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Chromone-3-aldehyde derivatives – sirtuin 2 inhibitors for correction of muscular dysfunction

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ABSTRACT

The aim of the study was to evaluate experimentally, the myoprotective effect of new chromone-3-aldehyde derivatives in conditions of muscular dysfunction and to establish a potential mechanism of myoprotective activity – the blockade of the function of sirutin 2.
Materials and methods. The effect of new chromone-3-aldehyde derivatives on the development of muscular dysfunction under the conditions of an electromiostimulation test, was studied. The degree of muscle fatigue was evaluated in the «grip-strength» and through test biochemical assays (determination of the activity of lactate dehydrogenase, creatine kinase, concentration of lactic and pyruvic acids, creatinine, myoglobin, and total protein) to determine the possible mechanism of action of the test compounds (5 new derivatives of chromone-3-aldehyde) and their effect on the function of sirtuin 2 was evaluated.
Results. The study showed that chromone-3-aldehyde derivatives have a pronounced myoprotective effect associated with low toxicity (class 5 toxicity according to the GHS classification), which was confirmed by the results of the «grip-strength» test and biochemical tests data. Test compounds under the X3AC1, X3AOAC and X3AN codes evince sirtuin 2 inhibitory activity, which was reflected in a decrease in its concentration by 63.6% ($p<0.05$); 130.2% ($p<0.05$) and 218.8% ($p<0.05$).
Conclusion. The study showed that chromone-3-aldehyde derivatives are promising subjects for further study with the goal of creating a drug with a high myoprotective effect and an optimal safety profile.

INTRODUCTION

Muscular dysfunction (MDn) is defined as a syndrome in which skeletal muscles are unable to perform their physiological functions. This is manifested by loss of muscle strength and endurance [1]. Most often, MDn is associated with muscle fatigue – a potentially reversible condition wherein there is a temporary deterioration of the contractility of the striated muscle [2]. To date, muscle fatigue is a fairly common pathological syndrome – annually from 20% to 50% of all cases of treatment in medical hospitals are in response to complaints of chronic malaise are due to muscular fatigue [3].

Deterioration of the functional state of the striated muscle has a negative impact on physical performance and physical mobility, significantly reducing the quality of life [4]. Patients suffering from medium-heavy and severe forms of chronic muscle fatigue are forced to resort to outside help even when solving simple everyday tasks [1]. The development of muscle fatigue affects negatively on the professional activity of a person, which is especially important, for example, for highly skilled athletes. In sport of higher achievements, the development of muscle fatigue is recognized as one of the leading factors that limit the achievement of the maximum sporting result. Especially susceptible to muscle fatigue are Olympic level cyclical sports – running, swimming for long distances, sport walking, etc. [5]. At the same time, insufficient functional activity of skeletal muscles leads to the stress of the regulatory systems, such as adrenal or cortisol, which will inevitably lead to their disruption and the athlete's exit from the competitive process [6]. Thus, the correction of muscular fatigue is of undoubted scientific and practical interest.

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Chromone derivatives are a naturally class of substances that are found in plants such as: *Ammi Majus* L.; *Pastinaca Sativa* L.; *Heracleum lanatum Michx*.; *Melilotus officinalis* L.; *Ficus carica* L.; *Psoralea corylifolia* L.. Chromone derivatives have a broad spectrum of pharmacological activity, and display on the genome, anti-inflammatory, antioxidant, anticytokine, antitumor activity, effects, hence modulating the function of histone deacetylases and ADP-ribosyl transferase (for example, sirtuin 2 (SIRT 2)) [7,8]. At the same time, structures containing a privileged chromone core have low toxicity and have an optimal safety profile [9], which makes these compounds promising objects for study with a view to expanding their pharmacological activity spectrum.

As a potential target for the action of chromone derivatives, our attention was drawn to SIRT 2. SIRT 2 is present in the cytoplasm and is involved in the deacetylation of tubulin and differentiation of skeletal muscles. Recently it was found that SIRT 2 accumulates in the neurons of the brain during aging, as well as in neurodegenerative diseases. In the peripheral nervous system, SIRT 2 regulates the processes of myelination of nerve fibers. [10] In addition, in muscle tissue, SIRT 2 negatively regulates the response to insulin and insulin-like growth factors and reduces the concentration of glucose and activates the production of free radicals [11]. Thus, SIRT 2 can be a promising pharmacotherapeutic target for the correction of muscle dysfunction.

