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Effect of key enzyme inhibition of glycolysis on synovial fibroblasts in Rheumatoid Arthritis

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Abstract

Objective: To observe effects of glycolysis on human rheumatoid arthritis Fibroblast-like synoviocytes (HFLS-RA) by inhibiting glycolysis.

Methods: Hexokinase inhibitor (3-bromopyruvate, 3-BrPa), 6-phosphofructokinase 1 inhibitor citric acid and pyruvate kinase inhibitor shikonin were applied to HFLS-RA respectively. Cell count 8 Kit detects cell proliferation activity, the activity of hexokinase, 6-phosphofructokinase 1, and pyruvate kinase, as well as the cellular glucose, lactate and ATP content were detected by kits, and the ELISA kit detects the expression of cellular inflammatory factors TNF-α and TGF-β.

Results: 10 μg/mL 3-BrPa, 160 μg/mL citric acid and 5 μg/mL shikonin significantly inhibited cell proliferation activity ($P<0.001$); and significantly inhibited HFLS-RA hexokinase and fructose 6-phosphate Kinase 1 and pyruvate kinase activity; Glucose, lactate and ATP content decreased; TNF-α expression decreased, while TGF-β expression increased.

Conclusion: This study explored the changes in glucose metabolism and the expression of inflammatory factors in HFLS-RA by inhibiting the key enzymes of glycolysis, further confirming the important role of glycolysis in HFLS-RA, and laying a theoretical basis for a deep understanding of the pathogenesis of RA.

Keywords: Rheumatoid arthritis; Fibroblast-like synoviocytes; Glycolysis; Key enzyme

1. Introduction

Rheumatoid Arthritis (RA) as a systemic inflammatory autoimmune disease that mainly affects the surrounding joints. RA without systemic treatment can persist for many years, eventually leading to joint deformities and loss of function. According to epidemiological investigations, the prevalence rate in my country is 0.32%-0.36%. For patients who have not been diagnosed and treated in time, the disability rate is 50% after 2 years, and 70% of patients have varying degrees of disability after 3 years. The quality of life severely affected [1]. Current studies have shown that the pathogenesis of RA is related to multiple mechanisms such as infection, heredity, immune mechanism disorders, and metabolic abnormalities [2-4]. However, it has not yet been definitively concluded, and there is still a lack of effective preventive and therapeutic drugs.

Human rheumatoid arthritis synovial fibroblasts (HFLS-RA) exhibit abnormal activation and proliferation in the RA joint cavity. They are the main effector cells that mediate synovial inflammation and joint destruction, and can secrete a variety of cytokines including interleukins, tumor necrosis factor, vascular growth factor [5], not only can respond to inflammation, it can also stimulate inflammation, and even form pannus to destroy articular cartilage and bone [6]. Therefore, it has become the target of RA treatment. Recently, in the synovial fluid and synovial membrane of RA, an

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increase in glycolytic enzyme activity and its product, lactic acid, has been observed, while glucose has decreased [7]. Further studies have shown that glucose metabolism in RA synthetic ovarian fibroblasts (RASFs) changes, turning to glycolysis to support its own high proliferation, invasion and angiogenesis [8]. It is undeniable that HFLS-RA has already experienced increased glycolytic activity, but the specific mechanism is still unclear. In this study, hexokinase inhibitors (3-bromopyruvate, 3-BrPa) and 6-phosphofructokinase 1 inhibitor lemon the acid and pyruvate kinase inhibitor shikonin acted on HFLS-RA respectively to detect the changes of sugar metabolites and the mechanism of action, in order to provide a theoretical basis for further understanding of the pathogenesis of RA.

