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BIOAVAILABILITY STUDY OF MAGNESIUM AND PHOSPHORUS COMBINED MEDICATION BASED ON CASEIN

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Aim. The Department of Biochemistry and Physiology of Animals, named after Academician Guly NUBIP of Ukraine, developed magnesium and phosphorus combined medication based on casein. Our aim was to test its bioavailability based on the ability to be hydrolyzed by a mixture of pancreatic digestive enzymes trypsin and chymotrypsin, also check the absence of cytotoxic effects on cell cultures.

Methods. To assess bioavailability, we used hydrolysis of the medication with a mixture of trypsin and chymotrypsin, followed by detection of hydrolysis products by polyacrylamide gel electrophoresis. A standard MTT-test performed on both MT-4 and Namalva cell lines was used to assess cytotoxic effects.

Results. Based on electrophoresis data, it was found that despite chemical modifications of the natural casein, the medication based on it is characterized by a high ability to hydrolyze by digestive enzymes under the same conditions as casein. Also, an MTT-test demonstrates that the medication has no cytotoxic properties against cell lines MT-4 and Namalva.

Conclusions. Since the negative effects of the drug associated with its digestibility and toxicity have not been observed, it is recommended to continue the study of its effects on living organisms.

Key words: magnesium, phosphorus, casein, chelate, *in vitro*, hydrolysis, cell culture, cytotoxicity, MTT reagent, NADH (nicotinamide adenine dinucleotide).

Magnesium is the fourth most common cation in the body, so it is indispensable for the organism, and its deficiency causes severe disorders [1]. The biological action of magnesium is mainly due to the formation of complexes with intracellular ligands and antagonism with calcium for binding with proteins and membrane structures. As mentioned above, properties determine the participation of magnesium in the synthesis of macromolecules such as nucleic acids and proteins. Over 300 enzymes require magnesium as a cofactor for their activity [2]. The participation of magnesium in the antioxidant defense of the organism is explained by the magnesium-dependent synthesis of glutathione [3, 4].

Medications containing phosphorus combined with organic radicals are well-known compounds. These compounds are used as a source of organic phosphorus to enhance mineral, carbohydrate, fat, and protein metabolism. Also, they are characterized by a high rate of metabolism and low toxicity [5, 6].

Macro- and microelements enter the body of animals and humans mainly orally. The lack of certain elements is corrected by the mineral and vitamin-mineral supplements in the diet and in case of acute deficiency — parenterally [7, 8]. The lack of certain elements is caused in humans by the impoverishment of the diet due to deep food processing, and in animals — the intensification of productivity compared to natural conditions [9]. A common problem for humans and animals is the lack or imbalance of some elements in geochemical zones [10].

Assimilation of magnesium in the body is carried out through the digestive system. Metal ions chelated by amino acids are absorbed in the small intestine similarly to dipeptides. The intensity of ion chelation directly correlates with the degree of their

assimilation [11]. After assimilation, chelates are hydrolyzed to release two amino acids. Then they are used for peptide synthesis and magnesium ion, which acts as a cofactor for apoenzymes [12–14].

Studies demonstrate numerous advantages of chelates of microelements and macroelements compared to inorganic and organic salts of these elements. The main advantage of chelates is higher bioavailability, which allows to decrease the dose and thus improves organoleptic parameters, and reduces the possibility of toxic or irritant effects due to overdose [15, 16]. The Department of Biochemistry and Physiology of Animals, named after Academician Guly NUBIP of Ukraine, developed magnesium and phosphorus combined medication based on casein [17].

The task of this study was to investigate the bioavailability of the developed medication, namely the possibility and rate of its hydrolysis by digestive enzymes, as well as its effect on cell viability, and to prove the absence of cytotoxic effects.

The important factor in the construction of the study scheme of the medication is the expected path of its metabolism with decomposition to peptides and individual amino acids. Due to the clarity of the metabolic pathway of the medication, the determination of the biotransformation of the drug in the classical sense is not required [18]. The study of the ability to hydrolyze peptide medication for oral administration is the main criteria for assessing the bioavailability of the latter because the rate of hydrolysis limits the rate of absorption [19]. To assess the bioavailability of the medication, the model hydrolysis with a mixture of digestive enzymes trypsin and chymotrypsin was used, followed by electrophoretic analysis of the obtained hydrolysis products. The comparison was carried out with the starting material for the synthesis of the medication, i. e. casein.

