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In vitro evaluation of chitosan cytotoxic properties

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ABSTRACT

Introduction. Chitosan immunomodulatory and mucoadhesive properties render it a promising vaccine adjuvant. Safety – particularly the absence of cytotoxicity – is a key requirement for adjuvant candidates. *In vitro* biocompatibility assessments enable evaluation of chitosan preparations prior to animal testing.

Objective. To evaluate low molecular weight chitosan solution at a concentration of 10 mg/mL for its cytotoxic effect on chicken embryo fibroblast (CEF) cultures and calf coronary artery epithelial-like cells (CCEC) to justify its further use as a vaccine adjuvant.

Materials and methods. Low molecular weight (LMW) chitosan (degree of deacetylation: 90%) prepared with a 1% glutamic acid solution (pH 6.9) was used. Cytotoxicity was comprehensively assessed using three methods: trypan blue vital staining (for cell viability), live-cell microscopy (for morphological evaluation), and calculation of the proliferation index after 72 hours of incubation at 37 °C in a 5% CO_2 atmosphere.

Results and discussion. Following 2-hour incubation with chitosan, viable CEF and CCEC were 97.4 and 98.7%, respectively, with no significant differences from controls (97.6 and 96.4%). Microscopy at 72 hours showed dense, homogeneous monolayers in test groups, free of cytopathic effects, vacuolization, or morphological changes – indistinguishable from controls. Proliferation indices aligned closely (CEF: 3.9 and 3.6; CCEC: 3.7 and 3.8), evidencing no cytostatic effect of the chitosan preparation.

Conclusion. Low-molecular-weight chitosan (10 mg/mL) exhibited no *in vitro* cytotoxic or cytostatic effects on the tested cell lines. The findings confirm its biocompatibility and justify advancement to *in vivo* studies for developing safe, effective vaccines for veterinary use.

Keywords: chitosan, cytotoxicity, adjuvant, cell viability, proliferation, cell cultures, primary chicken embryo fibroblasts (CEF), continuous calf coronary artery epithelial-like cells (CCEC), *in vitro*

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Цитотоксические свойства хитозана *in vitro*

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РЕЗЮМЕ

Введение. Хитозан, благодаря иммуномодулирующим и мукоадгезивным свойствам, является перспективным адъювантом для вакцин. Безопасность, в частности отсутствие цитотоксичности, – ключевое требование к адъювантам. Исследования *in vitro* позволяют определять биосовместимость препарата хитозана до тестирования на животных.

Цель исследований. Исследовать цитотоксическое действие раствора низкомолекулярного хитозана в концентрации 10 мг/мл на культурах фибробластов эмбриона кур и эпителиоподобных клеток коронарных сосудов теленка для обоснования его дальнейшего применения в качестве вакцинного адъюванта.

Материалы и методы. Применяли низкомолекулярный хитозан (степень деацетилирования – 90%) в 1%-м растворе глутаминовой кислоты, pH 6,9. Цитотоксичность определяли комплексно, используя метод витального окрашивания трипановым синим (оценка жизнеспособности), прижизненное микроскопическое наблюдение (оценка морфологии) и расчет индекса пролиферации после 72-часовой инкубации при температуре +37 °С в атмосфере 5%-го диоксида углерода.

Результаты и обсуждение. Количество жизнеспособных клеток фибробластов эмбриона кур и коронарных сосудов теленка после двухчасовой инкубации с хитозаном соответствовало значениям 97,4 и 98,7%, не имеющим статистически значимых отличий от контролей (97,6 и 96,4%). При микроскопическом наблюдении клетки в опытной группе через 72 ч инкубации формировали плотный однородный монослой без признаков цитопатического эффекта, вакуолизации, без изменений морфологии, аналогичный таковому в контрольных лунках. Индексы пролиферации в опытных и контрольных группах были сопоставимы: для фибробластов эмбриона кур – 3,9 и 3,6, для коронарных сосудов теленка – 3,7 и 3,8, что свидетельствует об отсутствии цитостатического действия изучаемого препарата.

