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Abstract

I. Background: The roles of antioxidants on the energy metabolism of disc cells are not clear. In this study, we clarified the roles of antioxidant N-acetylcysteine (NAC) on the energy metabolism of goat temporomandibular joint (TMJ) disc cells exposed to hypoxia.

II. Methods and Results: The Isolated and cultured goat TMJ disc cells were divided into control, NAC group, cobalt chloride (CoCl2) group, and CoCl2 with NAC group, exposed to 21% O2 and 2% O2. The glucose consumption, lactate content, intracellular ATP, ROS, HIF-1α, GLUT1, LDHA, PKM2 and PGK1 expressions were detected respectively. Under the hypoxia model, NAC inhibited the mRNA expression of HIF-1α, GLUT1, LDHA, PKM2, and PGK1. And it significantly decreased the lactate content in the culture supernatant and the intracellular ROS. However, NAC increased glucose consumption and simultaneously promoted the production of ATP in goat TMJ disc cells under hypoxia. We speculated that NAC would transfer energy metabolism toward oxidative phosphorylation under hypoxia. And NAC would become a potential therapeutic target for TMJ disc engineering and disease study.

III. Conclusions: The antioxidant NAC promoted the proliferation of hypoxia-inducible goat TMJ disc cells, and the CoCl2 group (21% O2) was promoted effectively ($P < 0.05$). NAC promoted glucose consumption in culture supernatants under hypoxia and significantly reduced lactate production ($P < 0.01$). NAC effectively eliminated intracellular ROS expression in hypoxia-inducible cells ($P < 0.001$) and promoted intracellular ATP production ($P < 0.05$). However, NAC inhibited the relative mRNA level of HIF-1α, GLUT1, LDHA, PKM2, and PGK1 ($P < 0.05$).

Keywords: Cobalt chloride; Hypoxia; N-acetylcysteine; Goat; temporomandibular joint disc cells; Energy metabolism

Abbreviations

TMJ	Temporomandibular joint
TMD	Temporomandibular disorders
$CoCl_2$	Cobalt chloride
ROS	Reactive oxygen species
OS	Oxidative stress
NAC	N-acetylcysteine
FBS	Fetal bovine serum
Vc	Ascorbic acid vitamin C
HIF-1α	Hypoxia-inducible factor 1α
GLUT1	Glucose transporter1
LDHA	Lactate dehydrogenase A
PKM2	Pyruvate kinase M2
PGK1	Phosphoglycerate kinase1
HK	Hexokinase
PFK-1	Phosphofructokinase-1
DCFH-DA	2',7'-dichlorofluorescein diacetate
h	hour
min	minute

Introduction

The temporomandibular joint (TMJ) disc is a fibrocartilage-like tissue located between the condyle of the mandible and the articular surface of the temporal bone [1], which is the main component of the TMJ and plays an essential role in the functional movements such as mastication, swallowing, and phonation [2]. Temporomandibular disorders (TMD) are painful or dysfunctional disorders involving certain areas of the Cranio-Cervical-Mandibular Complex (CCMC), such as the masticatory muscles, TMJ, and other related structures, as well as the postural muscles of the neck, so, TMD can be considered a subgroup of musculoskeletal disorders [3]. The management of TMD is aimed to reduce joint pain, increase joint function and improve the opening and prevent further joint damage, thereby improving the patient's overall quality of life and reducing the incidence of TMD-related diseases. The treatment of TMD follows the principles of non-invasive, minimally invasive, and surgical therapies [4-6]. Current therapies are difficult to reverse the TMJ disc that has been lesioned and cannot restore the intact structure and good function of TMJ, because of the weak regenerative repairability of TMJ disc exposed physiologically to an ischemic and hypoxic environment. Therefore, an in-depth study of the pathophysiological mechanisms of TMD is crucial and beneficial to the clinical treatment of TMD.

Oxygen plays a vital role in cellular energy metabolism and internal environmental homeostasis. And it is indispensable for maintaining the normal growth and development of the organism. Hypoxia can cause abnormalities in cellular energy supply, and lead to dystrophic diseases. However, the normal TMJ disc is nourished by the synovial fluid around the disc and the post-disc tissues, physiologically in an ischemic and hypoxic environment. Continuous loading can further restrict the nutrition and metabolism of the disc cells [7, 8]. TMJ disc may be particularly susceptible to degenerative changes because of its avascularity, steep oxygen, and glucose gradients. The combination of hypoxia and limited glucose concentration dramatically affects the synthesis of the extracellular matrix (ECM), limiting the ability to repair itself. Therefore, culturing and studying the TMJ disc cells in a similar hypoxic environment can help further understand the pathophysiology of TMJ and the development of potential clinical therapies for TMD.

Hypoxic environments can be simulated physically or chemically. Cobalt chloride ($CoCl_2$) is the most commonly used chemical hypoxia simulator and has been used to induce and simulate hypoxic conditions *in vitro* [9]. Hypoxia promotes the production of reactive oxygen species (ROS), which in turn triggers the oxidative stress (OS) effects [10]. OS is defined as the imbalance between the production of ROS and the endogenous antioxidant defense system [11]. OS has been associated with the pathophysiology of several diseases [12-14], such as TMD, which leads to changes in synovial fluid composition [15], and is also involved in pathological conditions of intra-articular inflammation and degeneration [16]. N-acetylcysteine (NAC), a sulfhydryl-containing antioxidant [17], is a precursor of glutathione (GSH) and acts as a powerful antioxidant and free radical scavenger *in vivo* [18, 19] However, the effects and mechanism of NAC on the energy metabolism of TMJ disc cells are currently unknown. Because of the high similarity in size, morphology, structure, and function between TMJ discs in goats and humans [20], we select the TMJ disc cells from a 3-month-old goat as our study subjects.

