

ИННОВАЦИОННЫЙ ПРЕПАРАТ АМОРФНОГО КАЛЬЦИЯ **CALCIVER®**



В перечне биологических эффектов кальция (Ca) в организме человека на одном из первых по значимости мест находится участие этого минерального вещества в построении зубов и костей. Важная роль Ca в проведении нервного импульса, свертывании крови, других фундаментальных

процессах жизнедеятельности обусловила наличие в организме такой системы регуляции обмена этого минерала, которая обеспечивает строго постоянную концентрацию Ca в крови.

Это обеспечивается, в основном, за счет потока ионов из полости кишки в кровь. При первичном (дефицит Ca в рационе) или вторичном (следствие заболеваний, побочные действия лекарств и др.) нарушении этого процесса, постоянство уровня Ca крови обеспечивается за счет разрушения костей и зубов.

Несмотря на распространенность Ca в природе, в т.ч. в продуктах питания, проблема дефицита кальция сохраняет свою актуальность. Говоря бытовым языком, эта проблема состоит в том, чтобы «заставить» Ca лучше всасываться в кишечнике и откладываться в костях и зубах, обеспечивая их нормальное функционирование. Таким образом, на первое место выдвигается проблема биодоступности Ca, содержащегося в продуктах питания (включая биологически активные добавки) и медикаментах. На рынке представлено несколько соединений Ca, но преобладает карбонат кальция, обладающий низкой биодоступностью. В целом, имеющиеся в продаже препараты Ca (чистые соли и их смеси) по своей усвояемости мало различаются между собой. Создание более эффективных продуктов требует принципиально нового подхода, и таковым является использование аморфного Ca.

Аморфный карбонат кальция (ACa), по сравнению со всеми другими формами CaCO_3 , обладает самой большой площадью активной поверхности. Будучи нестабильным в обычных условиях, ACa в природе на удивление широко распространен как предшественник кристаллических форм Ca, например, входящих в состав раковин моллюсков. Биогенный ACa, обычно, содержит

стабилизаторы, в частности ионы магния, предотвращающие нежелательный процесс кристаллизации.

Недавно химики разработали технологию производства АСа в промышленных масштабах, использованную как базовую для производства препарата Calciver®. Для повышения специфической эффективности АСа был использован не только карбонат Са, но также и другие соли – гидрокарбонат, цитрат, гидроцитрат, а в качестве стабилизатора- цитрат магния. Как известно, ионы Са из смесей солей всасываются лучше, чем из одной.

Специфическая активность препарата АСа оценивалась в экспериментах на классическом объекте – цыплятах. В частности, АСа и препараты сравнения вводили животным per os, после чего определяли содержание Са в печени и сыворотке крови.

Таблица 1. Содержание минеральных веществ в ткани печени и сыворотке крови цыплат через 45 мин после перорального введения разовой дозы соединений кальция.

Группа животных и форма препарата Са	Доза введенного Са, мг	Содержание минеральных веществ в :			
		печени	сыворотке крови		
			Са, мкг/г	Са, ммоль/л	Мg, ммоль/л
1. Контроль	0	0,021±0,011	2,82±0,06	0,67±0,16	37,23±0,72
2. Кальция хлорид	2,4	0,130±0,028*	3,02±0,17	0,81±0,20	38,48 ±1,13
3. Кальция карбонат		0,146±0,024*	2,96±0,11	0,96±0,23	31,57± 0,88
4. Аморфный кальций		0,164±0,022*	2,98±0,10	1,00±0,13	39,02± 0,70

* достоверность различий по сравнению с контролем $p < 0,05$

Как видно из таблицы 1:

- **АСа с точки зрения биодоступности равен или превосходит хлористый Са, который не может применяться длительно.**
- **АСа превосходит усвояемость карбоната Са на 126%.** К тому же, в отличие от последнего, АСа не приводит к снижению уровня железа в крови в результате образования в кишечнике нерастворимых комплексов с фосфатами и железом.