Possible cytotoxic and antiproliferative effects were investigated by standard methods for assessing cell viability and proliferation — the MTT-test and trypan blue staining [20, 21]. MT-4 and Namalva cell lines were used for the assay.

Materials and Methods

For the study, the magnesium and phosphorus combined medication based on casein was used, developed at the Department of Biochemistry and Animal Physiology named after Academician Guly NUBIP of Ukraine (patent 139705 UA dated January 10, 2020). The medication is a homogeneous powder, and chemically it is artificially phosphorylated casein from bovine milk as a ligand that chelates magnesium ions. The magnesium content is 10%, phosphorus — 12–15%, and the rest is protein [22]. For the experiment, the medication was synthesized in 2 identical parallels (medication-1 and medication-2).

Hydrolysis. For the hydrolysis of the medication, a digestive enzyme of the pancreas trypsin with chymotrypsin trace (≈3%) (FERAK, Germany) was used. The efficiency of the hydrolysis of the medication relative to casein (#C3400, Sigma, USA) was compared. The reaction mixture contained 50 mg/ml of the medication or 25 mg/ml of casein dissolved in 0.05 M Tris buffer solution, pH 7.4,containing 0.5 mg/ml of a mixture of trypsin and chymotrypsin [23]. The reaction was carried out for 90 min at a temperature of 37 °C in a water bath. Samples for electrophoretic analysis were taken after 0 min, 45 min, and 90 min from the beginning of the reaction. Samples were dissolved in the Laemmli buffer in a ratio of 1:1. They were added to the wells of the polyacrylamide gel approximately 100 µg of starting material per track [24]. The reaction products were analyzed with the analytical electrophoresis, in 12% of separative and 4% concentrating polyacrylamide gel to prove the hydrolysis process [25]. The analytical electrophoresis procedure was performed in a vertical electrophoresis chamber (Helicon); the molecular weight standard was estimated with a use of a pre-stained protein markers Page Ruler 26619 (10–180 kDa). The process of hydrolytic cleavage of the medication was determined densitometrically by the color intensity of tracks stained with Coomassie brilliant blue R-250. Interpretation of results based on the fact that the color intensity of fractions of the medication after trypsin treatment is inversely correlated with the number of peptide bonds in molecules subjected to hydrolytic cleavage [26]. Densitometric analysis was performed using Image J software.

Cytotoxicity. The studies were performed using cell lines MT-4 (culture of T-cell leukemia) and Namalva (B-cell line obtained with Burkitt's lymphoma). The following equipment was used, such as laminar (LS, laminar systems), CO_2 incubator (Medcenter Einrichtungen GmbH MMM-Group), centrifuge (K-26), multiwell spectrometer

(Labsystems Multiscan MS). The calculation and cell population visualization were performed using an inverted microscope AxioVert (Carl Zeiss) with Axio Vision software. Cells used in the studies were cultured in RPMI 1640 medium (Sigma, USA) containing 10% FBS (Sigma, USA).

The cultivation was performed in a humidified atmosphere with 5% CO_2 at a temperature of 37 °C. Cytotoxic activity was determined by the conventional method of determining mitochondrial activity by MTT-test, pre-determining the number of living cells by trypan blue staining and counting in the Goryaev's cytometer [20].

Cell suspensions were added to 96-well plates in the amount of 100 µl/well, at a concentration of $1 \cdot 10^5$/ml. The medication was applied to a final concentration of 0.031, 0.0625, 0.125, 0.25, 0.5, and 1 mg/ml in a culture medium in three independent parallel each. Four hours before the end of incubation of cells, 20 µl of MTT reagent (Sigma, USA) dissolved in PBS were added to a final concentration of 0.5 mg/ml. After four hours, the plates were centrifuged at 400 *g* for 10 minutes, and the supernatant was removed. 100 µl of DMSO (Serva, Czech Republic) was added to each well, after which the plates were placed on a shaker until the formazan crystals were dissolved. The optical density was determined at a wavelength of 540 nm.