2. Material and methods

2.1. Materials

Human rheumatoid arthritis synovial fibroblasts (HFLS-RA) were purchased from Beina Chuanglian Biotechnology Co., Ltd.; Fetal Bovine Serum (Sciencell); RPMI 1640 Medium (Gibco); 3-Bromopyruvate (3-BrPa) ; Citric acid and shikonin (Beijing Soleibao Bio); Cell counting kit-8 (Beijing Soleibao); Glucose content detection kit, ATP content detection kit, lactic acid content detection kit and hexokinase Activity detection kit (Beijing Soleibao Bio). Human TGF-β ELISA Kit (Wuhan Genebei), Human TNF-α ELISA Kit (Wuhan Genebei).

2.2. Methods

2.2.1. CCK-8 detects cell viability

The HUVEC in the logarithmic growth phase was digested, collected by centrifugation, counted, diluted with DMEM complete medium to 1×104/mL, 100μL per well was added to a 96-well plate, adhered overnight, and DMEM medium was added to dilute the concentration gradient 3 -BrPa (0, 5, 10, 20, 40, 80, 160 μg/mL), Citric acid (0, 20, 40, 80, 160, 320 μg/mL) and Shikonin (Shikonin) (0, 5, 10, 20, 40, 80, 160 μg/mL) culture 24, add 10 μl of CCK-8, and detect the absorbance at 490 nm with a microplate reader.

2.2.2. Determination of glucose (G) content

According to the instructions, the cell glucose content was detected by spectrophotometry. Calculated by the number of cells, glucose content (μmol/10^4 cells) = C× (A measuring tube-A blank tube)/ (A standard tube-A blank tube) × V sample / (500/V sample total × V sample) = 0.002× (A measuring tube-A blank tube)/(A standard tube-A blank tube).

2.2.3. Activity detection of hexokinase (HK), phosphofructokinase 1 (PFK-1) and pyruvate kinase (PK)

According to the instructions, the activity of HK, phosphofructokinase 1 and pyruvate kinase was detected by spectrophotometry. Calculated according to the number of cells, HK (U/10^4 cells) =2.226×ΔA; PFK-1 (U/10^4 cells) =0.9×ΔA; PK (U/10^4 cells) =5.226×ΔA.

2.2.4. ATP and lactic acid (LA) content detection

According to the instructions, spectrophotometric method was used to detect cell ATP and lactic acid content. Calculated by the number of cells, ATP (umol/10^6 cells) =0.125×ΔA determination /ΔA standard; LA (μmol/10^6 cells) =0.2375×X.

2.2.5. ELISA to detect the expression of TNF-α and TGF-β

Collect the drug-treated cell supernatant, set blank wells and sample wells for dilution standards, add 40μL sample diluent and 10μL sample to be tested into the sample wells, add 50μL enzyme-labeled reagent, seal the plate and incubate at 37°C 30min, wash 5 times with the washing solution, absorb the liquid with absorbent paper, add 50μL each of developer A and developer B, incubate at 37°C for 10min in the dark, add 50μL of stop solution, measure the absorbance value at 450nm, draw a standard curve, Calculate the actual concentration of the sample to be tested.

2.3. Statistical methods

Use SPSS12.0 and GraphPad Prism 5.0 statistical software to analyze. The data is expressed by $\bar{x}$±SEM, the comparison between the two groups was performed by *t* test, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. CCK-8 detects cell viability

In order to determine the optimal concentration of the drug on HFLS-RA, 3-BrPa (0, 5, 10, 20, 40, 80, 160 μg/mL), citric acid (0, 20, 40, 80, 160, 320 μg/mL) and shikonin (0, 5, 10, 20, 40, 80, 160 μg/mL) were applied to the cells for 24 hours, and the proliferation activity of the cells was detected by CCK-8. The results showed that 10 μg/mL 3-BrPa (Figure 1A), 160 μg/mL citric acid (Figure 1B), and 5 μg/mL shikonin (Figure 1C) significantly inhibited the proliferation activity of HFLS-RA.