The aim of our study, therefore, was to assess the myoprotective effect of new chromone-3-aldehyde derivatives, as well as to establish a possible mechanism of their action – the inhibition of SIRT 2 activity.

MATERIALS AND METHODS

Animals. Ethical approval

As a biological model, 220 male Balb/c mice weighing 23-25 grams, obtained from the PMFI vivarium, were used in the work. The study was carried out in accordance with the norms of international experimental ethics and complied with the requirements of the European Convention for the Protection of Vertebrates used for experimental and other scientific purposes (Strasbourg, 22 June 1998) and was approved by a local ethical committee (Protocol №12 from 13 April 2018).

Studied compounds. Administration of test substances

The compounds studied were obtained at the Department of Organic Chemistry of the Pyatigorsk Medical and Pharmaceutical Institute under the guidance of prof., PhD Oganesyan E.T. and were new substituted derivatives of chromone-3-aldehyde under the conventional codes X3ANO$_2$, X3AOAC, X3AN, X3AF and X3ACl.

The investigated substances were obtained in the cyclization of 2-hydroxyacetophenone with dimethylformamide, with the addition of phosphorus oxychloride (Fig. 1).

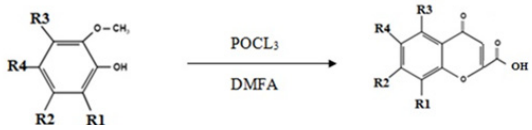


Figure 1. Scheme of synthesis of the test substances

The structures of the compounds were confirmed by NMR spectroscopy. During the study, test-compounds were administered orally.

Determination of single dose toxicity of the substances studied

To assess the single dose toxicity of the studied objects, 15 experimental groups of 10 individuals were formed (30 animals per compound). The studied substances were administered orally at doses of 1000 mg/kg, 2500 mg/kg and 5000 mg/kg divided in two-hour intervals. Further, the survival rate of the mice was assessed and the 14-day monitoring of the general condition of the animals was performed. LD50 was calculated by the Finney method [12].

Modeling of muscular dysfunction

Muscular dysfunction was reproduced by an electrical myostimulation method: under conditions of chloral hydrate anesthesia (350 mg/kg) to the experimental animals in the *m. biceps brachii*, electrodes were implanted and then after 24 hours electromyostimulation occurred in the mode: 100 Hz, 3 sec. (3 abbreviations) → 3 min. tedious contraction (sub-maximum stimulation, 40 HZ) → 100 Hz, 3 seconds. (3 abbreviations) [11]. In the course of the study, 7 groups of animals with 10 individuals were formed: the first group of mice positive control (PC) – without muscular dysfunction, the second group of animals negative control (NC) – with reproduced muscular dysfunction, but without pharmacological support. The remaining groups of mice were injected with the test compounds (per os prophylactically for 7 days at a dose of 1/100 of the LD50 value for each compound).

Evaluation of muscle strength

The muscle strength of the animals was evaluated by way of the «grip- strength» test (PANLAB device). The test procedure was performed three times: before the reproduction of muscle dysfunction (point T1), immediately after myostimulation (point T2) and 30 min. after myostimulation (point T3). Muscle strength was expressed in units of kg/f. The animals were then euthanized by dislocation of the cervical vertebrae, and biological material was collected (blood and muscle tissue).

Biomaterial sample preparation

Preliminary preparation of the biomaterial was carried out prior to conducting a series of biochemical tests: the citrated blood was centrifuged at 3500 RPM for 15 min to obtain a serum; the muscle tissue was homogenized in phosphate buffer (pH 7.4) in a ratio of 1:10, followed by the production of a supernatant (6000 g 10 min.). Herein serum levels were used to determine the concentration of myoglobin and creatinine, as well as the activity of lactate dehydrogenase (LDH) and creatine phosphokinase (CK). Changes in the concentration of lactic and pyruvic acids, as well as the level of total protein and SIRT 2 were evaluated in the supernatant of the muscle tissue.

