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BNIP3 Mediates the Different Adaptive Responses of Fibroblast-like Synovial Cells to Hypoxia in Patients With Osteoarthritis and Rheumatoid Arthritis

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Abstract

Background: Hypoxia is one of the important characteristics of synovial microenvironment in rheumatoid arthritis (RA), and it is very important in the process of synovial hyperplasia. Fibroblast-like synovial cells (FLSs) are relatively affected by hypoxia injury in cell survival, while FLSs from patients with RA (RA-FLSs) are particularly resistant to hypoxia-induced cell death. The purpose of this study was to evaluate whether FLSs in patients with osteoarthritis (OA) and RA-FLSs have the same adaptation to hypoxia.

Methods: CCK-8, flow cytometry and BrdU were used to detect the proliferation of OA-FLSs and RA-FLSs under different oxygen concentrations. Apoptosis was detected by AV/PI, TUNEL and Western blot, mitophagy was observed by electron microscope and Western blot, mitochondrial state was detected by reactive oxygen species (ROS) and mitochondrial membrane potential by flow cytometry, BNIP3 and HIF-1α were detected by Western blot and RT-qPCR. The silencing of BNIP3 is achieved by stealth RNA system technology.

Results: After hypoxia, the survival rate of OA-FLSs was reduced, and the proliferation activity of RA-FLSs was further increased. Hypoxia induced increased apoptosis and inhibited autophagy of OA-FLSs, but not in RA-FLSs. Hypoxia treatment led to a more lasting adaptive response. RA-FLSs showed a more significant increase in gene expression regulated by HIF-1α transcription. Interestingly, they showed higher BNIP3 expression than OA-FLSs, and showed stronger mitophagy and proliferation activities. The BNIP3 siRNA experiment in RA-FLSs confirmed the potential role of BNIP3 in the survival of FLSs. The inhibition of BNIP3 resulted in the decrease of cell proliferation and the decrease of mitophagy and the increase of apoptosis.

Conclusion: In summary, RA-FLSs maintained redox balance through mitophagy to promote cell survival under hypoxia. The mitophagy of OA-FLSs was too little to maintain the redox balance of mitochondria, leading to apoptosis. The difference of mitophagy between OA-FLSs and RA-FLSs under hypoxia is mediated by the expression of BNIP3.

Introduction

Hypoxia is divided into physiological hypoxia and pathological hypoxia. Embryonic development and adult brain tissue are in an environment with relatively low oxygen concentration, that is a physiological hypoxic environment, which is essential for exercise of the normal functions. The disturbance of normal oxygen supply leads to a series of diseases. In the microenvironment of excessive synovium hyperplasia in rheumatoid arthritis (RA) patients, severe hypoxia in local tissues caused by rapid cell proliferation is one of the important characteristics of RA (Guo et al., 2020). Different types of cells respond differently to hypoxia. Hypoxia induces adaptive response to maintain the homeostasis of intracellular environment and promote cell survival (Qureshi-Baig, et al., 2020). For some cells, hypoxia induces cell death by activating apoptosis (Ren et al., 2021). Studying the changes of metabolic function caused by hypoxia

and its mechanism will help people understand the pathogenesis of hypoxic diseases and promote the treatment of related diseases.

Hypoxia-inducible fator 1 (HIF-1) is a heterodimeric transcription factor that regulates the body's response under hypoxia, and its transcription activity is determined by the HIF-1α (Ikeda et al., 2021). Under hypoxia, HIF-1 can be stably expressed, thereby activating the expression of hundreds of target genes. HIF-1 is involved in the regulation of many pathological and physiological processes, such as angiogenesis, energy metabolism, embryonic development and tumor invasion (Wang et al., 2020; Wu et al., 2021). Studies have confirmed that HIF-1 activates mitophagy by up-regulating its target gene Bcl-2 and adenovirus E1B 19 kDa interacting protein 3 (BNIP3). The role of BNIP3 pathway in cell death is controversial. BNIP3 induces apoptosis and autophagy, or even stimulates cell proliferation (Lin et al., 2021; Gorbunova et al., 2020). But fundamentally, cell type and environment seem to determine the interdependence between autophagy and apoptosis. Studies have made it clear that mitophagy is an important hypoxic adaptive response. It can selectively identify and degrade damaged mitochondria, maintain cell redox balance and prevent cells from entering the death pathway. Studies have confirmed that different types of cells may have different adaptation to hypoxia by regulating the expression of BNIP3 protein.

As the most common inflammatory arthritis, RA is characterized by pannus formed by synovial cell layer and neovascularization,which protrude into the joint cavity or invade the cartilage, resulting in joint damage and dysfunction. Synovitis was previously thought to be driven immune inflammation mediated by T lymphocytes (Weyand et al., 2014). With the deepening of research, it is found that there is joint bone destruction mediated by RA-FLSs (Nygaard et al., 2020). RA-FLSs themselves have a series of transformation characteristics, the growth regulation mechanism is obstructed, and apoptosis is reduced, which leads to "tumor-like growth" and excessive proliferation (Wang et al., 2020). RA-FLSs proliferate rapidly, which is consistent with the pathological features of abnormal synovial hyperplasia. Their survival depends not only on the stimulation of growth factors in the synovial microenvironment, but also on hypoxia. Abnormal synovial hyperplasia is a persistent marker of RA progression. In fact, the excessive proliferation of RA-FLSs plays a crucial role in the progression of RA. It is reported that BNIP3 is widely expressed in RA-FLSs in patients with RA, and its expression is positively correlated with the severity of the disease (Kammouni et al., 2007). Therefore, in the pathogenesis of RA, "active invasion" RA-FLSs is regarded as the "trigger point" in the chronic inflammation of joints and the erosion and destruction of cartilage. An in-depth exploration of the characteristics of RA-FLSs may find new targets for the prevention and treatment of RA.

