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Original research article

Ethyl pyruvate attenuates cellular adhesion and proliferation of diffuse large B-cell lymphoma by targeting c-Jun

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Abstract
Diffuse large B-cell lymphoma (DLBCL) stands out as the most common type of malignant cancer, representing the majority of cases of non-Hodgkin's lymphoma. Ethyl pyruvate (EP) is a derivative of pyruvic acid and found to have potent anti-tumor properties. Despite its potential benefits, the impact of EP on DLBCL remains ambiguous. Our objective is to elucidate the role of EP in modulating the development of DLBCL. Analysis of cholecystokinin-8 (CCK-8) revealed that treatment with EP significantly diminished the viability of DLBCL cells. Furthermore, EP administration suppressed colony formation and hindered cell adhesion and invasion in DLBCL cells. Examination of cell cycle progression showed that EP treatment induced arrest at the G1 phase and subsequently reduced the S phase population in DLBCL cells. EP treatment consistently exhibited apoptosis-inducing properties in Annexin-V assays, and notably downregulated the expression of Bcl-2 while increasing levels of proapoptotic cleaved caspase 3 and BAX in DLBCL cells. Additionally, EP treatment decreased the overexpression of c-Jun in c-Jun-transfected DLBCL cells. Further, EP demonstrated DNA-damaging effects in TUNEL assays. *In vivo*, xenograft animal models revealed that EP treatment significantly mitigated DLBCL tumor growth and suppressed DLBCL cell adhesion to bone marrow stromal cells. In summary, these findings suggest that EP mitigates DLBCL progression by inducing apoptosis, inducing cell cycle arrest, and promoting DNA damage.

Keywords: Adhesion; c-Jun; Diffuse large B cell lymphoma; Ethyl pyruvate; Proliferation

Highlights:
- Ethyl pyruvate (EP), a derivative of pyruvic acid, possesses potent anticancer activities.
- EP treatment significantly reduced the cell viability and adhesion of Diffuse large B-cell lymphoma (DLBCL).
- EP treatment induced apoptosis and cell cycle arrest.
- EP treatment significantly reduced the tumor growth of xenografted DLBCL tumor *in vivo* and suppressed the adhesion to the bone marrow stromal cells.
- EP attenuated the cellular progression of DLBCL cells by regulating c-Jun signaling.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is a prevalent form of malignant lymphoma, representing the majority of cases within non-Hodgkin's lymphoma (Crombie and Armand, 2019; Li et al., 2018). DLBCL encompasses a diverse spectrum of tumors characterized by distinct clinical and morphological features, immunophenotypes, and molecular alterations, affecting individuals of all age groups, particularly the elderly (Ollila and Olszewski, 2018). Despite advancements in diagnostic and therapeutic strategies, DLBCL patients often face a grim prognosis, with a mortality rate of approximately 40% (Friedberg, 2011). Thus, there is a pressing need to unravel the molecular mechanisms underlying DLBCL and develop effective therapeutic interventions.

Increasing evidence has demonstrated that communication between malignant B cells and the surrounding microenvironment plays a pivotal role in the progression of B-cell lymphoma (Cioroianu et al., 2019; Ennishi et al., 2020). Lymphoid tissue organization is intricate, and characterized by a complex network of stromal cells that facilitate cell migration, survival, and homeostasis (Shen et al., 2020). Within lymphoma, this intricate stromal cell network and microenvironmental factors

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attract tumor cells, promoting tumor initiation and progression (Autio et al., 2021). Therefore, targeting the lymphoma microenvironment emerges as a promising strategy for addressing DLBCL.

c-Jun serves as a crucial transcription factor that typically forms a heterodimer with c-Fos, constituting the Activator Protein-1 (AP-1) complex (Tsiambas et al., 2020). Research suggests that c-Jun undergoes ubiquitination and degradation in resting cells but becomes phosphorylated and stabilized upon stimulation, often regulated by JNK kinase (Zenz et al., 2003). Importantly, c-Jun is influenced by its downstream target genes at the transcriptional level, creating a positive feedback loop (Yuen et al., 2001). Studies have indicated the upregulation of c-Jun in various malignancies, including lymphoma (Li et al., 2013; Zhang et al., 2019b). Furthermore, the depletion of c-Jun leads to a significant reduction in lymphoma cell adhesion to surrounding stromal cells and inhibits tumor growth (Blonska et al., 2015).

