EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 5
Issue: 5
64-71
Year: 2018

INFLUENCE OF GENISTIN ON ENERGY FORMATION, REACTIVE OXYGEN SPECIES AND OXIDASE SYSTEM ACTIVITY OF MITOCHONDRIAL MEMBRANE

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Article Received on 28/02/2018 **Article Revised on 21/03/2018** **Article Accepted on 11/04/2018**

ABSTRACT
Genistin dose-dependently increases ADP:O ratio in liver mitochondria. This process is especially noticeable in succinate oxidation. At low concentrations, genistin, does not affect on mitochondrial respiration, but with increasing of its concentration glutamate oxidation significantly decreases.Genistin inhibits lipid peroxidation and lytic enzymes activity in mitochondria. This means that genistin increasing bilayer membranes of mitochondria, stabilizes compactness of membranes. In this regard, cobra venom's exogenous PLA2 availability obstructed to phospholipids of mitochondrial membranes.

KEYWORDS: Genistin, liver, mitochondria, glutamate, succinate, ADP:O ratio, cobra venom, PLA2.

INTRODUCTION
Numerous in vitro research have shown that genistin is capable of qualitative and quantitative enhancing bone metabolism in the femoral-metaphyseal tissues of elderly rats.[1] The presence of genistin in the tissue culture caused a significant increase in alkaline phosphatase activity, deoxyribonucleic acid (DNA) and calcium contents. It is also revealed that genistin has a strong bone loss preventive activity on experimental rats, and is especially enhanced by combination with fructooligosaccharides.[2] The amount of new bone produced by grafting genistin in collagen matrix was compared to the bone produced by collagen matrix alone in New Zealand White rabbits, and was observed that genistin caused significant increase in bone formation.[3]

There are additional areas of gonadal synthesis of estrogen-stimulated genistin.[4] Genistin is the nuclear receptors that are activated by fatty acids and prostaglandins and function as a transcription factor.[5,6]

Genistin inhibits tumor necrosis factor, positively regulating prostaglandins.[6] In the research,[7,8] genistin showed antioxidant properties.

Mitochondria produce more than 90% of cell energy through oxidative phosphorylation. About 60% of the energy, released during the oxidation of substrates in mitochondria, dissipated as heat, maintaining a stable body temperature. Mitochondria are organelles not only provide the body with energy, but also maintain a dynamic relationship of metabolism. Mitochondria have all the basic functions of the cell: contractility, ions transport, heredity, etc.[9,13] During the normal process of oxidative phosphorylation from 0.4% to 4.0% of oxygen used in mitochondria is converted to superoxide radicals (O_2^-).[14,15] Mitochondria are the major regulators of calcium homeostasis, cells acid-base balance, the level of production of cellular activity regulators, as free radicals and nitric oxide. Many experimental data were obtained indicating that mitochondria are the primary targets of various pathological effects.[16,19] Moreover, mitochondria play a key role in apoptosis.[20,22]

Recently it was shown that the reduction in intracellular ATP levels by only 15-20%, intensity of all cell energetic functions fall to 75-80% of the initial value, which leads to the development of multisystem pathologies.[23] Mitochondria use about 85% of oxygen consumed by the cell, in the formation of ATP. In close dependence on intracellular ATP is the cells' ability to maintain their specific energetic function. Thus, mitochondria are organelles that are integrated functionally into the work of all life-support systems. Due to the abovementioned facts, considerable interest is study the effect of genistin to respiration, energy formation and reactive oxygen species of mitochondria.

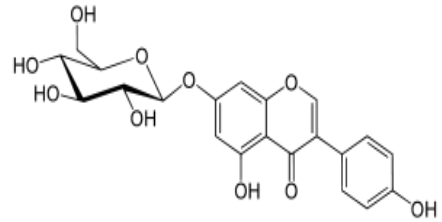
Study of the effect of genistin on respiration and oxidative phosphorylation, generation of reactive oxygen

species, oxidase systems activity of liver mitochondrial membrane are the aim of this research.