Lactate dehydrogenase activity (LDH)

The activity of lactate dehydrogenase was determined by the kinetic method for the ability to catalyze the reaction of the formation of lactate from pyruvate in the presence of coenzyme NADH. The incubation medium contained: phosphate buffer (pH 7.15) and tris (hydroxymethyl) -aminomethane 50 mmol/l, NADH 0.9 mmol/l. The volume of the sample was 100 µl. (set "Olvex" (Sweden)). The extinction of the samples was determined at 340 nm for 5 min. with a recording interval of 1 min. LDH activity was calculated by the formula:

$$U/L = 16030 \times \Delta E340/min.$$

Creatine phosphokinase activity (CK)

The level of CK activity was determined in a conjugated croatkinase-hexokinase-glucose-6-phosphate dehydrogenase reaction in the presence of NADP. The incubation medium consisted of imidazole buffer (pH 6.7), ADP 20 mmol/L, NADP 20 mmol/L, creatine phosphate 300 mmol/L, G6PDG 20,000 U/L, hexokinase 30,000 U/L. The volume of the sample was 100 µl. (set "Olvex" (Sweden)). The extinction of the samples was determined at 340 nm for 5 min. with a recording interval of 1 min. CK activity was calculated by the formula:

$$U/L = 4127 \times \Delta E340 /min.$$

Determination of the creatinine concentration

The creatinine concentration was evaluated by the method of Jaffe. The principle of the method consists of the formation of a colored complex of creatinine with picric acid in an alkaline medium with a maximum absorption at 505 nm. A standard set of "Olvex" reagents (Sweden) was used in the work. Calculation of the creatinine concentration was made using the formula:

$$C = Ex/E0 \times 177\ \mu mol/L,$$

where: Ex – absorbance of the test sample; E0 – absorbance calibration sample.

Lactic acid concentration

The lactate concentration was determined by way of an enzymatic reaction with the formation of quininomine, the concentration of which is proportional to the content of lactic acid in the sample. The incubation medium consisted of phosphate buffer (pH 6.8), Pipes 50 mmol/L, 4-chlorophenol 6 mmol/L, 4-AAP 0.4 mmol/l, 2000 U/L lactoxydase, U/L peroxidase. The volume of the test sample was 10 µl. Sampling was carried out at 500 nm. Calculation of lactic acid content was determined according to the formula:

$$C = Ex/E0 \times 3.34\ \mu mol/L,$$

where: Ex – absorbance of the test sample; E0 – absorbance calibration sample.

Total protein concentration

Protein content was determined by reaction with bromophenol blue. The method is based on the formation of a colored protein complex with bromophenol blue in an acidic medium, the intensity of which is proportional to the protein concentration in the sample. The incubation medium was buffer (pH 7.4) 180 µl and chromogen 20 µl. The volume of the sample was 40 µl. Sampling was carried out at 613 nm. Calculation of the content of lactic acid was carried out according to the formula:

$$C = Ex/E0 \times 0.25\ g/L,$$

where: Ex – absorbance of the test sample; E0 – absorbance calibration sample.

Pyruvic acid concentration

The content of pyruvic acid was determined by the decrease in NADH in the lactate dehydrogenase reaction. The incubation medium included Good's buffer 1000 µL, NADH 200 µL, LDH (2000 U/L) 20 µl. The volume of the sample was 600 µl. Samples were extruded at 340 nm. Calculation of the content of pyruvic acid was carried out according to the formula:

$$C = Ex/E0 \times 1.25\ \mu mol/L,$$

where: Ex – absorbance of the test sample; E0 – absorbance calibration sample.

Enzyme-linked immunosorbent assay

The change in the concentration of myoglobin and SIRT 2 was assessed through enzyme-linked immunosorbent assay. Myoglobin concentration was determined in the blood serum of the animals, the content of SIRT 2 in the supernatant of their muscle tissue. We used species-specific (mouse) sets of reagents for ELISA analysis by Cloud lone corp. (USA) manufacturing. The progress of the analysis was in accordance with the instructions attached to the kit.

Statistical methods

Statistical processing of the data was carried out using the software package "STATISTICA 6.0" (StatSoft, USA). The results were presented as M (median value) ± SD. To compare the groups of means, one-factor variant of ANOVA with the Newman-Keuls post-test was applied. Differences were considered statistically significant at $p<0.05$.