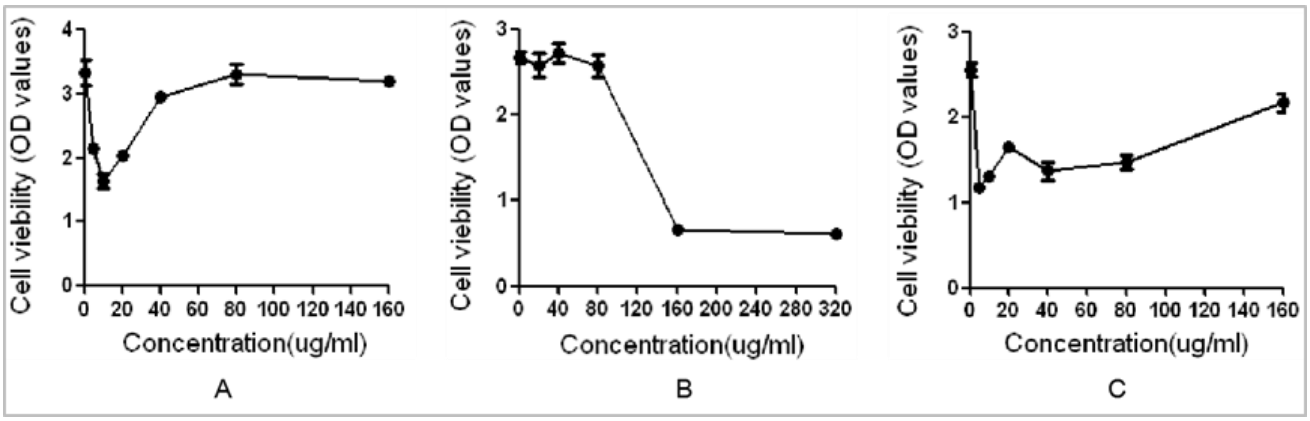

Figure 1 The effect of drugs on cell proliferation activity A: The effect of 3-BrPa on cell proliferation activity, with 10 μg/mL as the optimal concentration (P<0.001); B and C: citric acid and shikonin, respectively For the influence of cell proliferation activity, 160 μg/mL citric acid and 5 μg/mL shikonin are the best concentration.

3.2. Enzyme activity detection

In order to detect the effect of the drug on the key glycolysis enzymes in HFLS-RA, we treated the cells with inhibitors of the key glycolysis enzymes for 24 hours, and the kit was used to detect the enzyme activity. The results showed that compared with the control group, 3-BrPa, citric acid and shikonin significantly inhibited the expression activity of hexokinase (Figure 2A), 6-phosphofructokinase 1 (Figure 2B) and pyruvate kinase (Figure 2C).