Our study highlights the involvement of ethyl pyruvate (EP), derived from pyruvic acid, as a non-toxic endogenous metabolite effective in neutralizing reactive oxygen species (Yu et al., 2010). EP's anti-inflammatory properties have been documented in several cancer subtypes (Huang et al., 2020; Liang et al., 2009; Park et al., 2011; Yang et al., 2016). Notably, EP demonstrates promising antitumor effects as an adjunct to immune therapy by inhibiting the activation of IDO (indoleamine 2,3-dioxygenase), a key tolerogenic enzyme in various cancers, in both cellular and animal models (Muller et al., 2016).

Although EP holds the potential for beneficial effects, its impact on DLBCL remains uncertain. Thus, in this study, we thoroughly investigated the effects of EP on cell viability, migration, adhesion, apoptosis, cell cycle arrest, and DNA damage in DLBCL cells. Additionally, we examined the antitumor properties of EP in mice bearing DLBCL xenograft tumors.

Materials and methods

Animal model

SCID/nude male mice aged 5 to 6 weeks old were brought from Vital River Laboratory (China). OCI-Ly3 cells were suspended in PBS (1×10^7/100 μl) and subcutaneously injected into the left flank of mice. The mice were randomly divided into two groups and each group contained 10 animals (n = 10). Then, the treatment of 0.5% DMSO served as vehicle control and EP was intraperitoneally injected at a dose of 60 mg/kg BW twice a day for 24 days. Tumor size was checked every three days and determined following the formula: tumor volume = $0.5 \times \text{length} \times \text{width}^2$. After sacrifice, the tumors were collected and weighed. All experiments were authorized by the Animal Ethical Committee of the Department of Hematology, The First Affiliated Hospital of Gannan Medical University, Ganzhou, 341000, China.

Cell lines

Human DLBCL cell lines OCI-Ly3 and OCI-Ly7 were purchased from American Type Culture Collection (ATCC; USA), and cultured in RPMI 1640 medium that contains 10% fetal bovine serum (FBS; Hyclone, USA) and 1% penicillin: streptomycin antibiotic solution (Gibco, Thermo fisher scientific, USA) at humidified 37 °C atmosphere that incubated at 5% CO_2. EP was brought from Sigma and used at 2 mM in *in vitro* experiments (Birkenmeier et al., 2016; Huang et al., 2018) and 60 mg/kg BW in *in vivo* experiments (Moro and Sutton, 2010).

Cell transfection

The c-Jun overexpressing vectors (c-Jun OE) were custom-designed and synthesized by RiboBio (China). Cell transfections with plasmids were conducted using Lipofectamine 2000 as per the manufacturer's instructions. Successful transfections were validated through western blotting analysis.

Cell viability and colony formation assay

The viability and proliferation of DLBCL cells were assessed using the CCK-8 assay and colony formation assay. For the CCK-8 assay, cells were seeded in a 96-well plate and treated with EP at specified dose for varying durations. Subsequently, CCK-8 solution was added, and the cells were incubated for 2 h before measuring absorbance values using a microplate reader (Thermo, USA). To evaluate colony formation, cells were dissociated into single-cell suspensions and seeded in 12-well plates at a density of 500 cells per well. Then, the cells were treated with EP and incubated for 2 weeks to allow the colonies to form. Finally, the colonies were stained with crystal violet (Thermo, USA) and photographed using a digital camera (Nikon, Japan).

Cell adhesion

Cell adhesion to ECM was analyzed using the CultreCoat® Adhesion Protein Array Kit (Trevigen, China) following the manufacturer's instructions.

Cell invasion

Cells were seeded into Transwell inserts (Costar, USA) that were covered by Matrigel (Corning, USA), and incubated for 24 h. Then the images of invaded cells were captured by a microscope (Nikon, Japan).