Са всасывается в кишечнике двумя путями: активно, через эпителиальные клетки с потреблением энергии, и – пассивно, через межклеточные пространства. Витамин D₃ является основным стимулятором активного процесса всасывания Са. Поэтому Са и витамин D₃ являются классической комбинацией, входящей в состав практически всех современных препаратов Са, независимо от вида соединения Са. *В ходе оценки антирахитического действия на цыплятах АСа показал более выраженную, чем у комбинации карбоната Са и витамина D₃, способность восстанавливать минерализацию костей и ослаблять вторичный гиперпаратиреоз.*

Как отмечалось выше, более сложной задачей в профилактике и лечении метаболических остеопатий является стимуляция реминерализации скелета, которая актуальна при рахите, (включая врожденные рахитоподобные заболевания), остеопорозе, остеопорозе. В медицине с этой целью применяют аналоги пиро-фосфорной кислоты, т. н. бифосфонаты, которые достаточно дороги, не везде доступны, часто вызывают побочные реакции, и их действие нельзя назвать физиологичным. Поэтому непрерывно ведутся поиски натуральных веществ, в первую очередь, растительного происхождения, обладающих остеопротекторным действием. Речь идет о влиянии на дифференцировку и функции клеток костной ткани и хряща, процессы минерализации и др.

Самая большая библиография по теме «растения- остеопротекторы» посвящена

шалфею– *Salvia miltiorrhiza* (Шалфей краснокорневищный). Это растение давно используется в китайской народной медицине для лечения болезней костей. Научные данные свидетельствуют, что компоненты *S .miltiorrhiza* угнетают дифференцировку клеток-предшественников в остеокласты и их созревание (*Complemen .Alterna. Med., 2011 11, 120, 1472*), тем самым замедляя резорбцию костной ткани. Остеопротекторное действие экстракта шалфея продемонстрировано на экспериментальных моделях остеопороза: на

овариоэктомированных крысах (*Immunopharmacol. Immunotoxicol.*, 2004, 26, 1, 135), при стероидном остеопорозе (*PLoS One*, 2012, 7, 4, e34647), аллоксановом диабете у крыс (*Zhongguo Zhong Yao Za Zhi*, 2012, 37, 11, 1659). С учетом доказанной безопасности экстракта *S. miltiorrhiza* для взрослых (данных по детям и беременным недостаточно), этот фитодериват впервые был включен в рецептуру второй версии разрабатываемого препарата аморфного кальция в комбинации с витамином D₃ – Calciver Plus®.

Экспериментальная оценка специфического биологического действия Calciver Plus® проводилась на модели стероидного остеопороза у цыплят. Как известно, препараты глюкокортикоидов вызывают остеопороз, главным образом, за счет подавления образования органического матрикса кости, а также вторичного гиповитаминоза D₃ (*Tohoku J Exp Med*, 1980, 132, 3, 261).

В проведенном эксперименте ежедневное введение цыплятам преднизолона в течение двух недель вызвало выраженные изменения показателей фосфорно-кальциевого обмена: 5-кратное увеличение массы парашитовидных желез, гипофосфатемия, угнетение образования органической части костей (что привело к повышению процента зольности) и торможение их роста, снижение плотности, падение на 52% активности щелочной фосфатазы крови, как результат угнетения функций остеобластов.

Совместное введение цыплятам преднизолона и препарата Calciver Plus® в течение двух недель нормализовало уровень фосфатемии и массу парашитовидных желез, а также вызвало положительные изменения параметров костей (Таблица 2). Так, на 10% увеличилась длина б. берцовой кости, на 5,4% – ее плотность, нормализовалось содержание минеральных веществ, что свидетельствует о существенной нормализации структуры костной ткани под действием изучаемого препарата.

Таблица 2. Параметры большой берцовой кости цыплят получавших преднизолон (1,5 мг/день) при одновременном введении per os препарата Calciver Plus® или препарата сравнения (карбонат Ca + витамин D₃).

Группа	Параметры большой берцовой кости		
	Длина, см	Плотность, г/см ³	Содержание золы, %
Контроль	6,8±0,28	0,75±0,03	46,4±1,2
Преднизолон	5,6±0,55*	0,67±0,03*	49,8±0,7*
Преднизолон + CaCO ₃ + D ₃	5,8±0,91	0,69±0,02*	48,8±0,6*
Преднизолон + АСа	6,1±0,75	0,71±0,02	47,9±0,7

*достоверность различий по сравнению с контрольной группой p<0,05

Таким образом, включение в рецептуру препарата аморфного Са экстракта корневища шалфея (*S. miltiorrhiza*) привело к усилению остеопротекторного действия препарата за счет анаболического эффекта на органический матрикс кости, что проявилось у цыплят с экспериментальным стероидным остеопорозом.