Заключение. Хитозан низкомолекулярный в концентрации 10 мг/мл не проявляет цитотоксических или цитостатических свойств *in vitro* в отношении тестируемых клеток. Полученные данные подтверждают его биосовместимость и являются основанием для дальнейших исследований *in vivo* с целью разработки безопасных и действенных вакцин для ветеринарного применения.

Ключевые слова: хитозан, цитотоксичность, адъювант, жизнеспособность клеток, пролиферация, клеточные культуры, первичная культура фибробластов эмбриона кур, перевиваемая линия эпителиоподобных клеток коронарных сосудов теленка, *in vitro*

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INTRODUCTION

Avian infectious diseases such as avian influenza, Newcastle disease, infectious bronchitis, and avian metapneumovirus infection cause significant economic damage to poultry industry worldwide [1, 2]. Agents of these diseases are highly contagious and widespread, often causing mass mortality in poultry. This leads to significant economic losses and restrictions on international trade. Available commercial vaccines, although widely used, sometimes fail to confer sterilizing immunity and full protection for vaccinated flocks especially under the high pressure from field virus strains in intensive production system. This necessitates the exploration of novel approaches to enhance vaccine immunogenicity and prolong the duration of vaccine effectiveness [3, 4].

One of the promising areas of vaccinology is the development and application of modern adjuvants – substances capable of enhancing, prolonging, and modulating the immune response to a vaccine antigen when added to a vaccine formulation [5]. Modern adjuvants employ diverse mechanisms of action designed to activate innate immunity, ultimately leading to the development of a stronger and more potent adaptive immune response [6]. At the same time, safety, in particular the absence of cytotoxicity, remains a key requirement for any new adjuvant compositions [7, 8].

Chitosan is a natural biopolymer derived from the deacetylation of chitin, a structural component found in crustacean shells and fungal cell walls. Its key advantage is its low toxicity to warm-blooded organisms and its ability to degrade without harm to the environment [9, 10, 11]. Chitosan's favourable properties – coupled with its abundance of free reactive amino groups – underpin its extensive use in medicine and pharmacy. Key applications include wound dressings, drug delivery carriers, and, most notably, vaccine adjuvants [12].

In vaccinology, chitosan is considered as a multifunctional adjuvant, especially for mucosal use (intranasal, ocular, oral) [13, 14]. Its adjuvant properties are associated with a complex of mechanisms. Firstly, as a cationic polymer, chitosan can temporarily disrupt the integrity of tight junctions between epithelial cells in mucous membranes. This increases mucosal permeability and facilitates antigen penetration [15, 16]. Secondly, chitosan derivatives can form an antigen depot at the injection site, providing a prolonged release of the active substance. Thirdly, chitosan has a direct stimulating effect on innate immune cells, likely through interaction with pattern-recognition receptors such as TLR-2. This leads to the activation of antigen-presenting cells and the production of proinflammatory cytokines [17]. Foreign researchers have shown that chitosan is able to enhance both the humoral and cell-mediated immune response [16, 17, 18], which makes it particularly in demand for the development of new vaccines. It is important to note that the adjuvant effect may vary depending on the molecular weight and degree of polymer deacetylation [9, 14].

Use of LMW chitosan in avian vaccines, in particular against Newcastle disease is of particular interest. LMW fractions generally exhibit better solubility at physiological pH and, according to some studies, demonstrate lower potential toxicity compared to their high molecular weight counterparts [9]. However, prior to immunogenicity assessment, it is fundamentally important to establish the basic safety and absence of direct toxic effects of the tested product at the cellular level.

In vitro cytotoxicity determination in cell lines is the first mandatory step in preclinical studies of any new compound with potential biomedical or veterinary applications. This approach, governed by international standards like ISO 10993-5, allows for the quick, economical, and ethical collection of initial biocompatibility data in line with the 3R concept (Replacement, Reduction, Refinement). It thereby reduces or postpones the use of laboratory animals to later stages of the study [7]. This approach is widely used to assess the safety of a wide variety of compounds, from pesticides to pharmaceutical substances, which confirms its versatility and reliability [8, 19].