There are currently no studies documenting the adaptive response of FLSs to hypoxia in terms of survival, apoptosis and autophagy. Therefore, we decided to study and compare the effects of hypoxia on RA-FLSs and OA-FLSs. This study will help to clarify the mechanism of different FLSs adapting to hypoxia and the regulatory role of BNIP3 under hypoxic conditions, and provide a new direction for the treatment of RA diseases.

Materials & Methods

Cell culture and hypoxic treatment

MH7A is a cell line isolated from synovium of patients with RA, and it was purchased from BeNa Culture Collection (Suzhou, China). MH7A was inoculated in DMEM medium (Hyclone, New Zealand) containing 10% (v/v) fetal bovine serum (FBS, Biological Industries, Israel), 1% (v/v) 100 U/ml penicillin and 100 LG/ml streptomycin (Beyotime, Shanghai, China). OA-FLSs were isolated from the synovium of osteoarthritis (OA) patients and purchased from Bena Culture Collection (Suzhou, China). Cells were cultured at 37 ^{o}C with 5% CO_2. 2% and 21% O_2 were used as hypoxia and normoxia conditions. Cells was exposed to 2% O_2, 93% N_2, and 5% CO_2 in a hypoxic chamber for 24 h.

Cell proliferation assay

Cell viability was determined by CCK-8 assay. Berifly, OA-FLSs or RA-FLSs were inoculated in 96 well plates, and the cells were cultured in an incubator at 37 ℃ and 5% CO_2. The hypoxia condition of 2% O_2 was established by placing cells in an hypoxia device (Aipuins, USA). After the culture, 10 μl of CCK-8 (Solarbio, Beijing, China) was added to each well, cultured in a 5% CO_2 incubator at 37 ℃ for 1 h, and the optical density (OD) was measured at 450 nm.

Cell cycle analysis

According to different groups, OA-FLSs or RA-FLSs were cultured in the corresponding oxygen concentration of 35mm Petri dish for 24 h, and the cell precipitation was collected after trypsin digestion. After the cells were washed with PBS, 75% cold ethanol was added and fixed overnight at 4 ℃. Cells were centrifuged again, the precipitates were washed with PBS for 3 times and resuspended at 200 μl in PBS. 2μl RNase A with a concentration of 10mg/ml was added to the cell suspension for digestion for 30 min, and then 300 μl of propidium iodide (PI) at a concentration of 50 μg/ml incubated in dark for 30 min. The results of cell cycle were detected by flow cytometry.

BrdU assay

Cells were incubated with BrdU at room temperature for about 1.5 h. Cells were fixed by 4% paraformaldehyde at 4 ℃ for 20 min, and then infiltrated with 0.5% Triton X-100 for about 20 min. Finally, Hoechst staining was used for 15 min to label the nucleus. Subsequently, samples were observed under a fluorescence microscope, photographs were taken.

Annexin V-FITC/PI assay

Annexin V-FITC/PI double staining apoptosis detection kit was used to evaluate the induction of apoptosis. OA-FLSs or RA-FLSs were inoculated in 35 mm dishes. Cell precipitates were collected and stained with PI and AV-FITC according to the manufacturer's instructions (Bestbio, Shanghai, China), and samples were read by flow cytometry.

TUNEL assay

TUNEL cell apoptosis detection kit was used to detect the breakage of nuclear DNA in the late stage of apoptosis. TUNEL positive cells were detected according to the manufacturer's instructions (Servicebio, Wuhan, China). DAPI was used to label nuclei.

Mitochondrial membrane potential (MMP) polychromatic assay

MMP was measured with cationic JC-1 (5 ', 6,6' - tetrachloro-1,1 ', 3,3' - tetraethyl benzimidazolyl carbocyanine iodine) dye. Intact polarized mitochondrial membrane showed red signal (JC-1 aggregate), while green signal (JC-1 monomer) was depolarized mitochondrial membrane. After the cells were placed in a 35mm Petri dish for corresponding treatment, the cell precipitates were collected and stained with JC-1 dye according to the manufacturer's instructions (Solarbio, Beijing, China), and then the samples were examined by flow cytometry.

Reactive oxygen species (ROS) assay

The evaluation of cell ROS production is labeled with DCFH-DA probe (Beyotime, Shanghai, China), and the cells are added with DCFH-DA medium at a concentration of 10 μmol/ml and incubated at 37 °C for about 20 min. Then, the cells were washed three times with serum-free medium to remove the remaining probes. Cell precipitates were collected and each sample was analyzed by flow cytometry.

Western blot

To detect protein expression, Western blot was performed as previously described. First, the cells were washed three times under PBS at room temperature and then lysed in Ripa buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Subsequently, the cells were centrifuged at 4 °C for about 10 min to obtain the supernatant. Then, the protein concentration was detected by BCA protein quantitative kit. The target protein was separated by 12% SDS-polyacrylamide gel. About 50 mg of protein per sample was wet transferred to the NC membrane. The main antibodies used for immunoblotting are as follows: Bax (1:1000, Proteintech, No. 60267-1-ig), Bcl-XL (1:1000, Proteintech, No. 10783-1-ap), Beclin-1 (1:1000, Proteintech, No. 66665-1-ig), LC3 (1:1000, Proteintech, No. 14600-1-ap), BNIP3 (1:1000, ZEN BIO, 383308), HIF-1α (1:1000, ZEN BIO, 340462), cyclin D1 (1:1000, ZEN BIO, 382442), PCNA (1:1000, ZEN BIO, 385293).

