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**RYZHENKO G.F.**, PhD, associate
**GORBATYUK O.I.**, PhD, associate
**ANDRIYASCHUK V.A.**, PhD, associate
**ZHOVNIR O.M.**, researcher
*Institute of Veterinary Medicine NAAS*
**DYBKOVA S.M.,** PhD, senior research scientist, e-mail: sdybkova@gmail.com
**RIEZNICHENKO L.S.**, PhD, e-mail: Reznichenko_LS@mail.ru
**GRUZINA T.G.**, PhD, senior research scientist, e-mail: tgruzina@mail.ru
*F.D. Ovcharenko Institute of Biocolloidal Chemistry NASU*

# ESTIMATION OF GENOTOXICITY FOR PREPARATIONS OF VETERINARY VACCINES “Colisan”, “Colisan+AgNP”, “Velshicolisan”, “Velshicolisan+AgNP”, “Actinosan”, “Actinosan+AgNP”, “Multibovisan”, “Multibovisan +AgNP (1%)”, “Multibovisan +AgNP (0,5%)”

*Vaccines’ testing using genotoxicity estimation in vitro is very informative and high prognostic because of the possibility to predict malignant degeneration of the eukaryotic cells as well as risk for posterity health in the case of changes in the DNA of animals’ reproductive cells. By the Comet assay method in alkaline conditions using IMR-32 and MDVK cell cultures the absence of genotoxic influence for vaccines “Colisan”, “Colisan+AgNP”, “Actinocolisan Nadiya”, “Actinocolisan+AgNP”, “Velshicolisan”, “Velshicolisan+AgNP”, “Actinosan”, “Actinosan+AgNP”, “Multibovisan”, “Multibovisan+AgNP (1%)”, “Multibovisan+AgNP (0.5%)” has been revealed. The studied vaccines also have been characterized as biosafe according to the genotoxicity criterion under modulation of in vivo conditions using metabolic activation system (S9 microsomal fraction).*

***Keywords**: genotoxicity, veterinary vaccines, Comet assay method, cell culture, biosafety.*

**Introduction.** Animals’ vaccination is one of the most important procedures for farm and domestic animals in the prevention of their infectious diseases. Therefore, special attention should be paid to a comprehensive study of the veterinary vaccines’ safety during their development and implementation in practice of veterinary medicine [1].

Vaccines’ testing using the genotoxicity estimation *in vitro* (assessment of DNA-damages) is very informative and high prognostic because of the possibility to predict malignant degeneration of the eukaryotic cells as well as risk for posterity health in the case of changes in the DNA of animals’ reproductive cells.

On the modern stage of investigations Comet assay in alkaline conditions *in vitro* is high sensitive, expressed and informative method for estimation of genotoxicity of different chemical substances and physical agents [2□4]. It is based on registration of differences in migration of DNA and its fragments in

constant electrical field wherein cells were lysed. DNA breaks interrupt the structural organization of chromatin, which leads to relaxation of DNA macromolecule and formation of its fragments. Alkaline treatment of lysed cells' preparations stimulates untwisting of DNA duplex and allows individual threads to migrate in an electric field independently. Under these conditions DNA migrates to the anode with forming of electrophoretic track looks like "comet tail", which parameters are depend on the level of studied DNA damage [5, 6].

**The goal of the work** was estimation *in vitro* of genotoxicity parameters for vaccines "Colisan", "Colisan+AgNP", "Actinocolisan Nadiya", "Actinocolisan+AgNP", "Velshicolisan", "Velshicolisan+AgNP", "Actinosan", "Actinosan+AgNP", "Multibovisan", "Multibovisan +AgNP (1%)", "Multibovisan +AgNP (0.5%)".

**Materials and methods of research.** Veterinary vaccines "Colisan", "Colisan+AgNP", "Actinocolisan Nadiya", "Actinocolisan+AgNP", "Velshicolisan", "Velshicolisan+AgNP", "Actinosan", "Actinosan+AgNP", "Multibovisan", "Multibovisan +AgNP (1%)", "Multibovisan +AgNP (0.5%)" have been tested.

