ACTA MEDICA LITUANICA. 2009. Vol. 16. No. 3–4. P. 92–97
DOI: 10.2478/v10140-009-0013-9

Cytotoxicity of pharmaceutical and cosmetic gel-forming polymers, preservatives and glycerol to primary murine cell cultures

Aida Vaitkuvienė,

Vytautas Kašėta,

Giedrė Ramanauskaitė,

Genė Biziulevičienė

Laboratory of Immunopharmacology,
Institute of Immunology,
Vilnius University,
Vilnius, Lithuania

Introduction. In the development of regenerative medicine, an important role is designated to the search of nontoxic and biocompatible compounds to be used in cell treatment of skin injuries. We assume that compounds used in pharmaceutical and cosmetic industries, such as Natrosol 250 MR, Stabileze QM, Carbopol ultrez 20, sodium azide, thimerosal and glycerol, could be applied in cell technologies as well.

Materials and methods. The cytotoxicity of the compounds tested in our study was assessed by estimating spleen and bone marrow cell viability in light microscopy, using the trypan blue exclusion test.

Results. After treatment of primary cell cultures with Natrosol 250 MR, Stabileze QM, Carbopol ultrez 20, sodium azide and glycerol, the viability of cells was found to be decreased by 1–8%, while thimerosal caused death in 71–74% of these cells.

Conclusions. Our studies revealed that these gel-forming polymers are not toxic to bone marrow and spleen cells. They may slightly, if at all, influence the viability of cells. Furthermore, the use of the preservative sodium azide and glycerol in the development of cell culture technologies was proven to be quite acceptable. However, thimerosal cannot be applied in these technologies because of its high toxicity.

Key words: cytotoxicity, gel-forming polymers, mouse bone marrow and spleen cells

INTRODUCTION

The development of regenerative medicine, namely the treatment of skin injuries by using cells, made evident and necessary the search of non-toxic and biocompatible compounds. A potential source of such substances could be the gel-forming polymers that are already successfully applied by the pharmaceutical industry and cosmetics. Stabileze QM, Natrosol 250 MR and Carbopol ultrez 20 have become an issue for certain therapeutic products associated with cell technologies. As efficient stabilizers and thickeners, they are incorporated in the products and make 0.2% to 4.0%.

Natrosol 250 MR (hydroxyethylcellulose) as an additional component is found in the content of vitamins, drugs and cosmetics. This polymer is used in food industry, as well as in the production of dyes, glues, industrial coatings and even in the restoration of documents.

Correspondence to: Aida Vaitkuvienė, Laboratory of Immunopharmacology, Institute of Immunology, Vilnius University, Molėtų plentas 29, LT-08409 Vilnius, Lithuania. E-mail: aidavait@gmail.com

Stabileze QM (methylvinylether / maleic anhydride copolymer) could be found in gels, creams, lotions, hair and skin treatment formulas.

The acryl acid copolymer Carbopol ultrez 20 is used in pharmaceutical compositions (gels, healing plasters) and body care products. However, tests have proven its slightly toxic effect.

Sodium azide is a common preservative often used to store laboratory reagents and other biological liquids. It is a very toxic, mutagenic and potentially cancerogenic compound applied as a biocide. Exposure to sodium azide may cause hypotension, bradicardia and headache (1).

Thimerosal, a mercury-containing compound, is reported to have antimicrobial and antifungal activity. It is used as a preservative in biological products and vaccines at a concentration ranging from 0.003% to 0.1% (30–100 μg/ml) (2). An association between the use of thimerosal-containing vaccines and the disorders of the nervous system has been described. However, to prove this statement, additional investigations are necessary (3). Besides, it has also been noted that cosmetic products containing thimerosal may cause allergic reactions (4).

Still another compound used in pharmacy and food industry is glycerol. It is included in the composition of soaps, detergents, pesticides and other products. Data in the literature show its harmful effect on sensitizing eyes and skin, as well as being slightly toxic. Although this compound is neither cancerogenic nor does it express teratogenic properties, it may induce acute renal failure (5).