Cell lines were selected based on their representativeness. Primary CEF culture exhibits high sensitivity to toxic effects. Continuous CCEC line serves as a model for the cellular barrier encountered by an adjuvant upon mucosal administration. A concentration of 10 mg/mL was selected as it deliberately exceeds the estimated working concentrations in vaccines (1–2 mg/mL), allowing for stress testing and safety margin assessment.

Thus, the objective of this study was to evaluate the cytotoxic effect of a low molecular weight chitosan solution (10 mg/mL) on CEF and CCEC lines in order to justify its further use as a vaccine adjuvant.

MATERIALS AND METHODS

The study was carried out at the Department of Virology and Microbiology named after Academician V. N. Syurin of the Moscow State Academy of Veterinary Medicine and Biotechnology – MVA by K. I. Skryabin. All operations were performed in a laminar flow cabinet under sterile conditions, with strict adherence to standard aseptic techniques.

Preparation of chitosan solution. LMW water-soluble chitosan with a deacetylation degree of 90% (Bioprogress, Russia; dosage form – powder) was used. Working 2% (20 mg/mL) chitosan solution was prepared using 1% glutamic acid (Sigma-Aldrich, USA) in a ratio of 1:5 (w/v). The pH was adjusted to 6.9 ± 0.1 with a phosphate-buffered saline (PBS; BioloT, Russia) [9, 14].

Cell cultures and cultivation conditions. Two lines of adhesive cells of different origin were used for a comprehensive assessment of potential cytotoxicity:

– CCEC cells (BioloT Collection, Russia) are a continuous diploid line of epithelial-like cells derived from coronary vessels of calf. This cell line is characterized by stable growth and high sensitivity to external factors, making it a relevant model for toxicological studies;

– primary CEF culture was prepared in the laboratory from 11-day-old SPF embryo tissues using the standard trypsinization technique [19]. Cells at the 3rd passage, which retain high metabolic activity and sensitivity to toxic effects, characteristic of primary cultures were used [17].

The cells were cultivated under standard sterile conditions in an incubator (Binder, Germany) at a constant temperature of 37.0 ± 0.5 °C and 5% CO_2 to maintain a stable pH of the nutrient medium (7.2–7.4). The growth medium consisted of a 1:1 mixture of Eagle MEM and 199 media (BioloT, Russia), supplemented with 10% fetal bovine serum (St-Biol; BioloT, Russia) to provide essential adhesion and growth factors. Antibiotics were added to the medium: 100 IU/mL of penicillin and 0.1 mg/mL of streptomycin (BioloT, Russia), to prevent bacterial contamination. The medium was changed every 48–72 hours depending on the cell growth rate. When cell monolayers reached 80–90% confluence, cultures were passaged using standard trypsinization with 0.25% Trypsin-Versene solution (BioloT, Russia). Cells in the logarithmic growth phase were selected for tests.

Test design. The cells were inoculated into 6-well plates (Jet Biofil, China) at a density of 1×10^6 cells/mL for CEF and 8×10^5 cells/mL for CCEC, which provided a 70–80% monolayer density by the beginning of the test. Separate plates were used for each cell line:

– "positive control" plate: cells were cultured in a standard growth medium;

– "test" plate: cells were cultured in a growth medium supplemented with the chitosan solution (final concentration – 10 mg/mL).

Table 1
Viability of cells after 2-hour incubation with chitosan (M ± SD, *n* = 3)

Type of the effect on cells	Cell viability, %	
	CEF	CCEC
Chitosan, 10 mg/mL in standard growth medium (test)	97.4 ± 1.2	98.7 ± 0.9
Standard growth medium (positive control)	97.6 ± 0.8	96.4 ± 1.1
70% ethanol solution (negative control)	0*	0*

* $p < 0.05$ as compared to all other groups.

Three parallel wells of the plate ($n = 3$) were used for each variant to ensure statistical reliability. The incubation was started once the cell monolayer covered 70–80% of the well bottom.