Flow cytometry

Flow cytometry (BD FACSAria™ III, BD Bioscience) was utilized to analyze cell apoptosis and cell cycle progression following standard procedures. For cell cycle analysis, DLBCL cells were harvested and fixed in 70% ethanol at 4 °C for 2 days. Subsequently, the cells were treated with a mixture of propidium iodide (PI; 50 μg/ml, Sigma, USA), Triton-X 100 (1%), and RNase (100 μg/ml, Sigma, USA) for 30 minutes at room temperature before undergoing flow cytometry analysis.

For apoptosis assessment, samples were stained using dual Annexin V and PI staining (Beyotime, China). Cells were suspended in a binding buffer and then incubated with Annexin V and PI (5 μl) for 20 minutes at 37 °C before analysis using flow cytometry.

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay

The TUNEL assay was performed to assess cell apoptosis according to the manufacturer's instructions. Briefly, cells were initially fixed in 4% paraformaldehyde, then treated with 0.2% Triton X-100. Subsequently, the cells were incubated with FITC-labeled dUTP and terminal deoxynucleotidyl transferase. The apoptotic cells were visualized and captured using a confocal microscope (Olympus, Japan).

Western blotting

Total protein was extracted using ice-cold RIPA lysis Buffer (Sigma, USA), and the protein concentration was determined using a BCA assay kit (SolarBio, China). Subsequently, 50 μg of proteins were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto PVDF membranes. Following blocking with 5% nonfat milk, the membranes were incubated overnight at 4 °C with

primary antibodies, including anti-Bcl-2 (# MA5-11757), anti-Bax (# MA5-14003), anti-cleaved caspase-3 (# 43-7800), anti-c-Jun (# MA5-15881), and anti-β-actin (# AM4302) (Invitrogen, USA). The next day, the membranes were incubated with specific HRP-conjugated secondary antibodies (Sigma Aldrich, USA) (# 12-348) for one hour and visualized using an ECL reagent (Millipore, Germany).

Bone marrow infiltration

Lymphoma cells were stained using 5-chloromethylfluorescein diacetate (CMFDA; Thermo, USA) and then injected into the recipient mice. After 16 h, bone samples were harvested, and the percentage of infiltrated cells was determined using flow cytometry.

The infiltration of CD20+ cells was evaluated using the Automated Cellular Imaging System (ACIS III, Dako).

Adhesion to bone marrow cells

OCI-Ly3 cells that were stably transfected by Green Fluorescent Protein (GFP)-overexpressing vectors were labeled with CMFDA and then co-cultured in a 6-well plate that was previously coated with bone marrow stromal cells monolayer. After co-culture for 3 h, cells were observed, and the images were captured using the Automated Cellular Imaging System (ACIS III, Dako).

Statistics

All data were presented as mean ± SD of three replicates, and analyzed by a GraphPad Prism 7.0 software. Comparison between two or multiple groups was evaluated using Student's *t*-test and One-way ANOVA analysis, respectively. *P*-value < 0.05 was recognized as statistically significant.

Results

EP inhibits DLBCL cell proliferation and mediates cell cycle arrest in vitro

We postulated that EP might prevent DLBCL cell growth because it has been shown to have anti-tumor properties. Consequently, we investigated EP's role in DLBCL proliferation *in vitro*. The administration with EP (2 mM) decreased the cell viability of OCI-Ly3 and OCI-Ly7 cells, according to CCK-8 analysis (Fig. 1A and B). The treatment of EP suppressed the col-

