that more than 90% of the cells of both the intact culture and the culture after 15 min incubation with the buffer solutions appropriately containing the nuclease displayed intracellular esterase activity that was evidenced by their green fluorescence and served us as a proof of their viability. Both in the presence and in the absence of the nuclease about 9% of the examined culture was represented by dead or damaged cells differing from the prevailing majority of fluorescent with green cells by red fluorescence in the nucleus or cytosol. After incubation with the buffer solutions the amount of damaged cells near 2-fold increased relatively the intact culture independently on the nuclease activity that served as evidence of lack of the tumoricidal effect of Sma nuc nuclease itself towards the rat hepatoma cells but a weak effectiveness of the buffer solution towards the cells H4-II-E-C3.

Although we did not observe the tumoricidal effect of Sma nuc nuclease itself we decided to move further and examine a tumoricidal effectiveness of a combination of Betadine® and the nuclease solutions. As Betadine® is known to pore the cell's membranes [12] and the nuclease powerfully hydrolyzes nucleic acids we have expected to degrade the intracellular polynucleotides with Sma nuc nuclease that could seep through the pores after the cells damage with Betadine®.

Tumoricidal effectiveness of a combined action of the Betadine® and the nuclease solutions

In order to realize the proposed idea, first of all, we studied a compatibility of Betadine® and Sma nuc nuclease because a iodophor of Betadine® oxidizingly effects on functional groups of amino acids in proteins that resulted in change of both the protein conformation and its catalytic activity. The result of change in Sma nuc nuclease activity under the action of Betadine® is shown in Figure 5. As seen from Figure 5A, 3 min incubation of Sma nuc nuclease water solution with diluted Betadine® containing 5 % PVP-I reduced the enzymatic activity by 30-40%. 2-fold increasing incubation time resulted in 2–fold reducing the activity additionally. Further extension of the incubation time to 20 min unremarkably changed the residual nuclease activity. The residual nuclease activity was similar after 6 min incubation with the diluted Betadine® containing 5 % PVP-I as in the presence and in the absence of 0.1 M Tris-HCl buffer, pH 8.5, containing 0.01 M Mg^{2+} (Figure 5B). Thus examination of Betadine® effect on Sma nuc nuclease showed that 20 min incubation with the Betadine® solution containing 5 % PVP-I resulted in diminishing the nuclease activity by 30-60 %. Besides the maximal rate in diminishing the activity was found at the first 3-6 min of incubation.