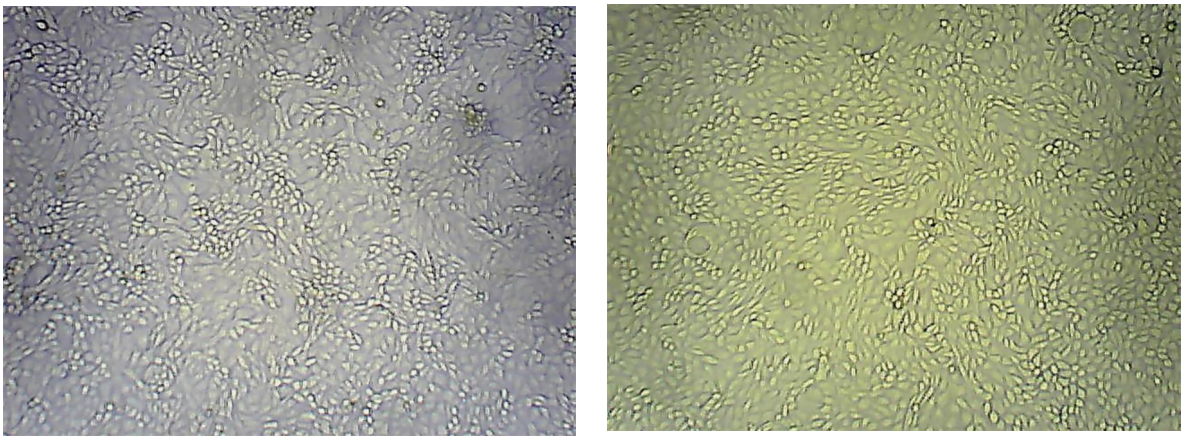
Determination of cell viability. Cell viability were assessed after 2-hour incubation with the test solutions using vital staining with 0.4% trypan blue solution (BioloT, Russia).

The counting was carried out in Goryaev counting chamber. Only cells with intact membranes (unstained) were taken into account [19]. Cells placed in a standard growth medium were used as a positive control, and cells treated with a 70% ethanol solution for 10 minutes were used as a negative control.

Morphological analysis (vital examination method). The cells were cultured with chitosan for 72 hours. Visual examination of the monolayer, cell morphology, and cytopathic effect manifestations was performed every 24 hours using Axio Observer A1 inverted microscope (Carl Zeiss, Germany) equipped with phase contrast optics and Axiocam 305 digital camera. Microscopy was performed at 120× magnification. The assessment parameters included adhesion degree, monolayer density, cytoplasmic vacuolization, shape alterations (rounding), substrate detachment, and lysis.

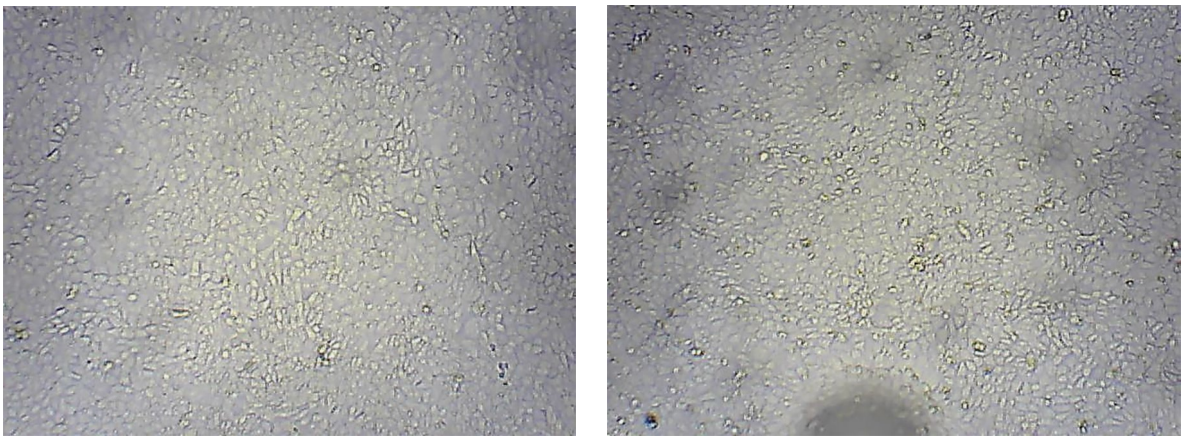
Determination of proliferative activity. The cells were inoculated into 6-well plates at known concentration (N_0). After 72 hours of co-cultivation with chitosan, the cells were dispersed with a 0.25% trypsin solution (from pig pancreas, activity: 1:250; BioloT, Russia) prepared using Versene solution (BioloT, Russia), and counted (N_{72}) in Goryaev counting chamber. The proliferation index (PI) was calculated using the formula: $PI = N_{72} / N_0$. To eliminate artifacts associated with potential errors during inoculation, the initial cell count (N_0) was performed in three additional wells immediately after cell adhesion, i.e., 4 hours post-inoculation.