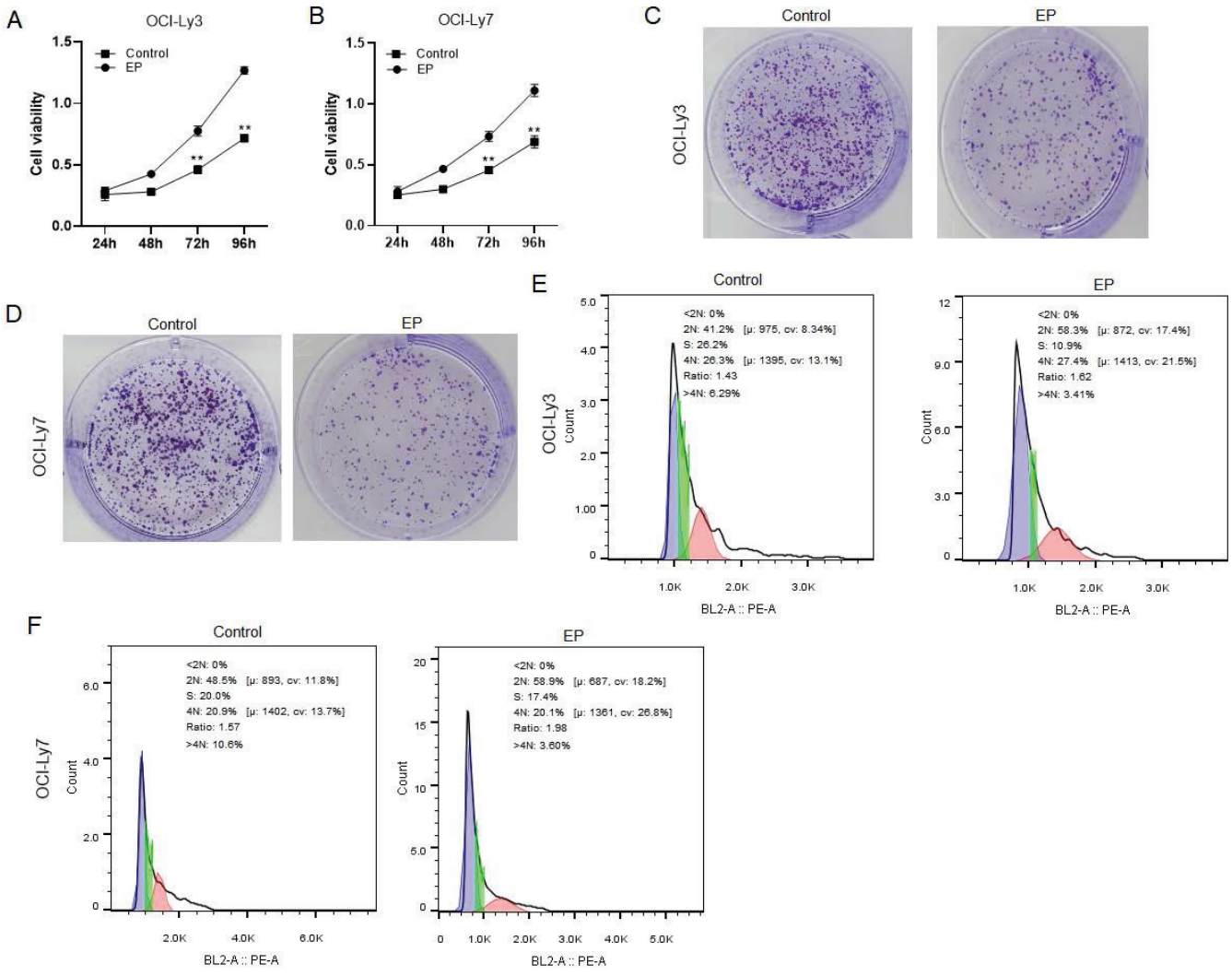

Fig. 1. EP inhibits DLBCL cell proliferation and mediates cell cycle arrest *in vitro*. (**A–F**) The OCI-Ly3 and OCI-Ly7 cells were treated with EP. (**A, B**) The cell viability was analyzed by CCK-8 assay. (**C, D**) The cell proliferation was detected by colony formation assays. (**E, F**) The cell cycle was determined by flow cytometry analysis. ** $P < 0.01$.

ony formation number of OCI-Ly3 and OCI-Ly7 cells (Fig. 1C and D). Cell cycle analysis revealed that EP treatment significantly arrested the cells in the G1 phase of the cell cycle, and ultimately reduced the cell population in the S phase of the cell cycle in OCI-Ly3 and OCI-Ly7 cells (Fig. 1E and F). These findings suggested that EP prevents the growth of DLBCL cells.