В состав Calciver® входят вода, комплекс солей аморфного кальция (гл. образом, карбоната), загуститель- ксантановая камедь, стабилизатор- цитрат магния, натуральный подсластитель- стевиогликозид, холекальциферол, ванильный ароматизатор. Содержание (в 20 г) кальция – 500 мг, витамина D₃ – 25 мкг. Препарат имеет статус биологически активной пищевой добавки. Продукт по консистенции и вкусу напоминает йогурт или крем, выпускается в тубах по 200 г. Calciver Plus® дополнительно содержит экстракт корневища *Salvia miltiorrhiza* 7 mg

Таким образом, разработанный препарат аморфного кальция существенно отличается от имеющихся на рынке препаратов Са более высокой биодоступностью, специфической активностью, меньшей вероятностью побочных действий (в частности, развития железо-дефицитной анемии), удобной формой, особенно подходящей для детей и людей в возрасте.

Способ применения: **Calciver**® рекомендуется всем. Детям - 1 чайная ложка в день, что эквивалентно 250 мг кальция и 2,5 мкг витамина D₃, перед едой, запивая водой. Дозировка для взрослых, в т.ч. беременных женщин, - 1 десертная ложка в день (500 мг кальция, 5 мкг витамина D₃). Препарат применяется для профилактики рахита, кариеса у детей. Calciver® также является эффективным источником высоко-усвояемого кальция, столь необходимого при беременности для обеспечения здоровья матери и плода.

Способ применения: **Calciver Plus**® показан взрослым (40+) в дозе, указанной выше. Показания – остеопороз любой этиологии, переломы костей, длительный прием препаратов глюкокортикоидов. Препарат не рекомендуется детям, подросткам и беременным женщинам.

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Amorphous Calcium Salt Composition Bioavailability Evaluation in Chickens

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Abstract

Despite the market saturation with a wide range of calcium preparations in dietary supplements as well as in pharmaceuticals, calcium product safety and efficacy remain an item for further optimization. Bones and teeth mainly consist of calcium phosphate, but tablets and capsules are predominantly produced from calcium carbonate. On the other hand, in human food Ca^{2+} is bound with a lot of anions—carbonates, sulphates, chlorides, phosphates, citrates, oxalates etc. It seems that traditional calcium formulations due its low bioavailability cannot provide all spectra of biological calcium-dependant effects and dietary calcium might be safer and more effective than traditional calcium supplements and medicines. To increase calcium salt efficacy a new formulation of calcium salts has been developed. It is a mixture (water suspension) of amorphous calcium carbonate, citrate, hydrocarbonate, as well as magnesium hydrocarbonate and hydrocitrate as stabilizers. The capacity of a cockerel's duodenal mucosa to absorb Ca^{2+} after peroral ingestion of the mentioned composition *in vivo* was as much as 126% higher in comparison with CaCO_3 suspension intake. Vitamin D_3 enhanced the amorphous antirachitic activity of calcium salts. The aim of the study was to compare original amorphous calcium salt composition bioavailability with Ca salts most often in food and medicines, as well as to evaluate the antirachitic activity of the mentioned composition in combination with vitamin D_3 in chickens.

Keywords

Calcium Absorption, Amorphous Calcium, Experimental Rickets, Chicken

1. Introduction

Due to the dependence of a huge number of the organism's essential biochemical

and physiological processes of calcium (Ca), the complex system of Ca metabolism regulation in mammals has been developed during evolution. This mechanism regulates Ca intestinal absorption, supporting the constant cations blood concentration in any physiological periods. In the case of inadequate Ca absorption from the gastrointestinal tract, stable calcemia is supported on the account of the destruction of the skeleton due to hyperactivity of the parathyroid glands [1].

Despite the wide prevalence of calcium minerals in nature, there is a global problem of Ca deficiency for livestock and humans. Of great importance is not only Ca content in the diet, but also Ca bioavailability. Inadequate consumption of dairy products, excessive drinking water purification from minerals and growing popularity of ortho-phosphate containing light drinks are tending toward Ca deficiency [2]. But medicinal technological progress, prolongation of human life, and the increase of the number of patients with earlier incurable gastroenterological and endocrinological diseases promote the prevalence of secondary Ca deficiency.

Intestinal Ca absorption occurs through the transcellular and paracellular pathways. The first route comprises the entrance of Ca across the brush border membranes (BBM) of enterocytes through special epithelial Ca channels; Ca movement from BBM to basolateral membranes by binding proteins with high Ca affinity (as calbindin) and Ca extrusion into the blood. Plasma membrane Ca-ATPase and a sodium calcium exchanger are involved in the exit of Ca from enterocytes. Paracellular Ca transport includes the movement of the ions through transmembranes of tight junction structures and depends on the electrochemical gradient of the intestinal epithelium. There is evidence of crosstalk between both pathways in intestinal Ca transport. Under the impact of pathogenic factors (oxidative stress) due to the decrease in expression of several molecules of both pathways, Ca absorption is inhibited [3]. Vitamin D, more precisely, its active metabolite $1.25(\text{OH})_2\text{D}_3$ or calcitriol, which is the principal promoter of Ca intestinal absorption [4], acts in the opposite direction—increases gene and protein expression, involved in both pathways.