General requirements for the production of cosmetics and pharmaceuticals include the safety of the compounds used, but there is only scarce data on the toxicity of the above-described substances. Even less is known about their effect on cell cultures. Routine cytotoxicity tests are performed *in vitro* using cell lines, although primary cell cultures (which were used in our investigation) have been proven to be more sensitive to the influence of the toxic compounds (6). In this study, we examined the effect of the above-described compounds on primary murine cell cultures. We failed to find any data on similar investigations of the cytotoxicity of the compounds under study.

MATERIALS AND METHODS

Isolation of mouse bone marrow cells

Isolation of mouse bone marrow cells was performed as described by Joupperi (7) with some modifications. BALB/c mice, 6–8 weeks old, were sacrificed by cervical dislocation. Their femurs and tibias were removed aseptically and placed on a Petri dish containing Hanks' balanced salt solution (HBSS). Bone marrow was obtained by flushing with sterile HBSS through one of the femoral epiphyses, using a syringe needle (27-gauge). The bone marrow cells were collected in sterile HBSS and washed three times by centrifugation for 6 min at 300 g. Mononuclear cells were obtained by separation of mouse bone marrow cells in Ficoll–Paque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) density gradient; 3 ml aliquot of Ficoll–Paque was placed into a plastic tube, and the same amount of bone marrow cell suspension in RPMI was layered on the top. This was centrifuged for 40 min at 400 g. The cell fraction harvested from the interface between these two media was washed immediately and suspended in HBSS.

Isolation of mouse spleen cells

BALB/c mice were sacrificed by cervical dislocation. Spleens were removed aseptically and placed on a Petri dish containing 3 ml HBSS. Spleens were perfused through one cutting end. Subsequently, the spleen cells were flushed into a Petri dish. Cells and fragments were dispersed by repeated pipetting. In order to remove stroma residual, the cells were passed through a mesh. Spleen cells were washed three times by centrifugation for 6 min at 300 g. Mononuclear cells were obtained by the same procedure as in the case of bone marrow cells.

Evaluation of cell concentration

Cells were counted using a hemocytometer according to equation: $X = a \times b \times 10^4$, where X is the number of cells per ml, a is the mean number of cells in the one square, and b is the dilution rate.

Preparation of test compouds

0.5, 1, 3 and 5% solutions of the test polymers were prepared. The compound-forming gels were dispensed in deionized water. If needed, neutralization was performed with sodium hydroxide. To accelerate the process, the solutions were stired and heated in a water bath (to 80 °C). Besides, 0.1, 0.01 and 0.003% solutions of glycerol, sodium azide and thimerosal were prepared. About 500 µl gel and the test compound solutions were added into a plate with 48 wells. All experiments were performed at least in triplicate.

Cell cultivation and viability evaluation

Bone marrow and spleen mononuclear cell suspensions (2×10^7 cell/ml) were prepared in RPMI medium supplemented with 10% fetal bovine serum; 400 µl of each suspension was added on the top of gels tested. The other compounds (glycerol, thimerosal and sodium azide) were stired with 400 µl of cell suspension. After 4 h of incubation in a CO_2 termostat at 37 °C, cell viability was tested. To determine the percentage of viable cells in a population, the cell suspension was mixed with trypan blue. The percentage of viable cells was counted and compared with the control group in which cell viability was equaled to 100%.

Statistical analysis

Statistical analysis was performed using Microsoft Excel (version 7.0). Differences between mean values were determined using Student's t test. Differences were considered significant at $p < 0.05$.

RESULTS

Mononuclear cells isolated from spleen and bone marrow were incubated for four hours in CO_2 thermostat in the presence of the compounds investigated and without them. After treatment of primary spleen cell cultures with polymer Natrosol 250 MR (hydroxyethylcellulose), the strongest effect was achieved at the polymer concentration of 1%. At this concentration the viability of cells decreased by 7%. However, low concentrations of this gel (0.5% and 0.25%) had almost no effect. The cell viability was approximately 98 and 96%, respectively. The results presented in Fig. 1 show an even lesser effect of Natrosol 250 MR on bone marrow cells. The cell viability ranged within 96–97% in the presence of this compound.

The effect of Stabileze QM on spleen and bone marrow cells was not significant, either (Fig. 2). The viability of spleen cells was decreased by 3–5% and of bone marrow cells by 3–6%.