The qPCR and RNA interference

qPCR analysis was performed according to our previous study. Primers for polymerase chain reaction are as follows: BNIP3, forward, TCCAGCCTCGGTTTCTATTT and reverse, AGCTCTTGGAGCTACTCCGT, GAPDH, forward, GCGGGAAATCGTGCGTGAC and reverse, CGTCATACTCCTGCTTGCTG. The mRNA level of each independently prepared RNA was determined by qRT-PCR in triplicate and normalized to GAPDH expression level. SYBR Green (Bimake, America) was used to quantify gene expression. The mRNA level of each independently prepared RNA was determined by qRT-PCR in triplicate and normalized to GAPDH expression level.

BNIP3 siRNA

The low expression of BNIP3 was achieved by invisible RNA system technology (Hanbio). The siRNA sequence of the control virus vector is as follows: SiRNA sequence: TTCTCCGAACGTGTCACGTAA. The siRNA sequence of the target gene is GGAATTAAGTCTCCGATTA. The transfection process was carried out according to the manufacturer's instructions. RA-FLSs were infected with virus with MOI value of 30 and cultured for 24 h. The culture medium containing virus was absorbed and replaced with fresh culture medium for 24 h. After transfection, the cells were exposed to normoxia or hypoxia for 24 h. Cell proliferation, apoptosis and autophagy were analyzed by real-time PCR and Western blot.

Statistical analysis

The statistical significance between the means was determined by t-test. The value of $P<0.05$ was considered statistically significant.

Reslults

Hypoxia had different effects on the proliferation of OA-FLSs and RA-FLSs

The redox state and survival state of cells are significantly different under hypoxic and normoxic conditions. In addition to apoptosis affecting the final survival of cells, the effect of hypoxia on cell proliferation also affects the final survival ability of cells. Therefore, we investigated the different effects of hypoxia on the proliferation of RA-FLSs and OA-FLSs. It was found that the viability of RA-FLSs increased with the decrease of oxygen concentration. When the oxygen concentration was lower than 2%, the proliferation viability decreased. Therefore, we selected 2% O2 as the hypoxic condition of subsequent experiments (Fig. 1A). We compared the effects of hypoxia on the survival of OA-FLSs and RA-FLSs through CCK-8 assay. After continuous exposure to hypoxia for 24 h, the cell proliferation of OA-FLSs decreased significantly, while the proliferation of RA-FLSs was not affected, and its cell viability increased significantly (Fig. 1B). Hypoxia relatively affected the proliferative activity of OA-FLSs, but RA-FLSs completely tolerated hypoxia. We observed the proliferation of OA-FLSs and RA-FLSs under different oxygen concentrations by BrdU incorporation method: the proliferation of RA-FLSs increased significantly after moderate hypoxia, while the positive rate of BrdU in OA-FLSs decreased significantly after hypoxia (Fig. 1C). The results of flow cytometry (Fig. 1D-E) were consistent with the results measured by BrdU. After hypoxia, the cell proliferation of OA-FLSs had no significant change compared with that of normoxia group (11.24% vs 11.42%), while the proliferation of RA-FLSs further increased, and its G2/M increased from 9.62% to 24.35%. We also measured the protein expression of cyclin D1 and PCNA. Cyclin D1 is G1/s-specific cyclin-D1, which is necessary for the progression of cell cycle from G1 phase to S phase. PCNA is not significantly expressed in G0-G1 phase cells. In late G1 phase, its expression increased significantly, peaked in S phase and decreased significantly in G2-M phase. The expressions of cyclin D1 and PCNA were significantly increased in hypoxic RA-FLSs and significantly decreased in OA-FLSs (Fig. 1F), which confirmed the resistance of RA-FLSs to hypoxic injury.

RA-FLSs were more resistant to hypoxia-induced apoptosis than OA-FLSs

We further observed the effects of different oxygen concentrations on the apoptosis of OA-FLSs and RA-FLSs by AV/PI double staining experiment. The experimental results showed that (Fig. 2A-B), RA-FLSs had a low apoptosis rate (5.7%, 5.0%) under normoxia and moderate hypoxia, while the apoptosis rate of OA-FLSs increased significantly after hypoxia treatment (26.3% vs 8.3%). We further detected the apoptosis rate and the expression of its related proteins by TUNEL double staining and Western blot. The results also showed that hypoxia significantly induced the apoptosis of OA-FLSs, increased the expression of pro-apoptotic protein Bax and significantly decreased the anti-apoptotic protein Bcl-XL, but there was no significant change in the apoptosis rate of RA-FLSs. The expression of anti-apoptotic protein Bcl-XL increased and the expression of pro-apoptotic protein Bax decreased in RA-FLSs (Fig. 2C-D).