EP induces DLBCL cell apoptosis in vitro

Inhibition of apoptosis is a crucial phenotype in DLBCL. We ascertained EP's function in controlling DLBCL cells' apoptosis. Flow cytometry results demonstrated that treatment with EP promoted apoptosis in OCI-Ly3 and OCI-Ly7 cells (Fig. 2A and B). TUNEL staining consistently showed that the treatment with EP increased the number of TUNEL-positive lymphoma cells (Fig. 2C and D). In addition, treatment with EP increased the levels of Bax and cleaved caspase-3 while suppressing the Bcl-2 level in lymphoma cells (Fig. 2E and F), suggesting that EP can cause DLBCL cell apoptosis *in vitro*.

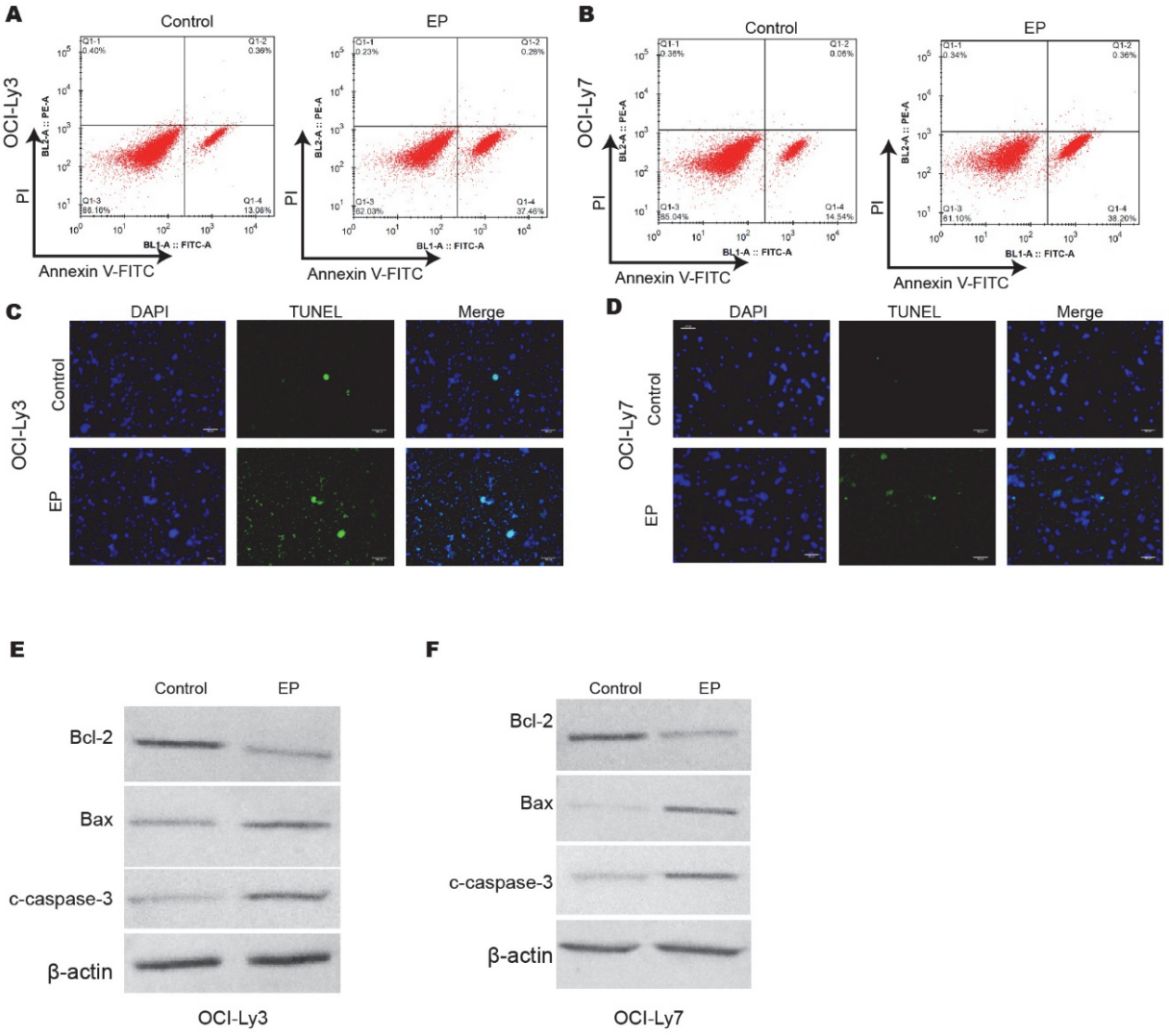

Fig. 2. EP induces DLBCL cell apoptosis *in vitro*. (**A–F**) The OCI-Ly3 and OCI-Ly7 cells were treated with EP. (**A, B**) The cell apoptosis was detected by flow cytometry analysis. (**C, D**) The cell apoptosis was determined by TUNEL analysis. (**E, F**) The expression of Bcl-2. Bax, cleaved caspase-3 (c-caspase3) was tested by Western blot analysis. Scale bar = 100 μm.

EP decreases DLBCL cell adhesion in vitro

