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Original article

# Predicted oxaloacetate activity, gene expression and viability of human dermal fibroblasts

Nataliya A. Kolotyeva, Larisa V. Limareva, Frida N. Gilmiyarova, Violetta V. Boltovskaya, Pavel V. Ilyasov, Oksana A. Gusyakova, Vasily V. Remizov

Samara State Medical University, Samara, Russia

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**Abstract:** *Rationale* — The impact of metabolic pathway intermediates on biological processes and cell viability is a topic of fundamental research: functional potential of low-molecular weight biologically active compounds as regulators of metabolic processes, signal molecules, transmitters of intra- and intercellular signals are of special interest; however, this field remains poorly studied.
*Objective* — To perform computer modeling of oxaloacetate biological activity and to evaluate its effect on human dermal fibroblast culture.
*Material and Methods* — We predicted biological activity of oxaloacetate using in silico methods. The obtained data demonstrated that oxaloacetate could influence proliferative properties and cell culture viability of dermal fibroblasts. We studied metabolic parameters and enzymatic activity in supernatant and cell culture lysate. Cell viability was assessed via methyl tetrazolium test (MTT).
*Results* — We established that oxaloacetate had a stimulating effect on human dermal fibroblast culture.
*Conclusion* — Our results demonstrated the stimulating role of oxaloacetate in the form of changes in the level of metabolites and activity of enzymes, such as gamma-glutamyl transpeptidase ($p=0.0019$) and glycerol-3-phosphate dehydrogenase ($p<0.0001$). We also established that oxaloacetate increased the percentage of viable cells, compared with the control ($p=0.028$).

**Keywords:** Oxaloacetate, computer modeling, human dermal fibroblasts, viability.

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*Correspondence to* Nataliya A. Kolotyeva. Address: Department of Fundamental and Clinical Biochemistry with Laboratory Diagnostics, Samara State Medical University, 89 Chapaevskaya St., Samara 443099, Russia. Phone: + 78463370463. E-mail: bio-sam@yandex.ru.

## Introduction

Whether metabolic intermediates could affect biological processes and alter cell viability is not unambiguously studied so far. The focus of this article is a small molecule of oxaloacetate, the so-called molecular switch, which plays an important role in the cell energy metabolism. There are some scarce studies demonstrating use of oxaloacetate as a therapeutic bioenergetic agent [1]. According to published data, oxaloacetate prevents neuroinflammation and neurodegeneration by reducing the concentration of glutamate in the brain via activating the blood enzyme glutamate-oxaloacetate transaminase [2]. The neuroprotective effect of oxaloacetate (activation of mitochondrial biogenesis in the brain) is currently studied in preclinical trials (1) as a remedy for Alzheimer's disease, since in this ailment, the parameters of mitochondrial function and the level of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) decrease [3]; and (2) as the first-line treatment for ischemic and hemorrhagic brain stroke [4]. Oxaloacetate also significantly enhances the neuroprotective effect of adipose stromal cell-conditioned medium [5]. Moreover, there is evidence that oxaloacetate stimulates the growth of hippocampal gyrus neurons with a beneficial effect on cognitive processes and mnemonic functions [6].

It is not clear yet how natural metabolites integrate into central metabolic systems and what happens when they are part of an isolated system. Bioenergetic and mitochondrial medicine demonstrate that these low-molecular weight ligands can act as medications affecting one of the earliest stages of the organization of life, thereby increasing an effectiveness of the impact. Use of computer simulation of the compound under study makes it possible to reduce the amount of necessary experimental verification by a factor of ten compared to blind search [7]. Contemporary technologies, programs and algorithms based on the analysis of numerous data libraries help the researcher conducting a preliminary search for possible interactions of small molecules with proteins, mapping targets, and selecting the most relevant field of study. Accordingly, we performed computer modeling of oxaloacetate molecule, a protein-carbohydrate and carbohydrate-protein metabolic switcher.

At present, insufficient knowledge was accumulated on the effect of oxaloacetate molecule on cell cultures. There are few publications on the direct effect of oxaloacetate on cells. It was confirmed that oxaloacetate causes a decrease in the energy metabolism in hepatocellular carcinoma cells (HepG2) [8]. Further in vivo study of the oxaloacetate effect on biological processes in dermal fibroblasts may reveal an important and still unexplored potential of small molecules in intermolecular processes.