RA-FLSs was more prone to hypoxia-induced mitophagy than OA-FLSs

Direct observation of subcellular structural changes by electron microscope is the gold standard for the detection of autophagy and mitophagy. Under normoxic conditions, the nuclear membranes of OA-FLSs and RA-FLSs were relatively smooth, the cytoplasmic hollow vesicular structure was less, and there were a large number of mitochondria with clear double-layer membrane structure, which were spindle or oval, rich in contents and clear cristae structure. Under hypoxic conditions, the nuclear membrane of RA-FLSs was smooth and complete, and a large number of mitochondria were oval and have normal structure and morphology, but the vacuolar structure increased, and many vacuolar structures can be observed or contacted with mitochondria. The phenomenon of enveloping mitochondria, in which vacuoled with a double membrane structure were observed to envelop darker mitochondria, that is, mitophagy. The nuclear membrane of OA-FLSs was irregularly curved, and vacuolated structures can be seen everywhere. Mitochondria with a double-layer membrane structure can be observed, but the inclusions and cristae structure almost disappeared, which indicated that the mitochondria are severely damaged and their normal structures are destroyed (Fig. 3A). After treatment with different oxygen concentrations, Western Blot test results showed that compared with the normoxia group, the levels of LC3-II/LC3-I and Beclin-1 in RA-FLSs and OA-FLSs induced by hypoxia increased, while compared with RA-FLSs, LC3-II/LC3-I and Beclin-1 increased in OA-FLSs at a lower level (Figure 3B). The above results indicated that hypoxia induced the occurrence of mitophagy in RA-FLSs, while mitophagy alleviated the apoptosis caused by hypoxia. Although the level of mitophagy in OA-FLSs has increased, it was still not enough to eliminate the occurrence of hypoxia-induced apoptosis, which may be the reason for the significant difference in the survival rate of the two cells under hypoxia.

Hypoxia-induced responses were more pronounced in OA-FLSs than in RA- FLSs

Hypoxia seems to have different effects on the proliferation, apoptosis and autophagy of OA-FLSs and RA-FLSs. To determine whether these observations are related to different adaptive responses to hypoxia, we evaluated the characteristics of hypoxia in two cell types. After normoxia and hypoxia treatment, flow cytometry is used to analyze the fluorescence intensity of cells stained by ROS probe molecules. The

results showed that after hypoxia treatment, the fluorescence intensity of OA-FLSs stained with ROS probe molecules increased significantly, while the fluorescence intensity of RA-FLSs stained with probe molecules did not change significantly (Fig. 4A). We used the fluorescent probe JC-1 to explore the changes of MMP(△Ψm) under different oxygen concentrations. The experimental results showed that there was no significant change in MMPin RA-FLSs after moderate hypoxia treatment compared with normoxia group, while the MMPdecreased significantly after OA-FLSs treatment (Fig. 4B). In conclusion, under hypoxic conditions, the content of ROS in RA-FLSs did not increase significantly, the MMPremained stable, and the redox balance of cells remained stable. The content of ROS in OA-FLSs increased significantly and the MMPdecreased, resulting in the disorder of redox balance.

BNIP3 is a marker molecule for mitophagy. Since HIF-1α is the core transcription factor under hypoxic conditions and the upstream molecule that regulates the transcriptional activity of BNIP3, we tested the expression of HIF-1α protein and mRNA under different oxygen concentrations. The experimental results showed (Fig. 4C-D) the expression of HIF-1α in OA-FLSs and RA-FLSs was very low under normoxia, after moderate hypoxia, the protein expression of HIF-1α in OA-FLSs and RA-FLSs was induced to increase, however, HIF-1α accumulated in RA-FLSs. Meanwhile, we further tested the protein expression of the HIF-1α target gene BNIP3. BNIP3 was only slightly expressed in OA-FLSs and RA-FLSs under normoxia. After hypoxia treatment, the expression of BNIP3 in RA-FLSs increased significantly and continuously compared with the normoxia group. In OA-FLSs, exposure to hypoxia caused a moderate increase in BNIP3 expression, which increased after 8 h and then decreased. The expression of BNIP3 protein may not only be affected by HIF-1α transcriptional regulation. These results clearly indicated that RA-FLSs were easier to adapt to hypoxia than OA-FLSs, and the regulation of BNIP3 expression may play a role in the adaptive response of cells.

Inhibition of BNIP3 reduced RA-FLSs survival under hypoxia

In order to better determine the role of BNIP3 in the survival of RA-FLSs under hypoxic conditions, we suppressed the expression of BNIP3 by siRNA technology to observe its effects on the proliferation, apoptosis and mitophagy of RA-FLSs. Under normoxia and hypoxia, the inhibition of BNIP3 resulted in a significant decrease in the expression of BNIP3 protein, while hypoxia still significantly induced the expression of BNIP3 in the Ctr siRNA group. Inhibition of BNIP3 expression is associated with decreased cell proliferation (Fig. 5A, B, C, D) and enhanced apoptosis (Fig. 5 E, F, G, H), and is closely related to the rBrdUction of autophagy process caused by the inhibition of BNIP3 in RA-FLSs under hypoxia (Fig. 6 A, B). In fact, the ratio of LC3B-II/LC3B-I in the hypoxic RA-FLSs silenced by BNIP3 was significantly lower than that of the hypoxic Ctr siRNA group (Fig. 6 C, D, E). Our results again indicated that BNIP3 played a key role in the survival of pathological and hyperproliferative RA-FLSs.

