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EFFECT OF HYDROCHLORIC POLYHEXAMETHYLENE GUANIDINE (PHMGH) AND POLYHEXAMETHYLENE BIGUANIDINE (PHMBH), ALSO IN COMBINATION WITH PLANT ESSENTIAL OILS AND ZnO NANOPARTICLES ON SOME EUKARYOTIC CATTLE AND PIG CELLS

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Aim. To investigate both toxic (hemolytic), and stimulating effects of two polymeric derivatives of guanidine, in particular, polyhexamethylene guanidine (PHMG) and polyhexamethylene biguanidine (PHMB) both in the hydrochloride form, on eukaryotic cells depending on the concentration of the preparation; to study the possibility of using wound-healing and stimulating properties of these preparations in veterinary medicine. **Methods**. The hemolytic activity (toxicity) of PHMGH and PHMBH preparations in the concentration of 0.1% towards cattle and pig erythrocytes was determined by titration. Primary cell cultures of fetal kidney cells of calves and piglets were used to determine the influence of PHMGH and PHMBH both alone and in combination with the following biologically active substances: essential oils of *Pinus sylvestris*, *Eucalyptus globulus*, *Citrus sinensis*, *Monarda didyma*, ZnO nanoparticles (size c. 25 nm), and electrochemically activated water – anolyte (Eh = –800 mV, pH 6.5–7.0). The concentration of the cells in the nutrient medium was determined via photocolorimetry. **Results.** It was found that depending on the concentration, PHMGH, and PHMBH preparations can cause the lysis of erythrocytes, and stimulate cell proliferative activity, including the formation of a monolayer of kidney cells of calves and piglets They cause hemolysis of cattle erythrocytes in the concentrations commonly used for disinfection, i.e., about 0.1 %, in the average titers of 1 : 7 for PHMGH and 1 : 2.5 for PHMBH. Therefore, PHMBH shows greater hemolytic (biocidal) activity for cattle erythrocytes than PHMGH (in ≈2.8x). The high molecular weight fraction of PHMBH (M_2 ≈ 2,000–7,000 Da) demonstrated a lower (in ≈2.4x) hemolytic activity than the low molecular weight basic fraction (M_1 ≈ 500–2,000 Da). The experiments on the kidney cell cultures of pigs and cattle have shown that at non-toxic concentrations (10^{-5} %) PHMBH can effectively stimulate (from 27 to 65 % increase) the proliferative activity of eukaryotic cells and accelerate the formation of a monolayer of cells. The combinations of PHMGH with some essential oils of medicinal plants also show a good effect (from 52 to 95 % increase), and PHMBH shows a good effect with oil of pine for pig kidney cells (20 % increase) and oil of horsemint for cattle kidney cells (67 % increase). **Conclusions.** PHMGH and PHMBH can possibly be used in agricultural production not only as disinfectants or antiseptics, but also in wound healing. Although their toxicity is also significant to eukaryotic cells, yet they can possibly be used in veterinary medicine in low concentrations (0.005–0.5 %) for the treatment of wounds of various origin, including burns, in the composition of ointments, gels, bandages, or plasters, which we have presently in investigation.

Key words: polyhexamethylene guanidine, essential oils of medicinal plants, cell culture, disinfection, toxicity, growth stimulation.

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INTRODUCTION

The polymeric guanidine derivatives have long proven to be efficient disinfectants and antiseptics. Most common these are polyhexamethylene guanidine hydrochloride (PHMGH) and its analog, polyhexamethylene biguanidine hydrochloride (PHMBH). They have clearly expressed bactericidal, virucidal, fungicidal, and algicidal properties (Lysytsya AV et al, 2015; Mashat BH, 2016; Oule MK et al, 2017). There is much information on the application of polymeric guanidine derivatives in the composition of different disinfectants and antiseptics, for instance, e.g. in the treatment of hospital-acquired infections (biofilms) (Oule MK et al, 2017; Vitt A et al, 2017; Moshynets OV et al, 2022). In addition, PHMGH, has also been used with some effect in the purification of sewage and underground waters (Kvartenko O et al, 2018; Kvartenko O et al, 2021). It was long believed that PHMGH and PHMBH hads low toxicity for higher organisms, including humans (Oule MK et al, 2015; Oule MK et al, 2017; Lysytsya A, 2017). The use of PHMGH in air humidifiers demonstrated that under long-term inhaling, the preparation has a very negative impact on human breathing organs, including the induction of lung fibrosis (Kim HR et al, 2016; Jeong MH et al, 2021; Kang MS al, 2022; Lee H al, 2022). An initial step in the development of the fibrosis proved to be a programmed cell death (apotosis) triggered by PHMG-p (Jeong MH et al, 2021). The mechanisms of possible impact PHMGH on cells have been and still are under active investigation (NB: citations not relevant, action on micro-organisms, please delete; Paliienko KO et al, 2019; Kamenieva TM et al, 2020; Sowlati-Hashjin S et al, 2020; NB: citation not relevant, spread of e-DNA, gene transfer microorganisms; Song J et al, 2022; Lee H et al, 2022).