Therefore, in this study, we simulated the physiological hypoxic environment of goat TMJ disc cells by chemical hypoxia with $CoCl_2$, physical hypoxia with 2% O_2, and physical-chemical dual hypoxia. Cell proliferation, glucose consumption, and lactate production in the culture supernatant, intracellular ATP production, ROS expression, and relative mRNA level of HIF-1α and four key enzymes in the glycolytic process of hypoxia-inducible goat TMJ disc cells were detected respectively. To investigate

the effects and regulatory mechanisms of NAC on the energy metabolism of goat TMJ disc cells under hypoxia, which can help further understand and appreciate the pathophysiological mechanisms of TMD, and provide a basis for clinical treatment.

Results

NAC promoted cell proliferation under hypoxia

5 mM NAC had no significant effects on the proliferation of goat TMJ disc cells in the control group under 21% O_2 for 24 and 48 h ($P > 0.05$), while it promoted the proliferation of goat TMJ disc cells in the $CoCl_2$ group (21% O_2), with a significant effect at 24 h ($P < 0.05$) and no statistical significance at 48 h ($P > 0.05$) (**Fig. 1 A)**. In the control group (2% O_2), there was no significant effect after adding NAC for 24 and 48h. However, NAC significantly promoted the proliferation of goat TMJ disc cells in the $CoCl_2$ group (2% O_2) at 24 and 48 h ($P < 0.001$) (**Fig. 1 B**).

NAC increased glucose consumption and reduced lactate production

NAC increased the glucose consumption in the culture supernatant of goat TMJ disc cells exposed to hypoxia (**Fig. 2 A**). Unlike the NAC group, glucose consumption decreased under hypoxia compared with the normoxic groups. While the addition of NAC could increase glucose consumption to some extent, the promotion effect was the most obvious under the physicochemical double hypoxia ($P < 0.05$).

NAC significantly reduced the lactate content in the culture supernatant of goat TMJ disc cells (**Fig. 2 B**). Lactate content in the culture supernatant exposed hypoxia was higher than that of the normoxic groups, at the same time, the treatment of NAC significantly reduced the lactate content in the supernatant of cultures ($P < 0.001$).

NAC enhanced the intracellular ATP production

NAC enhanced the intracellular ATP production in goat TMJ disc cells (**Fig. 3 A**). Intracellular ATP synthesis in goat TMJ disc cells was significantly decreased under hypoxia ($P < 0.001$). In the control group (2% O_2), $CoCl_2$ group (21% O_2), and $CoCl_2$ group (2% O_2), the intracellular ATP synthesis was significantly increased after the treatment of NAC ($P < 0.01$).

NAC effectively eliminated the intracellular ROS

NAC effectively eliminated ROS in goat TMJ disc cells (**Fig. 3 B**). ROS expression was elevated in goat TMJ disc cells under hypoxia, with the most significant increase in the $CoCl_2$ group (2%O_2) ($P < 0.001$), while the addition of NAC significantly eliminated ROS expression ($P < 0.001$).

NAC inhibited the mRNA expression of HIF-1α and four key enzymes of glycolysis

NAC inhibited the mRNA expression of HIF-1α in goat TMJ disc cells (**Fig. 4 A**). The relative mRNA level of HIF-1α was reduced in goat TMJ disc cells exposed to hypoxia, and HIF-1α mRNA expression was further inhibited after the addition of NAC, which was significantly reduced in the control group (2% O_2) ($P < 0.01$), and the $CoCl_2$ group (2% O_2) ($P < 0.001$).

NAC suppressed the mRNA expression of key enzymes in the glycolysis process of goat TMJ disc cells under hypoxia (**Fig. 4 B-E**). Under hypoxia, except for LDHA mRNA expression increased, the relative mRNA level of GLUT1, PKM2, and PGK1 were suppressed in goat TMJ disc cells. While the expression of GLUT1, LDHA, PKM2, and PGK1 mRNA were significantly reduced in goat TMJ disc cells after NAC was added ($P < 0.001$).

Discussion

The normal temporomandibular joint disc is located in a physiological environment of ischemia and hypoxia [7]. In this study, we used the chemical hypoxia inducer cobalt chloride, 2% O_2 physical hypoxia, and physicochemical double hypoxia to simulate an ideal oxidative stress model under hypoxia of goat TMJ disc cells.

Carbohydrate metabolism is the energy source for most cells, and glucose metabolism is mainly divided into oxidative phosphorylation, glycolysis, and pentose phosphate pathway etc[21] (**Fig. 5**). The pentose phosphate pathway without ATP production plays an essential role in the body, which includes the precursors for nucleotide synthesis and the provision of nicotinamide adenine dinucleotide phosphate (NADPH) [22]. Glucose produces ATP through the tricarboxylic acid cycle (TCA cycle) with adequate oxygen and glycolysis without oxygen or anaerobic conditions. Compared to oxidative phosphorylation, glycolysis is a fast but inefficient process to produce ATP (i.e., 1 mol of glucose produces 2 mol of ATP by glycolysis, whereas 1 mol of glucose can produce 30 or 32 mol of ATP by oxidative phosphorylation), so the cellular energy supply is mainly provided by oxidative phosphorylation [23]. However, in cancer cells or immune cells, even under aerobic conditions, glycolysis is still used as the primary form of energy supply, also known as aerobic glycolysis (Warburg effect) [24, 25]. Glycolysis is the main carbohydrate metabolic pathway in normal intervertebral disc nucleus pulposus cells [26]. Glucose transporter1 (GLUT1), lactate dehydrogenase A (LDHA), pyruvate kinase M2 (PKM2), and phosphoglycerate kinase 1 (PGK1) are critical enzymes in glycolysis [27]. GLUT family mediates the first step for cellular glucose usage. GLUT1 is one of the GLUT family, a transmembrane protein, which transports glucose from the extracellular to the cell [28]. The glucose was transferred to glucose-6-phosphate with hexokinase (HK), and then converted to fructose-6-phosphate [29]. Fructose-1,6-bisphosphate is generated from fructose-6-phosphate by the action of phosphofructokinase-1 (PFK-1), and ultimately, two molecules of propane phosphate are produced [30]. The first stage of ATP synthesis during glycolysis comes from the synthesis of glycerate-3-phosphate by glycerate-1,3-bisphosphate with PGK1[31]. And the second stage occurs in the production of pyruvate by phosphoenolpyruvic acid with PKM2[32]. Finally, pyruvate changes to lactate under LDHA [33].