MATERIALS AND METHODS

Experiments were carried out on Wistar rats with an initial body weight of 180-200 g. Mitochondria from rat liver cells were isolated by the method of Schneider and Hogeboom.[24] All procedures were performed on isolated mitochondria at 0-2°C. Study of the energy parameters of isolated mitochondria was performed by Chance and Williams.[25] The experiments were performed with the addition of genistin into the polarographic cell. Study of activity of rotenone sensitive and insensitive NADH-oxidase systems, as well as the mitochondrial succinate oxidase carried by Almatov K.T. et al.[26] Lipid peroxidation of mitochondria was determined by the method of.[27] A principle of the method based on the reaction of thiobarbituric acid with malondialdehyde formed on peroxidation of unsaturated fatty acids having 2-3 diene bonds.

Malondialdehyde content was measured at a wavelength of 535 nm on a spectrophotometer (SF-16) vs. control. Calculation of the products content which react with thiobarbituric acid was performed based malondialdehyde molar extinction equals $1{,}56\times10^{6}$ µ/ cm^{-1} and expressed as nanomoles of malondialdehyde/mg protein per min. Genistin was used as glycerol solution and carried into the polarographic cell (at a final concentration of 20, 40, 60 microgram/milligram protein of mitochondria) and studied features of changes in the functional state of mitochondria. Mitochondrial protein content was determined by Lowry et al. [28]. The results were processed by a parametric Student's *t*-criterion and its determining of arithmetic mean value of M and its standard error *m*. Genistin was purchased by Shijiazhuang Zulei commerce Co., Ltd (China).



Genistin. 5-hydroxy-3-(4-hydroxyphenyl)-7-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-hydroxymethyl)oxan-2-yl]oxychromen-4-one.
Other names
Genistoside
Genistine
Genistein 7-glucoside
Genistein glucoside
Genisteol 7-monoglucoside
Glucosyl-7-genistein
Genistein 7-O-beta-D-glucoside
Chemical formula: $C_{21}H_{20}O_{10}$
Molar mass: 432.37 g/mol

RESULTS AND DISCUSSION

The data presented in Fig. 1 show that genistin, dissolved in glycerol at low concentrations (20 µg/mg of protein) does not influence on glutamate oxidation in liver mitochondria. At the same time after genistin administration at a dose of 60 µg/mg of protein, mitochondrial oxidation rate of glutamate in the metabolic state of V_2, V_3 and V_4 slightly (10.8, 14.0 and 11.5% respectively of control level) decreases. Genistin does not affect respiratory control rate of Chance and dinitrophenol stimulated mitochondrial respiration. However, genistin dose-dependently increases ADP/O ratio. Introduction of genistin into the polarographic cell of 20, 40 and 60 µg/mg of mitochondrial protein leads to increase of ADP/O ratio respectively by 8.6, 11.5 and 15.6% of control levels.