The presence of two pathways of intestinal Ca absorption has an important biological meaning. The paracellular pathway provides a passive basic flow of Ca into the organism's internal environment. The transcellular pathway is active, energy dependent and provides "fine" Ca absorption regulation, its adaptation to the mineral content in the diet, bioavailability, organism physiological status etc. Wherein the effects on Ca intestinal absorption of other hormones—thyroid, parathyroid, growth hormones, estrogens, prolactin—are mediated via calcitriol synthesis [5].

The above algorithm of Ca absorption regulation implies an important practical conclusion. In the case of Ca deficiency, it is rational to stimulate both Ca intestinal transport pathways. So, the combination of Ca preparations and vitamin D became the "golden standard" for metabolic bone disease prevention and treatment [6] [7].

Thus, the main factors which determine Ca intestinal absorption are vitamin D status, Ca content in the diet as well as mineral bioavailability. In general, Ca bioavailability depends on the corresponding salt solubility, capability to provide absorbable Ca ions. Ca salts of most frequent use, carbonate, citrate, pidolate, dobesilate, gluconate, phosphate and lactate, contribute different quantities of elementary Ca [8]. The percentage content of Ca is minimal for lactate and gluconate. Ca absorption from calcium citrate is consistently significantly higher than from calcium carbonate by 27.2% on an empty stomach, and by 21.6% with meals [9], but calcium carbonate has better cost effectiveness [10].

A lot of natural compounds modulate Ca salt solubility. Thus, sodium gluconate can increase the solubility of calcium lactate [11]. Amino acids and bile components reduce Ca salt precipitation in the intestine, increasing ion bioavailability [12]. It should be noted that Ca from dairy products as well as high-calcium mineral waters have high absorbability [13].

It seems the search for new Ca containing substances with high bioavailability has reached a certain impasse. Food does not contain pure Ca compounds but is the mixture of a lot of chemicals. On the other hand, Ca from salt mixtures has higher bioavailability in comparison with mono salts. In addition, food Ca toxicity is less than that of pure Ca chemicals at the same dosage [14]. This allows us to conclude that to develop highly bioavailable Ca compounds we should return to nature. From this point of view, amorphous Ca salts seem promising.

Amorphous calcium carbonate (ACC) has the highest reported specific surface area of all current forms of calcium carbonate [15]. Although unstable under ambient conditions, ACC is surprisingly widespread in biology either as a precursor phase, which later transforms into more crystalline forms, or as a stabilized phase sometimes coexisting with crystalline polymorphs [16]. Biogenic ACC often contains stabilizers to avoid or delay unwanted crystallization. Magnesium is commonly present as one of the stabilizing agents [17]. A study on rats revealed that Ca absorption from ACC preparations was up to 40% higher than from calcium carbonate [18]. Recent technology allows the synthesis of a stable amorphous calcium salt, containing different anions. The method of preparation of the composition of amorphous Ca and Mg salts has been developed recently [19]. Water and inorganic additives: calcium carbonate, calcium citrate, calcium hydrocitrate, magnesium citrate were used to stabilize the ACC.

The aim of the study was to compare original amorphous calcium salt composition bioavailability with Ca salts most often in food and medicines, as well as to evaluate the antirachitic activity of the mentioned composition in combination with vitamin D₃ in chickens.

2. Materials and Methods

2.1. Ethics Statement

All experimental procedures were approved by the local Animal Ethics Committee.

2.2. Amorphous Calcium Salt Composition (ACaSC)

ACaSC was prepared using our previously developed method [19] [20]. ACaSC is a thick white suspension and contains calcium carbonate, calcium citrate, calcium hydrocitrate and magnesium citrate. The total calcium content in the mixture was 50 mg/g of calcium and magnesium-0.64 mg/g. ACaSC pH (diluted with water 1:1) is 7.53.

2.3. Experimental Design and Animals

Lohmann Brown cockerels were used for the laboratory investigations. Chickens were delivered from the Latvian poultry company “Balticovo Ltd.” and housed in cage units with free access to food and water. The animals were fed a wheat-barley full-fed diet, containing all necessary nutrients. Calcium content in the diet was 7.75 mg/kg.