A similar picture was noted while analyzing the effect of Carbopol ultrez 20 (acryl acid copolymer) on primary cell cultures (Fig. 3). The viability of spleen cells treated with this

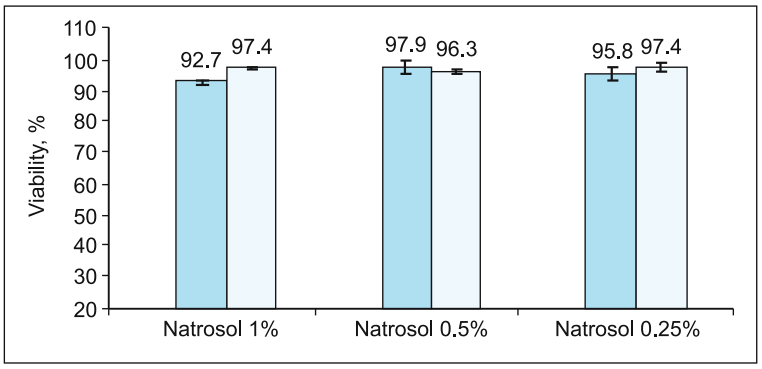

Fig. 1. The effect of Natrosol 250 MR on the viability of spleen (■) and bone marrow (□) cells

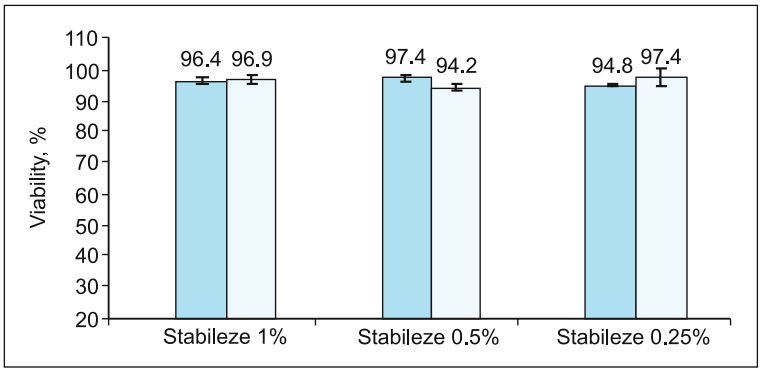

Fig. 2. The effect of Stabileze QM on the viability of spleen (■) and bone marrow (□) cells

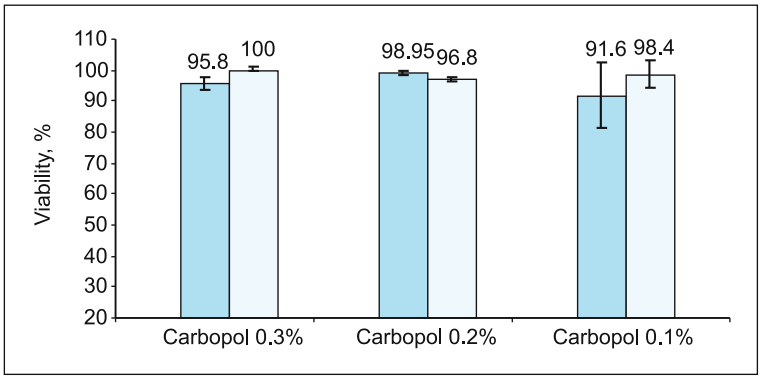

Fig. 3. The effect of Carbopol ultrez 20 on the viability of spleen (■) and bone marrow (□) cells

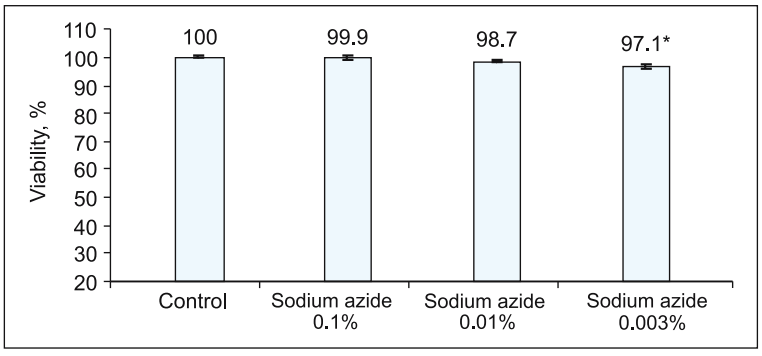

Fig. 4. The effect of sodium azide on the viability of spleen cells. * The difference between the control and the test group is statistically significant ($p < 0.05$)

copolymer ranged from 92% to 99%. The gel of this polymer decreased cell viability by 8%. The viability of bone marrow cells in the presence of Carbopol ultrez 20 reached 98–100%.