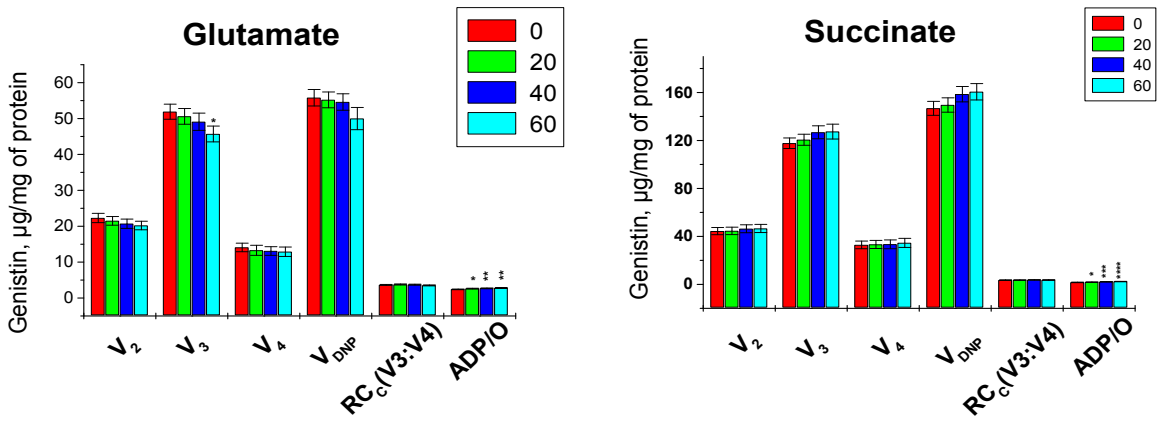


Fig. 1: Influence of genistin on respiration and oxidative phosphorylation of liver mitochondria.

In low concentrations, genistin does not affect the oxidation rate of succinate in different metabolic states of mitochondria. It should be noted that genistin dose-dependent increases ADP/O ratio. For example, if after adding genistin a dose of 20 µg/mg of mitochondrial protein, ADP/O ratio increases by 11.0% compared to control, after the introduction of 40 and 60 µg /mg of protein - 29.6 and 42.4%. In the presence of genistin, increase of ADP/O ratio with succinate in liver mitochondria, in our opinion due to the phenomenon of "reverse electron transfer" (on restoration of NAD^+).[29] A significant advantage of succinate in maintaining a high level of energy-dependent NADH compared with NAD-dependent substrates as the main factor determining the specific role of succinate in energy supply. Its advantage in this respect is obvious when we consider the 10-100 fold difference values K_M restore NAD^+ for succinate and NAD-dependent substrates. This leads to the existence of the fund capacious pyridine nucleotides, depending on succinate and inaccessible under normal conditions for NAD-dependent substrates. This fund is a good example of dynamic compartmentalization of mitochondria, when the realization of various metabolic pathways are not determined by morphological (structural), but kinetic parameters. The high and the maximum level of energy-rich compounds NADH and related transhydrogenase and biosynthetic reactions can not be achieved at low speed energy production of NAD-dependent substrates and realized only at the expense of the respective high speeds associated with the oxidation of succinate.[30] In our opinion, the increase in ATP content in mitochondria leads to the closure of cyclosporin A sensitive pores. In the work[31] it has been shown that at binding of ATP (to a lesser extent ADP and AMP) channel is rapidly closing.

According to the viewpoint of supporters of the translocase hypothesis of respiration, it is interesting to study the natural physiological metabolites that affect the translocase activity and thus act as physiological regulators of cell bioenergetics.[32] In our opinion, genistin enhances the translocase activity. It is known that the exchange of adenine nucleotides (ATF^{4-}/ADF^{3-}) between the mitochondrial matrix and the cytosol, carried out by a special transport system - translocase, determines the gross rate of respiration.The most significant feature of the translocase is its electrogenic nature. This means that nucleotides transport in energized mitochondria occurs always in one direction: ADP from the cytosol to the mitochondria, ATP - from mitochondria to the cytosol, where K_M for exogenous ATP in more than 100 times higher than for exogenous ADP; the ratio of ATP/ADP of the cytosol and ATP/ADP of mitochondria is a linear dependence from the size of the membrane potential. Adenine nucleotide translocase, working synchronously with the H^+ -ATP-synthase system and oxidative enzymes[33] is under control intramitochondrial pool of adenine nucleotides and a linear dependence of the size of this pool.[34]

Data on alterations in lipid peroxidation during incubation of mitochondria under in vitro conditions (at 36.7°C in ischemic state) by genistin are given in Fig. 2.