The objective of our study was to perform computer modeling of oxaloacetate biological activity and to evaluate its effect on human dermal fibroblast culture.

## Material and Methods

### *Computer modeling of oxaloacetate biological activity via PASS*

Computer modeling of oxaloacetate biological activity was performed via the Prediction of Activity Spectra for Substances (PASS) software, based on structural formula and using unified descriptors of the chemical structure and a mathematical algorithm for assessing the structure-activity relationship. The range of biological activity includes pharmacological effects, molecular mechanisms of action, specific toxicity, side effects, metabolism, along with effects on targets, molecular transport, and gene expression [4]. The result of predicting biological activity is presented in the form of probability of being active, Pa, or being inactive, Pi. We analyzed the predicted activity spectrum Pa>Pi, especially at Pa>0.5.

### *Participants and research design*

We chose oxaloacetate (Sigma, USA) as the small molecule for our study and prepared its solution with PBS buffer (Sigma, USA) pH=7.4. After that, pH was controlled using Mettler Toledo pH-meter (USA).

We used a cell culture of human dermal fibroblasts as a model to evaluate the effects of oxaloacetate. Fibroblasts were grown employing the primary explant technique [9] in complete cell growth medium (Biolot LLC, Russia) in $CO_2$ incubator MSO-17AI (Sanyo, Japan) at a constant temperature (37 °C) and humidity with 5% $CO_2$. Prior to the study, the grown culture was identified and characterized by morphological and molecular genetic methods. Evaluated cells were classified as embryonic fibroblasts. PCR study showed that the culture was not contaminated with infectious agents, including mycoplasma and cytomegalovirus. The cells were seeded in a 96-well plate at a dose of $2\times10^4$ cells/cm$^2$, and the test compound was added to the cells at a final concentration of 1.5 mM. The cells were incubated with a solution of oxaloacetate for 120 hours at 37 °C in a $CO_2$ atmosphere (fibroblasts with oxaloacetate). The experiments were carried out in four repetitions. When determining the metabolic profile of the fibroblast supernatant, pure culture medium without fibroblasts (control medium) and culture medium of incubated fibroblasts without small molecules (fibroblast control) served as controls. When studying the activity of fibroblast enzymes in the cell lysate, the culture medium of fibroblasts incubated without small molecules (fibroblast control) served as a control sample.

### *Biochemical parameter assessment*

Biochemical parameters in the fibroblast supernatant (cholesterol, glucose, lactate, pyruvate), along with enzyme activity of glycerophosphate dehydrogenase, alkaline phosphatase, and gamma-glutamyl transpeptidase in cell lysate, were evaluated on the automated biochemistry analyzer Cobas Integra 400+ (Roche Diagnostics, Switzerland), reagents by Roche Diagnostics.

### *Determining viability of human dermal fibroblasts*

Cell viability was assessed using methyl tetrazolium test (MTT), based on reduction of tetrazolium salts with oxidoreductase enzymes of mitochondria to formazan purple crystals. The reduction capacity of mitochondria is measured by the intensity of coloration identified via spectrophotometric analysis [10]. After cultivation with oxaloacetate, 20 µl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Merck KGaA, Darmstadt, Germany) was added to each well with subsequent cultivation for 2 hours. Then the medium was removed, and 100 µl measured with Tecan Infinite M200 PRO Multi-Detection Microplate Reader (Tecan Austria GmbH, Austria), at the wavelength of 570 nm.