The cytotoxic effect of the preservative sodium azide on spleen cells was not significant, either. While decreasing its concentration, cell viability changed insignificantly. The lowest concentration of sodium azide (at 0.003%) had the greatest impact on cells. As is seen in Fig. 4, the viability of cells, as compared to the control group, was decreased only by 3% (at 0.003% concentration of sodium azide). However, this decrease was statistically significant.

Analysis of the effect of thimerosal on spleen cells revealed its toxicity. Of all the compounds studied, it showed the greatest impact on cells. Data on cell viability after treatment with thimerosal are given in Fig. 5. At the concentrations of 0.003, 0.01 and 0.1%, it significantly decreased and remained only at a level of 26–29%.

Assessment of glycerol cytotoxicity showed that higher amounts (10% and 5%) of this compound decreased cell viability by 8 and 6%, respectively, whereas low concentrations (1% and 0.01%) had no significant effect (Fig. 6).

DISCUSSION

Data in scientific literature indicate that products created on the basis of cellulose polymer are not toxic (8) or express only an average cytotoxicity (9, 10). Furthermore, such polymer as carboxymethylcellulose is used in preparing eye drops (artificial tears). Because of a higher gel viscosity it prolongs tear-retention time in the eye and even enhances its positive effect (11). Interacting with corneal cells, it facilitates the convalescence of injuries in corneal epithelium [12]. Our results show that Natrosol 250 MR only insignificantly influences the viability of cells and thus confirm the literature data on the nontoxicity and biocompatibility of cellulose compounds. However, maleic anhydride polymers were found to be cytotoxic and anti-tumorous (13). Because of their biodegrading adhesive properties these polymers are used in pharmacy. Besides, methylvinylether / maleic acid inhibits alkaline phosphatase in *Escherichia coli* (14). The

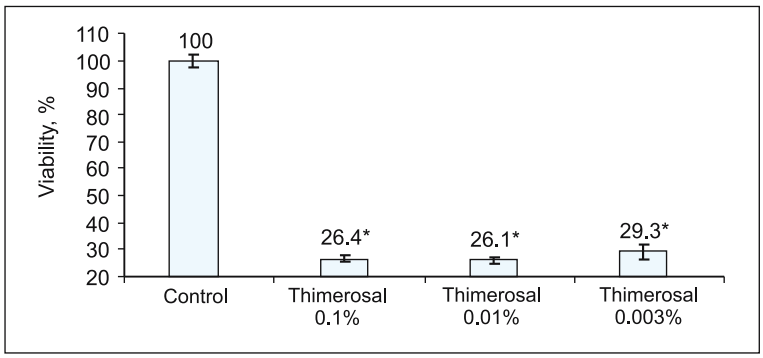

Fig. 5. The effect of thimerosal on the viability of spleen cells. * The difference between the control and the test groups is statistically significant ($p < 0.05$)

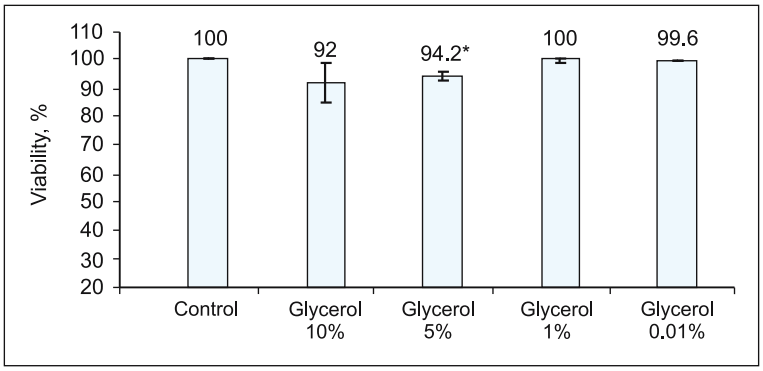

Fig. 6. The effect of glycerol on the viability of spleen cells. * The difference between the control and the test groups is statistically significant ($p < 0.05$)