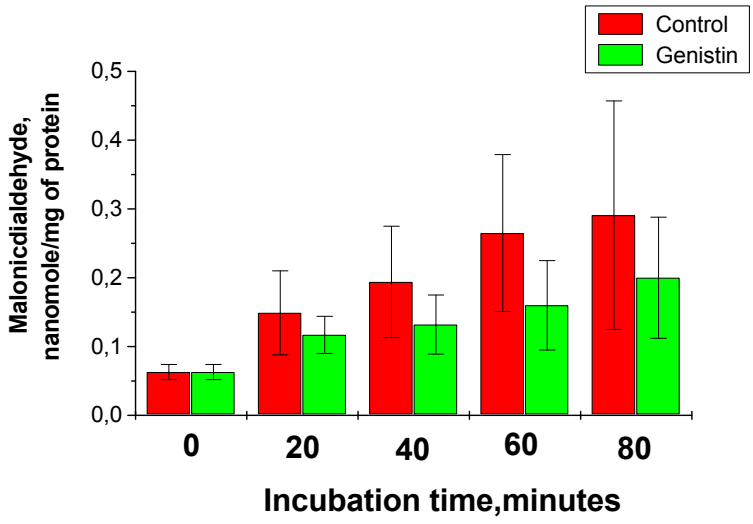


Fig. 2: Influence of genistin on lipids peroxidation in mitochondria.

Note: Lipid peroxidation in mitochondria was measured by addition of 20 micromole of $FeSO_4$+0,2 micromole of ascorbate. Aggregation of malondialdehyde was determined at 532 of optic density. Measuring medium: KCl – 115 mM, NaH_2PO_4 – 1mM, tris-HCl – 5 mM (pH – 7,4) at 36,7°C.

At incubation of mitochondria, in 20, 40, 60 and 80 minutes of the experiment formation of malondialdehyde

reinforced to 2.37, 3.08, 4.21 and 4.62, with genistin barely reinforced to 1.86, 2.09, 2.53 and 3.17. Consequently, genistin significantly reduces lipids peroxidation in the mitochondria. Lipids peroxidation in biological membranes, including in mitochondrial membranes, occurs with the formation of aldehydes of different structures - they are the products of the decomposition of polyunsaturated fatty acids and squalene peroxides. Approximately 40% of the total carbonyl compounds fall to share of malondialdehyde. Malondialdehyde is very sensitive to amino groups of proteins and amino acids and forms internal and intermolecular bonds.[35]

Influence of various biologically active compounds on the mitochondrial membranes can be studied by means of alterations in the activities of the retention-sensitive and rotenone-insensitive NADH oxidase, succinic oxidase. The physiological significance of these enzymes is not only their biotransformation of energy and participation in the process of electron transport through the respiratory chain to molecular oxygen, but also in the regulation of the rate of intake of various substances and the removal of metabolites, changes in conductivity, and contractility of mitochondrial membranes.[36]

Genistin in small amounts does not affect the activity of mitochondrial oxidases (Table 3). An increase in the content of genistin, added to the mitochondrial suspension slightly reduces NADH oxidase activity, but increases succinate oxidase activity. Therefore, genistin reduces NADH oxidase activity and increases succinate oxidase activity. The change in the oxidation of NAD-dependent substrates to succinate oxidation is such a mechanism that, under the influence of external stimuli, translates the consumption of energy-rich substances to a more economical state. This energy is subsequently used to restore cell activity. To enhance the synthesis of proteins and lipids, a high-energy potential of the mitochondria is required. No oxidation substrates are compared with succinic acid in the creation of energy-rich compounds and the reduction of pyridine nucleotides.[37] Therefore, genistin, inducing the formation of a high-energy potential in mitochondria, accelerates the synthesis of proteins and fats in the cell by enhancing the oxidation of succinate.