Genotoxicity estimation *in vitro* for the preparations of veterinary vaccines has been analyzed by the Comet assay method using eukaryotic cell cultures IMR-32 (neuroblastoma) and MDVK (calf kidney cell culture) from the Bank of Cell Lines from Human and Animal Tissues of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of NAS of Ukraine.

The cells of IMR-32 culture have been grown in 96-well plates ("Costar", Great Britain) by the incubation in thermostat in water saturated atmosphere with 5% $CO_2$ at 37°C in the RPMI 1640 medium ("SIGMA", USA), which contained 4 mM/L L-glutamin ("SIGMA", USA), 10% fetal calf serum ("SIGMA", USA), 40 μg/ml gentamicin up to optimal cell number $(2.0–4.0)\times10^4$ cells/cm$^2$.

The cells of MDVK line have been grown in 96-well plates ("Costar", Great Britain) by the incubation in thermostat in water saturated atmosphere with 5% $CO_2$ at 37°C in the growth medium 199 (SDM "Bio-Test-Laboratory", Ukraine) which contained 10% fetal calf serum warmed at 56°C within 30 min, 25 mM HEPES ("SIGMA", USA), 100 U/ml kanamycin up to optimal cell number $(2.0–4.0)\times10^4$ cells/cm$^2$.

The cells have been removed from the nutrient medium using a mixture of trypsin-EDTA and washed by fresh nutrient medium. The total cells number and percentage of viable cells have been assessed using staining by 0.3% trypan blue solution.

The eukaryotic cells have been treated by vaccines preparations within 18 hours under the cell titer $5\times10^5$ cells/ml for both IMR-32 and MDVK cell lines.

The quantity of vaccine preparations in the samples was 20% by volume.

For positive control samples obtaining IMR-32 and MDVK cell cultures have been treated within 18 hours by 1 mM N-nitrosomethylurea – well known mutagen for positive control preparation.

As negative control samples the intact cell cultures have been used. The cells of negative controls have been grown up to the cell titer $5\times10^5$ cells/ml at 37°C within 24 hours.

The genotoxic influence of vaccines under modulation of *in vivo* conditions using metabolic activation at the presence of S9 microsomal fraction has been also studied. It is necessary for registration of direct and indirect mutagens, which activity is determined by the formation of genotoxic metabolites. In the experiments with metabolic activation the incubation mixture contained: 500 µl of the medium, 200 µl of the cell suspension, 200 µl of the investigated vaccine solution and 100 µl of S9 microsomal fraction.

For estimation of the vaccines genotoxicity by the Comet assay method in alkaline conditions the micropreparations have been made as describe below. Micropreparations were formed on the microscopic slides with agarose plate (1% agarose gel of normal molten agarose ($T_{mel}$ <65 $^0$C)), on which 60 µl of treated cell suspension and 60 µl of 0.5% agarose gel have been spread. On the next stage slides with immobilized in agarose preparations were immersed in freshly prepared cold lysis solution (10 mM Tris-HCl (pH 10.0), 2.5 M NaCl, 100 mM EDTA-$Na_2$, 1% Triton X-100 and 10% DMSO) for 3 hours at 4 $^0$C. After finishing the lysis procedure the slides were placed in a horizontal gel electrophoresis tank filled with fresh cold electrophoresis solution (300 mM NaOH, 1 mM EDTA-$Na_2$, (pH>13.0), 4$^0$C) for the alkaline DNA denaturation (20 min under switched off apparatus). Distribution of denatured DNA has been conducted by gel-electrophoresis during 20□30 min under field strength 1 V/cm and current intensity not more than 250 mA. The preparations have been fixed using 70% ethanol solution during 15 min after electrophoresis finishing. Obtained micropreparations have been stained by acridine orange fluorescent dye within 30 min. “DNA-comets” have been visualized using fluorescent microscope “LUMAM R8” (exciting filter 490 nm, dichroic mirror 510, reflective filter 530 nm, magnification X200–400).