Two experiments have been done to study:

- 1) Dependence of Ca absorption in chicken duodenum on the chemical composition of ingested Ca salt, its dosage and exposition time;
- 2) Antirachitic efficacy of amorphous salt composition (ACaSC) with and without cholecalciferol (vitamin D₃) supplementation.

At the end of the experiment the cockerels were weighted and sacrificed by decapitation, in accordance with the recommendations of the European Convention [21] for the euthanasia of experimental animals.

Experiment 1. 30-day-old chickens with a body weight of 289.3 ± 29.5 g were randomized in 7 experimental groups of 7 heads each. Ca absorption in chicken duodenum was studied *in vivo*. 18 hours before the experiment the food was removed from the cages; drinking water was available. Different Ca salts—carbonate, chloride (as hexahydrate) and a tested amorphous Ca composition suspended (diluted) in 2 ml of distilled water were ingested *per os* 45 or 60 min before decapitation. The amount of administrated Ca with all kinds of salts was 1.2 or 2.4 mg. The chickens of control group received drinking water only.

At the end of the exposition period, vein (*v. jugularis*) blood sampling was done. The chickens were decapitated, the liver and isolated intestine segments removed. Duodenum content was drained into a test-tube; segments were rinsed with 1 ml of 154 mM NaCl and put into a separate test-tube. The duodenum (intestinal content and mucosa) was analyzed for Ca content. The amount of Ca absorbed was calculated as the difference between Ca content just after ingestion infusion of the tested compounds and after the exposition period and expressed as nmol/g tissue.

Experiment 2. 2-day-old Lohmann Brown cockerels were used in the study. The birds were weighed and randomized in 6 groups (n = 7). The chickens were fed *ad libitum* vitamin D-free feed, containing: corn flour-40%, barley-30%, soybean meal-15%, casein-10%, fodder yeast-3%, dicalcium phosphate-0.5%, chalk-0.25%, salt-0.5%, Ca content-0.81%, phosphorus-0.64% (basic ration). The

control group was provided the mentioned diet (control) but enriched with cholecalciferol (400 IU/kg). The experimental algorithm is as follows (**Table 1**).

At the age of 4 weeks venous blood sampling was done, the chickens were decapitated, the parathyroid glands were extirpated and weighted, the femurs collected for subsequent analysis. Parathyroid relative weight was calculated as a gland and body weight ratio. In blood serum calcium, phosphorus concentration and alkaline phosphatase activity were measured. The gravity of defatted and dried femur was measured; also, calcium and phosphorus content in bone was analyzed.

Three days before the decapitation of the birds, the daily balance of minerals in the chickens' bodies was evaluated. For 3 days, the food eaten every 24 h as well as the birds' litter were controlled (weighted, dried and analyzed for Ca content).

2.4. Laboratory Determinations

At the end of the experiment, Fe, Mg and Ca concentrations in organs and tissues were estimated using a flame atomic absorption spectrophotometer (Perkin-Elmer, model AAnalyst 700) [22]. Duodenal mucosa were homogenized in a solution of ice-cold tris-buffer (pH 7.4) using a glass homogenizer and a motor-driven Teflon pestle at 3000 rpm for 1 min to yield a 20% (w/v) homogenate. Proteins were denatured with trichloroacetic acid (120 g/l) and removed by centrifugation for 10 min at 600 g.

The liver samples were dried at 1050 C to constant weight, ashed in a muffle furnace at 6500 C, and subsequently dissolved in 6 N-HCl.

Blood serum was analyzed for Fe, Mg, Ca and alkaline phosphatase performed on ILAB 300+ analyzer (Instrumentation Laboratory, USA).

Table 1. Calcium salt antirachitic activity study in chickens.

Experimental group	Characteristic of group
1) Control	Basic ration + vitamin D ₃
2) Rickets	Basic ration (vitamin D ₃ free)
3) Basic ration + CaCO ₃	Basic ration (vitamin D ₃ free); starting from the age of 14 days the chickens received per os calcium carbonate for two weeks (Ca ²⁺ 5 mg/day)
4) Basic ration + CaCO ₃ + D ₃	Basic ration (vitamin D ₃ free); starting from the age of 14 days the chickens received per os calcium carbonate for two weeks (Ca ²⁺ 5 mg/day) and cholecalciferol 0.25 µg/day
5) Basic ration + ACaSC*	Basic ration (vitamin D ₃ free); starting from the age of 14 days age the chickens received per os amorphous calcium salts for two weeks (Ca ²⁺ 5 mg/day)
6) Basic ration + ACaSC + D ₃	Basic (vitamin D ₃ free) ration; starting from the age of 14 days the chickens received per os amorphous calcium salts for two weeks (Ca ²⁺ 5 mg/day) and cholecalciferol 0.25 µg/day

*Amorphous calcium salt composition contains calcium carbonate, calcium citrate, calcium hydrocitrate, magnesium citrate.