Stabileze (methylvinylether / maleic acid) polymer included in the composition of pesticides prolongs the viability of the microorganisms (15) and effectively stabilizes bacterial cultures (16, 17). The effect of Stabileze QM on spleen and bone marrow mononuclear cells, as determined in our study, was not significant; however, according to other investigators, maleic anhydride polymers are cytotoxic (13). Our results show that low concentrations of this polymer do not exert a significant influence on the viability of cells.

Literature data on the toxicity of acryl acid copolymers describe their effect as slightly toxic and potentially irritating. Nevertheless, they are used as thickeners in the production of different pharmaceutical products (gels, healing plasters and body care products). Our tests revealed Carbopol ultrez 20 gels to show no cytotoxicity at the concentrations of 0.3, 0.2 and 0.1%.

From literature we know that the most commonly reported effects of sodium azide exposure are hypotension, bradicardia and headache (1). However, sodium azide combines with water to form the highly volatile hydrazoic acid. Both substances are known to produce hypotension in laboratory animals and humans because they form strong complexes with hemoglobin and block oxygen transport in the blood. The dose of ≤700 mg (10 mg/kg) is lethal for humans (18). On the cellular level, this compound blocks ATP production by inhibiting the breathing tract, i. e. blocking the activity of cytochrome oxidase (19). However, our results show that low concentrations of sodium azide are of low cytotoxicity, and therefore their influence on cell viability is not significant.

Analysis of the effect of thimerosal on spleen cells exhibited its toxicity. Other investigators revealed thimerosal to be toxic to different cell cultures as well. The negative effect of this compound on human neurons and fibroblasts has been reported. Micromole doses of thimerosal (1–250 μM) may damage cell membrane and DNR as well as initiate caspase-3-dependent apoptosis (20). It can also affect human osteosarcoma cells by increasing the concentration-dependent amount of free calcium in the citosol. At concentrations of 5, 10 and 20 μM, thimerosal can cause the death of 33, 55 and 100% of cells, respectively (21). Different human neuroblastoma cell lines while treated with thimerosal show a decrease in cell viability, depending on the dose of the compound and the time of exposure. It can also induce cell apoptosis and oncosis / necrosis (22, 23). Besides, mercury is reported to increase the production of reactive oxygen radicals and to decrease the content of thiols in the endothelium of blood cells, thus activating phospholipase D (24).

As regards glycerol, only higher amounts of this compound exhibited a weak cytotoxicity. Data in literature show that glycerol concentrations higher than 100 μM are not toxic to mammalian cells. Experiments *in vivo* proved that administering glycerol to mice *per os*, the LD_{50} is 4090 mg/kg, whereas when subcutaneously injected it is 91 mg/kg. Tests on rats showed that glycerol could induce the sensitivity to endotoxin (25). Besides, it may stabilize the membranes and inhibit the effect of exotoxins (26). Tests *in vivo* also revealed the subsidiary effect of glycerol in restoring the structure of heat-denaturated luciferase and protecting cells from heat-induced cytotoxicity (27). Our results are confirmed by literature data on a weak cytotoxic effect of glycerol on mammalian cells.

CONCLUSIONS

To summarize the results, we could assume that all gel-forming polymers at the given (tested) concentrations are not toxic to primary spleen and bone marrow mononuclear cells. They either do not influence or only slightly affect the viability of these cells and therefore could be used in the innovative technologies for preparing cell / gel samples. According to the literature data, the most effective is Natrosol 250 MR (hydroxyethylcellulose polymer) because of its well-expressed biocompatibility. The use of the preservative

sodium azide and glycerol in cell cultures is quite acceptable, too, because these compounds are not cytotoxic at the concentrations tested. However, the use of thimerosal, because of its high cytotoxicity, is not advisable. Our results prove the potential use of the test polymers in creating cell-based healing technologies.