Statistical analysis. Statistical processing of the results was performed by the conventional method of variation statistics using MS Excel software. It determined the mean (*M*), deviations of each measured value from M (a), quadratic deviations for each group (σ), and mean error (*m*). Statistic significance of the results was determined by the Student's *t*-test and the degree of probability of the difference between the values of *P*. The results with $P < 0.05$ were reliable.

Results and Discussion

The electrophoregram (Fig. 1, *A*) shows the comparability of the molecular weights of the casein fraction, the starting material for synthesis, and the final medication (*1, 2, 4, 7*).

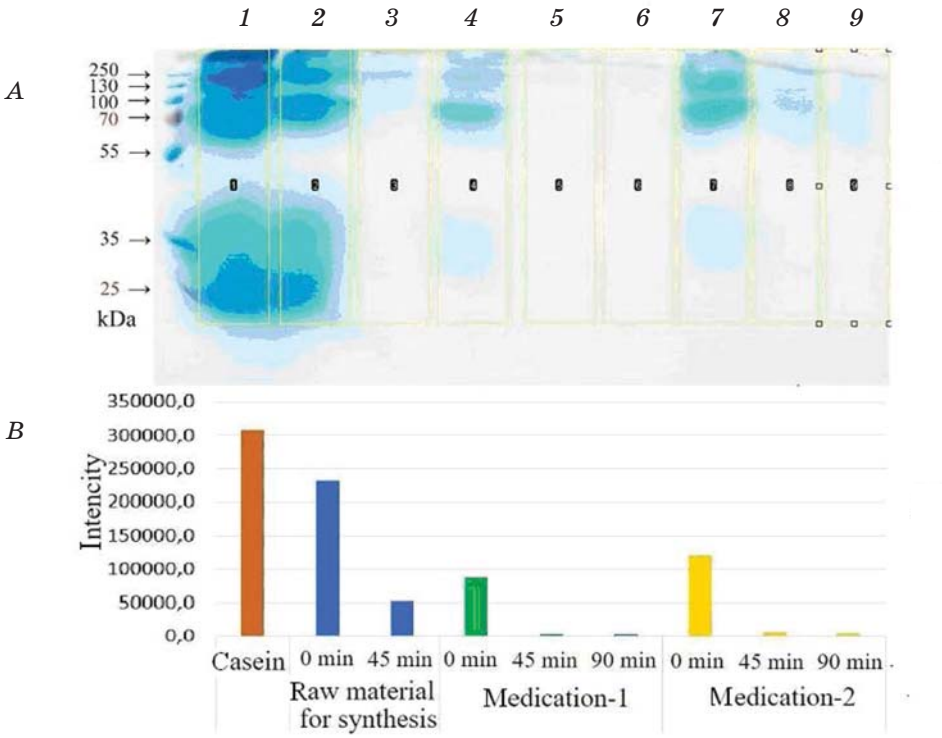

Fig. 1. A — **electrophoregram of the hydrolysate products of the magnesium and phosphorus combined medication based on casein:**

1 — casein, *2* — starting material for synthesis, *3* — starting material after treatment with trypsin 45 min, *4* — medication-1 before treatment with trypsin, *5* — medication- 1 after trypsin treatment 45 min, *6* — medication-1 after trypsin treatment 90 min, *7* — medication-2 before trypsin treatment, *8* — medication-2 after trypsin treatment 45 min, *9* — medication-2 after trypsin treatment 90 min;

***B* — the results of densitometric analysis of electropherogram *A*:**

the intensity of the color of the tracks in conventional units

Reducing the color intensity of tracks *3, 5, 6, 8, 9* obviouslyindicates that the medication has no worse ability to hydrolyze trypsin than the starting material for synthesis.

Hydrolysis followed by the detection of protein fractions in the polyacrylamide gel demonstrated that despite modification of the peptide bonds of the casein molecule, the medications retained the ability to be efficiently hydrolyzed by trypsin and chymotrypsin. The presence of high molecular weight zones (more than 30 kDa) can be explained by the ability of κ-casein to form stable complexes. The formation of these complexes depends on exposure to high temperatures. In the literature, chemical complexes between milk proteins are known as milk protein coaggregates [27, 28]. Due to the formation of stable coaggregates of casein, hydrolysis followed by visualization by electrophoresis demonstrates a particularly good visualization of the process.