2.5. Statistical Analysis

All statistics were performed using the software Statistica 7. Results of investigated parameters are presented as means \pm SE. Multiple group comparison was done using one-way ANOVA and Post-hoc Tukey HSD test.

3. Results and Discussion

The aim of the first experiment was to compare the impact of different ingested Ca salts on Ca content in duodenal mucosa as well as in blood serum and in the liver.

ACaSC specific activity was compared with Ca chloride, which is characterized by high Ca bioavailability, as well as with calcium carbonate – the most commonly used Ca salt in food and medicines but having low solubility and bioavailability. In dose 1.2 mg Ca all tested preparations did not induce statistically significant changes neither in the duodenal mucosa, nor the blood serum (Table 2 and Table 3). However, induced Ca accumulation in the liver was found by all formulations, but it was the same in all groups.

Table 2. Mineral content in chickens duodenal mucosa after peroral ingestion of amorphous calcium salt composition (ACaSC) and comparable calcium salts (ingesting period 60 min).

Ca formulation in experimental group	Ca dose, mg	Mineral content in duodenal mucosa, $\mu\text{g/g}$		
		Ca	Fe	Mg
1) Control	0	0.53 \pm 0.13 ^{a*}	0.11 \pm 0.03 ^a	0.55 \pm 0.10 ^b
2) Calcium chloride	1.2	0.70 \pm 0.20 ^{bc}	0.12 \pm 0.03 ^a	0.60 \pm 0.13 ^{ab}
3) Calcium carbonate	1.2	0.68 \pm 0.14 ^c	0.10 \pm 0.01 ^a	0.89 \pm 0.16 ^a
4) ACaSC**	1.2	0.73 \pm 0.18 ^{bc}	0.09 \pm 0.04 ^a	0.81 \pm 0.25 ^{ab}
5) Calcium chloride	2.4	0.98 \pm 0.25 ^{bc}	0.09 \pm 0.01 ^a	0.58 \pm 0.20 ^{ab}
6) Calcium carbonate	2.4	1.08 \pm 0.22 ^b	0.11 \pm 0.04 ^a	0.70 \pm 0.34 ^{ab}
7) ACaSC	2.4	1.63 \pm 0.26 ^a	0.09 \pm 0.01 ^a	0.56 \pm 0.18 ^{ab}

*Statistically different or similar within column according to Post-hoc Tukey HSD test ($p < 0.05$);

**Amorphous calcium salt composition contains calcium carbonate, calcium citrate, calcium hydrocitrate, magnesium citrate.

Table 3. Mineral content in chicken liver and blood serum after peroral ingestion of amorphous calcium salts composition (ACaSC) and comparable calcium salts (ingesting period 45 min).

Ca formulation in experimental group	Ca dose, mg	Ca content in liver, $\mu\text{g/g}$	Mineral content in blood serum		
			Ca, mmol/L	Mg, mmol/L	Fe, mg/L
1) Control	0	0.021 \pm 0.011 ^{b*}	2.82 \pm 0.06 ^a	0.67 \pm 0.16 ^b	2.08 \pm 0.06 ^{ab}
2) Calcium chloride	1.2	0.163 \pm 0.063 ^a	2.77 \pm 0.13 ^a	0.82 \pm 0.11 ^{ab}	2.12 \pm 0.06 ^a
3) Calcium carbonate	1.2	0.150 \pm 0.016 ^a	2.89 \pm 0.07 ^a	0.69 \pm 0.08 ^b	1.98 \pm 0.10 ^{bc}
4) ACaSC**	1.2	0.144 \pm 0.026 ^a	2.81 \pm 0.11 ^a	0.70 \pm 0.05 ^b	2.03 \pm 0.05 ^b
5) Calcium chloride	2.4	0.130 \pm 0.028 ^a	3.02 \pm 0.17 ^a	0.81 \pm 0.20 ^{ab}	2.15 \pm 0.08 ^a
6) Calcium carbonate	2.4	0.146 \pm 0.024 ^a	2.96 \pm 0.11 ^a	0.96 \pm 0.23 ^{ab}	1.82 \pm 0.08 ^c
7) ACaSC	2.4	0.164 \pm 0.022 ^a	2.98 \pm 0.10 ^a	1.00 \pm 0.13 ^a	2.18 \pm 0.05 ^a

*Statistically different or similar within column according to Post-hoc Tukey HSD test ($p < 0.05$);

**Amorphous calcium salt composition.

At a dose of 2.4 mg Ca, the ingestion of Ca salts caused an increase of Ca content in intestinal mucosa. The effect of ingested Ca salts—Ca chloride, Ca carbonate and ACaSC was 85%, 103% and 207% stronger in comparison with control, respectively. Taking into account the fact, that Ca absorption by duodenal mucosa is the best indicator of intestinal absorption of ions [1] we can conclude that ACaSC bioavailability exceed calcium chloride and calcium carbonate by 66% and 51%, respectively.

