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ULTRASTRUCTURE AND BIOLOGICAL PROPERTIES OF AVIAN INFLUENZA VIRUSES FOLLOWING CRYOPRESERVATION

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Summary. Ultrastructure and infectious activity of avian influenza virus (strain A/Chicken/Sivash/02/05 (H5N1)) following cryopreservation and low temperature storage at –20, –70, and –196°C during various terms from 25 days up to 143 months using electron microscopy, serological and virological methods were investigated. Avian influenza viruses strain A/Chicken/Sivash/02/05 (H5N1) is stored in the Collection of Pathogens of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (Kharkiv, Ukraine), which was granted the National Endowment of Ukraine status. The conducted study allowed to reveal on electronograms the ultrastructural changes in AIV during long term storage (18 months) at moderately low temperature (–20°C), in particular loss of glycoprotein of peplomers in the majority of virions. The changes in ultrastructure of the virus samples were accompanied by a loss of hemagglutinating activity during long-term storage of AIV samples at moderately low temperature of –20°C. When storing the AIV samples at –70 and –196°C the virions generally remain negatively contrasted, keep peplomers for the studied storage duration

Keywords: avian influenza virus, morphology, ultrastructure, virion, cryopreservation, cryobank, cryodamage

Introduction. Avian influenza viruses strains isolated during infectious disease outbreaks from the brain of chickens, in the AR Crimea in 2005, are stored at the Cryobank of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' for further investigation of their biological properties, patenting, development of the preventive measures and therapy of viral infections in birds (Stegniy B., 2012; Stegniy M., 2006). Avian influenza viruses are the part of the Collection of Pathogens of the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine', which was granted the National Endowment of Ukraine status.

As classified by the International Committee on Taxonomy of Viruses (Van Regenmortel et al, 2000) this virus belongs to the family Orthomyxoviridae, genus *Influenza virus A;* affects mainly birds of all ages. Humans are also susceptive to the avian influenza virus (AIV). Avian influenza is the most dangerous disease of birds, mammals and humans. Herewith the disease proceeds with the symptoms of injury of upper respiratory tract. In birds, the avian influenza virus causes respiratory and intestinal syndromes (Stegniy B., 2012).

High mortality due to avian influenza is observed in birds of about from 10 to 100%. Respiratory syndrome manifests itself in birds by a cough, conjunctivitis, rhinitis and sinusitis, hyperemia of mucous membranes and edema. Thus, from the foregoing it can be concluded that the avian influenza viruses play an important role in infectious pathology of humans and animals and are capable of overcoming the species barrier.

Avian influenza viruses possess a lipoprotein envelope, viruses bind to the receptors containing the sialic acid and exhibit a neuraminidase activity (Stegniy B., 2012; Stegniy M., 2006). The features of the family Orthomyxoviridae are: average size of virions from 80 to 150 nm; the presence of RNA; a membrane comprising the lipids; may occur filamentous form of virions.

The aim of the work was to assess the disorders in structural integrity of the virions, in particular viral shells and nucleocapsid during their low temperature storage in the Collection of pathogens.

Materials and methods. The research object was the AIV strain A/Chicken/Sivash/02/05 (H5N1), obtained from the collection of the Department of Avian Viral Diseases of the the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine'.

Freezing and storage of the collection samples of extra-embryonic virus-containing liquid was performed in a domestic refrigerator (–20 ± 0.1°C), low-temperature freezer (–70 ± 0.4°C) for various time periods: from 25 days and up to 143 months. Viruses are known to be generally more resistant to environmental and cryopreservation factors, therefore in many laboratories and at bioindustrial enterprises for storage of viruses there are used moderately low temperatures (–20 … –22°C).

This stipulated the choice of the range of cryopreservation and storage regimens of viral material.

Virus-containing liquid was packed into 1.8 and 2.0, 4.5 cm^3 cryovials ('Nunc', Denmark). Cooling of biological samples was performed using a refrigerator chamber; the samples were cooled passively in the refrigerator chamber. Temperature conditions were monitored by thermo gauge placed into the cooled object, or by low-temperature thermometer. The samples were either slowly thawed on air or in a water bath at 38–39°C for 1–2 min.