As a powerful oxidant, NAC is used clinically to treat heart injury, lung disease, kidney injury, influenza, and cancer et al [34-37]. NAC can exert antioxidant effects through the free sulfhydryl groups and the increasing concentration of glutathione (GSH) in the body [38], which could protect the cells from oxidative stress damage. The study of broilers showed that NAC inhibited the production of ROS *in vivo* through sulfhydryl groups, thereby reducing the peroxidation of the mitochondrial membrane and protecting the structure of the mitochondrial membrane, then protecting oxidative phosphorylation and promoting ATP synthesis [39]. The study in the rat model of OA revealed that the development of OA was significantly antagonized by oral NAC administration [40]. NAC also had protective effects on hydrogen peroxide-inducible TMJ chondrocytes, which would promote TMJ chondrocyte proliferation, reduce apoptosis considerably, promote extracellular matrix secretion, and greatly enhance the antioxidant capacity of the body [41]. In the most recent retrospective analysis, it emphasized the numerous beneficial effects of the administration of antioxidants for the treatment of OA, which included the rebalancing of redox; alleviation of oxidative stress and inflammation that are detrimental to the body; and the appropriate regulation of growth and apoptosis [42].

In our study, we explored the effects of NAC on the proliferation and energy metabolism of goat TMJ disc cells. The results showed that NAC promoted the proliferation of hypoxia-inducible TMJ disc cells, and may also contribute to the shift in glucose metabolism exposed to hypoxia from the glycolysis

to oxidative phosphorylation. The proliferation of cells under hypoxia slowed down due to oxidative stress by accumulating large amounts of ROS. The application of NAC effectively alleviated the inhibitory effect of hypoxia on the proliferation of cells, with a significant impact under the dual hypoxia of cobalt chloride and 2% O_2.

Glycolysis is the central glucose metabolism in the TMJ disc cells; thus, the lactate content was significantly increased, while the addition of NAC significantly decreased the lactate content. Correspondingly, NAC inhibited the expression of HIF-1α and glycolytic enzymes GLUT1, LDHA, PKM2, and PGK1 in goat TMJ disc cells under hypoxia, which in turn, inhibited the glycolytic process.

However, NAC increased glucose consumption in the culture supernatant exposed to hypoxia, with the evident impact in physicochemical double hypoxia, which may be related to the increasing number of cells. ATP is the most direct energy source in the organism, and intracellular ATP synthesis was significantly reduced in goat TMJ disc cells under hypoxia, whereas NAC could increase intracellular ATP production.

The results of ROS illustrated that ROS was significantly increased in hypoxia, but it was reduced considerably with NAC, as an effective scavenger of ROS. NAC can effectively alleviate the oxidative stress damage caused by hypoxia on cells, which has a protective effect on goat TMJ disc cells. Meanwhile, NAC may shift the cellular energy metabolism toward aerobic phosphorylation, which is similar to the results of Li [39], who reported that the addition of NAC to the diet improved the energy reserves and antioxidant function of broiler ducks.

Limitations

The present study is not without limitations. On the one hand, the protein levels of HIF-1α, GLUT1, LDHA, PKM2, and PGK1 were not apparent in our study. On the other hand, oxidative stress indicators only included the ROS assay and lacked antioxidant detection, such as superoxide dismutase (SOD) and glutathione (GSH).

In addition, lactate, LDHA, and ROS showed a homothetic trend in TMJ disc cells in this study. The studies in knee osteoarthritis have shown that LDHA binds to NADH and promotes ROS-induced catabolic changes by stabilizing IκB-ζ (a key pro-inflammatory mediator) in chondrocytes [35]. It requires our further study, whether the same pathway exists in TMJ disc cells.

Conclusions

In conclusion (Fig. 6), as a powerful antioxidant, NAC could effectively reduce ROS content in TMJ disc cells under hypoxia, promote cell proliferation, reduce lactate content and mRNA expression of HIF-1α and key enzymes of glycolysis GLUT1, LDHA, PKM2, and PGK1, promoted ATP production, and facilitated the provision of energy to cells. NAC effectively reversed the oxidative stress damage caused by ROS and had protective effects on TMJ disc cells under oxidative stress. Also, NAC may become a potential clinical therapeutic target for TMJ disc diseases and TMD.