**Table 1. Fibroblast supernatant metabolic profile after small-molecule cultivation**

| *Parameter* | *Control medium* | *FB control* | *FB with oxaloacetate* | *p-value (control medium – FB control)* | *p-value (FB control – FB+OA)* |
|---|---|---|---|---|---|
| Cholesterol, mmol/L, M±m (95% CI) | 0.098±0.002 (0.09–0.103) | 0.11±0.0067 (0.09-0.13) | 0.08±0.006 (0.055-0.1) | 0.226 | 0.03 |
| Glucose, mmol/L, M±m (95% CI) | 6.0±0.09 (5.8-6.2) | 4.3±0.1 (4.05-4.5) | 3.9±0.18 (3.4-4.4) | <0.0001 | 0.068 |
| Pyruvate, mmol/L, M±m (95% CI) | 0.0039±0.00013 (0.0034-0.0045) | 0.118±0.0001 (0.118-0.119) | 0.62±0.0024 (0.61-0.624) | <0.0001 | <0.0001 |
| Lactate, mmol/L, M±m (95% CI) | 2.58±0.1 (2.35-2.82) | 6.8±0.4 (5.83-7.78) | 6.63±0.18 (5.87-7.40) | <0.0001 | 0.8137 |
| Glucose/lactate ratio, M±m (95% CI) | 2.38±0.045 (2.24-2.52) | 0.57±0.063 (0.37-0.77) | 0.55±0.0054 (0.52-0.57) | <0.0001 | 0.7652 |
| Glucose/pyruvate ratio, M±m (95%CI) | 1480.12±34.25 (1332.74-1627.5) | 34.26±1.04 (29.77-38.75) | 5.87±0.12 (5.34-6.39) | <0.0001 | <0.0001 |

95% CI, 95% confidence interval; FB, fibroblasts; M±m, arithmetic mean and its standard error; OA, oxaloacetate; *p*, statistical significance of intergroup comparisons.

**Table 2. Fibroblast enzymatic activity in cell lysate after cultivation with oxaloacetate**

| *Parameter* | *FB control* | *FB with oxaloacetate* | *p-value* |
|---|---|---|---|
| Alkaline phosphatase, U/L, M±m (95% CI) | 0.8±0.12 (0.43-1.17) | 0.55±0.029 (0.46-0.64) | 0.0804 |
| Gamma-glutamyl transpeptidase, mmol/L, M±m (95% CI) | 3.3±0.15 (2.8-3.77) | 2.025±0.19 (1.41-2.64) | 0.0019 |
| Glycerol-3-phosphate dehydrogenase, mmol/L, M±m (95% CI) | 0.284±0.0026 (0.28-0.29) | 0.35±0.0002 (0.349-0.3503) | <0.0001 |

95% CI, 95% confidence interval; FB, fibroblasts; M±m, arithmetic mean and its standard error; *p*, statistical significance of intergroup comparisons.

**Table 3. MTT test after fibroblast small-molecule cultivation incubation**

| *Parameter* | *FB control* | *FB with oxaloacetate* | *p-value* | *Viable cells, %* |
|---|---|---|---|---|
| MTT test, M±m (95% CI) | 0.55±0.03 (0.45-0.66) | 0.74±0.09 (0.49- 0.99) | 0.028 | 133.8 |

95% CI, 95% confidence interval; FB, fibroblasts; M±m, arithmetic mean and its standard error; MTT test, methyl tetrazolium test; *p*, statistical significance of intergroup comparisons.

***Determining oxaloacetate cytotoxicity***

Lactate dehydrogenase-test (LDH test) was carried out in a culture fluid after its centrifugation (lysis of cells that were exposed to investigated factor was estimated), and lysis of cells (the number of viable cells that were exposed to investigated factor) was estimated; then the percentage of dead cells of the total cell number of cells after lysis and viable cells was calculated [11]. To perform this test, we employed the spectrophotometer SF-56 (LOMO-Spectrum, St. Petersburg, Russia), the wavelength of 340 nm.

***Statistical data processing***

Statistical analyses were performed with SPSS 21 software (IBM SPSS Statistics, USA, license number 20130626-3). The calculated features had a normal distribution, confirmed by the Kolmogorov-Smirnov test. Hence, we used parametric tests. We calculated mean (M), error of the mean (m), standard deviation (SD), and 95% confidence interval (95% CI). Intergroup comparisons were performed using the Student's t-test for two independent samples, along with analysis of variance.

**Results**

We revealed the effect of oxaloacetate on gene expression using the PASS computer environment. It is well-known that oxaloacetate enhances the expression of TP53 gene (Pa=0.599). We predicted the inhibitory effect of oxaloacetate on expression of MMP9 gene (Pa=0.601), a protein belonging to the family of matrix metalloproteinases. In addition, oxaloacetate was predicted to reduce the expression of the gene of tumor necrosis factor (TNF) (Pa=0.514). The software projected an effect of inhibition of HIF1A factor (Pa=0.506). Oxaloacetate enhanced the expression of HMOX1 gene encoding heme oxygenase protein-1 (Pa=0.574) and JAK2 gene (Pa=0.661).