PHMG is also known for its anti-inflammatory and wound-healing properties and to act as an anti-oxidant, so it was found to be useful in the treatment of chronic wounds and thermal burns (Lebedeva et al, 2017; Han et al, 2019; Kamenieva TM et al, 2020; Dias FGG et al, 2021), PHMBH is also known (Gray et al, 2010; Paydar et al, 2017; Oluwole et al, 2022).

Our previous experiments demonstrated that relative non-toxic concentrations of PHMGH for the already formed monolayer of cattle kidney cells (not causing the destruction of the monolayer) were from 10^{-4}% (1.0 μg/ml) and lower (Kryvoshyya P et al, 2021), for other types of cells, non-toxic concentrations of PHMGH were usually from 10^{-5}% (0.1 μg/ml) and lower (Mandygra MS and Lysytsya AV, 2014). It should be considered that, for instance, in bacteria, the suspension cultures are one order more sensitive to PHMGH than bacteria in biofilms (Moshynets OV et al, 2022). Thus, the suspension of eukaryotic cells may be more sensitive than the already formed monolayer of cells.

The impact of PHMGH on eukaryotic cells, however, is ambiguous, which, in our opinion, is explained due to the fact that the main target of this compound is the cytoplasmic membrane and the endoplasmatic reticulum. Since the membrane structures of prokaryotes and eukaryotes are considerably different, there are also differences in the impact of PHMGH on them (Paliienko KO et al, 2019; Lysytsya AV et al, 2022). It can also be assumed that other biologically active compounds, such as essential oils of medicinal plants (Park CM et al, 2022) or ZnO nanoparticles by analogy with nanosilica (Zaychenko GV et al, 2019), will affect the biological activity of PHMGH and PHMBH.

Our study was aimed at investigating both toxic and stimulating impact of PHMGH and PHMBH on eukaryotic cells depending on the preparation concentration. *The object of the study*: erythrocytes of cattle and pigs, kidney cell cultures of calves and pigs. *The topic of the study:* hemolytic activity of PHMGH and PHMBH and their impact on the proliferation of kidney cells and the formation of a monolayer of cells, and the effect of some the biologically active compounds in combinations with PHMGH and PHMBH.

MATERIALS AND METHODS

Polyhexamethylene guanidine hydrochloride (PHMG) and polyhexamethylene biguanidine hydrochloride (PHMB) were synthesized at a private company "Termit" (Rivne, Ukraine) by polycondensation of hexamethylenediamine and dicyandiamide with the addition of ammonium chloride (Sinopharm Chemical Reagents Co. Ltd., Shanghai, China). The molecular weights of PHMG and PHMB polymers determined by the viscosity of PHMG-containing solutions exhibited their distribution within the range of about 1,000–2,000 Da (8–16 repeat units), PHMB – 500–7,000 Da. The estimates of kinematic and reduced viscosities were carried out by Ostwald viscometer VPZH-2 (LLC "Inlex-Kvadro", Kyiv, Ukraine) with a capillary diameter of 0.56 mm.

PHMGH was dissolved in a phosphate buffered saline (pH 7.4) and after filtration, the preparation solutions with the mass concentration from 10^{-5} % to 10^{-1} % (from 0.1 mg/l to 1 g/l) were used. It amounts to ≈ 7×10^{-7}–7×10^{-3} M/l, where one monomer is ≈141

Dalton. During the process of synthesis and purification, we divided PHMBH into two fractions: the main one, containing mostly low molecular monomers with an average molecular mass from 500 to 2,000 Da ($M_1 \approx$ 500–2,000 Da), since the mass of one monomer – ≈183 Da, then the maximal degree of polymerization is n = 11. The second one is a high molecular fraction with an average molecular mass of up to 7,000 Da ($M_2 \approx$ 2,000–7,000 Da); the degree of polymerization was within n = 11–38. In all experiments, except those with pig erythrocytes, we used the first main, low molecular fraction of PHMBH.

The following compounds, used in combination with PHMGH and PHMBH were used: nanoparticles of zinc oxide (ZnO) of ≈25 nm (produced according to Danilevskaya NB et al, 2018), and electrolyzed water – anolyte with Eh = –800 mV, pH 6.5–7.0 (produced according to Mandygra MS et al, 2020) on the "STEL-ANK-SUPER" device (Envirolyte Industries International Ltd., Tallinn, Estonia), glutaric aldehyde ($C_5H_8O_2$, *Glutaraldehyde* CAS RN: 111-30-8) (produced by Hebei Junyu Pharmaceutical Co., Ltd, Hebie, China) and pharmacopeial preparations of essential oils of medicinal herbs (EOMH): common pine (*Pinus sylvestris* L.), oil obtained from pine needles (produced by Frey + Lau GmbH, Germany), eucalyptus (*Eucalyptus globulus* Labill.), oil from young shoots (produced by Frey + Lau GmbH, Germany), orange (*Citrus sinensis* L.), cold-pressed oil from orange skin (produced by LLC CPC GREEN PHARM COSMETIC, Ukraine), horsemint (*Monarda didyma* L.), oil obtained from flowers by steam distillation (produced by AZ-M Aroma-Zone, France).