Materials and Methods

Experimental reagents

Cobalt chloride (Guangdong Chemical Reagent Engineering Technology Research and Development Center, China), N-acetylcysteine (MCE, USA). DMEM/F-12 (Hyclone, USA), FBS (BI, Israel), collagenase type I (Sigma, USA). Glucose assay kit, lactate assay kit, ATP assay kit, and reactive oxygen species assay kit (all from Solarbio, China), CCK-8 solution, FastPure® Cell/Tissue Total RNA Isolation Kit, HiScript® III RT SuperMix for qPCR and ChamQ Universal SYBR qPCR Master Mix (all

from Vazyme, USA).

Experimental instruments

Microscope (CX31, Olympus, Japan), Maworde DY-S workstation (Qiqihar Maworde Industry & Trade Co., Ltd., China), ultrasonic cell pulverizer (SCIENTZ-950E, Ningbo Xinzhi Biotechnology Co. Ltd. China), Peiou enzyme standardizer (Shanghai Peiou Analytical Instruments Co., Ltd.), Nano ultra-micro UV Spectrophotometer (Mettler UV5Nano, Switzerland), PCR reaction amplification instrument (BORI LifeECO, China), real-time fluorescence quantitative PCR instrument (QuantStudio 3, ABI, USA), multi-dimensional panoramic flow cytometer (FlowSight, USA).

Isolation, extraction, and culture of goat TMJ disc cells

The TMJ discs were dissected from a 3-month-old healthy goat, rinsed three times in PBS with a bit of penicillin-streptomycin, shredded, and treated with 2 mg/mL collagenase I about 8 mL for 16 h at 37 ℃ constant temperature water shaker. Then transferred to centrifuge tubes and added the DMEM/F12 to 10 mL, centrifuged three times (the first time, 13 min at 1,300 rpm, the second time, 10 min at 1,000 rpm, the third time, 5 min at 800 rpm). Lastly, resuspended cells with the complete medium (88% DMEM/F12, 10% FBS, 1% penicillin-streptomycin solution, and 1% Vc), were lastly plated in the cell culture flasks. When the cell fusion rate reached 80-90%, the cells were trypsinized with 0.25% trypsin and passaged to the next generation. The P_2 cells were mainly used in this study.

Experimental groups and interventions

21% O_2 group: control group, NAC group, $CoCl_2$ group, $CoCl_2$ + NAC group

2% O_2 group: control group, NAC group, $CoCl_2$ group, $CoCl_2$ + NAC group

The control group was cultured with complete culture medium, the NAC group was cultured with 5 mM NAC medium, the $CoCl_2$ group was cultured with 300 μM $CoCl_2$ medium, and the $CoCl_2$ + NAC group was cultured with 300 μM $CoCl_2$ + 5 mM NAC medium.

Determination of cell viability

The goat TMJ disc cells of P_2 generation were plated in 96-well plates at $6*10^4$ cells/mL, 100 μL per well, five replicate wells in each group, incubated at 37 ℃, 21% O_2, 5% CO_2 incubator for 24 h. Then the cells were treated with the corresponding culture medium respectively, and incubated in 21% O_2 and 2% O_2 incubator for 24 and 48 h. 10 μL CCK-8 solution was added to each well, and was set in the original incubator for three h. The OD value of each well was measured at 450 nm.

Glucose consumption and lactate content measurements

The goat TMJ disc cells of P_2 generation were plated in 6-well plates at $1*10^5$ cells/mL with 2 mL per well. After 24 h of incubation, the cells were treated with the corresponding culture medium respectively. Then the culture medium was collected into centrifuge tubes at 21% O_2 and 2% O_2 for 48 h respectively, at 1,000 rpm, 5 min, and the supernatants were carefully collected into EP tubes for subsequent assays according to the glucose assay kit, and the lactate assay kit.

Measurement of ATP

Experimental groups and cell culture were the same as above. The cells were collected into the centrifuge tubes for centrifugation. Then added 500 μL extraction solution and broken by ultrasound for 1 min (ice bath, the intensity of 20% or 200 W, works for 2 s, stops for 1 s), then centrifuged at 10,000 g at 4 ℃ for 10 min. Extracted the supernatant into another EP tube, added 500 μL chloroform mixed well,

and centrifuged at 10,000 g for 3 min at 4 ℃, then took the supernatant and detected according to the ATP content assay kit.

Detection of the intracellular ROS expression with the DCFH-DA probe

Experimental groups and cell culture were the same as above. The cells were collected into a centrifuge tube. And then, the cells were resuspended with the DCFH-DA dilution at a final concentration of 10 μM and incubated at 37 °C for 15-20 min, mixed upside down every 4 min. After centrifugation, the cells were washed 3 times with DEME/F12 culture medium to remove the DCFH-DA that had not entered the cells. Finally, the cells were resuspended and detected on FlowSight (488 nm excitation wavelength, 525 nm emission wavelength).

RNA isolation and RT-qPCR

The experimental groups and cell culture were the same as above. RNA was isolated from goat TMJ disc cells with RNA extraction kit FastPure® Cell/Tissue Total RNA Isolation Kit (Vazyme, USA). Then 2.5 μL of mRNA was taken immediately after extraction and measured by UV5Nano ultra-micro. Suppose the sample ratio was $1.8 \leq A260/A280 \leq 2.0$, the mRNA purity was suitable for subsequent experiments. The cDNA was synthesized by reverse transcription using HiScript® III RT SuperMix for qPCR assay kit, and then was used as the template for PCR amplification. RT-PCR was performed using ChamQ Universal SYBR qPCR Master Mix kit and QuantStudio 3 RT-PCR instrument. Target gene levels, hypoxia-inducible factor1-α (HIF-1α), glucose transporter1 (GLUT1), lactate dehydrogenase A (LDHA), pyruvate kinase M2 (PKM2), phosphoglycerate kinase1 (PGK1) and the reference gene β-actin were carried out on the machine respectively. The relative expression of the target genes was calculated using the formula $F = 2^{-\Delta\Delta CT}$. The primer sequences were shown in table 1.