Visual analysis of “DNA-comets” has been provided. For each micropreparation 200 “DNA-comets” without “tails” overlays have been analyzed. Herewith the “DNA-comets” have been divided on 5 relative types with appropriate number from 0 to 4 for each “DNA-comet” (Fig.1).

The level of DNA damage in this case has been defined like “DNA-comet” index ($I_{DNA}$), which calculated by the formula:

$$\mathbf{I_{DNA} = (0n_0+1n_1+2n_2+3n_3+4n_4)/\Sigma,}$$ where

$n_0$–$n_4$ – number of each type “DNA-comets”, Σ – sum of “DNA-comets”.

The experiments have been done in two parallels. Statistical analysis of obtained results has been performed comparing the indexes of DNA damage in experimental and control groups. The data of two replications have been combined and average parameter for each group has been calculated. Statistically significant high indexes of DNA damage (data close to positive control) serve as criteria of positive result. The differences $p<0.05$ were considered as significant.

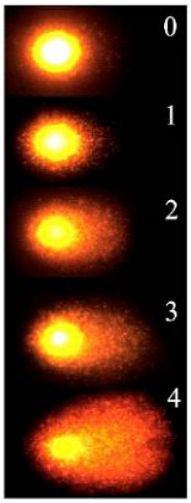


**Fig. 1. “DNA-comets” with different level of DNA damage**

**Results of research and discussion.** The investigations of the veterinary vaccines’ genotoxic properties using test cultures of eukaryotic cell lines IMR-32 and MDVK allow certify the following effect.

When using the Comet assay method in alkaline conditions for testing of veterinary vaccines’ genotoxicity the electrophoretic tracks of “DNA-comets” type have been obtained in the positive control samples, where IMR-32 (Fig. 2) and MDVK (Fig. 3) cell cultures have been treated by 1 mM N-nitrosomethylurea.

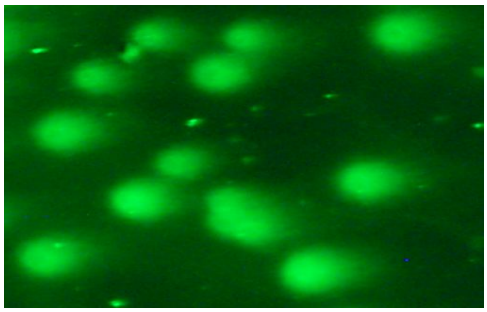

**Fig. 2. Electrophoretic images of DNA-comets for the positive control sample of IMR-32, where cells have been treated by 1 mM N-nitrosomethylurea**

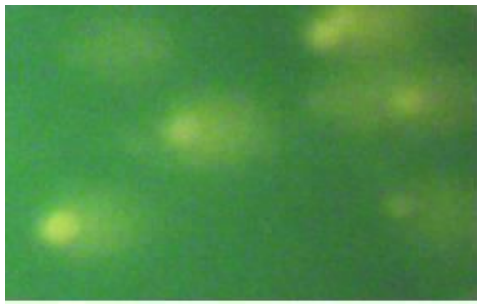

**Fig. 3. Electrophoretic images of DNA-comets for the positive control sample of MDVK culture, where cells have been treated by 1 mM N-nitrosomethylurea**

In the negative control samples the electrophoretic tracks of “DNA-comets” type have been absent. Only intact nuclei of eukaryotic IMR-32 and MDVK cells have been observed (Fig. 4, 5).