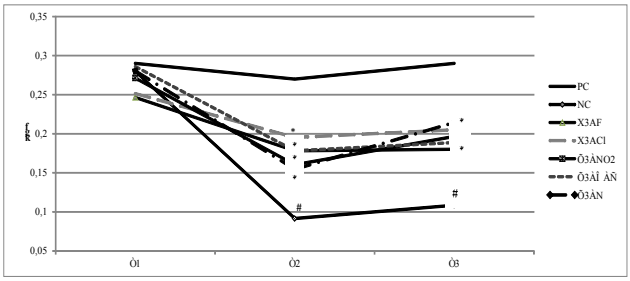
RESULTS

During the first series of experiments devoted to the study of single dose toxicity of the investigated chromone-3-aldehyde derivatives, it was found that the LD50 for the compound X3AF was 2885.04 mg/kg, for the substance X3ACl 3028, 46 mg/kg, and for compounds $X3ANO_2$, X3AOAC and X3AN – 3558.44 mg/kg; 4012.51 mg/kg and 3874.42 mg/kg, respectively. These results, according to the GHS classification (New-York and Geneva, 2011), corresponds to the 5^{th} toxicity class, and indicates a low toxicity

of test-substances. Thus, for the second stage of the experimental study, the effect of the derivatives of chromone-3-aldehyde on the development of muscle dysfunction in mice involved administered doses of substances: X3AF, X3ACl $X3ANO_2$, X3AOAC and X3AN substances at 28.9 mg/kg; 30.3 mg/kg; 35.6 mg/kg; 40.12 mg/kg and 38.7 mg/kg, respectively.

During the second stage of the study, we established that the background muscle strength in all experimental groups of animals was comparable (Fig. 2). Indeed, in the PC group of mice, the muscle tone did not significantly change any registration points (point T1-T3).

Prophylactic administration of test compounds promoted an increase in the grip strength of mice at point 2 (Fig. 2). Thus, against the background of the use of compounds $X3ANO_2$, X3AOAC, X3AN, X3AF and X3ACl, the grip strength of animals exceeded that of the NC group of mice by 76.9% ($p<0.05$), 84.5% ($p<0.05$), 69.3% ($p<0.05$), 94.6% ($p<0.05$), and 113.5% ($p<0.05$), respectively. At point 3, the muscle strength of the animals receiving $X3ANO_2$, X3AOAC, X3AN, X3AF, and X3ACl was statistically significantly higher than that of the NC group of mice by 81.1 ($p<0.05$), 73.9% ($p<0.05$), 97.6% ($p<0.05$), 66.1% ($p<0.05$), and 88.9% ($p<0.05$), respectively. At the same time, the grip strength of the mice that obtained X3AOAC, X3AF and X3ACl was statistically significant with respect to the parameters after miostimulation did not change. In contrast, at 30 min post-myostimulation, in the mice that were injected with $X3ANO_2$ and X3AN, muscle tone was higher than that at point 2 by 22.5% ($p<0.05$) and 38.6% ($p<0.05$), respectively (Fig. 2).



Note: PC is a group of animal positive controls; NC – group of animals of negative control;
* – statistically significant relative to the NC group of animals;
# – statistically significant relative to the PC group of animals

Figure 2. Effect of chromone-3-aldehyde derivatives on the muscle strength of animals in the «grip-strength» test

When carrying out the biochemical tests shown in Table 1, we established that muscular dysfunction, reproduced by electromyostimulation, is accompanied by an increase in the NC group of mice in the activity of LDH and CK in serum by 119.6% ($p<0.05$) and 101.6%, as well as an increase in serum myoglobin concentration by 257.8% ($p<0.05$) and a decrease in serum creatinine by 125.8% ($p<0.05$). We also noted an increase in the concentration of lactic and pyruvic acids, and a decrease in the protein concentration in the homogenate of muscle tissue by 245% ($p<0.05$), 373.7% ($p<0.05$), and 107.4 % ($p<0.05$), respectively.

When using the studied substances $X3ANO_2$, X3AOAC and X3AN, the concentration of lactate in the muscle homogenate of experimental animals decreased by 68.3% ($p<0.05$), 40.8% ($p<0.05$) and 81.5% ($p<0.05$), respectively, when compared to the NC group of mice. The content of pyruvate also decreased by 260% ($p<0.05$); 200% ($p<0.05$) and 291.3% ($p<0.05$), respectively. The muscle protein concentration against the background of the use of the compounds $X3ANO_2$, X3AOAC and X3AN, in relation to the NC group of mice, increased by 57.1% ($p<0.05$); 31.8% ($p<0.05$) and 69.1% ($p<0.05$), respectively. In addition, when the animals were administered $X3ANO_2$, X3AOAC, and X3AN, LDH activity decreased by 75.3% ($p<0.05$), 102.5% ($p<0.05$), and 130.9% ($p<0.05$), respectively. Furthermore, the activity of CK also decreased by 61.7% ($p<0.05$); 74% ($p<0.05$) and 136.8% ($p<0.05$), respectively. In addition, the concentration of myoglobin in the blood serum of mice against the background of the correction of muscle dysfunction by way of administering the compounds $X3ANO_2$, X3AOAC and X3AN, decreased by 41% ($p<0.05$); 97.8% ($p<0.05$) and 110.5% ($p<0.05$), respectively, while the creatinine concentration increased by 67.1% ($p<0.05$), 74% ($p<0.05$), and 93.4% ($p<0.05$), respectively.