Experiments to study the impact of PHMGH and PHMBH with other biologically active substances on the formation of the monolayer (activation or inhibition) of cattle kidney cells: we mixed PHMGH and PHMBH in the concentrations of 10^{-5}% with EOMH, anolyte, and ZnO nanoparticles. The volumetric proportion of PHMGH (or PHMBH) with EOMH or ZnO nanoparticles was 50 : 1, and with anolyte – 1 : 1. The final concentrations of PHMBH or PHMGH were 10^{-5}%, in some cases – 10^{-4}%, i.e. those not manifesting even minimal bacteriostatic properties usually and 1,000–10,000 times lower than the concentrations used for disinfection (Lysytsya AV et al, 2015).

Sample #

1c – anolyte,
2c – PHMGH 0.0001 %,
3c – PHMBH 0.00001 %,
4c – PHMGH 0.00001 % + anolyte (the ratio is 1 : 1),
5c – PHMGH 0.00001 % + oil of horsemint (the ratio is 200 : 1),
6c – PHMGH 0.00001 % + oil of eucalyptus (the ratio is 200 : 1),
7c – PHMGH 0.00001 % + oil of orange (the ratio is 200 : 1),
8c – ZnO nanoparticles + oil of eucalyptus (the ratio is 100 : 1),
9c – PHMGH 0.00001 % + oil of pine (the ratio is 200 : 1),
10c – PHMBH 0.00001 % + oil of horsemint (the ratio is 200 : 1),
11c – anolyte + oil of pine (the ratio is 100 : 1),
12c – anolyte + ZnO nanoparticles + oil of eucalyptus (the ratios are 200 : 100 : 1),
13c – PHMGH 0.00001 % + ZnO nanoparticles + + anolyte + oil of pine (the ratios are 200 : 10 : 200 : 1),
14c – PHMGH 0.00001 % + anolyte + oil of pine (the ratios are 100 : 100 : 1).

Experiments to study the impact of PHMBH with other biologically active substances on the formation of the monolayer (activation or inhibition) of pig kidney cells:

Sample #

1p – anolyte,
2p – PHMBH 0.0001 %,
3p – PHMBH 0.00001 %,
4p – PHMBH 0.00001 % + anolyte (the ratio is 1 : 1),
5p – PHMBH 0.00001 % + oil of horsemint (the ratio is 200 : 1),
6p – PHMBH 0.00001 % + oil of eucalyptus (the ratio is 200 : 1),
7p – PHMBH 0.00001 % + oil of orange (the ratio is 200 : 1),
8p – glutaric aldehyde 0.1 %,
9p – PHMBH 0.0001 % + glutaric aldehyde (the ratio is 100 : 1),
10p – PHMBH 0.00001 % + anolyte + oil of pine (the ratios are 100 : 100 : 1),
11p – PHMBH 0.00001 % + oil of pine (the ratio is 200 : 1),
12p – PHMBH 0.00001 % + ZnO nanoparticles + + anolyte +oil of pine (the ratios are 200 : 10 : 200 : 1),
13p – PHMBH 0.00001 % + ZnO nanoparticles (the ratios are 20 : 1),
14p – PHMBH 0.0001 % + glutaric aldehyde + oil of pine (the ratios are 200 : 2 : 1).

Preparation of erythrocytes and hemolytic effect of PHMGH and PHMBH on cattle and pig erythrocytes.

Table 1. The ratio between the concentration of erythrocytes in the sample and the degree of their dilution (titer)

The dilution of erythrocytes (titer)					
1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 :64
The quantity of erythrocytes in 1 ml					
$\approx 8 \times 10^6$	4×10^6	2×10^6	1×10^6	5×10^5	2.5×10^5

To obtain erythrocytes blood samples of cattle (*Bos taurus taurus* L., 1758) and domestic pigs (*Sus domesticus* Erxleben, 1777) were defibrinated according to Wilson JD et al, 1968. Prior to determining the hemolytic titer of PHMGH, PHMBH and mixtures with biological active compounds described earlier, the erythrocyte samples were washed thrice (by centrifugation for 10 min at 2,000 g) using phosphate buffered saline (1M PBS, 0.9 % NaCl, pH 7.4).

Erythrocyte counting was performed using a binocular microscope XS-5520 LED (MICROmed, Poltava, Ukraine) at 100x magnification and a hemocytometer (Gorjaev's chamber, EximLab, China). The erythrocyte samples were mixed 1 : 1 with PBS, and counted (Batra S, 2018).

The hemolytic titer under different concentrations of cattle and pig erythrocytes, and doses of disinfectants was determined by multiple titration of the concentrate of erythrocytes in 72-well polystyrene microplates (Fisher Scientific, USA), starting with 1 : 2 to 1 : 64. The same dose of 0.1 ml of disinfectants was added to all wells with diluted erythrocytes (PHMG hydrochloride or PHMB hydrochloride in PBS) in the concentration of 1.0 %. Thus, the dilution of disinfectants with erythrocytes was 1 : 10 (Table 1).