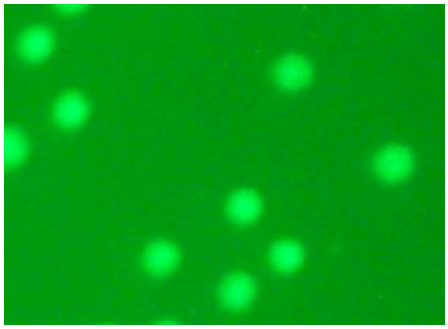

**Fig. 4. Electrophoretic images of IMR-32 cells intact nuclei (negative control)**

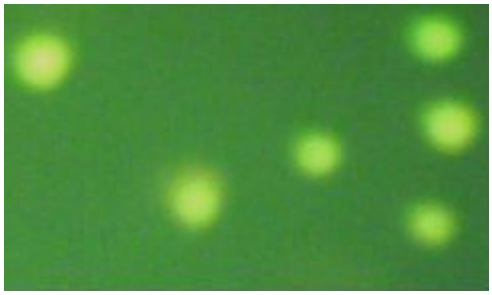

**Fig. 5. Electrophoretic images of MDVK cells intact nuclei (negative control)**

Table 1 demonstrates the parameters of genotoxic influence for the investigated veterinary vaccines compared with positive and negative control samples under the conditions where eukaryotic cells of IMR-32 line have been used like test culture.

*Table 1*

**The genotoxicity indexes of the investigated veterinary vaccines compared with positive and negative control samples using IMR-32 cells like test culture**

| Name of investigated vaccine | Number of viable cells, % | Genotoxicity index «$I_{DNA}$» | Genotoxicity index «$I_{DNA}$» with S9 usage |
|---|---|---|---|
| “Colisan” | 87 | 0.21±0,02 | 0.34±0,04 |
| “Colisan+AgNP” | 86 | 0.33±0.04 | 0.32±0.05 |
| “Actinocolisan Nadiya” | 91 | 0.34±0.03 | 0.23±0.01 |
| “Actinocolisan+AgNP” | 90 | 0.14±0.01 | 0.25±0.03 |
| “Velshicolisan” | 92 | 0.12±0.01 | 0.23±0.01 |
| “Velshicolisan+AgNP” | 92 | 0.18±0.01 | 0.32±0.04 |
| “Actinosan” | 88 | 0.23±0.01 | 0.21±0.01 |
| “Actinosan+AgNP” | 88 | 0.25±0.04 | 0.22±0.01 |
| “Multibovisan” | 90 | 0.23±0.01 | 0.22±0.01 |
| “Multibovisan +AgNP (1%)” | 90 | 0.31±0.03 | 0.24±0.01 |
| “Multibovisan +AgNP (0.5%)” | 91 | 0.17±0.03 | 0.27±0.02 |
| Negative control | 92 | 0.17±0.03 | 0.17±0.02 |
| Positive control | 88 | 1.94±0.11 | 2.34±0.12 |

According to the data of Table 1, all investigated veterinary vaccines were not genotoxic even under the modulation of *in vivo* conditions in which the metabolic activation system has been used.

Table 2 shows genotoxicity indexes for the investigated veterinary vaccines in compare with positive and negative control samples, which have been determined using MDVK cells as test culture in Comet assay analysis.

Obtained results demonstrate the absence of genotoxic influence of all investigated vaccines both under the conditions *in vitro* and in the experiments with using of metabolic activation system (table 2). The genotoxicity indexes «$I_{DNA}$» for all investigated veterinary vaccines were at the level of negative control parameters.

*Table 2*

**The genotoxicity indexes of the investigated veterinary vaccines compared with positive and negative control samples using MDVK cells like test culture**

| **Name of investigated vaccine** | **Number of viable cells (%)** | **Genotoxicity index «$I_{DNA}$»** | **Genotoxicity index «$I_{DNA}$» with S9 usage** |
|---|---|---|---|
| “Colisan” | 90 | 0.33±0.03 | 0.75±0.03 |
| “Colisan+AgNP” | 90 | 0.44±0.02 | 0.74±0.04 |
| “Actinocolisan Nadiya” | 90 | 0.36±0.01 | 0.56±0.04 |
| “Actinocolisan+AgNP” | 91 | 0.45±0.02 | 0.61±0.02 |
| “Velshicolisan” | 92 | 0.49±0.04 | 0.59±0.03 |
| “Velshicolisan+AgNP” | 91 | 0.61±0.03 | 0.57±0.01 |
| “Actinosan” | 90 | 0.57±0.02 | 0.68±0.02 |
| “Actinosan+AgNP” | 90 | 0.48±0.02 | 0.67±0.02 |
| “Multibovisan” | 91 | 0.51±0.02 | 0.76±0.01 |
| “Multibovisan +AgNP (1%)” | 91 | 0.48±0.04 | 0.79±0.01 |
| “Multibovisan +AgNP (0.5%)” | 91 | 0.59±0.04 | 0.71±0.01 |
| Negative control | 91 | 0.36±0.01 | 0.56±0.04 |
| Positive control | 90 | 2.28±0.18 | 2.71±0.15 |