Both medications showed identical results in the test for hydrolysis, so further there was used a homogenized mixture of them (hereinafter the medication).

The data obtained by vital staining of cells line MT-4 with trypan blue did not show a significant increase or decrease in proliferation compared to control (Table). Since the MTT-test did not show a significant difference in the effect of the drug on the MT-4 and Namalva cell lines, vital staining of the Namalva line with trypan blue was not performed.

Similar results were demonstrated by the MTT-test (Fig. 2).

The studies of the cytotoxic properties of the studied protein preparation showed the absence of obvious toxic influence against the cell lines MT-4 and Namalva in the all above-mentioned concentrations, so the data are presented for a concentration of 1 mg/ml as potentially the most toxic one (Fig. 2). The choice of MT-4 and Namalva cell lines as models for the evaluation of cytotoxic properties is explained by the subsequent test plan of the drug as an immunostimulant. The proliferative activity of the MT-4 line in the presence of the medication was slightly higher than in control, and the Namalva line was lower. However, a significant difference between the proliferative effect of the

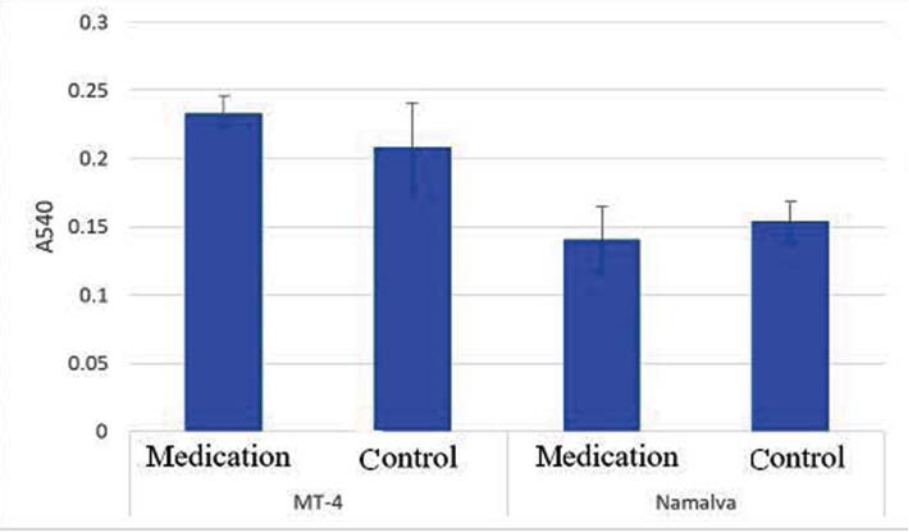

Fig. 2. **The results of indicating no cytotoxic effects of the studied protein preparation in MT-4 and Namalva cell lines ($n = 3$)**

The results of determining the number of living MT-4 cells by trypan blue staining

	Control	Medication
Concentration of living cells MT-4	$34.4 \cdot 10^3 \pm 1.2 \cdot 10^3$	$37.4 \cdot 10^3 \pm 5.6 \cdot 10^3$
Concentration of dead cells MT-4	$2.6 \cdot 10^3 \pm 0.7 \cdot 10^3$	$3.8 \cdot 10^3 \pm 2.2 \cdot 10^3$
% of dead cells MT-4	7.02*	9.22*

Note: * — $P < 0.05$ compared to control.

medication on cell cultures, compared with controls, was not observed. Therefore, it was found that the magnesium and phosphorus combined medication based on casein does not inhibit cellular respiration and does not have a marked cytotoxic effect on cells.

The electrophoregram of the medication fractions before and after hydrolysis shows a significant decrease in the staining intensity of the fractions inherent in the starting material after hydrolysis. It indicates the ability to hydrolyze the medication by the digestive enzymes under the same conditions as bovine milk casein. Casein has high bioavailability, so the medication with the similar properties can be characterized as bioavailable [29].

An important factor indicating the high bioavailability of the medication is that natural casein is used for synthesis, not a mixture of artificial amino acids. The presence of D-amino acids in mixtures of enantiomers can reduce bioavailability, in contrast to raw materials of natural origin containing only L-amino acids [30].