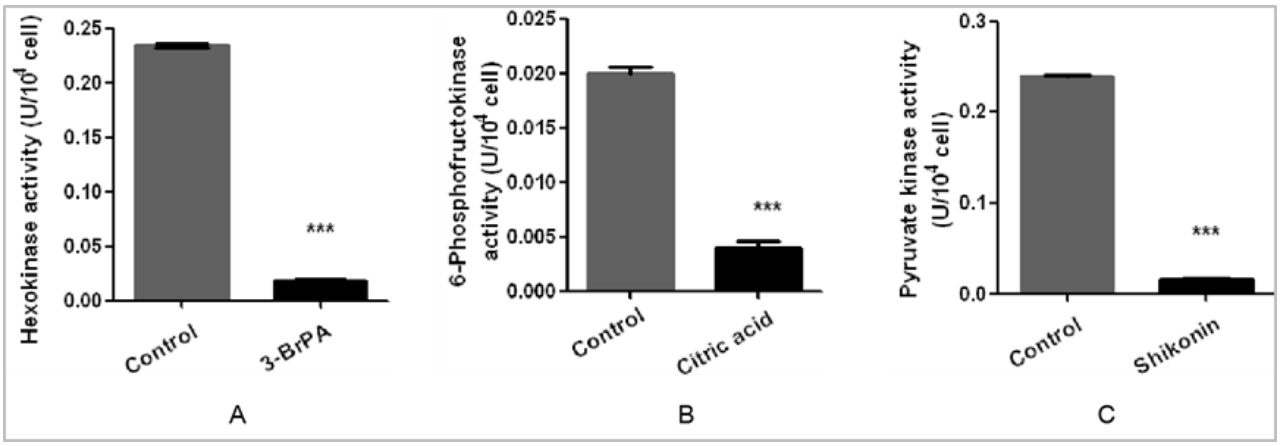

Figure 2 Enzyme activity detection of HFLS-RA. A: Activity of of hexokinase; B: 6-phosphofructokinase 1 activity of; C: Pyruvate kinase activity of (n=3, ***P<0.001)

3.3. Detection of glucose, ATP and lactic acid content

For further understand the glucose metabolism in HFLS-RA treated with different inhibitors, we detected the content of glucose, ATP and lactic acid in the cells. The results showed that compared with the control group, the hexokinase inhibitor and the pyruvate kinase inhibitor significantly reduced the glucose, ATP and lactate content; however, the 6-

phosphofructokinase 1 inhibitor increased the glucose, ATP and lactate content; both Combined with three inhibitors, the content of glucose and lactic acid was reduced. Show in Figure 3.

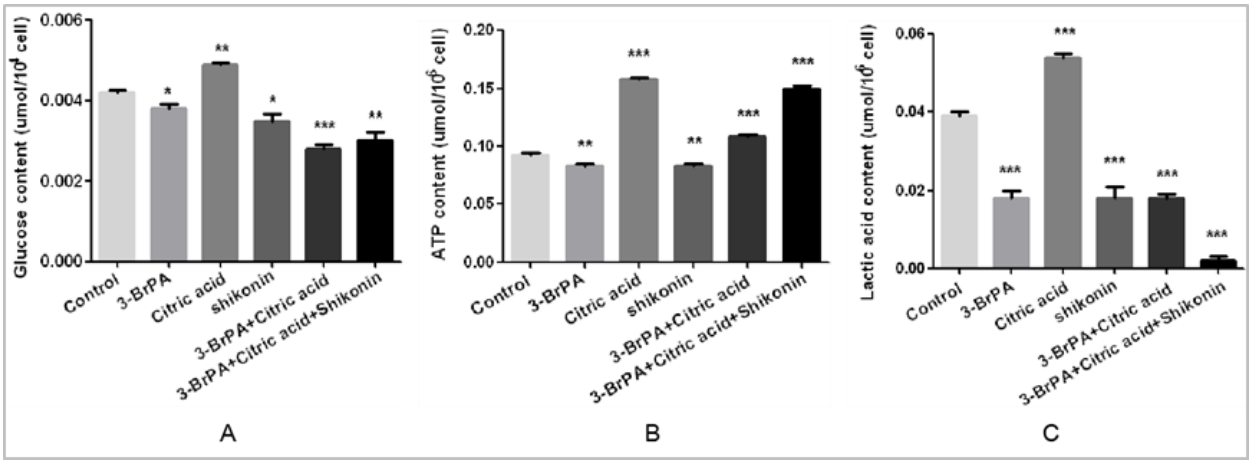

Figure 3 Detection of glucose, ATP and lactic acid content. A: Glucose content detection; B: ATP content detection; C: Lactic acid content detection (n=3, **P*<0.05, ***P*<0.01, ****P*<0.001).

3.4. ELISA to detect the expression of cell inflammatory factors

To further understand the pathogenesis of RA, we tested the expression of inflammatory factors in HFLS cells. The results showed that, compared with the control group, the expression of TNF-α in the inhibitor group generally decreased, and the expression of TGF-β increased, as shown in Figure 4.