Statistical analysis. Statistical processing was performed using Student's *t*-test for independent samples in Microsoft Excel 2019 and Statistica 10.0 (StatSoft, USA) software packages. The cell viability data obtained by direct counting in Goryaev counting chamber were analysed with a preliminary check of the data compliance with the normal



A B

Fig. 1. Morphology of CEF cells after 72 hours of cultivation (magnification 120×): A – control, B – test group (chitosan 10 mg/mL)



A B

Fig. 2. Morphology of CCEC after 72 hours of cultivation (magnification 120×): A – control, B – test group (chitosan 10 mg/mL)

distribution using the Shapiro – Wilk test (at $n = 3$). The uniformity of variances in the compared groups was checked using the F-test (Fisher's criterion). The following parameters were incorporated into the analysis: viability assessment was performed using absolute values of live and dead cell counts, from which the viability percentage was calculated; proliferative activity was analysed on the basis of absolute cell counts before and after incubation. For each test variant, data for three independent biological replicates ($n = 3$) were used, in each of which cell counts were performed in two analytical replications. Morphological examination findings were statistically analysed by qualitative assessment of a series of micrographs taken under identical conditions for all test groups. Quantitative results are presented in the following format: mean value ± standard deviation (M ± SD). The differences at the $p < 0.05$ level were considered statistically significant. All calculated *t*-test values and the corresponding *p*-significance levels were recorded in summary tables for further analysis.

RESULTS AND DISCUSSION

In the study, the cytotoxic effect of a low molecular weight chitosan solution was evaluated multilaterally using three complementary methods. At the first stage, cell viability was assessed after short-term incubation with the preparation. Then, dynamic microscopic examination of the cell morphology and monolayer state was carried out. At the final stage, the effect of chitosan on cell proliferative activity was assessed.

Cell viability. Results of tests of cells for their viability after 2-hour incubation are given in Table 1.

Obtained data indicate the absence of cytotoxic effects of low molecular weight chitosan (deacetylation rate – 90%) at a concentration of 10 mg/mL on two different cell models. For the CEF line, the viability index was 97.4 ± 1.2% versus 97.6 ± 0.8% in the control ($p > 0.05$). For CCEC line the values were also comparable: 98.7 ± 0.9% for cells in test plate and 96.4 ± 1.1% for the control ($p > 0.05$). Consequently, there were no statistically significant differences between the test and control groups for both tested cell lines.

High viability indices, intact morphology, and unaltered proliferative activity conclusively establish the biocompatibility of this preparation with animal cells.

Analysis of the data obtained (Table 1) allows us to conclude that the chitosan preparation under study does not have any cytotoxic effect under short-term incubation conditions. The minimal spread of values (SD = 0.8–1.2%) is noteworthy, as it indicates both high reproducibility of the results and uniformity of the cell populations. Complete

cell death was recorded in the negative control (70% ethanol treatment), thereby confirming both the adequacy of the applied technique and the sensitivity of the test system. Comparative analysis of the data for the two cell lines demonstrates that the response to chitosan exposure is neither species-specific nor tissue-specific. The comparably high viability indices observed in both the primary CEF line and the continuous CCEC line suggest that the biocompatibility of the preparation under study is universal.

Morphology of cells. Results of live-cell microscopic examination of cell cultures over time (after 24, 48, and 72 hours of incubation) clearly demonstrate the absence of any negative effect of chitosan on the morphofunctional state of the cells. Visual examination after 72 hours of incubation showed that cells in the test groups retained their typical morphology and demonstrated an active ability to form a monolayer, completely similar to control cells (Fig. 1, 2).