The increase in cell proliferation under the action of the medication was not expected from the beginning, because the cultural medium RPMI 1640 is balanced by the concentration of magnesium and phosphorus. Thus, the absence of antiproliferative effects is a promising result for future application of the studied preparation. Cytotoxicity studies can be used to predict toxic effects on the whole organism [31].

Conclusions

As a result of a set of studies, it was found that the magnesium and phosphorus combined medication based on casein is hydrolyzed by a mixture of trypsin and chymotrypsin and does not show an obvious cytotoxic effect on cell lines MT-4 and Namalva. Studies of possible cytotoxic properties did not show a statistically significant difference in the proliferation of cells under the action of the medication. Thus, the medication can potentially be used for animals after *in vivo* studies.

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Conflicts of Interest. The authors declare no conflicts of interest.

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ДОСЛІДЖЕННЯ КОМБІНОВАНОГО ПРЕПАРАТУ МАГНІЮ І ФОСФОРУ НА ОСНОВІ КАЗЕЇНУ

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Мета. Перевірити біодоступність розробленого комбінованого препарату магнію і фосфору на основі казеїну, ґрунтуючись на здатності гідролізуватися сумішшю травних ензимів підшлункової залози — трипсину і хімотрипсину, та можливість цитотоксичного впливу на культури клітин.

Методи. Для досягнення поставленої мети використовували гідроліз препарату сумішшю трипсину і хімотрипсину з подальшою детекцією продуктів гідролізу методом електрофорезу в поліакриламідному гелі. Для оцінювання цитотоксичних ефектів застосовували стандартний МТТ-тест на культурах клітин МТ-4 і Namalva.

Результати. Досліджено вплив ультразвукової дезінтеграції на лігноцелюлозну сировину з подальшим її використанням для отримання біопалива за допомогою мікробіологічної конверсії. Показано можливість використання отриманих компонентів лігноцелюлози як субстрату після ультразвукової дезінтеграції для мікробіологічного синтезу бутанолу. Встановлено, що найбільше накопичення бутанолу (2,4 г/л) отримано за 5% вмісту сухої речовини у середовищі, 5 хв оброблення та питомій потужності ультразвукової дезінтеграції 0,72 Вт/мл.

Висновки. Оскільки негативних ефектів препарату, пов'язаних з його перетравністю і токсичністю, не виявлено, рекомендовано продовжити вивчення його впливу на моделі *in vivo*.

Ключові слова: магній, фосфор, казеїн, хелат, гідроліз, культура клітин, цитотоксичність, МТТ-реагент, NADH (нікотинамідаденіндинуклеотид).

ИССЛЕДОВАНИЕ КОМБИНИРОВАННОГО ПРЕПАРАТА МАГНИЯ И ФОСФОРА НА ОСНОВЕ КАЗЕИНА

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Цель. Проверить биодоступность разработанного комбинированного препарата магния и фосфора на основе казеина, основываясь на способности гидролизоваться смесью пищеварительных энзимов поджелудочной железы трипсином и химотрипсином, и возможность цитотоксического воздействия на культуры клеток.

Методы. Для достижения поставленных целей был использован гидролиз исследуемого препарата смесью трипсина и химотрипсина с последующей детекцией продуктов гидролиза методом электрофореза в полиакриламидном геле. Для оценки цитотоксических эффектов использовали стандартный МТТ-тест на культурах клеток МТ-4 и Namalva.

Результаты. Исследовано влияние ультразвуковой дезинтеграции на лигноцеллюлозное сырье с дальнейшим использованием для получения биотоплива с помощью микробиологической конверсии. Показана возможность применения полученных компонентов лигноцеллюлозы после обработки ультразвуковой дезинтеграцией как субстрата для микробиологического синтеза бутанола. Установлено, что наибольшее накопление бутанола (2,4 г/л) получено при 5% содержании сухого вещества в среде, 5 мин обработке и удельной мощности ультразвуковой дезинтеграции 0,72 Вт/мл.

Выводы. Поскольку отрицательных эффектов препарата, связанных с его переваримостью и токсичностью, не выявлено, рекомендуется продолжить изучение его влияния на модели *in vivo*.

Ключевые слова: магний, фосфор, казеин, хелат, гидролиз, культура клеток, цитотоксичность, МТТ реагент, NADH (никотинамидадениндинуклеотид).