**Conclusions and prospects for further research.** By the Comet assay method using IMR-32 and MDVK cell cultures the absence of genotoxic influence for vaccines “Colisan”, “Colisan+AgNP”, “Actinocolisan Nadiya”, “Actinocolisan+AgNP”, “Velshicolisan”, “Velshicolisan+AgNP”, “Actinosan”, “Actinosan+AgNP”, “Multibovisan”, “Multibovisan +AgNP (1%)”, “Multibovisan +AgNP (0.5%)” has been revealed.

Studied veterinary vaccines are also not genotoxic in the experiments, where *in vivo* conditions have been modulated using the metabolic activation system.

Investigated veterinary vaccines are biosafe according to the genotoxicity parameter.

Fulfilled investigations open perspectives for next improvement of the system for estimation of domestic immunobiological preparations’ safety according to the parameters of their influence on animals’ genetic apparatus.

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**ОЦЕНКА ГЕНОТОКСИЧНОСТИ ПРЕПАРАТОВ ВЕТЕРИНАРНЫХ ВАКЦИН «Колисан», «Колисан+AgNP», «Актиноколисан Надия», «Актиноколисан+ AgNP», «Вельшиколисан», «Вельшиколисан+ AgNP», «Актиносан», «Актиносан+ AgNP», «Мультибовисан», «Мультибовисан+ AgNP (1%)», «Мультибовисан+ AgNP (0,5%)» /** Рыженко Г.Ф., Горбатюк О.И., Андриящук В.А., Жовнир А.М., Дыбкова С.Н., Резниченко Л.С., Грузина Т.Г.

*Тестирование вакцин in vitro по параметру генотоксичности является чрезвычайно информативным и высокопрогностичным, поскольку позволяет предсказать злокачественное перерождение эукариотических клеток, а в случае изменений ДНК в половых клетках животных – опасность для здоровья потомков. Методом ДНК-комет в щелочных условиях с использованием эукариотических клеток линий IMR-32 и МДВК показано отсутствие генотоксического действия препаратов ветеринарних вакцин «Колисан», «Колисан+AgNP», «Актиноколисан Надия», «Актиноколисан+AgNP», «Вельшиколисан», «Вельшиколисан+AgNP», «Актиносан», «Актиносан+AgNP», «Мультибовисан», «Мультибовисан+AgNP(1%)», «Мультибовисан+AgNP(0,5%)». Установлена безопасность исследованных вакцин по параметру генотоксичности и при моделировании условий in vivo с использованием системы метаболической активации (микросомальной фракции S9).*

***Ключевые слова:*** *генотоксичность, ветеринарные вакцины, метод ДНК-комет, культура клеток, биобезопасность.*

**ОЦІНКА ГЕНОТОКСИЧНОСТІ ПРЕПАРАТІВ ВЕТЕРИНАРНИХ ВАКЦИН «Колісан», «Колісан+AgNP», «Актиноколісан Надія», «Актиноколісан+AgNP», «Вельшиколісан», «Вельшиколісан+AgNP», «Актиносан», «Актиносан+AgNP», «Мультибовісан», «Мультибовісан+AgNP (1%)», «Мультибовісан+AgNP (0,5%)» /** Риженко Г.Ф., Горбатюк О.І., Андріящук В.О., Жовнір О.М., Дибкова С.М., Рєзніченко Л.С., Грузіна Т.Г.