Typical fusiform (fibroblast-like) and stellate (epithelial-like) cells with long cytoplasmic protrusions firmly attached to the substrate were observed in the test group of CEF culture. The cells had smooth, well-defined margins and a homogeneous, non-vacuolated cytoplasm. The nuclei were clearly visible and had a regular oval or rounded shape without pyknosis or karyorrhexis signs. The resulting monolayer was dense and homogeneous, displaying the ordered cell arrangement characteristic of fibroblasts.

Both in the test group and the control group, CCEC cells had a flattened polygonal shape and formed a typical monolayer resembling "cobblestone pavement". Intercellular contacts were well developed, and there were no signs of cytoplasmic contraction or detachment from the plastic surface. Importantly, no cytopathic effects (CPE) were observed across any test groups: monolayers showed no cytoplasmic vacuolization, rounded or shrivelled cells, lysis, or zones of degeneration. Dynamic observation showed that the monolayer formed at the same rate in the test groups as in the control, reaching 90–95% confluence by 72 hours of cultivation. The morphological findings were entirely consistent with the viability and proliferative activity data, collectively confirming that chitosan exerts no cytotoxic effect at the concentration tested.

Proliferative activity. To evaluate the potential effect of chitosan on cell division, quantitative analysis of proliferative activity was carried out during a 72-hour period. Data given in Table 2 show that low molecular weight chitosan at a concentration of 10 mg/mL did not have any inhibitory effect on the proliferation of the tested cell cultures. Quantitative analysis showed that the proliferation indices in the test groups remained at a high level: for CEF – 3.9 ± 0.4, for CCEC – 3.7 ± 0.3. These values did not differ statistically ($p > 0.05$) from the control values, which were 3.6 ± 0.4 and 3.8 ± 0.3, respectively. Absolute cell counts confirmed that the cultures actively proliferated in the presence of chitosan: the CEF counts increased from 1.4×10^6 to 5.4×10^6 cells/mL, CCEC counts – from 1.2×10^6 to 4.4×10^6 cells/mL.

Comparative analysis of the two cell lines for their proliferative activity revealed an interesting trend for stimulation of CEF culture proliferation (3.9 in the test group vs. 3.6 in the control), though the difference was not statistically significant. CEF proliferation indices in the test and in the control were almost identical.

Thus, comprehensive proliferative activity analysis showed that chitosan at 10 mg/mL exhibits no cytostatic effects and fully preserves mitotic capacity and growth potential of cells.

Table 2
Effect of chitosan on cell proliferative activity (M ± SD, *n* = 3)

Cell culture	Parameters	Test (chitosan, 10 mg/mL)	Positive control
CEF	Number of cells added, cells/mL	1.4×10^6	1.4×10^6
	Number of cells after 72 hours, cells/mL	$(5.4 \pm 0.6) \times 10^6$	$(5.1 \pm 0.5) \times 10^6$
	Proliferation index	3.9 ± 0.4	3.6 ± 0.4
CCEC	Number of cells added, cells/mL	1.2×10^6	1.2×10^6
	Number of cells after 72 hours, cells/mL	$(4.4 \pm 0.4) \times 10^6$	$(4.6 \pm 0.3) \times 10^6$
	Proliferation index	3.7 ± 0.3	3.8 ± 0.3

Notably, all three methods employed to assess cytotoxicity – vital staining, morphological analysis, and proliferation assessment – produced consistent results, thereby strengthening the reliability of the conclusions. The revealed absence of toxic effects is consistent with the data of other authors, who also note the low cytotoxicity of chitosan and its oligomers [9, 10, 11]. It should be particularly noted that chitosan at a relatively high concentration (10 mg/mL) was used for our study. This concentration is much higher than the typical working concentrations of adjuvants in vaccine formulations, which are usually in the range of 0.1–2.0 mg/mL [14, 15, 18]. Despite this, the cells retained their normal viability and functional activity. Low molecular weight fractions of chitosan that are similar in physicochemical characteristics to the test sample typically exert the least toxicity, which is attributed to their milder interaction with cell membranes [11, 17]. This finding is especially significant for vaccine development, in which avoiding even minimal cellular damage is critically important. Chitosan's ability – at the concentration used – to preserve cell membrane integrity and not suppress proliferation is a key factor in its suitability as an adjuvant [12]. Furthermore, the lack of effect of chitosan on proliferative characteristics suggests that it does not interfere with the basic mechanisms of cell division and poses no risk of inducing pathological changes in proliferating tissues.