Ca content in the liver of animals of the 7th group did not differ from that of groups 2 - 4, the calcemia level tended to increase, but not significantly. Attention is drawn to the fact of the decrease of iron concentration in blood serum in animals of group 6 (received calcium carbonate), although this fact was not observed in groups 5 (calcium chloride) and 7 (ACaSC) (Table 1). The probable mechanism of such a phenomenon is a luminal event: calcium carbonate with phosphates and iron ions form an insoluble complex, which decreases Fe bioavailability [23] and can cause iron deficiency anaemia [24]. From the point of view of practical application, ACaSC has an advantage in comparison with calcium chloride, which is not indicated for long term use.

In the second experiment antirachitic efficacy of ACaSC *per os*, as well as in combination with vitamin D₃, was tested. At the age of 4 weeks the rachitic chickens' body weight was 54% of the control. The birds had an untidy appearance, a soft and deformed beak. Hypocalcemia and hypophosphatemia were detected in the blood serum, the activity of alkaline phosphatase increased more than 2.5 times; hyperplasia of parathyroid glands was found: its relative weight exceeded the control 10 times (Table 4). The ash content in the femur was significantly less in comparison with the control (Table 5). This is the result of impaired bone mineralization due to primary calcium and vitamin deficiency as well as secondary hyperparathyroidism [1].

Under the influence of diet enrichment with calcium carbonate manifestations of rachitic in Group 3 weakened (increased blood serum Ca, diminished alkaline

Table 4. Impact of calcium preparations and vitamin D₃ peroral ingestion on biochemical indices of blood serum and parathyroid gland relative weight of chickens with rickets.

Experimental group	Blood serum			Parathyroid gland relative weight, mg/100 g BW
	Ca, mmol/L	P, mmol/L	Alkaline phosphatase, IU	
1) Control (D ₃)	2.66 ± 0.13 ^{ab}	1.52 ± 0.07 ^a	14.2 ± 2.18 ^c	0.53 ± 0.10 ^d
2) Rickets (basic ration)	1.98 ± 0.21 ^c	1.04 ± 0.06 ^c	38.7 ± 3.31 ^a	5.24 ± 0.45 ^a
3) Basic ration + CaCO ₃	2.12 ± 0.08 ^c	1.14 ± 0.05 ^c	33.7 ± 3.13 ^a	3.50 ± 0.15 ^b
4) Basic ration + CaCO ₃ + D ₃	2.55 ± 0.11 ^{ab}	1.54 ± 0.09 ^a	17.3 ± 1.59 ^{bc}	0.72 ± 0.22 ^{cd}
5) Basic ration + ACaSC**	2.34 ± 0.11 ^b	1.30 ± 0.05 ^b	21.1 ± 2.37 ^b	0.80 ± 0.12 ^c
6) Basic ration + ACaSC + D ₃	2.70 ± 0.15 ^a	1.53 ± 0.09 ^a	13.9 ± 1.74 ^c	0.50 ± 0.09 ^d

*Statistically different or similar within column according to Post-hoc Tukey HSD test (p < 0.05);

**Amorphous calcium salt composition.

Table 5. The effect of calcium salts and vitamin D₃ administration on body weight dynamic and content of ash in femur in chickens with rickets.

Experimental group	Chicken body weight, g			Ash content in the femur, %
	1 day	14 day	28 day	
1) Control	43.66 ± 7.23 ^{a*}	158.3 ± 10.9 ^a	337.5 ± 31.7 ^a	48.93 ± 3.84 ^a
2) Ricket (basic ration)	44.02 ± 7.49 ^a	128.1 ± 20.7 ^{ab}	180.5 ± 10.9 ^c	22.85 ± 2.35 ^c
3) Basic ration + CaCO ₃	44.50 ± 6.92 ^a	131.5 ± 15.9 ^{ab}	219.1 ± 16.2 ^b	29.86 ± 1.76 ^b
4) Basic ration + CaCO ₃ + D ₃	43.46 ± 8.44 ^a	138.5 ± 7.07 ^b	302.4 ± 17.7 ^a	46.85 ± 4.47 ^a
5) Basic ration + ACaSC**	42.56 ± 6.41 ^a	130.1 ± 17.3 ^{ab}	236.1 ± 12.0 ^b	30.77 ± 1.42 ^b
6) Basic ration + ACaSC + D ₃	45.36 ± 4.86 ^a	133.1 ± 9.91 ^b	317.0 ± 11.3 ^a	49.11 ± 2.35 ^a