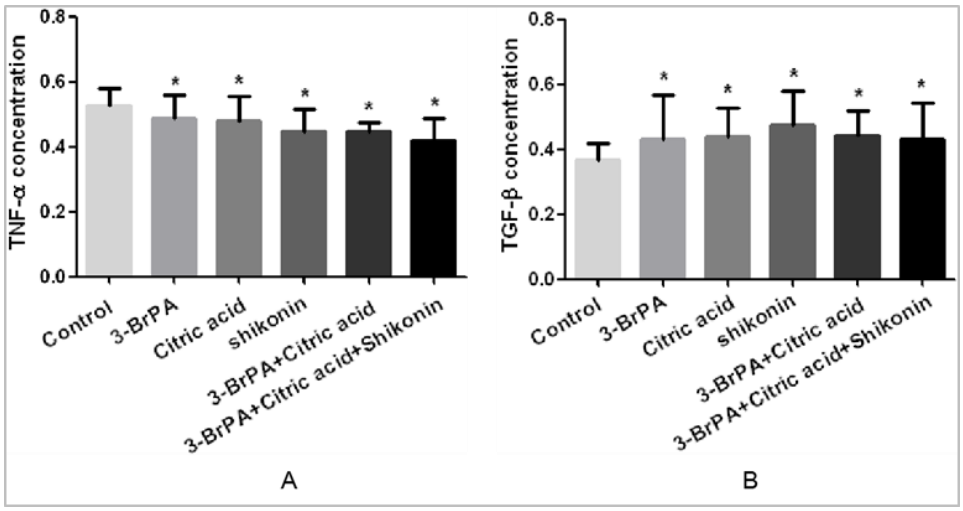

Figure 4 Detection of cell inflammatory factor expression. A: TNF-α expression detection; B: TGF-β expression detection (n=6, *P<0.05).

4. Discussion

HFLS-RA plays a crucial role in the pathogenesis of RA. FLS is a kind of stromal cells that can bestow synovial lining structure, and promote by invading and promoting cartilage destruction, secreting pro-inflammatory cytokines and chemokines the pathological process of RA [9-11]. At present, there have been studies focusing on the sugar metabolism of RA synovial stromal cells (such as FLS), but the influence of multiple key glycolysis enzymes on HFLS-RA is rarely reported. The first step of glycolysis is the phosphorylation of glucose, which is catalyzed by hexokinase (HK). Currently reported that 2-deoxy-D-glucose (2-DG), 3-bromopyruvate and Lonidamine as carriers of HK inhibitors [12,13] are limited to the first step of glycolytic pathway hexokinase catalyzes glucose as Inhibition of the first rate-limiting reaction of 6-phosphate-glucose has achieved certain results in the treatment of inflammation in experimental autoimmune model mice, but the inhibition of hexokinase makes the glycolytic pathway in the complex regulation of the body not completely resolved. Instead, the second key enzyme for irreversible reaction, 6-phosphofructokinase, catalyzes 6-

phosphate-fructose to 1.6-diphosphate fructose, which is the most critical enzyme for regulating glycolysis flow, because it can be allosteric with ATP and citric acid. Inhibitors, AMP, ADP and fructose 2,6-diphosphate are effective allosteric activators, that is, ATP is both a substrate and an inhibitor, and glycolysis is constantly using AMP and ADP to synthesize energy due to lack of ATP. Therefore, the glycolysis pathway is continuously stimulated [14]. Therefore, the inhibition of only one key enzyme cannot achieve true treatment of RA. In this study, we used inhibitors to inhibit the three key rate-limiting enzymes of glycolysis and detect the changes in glucose metabolism in HFLS-RA cells, in order to further understand RA. The pathogenesis provides a theoretical basis.