Since DLBCL cells exhibit strong cell adhesion, we therefore investigated the role of EP in this phenomenon. We discovered that OCI-Ly3 and OCI-Ly7 cells' capacity to adhere to the surface was significantly suppressed by EP treatment (Fig. 3A and B). These findings suggest that EP can reduce DLBCL cell adhesion.

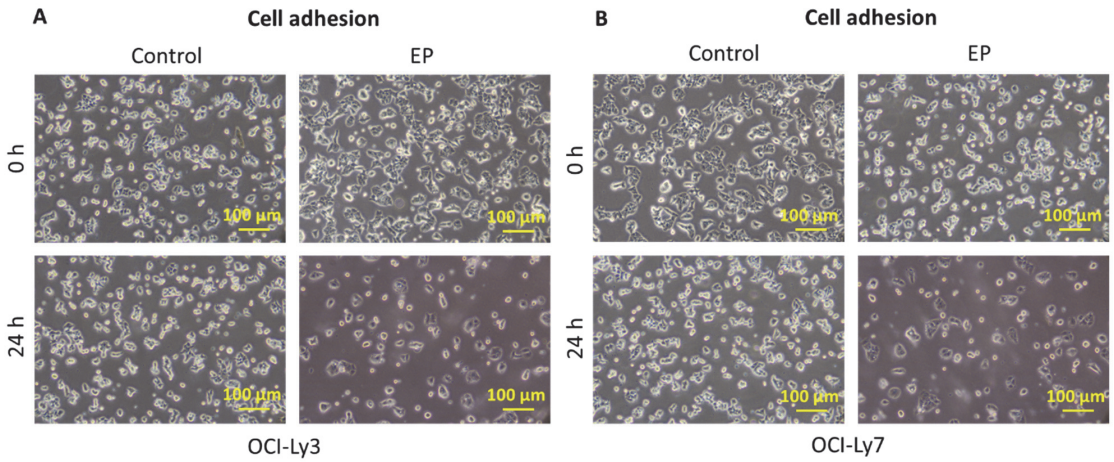

Fig. 3. EP inhibits DLBCL cell adhesion *in vitro*. (**A, B**) The OCI-Ly3 and OCI-Ly7 cells were treated with EP and the images were captured initially at 0 h and then 24 h for cell adhesion detection.

EP inhibits DLBCL progression by targeting c-Jun in vitro

We postulated that EP regulates DLBCL cells via targeting c-Jun since c-Jun is important for DLBCL formation. In DLBCL cells, c-Jun was overexpressed as a result of c-Jun plasmid transfection. We found that treating OCI-Ly3 and OCI-Ly7 cells with EP reduced the amount of c-Jun overexpression (Fig. 4A–D). Treatment with EP caused OCI-Ly3 and OCI-Ly7 cells to undergo cell apoptosis. The administration with EP consistently resulted in the induction of Bax and cleaved caspase-3 levels while maintaining the repression of the Bcl-2 level. However, c-Jun overexpression prevented the cells from exhibiting their characteristic (Fig. 4E and F).