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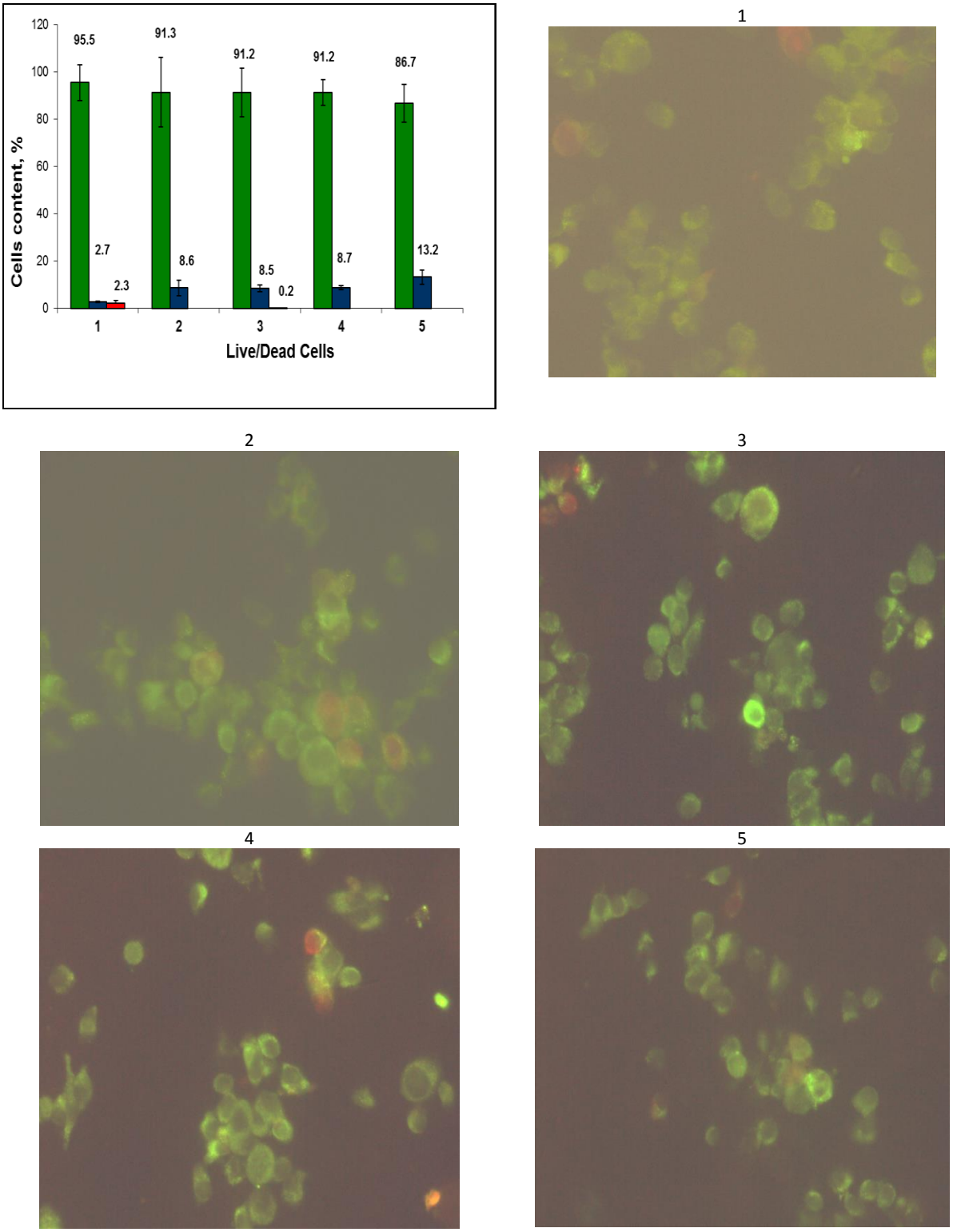

Figure 4: Tumoricidal effect of the nuclease buffer solutions towards H4-II-E-C3 cells. Variation of fluorescence at 490 nm characterizing the activity of intracellular esterases (live cells, green boxes/cells) or cellular membrane damage (dead cells, red boxes/cells) in the cell culture after 15 min incubation with 0.1 M Tris-HCl buffer, pH 8.5, containing 0.01 M Mg^{2+} (2) and additionally Sma nuc nuclease at the activity of 6440 U/ml (3), 1288 U/ml (4) or 12.88 U/ml (5). (1) - the variation of fluorescence without addition of the buffer solution, intact culture. Cells stained with green and red at once considered as damaged (dark boxes at the diagram).