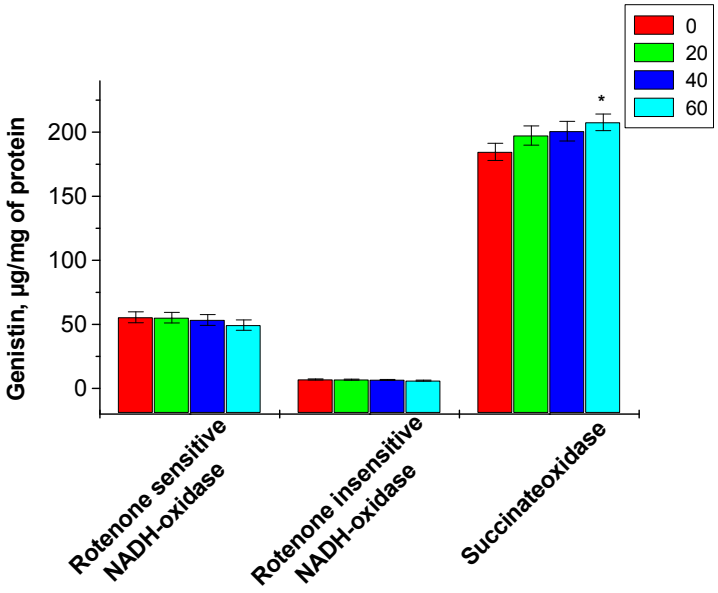


Fig. 3: Influence of genistin on rotenone-sensitive and rotenone-insensitive NADH-oxidase and succinic oxidase.

The increase or decrease of membrane-bound oxidases activity is due to the acceleration or vice versa slowing down of NADH and succinate reach to the enzyme activity center, and this is checked by determining the changes in enzyme activity by incubation of endogenous lipolytic and proteolytic enzymes under activated conditions. If genistin is actually a membrane stabilizer, then oxidases activity should undergo smaller changes in comparison with the control rates. Incubation was performed by adding 40 µg of genistin to per mg of mitochondrial protein (under conditions of ischemia at 36.7°C). After the 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220 and 240th minutes, changes in the activity of mitochondrial oxidases were detected. The results are shown in Table 1.

If at the incubation of control mitochondria at the 20, 40 and 60th minutes, rotenone-sensitive NADH oxidases activity increased by 12.8; 17.4 and 18.9%, in the presence of genistin increased by 5.8, 7.9 and 11.2%. After the 80th minutes in the mitochondria of both groups, the activity of the rotenone-sensitive NADH oxidase began to decrease in comparison with that in the 60 and 100th minutes it began to equal the previous index

before incubation. After the 140th minutes, the activity of this oxidase began to decrease gradually in both groups. Reduction of oxidase activity was more noticeable in control mitochondria. If the activity of control mitochondria decreased by 11.1, 18.0, 24.4, 32.3, 45.8 and 54.4% in the 140, 160, 180, 200, 220 and 240th minutes of the experiment, then, with the participation of genistin, it decreased only by 5.5, 8.4, 11.8, 17.6, 23.4 and 30.1%.

At the incubation of mitochondria at 36.7°C, the alterations observed in the activity of the rotenone-sensitive NADH oxidase are also observed in the activity of succinic oxidase. An increase in oxidase activity at the beginning of incubation indicates an increase in the effect of endogenous lytic enzymes and free radicals on membranes and facilitating the reach of substrates to the active site of oxidases because of an increase in "non-bilayer" membrane sites.[38] Subsequently, enhancement of endogenous lytic enzymes activity and free radicals accelerates membrane damage. Because of these changes, dysfunction of the activity of cytochrome c, coenzyme Q and other compounds located in the inner membrane of mitochondria and a decrease in the activity of oxidases is observed.

Table 1: Dynamics of oxidases activity alterations at incubation of mitochondria by genistin at 36.7ºC (M±m; n = 5-6).