Discussion

Some studies have confirmed that RA-FLSs have special resistance to hypoxic stress, and RA-FLSs shows abnormal proliferation activity in the joint hypoxic microenvironment of RA (Yu et al., 2021). Our

research showed that RA-FLSs were functionally different from OA-FLSs, which was related to the abnormal proliferation of joint synovium in the progression of RA. We compared the adaptive response of FLSs from OA patients and RA patients to hypoxia. This is the first report that RA-FLSs are more resistant to hypoxic stress than OA-FLSs in terms of cell viability, and are more prone to mitophagy and less prone to apoptosis. In our study, we determined that OA-FLSs are less resistant to cell death than RA-FLSs due to long-term exposure to hypoxia. Actually, short-term exposure to hypoxia did not induce cell death of two cell types, that is, OA-FLSs resisted hypoxia induced cell death after 24 h of exposure. However, after 24 h of hypoxia treatment, the survival rate of OA-FLSs decreased, but RA-FLSs continued to resist hypoxia. RA-FLSs expressed higher levels of Cyclin D1 and PCNA compared with OA-FLSs. Cyclin D1 is G1/s-specific cyclin-D1, which is necessary for the progression of cell cycle from G1 phase to S phase. PCNA is closely related to cell DNA synthesis, plays an important role in the initiation of cell proliferation, and is a good indicator to reflect the state of cell proliferation (Converse et al., 2021; Tao et al., 2021). The results showed that the increase of Cyclin D1 and PCNA expression in RA-FLSs were closely related to the increase of cell proliferation. Regarding the detection of apoptosis, we observed that the change in the ratio of Bax/Bcl-xL protein was negatively correlated with cell survival. Our results indicated that the hypoxia-induced death of OA-FLSs may be attributed to apoptosis, which was consistent with the hypothesis that RA-FLSs were more resistant to hypoxia-induced cell death. It is important to emphasize the significant increase in Beclin-1 expression and LC3B-II/LC3B-I ratio in hypoxic RA-FLSs. As mentioned earlier, mitophagy may be considered as a pro-survival adaptive response in several cell types. This may further explain why OA-FLSs is less resistant to hypoxia-induced cell death than RA-FLSs, which are more prone to mitophagy.

Previous studies have clearly illustrated how cells adapt to hypoxia by expressing genes closely regulated by HIF-1α, including BNIP3, GLUT-1, CAIX and MCT-4, in order to survive in an unfavorable microenvironment (Filippi et al., 2018). OA-FLSs accumulated less HIF-1α than RA-FLSs, and RA-FLSs expressed higher levels of BNIP3 than OA-FLSs, which supported the hypothesis that RA-FLSs showed better metabolic and mitophagy adaptive response to hypoxia stress. In fact, BNIP3 is described as a key molecule induced by hypoxia, participating in the switch of mitophagy, promoting cell survival and avoiding apoptosis (Tang et al., 2019). Our results are completely consistent with previous reports, indicating that hypoxia induced mitophagy is a survival process and is related to the expression of BNIP3. It should be emphasized that even under normoxic conditions, the expression of BNIP3 in RA-FLSs exceeded that of OA-FLSs, and our results clearly defined its overexpression in RA-FLSs under hypoxic conditions. When using siRNA to make BNIP3 low expression in RA-FLSs, we observed the decrease of proliferation activity and increase of apoptosis of RA-FLSs, which further confirmed the protective effect of BNIP3 on RA-FLSs in hypoxic environment. This study showed that when BNIP3 was inhibited in RA-FLSs, apoptosis increased. In conclusion, our results suggested that understanding the mechanism of hypoxia adaptation of different FLSs may have important therapeutic implications.

BNIP3 is an outer mitochondrial membrane protein that is localized in the mitochondria after expression. It is also an important signal molecule in hypoxia-induced activation of mitophagy. How do they activate mitophagy? After a series of studies, it is found that Beclin-1 plays an important role in BNIP3-mediated

induction and activation of the autophagy pathway (Lee et al., 2020). Beclin-1 is the main inducer of autophagy. It plays an important role in the formation of autophagy precursor structure and regulates the formation of autophagy precursor (Hill et al., 2019). Beclin-1 and BNIP3 are both members of the Bcl-2 family, both of which are BH3-only subfamily proteins with BH3 domains. Therefore, Beclin-1 binds to the pro-survival protein Bcl-2 or Bcl-XL through its BH3 domain. When hypoxia induces high expression of BNIP3, BNIP3 competes with Beclin-1 through its BH3 structure and binds to Bcl-2 or Bcl-XL. Therefore, Beclin-1 is released from the Bcl-2/Beclin-1 or Bcl-XL/Beclin-1 complex on the mitochondria, and the free Beclin-1 forms a PI3K complex with Vps34, Ambra1 and other proteins to activate it. The PI3K/Akt pathway regulates the location of downstream autophagy-related ATG proteins in the autophagy precursor and isolation membrane, thereby activating the occurrence of mitophagy (Niu et al., 2019; Won et al., 2015). At present, the understanding of BNIP3 in the regulation of mitophagy is not only to regulate Beclin-1 of BH3 only family through BH3 domain to activate autophagy. BNIP3 also activates mitophagy by inducing MMPloss or depolarization and directly interacting with autophagy connecting molecule LC3 (Zeng et al., 2021; Shi et al., 2014). When autophagy is activated, the cytoplasmic LC3B-I protein is cleaved, fatty and inserted into the autophagosome membrane as LC3-II. Therefore, the increase in the number of LC3-II proteins with small molecular weight and the increase in LC3-II/LC3-I ratio are markers of autophagy and are related to the increase in the number of autophagosomes.