ACKNOWLEDGEMENTS

This work was in part supported by the Lithuanian State Science and Studies Foundation.

Received 8 October 2009
Accepted 30 October 2009

References

1. Pham T, Palmieri TL, Greenhalgh DG. Sodium azide burn: a case report. J Burn Care Rehabil 2001; 22: 246–8.
2. Ball LK, Ball R, Pratt RD. An assessment of thimerosal use in childhood vaccines. Pediatrics 2001; 107: 1147–54.
3. Geier DA, Geier MR. An assessment of downward trends in neurodevelopmental disorders in the United States following removal of Thimerosal from childhood vaccines. Med Sci Monit 2006; 12: CR231–9.
4. Kiec-Swierczynska M, Krecisz B, Swierczynska-Machura D. Contact allergy to preservatives contained in cosmetics. Med Pr 2006; 57: 245–9.
5. Vlahović P, Cvetković T, Savić V, Stefanović V. Dietary curcumin does not protect kidney in glycerol-induced acute renal failure. Food Chem Toxicol 2007; 45: 1777–82.
6. Bourdeau P, Somers E., Richardson GM, Hickman JR. (eds.). Short-term toxicity tests for non-genotoxic effects. Scope 41, IPCS Joint Symposia 8; 1990.
7. Juopperi TA, Schuler W, Yuan X, Collector MI, Dang CV, Sharkis SJ. Isolation of bone marrow-derived stem cells using density-gradient separation. Exp Hematol 2007; 35: 335–41.
8. Janvikul W, Uppanan P, Thavornyutikarn B, Prateepasen R, Swasdison S. Fibroblast interaction with carboxymethylchitosan-based hydrogels. J Mater Sci Mater Med 2007; 18: 943–9.
9. Diebold Y, Herreras JM, Callejo S, Argüeso P, Calonge M. Carbomer- versus cellulose-based artificial-tear formulations: morphologic and toxicologic effects on a corneal cell line. Cornea 1998; 17: 433–40.
10. Ito T, Yeo Y, Highley CB, Bellas E, Benitez CA, Kohane DS. The prevention of peritoneal adhesions by *in situ* cross-linking hydrogels of hyaluronic acid and cellulose derivatives. Biomaterials 2007; 28: 975–83.
11. Simmons PA, Vehige JG. Clinical performance of a mid-viscosity artificial tear for dry eye treatment. Cornea 2007; 26: 294–302.
12. Garrett Q, Simmons PA, Xu S, Vehige J, Zhao Z, Ehrmann K, Willcox M. Carboxymethylcellulose binds to human corneal epithelial cells and is a modulator of corneal epithelial wound healing. Invest Ophthalmol Vis Sci 2007; 48: 1559–67.
13. Uglea CV, Panaitescu L, Spridon D, Ursu D, Popa I, Ottenbrite RM. Biological activity of maleic anhydride copolymers. I. Biocompatibility and antitumoural effects of maleic anhydride – vinyl acetate, maleic anhydride – methyl methacrylate and maleic anhydride – styrene copolymers. J Biomater Sci Polym Ed 1996; 8: 269–80.
14. Afflitto J, Smith KA, Patel M, Esposito A, Jensen E, Gaffar A. 113Cd nuclear magnetic resonance (NMR) study of the inhibitory effect of methylvinylether / maleic acid (PVM/MA) copolymer on the alkaline phosphatase of *Escherichia coli*. Pharm Res 1991; 8: 1384–8.
15. Stelljes KB. New process keeps biocontrols alive longer – Stabileze, new prevention process for crop fungi and bacteria. Agricultural Research 1997. www.nal.usda.gov/wqic
16. Quimby PC, Zidack NK, Boyette CD, Grey WE. A simple method of stabilizing and granulating fungi. Biocontrol Sci Technol 1999, 9: 5–8.
17. Zidack NK, Quimby PC. Formulation of bacteria for biological weed control using the Stabileze method. Biocontrol Sci Technol 2002; 12: 67–74.
18. Chang S, Lamm SH. Human health effects of sodium azide exposure: a literature review and analysis. Int J Toxicol 2003; 22: 175–86.
19. Bennett MC, Mlady GW, Fleshner M, Rose GM. Synergy between chronic corticosterone and sodium azide treatments in producing a spatial learning deficit and inhibiting cytochrome oxidase activity. Proc Natl Acad Sci USA 1996; 93: 1330–4.
20. Baskin DS, Ngo H, Didenko VV. Thimerosal induces DNA breaks, caspase-3 activation, membrane damage, and cell death in cultured human neurons and fibroblasts. Toxicol Sci 2003; 74: 361–8.
21. Chang HT, Liu CS, Chou CT, Hsieh CH, Chang CH, Chen WC et al. Thimerosal-induced cytosolic Ca^{2+} elevation and subsequent cell death in human osteosarcoma cells. Pharmacol Res 2005; 52: 328–33.
22. Humphrey ML, Cole MP, Pendergrass JC, Kiningham KK. Mitochondrial mediated thimerosal-induced apoptosis in a human neuroblastoma cell line (SK-N-SH). Neurotoxicology 2005; 26(3): 407–16.
23. Parran DK, Barker A, Ehrich M. Effects of thimerosal on NGF signal transduction and cell death in neuroblastoma cells. Toxicol Sci 2005; 86: 132–40.
24. Hagele TJ, Mazerik JN, Gregory A, Kaufman B, Magalang U, Kuppusamy ML et al. Mercury activates vascular endothelial cell phospholipase D through thiols and oxidative stress. Int J Toxicol 2007; 26: 57–69.
25. Zurovsky Y, Eligal Z, Grossman S, Bergman M, Gafter U. Glycerol-induced augmentation of sensitivity to endotoxin in rats. Toxicon 1994; 32: 17–26.
26. Peterson ML, Schlievert PM. Glycerol monolaurate inhibits the effects of Gram-positive select agents on eukaryotic cells. Biochemistry 2006; 45: 2387–97.
27. Deocaris CC, Shrestha BG, Kraft DC, Yamasaki K, Kaul SC, Rattan SI, Wadhwa R. Geroprotection by glycerol: insights to its mechanisms and clinical potentials. Ann NY Acad Sci 2006; 1067: 488–92.