When comparing the content of metabolites in the complete medium without cells and in the medium of fibroblast culture without intermediate, significant changes in the content of glucose ($p<0.0001$), lactate ($p<0.0001$) and pyruvate ($p<0.0001$) were detected, while glucose level reduction was accompanied by lactate accumulation and increase of pyruvate content. It was noted that addition of oxaloacetate solution into the cultivation medium with fibroblasts led to changes in the metabolic profile (*Table* 1). Oxaloacetate reduced cholesterol levels by 27%, compared with control samples ($p=0.03$). The addition of oxaloacetate to the medium with growing fibroblasts promoted a lower accumulation of lactate in the supernatant of 6.63±0.18 mmol/L, compared with the cell control (6.8±0.4 mmol/L), but these values were higher than the baseline control of 2.58±0.1 mmol/L (nutrient medium without cells) ($p<0.0001$). When cells grew with oxaloacetate solution, we also observed a 5.2-fold increase in pyruvate content ($p<0.0001$) vs. the control values of the medium with fibroblasts.

Further on, we evaluated the activity of intracellular fibroblast enzymes (*Table* 2). Growth of fibroblasts with the addition of oxaloacetate solution reduced the activity of alkaline phosphatase, gamma-glutamyl transpeptidase ($p=0.0019$), and increased the activity of glycerol-3-phosphate dehydrogenase ($p<0.0001$) in the cell lysate.

Oxaloacetate improved fibroblasts viability by 33.8% ($p=0.028$) (*Table* 3). The differences in LDH test parameters between the control and experimental samples were not statistically significant.

**Discussion**

Our study was devoted to the role of a small molecule in intermolecular interactions. We revealed the biological activity of oxaloacetate via computer modeling. It should be specifically noted that oxaloacetate enhances the expression of TP53 gene that acts as a suppressor of malignant tumor formation, and is considered antioncogene [12]. In addition, oxaloacetate was predicted to reduce the expression of TNF gene encoding multifunctional proinflammatory cytokine, which is mainly secreted by macrophages and participates in the regulation of a wide range of biological processes. We predicted the effect of inhibition of HIF1A factor induced by hypoxia 1, which acts as the main regulator of transcription of adaptive response to hypoxia. Use of natural metabolite as a therapeutic agent for the treatment of phobic disorders is well-known, which is associated with the manifestation of neuroprotective action [2]. Biologically active compound under study enhances the expression of HMOX1 gene encoding heme oxygenase protein-1 and JAK2 gene, which regulates viability, proliferation and differentiation of many cell types. Thus, a small molecule plays a coordinating role in the functioning and modulation of mediator, receptor responses, immunological, inflammatory, antibacterial and antiviral reactions, having an anticarcinogenic effect [13, 14]. Thus, small molecule plays a coordinating role in the functioning and modulation of the mediator, in receptor responses; in immunological, inflammatory, antibacterial and antiviral reactions; and has anticarcinogenic properties [13, 14]. The study of an array of computer modeling data using the PASS platform made it possible to form a general awareness of the effects, and intermolecular and regulatory mechanisms of oxaloacetate action. Further study of oxaloacetate using the cell culture method is of a definite interest, as is the possibility to evaluate its proliferative properties.

We consider metabolites in dermal fibroblast cell culture a phenotypic response of biological systems to changes occurring in their microenvironment. According to L. Willmann, cell culture metabolites consist of intracellular metabolites (endometabolome) and metabolites released into the extracellular environment (exometabolome) [15]. While studying cell cultures, it is necessary to consider general metabolic status of the cells used in vitro, relative intensity of different metabolic processes, and changes caused by the factors under study. To assess the metabolic profile of endometabolome, we used fibroblast cell culture lysate, whereas extracellular medium was evaluated in the supernatant, in which the cells were growing.