Conclusion

In summary, this study proved that RA-FLSs cleared excess ROS in cells through mitophagy under hypoxia to maintain redox balance, rBrdUce apoptosis and promote cell survival. While the mitophagy in OA-FLSs was not enough to remove excess ROS, and the redox imbalance in the cell induced the increase of apoptosis and the decrease of cell viability. The difference in mitophagy between OA-FLSs and RA-FLSs under hypoxia was mediated by differences in the expression of BNIP3.

Abbreviations

BNIP3: Bcl-2 and adenovirus E1B 19 kDa interacting protein 3; FLSs: Fibroblast-like synovial cells; HIF-1: Hypoxia-inducible fator 1; MMP: Mitochondrial membrane potential; OA: osteoarthritis; OA-FLSs: Fibroblast-like synovial cells from patients with osteoarthritis; RA: Rheumatoid arthritis; RA-FLSs: Fibroblast-like synovial cells from patients with rheumatoid arthritis; ROS: Reactive oxygen species;

Declarations

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Author Contributions

HW and RD designed this study. RD performed the main experimental procedures in vitro. YWandYHB carried out partial experiments. RD and YW performed the statistical analysis. RD and HW wrote this paper. All the authors read and approved the final paper.

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Availability of data and material

The data and materials used to support the fndings of this study are available

from the corresponding author upon request.

Ethics approval and consent to participate

The experimental protocol for animal studies was reviewed and approved by ethics

committee of Anhui University of Chinese Medicine

Consent for publication

All authors agree to publish.

Competing interest

The authors declare no conflict of interest.

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Figures

Figure 1

Effects of hypoxia on the proliferation of OA-FLSs or RA-FLSs. A: The effects of different oxygen concentrations on the viability of FLSs were detected by CCK-8 method; B: The effects of hypoxia on the viability of different FLSs were detected by CCK-8 method; C: BrdU was incorporated into OA-FLSs and RA-FLSs, cells were exposed to 20% and 2% oxygen for 24 h, and then BrdU (green) and DAPI (blue) immunostaining were detected; D: Flowcytometry analysized the cell proliferation with PI staining after cells were treated with different oxygen concentrations for 24 h; E: Histogram depicts quantitative data from the flow cytometric analysis; F: After exposure to different oxygen concentrations for 24 h, the expression of cyclin D1 and PCNA was analyzed by Western blot. Error bars represent SD. The values are expressed as the mean±SD. #P<0.05, ##P<0.01 represents the comparison between OA-FLSs N group and OA-FLSs H; *P<0.05, **P<0.01 represents the comparison between RA-FLSs N group and RA-FLSs H, +P<0.05, ++P<0.01 represents the comparison between OA-FLSs H group and RA-FLSs H.

Figure 2

Effects of hypoxia on the apoptosis of OA-FLSs or RA-FLSs. A: After exposed to different oxygen concentrations for 24 h, cells were analyzed by FCM using Annexin V/PI double-staining; B: Histogram depicts the quantitative data from the AV/PI double-staining; C: Representative fluorescence images of cells stained with TUNEL and Propidium Iodide to visualize apoptosis cells (Green) and nucleus (Bule), respectively; D: The expression of Bax and Bcl-XL was analyzed by Western blot. The values are expressed as the mean±SD. #P<0.05, ##P<0.01 represents the comparison between OA-FLSs N group and OA-FLSs H; *P<0.05, **P<0.01 represents the comparison between RA-FLSs N group and RA-FLSs H, +P<0.05, ++P<0.01 represents the comparison between OA-FLSs H group and RA-FLSs H.

Figure 3

Effects of hypoxia on the mitophagy of OA-FLSs or RA-FLSs. A: After exposed to 20% O2, 2% O2 for 24 h, cells were analysed by electron microscopy. B: The expression of Beclin-1 and LC3 was analyzed by Western blot. The values are expressed as the mean±SD. #P<0.05, ##P<0.01 represents the comparison between OA-FLSs N group and OA-FLSs H; *P<0.05, **P<0.01 represents the comparison between RA-

FLSs N group and RA-FLSs H, +P<0.05, ++P<0.01 represents the comparison between OA-FLSs H group and RA-FLSs H.

Figure 4

Effect of hypoxia on oxidation indexes in OA- FLSs and RA- FLSs. A: Cells were exposed to 20% O2 or 2% O2 for 24 h and assessed the ROS content by FCM (flowcytometry) using 10 μM CM-H2DCFDA as a ROS probe; B:JC-1 was used to detect the effect of hypoxia on MMPof OA- FLSs and RA- FLSs; C: The expression of HIF-1α and BNIP3 was analyzed by Western blot; D: The expression of HIF-1α and BNIP3 was analyzed by RT-qPCR. The values are expressed as the mean±SD. #P<0.05, ##P<0.01 represents the comparison between OA-FLSs N group and OA-FLSs H; *P<0.05, **P<0.01 represents the comparison between RA-FLSs N group and RA-FLSs H, +P<0.05, ++P<0.01 represents the comparison between OA-FLSs H group and RA-FLSs H.