Data analysis and statistics

All experiments were repeated at least three times with similar results, and the experimental data was displayed as mean ± S.D. Then the data were analyzed by one-way ANOVA and two-way ANOVA with SPSS 23.0 statistical software. $\alpha = 0.051$, $P < 0.05$ was considered statistically significant difference.

Ethical approval

The disc cells of temporomandibular joint of 3-6 months old healthy fresh sheep head (purchased from the slaughter house of Qilihe district, Lanzhou City) used in this study were cultured, prepared, and primed by the Key Lab of Stomatology with the approval of the Lab Animal Ethical Commission of College of Stomatology, Lanzhou University (No. LZUKQ-2019-057). ----[Roles and Regulation of Low-dose YC-1 and NAC on Energy Metabolism in Hypoxia-inducible Goat Temporomandibular Joint disc cells]

Data Availability

All data associated with this study are presented in the paper.

Acknowledgments

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Author contribution

JL, GB, and HK conceived and designed the study and experiments. JL finished the experiments, performed the statistical analysis, and drafted the manuscript, and all authors took part in revising and approving the manuscript before submission.

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Declarations

Conflict of interest The authors declared that there are no conflicts of interest.

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Legends for figures and table:

Fig. 1 NAC promoted cell proliferation of goat temporomandibular joint disc cells under hypoxia. The cell proliferation under 21% O_2 (**A**) and 2% O_2 (**B**). The cell viability% = (OD value of experiment group - OD value of blank group) / (OD value of control group - OD value of blank group) *100%. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs*. the control group (21% O_2); $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$; 21% O_2, groups, F = 28.500, $P < 0.001$, times, F = 4.757, $P = 0.039$, 2% O_2, groups, F = 41.106, $P < 0.001$, times, F = 9.631, $P = 0.005$

Fig. 2 NAC increased glucose consumption and reduced lactate content in culture supernatant. **A** The consumption of glucose in goat TMJ disc cells medium (groups, F = 4.242, $P < 0.01$) **B** The production of lactate in goat TMJ disc cells medium (groups, F = 272.166, $P < 0.001$) $^{**}P < 0.01$, $^{***}P < 0.01$*vs.* the control group (21%O_2); $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, no statistical significance/*NS*, $P > 0.05$

Fig. 3 NAC enhanced the intracellular ATP production and effectively eliminated the intracellular ROS under hypoxia. **A** The ATP content in goat temporomandibular joint disc cells (groups, F = 905.445, $P < 0.001$) **B** The fluorescence intensity of intracellular ROS in goat temporomandibular joint disc cells medium (groups, F = 6442.181, $P < 0.001$). $^{***}P < 0.001$ *vs.* the control group (21%O_2); $^{\#\#}P < 0.01$，$^{\#\#\#}P < 0.001$

Fig. 4 RT-qPCR analysis of selected genes. **A** Hypoxia inducible factor-1α, HIF-1α (groups, F = 61.743, $P < 0.001$), **B** glucose transporter1,GLUT1 (groups, F = 75.921, $P < 0.001$), **C** lactate dehydrogenase A, LDHA (groups, F = 210.932, $P < 0.001$), **D** pyruvate kinase M2, PKM2 (groups, F = 45.253, $P < 0.001$), **E** phosphoglycerate kinase1, PGK1 (groups, F = 145.655, $P < 0.001$) mRNA expression in goat temporomandibular joint disc cells with the treatment of NAC 48h. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* the control group (21%O_2)；$^{\#}P < 0.05$，$^{\#\#}P < 0.01$，$^{\#\#\#}P < 0.001$，no statistical significance/NS, $P > 0.05$

Fig. 5 Schematic illustration of glucose metabolism. The glucose metabolism is mainly divided into oxidative phosphorylation, glycolysis, and the pentose phosphate pathway. The pentose phosphate pathway, without adenosine triphosphate (ATP) production, provides precursors for nucleotide biosynthesis, which includes ribose phosphate and nicotinamide adenine dinucleotide phosphate (NADPH). Glucose is catalyzed by the action of glycolytic enzymes to produce lactic acid, which has less ATP. Most of the cellular energy supply is provided by oxidative phosphorylation, which is an approach to in which glucose is wholly oxidized through the tricarboxylic acid cycle (TCA cycle) to produce large amounts of ATP for the body. phosphofructokinase-1 (PFK-1), phosphoglycerate kinase1 (PGK1), pyruvate kinase M2 (PKM2), lactate dehydrogenase A (LDHA)

Fig.6 Diagram of the suggested mechanism by which NAC controls the energy metabolism of goat TMJ disc cells under hypoxia. The glycolysis of TMJ disc cells is inhibited under hypoxia. NAC contributes to the transition in energy metabolism toward oxidative phosphorylation by promoting glucose consumption and ATP synthesis and inhibiting glycolysis-related genes.

Table1 Gene names and primer sequences.