Titers of AIV infectious activity were determined by titration in 9–10-day-old chicken embryos, the embryonic virus infecting dose (EID_{50}/ml) was calculated according to the standard procedure (Stegniy B., 2012; Stegniy M., 2006). AIV hemagglutination titers were studied in hemagglutination test. The effect of low temperatures and deep cooling on the AIV viral ultrastructure was investigated using electron microscopy. The samples were inactivated with 0.5% formaldehyde solution.

The inactivation completeness was tested by means of three successive passages in 9-day-old chicken embryos. The virus samples were used for the electron microscopy studies if there was no infectious activity. The neutralization reaction (NR) in chicken embryos and assessment of antigen specificity of the AIV virus after cryopreservation was performed with different virus dilutions and constant dose of a specific serum. Once infected with a mixture of serum and virus the 8–10-day-old chicken embryos were incubated at 37°C for 8 days with daily ovoscopy.

The embryo death in the first 24 h was not taken into account because considered as non-specific. Neutralization was considered positive when no death of embryos was found as well as no changes characteristic for AIV resulting from neutralization of virus virulence by antibodies. The results were evaluated by the neutralization index: if higher than 2 lg — the reaction was considered as positive; 1.5–2 lg — doubtful; 1 lg — negative.

Electron microscopy was performed in the samples which were stored from 25 days up to 143 months at moderately low (–20°C), low (–70°C) and liquid nitrogen temperatures (–196°C). The copper grid platforms covered with formvar film were used. Adsorption of virus particles, preliminary purified in sucrose density gradient on the film substrate was carried out within 3–5 min. The resulted specimens were contrasted using phosphotungstic acid (Turcu et al., 1994; Ponomarev and Mishchenko, 2005; Ponomarev, 2001). Studies were performed with 'PEM-125' electron microscope using the 60–100 thousand times magnification at the premises of Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine.

Performing statistical analysis of the research results we calculated mean and standard deviation. Significance of differences between samples was assessed according to the Student-Fisher's test (Van Emden, 2019).

Results and discussion. Electron microscopic study of the post-thaw AIV samples, stored from 25 days at –20, –70, and –196°C to 143 months at –70 and –196°C demonstrated a moderate number of circular, elliptical and filamentous form virions with polymorphism and the sizes of 80–180 nm. Ultrastructure of AIV viruses' strain A/chicken/Sivash/02/05 (H5N1), was presented with a two-layer lipoprotein membrane with spicules of hemagglutinin and neuraminidase up to 12 nm and nucleocapsid of 90 nm diameter The central part of the circular virions from the mentioned samples was characterized by the concave presence (Fig. 1).

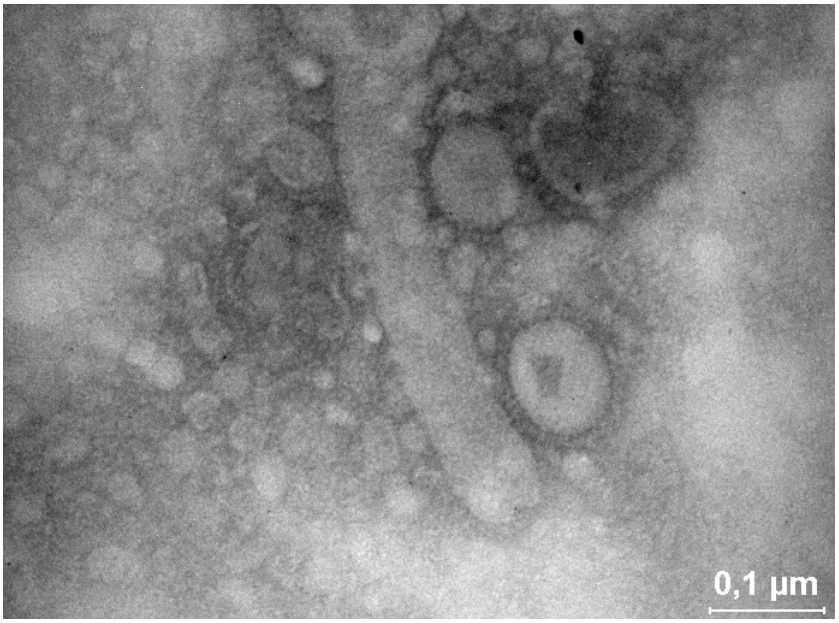

Fig. 1. Ultrastructure of the no frozen AIV virus samples (filamentous form, circular virions) on the fifth passage cultivation in chicken embryos.