Changes in glucose content under the influence of a small molecule were statistically insignificant. It is noteworthy that the addition of oxaloacetate in a medium with growing fibroblasts did not lead to statistically significant changes in lactate levels; the concentration of this metabolite decreased, but remained close to the control values, which could have implied that relatively constant lactate level was a necessary condition for normal functioning of physiological systems. There are some data that lactic acid concentration can increase up to 10-18 times, compared with the control medium during cell cultivation. The release of a relatively large amount of lactate indicated high activity of anaerobic glycolysis processes in growing fibroblasts, while the in

vitro activity of Krebs cycle processes in dermal fibroblasts was lower [16]. The analysis of pyruvate level demonstrated an increase of pyruvate content, which could be explained by ease of oxaloacetate conversion into pyruvate, because these compounds have structural similarity: both of them are ketoacids and differ from each other by one carboxylic group. The glucose/lactate index remained within the control values, while glucose/pyruvate index exhibited significant decrease, which could probably be explained by structural transition of one metabolite into another due to their similar chemical organization the regulation of tricarboxylic acid cycle enzyme activity in excess of oxaloacetate [17]. A statistically significant reduction in cholesterol suggests incorporation of this molecule into membranes and corresponding cell growth.

Gamma-glutamyl transpeptidase is habitually involved in the prooxidant-antioxidant balance; under conditions created by an increase in the concentration of oxaloacetate, the enzyme can also have antioxidant effect and reduce the cascade of free radical reactions, and trigger proliferative processes in the cell [18, 19]. The growth of fibroblasts with the addition of oxaloacetate solution reduces the activity of alkaline phosphatase in cell lysate, which probably implies the protective action of this metabolite and participation in the structural formation of cellular components in the fibroblast culture. There is information about the participation of alkaline phosphatase of fibroblasts in the processes of collagen degradation, associated with the provision of calcium ions necessary for collagenase activity [20]. The study of glycerol-3-phosphate dehydrogenase activity is required for understanding metabolic processes linking energy and lipid metabolisms. The reverse directed effect of a small molecule on the activity of glycerol-3-phosphate dehydrogenase is noted; oxaloacetate increases the activity of this enzyme in the cell lysate, resulting in an increase of the energy metabolism effectiveness.

Hence, demonstrated changes in enzymatic activity and viability of fibroblasts under impact of oxaloacetate imply that intermediates may play a regulatory role and effectively switch the cell energy flows, preserving internal homeostasis and optimizing metabolism in response to external factors. As natural endogenous cell compound, even when introduced exogenously, the studied biologically active substance has a low cytotoxicity profile and, thereby, can be used for further examination as a therapeutic agent, which certainly opens up opportunities for further research.

## Conclusion

The study provides important information on oxaloacetate capability to influence the proliferative properties of human dermal fibroblasts, which is reflected by changes in the content of metabolites in the cell culture supernatant and enzyme activity in the cell lysate.

## Study limitations

1. Examination of oxaloacetate in other concentrations is required.

2. It is necessary to evaluate the expression of GPD1, GPD2, as well as enzymes of glycolysis and oxidative phosphorylation of human dermal fibroblasts.

3. In the future, it is worth evaluating the effect of oxaloacetate on various moderated cell states, such as hypoxia.

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## Conflict of interest

The authors declare that they have no conflicts of interest.

## Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

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*Authors:*

**Nataliya A. Kolotyeva** – MD, DSc, Professor, Department of Fundamental and Clinical Biochemistry with Laboratory Diagnostics, Samara State Medical University, Samara, Russia. https://orcid.org/0000-0002-7853-6222.

**Larisa V. Limareva** – DSc, Associate Professor, Director of the Research Institute for Experimental Medicine and Biotechnology, Samara State Medical University, Samara, Russia. https://orcid.org/0000-0003-4529-5896.

**Frida N. Gilmiyarova** – DSc, Professor, Department of Fundamental and Clinical Biochemistry with Laboratory Diagnostics, Samara State Medical University, Samara, Russia. https://orcid.org/0000-0001-5992-3609.

**Violetta V. Boltovskaya** – PhD, Senior Researcher, Research Institute for Experimental Medicine and Biotechnology, Samara State Medical University, Samara, Russia. https://orcid.org/0000-0003-3457-8524.

**Pavel V. Ilyasov** – PhD, Senior Researcher, Research Institute for Experimental Medicine and Biotechnology, Samara State Medical University, Samara, Russia. https://orcid.org/0000-0002-1532-0272.

**Oksana A. Gusyakova** – MD, DSc, Associate Professor, Head of the Department of Fundamental and Clinical Biochemistry with Laboratory Diagnostics, Samara State Medical University, Samara, Russia. https://orcid.org/0000-0002-5619-4583.

**Vasiliy V. Remizov** – 6th year student, Institute of Clinical Medicine, Samara State Medical University, Samara, Russia. https://orcid.org/0000-0002-1004-5443.