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Metabolomics and viability of fibroblasts on the influence of the components of the malate dehydrogenase shuttle system in vitro

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The aim of the research. The aim is to evaluate the metabolic profile and viability of human dermal fibroblasts under the influence of malate and oxaloacetate in vitro.

Material and methods. Fibroblasts grown by the method of primary explants were incubated with solutions of malate and oxaloacetate. After that, the biochemical profile and enzyme activity relative to intact fibroblasts in the cell culture supernatant and lysate were determined. Cell viability was determined using an methyltetrazolium test (MTT-test). Statistical processing of the obtained research results was carried out in the environment of IBM SPSS Statistics 21 application programs.

Results. After incubation of fibroblasts with malate solution, the level of glucose, pyruvate and cholesterol in the supravascular fluid; pyruvate, glycerophosphate dehydrogenase and gamma-glutamyltranspeptidase in the cell lysate significantly changed. The effect of oxaloacetate is confirmed by a significant change in the content of pyruvate and cholesterol in the cell culture supernatant; pyruvate, glycerophosphate dehydrogenase and gamma-glutamyltranspeptidase in fibroblast lysate. The use of the MTT-test revealed an increase in the viability of fibroblasts by 27,31% (p=0,019) and 33,83% (p=0,028) when growing in an environment with malate and oxaloacetate, respectively.

Conclusion. The data obtained by us indicate that the components of the malate dehydrogenase shuttle can play a regulatory role, rearrange the metabolomics of fibroblasts and have a protective effect on cell viability.

Key words: malate dehydrogenase shuttle, impact, fibroblasts, metabolome, MTT-test, survival.

Conflict of interest. The authors declare the absence of obvious and potential conflicts of interest associated with the publication of this article.

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Introduction

Energy supply of cells, as you know, directly depends on the efficiency of the mitochondrial respiratory chain [1]. Aerobic and anaerobic respiration are not separate processes. Malate-aspartate shuttle is a key mechanism linking both types of oxidation. There is a transfer of nicotinamide adenine dinucleotide reduced (NADH), reduced as a result of glycolysis, into the mitochondria for further oxidation by the enzymes of the respiratory chain. Nicotinamide adenine dinucleotide oxidized (NAD^+) is transported simultaneously to the cytosol to continue glycolysis [2]. The key enzymes of the malate-aspartate system are cytosolic and mitochondrial malate dehydrogenase, metabolites are malate and oxaloacetate, as a result of their metabolism the NAD^+ / NADH ratio and electron transfer are maintained [3]. Malate, in addition to a transporter between the cytosol and mitochondria, is involved in many biochemical processes, for example, the amino acids synthesis, β-oxidation of fatty acids [4]. Oxaloacetate is a unique metabolite and it is involved in a huge number of biochemical processes such as protein metabolism, including amino acid metabolism, urea cycle, carbohydrate metabolism, gluconeogenesis, glyoxylate cycle [5]. There is a limited number of researches showing the role of oxaloacetate as a therapeutic bioenergetic agent [6].

Metabolomics specializes in the study of specific low molecular weight metabolic profiles that function in living cells under various conditions [7]. Our focus is on the culture of human dermal fibroblasts. Fibroblasts creating a microenvironment for other cells affect them through the release of growth factors, cytokines into the intercellular space. Fibroblasts play an important role in the process of tissue damage, the pathogenesis of inflammation and systemic response, as well as in cardiovascular and oncological diseases [8]. They are a universal biological model for studying the molecular processes in vitro underlying metabolism, transformation of intra- and extracellular signals, as well as cell growth and cell proliferation [9]. The malate dehydrogenase shuttle system with its enzymes and components functions inside cells, is a preparatory stage for oxidative phosphorylation, a link of antioxidant protection, participates in the synthesis of neurotransmitters and insulin [3]. It has been proved that the activity of a number of glycolytic enzymes, including malate dehydrogenase, decreases in aging human fibroblasts [10]. There are studies on the improvement of fasting blood glucose regulation and a decrease in insulin resistance in L6 myocytes under the influence of malate and its derivatives. This occurs, apparently, due to an increase in insulin-regulated glucose transporter (GLUT-4) secretion and a decrease in the level of p-Irs-1 in L6 cells. In addition, the malate derivative exhibits direct hypoglycemic activity in vitro [11]. It is relevant to study the effect of the components of the malate dehydrogenase system acting on the proliferation and viability of human dermal fibroblasts.

The aim of this study is to evaluate the metabolic profile and viability of human dermal fibroblasts under the influence of malate and oxaloacetate in vitro.

Material and methods

In our work, Sigma reagents (USA) malate and oxaloacetate which were diluted in Phosphate buffered saline (PBS) (Sigma, USA) pH=7,4 were used. pH control was performed using a Mettler Toledo pH meter (USA) after the preparation of solutions.