Lethal virus activity after freezing of the samples stored not longer than 25 days was 8.31 ± 0.02 ELD_{50}/0.2 ml. Hemagglutination titers within these storage periods at –20°C did not differ from the baseline. Lethal virus activity after freezing –70 ± 2°C of the samples stored 143 months was 8.0 ± 0.01 and 7.5 ± 0.01 ELD_{50}/0.2 ml.

In all the cases of storage temperature and duration we identified such a feature of AIV infecting the chicken embryos as hemorrhages in serous and mucous membranes and organs of chicken embryos, regarded as a pathognomonic sign of AIV (Fig. 2).

Hemagglutination virus activity after freezing –20 ± 0.5, –70 ± 0.5, –196 ± 0.1°C of the samples stored for 25 and 115 days was (1:512), it did not differ from the baseline. After 18 months at –20 ± 0.5°C viral titer in hemagglutination was (1:256). After 210 days at –20 ± 0.5°C viral titer in hemagglutination was zero.

Ultrastructure of AIV virus samples stored for 18 months at –20°C presented viruses with damaged spicules of hemagglutinin on the surface of virions (Fig. 3).

Hemagglutination virus activity after freezing –70 ± 2°C of the samples stored 143 months was (1:128), it differed from the baseline (1:1024).

Infectious virus activity after thawing of the samples stored for 143 months at –70 ± 2°C was slightly reduced and made 8.0 ± .03 EID_{50}/ml.

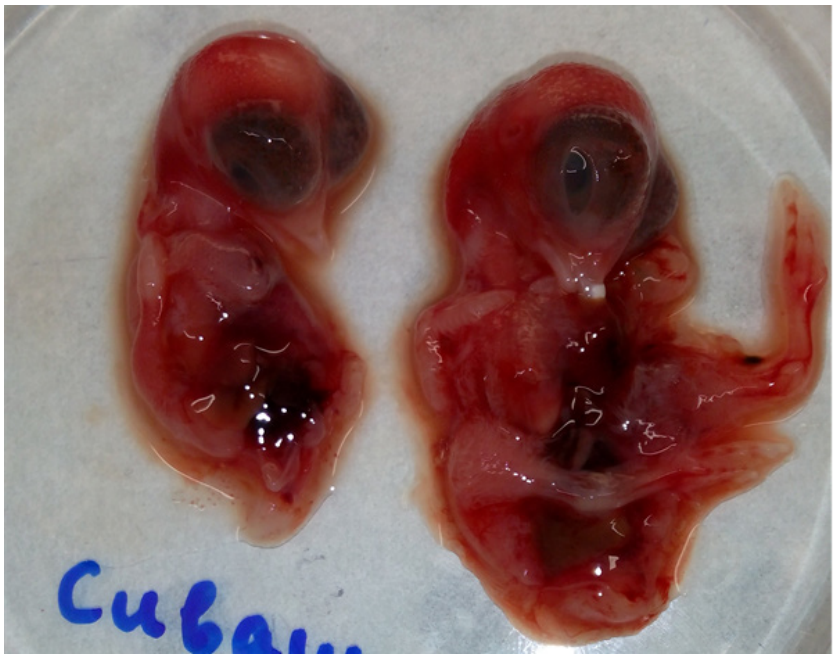

Fig. 2. Hemorrhages in serous and mucous membranes and organs of AIV infected chicken embryos.