Control erythrocytes to check their ability to spontaneous hemolysis were made in parallel without the addition of drugs with PHMGH or PHMBH. A plate was carefully shaken and left at room temperature (20 ± 1°C) for 24 h. The hemolytic titer was defined visually as the highest dilution of erythrocytes when clear hemolysis of erythrocytes was observed. The concentrate of pig erythrocytes was prepared similarly to the concentrate of cattle erythrocytes. Each experiment was conducted in four repeats.*Preparation of fetal kidney cell cultures and measuring effects of disinfectants.* The initial cell cultures from kidneys of calves and domestic pigs were obtained by methods described in Golubev DB et al, 1976; Adams R, 1983; Freshni R, 1989; Sergeyev VA and Sobko YA, 1990. The principles of biological ethics were followed according to (Beauchamp TL and Childress JF, 1979). Organ samples were selected in slaughterhouses and meat-processing enterprises. The cells from embryonic calve and piglet kidneys were sown in the concentration of 3–4 × 10^5 cells per 1 ml according to the abovementioned recommendations. The *concentration* of cells in the culture medium was determined using a photocolorimeter KFK-3 (Zagorsk Optical and Mechanical Plant, Russia) according to Margis R and Borojevic R, 1989. During the studies, nutrient media were used: Dulbecco's modified Eagle's Medium (DMEM, D 5468, Sigma), 199 medium (M 5017, Sigma) and enriched Eagle's minimal medium (MEM, M3024, Sigma). We used a mixture of DMEM and 199 media in a 1 : 1 ratio with the addition of 10 % cattle blood serum («Sangva», Lviv, Ukraine). The subcultures were obtained by re-sowing the cells 3–8 days later with the formation of the monolayer of cells. The monolayer was suspended and diluted in Versene solution (BIOWEST, France, L-0630-100) with trypsin («Biopharma», Kyiv, Ukraine) in the 3:1 ratio. The viability of cells was determined by staining them with 0.2 % solution of trypan blue («Bio-Rad», USA, #1450021) and observation under a binocular microscope XS-5520 LED («MICROmed», Poltava, Ukraine). The corresponding concentration of cells from 0.5 × 10^5 to 12 × 10^5 in 1 ml was added in the volume of 2 ml per each tube for the formation of the monolayer. Then 0.2 ml of the relevant preparation or mixtures under investigation was added i.e. the dilution of the experimental sample was 1:10.

RESULTS

The effect of PHMGH and PHMBH and their compositions with anolyte and essential oils of medicinal herbs (EOMH) on cattle and pig erythrocytes. Using cattle erythrocytes, it was found that the samples with PHMBH demonstrated higher (on average 2.5-3x higher) hemolytic activity than those with PHMGH in a similar concentration (table 2). The pig erythrocytes

were found to be more stable to the impact of PHMGH and PHMBH. The preparations, diluted PBS with the addition of anolyte in the ratio of 1 : 1, demonstrated somewhat higher hemolytic activity. The final concentrations of PHMBH and PHMGH were 0.1 % i.e. the ones commonly used for disinfection. Table 2 presents the titers of erythrocytes when the introduced dose (standard 0.1 % concentration) of the preparation has already acted i.e. induced the hemolysis of blood cells.

PHMB samples with different molecular mass were also tested in the experiments with hemolysis of pig erythrocytes. Higher hemolytic activity (and thus toxicity) demonstrated the main sample with the prevailing low molecular oligomers with an average molecular mass up to 2 kDa ($M_1 \approx 500$–$2{,}000$ Da). The high molecular fraction of PHMB ($M_2 \approx 2{,}000$–$7{,}000$ Da) was found to be less toxic (≈ in 2.4x) regarding pig erythrocytes.

The impact on the formation of the monolayer of cattle kidney cells. The results of measuring the length of the monolayer being formed in the experimental samples after the completion of the formation of the cell monolayer in the control samples is summarized in Table 3. The observation time was 96 h.

The results demonstrated that the samples containing PHMBH (sample #3 c) and compositions of PHMBH or PHMGH with essential oils of medicinal plants (# 5c, 6c, 7c, 9c, 10c), stimulate the proliferation and development of a monolayer of cattle kidney cells. PHMGH in the concentration of 10^{-4}% (# 2c) had a negative impact on the proliferation of cells and the development of the monolayer. All the compositions containing anolyte had either negative (# 1c, 11c, 12c) or neutral (# 4c, 13c, 14c) impact on the formation of the monolayer of cells. In samples # 13c and 14c, it may be the result of the mitigating effect of the essential oil of pine.

The impact on the formation of the monolayer of pig kidney cells. Similar studies were conducted to determine the effect of experimental compositions containing PHMB and other biologically active substances on the formation of the monolayer of pig kidney cells (Table 4). To check the possibilities of enhancing the biocidal properties of PHMB, some samples (# 8p, 9p, 14p) were added glutaric aldehyde in the concentrations of 0.01–0.1 %.

The results demonstrate that PHMBH in the concentration of 0.00001 % (sample # 3p) stimulates the formation of the monolayer of cells. Even its compositions with anolyte (# 4 p, 10p, 12p) have a positive impact on the proliferation of cells. Good results were also demonstrated by the mixtures of PHMBH with oil of pine (# 11p) and ZnO nanoparticles (#13p).