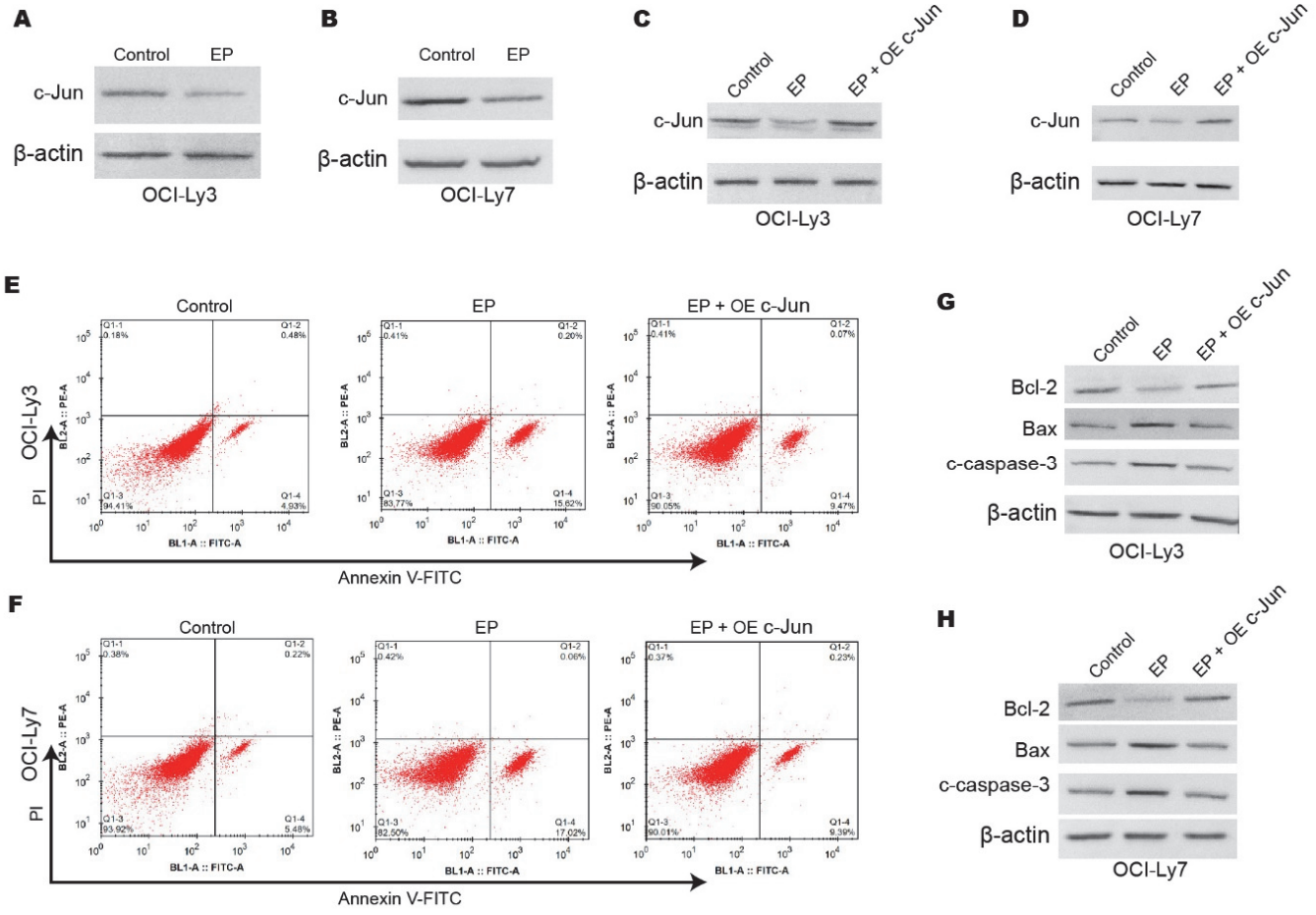

Fig. 4. EP decreases DLBCL cell progression and induces apoptosis *in vitro*. (**A, B**) The OCI-Ly3 and OCI-Ly7 cells were treated with EP. The expression of c-Jun was tested by Western blot analysis. (**C–H**) The OCI-Ly3 and OCI-Ly7 cells were treated with EP, or co-treated with c-Jun overexpression plasmids. (**E, F**) The cell apoptosis was detected by flow cytometry analysis. (**G, H**) The expression of Bcl-2, Bax, cleaved caspase-3 (c-caspase3) was tested by Western blot analysis.