Incubation period, min	Activity, nanogram of O_2 atom/min to mg of protein					
	Rotenon sensitive NADH-oxidase		Rotenon insensitive NADH-oxidase		Succinate-oxidase	
	Control	Genistin	Control	Genistin	Control	Genistin
Control	55,54±4,27	55,54±4,27	7,05±0,42	7,05±0,42	184,6±6,7	184,6±6,7
20	62,65±4,86	58,76±4,22	7,39±0,43	7,17±0,41	215,4±7,9	189,4±6,9
40	65,20±4,97	59,93±4,20	7,60±0,41	7,30±0,38	224,5±8,5	196,4±7,6
60	66,04±4,88	61,76±4,12	7,73±0,39	7,44±0,46	237,0±9,0	204,5±8,2
80	62,42±4,23	58,70±4,04	7,95±0,48	7,50±0,48	222,1±7,7	196,9±6,8
100	57,54±4,08	55,70±3,98	8,10±0,53	7,60±0,45	201,7±8,9	191,0±7,7
120	53,76±3,97	55,20±3,60	8,29±0,52	7,67±0,49	189,9±8,5	188,3±7,5
140	49,37±4,01	52,48±3,77	8,44±0,57	7,75±0,52	178,5±6,9	184,8±6,8
160	45,54±3,84	50,87±4,01	8,74±0,62	7,99±0,47	168,7±7,7	183,6±7,5
180	41,99±3,42	48,98±3,78	9,07±0,66	8,23±0,49	161,0±7,3	182,0±6,9
200	37,60±3,24	45,76±2,55	9,71±0,72	7,24±0,52	154,3±6,5	178,5±6,4
220	30,10±2,27	42,54±2,67	10,34±0,76	8,49±0,56	139,7±6,2	171,7±6,0
240	25,32±2,30	38,82±2,89	10,74±0,79	8,73±0,59	125,7±6,0	167,0±7,1
260					114,2±5,7	162,2±6,2
280					104,3±6,1	157,3±6,5
300					91,0±5,2	147,3±5,4

Cytochrome c is heme-containing protein, which is found in large quantities in the cells of humans and animals. The main function of this hemoprotein is participation in electron transport in the mitochondrial respiratory chain. Relatively recently it was opened no less important role of cytochrome c in the process of apoptosis induction.[39] It is believed that mitochondria contain two pools of cytochrome c: weakly and firmly bound to the membrane.[40] Proteins belonging to the first pool carry out the reaction of electron transport and are responsible for catalysis of reactive oxygen species in the intermembrane space. Hemoproteins of the second pool do not participate in the transport of electrons of the respiratory chain. Their function is mitochondrial membrane destruction in cases when the mitochondrial antioxidant system is not able to recycle accumulated hydro peroxides of lipids in these organelles.

Further intensification of the above-mentioned alterations at the end of the incubation causes a sharp decrease in the activity of oxidases. The slowing down of the aforementioned abnormalities of the mitochondrial membrane by genistin leads to a decrease in the changes in the activity of NADH oxidase and succinic oxidase during incubation as compared to the control readings. Therefore, genistin can be considered a membrane stabilizer.

During incubation, genistin significantly slows down rotenone-insensitive NADH oxidase activity. If in the 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220 and 240th minutes of the control incubation, rotenone-insensitive NADH oxidase activity increased by 4.9; 7.8; 9.7; 12.8; 14.9; 17.6; 19.7; 24.0 28.7; 37.8; 46.7 and 52.4%, with the participation of genistin increased by 1.7; 3.6; 5.6; 6.4; 7.8; 8.9; 10.0; 13.4; 16.7; 18.4; 20.4 and 23.8%. Therefore genistin significantly slows desorption of cytochrome c from the inner membrane of the mitochondria to the intermembrane space. According to experimental data, the outflow of cytochrome c on the intermembrane space leads to "apoptosis" of the cell[40,42] The outflow of cytochrome c from the intermembrane space to the outer part of the mitochondria can occur in two ways. The first is the rupture of the outer membrane

as a result of swelling of mitochondria[40] and in the second path the outer membrane remains intact and cytochrome c emerges through the giant pores of the outer membrane, which in the open state passes molecules up to 1.5 kDa.[42]

Usually the stability of the membrane is determined by the ratio of "bilayer" and "non-bilayer" regions and phospholipid/phospholipid and phospholipid/proteins.[43] Alterations in the above mentioned readings lead to an increase or decrease in the bilayer and nonbilayer regions of the membrane and as a result, the hydrolytic activity of phospholipases and proteases concerning phospholipids and proteins decreases or increases. To test the effect of genistin on this process (with the addition of 40 μg of genistin for each mg of the mitochondrial protein at 20°C), in our next experiment, changes are determined in the activity of mitochondrial oxidases at the 20, 40, 60 and 80th minutes of incubation after addition of 0.08 μg of PLA2 of Central Asian cobra *Naja naja Oxiana Echwald* for each mg of the mitochondrial protein (Table 2).