The development of the monolayer was inhibited by the samples with glutaric aldehyde (#8p, 9p, 14p), which was another confirmation of its biocidal properties and may be used as a substantiation of the reasonability of producing disinfectants containing both PHMGH (or PHMBH) and glutaric aldehyde. In sam-

Table 2. The comparative hemolytic activity of PHMGH and PHMBH regarding cattle and pig erythrocytes, n = 4

Experiment No.	PHMGH 0.1 %	PHMGH 0.1 % in anolyte	PHMBH 0.1 % ($M_1 \leq$ 2,000 Da)	PHMBH 0.1 % ($M_1 \leq$ 2,000 Da)	PHMBH 0.1 % ($M_2 \approx$ ≈ 2,000–7,000 Da)	PHMBH 0.1% in anolyte ($M_2 \approx$ ≈ 2,000–7,000 Da)	Control, 0.1M PBS, pH 7.4
	Titer (concentration) of cattle erythrocytes, which enables their complete hemolysis			Titer (concentration) of pig erythrocytes, which enables their complete hemolysis			Titer of cattle and pig erythrocytes to reveal spontaneous hemolysis
1	1 : 8	1 : 4	1 : 2	1 : 32	1 : 32	1 : 32	–
2	1 : 8	1 : 4	1 : 4	1 : 16	1 : 64	1 : 16	1 : 64
3	1 : 8	1 : 8	1 : 2	1 : 16	1 : 16	1 : 16	–
4	1 : 4	1 : 8	1 : 2	1 : 8	1 : 64	1 : 16	–
average value*	1 : 7	1 : 6	1 : 2.5	1 : 18	1 : 44	1 : 20	spontaneous hemolysis of erythrocytes is practically absent

Note. * the estimated average value of the titer is presented only to facilitate the comparison.

ple # 14p, even the introduction of the essential oil of pine did not mitigate the toxic effect of the composition of PHMBH with glutaric aldehyde.

Contrary to the experiments with cattle cells, the pig cell samples treated with PHMGH with EOMH of horsemint, eucalyptus, orange (# 5p, 6p, 7p) did not have a considerable impact on the growth of the monolayer. PHMBH in the concentration of 0.0001 % (# 2p) and anolyte (# 1p) did not have any considerable impact on the rate of the formation of the monolayer of pig kidney cells either.

DISCUSSION

The results of our orientation study on the resistance of cattle and pig erythrocytes to the PHMBH demonstrated that cattle erythrocytes were found less stable (in ≈7x) to hemolysis as compared to pig erythrocytes (Table 2). Though pig erythrocytes were more resistant to hemolysis, there were manifestations of the phenom-

Table 3. The impact of PHMGH and PHMBH with other biologically active substances on the formation of the monolayer (activation or inhibition) of cattle kidney cells (*Bos taurus taurus* Linnaeus, 1758), n = 4

The initial concentration of cells in 1 ml	Number of an experimental sample														Control cultures
	1c	2c	3c	4c	5c	6c	7c	8c	9c	10c	11c	12c	13c	14c	
	The length of the monolayer of cattle kidney cells in the test tube (*in vitro*), cm*														
	1st series of experiments														
12×10^5	0	0	9	6	10	9	9	7	9	10	6	5	6	7	7
6×10^5	0	0	8	4	9	7	8	6	8	9	4	4	6	5	6
3×10^5	0	0	6	3	8	5	6	6	5	6	2	2	5	4	3
1×10^5	0	0	4	2	4	2	4	2	3	4	0	0	1	2	1
0.5×10^5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2nd series of experiments															
12×10^5	0	0	10	7	11	10	12	8	10	11	5	4	9	8	8
6×10^5	0	0	9	6	9	9	10	6	9	10	3	4	6	6	7
3×10^5	0	0	7	4	8	6	8	5	6	7	0	3	3	5	4
1×10^5	0	0	4	2	6	3	4	2	4	3	0	0	2	1	2
0.5×10^5	0	0	0	0	3	0	2	0	0	0	0	0	0	0	0
3rd series of experiments															
12×10^5	0	0	9	7	10	9	10	5	8	9	4	4	5	6	5
6×10^5	0	0	8	3	8	7	7	5	7	8	3	2	4	3	4
3×10^5	0	0	6	1	8	6	5	2	5	7	1	1	3	1	2
1×10^5	0	0	2	0	5	2	3	0	2	2	0	0	0	2	0
0.5×10^5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4th series of experiments															
12×10^5	0	0	9	6	10	9	11	7	9	10	4	5	8	7	7
6×10^5	0	0	8	5	8	8	8	4	8	7	2	3	4	5	5
3×10^5	0	0	6	3	7	5	7	3	5	5	2	1	4	3	4
1×10^5	0	0	4	2	5	3	2	2	2	2	0	0	1	1	1
0.5×10^5	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0
Effect**	–	–	+	0	+	+	+	0	+	+	–	–	0	0	
The averaged relative % of increase (or decrease)	–100	–100	+65		+95 (without the last concentration)	+52	+73 (without the last concentration)		+67	+67	–45	–44			

Note. * – the deviation in measuring the monolayer sizes ± 0.5 cm. ** – the negative impact on the formation of the cell monolayer, + positive, 0 neutral.

enon of the agglutination of erythrocytes which was not observed in cattle erythrocytes.