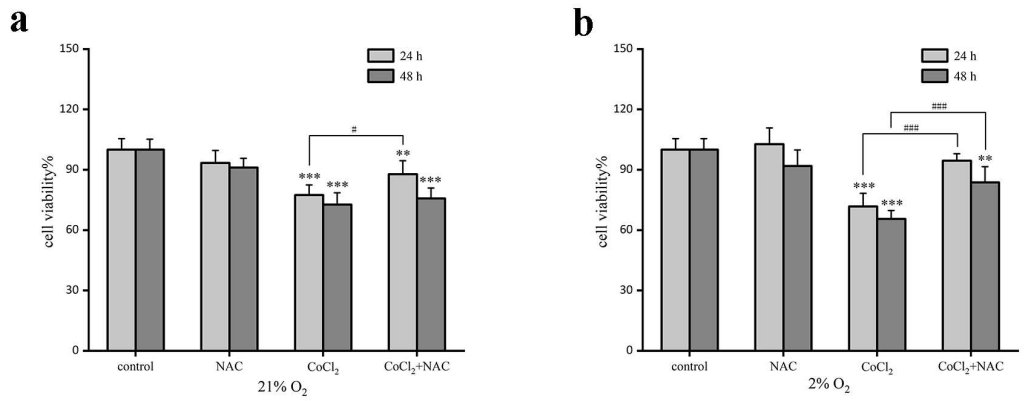
a
b
24 h
48 h
cell viability%
150
120
90
60
30
0
control
NAC
CoCl2
CoCl2+NAC
21% O2
2% O2
#
###
**
***

Fig 1

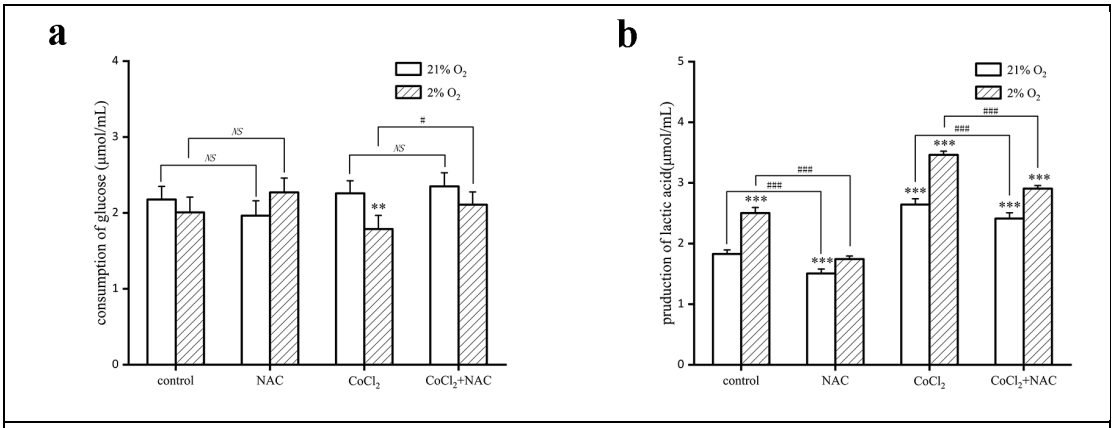
a
b
consumption of glucose (μmol/mL)
pruduction of lactic acid(μmol/mL)
21% O2
2% O2
control
NAC
CoCl2
CoCl2+NAC
NS
#
##
###
***

Fig 2

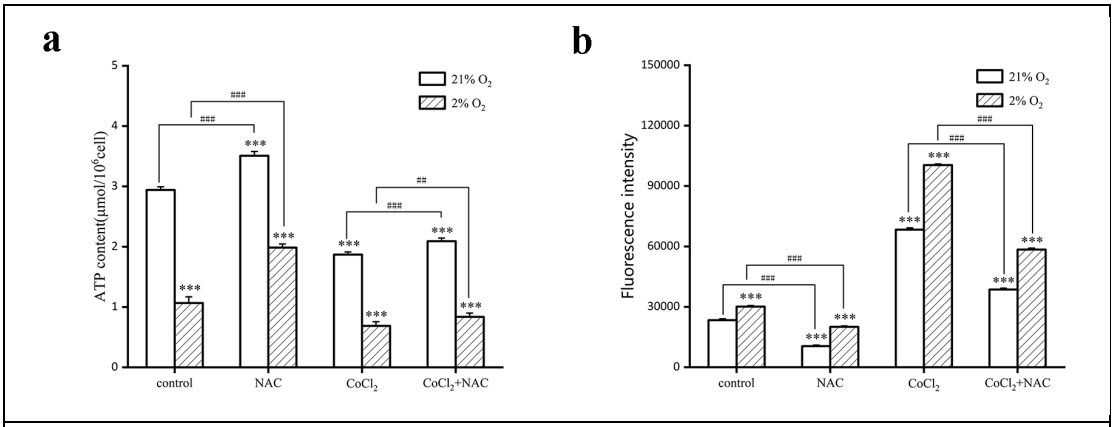
a
b
ATP content(μmol/10⁶cell)
Fluorescence intensity
21% O2
2% O2
control
NAC
CoCl2
CoCl2+NAC
##
###
***