Cultivation of fibroblasts was performed according to the method of primary explants [12] using a complete growth medium (medium 199 with the addition of 10 % fetal calf serum and 40 μg/ml gentamicin) in the Sanyo MCO-17AI CO_2 Incubator (Sanyo, Japan) at a constant temperature of 37°C and humidity with 5 % of CO_2. The grown culture before further research was identified and characterized using morphological and molecular genetic methods. It was revealed that the cells are deterministic and they belong to the fibroblastic diferon. The PCR analysis showed the absence of contamination of the culture with infectious agents, including mycoplasmas and cytomegalovirus. Next, the cells were seeded in a 96-well plate at a dose of 2×10^4 cells/cm^2; solutions of the test compounds were added to the cells at a final concentration of 1,5 mmol/L during plating. The cells were incubated with a solution of malate and oxaloacetate during 120 hours at 37°C in a CO_2 atmosphere. The experiments were performed four times. A complete culture medium without fibroblasts and a culture medium of fibroblasts incubated without small molecules were the control.

Determination of biochemical parameters of cell metabolism (glucose, lactate, pyruvate, cholesterol) and enzyme activity of glycerophosphate dehydrogenase, alkaline phosphatase, gamma-glutamyltransferase in the supernatant and fibroblast lysate was carried out using an automatic biochemical analyzer Cobas Integra 400+ (Roche Diagnostics, Switzerland) with reagents Roche Diagnostics (Switzerland).

Cell viability was determined by screening method using MTT assay based on the reduction of tetrazolium salts by oxidoreductase enzymes of mitochondria into purple formazan crystals. The reducing ability of mitochondria was assessed by the color intensity registered by spectrophotometric [13]. After incubation of cells with malate and oxaloacetate, 20 μl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Merck KGaA, Darmstadt, Germany) was added to each well and incubated for 2 hours. Then the medium was removed, 100 μL of DMSO was added to the plates to dissolve the formazan crystals. Optical density was determined using the Tecan Infinite M200 PRO Multi-Mode Reader (Tecan Austria GmbH, Austria), wavelength 570 nm.

The lactate dehydrogenase test (LDH-test) was performed after centrifugation in culture fluid (cell lysis under the action of the analysed compounds was evaluated) and lysate of attached cells (the number of viable cells after exposure to the analysed compounds was evaluated), then the percentage of dead cells from the total number of lysed and viable cells was calculated [14]. This test was performed using the SF-56 spectrophotometer (LOMO-Spektr, St. Petersburg, Russia), wavelength 340 nm.

Statistical processing of the obtained research results was carried out in the SPSS 21 software environment (IBM SPSS Statistics, USA, license No. 20130626-3). The calculated parameters had a normal distribution, proved using the Kolmogorov-Smirnov test. Consequently, we used parametric methods of statistical analysis. The mean (M), mean error (m), 95 % confidence interval (95 % CI) were found. Intergroup comparisons for independent samples were performed using the Student test, as well as using analysis of variance.

All studies were carried out in accordance with the decision of the local Ethical Committee on Bioethics at Samara State Medical University (Protocol No. 201 of 11.09.2019)

Results and discussion

At the first stage of the research, we evaluated the change in the activity of a number of enzymes and the concentration of metabolites in control samples: a nutrient medium without cells and a medium in which fibroblasts were cultivated. There was a significant increase in the activity of glycerophosphate dehydrogenase (p=0,047) and alkaline phosphatase (p=0,0002); a significant decrease in glucose levels (p=0,0001), an increase in lactate (p=0,0001) and pyruvate (p=0,0001). Focusing on a significant decrease in the glucose / lactate index, which was 0,57±0,063 (p=0,0001) compared to the control value 2,38±0,045, it can be concluded that the metabolic needs of human dermal fibroblasts are mainly realized due to anaerobic processes.

After that, we performed experiments with the addition of the analysed compounds to the growth medium with fibroblasts. The metabolic panel of the supernatant after incubation of fibroblasts for 120 hours with and without small molecules was analysed (tabl. 1).