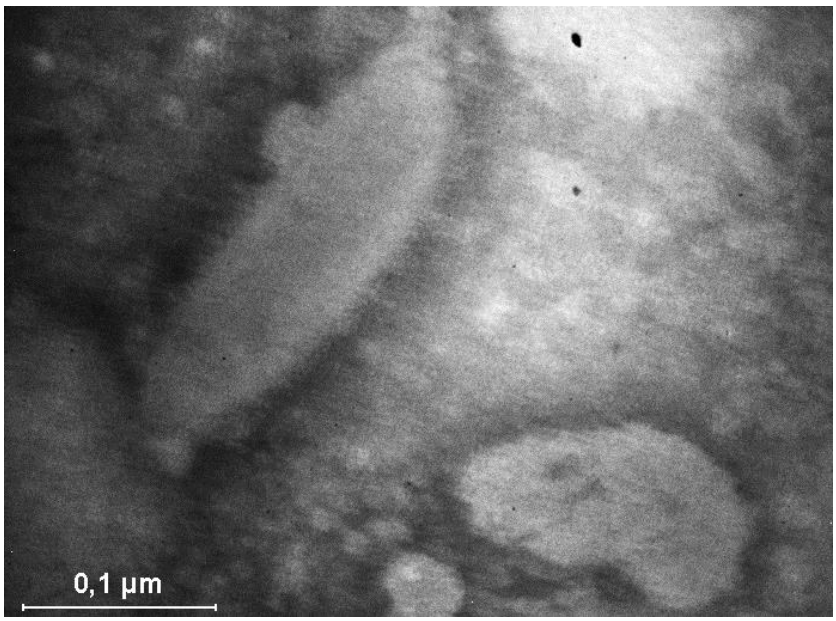

Fig. 3. Ultrastructure of AIV virus samples stored for 18 months at –20°C (viruses with the partly damaged hemagglutinin on the surface of virions).

The analysis of electronograms of the viruses, stored for 18 months at –20°C, showed the presence of polymorphic virions with a distinct bilayer lipoprotein envelope, but the glycoprotein processes, forming the spicules of hemagglutinin were absent (Fig. 3).

During freezing the virions possessing a total electric charge of a certain value (Oparin, 1996) occur to the water-ice phase transition zone, where they are exposed to an external electric field effect. It can be assumed that during long-term storage of AIV at –20°C the structure of virions could be destabilized because of the conformational changes associated not only with an alteration in their shape, but also with intramolecular mobility of structural components of the virion protein molecules.

The researches of Lugovoy (1985) on the mechanisms of freezing damaging effect on proteins, in particular enzymes, have shown that one of the main causes of the disordered structural and functional properties of enzyme proteins are high concentrations of inorganic salts, appearing during water crystallization in the solution, which possess the lyotropic properties as well as provide the pH shifts during the water freezing-out. The author believed that the structural basis of cryostability or cryolability in enzyme proteins was stipulated by an unequal degree of conformational mobility and rigidity of macromolecules, which, in turn, was caused (in accordance with the principle of thermo-dynamic free energy minimum) by different features of the amino acid composition of the protein molecule polypeptide chain.

The absence of peplomers on the electronograms of AIV stored for 18 months at –20°C, explains the total loss of hemagglutinating properties.

The ultrastructure of AIV, long-term-stored at low temperatures (–70 and –196°C) was more intact than after storage at –20°C and was close to the ultrastructure of the virus which was shortly stored (115 days) at a temperature of refrigerator.

All this testifies to the fact that the lower the storage temperature of cryopreserved viruses is, the more fully their ultrastructure and biological properties are retained. Electron microscopy studies allowed a visual assessment of the contribution of disordered structural integrity of the virions in the cryoinjuries of viruses during their cryopreservation and low temperature storage as exemplified by AIV.

Conclusions. The conducted study allowed to reveal on electronograms the ultrastructural changes in AIV during long term storage (18 months) at moderately low temperature (–20°C), in particular loss of glycoprotein of peplomers in the majority of virions.

The changes in ultrastructure of the virus samples were accompanied by a loss of hemagglutinating activity during long-term storage of AIV samples at moderately low temperature of –20°C.

When storing the AIV samples at –70 and –196°C the virions generally remain negatively contrasted, keep peplomers for the studied storage duration.

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