PHMBH was found to be more toxic (in ≈2.8x) regarding cattle erythrocytes than PHMGH. As for pig erythrocytes, it was found that the high molecular fraction of PHMBH (M_2 ≈ 2,000–7,000 Da) demonstrated lower toxicity (in ≈2.4x) than the main fraction, containing low molecular oligomers (M_1 ≤ 2,000 Da). It is noteworthy that in most cases, low molecular oligomers of PHMGH and PHMBH are synthesized and used in the composition of disinfectants. Thus, in our opinion, to decrease total toxicity for antiseptics and wound-healing means, it is reasonable to use more high molecular fractions of these polymers.

The solutions of PHMGH and PHMBH in anolyte demonstrated higher hemolytic activity than in PBS (table 2). Still the antioxidant activity of PHMGH (Kamenieva TM et al, 2020) in general raises doubts regard-

Table 4. The impact of PHMB compositions with other biologically active substances on the formation of the monolayer (activation or inhibition) of pig kidney cells (*Sus domesticus* Erxleben, 1777), n = 4

The initial concentration of cells in 1 ml	Number of an experimental sample														Control cultures
	1p	2p	3p	4p	5p	6p	7p	8p	9p	10p	11p	12p	13p	14p	
	The length of the monolayer of pig kidney cells in the test tube (*in vitro*), cm* 1st series of experiments														
12×10^5	10	9	11	10	9	8	9	0	0	11	11	10	10	0	9
6×10^5	7	7	10	10	8	8	7	0	0	10	11	9	10	0	8
3×10^5	4	6	8	7	5	6	5	0	0	7	8	7	8	0	5
1×10^5	2	2	4	4	1	2	3	0	0	4	5	4	5	0	2
0.5×10^5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2nd series of experiments															
12×10^5	9	9	11	10	9	10	9	0	0	0	10	11	10	0	10
6×10^5	8	7	10	10	8	9	8	0	0	0	9	10	10	0	8
3×10^5	7	6	8	8	8	7	5	0	0	0	7	6	7	0	5
1×10^5	2	3	6	5	3	3	3	0	0	0	4	5	4	0	3
0.5×10^5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3rd series of experiments															
12×10^5	9	10	11	10	9	10	10	0	0	11	11	10	11	0	10
6×10^5	8	9	10	10	8	9	9	0	0	10	9	9	10	0	9
3×10^5	8	5	9	8	8	7	6	0	0	8	7	9	9	0	6
1×10^5	4	4	5	5	3	3	4	0	0	5	5	5	6	0	3
0.5×10^5	0	0	1	0	0	0	0	0	0	0	1	0	1	0	1
4th series of experiments															
12×10^5	10	9	11	11	9	9	10	0	0	11	11	11	10	0	9
6×10^5	8	8	9	10	8	8	8	0	0	10	9	10	10	0	9
3×10^5	7	8	9	9	8	7	8	0	0	9	8	8	9	0	7
1×10^5	4	4	5	6	3	5	3	0	0	5	5	6	5	0	4
0.5×10^5	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
Effect**	0	0	+	+	0	0	0	–	–	+	+	+	+	–	
The averaged relative % of increase (or decrease)			+27	+21				–100	–100	+24	+20	+20	+25	–100	

Note. * the deviation in measuring the monolayer sizes ± 0.5 cm. ** – the negative impact on the formation of the cell monolayer, + positive, 0 neutral.

ing the reasonability of its combination with anolyte in one disinfectant. The shelf life of this disinfectant will be very limited, probably, free active radicals in the anolyte will quickly lose their bactericidal properties under the influence of PHMGH, it increases the general mineralization of solutions. The active components in the anolyte are peroxide compounds ($HO^{\bullet}$, HO_2^-, $^1O_2$, O_2^-, O_3, $O^{\bullet}$) and chlorine-oxygen compounds (HClO, ClO^-, $ClO^{\bullet}$, ClO_2) are neutralized (Mandygra MS et al, 2020).

The experiments on the cell cultures of calf kidneys demonstrated that the lowest toxicity and the best growth-stimulating effect were shown by PHMGH compositions in the concentration of 0.00001 % with EOMH (Table 3); these are samples # 5c, 6c, 7c, 9c (from 52 to 95 % increase). Samples #3c and 10c demonstrate that PHMBH has a positive impact on the proliferation of cells both independently and in combination with the oil of horsemint (which has its own specific biocidal properties) in the concentration selected by us (65–67 % increase). PHMGH samples with anolyte (# 1c, 11c, 12c etc.) have a cytotoxic effect (full or to 45 % decrease). Similarly, this combination is not suitable for growth stimulation; the results of the impact on the growth of the monolayer as is clear from samples #4c, 13c, 14c.

The experiments on the pig kidney cell cultures demonstrated that PHMBH in the concentration of 0.00001 % (sample # 3p) had a positive impact on the formation of the monolayer of cells (27 % increase). It is also noteworthy that the composition of PHMB with anolyte and oil of pine (#10p) has a good stimulating effect (24 % increase), a little better than separate compositions of PHMB + anolyte (#4p) (21 % increase) or PHMB + oil of pine (#11p) (20 % increase). As for ZnO nanoparticles, the result is ambiguous, but it seems that it may somewhat enhance the stimulating effect of PHMB on the growth of the monolayer (#13p) (25 % increase). As expected, anolyte (#1p) demonstrated its inhibiting effect, but in the composition with PHMB (#4p) it had a contrary stimulating action, similar to samples #10p and 12p (from 20 to 24 % increase). The samples with glutaric aldehyde (#8p, 9p, 14p) inhibit the development of the monolayer of cells (full decrease). Thus, in principal, the disinfecting (biocidal) preparations of PHMB with glutaric aldehyde could be possible.