In this study, we applied 3-BrPa, citric acid and shikonin to HFLS-RA according to the literature [15-19]. The results showed that 3-BrPa, citric acid and shikonin significantly inhibited the proliferation activity of HFLS-RA cells, and inhibited the activities of hexokinase, 6-phosphofructokinase 1 and pyruvate kinase, respectively. In the detection of glucose, ATP and lactic acid content, the citric acid group was significantly higher than the control group, while the other drug inhibition group was significantly lower. For synovial tissue in RA, the purpose of adjusting the flow is to adapt to its energy demand. When the intracellular ATP/AMP ratio decreases, both phosphofructokinase-1 and pyruvate kinase are activated, and glucose decomposition is accelerated. Citric acid can inhibit phosphofructokinase catalyzing 6-phosphate fructose to 1,6-diphosphate fructose, and at the same time, as an intermediate product of the tricarboxylic acid cycle, it inhibits the action of succinate dehydrogenase. It is also related to the metabolic process of mitochondria and inhibits acetone. The action of acid dehydrogenase prevents the production of acetyl-CoA from pyruvate. But as an activator of gluconeogenesis, it promotes the production of glucose from muscle glycogen [20].

To further understand the expression of inflammatory factors in cells, we tested TNF-α and TGF-β. Studies have found that TNF-α is one of the pathogenic factors that play a decisive role in the pathogenesis of rheumatoid arthritis. It can induce the expression of endothelial cell adhesion molecules and promote the occurrence of local inflammation caused by the adhesion and penetration of leukocytes and vascular endothelium [21]. TNF-α can stimulate synovial fibroblasts and chondrocytes to secrete prostaglandin E2 (PGE2) and collagenase, promote bone destruction, bone resorption and fibroblast proliferation, inhibit collagen synthesis, increase synovial endothelial cells and The release of fibroblast growth factor promotes the production of inflammatory factors by synovial cells, fibroblasts, etc., accelerates the process of arthritis damage, and then aggravates tissue damage [22]. TGF-β is a bidirectional, pleiotropic network cytokine, which can promote inflammation and inhibit inflammation. We tested TGF-β to explore its expression in HFLS-RA after glycolysis inhibition. At present, most studies believe that TGF-β can regulate the proliferation of T cells, can also inhibit the effect of B lymphocytes, reduce the secretion of immunoglobulin IgG and IgM, and inhibit humoral immune response, thereby exerting the effect of negative regulators [23]. Recent study [24] have shown that, the level of TGF-β in RA patients is significantly reduced, and the changes in the active phase are more significant. It is believed that TGF-β mainly plays a negative regulatory role in the pathogenesis of RA and inhibits the progression of the disease. With the development of rheumatoid arthritis, serum TGF-β1 can invade the joint synovium in a latent form. Synovial TGF-β1 promotes synovial hyperplasia by inhibiting Fas antigen-mediated apoptosis, thereby accelerating the occurrence of the disease Development [25]. In this study, the expression of TNF-α in HFLS-RA cells decreased, which is consistent with the above study, while the expression of TGF-β increased after glycolysis inhibitors, suggesting that TGF-β mainly plays a negative role in the pathogenesis of RA Function to inhibit inflammation of RA synovium.

5. Conclusion

In summary, 3-BrPa, citric acid, and shikonin, respectively, as inhibitors of hexokinase, 6-phosphofructokinase 1 and pyruvate kinase, significantly inhibited the proliferation of HFLS-RA cells, and at the same time caused glucose, the content of ATP and lactic acid is reduced. The decrease of TNF-α expression in the cells indicates that the inhibition of glycolysis reduces the expression of HFLS-RA inflammatory factors and inhibits the occurrence and development of RA synovial inflammation. The increase of TGF-β mainly plays a negative regulatory role in RA synovial fibroblasts and inhibits RA synovial inflammation. This study explored the proliferation, enzyme activity, intracellular glucose, ATP, and lactic acid content and cytokine expression of HFLS-RA through the inhibition of multiple key enzymes of glycolysis, confirming that HFLS-RA cells rely on glycolysis to satisfy their pathological acidity. Get energy from the environment, hoping to provide a theoretical basis for further understanding of the pathogenesis of RA.

Compliance with ethical standards

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Disclosure of conflict of interest

None.

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