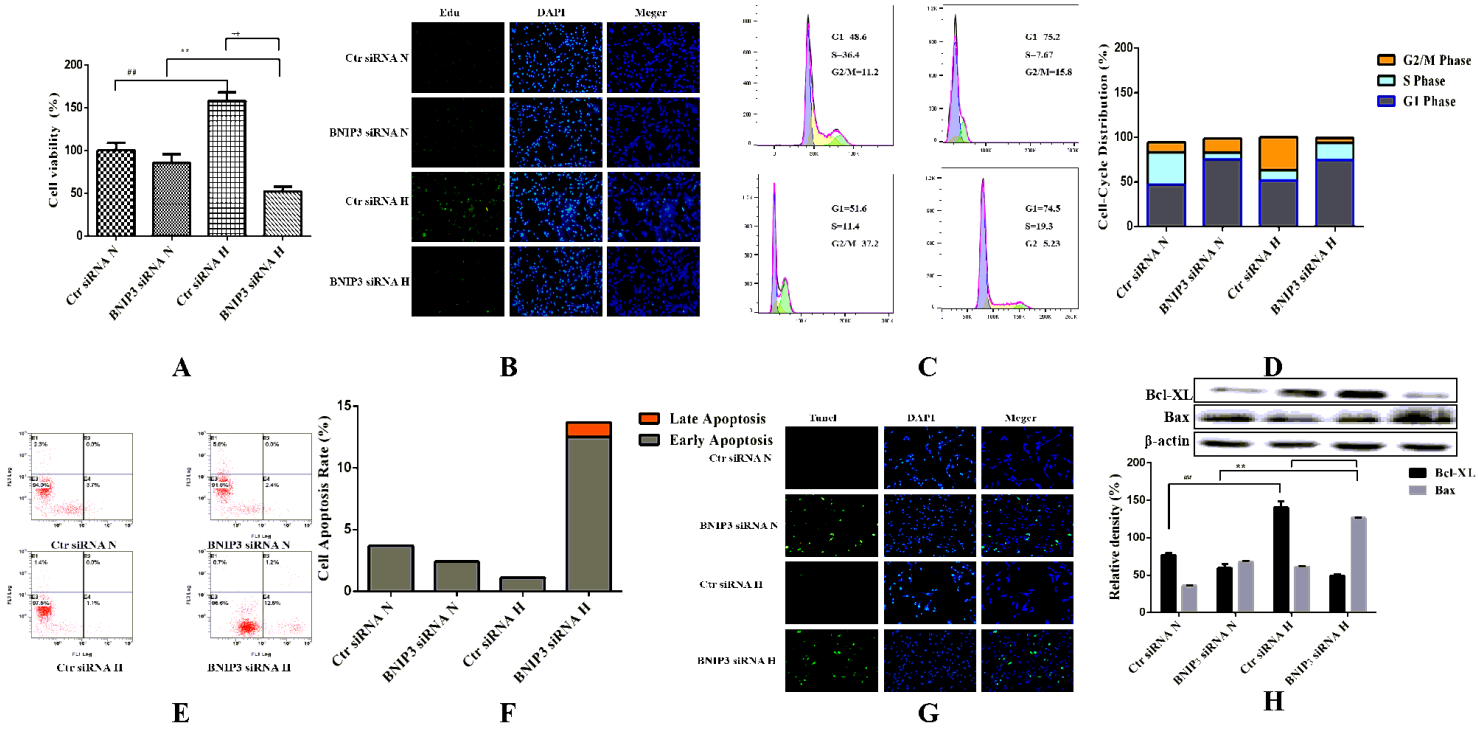

Figure 5

Effects of low expression of BNIP3 on proliferation and apoptosis of FLSs. A: The effects of low expression of BNIP3 on the viability of FLSs were detected by CCK-8 method; B: BrdU was incorporated into OA-FLSs and RA-FLSs, the cells were exposed to 20% and 2% oxygen for 24 h, and then BrdU (green) and DAPI (blue) immunostaining were detected; C: Flowcytometry analysized the cell proliferation with PI staining after cells were treated with different oxygen concentrations for 24 h; D: Histogram depicts quantitative data from the flow cytometric analysis; E: Cells were analyzed by FCM using Annexin V/PI double-staining; F: Histogram depicts the quantitative data from the AV/PI double-staining; G: Representative fluorescence images of cells stained with TUNEL and Propidium Iodide to visualize

apoptosis cells (Green) and nucleus (Bule); H: The expression of Bax and Bcl-XL was analyzed by Western blot. The values are expressed as the mean±SD. #P<0.05, ##P<0.01 represents the comparison between Ctr siRNA N group and Ctr siRNA H; *P<0.05, **P<0.01 represents the comparison between BNIP3 siRNA N group and BNIP3 siRNA H, +P<0.05, ++P<0.01 represents the comparison between Ctr siRNA H group and BNIP3 siRNA H.