It should also be noted that in general, pig kidney cells were found to be less sensitive to the impact of the investigated preparations than kidney cells of calves (similarly for erythrocytes). The PHMBH has not worse stimulating effect on the proliferation activity and monolayer growth than PHMGH in similar concentrations. Both PHMGH and PHMBH have been suggested as possible candidates to be included in growth stimulators or wound-healing medicines (Kamenieva TM et al, 2020; Dias FGG et al, 2021).

The observation of the development of cattle kidney cell monolayer confirmed the assumption that the addition of the essential oils of medicinal plants used in our study to PHMGH decreased its toxicity and accelerated the proliferation of cells i.e. it might enhance wound-healing properties of PHMGH. It does not contradict the data of other authors regarding pycnogenol (French maritime pine bark extract) (Park CM et al, 2022). It may be used while developing new medical means for antiseptics and for the acceleration of wound healing, for instance, antiseptic bandages and plasters. In a study comparing PHMGH with some traditional disinfecting means demonstrated that it is actually rather toxic as compared to ethyl alcohol or even glutaric aldehyde (Kryvoshyya P et al, 2021). So, its combination with EOMH to decrease the toxicity could be promising.

While developing veterinary means to improve wound healing, it is necessary to select optimal (compromise) concentrations that would have bacteriostatic properties and promote the division (renewal) of cells on damaged parts of the skin at the same time. Based on our study these may be PHMGH concentrations of 0.005–0.5 %.

Considering that apart from disinfecting properties, PHMG salts have the potential ability to accelerate the proliferation of cells and heal wounds, we have developed and tested an antiseptic topical preparation, Epidez-plaster. One of the main active substances of the plaster on a hydrogel basis is PHMG succinate. The preparation belongs to the group of topical antiseptic dressing means and may be used both in treatment and prophylaxis institutions and in household or field conditions for external application as a medicinal means for the treatment of burns (thermal, chemical) and wounds (open, post-surgical, trophic), dermatitis, eczema, and skin necrosis. The laboratory investigations and preliminary industrial testing were done in the veterinary clinic and testing laboratory of the Experimental station of epizootiology at the Institute of Veterinary Medicine, NAAS of Ukraine. Further on, we plan to optimize the composition of active and supplementary substances and continue the studies according to the

approved schemes of testing veterinary preparations (Kotsyumbas IYa et al, 2013.)

As for the capability of PHMGH to induce the damage to breathing organs under long-term inhalation, including lung fibrosis (Kim HR et al, 2016; Kang MS et al, 2022), one of the reasons may be found in the possible use of PHMGH preparations, containing low molecular oligomers (polymerization degree n ≈ from 3 to 7, the molecular mass did not exceed 1,000 Da) in air humidifiers (Park DU et al, 2021). Usually, this poly-cation is used in disinfectants with PHMGH with oligomers, the molecular mass of which fluctuates in rather a wide range from several hundred to several thousands of Da. But this question needs further study. Hemolytic activity of PHMBH with a higher molecular weight regarding pig erythrocytes is 2.4x less than PHMBH with short oligomeric chains (Table 2).

The perspectives of internal use of PHMG (or PHMB) (orally or by inhaling) are problematic. Yet, since PHMGH still can penetrate inside the organism and get distributed unevenly, with possibly different effects, in different tissues and organs (Mushtaq S et al, 2022), in the future, we plan on conducting similar studies using cell cultures of other tissues and organs of animals.

It should also be noted that our earlier studies indicated the possibility of using PHMGH not only in veterinary medicine and animal breeding but also in plant cultivation, for instance, to to stimulate seed germination and germination energy (Lysytsya A et al, 2013; Lyoshyna L et al, 2020).

CONCLUSIONS

Depending on the dose (concentration), PHMGH and PHMBH preparations cause a biocidal effect (lysis of cells) and excert a cell proliferative stimulation activity. The PHMGH and PHMBH in the concentrations of 0.1 % (similar concentrations are usually used for disinfection (Lysytsya AV et al, 2015; Mashat BH, 2016; Oule MK et al, 2017)) trigger the hemolysis of cattle erythrocytes in titers (in an average way) – 1 : 7 for PHMG, and 1 : 2.5 for PHMB. PHMB shows greater hemolytic activity. The pig erythrocytes were more resistant to the action of the preparation, the high molecular weight fraction of PGMB (M_2 ≈ 2,000–7,000 Da) demonstrated a lower hemolytic activity (the averaged titer 1 : 44) than the low molecular weight basic fraction (M_1 ≈ 500–2000 Da), where the averaged titer was 1 : 18.