*Тестування вакцин на генотоксичність in vitro (здатність викликати первинні ушкодження ДНК) є надзвичайно інформативним та високопрогностичним оскільки такі*

*дані дають можливість передбачити злоякісне переродження еукаріотичних клітин, а в разі змін ДНК у статевих клітинах тварин і небезпеку для здоров’я нащадків. Метою роботи була оцінка генотоксичності in vitro препаратів ветеринарних вакцин «Колісан», «Колісан +AgNP», «Актиноколісан Надія», «Актиноколісан +AgNP», «Вельшиколісан», «Вельшиколісан +AgNP», «Актиносан», «Актиносан +AgNP», «Мультибовісан», «Мультибовісан +AgNP (1%)», «Мультибовісан +AgNP(0,5%)». Оцінку генотоксичності препаратів ветеринарних вакцин здійснювали in vitro методом ДНК-комет в лужних умовах із застосуванням еукаріотичних клітин ліній IMR-32 (нейробластоми) та МДВК (культури клітин нирки теляти). Для реєстрації дії прямих і непрямих мутагенів, активність яких пов’язана з утворенням генотоксичних метаболітів, проводили також тестування з використанням метаболічної активації в присутності мікросомальної фракції S9. Методом ДНК-комет в лужних умовах показана відсутність генотоксичної дії досліджуваних ветеринарних вакцин «Колісан», «Колісан +AgNP», «Актиноколісан Надія», «Актиноколісан+AgNP», «Вельшиколісан», «Вельшиколісан+AgNP», «Актиносан», «Актиносан +AgNP», «Мультибовісан», «Мультибовісан+AgNP(1%)», «Мультибовісан +AgNP(0,5%)». Досліджувані ветеринарні вакцини є не генотоксичними також і в експериментах, наближених до умов in vivo, за використання системи метаболічної активації. Досліджені ветеринарні вакцини є біобезпечними за показником генотоксичності. Виконані дослідження відкривають перспективи подальшого удосконалення системи оцінки безпечності вітчизняних імунобіологічних препаратів за показниками їх впливу на генетичний апарат тварин.*

***Ключові слова:*** *генотоксичність, ветеринарні вакцини, метод ДНК-комет, культура клітин, біобезпека.*

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**RYZHENKO G.F.,** PhD, associate, e-mail: anaerob12@ukr.net
**GORBATYUK O.I.,** PhD, associate, e-mail: anaerob12@ukr.net
**ANDRIYASCHUK V.A.,** PhD, associate, e-mail: anaerob12@ukr.net
**ZHOVNIR O.M.,** researcher, e-mail: anaerob12@ukr.net
*Institute of Veterinary Medicine NAAS*
**DYBKOVA S. M.,** PhD, senior research scientist, e-mail: sdybkova@gmail.com
**RIEZNICHENKO L.S.,** PhD, e-mail: reznichenko_ls@mail.ru
**GRUZINA T.G.,** PhD, senior research scientist, e-mail: tgruzina@mail.ru
*F.D. Ovcharenko Institute of Biocolloidal Chemistry of NAS of Ukraine*

## EVALUATION OF CYTOTOXICITY OF VETERINARY VACCINES “Colisan”, “Colisan+AgNP”, “Velshicolisan”, “Velshicolisan+AgNP”, “Actinosan”, “Actinosan+AgNP”, “Multibovisan”, “Multibovisan +AgNP (1%)”, “Multibovisan +AgNP (0,5%)”

*Research efforts regarding veterinary vaccines “Colisan”, “Colisan+AgNP”, “Velshicolisan”, “Velshicolisan+AgNP”, “Actinosan”, “Actinosan+AgNP”, “Multibovisan”, “Multibovisan +AgNP (1%)”, “Multibovisan +AgNP (0,5%)” showed a low level of their cytotoxic effect. The results of cytotoxicity testing of vaccines regarding parameters of viability of passaged eukaryotic cells of line L929. Using microscopic analysis of cell*