*Statistically different or similar within column according to Post-hoc Tukey HSD test ($p < 0.05$);

**amorphous calcium salt composition.

phosphatase activity and parathyroid relative gland weight), but did not disappear completely. This was achieved using calcium carbonate in combination with cholecalciferol (Group 4). Based on body weight gain during the experiment, chicken groups may be arranged as follows: control/Group 1 = Group 6 > Group 4 > Group 5 > Group 3 > Group 2 (Table 5).

How did rachitic chickens (Group 5) respond to the administration of ACaSC, which has a higher than calcium carbonate bioavailability? All tested indices show, that ACaSC efficacy was better than for CaCO₃ (Group 3), but worse than for CaCO₃ in combination with vitamin D₃ (Group 4). Complete normalization of all analyzed indices was achieved in chickens of Group 6, received ACaSC and vitamin D₃. It seems logical, because vitamin D functions are not limited by influence on calcium intestinal absorption regulation [25].

In this experiment the different severity of rickets in bird groups is apparent: between the “classical” picture of D-avitaminosis and the healthy birds (Control). Ingestion of calcium carbonate, vitamin D₃ and ACaSC to varying degrees normalized tested parameters. In general, the final outcomes, *i.e.* antirachitic efficacy, was practically the same in two groups receiving calcium carbonate + vitamin D₃ (Group 4) and ACaSC + vitamin D₃ (Group 6).

A calcium balance study did not identify significant differences between Ca accumulation only in the chickens of groups 4 and 6 (Table 6).

In case ACaSC was used without vitamin D, its specific activity exceeded CaCO₃ in all tested physiological and biochemical parameters. This fact coincides with information, that in a double-blind randomized crossover clinical trial in postmenopausal women, it was shown that fractional Ca absorption of amorphous Ca was doubled on average compared to that of crystalline calcium carbonate [26]. The differences between CaCO₃ and ACaSC biological activity in our experiment were “smoothed” by vitamin D.

Nevertheless, there is evidence of an amorphous calcium salt composition advantage in comparison with crystalline calcium carbonate. It was shown that ACaSC is more effective regarding parathyroid gland function suppression, not

Table 6. The effect of calcium salts and vitamin D₃ administration on chicken feed consumption and calcium daily balance.

Experimental group	Consumpted feed quantity g/100g BW/day	Calcium balance* in one day mg/per one bird
1) Control	9.9 ± 2.1 ^{a**}	
2) Ricket (basic ration)	5.3 ± 1.0 ^b	(-) 24 ± 3
3) Basic ration + CaCO ₃	6.4 ± 1.3 ^b	(+) 31 ± 5
4) Basic ration + CaCO ₃ + D ₃	9.9 ± 1.7 ^a	(+) 76 ± 4
5) Basic ration + ACaSC	6.9 ± 1.3 ^{ab}	(+) 37 ± 2
6) Basic ration + ACaSC + D ₃	10.1 ± 1.9 ^a	(+) 79 ± 4

*(-) lost calcium quantity (+) accumulated calcium quantity; **Statistically different or similar within column according to Post-hoc Tukey HSD test (p < 0.05).

impacting blood level of phosphates. In case ACaSC is used without vitamin D, its specific activity excels CaCO₃ in all tested physiological and biochemical parameters. No less important is the ACaSC formulation: the fluid product is more suitable than calcium carbonate tablets and capsules for use by children and seniors.

The current paper contains only the initial part of the wider experiment's data. In the nearest future the information about combination's of ACaSC and vitamin D ricket's curative efficacy will be presented.

4. Conclusion

The studied composition of amorphous calcium salts seems prospective for new health food and pharmaceutical development. The product has a significantly higher bioavailability than calcium carbonate, suitable formulation for use by children and seniors. Combination with vitamin D increases ACaSC antirachitic effect. There is a reason to suppose that ACaSC together with some phytochemicals can provide significant osteoprotective activity in children and adults, including pregnant women.

Conflicts of Interest

The authors declare no competing interest concerning any commercial associations or patent licenses.

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