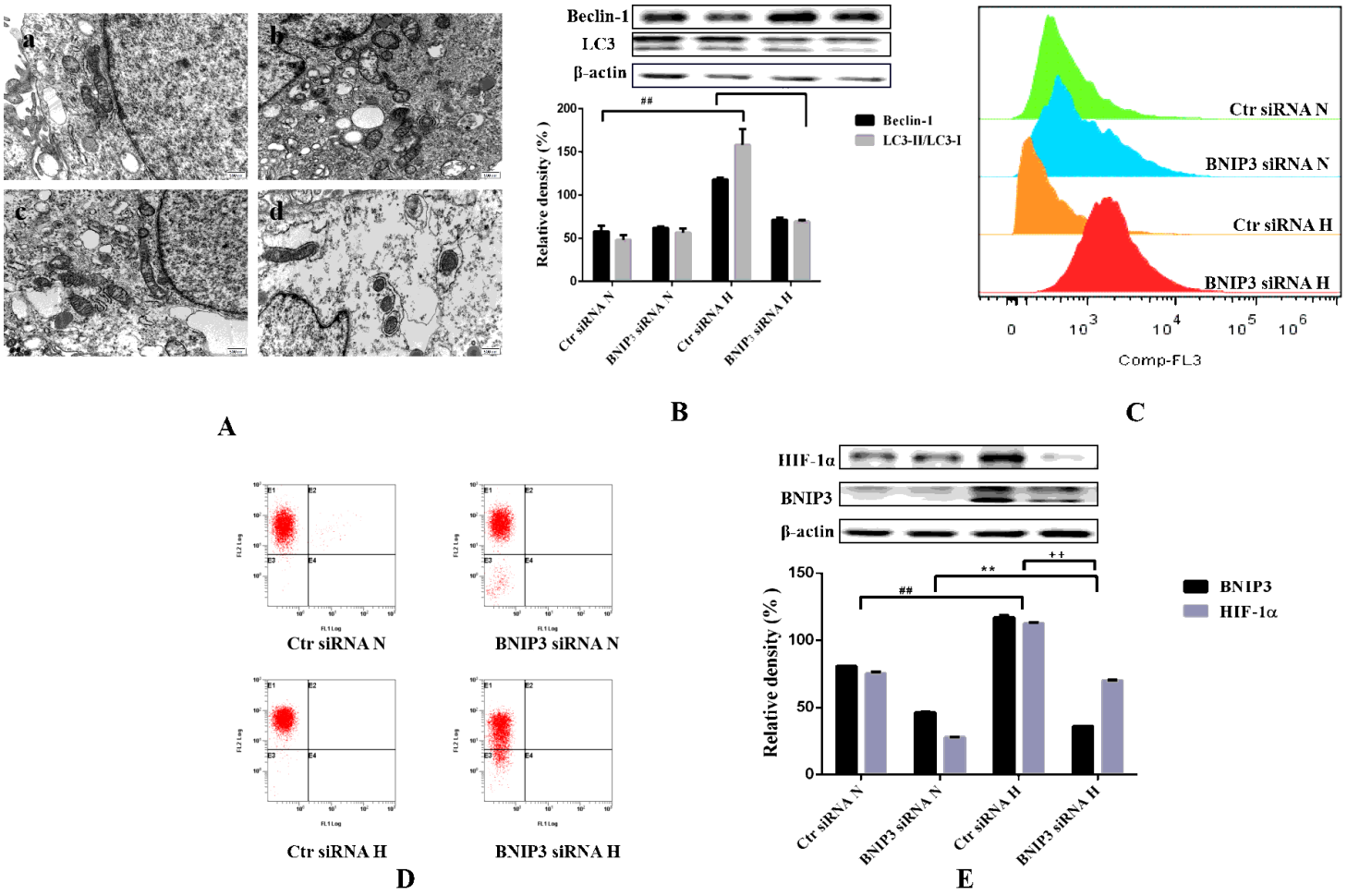

Figure 6

Effects of low expression of BNIP3 on the mitophagy and oxidation indexesof RA-FLSs. A: Cells were analysed by electron microscopy. B: The expression of Beclin-1 and LC3 was analyzed by Western blot; C: Cells were exposed to 20% O2 or 2% O2 for 24 h and assessed the ROS content by FCM (flowcytometry) using 10 μM CM-H2DCFDA as a ROS probe; D: JC-1 was used to detect the effect of hypoxia on MMPof OA-FLSs and RA-FLSs; E: The expression of HIF-1α and BNIP3 was analyzed by Western blot. The values are expressed as the mean±SD. #P<0.05, ##P<0.01 represents the comparison between Ctr siRNA N group and Ctr siRNA H; *P<0.05, **P<0.01 represents the comparison between BNIP3 siRNA N group and BNIP3 siRNA H, +P<0.05, ++P<0.01 represents the comparison between Ctr siRNA H group and BNIP3 siRNA H.

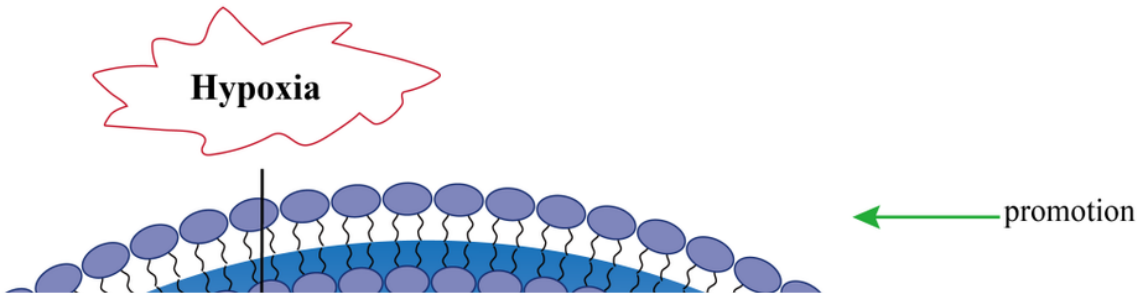

Figure 7

Under hypoxic conditions, the occurrence of mitophagy and the regulation of BNIP3. Under hypoxia, RA-FLSs mediated mitophagy through high expression of BNIP3 to eliminate excess ROS in cells, maintain redox balance, reduce the occurrence of apoptosis, and promote cell survival. The expression of BNIP3 in OA-FLSs was relatively small, and the mitophagy mediated by it was not enough to eliminate excess ROS in cells, resulting in increased apoptosis and decreased cell survival.