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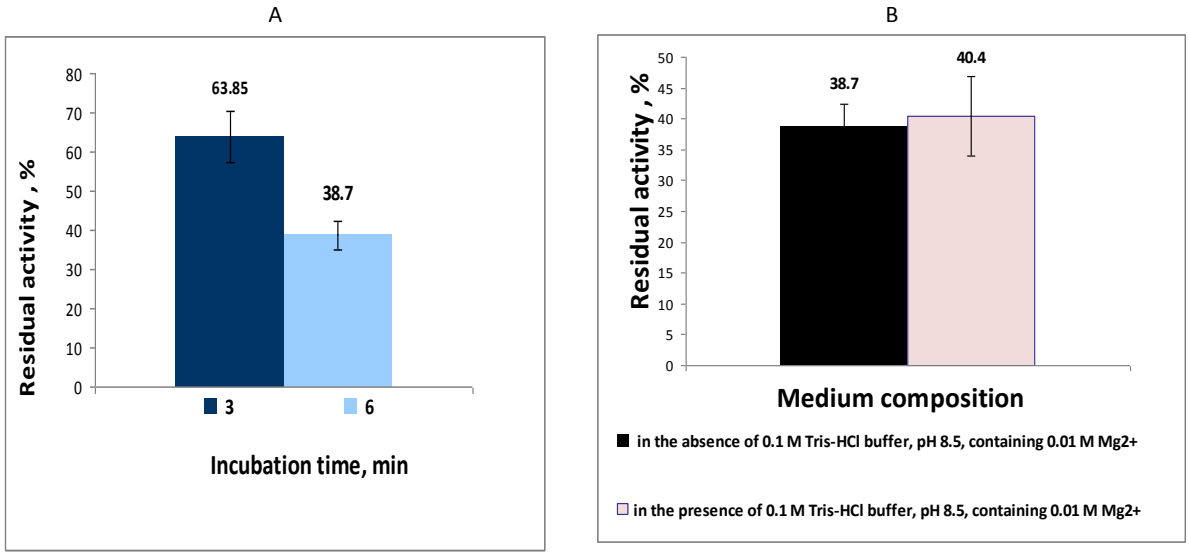

Figure 5: Changes in Sma nuc nuclease activity as a result of 3 (A) or 6 (A,B) min incubation with the diluted Betadine® solution containing 5 % PVP-I in the presence (B) or in the absence (A,B) of 0.1 M Tris-HCl buffer, pH 8.5, containing 0.01 M Mg^{2+}. The nuclease activity before mixing with the Betadine® was taken as 100%.

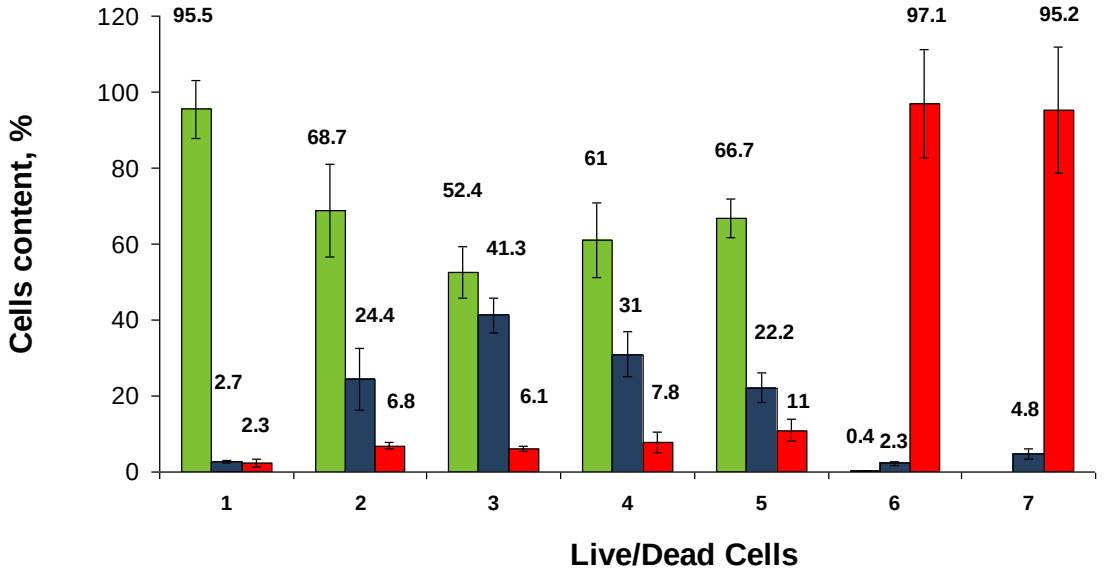

Figure 6: A combined tumoricidal effect of Betadine® water solutions with the nuclease buffer solutions. Variation of live/dead cells in the culture (H4-II-E-C3) characterizing a survivability of the hepatomic cells after 6 min incubation with the Betadine® solutions containing 0.005% PVP-I (boxex 2-5) or 1% PVP-I (boxes 6-7) and additional 15 min incubation after addition of the nuclease solutions at the activity of 12.88U/ml (boxes 3), 1288U/ml (boxes 4 and 7) or 6440 U/ml (boxes 5). Boxes 1- the variation of fluorescence of the intact culture.
Red boxes- dead cells, green boxes- live cells, dark boxes – damaged cells.