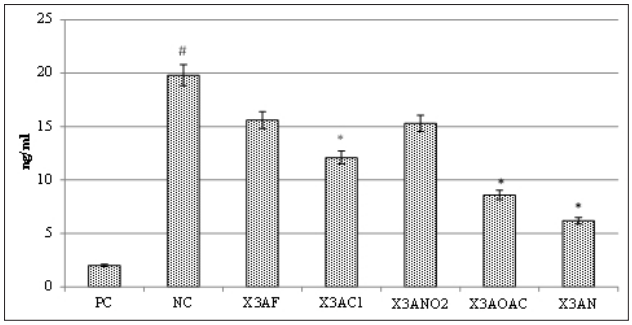
Against the background of administration of X3AF compounds to the experimental animals, compared with the NC group of mice, a decrease in LDH and CK activity in blood serum was observed at 125.8% ($p<0.05$) and 65% ($p<0.05$), respectively, while myoglobin concentration also decreased by 63.7% ($p<0.05$). The creatinine content, by contrast, increased by 82.7% ($p<0.05$). When using the compound X3ACl, the activity of LDH, CK, myoglobin concentration was decreased and the creatinine level saw an increase in the mouse blood serum relative to the NC group of animals by 83.3% ($p<0.05$); 91.8% ($p<0.05$); 22.1% ($p<0.05$) and 82.5% ($p<0.05$), respectively. In the muscle tissue, against the background of administration of the compound X3AF, a decrease in the concentration of lactate and pyruvate was also observed, as well as an increase in the protein content as compared to the mice of the NC group by 86.5% ($p<0.05$); 233.3% ($p<0.05$) and 53.3% ($p<0.05$), respectively. In animals that received compound X3ACl, the concentration of lactate, pyruvate and protein in the muscle tissue did not statistically significantly differ from the indices of the NC group of mice (Table 1).

Table 1. Biochemical parameters against the background of correction of muscle dysfunction with chromone-3-aldehyde derivatives

Group	PC	NC	X3AF	X3ACl	X3ANO2	X3AOAC	X3AN
Lactic acid, mmol/g	0.20 ±0.003	0.69 ±0.06#	0.37 ±0.023*	0.59 ±0.033	0.41 ±0.035*	0.49 ±0.039*	0.38 ±0.019*
Piruvic acid, mmol/g	0.019 ±0.001	0.09 ±0.038	0.027 ±0.001*	0.039 ±0.002	0.025 ±0.002*	0.03 ±0.002*	0.023 ±0.001*
Total protein, g/l	15.01 ±0.325	7.24 ±0.547#	11.1 ±0.081*	10.57 ±1.539	11.37 ±0.497*	9.54 ±0.518*	12.238 ±0.45*
Myoglobin, ng/ml	11.21 ±0.773	40.11 ±1.237#	24.49 ±4.867*	32.85 ±5.815*	28.44 ±2.265*	20.28 ±1.031*	19.05 ±1.308*
LDH, U/L	1020.79 ±82.462	2241.61 ±92.176#	992.67 ±28.062*	1222.62 ±60.116*	1300.47 ±120.196*	1110.33 ±148.233*	971 ±102.438*
CPK, U/L	606.45 ±36.523	1222.70 ±35.473#	740.81 ±24.933*	637.38 ±47.299*	756.28 ±77.181*	860.33 ±106.826*	516.36 ±32.187*
Creatinine, mmol/l	90.65 ±4.579	40.14 ±3.414#	72.89 ±5.914*	73.27 ±2.272*	67.06 ±1.841*	69.85 ±6.159*	77.64 ±5.519*

Note: PC is a group of animal positive control;
NC – group of animals of negative control;
* – statistically significant relative to the NC group of animals;
#- statistically significant relative to the PC group of animals

In the conditions of muscular dysfunction, an increase in the concentration of SIRT 2 (Fig. 3) of 9.8 times ($p<0.05$) in the muscular tissue was noted, in comparison with the group of animals of the positive control in the NC group of mice. What is more, when the test compounds X3AC1, X3AOAC and X3AN were used, the SIRT 2 content relative to the NC group of mice was 63.6% ($p<0.05$); 130.2% ($p<0.05$) and 218.8% ($p<0.05$), respectively, while against the background of the introduction of X3AF and $X3ANO_2$ to experimental animals, a significant effect on the change of SIRT 2 concentration was not observed (Fig. 3).