Table 2: Dynamics of oxidases activity alterations at incubation of mitochondria by genistin and PLA2 (M±m; n = 4-5).

Incubation period, Min	Activity, nanogram of O_2 atom/min to mg of protein					
	Rotenon sensitive NADH-oxidase		Rotenon insensitive NADH-oxidase		Succinate-Oxidase	
	PLA2	Genistin + PLA2	PLA2	Genistin + PLA2	PLA2	Genistin + PLA2
Control	53,7±3,8	53,7±3,8	7,00±0,38	7,00±0,38	182,9±6,5	182,9±6,5
20	67,6±4,5	58,1±3,7	8,79±0,42	7,88±0,35	140,3±6,2	163,1±5,8
40	54,2±3,9	56,1±3,5	9,72±0,54	8,43±0,37	100,2±5,6	150,9±4,7
60	28,0±3,3	43,4±2,8	11,02±0,58	9,01±0,43	66,7±3,4	120,3±4,5
80	12,1±2,2	32,4±2,5	14,05±0,67	9,69±0,48	46,1±2,3	103,5±3,1

In the 20th minutes of the experiment, rotenone-sensitive NADH oxidase activity increased by 25.9% under the influence of PLA2 and only 8.2% under the influence of genistin. Starting from the 40th minutes of the experiment, rotenon-sensitive NADH oxidase activity is restored to the control state. Further incubation leads to a decrease in the activity of oxidases and this process is accelerated according to the course of incubation. Genistin significantly slows down the decrease in oxidase activity. If by the 60 and the 80th minutes of the experiment rotenone-sensitive NADH oxidase activity decreased by 47.8 and 77.5% compared to the control, with the participation of genistin by only 19.2 and 39.6%.

Under the influence of PLA2, rotenone-insensitive NADH oxidase activity increased and this process is accelerated according to the course of incubation. Genistin significantly reduces the change of rotenone-insensitive NADH oxidase activity under the influence of PLA2. If in the 20, 40, 60 and 80th minutes of incubation rotenone-insensitive oxidase activity of control increased by 25.6, 38.9, 57.5 and 100.7%, with genistin increased by only 12.6, 20.4, 28.7 and 38.5%. This means that because of hydrolysis of the phospholipids of the inner mitochondrial membrane with PLA2, the activity of the rotenone-insensitive NADH oxidase increases due to an increase of NADH reaches to the active center of NADH oxidase and this process is accelerated according to incubation, i.e. enhances the hydrolysis of phospholipids.[43]

PLA2 begins to decrease succinic oxidase activity located in the inner membrane of the mitochondria at the beginning of the incubation. Genistin also significantly decreases the hydrolytic activity of PLA2 relative to succinic oxidase. If at 20, 40, 60 and 80 minutes succinic oxidase activity is reduced by 23.3, 45.2, 63.5 and 74.8%, with genistin only decreased by 10.8, 17.5, 34.2 and 43.4%. Genistin significantly inhibits the hydrolytic activity of PLA2 relative to the phospholipids of the inner mitochondrial membrane.

CONCLUSION

Analyzing the results it can be concluded that genistin increases dose-dependent ratio of ADP/O in liver mitochondria. This process occurs particularly noticeable in the succinate oxidation. At low concentration genistin does not affect the mitochondrial respiration, but glutamate oxidation decreases significantly with increase of its concentration. Genistin inhibits lipid peroxidation and lytic enzymes activity of mitochondria. This means that genistin increasing a strength of bilayer areas of mitochondrial membranes, stabilizes a membranes compactness. In this connection, an availability of exogenous PLA2 of cobra venom to phospholipids of mitochondrial membranes is obstructed.

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