It is important to note that CEF and CCEC cultures are representative for *in vivo* modelling. CEF as a primary culture is more sensitive to toxic effects [8, 19]. The use of a primary fibroblast culture precludes artifacts associated with extended adaptation of cells to *in vitro* conditions – a phenomenon characteristic of continuous cell lines. Epithelial-like calf coronary cells (CCEC) are the first target for mucosal adjuvants [13]. Mucous epithelial cells represent the first barrier encountered by mucosally applied vaccines; therefore, preserving their integrity and functional activity is essential for effective vaccination. The lack of adverse effects on both cell lines suggests that chitosan will be well tolerated at the whole-organism level.

According to the literature, chitosan may exert its adjuvant effect not through direct cytotoxicity, but through

mild activation of cells and induction of chemokines and cytokines [14, 15]. Our findings on the preservation of cell morphology and proliferative activity are fully consistent with this concept, since they rule out non-specific cell damage as a mechanism underlying immunostimulation.

The immunostimulatory effect of chitosan is thought to be mediated by activation of innate immune signalling pathways [17, 18], a process that does not involve cell damage at the injection site. This mechanism – whereby a "beneficial" immunological effect is achieved without cell damage – is preferable for the development of modern, safe adjuvants [6]. Our findings indirectly confirm this hypothesis. Further studies should focus on the effects of chitosan on the functional activity of immunocompetent cells, specifically their antigen-presenting ability and cytokine production. Another area of interest is the evaluation of chitosan's synergistic effects in combination with other well-known adjuvants within combined adjuvant systems.

This study is limited by the testing of a single chitosan concentration. Further studies should involve the generation of a full concentration-response curve to accurately determine the toxicity threshold, that is consistent with modern paradigms for the preclinical safety evaluation of biomaterials [7, 8]. Notwithstanding this limitation, it can be confidently concluded that within the concentration range typically employed in vaccinology (generally not exceeding 1–2 mg/mL), chitosan demonstrates an excellent safety profile.

CONCLUSION

This comprehensive study demonstrates that low molecular weight chitosan (90% deacetylation) exhibits no cytotoxic or cytostatic effects *in vitro* on CEF and CCEC lines at a concentration of 10 mg/mL. These findings were corroborated by a range of complementary methods. Cell viability in the test groups remained between 97.4 and 98.7%, with no statistically significant difference from control values. Morphological analysis showed preserved normal cellular architecture and the capacity to form a dense, homogeneous monolayer. Proliferative activity assessment determined high proliferation indices (3.7–3.9), which were comparable to those of the control. The obtained data on the high chitosan biocompatibility are consistent with the results of other studies [9, 11, 14, 15] and provide justification for conducting further *in vivo* experiments to study its adjuvant activity. Importantly, the concentration of the tested chitosan preparation is significantly higher than the estimated working concentrations of adjuvants used in vaccines, suggesting a broad therapeutic range and a favourable safety profile for the preparation under study.

The inclusion of low molecular weight chitosan in vaccines against Newcastle disease and other avian infections, followed by assessment of the specific immune response, is a promising direction for further studies [4]. Future work should examine the adjuvant properties of chitosan administered by different routes (intranasal, oral, intramuscular) and its synergistic action with other immunostimulants. Further studies may be aimed at establishing the optimal working concentrations of chitosan in vaccine formulations, elucidating its impact on cellular and humoral immunity, and characterizing the duration of the resulting post-vaccination immune response.

The data obtained serve as the basis for the development of new safe and effective adjuvants based on low molecular weight chitosan. The proposed preparation meets modern requirements for the biocompatibility of immunostimulating products for use in veterinary medicine.

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