Malate and oxaloacetate equally reduced the cholesterol value, which turned out to be at the level of 0,08±0,004 mmol/L (p=0,004) and 0,08±0,006 mmol/L (p=0,033), respectively. This may indicate an increased consumption of cholesterol during the construction of cell membranes, which occurs in response to active proliferation. The metabolites introduced by us acted unidirectionally concerning glucose which fibroblasts, apparently, utilize at a higher rate under new conditions of existence. However, a significant decrease in its level by 1,2 times turned out to be only with the addition of malate (p=0,0017). No significant changes were detected during studying the lactate value. The introduction of biologically active compounds analysed by us leads

Table 1

Metabolic panel of fibroblast supernatant after incubation with components of the malate dehydrogenase system

	Control medium without FB	Control medium with FB	FB with the addition of	
			Malate	Oxaloacetate
Glucose, mmol/L, M±m	6,0±0,09	4,3±0,1	3,64±0,14	3,9±0,18
95 % CI	5,8–6,2	4,05–4,5	3,31–3,97	3,4–4,4
p (control medium without FB)		<0,0001		
p (control medium with FB)			0,0017	0,0686
Lactate, mmol/L, M±m	2,58±0,1	6,8±0,4	6,78±0,42	6,63±0,18
95 % CI	2,35–2,82	5,83–7,78	5,84–7,90	5,87–7,40
p (control medium without FB)		<0,0001		
p (control medium with FB)			0,9087	0,8137
Pyruvate, mmol/L, M±m	0,0039±0,00013	0,118±0,0001	0.175±0.004	0,62±0,0024
95 % CI	0,003–0,004	0,118–0,119	0,13–0,22	0,61–0,624
p (control medium without FB)		<0,0001		
p (control medium with FB)			0,0003	<0,0001
Cholesterol, mmol/L, M±m	0,098±0,002	0,11±0,0067	0,08±0,004	0,08±0,006
95 % CI	0,09–0,103	0,09–0,13	0,07–0,09	0,055–0,1
p (control medium without FB)		0,226		
p (control medium with FB)			0,004	0,033
Glucose / Lactate, M±m	2,38±0,045	0,57±0,063	0,6±0,007	0,2±0,0052
95 % CI	2,24–2,52	0,37–0,77	0,57–0,63	0,197–0,23
p (control medium without FB)		<0,0001		
p (control medium with FB)			0,6985	0,0014
Glucose / Pyruvate, M±m	1480,1±34,2	34,26±1,04	20,76±0,53	177,82±2,8
95 % CI	1332,74–1627,5	29,77–38,75	14,08-27,44	165,63-190
p (control medium without FB)		<0,0001		
p (control medium with FB)			0,0024	<0,0001

*Note: * 95 % CI – 95 % confidence interval, FB – fibroblasts, M±m – arithmetic mean and standard error of mean, p – level of intergroup significance.*

to a slight decrease in the lactate level, which remains at the level of control values of 6,8±0,4 mmol/L. It is possible that mechanisms are activated that maintain the lactate level at a relatively stable level for the adequate functioning of processes in fibroblasts. Analysis of the level of the substrate of pyruvate carbohydrate metabolism showed a fairly high increase of 5,2 times with the addition of oxaloacetate solution (p=0,0001) at a final concentration of 1,5 mM. This can be explained by the conversion transition of two ketoacids into each other. They differ only in the presence of one additional carboxyl group in oxaloacetate. The same analysis showed an increase of 1,5 times with the addition of malate solution (p=0,0003). Changes in the enzyme profile of the supernatant were registered in the form of a decrease in the activity of alkaline phosphatase (p=0,0259) and gammaglutamyl transpeptidase (p=0,2283) after incubation of human dermal fibroblasts with malate at a final concentration of 1,5 mmol /L relative to an intact cell culture. It probably stabilizes the cell membranes of fibroblasts, since along with other connective tissue cells, fibroblasts are sources of these enzymes, which exit into the paracellular space when the cell membranes are destroyed [15, 16]. The activity of glycerophosphate dehydrogenase decreased by 1,12 times and slightly deviated from the control values of 0,057±0,019 E/L .

Further, the components of the fibroblast endometabolome were evaluated (tabl. 2).

The growth of fibroblasts with the addition of malate and oxaloacetate solutions leads to a decrease in the level of glucose inside the cells. Based on this, it can be concluded that glycolytic processes are more characteristic of cultured fibroblasts. The low-molecular-weight bioactive compounds introduced by us have a multidirectional effect on the intracellular content of pyruvate. Malate significantly reduces the level of pyruvate in the cell lysate to 0,00016±0,00004 mmol/L (p<0.0001), and oxaloacetate increases $5{,}1 * 10^{-3} \pm 5{,}8 * 10^{-5}$ mmol/L (p<0,0001) relative to the control level, which was $4{,}1 * 10^{-3} \pm 6 * 10^{-5}$ mmol/L. In this regard, a significant decrease in the glucose/pyruvate ratio during incubation of fibroblasts with malate solution can be explained. An interesting fact is that the level of lactate in both cases decreases exactly 1,6 times in comparison with the control in the cell lysate and come to 0,016±0,004 mmol/L. Utilization of lactate, apparently, occurs due to an insufficient amount of other hexose substrates, for example, glucose,

Table 2

Metabolic panel of human dermal fibroblast lysate after cultivation with malate dehydrogenase components