The experiments on the kidney cell cultures of cattle and pigs showed that at non-toxic concentrations (10^{-5}% and below) PHMGH and PHMBH could effectively stimulate the proliferative activity of eukaryotic cells. It indicates their wound-healing properties and therefore they could be included in disinfectants, antiseptics, and stimulators of wound healing. It is noteworthy that during the formation of the monolayer of kidney cells of calves, the combinations of PHMGH with essential oils of medicinal plants accelerated the rate of cell proliferation (from 52 to 95 % increase). It was observed that in addition to healing properties of some plant essential oils, the latter can also decrease the toxicity of PHMGH.

Therefore, PHMGH and PHMBH can possibly be used in the development of wound-healing means to treat wounds of different origins, including burns, in veterinary medicine. For instance, they can be used in the composition of ointments, gels, bandages, or plasters in the concentrations of 0.005–0.5 %.

While developing new disinfectants, it is possible to combine PHMGH with anolyte, but due to the quick anolyte deactivation during long-term storage, active radicals such as the peroxide compounds and chlorine-oxygen compounds are neutralized. It may also be possible to combine PHMGH with glutaric aldehyde, which will enhance the bactericidal properties of the preparation but in our hands also increased its toxicity for animals.

In cases where PHMG or PHMB would still be allowed to be used in air humidifiers, it is imperative to decrease the risk of damaging breathing pathways, including the development of lung fibrosis to use these poly-cations with a higher degree of polymerization and larger average masses of oligomers, for instance, several thousand Da.

Adherence to ethical principles. All the experiments, described in this paper, did not involve animals.

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Вплив полігексаметиленгуанідину (ПГМГ) і полігексаметиленбігуанідину (ПГМБ) самостійно та у поєднанні з рослинними ефірними оліями та наночастинками ZnO на деякі еукаріотичні клітини великої рогатої худоби та свиней

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Мета. Дослідити як токсичну (гемолітичну), так і стимулюючу дію двох полімерних похідних гуанідину, зокрема полігексаметиленгуанідину (ПГМГ) та полігексаметиленбігуанідину (ПГМБ) обох у формі гідрохлориду, на еукаріотичні клітини залежно від концентрації препарату; а також вивчити можливість використання ранозагоювальних стимулюючих властивостей цих препаратів у ветеринарній медицині. **Методи**. Методом титрування визначали гемолітичну активність (токсичність) препаратів ПГМГ і ПГМБ у концентрації 0,1 % щодо еритроцитів великої рогатої худоби та свиней. Для визначення стимулюючого впливу цих препаратів брали первинні клітинні культури клітин фетальних нирок телят і поросят. ПГМГ та ПГМБГ брали як окремо, так і в комбінації з такими біологічно активними речовинами: ефірні олії – *Pinus sylvestris*, *Eucalyptus globulus*, *Citrus sinensis*, *Monarda didyma*, наночастинки ZnO (розмір бл. 25 нм), і електрохімічно активовану воду – аноліт (Eh = –800 мВ, pH 6,5–7,0). Концентрацію клітин у живильному середовищі визначали методом фотоколориметрії. **Результати**. З'ясовано, що залежно від концентрації препарати ПГМГ та ПГМБ можуть викликати як лізис клітин, зокрема еритроцитів, так і стимулювати їхню проліферативну активність, зо-крема формування моношару клітин нирки теляти і свині. В концентраціях, які зазвичай використовують для дезінфекції, тобто близько 0,1 % полімерні похідні гуанідину викликають гемоліз еритроцитів ВРХ у титрах (усереднено) для ПГМГ 1 : 7, а для ПГМБ 1 : 2,5. Тобто, ПГМБ проявляє більшу гемолітичну (біоцидну) активність ніж ПГМГ (приблизно у 2,8 рази). Еритроцити свині виявилися стійкішими до його дії, при цьому високомолекулярна фракція ПГМБ (M_2 ≈ ≈ 2000–7000 Da) показала нижчу гемолітичну активність, ніж низькомолекулярна основна фракція (M_1 ≈ 500–2000 Da) (приблизно у 2,4 рази). Досліди на культурах клітин нирки ВРХ і свині довели, що за нетоксичних концентрацій (10^{-5}%) полімерні похідні гуанідину можуть ефективно стимулювати проліферативну активність клітин еукаріот і прискорювати формування моношару клітин. Комбінації ПГМГ з деякими ефірними оліями лікарських рослин показують також хороший ефект (збільшення від 52 до 95 %), аналогічно і ПГМБ проявив стимулюючу дію з олією сосни для клітин нирок свиней (збільшення на 20 %) та олією монарди для клітин нирок великої рогатої худоби (збільшення на 67 %). **Висновки**. Отже, ПГМГ і ПГМБ можуть використовуватися в сільськогосподарському виробництві не тільки як дезінфікуючі або антисептичні засоби, а й для загоєння ран. Хоча їхня токсичність також значна для еукаріотичних клітин, все ж їх можливо застосовувати у ветеринарній медицині в невисоких концентраціях (0,005–0,5 %) для лікування ран різного походження, в тому числі опіків, наприклад у складі мазей, гелів, пов'язок або пластирів.

Ключові слова: полігексаметиленгуанідин, олії лікарських рослин, культура клітин, дезінфекція, токсичність, стимулювання росту.

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