Aida Vaitkuvienė, Vytautas Kašėta, Giedrė Ramanauskaitė, Genė Biziulevičienė

GELĮ FORMUOJANČIŲ POLIMERŲ, KONSERVANTŲ IR GLICEROLIO POVEIKIS PIRMINĖMS PELIŲ LĄSTELIŲ KULTŪROMS

Santrauka

Įvadas. Plėtojantis regeneracinei medicinai bei tobulėjant odos pažeidimų gydymo technologijoms, susijusioms su ląstelių panaudojimu, tampa aktuali netoksiškų ir biosuderinamų junginių paieška. Manome, tam galėtų praversti kosmetikos ir farmacijos pramonėse jau naudojamos medžiagos, tokios kaip Stabileze QM, Natrosol 250 MR, Carbopol ultrez 20, natrio azidas, timerosalis ir glicerolis.

Medžiagos ir metodai. Visų tiriamųjų medžiagų citotoksiškumą nustatėme įvertinę tripano mėliu dažytų blužnies ir kaulų čiulpų ląstelių gyvybingumą šviesiniu mikroskopu.

Rezultatai. Natrosol 250 MR, Stabileze QM, Carbopol ultrez 20, natrio azidas ir glicerolis ląstelių gyvybingumą sumažino 1–8 %, tuo tarpu timerosalis – net 71–74 %.

Išvados. Mūsų tyrimai rodo, kad šie gelį formuojantys polimerai nėra toksiški blužnies ir kaulų čiulpų ląstelėms, t. y. neveikia ar tik silpnai veikia šių ląstelių gyvybingumą. Be to, nustatėme, kad konservanto natrio azido ir glicerolio panaudojimas kuriant su ląstelių kultūromis susijusias technologijas yra visiškai priimtinas, kadangi šios medžiagos nebuvo citotoksiškos, tuo tarpu timerosalis dėl per didelio toksiškumo šiam tikslui nėra tinkamas.

Raktažodžiai: citotoksiškumas, gelį formuojantys polimerai, pelių kaulų čiulpų ir blužnies ląstelės