	Control medium with FB	FB with the addition of	
		Malate	Oxaloacetate
Glucose, mmol/L, M±m	0,02±0,0045	0,016±0,0025	0,012±0,002
95 % CI	0,0075–0,032	0,0092–0,023	0,0065–0,018
p control medium with FB		0,45	0,14
Lactate, mmol/L, M±m	0,026±0,0025	0,016±0,004	0,016±0,004
95 % CI	0,019–0,033	0,0049–0,027	0,0049–0,027
p control medium with FB		0,0656	0,0656
Pyruvate, mmol/L, M±m	0,0041±0,00006	0,00016±0,00004	0,0051±0,00006
95 % CI	0,0039–0,004	0.00005–0.0002	0,0049–0,005
p control medium with FB		<0,0001	<0,0001
Glucose / Lactate, M±m	0,8±0,21	0,8±0,12	0,6±0,1
95 % CI	0,23–1,37	0,46–1,14	0,32–0,88
p control medium with FB		1,0000	0,4094
Glucose / Pyruvate, M±m	4,88±1,4	0,8±0,12	1,96±0,02
95 % CI	0,39–9,37	0,46–0,14	1,89–2,0
p control medium with FB		0,0136	0,0840
GPDH, U/mL, M±m	0,284±0,0026	0,143±0,001	0,35±0,0002
95 % CI	0,28–0,29	0,138–0,147	0,349–0,3503
p control medium with FB		<0,0001	<0,0001
ALP, U/L, M±m	0,8±0,12	0,65±0,02	0,55±0,029
95 % CI	0,43–1,17	0,56–0,74	0,46–0,64
p control medium with FB		0,2544	0,0804
GGT, U/L, M±m	3,3±0,15	2,05±0,29	2,025±0,19
95 % CI	2,8–3,77	1,13–2,97	1,41–2,64
p control medium with FB		0,0085	0,0019

*Note: * 95 % CI – 95 % confidence interval, ALP – alkaline phosphatase, FB – fibroblasts, GGT – gamma-glutamyltransferase, GPDH – glycerophosphate dehydrogenase, M±m – arithmetic mean and standard error of mean, p – level of intergroup significance.*

the sufficient supply of which is limited due to the increased content of lactate which is actively released into the culture medium of fibroblast cultivation as a result of anaerobic processes [17]. The tendency of inhibition of the activity of intracellular enzymes such as alkaline phosphatase and gamma-glutamyltransferase during the cultivation of fibroblasts with malate and oxaloacetate solutions coincides with that in the cell supernatant. The decrease in the activity of these enzymes can be explained by the protective role of the studied molecules on the structure formation of components inside human fibroblasts. However, after adding a malate solution (p<0,0001), the activity of glycerophosphate dehydrogenase decreases to 0,143±0,001 U/ml, which characterizes mitochondrial-cytoplasmic metabolism and the growth of fibroblasts with oxaloacetate solution (p<0,0001) leads to an increase in the activity of glycerophosphate dehydrogenase 0,35±0,0002 E/ml which is significantly different from the control level of 0,284±0,0026 E/ml. Probably, the multidirectional effect of the metabolites analysed by us is associated with the activity of energy metabolism in mitochondria, the number of which varies depending on the state and needs of the cells [18].

The described changes in the exo- and endo-metabolome of human dermal fibroblasts under the influence of the components of the malate-aspartate shuttle are confirmed by tests characterizing cell viability. Determining the mitochondrial activity of cells using the MTT-test, it was found that the viability of fibroblasts increased by 27,31 % (p=0,019) and 33,83 % (p=0,028) during growth with the addition of malate and oxaloacetate solutions, respectively. An additional LDH test did not show significant differences between the control and experimental values.

Conclusion

An important problem in the study of cell cultures is the study of cell adaptation to the action of various factors, both local and systemic. Our results indicate that the components of the malate-aspartate shuttle system, introduced exogenously, exhibit a regulatory role, can change the course and efficiency of metabolic pathways, and also affect the viability of human dermal fibroblasts. This can be seen in a stable decrease in the level of glucose and lactate, inhibition of the activity of alkaline phosphatase and gamma-glutamyltransferase, an increase in the content of pyruvate. This tendency is typical for both fibroblast supernatant and cell

lysate. A stimulating effect on the culture of human dermal fibroblasts of malate (p=0,019) and oxaloacetate (p=0,028) was revealed. They also have a low cytotoxicity profile and can be used as therapeutic compounds, which certainly opens up prospects for further research.

Thus, changes in the exo- and endo-metabolome of human dermal fibroblasts are significantly altered by metabolites and enzyme profile under the action of malate and oxaloacetate, which in turn affects cell viability, increasing it.

It can be assumed that the metabolic profile of fibroblasts will be similar under the action of these substances *in vivo*.

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