Fig 3

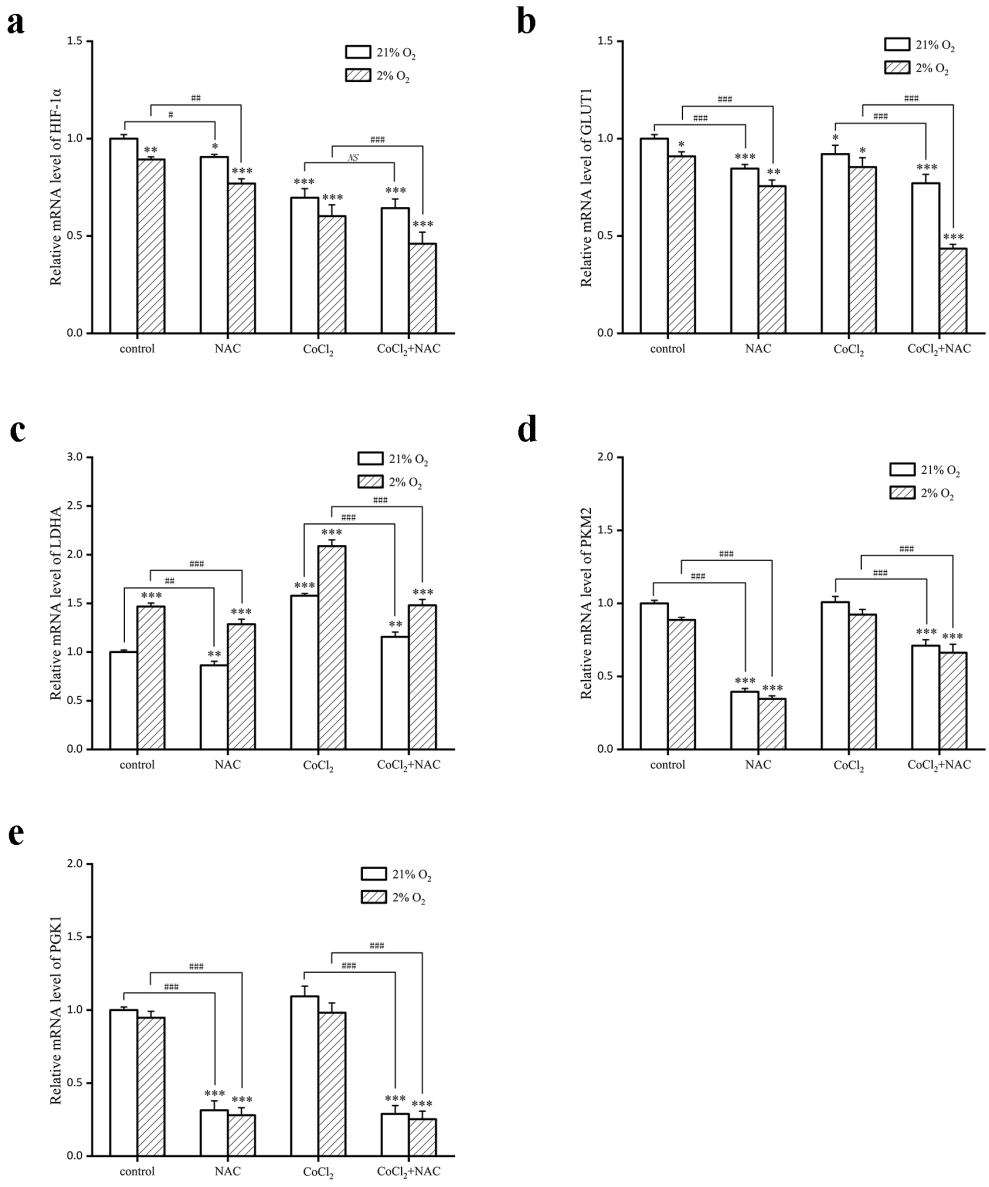
a
Relative mRNA level of HIF-1α
21% O2
2% O2
control
NAC
CoCl2
CoCl2+NAC
b
Relative mRNA level of GLUT1
c
Relative mRNA level of LDHA
d
Relative mRNA level of PKM2
e
Relative mRNA level of PGK1

Fig 4

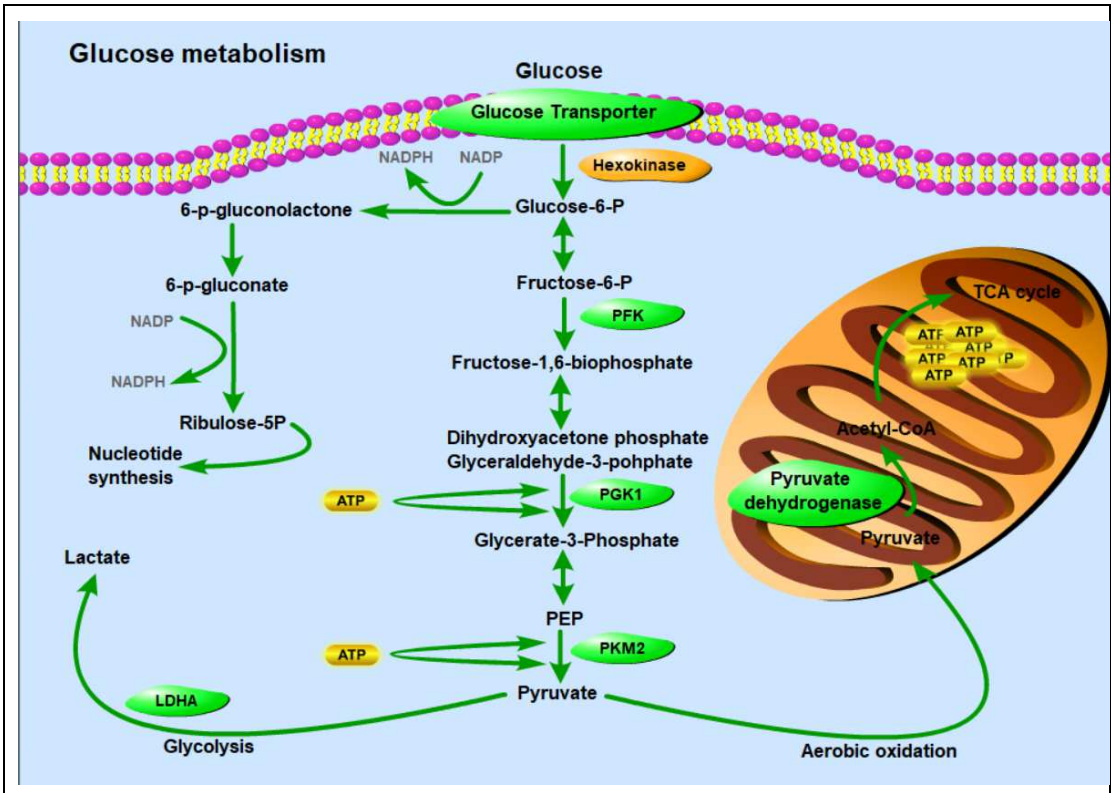
Glucose metabolism
Glucose
Glucose Transporter
NADPH
NADP
Hexokinase
6-p-gluconolactone
Glucose-6-P
6-p-gluconate
Fructose-6-P
NADP
PFK
NADPH
Fructose-1,6-biophosphate
Ribulose-5P
Dihydroxyacetone phosphate
Glyceraldehyde-3-pohphate
Nucleotide
synthesis
ATP
PGK1
Glycerate-3-Phosphate
Lactate
PEP
ATP
PKM2
LDHA
Pyruvate
Glycolysis
Aerobic oxidation
TCA cycle
Acetyl-CoA
Pyruvate
dehydrogenase
Pyruvate