On the bases of found results the scheme of examination of a tumoricidal effectiveness of a combination of Betadine® and Sma nuc nuclease solutions contained the next steps. First, the cell culture was incubated for 6 min with the diluted Betadine®. Second, the nuclease buffer solution containing Mg^{2+} was added into the incubation medium. Then the cell culture was incubated for 15 min with the prepared mixture of Sma nuc and the Betadine® solutions. The results of combined action of Betadine® and Sma nuc nuclease solutions are shown in Figure 6. As found, the combined action of the Betadine® and Sma nuc nuclease solutions depended on both the PVP-I concentration and the nuclease activity. When the PVP-I concentration

corresponded to 0.005% the increasing nuclease activity in the mixture followed with the increasing number of dead cells. At once the number of damaged cells diminished (Figure 6, boxes 3-5). A similar trend was observed when the PVP-I concentration corresponded to 1%. Moreover, when the initial PVP-I concentration was 1% and the nuclease activity at 1288U/ml (in the mixture, respectively 0.5% and 644 U/ml) the combined treatment led to the absence of alive cells in culture at all (Figure 6, boxes 7). After the treatment the culture contained only the dead and damaged cells (near 5%) at observation of over 10 fields of view.

CONCLUSION

The found results show that water solutions of Betadine® containing 0.00003, 0.003 % PVP-I displays cytotoxic effect towards the human embryonic kidney cells HEK293. The water solutions of Betadine® also demonstrated a tumoricidal activity. As a test culture we used the rat hepatoma cells H4-II-E-C3. The intact H4-II-E-C3 culture contained more than 90% of alive cells and about 9% of dead or damaged cells. After incubation with 0.005% Betadine® the number of alive cells 1.3-fold diminished and number of dead cells 3-fold increased comparatively the intact H4-II-E-C3 culture, used as a control. The pronounced changes were observed when PVP-I amount raised to 1%. More than 90% of the cells in culture were considered as dead after 6 min incubation with 1% Betadine®. However some cells in the culture remained alive or damaged, respectively about 0.5 or 2.4 %.

Vice versa, Sma nuc nuclease buffer solutions containing the activating Mg^{2+} caused a weak both the cytotoxic effect depending on the enzymatic activity, and the tumoricidal effect independent on the enzymatic activity. However when we combined Sma nuc nuclease solutions with the Betadine® solutions the tumoricidal action appeared to depend on the enzymatic activity. In the presence of 0.005% PVP-I the number of dead cells increased with the increasing nuclease activity in the mixture composed of the Betadine® and Sma nuc nuclease solutions. The similar trends were revealed for 1% PVP-I. Moreover after the incubation in the presence of Sma nuc nuclease with initial activity of 1288U/ml (644 U/ml of the composition) the alive cells in culture were not found at all.

Taken together, the results of the study of combined effect in vitro of the diluted Betadine® and Sma nuc nuclease on viability of rat hepatoma cells H4-II-E-C3 indicate:

- Water solutions of Betadine® containing 0.005 -1% of PVP-I display the PVP-I dependent tumoricidal action towards the test cell culture.
- Although 3-6 min incubation of Betadine® water solutions with the buffer solutions of Sma nuc nuclease diminishes the nuclease activity the Betadine® water solutions are compatible with the Sma nuc solutions since about a half of the initial nuclease activity remains after the incubation with the solutions containing up to 5% of PVP-I.
- A combination of Betadine® and Sma nuc nuclease solutions including, first, the cell culture incubation with the diluted Betadine® and, second, with the composition containing the Betadine®, Sma nuc nuclease, Tris and Mg^{2+} at selected concentrations enhances a tumoricidal effectiveness of the diluted Betadine® and can be clinically feasible in cancer surgery.

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