Note: PC – group of animal positive control;
NC – group of animals of negative control;
* – statistically significant relative to the NC group of animals;
# – statistically significant relative to the PC group of animals

Figure 3. Change in the concentration of sirtuin 2 in the muscle tissue of animals under using the test compounds in conditions of muscular dysfunction

DISCUSSION

MDn underlies the limitation of physical activity, which can adversely affect the work of organs and systems, primarily that of the cardiovascular and respiratory. The decrease of physical activity then contributes to the increase in the number of cases of hypertension, coronary heart disease, bronchial asthma – diseases with a high risk of complications, including death [13]. In addition, muscle fatigue is the main result-limiting factor in professional sports [5]. The above makes recovery of muscle tone and associated physical activity to an optimal level of one of the topical problems of modern pharmacology. The study showed that the course application of new derivatives of chromone-3-aldehyde contributed significantly lower expression of MDn, compared with animals receiving no pharmacological support under the conditions of the electromiostimulation test, which was confirmed by biochemical studies. At the same time, according to the totality of the studied indices characterizing the MDn, the test compounds can be arranged in the following activity range: X3AN> X3AOAC> X3ACl> $X3ANO_2$ > X3AF. It is important to underline that along with the high pharmacological activity, the compounds studied have low toxicity (class 5 toxicity according to the GHS classification).

In assessing potential mechanisms to implement a mioprotective effect, we investigated derivatives of chromone-3-aldehyde based on an analysis of research by other authors. We then postulated that the basis of the positive effect of tested compounds on the state of striated muscle in conditions muscle dysfunction lie their inhibitory activity against SIRT 2. SIRT 2 is representative of the superfamily NAD+ – dependent deacetylases, which exerts a multifaceted influence on the functions of the cell through interaction with chromatin [14]. The study showed that three of the five objects: X3ACl, X3AOAC and X3AN potentially exhibit the properties of down-regulating the function of SIRT 2 in the muscle tissue of the experimental animals. This, in turn, prevents degradation of contractile proteins of the striated musculature, as evidenced by the reduction in the concentration of myoglobin and the activity of LDH and CK in the blood serum of mice [7].

In addition, inhibition of SIRT2 promotes blockade of the PAR-3 → aPKC pathway. As a result, skeletal muscle insulin receptor density increases, anabolic processes are intensified and the accumulation of energy substrates is enhanced, thus preventing depletion of the muscle [15,16]. This conlcusion is indirectly supported by the increase in the total protein concentration in the muscle tissue. Furthermore, it is shown that the blockade of SIRT2 contributes to the antiapoptotic action by inhibiting pro-apoptotic genes and p53 protein [17,18].

In the study by Choi *et al*., the change in the activity of SIRT 2 significantly affects the protein concentration in muscle tissue through ERK1/2 signaling. Thus, activation of SIRT 2 reduces anabolic processes in muscles [19].

In another study, data were obtained concerning the effect of SIRT 2 activity on the change in glucose concentration in muscle tissue. The researchers saw that the inhibition of SIRT 2 provided intensification of de novo synthesis of the GLUT1 glucose transporter. This increases the concentration of glucose in muscles and increases their functional reserve [20]. Based on the results of the work [21], it can be stated that a decrease in the activity of SIRT 2, increases the acetylation of FOXO1 and eliminates the repression of PPARγ, thus increasing lipolysis and the concentration of free fatty acids in the muscles, thereby reducing energy deficit.

CONCLUSION

The study showed that preventive administration of new derivatives of chromone-3-aldehyde contribute to the elimination of the manifestation of MDn, normalize energy and plastic metabolism in the muscle tissue. The studied compounds are low-toxic substances. Based on the findings, it can be assumed that further study of chromone derivatives is a promising direction in finding means to correct MDn.

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CONFLICTS OF INTEREST

The authors statement no conflict of interest with the submitted manuscript.

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