Fig 5

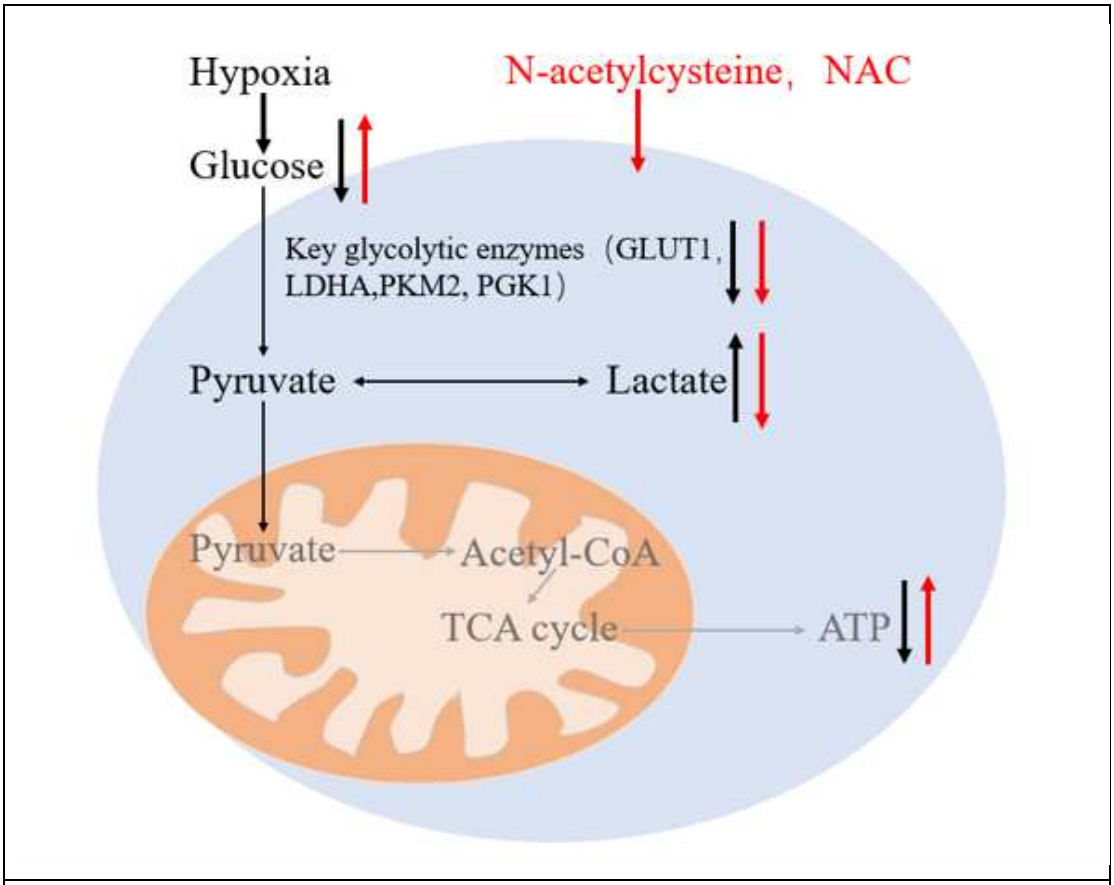
Hypoxia
N-acetylcysteine, NAC
Glucose
Key glycolytic enzymes (GLUT1, LDHA,PKM2, PGK1)
Pyruvate
Lactate
Pyruvate
Acetyl-CoA
TCA cycle
ATP

Fig 6

Table 1

gene	Forward primer (5’-3’)	Reverse primer (5’-3’)
β-actin	ATCACCATCGGCAATGAGC	CCGTGTTGGCGTAGAGGT
HIF-1α	TGAAGTGTACCCTAACTAGCCGG	GGATGAGGAATGGGTTCACAAA
GLUT1	GTGGGCCTTTTTGTTAACCGT	GCACAAAGCCAGTGGTCAGG
LDHA	CAGCAAGAGGGAGAGAGTCGTC	TCTTCCAAGCCACATAGGTCAA
PKM2	GGAATGAATGTGGCTCGTTTG	TGTAGGCATTGTCCAGGGTGAT
PGK1	AGAAGTATGCTGAGGCTGTTGC	ACTGACTTTATCCTCCGTGTTCC