EP inhibits DLBCL xenograft tumor growth and cell invasiveness in vivo

To assess the inhibitory effect of EP on DLBCL tumor growth, we subsequently established an animal model bearing xenografts of DLBCL tumors. We found that treating the mice with EP (60 mg/kg BW) considerably suppressed the growth of the OCI-Ly3 tumor (Fig. 5A). In addition, the EP treatment prevented the OCI-Ly3 cell adherence to the bone marrow stromal cells (Fig. 5B). These findings verified that EP impeded the growth of xenograft tumors and the *in vivo* invasiveness of lymphoma cells.

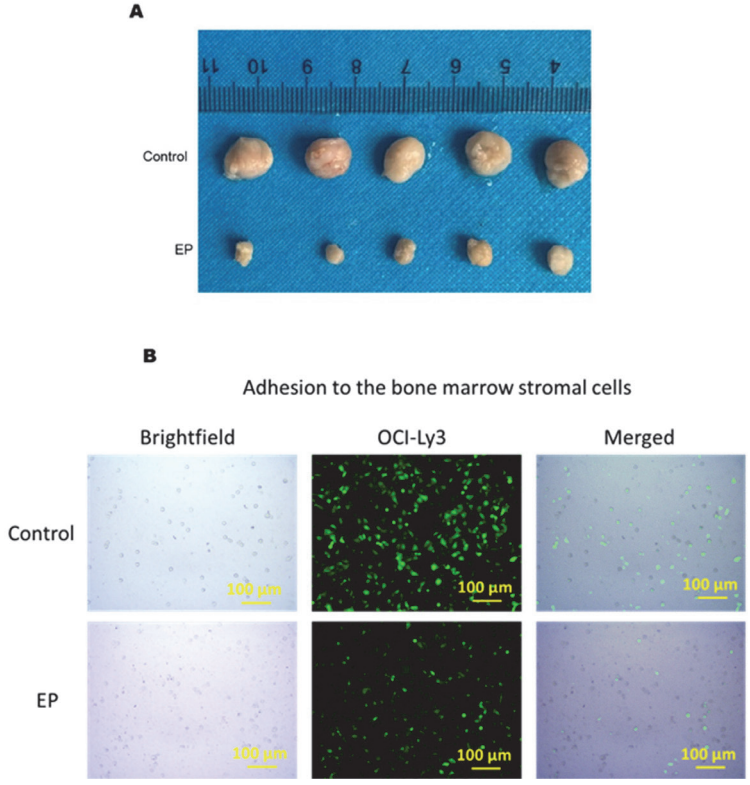

Fig. 5. EP inhibits tumor size in the xenograft model of DLBCL and lymphoma cell invasiveness *in vivo*. (**A**) OCI-Ly3 cells were suspended in PBS (1×10^7/100 μl) and subcutaneously injected into the left flank of SCID/nude mice. The cells were allowed to grow as a solid tumor and then EP was intraperitoneally injected at a dose of 60 mg/kg twice a day. (**B**) The OCI-Ly3 cells were tested for adhesion to the bone marrow stromal cells.

Discussion

Diffuse large B-cell lymphoma (DLBCL) represents a heterogeneous group of lymphoid malignancies with diverse clinical behaviors and responses to therapy (Li et al., 2018). Our study focused on understanding the potential therapeutic role of ethyl pyruvate (EP) in the treatment of DLBCL.

EP exhibits potent anti-inflammatory properties, which can be beneficial in cancer treatment. Chronic inflammation has been associated with several stages of carcinogenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis. Previous studies have revealed the tumor inhibiting role of EP in cancers (Huang et al., 2018; Zhang et al., 2019a). EP may help suppress tumor-promoting inflammation and create an unfavorable microenvironment for cancer cells (Park et al 2011). Moreover, our study adds to the growing body of evidence highlighting the importance of exploring novel therapeutic approaches for DLBCL. Despite advances in treatment modalities, including chemoimmunotherapy and targeted therapies, a significant proportion of DLBCL patients experience relapse or refractory disease, underscoring the need for alternative treatment strategies. EP presents itself as a promising candidate, offering the potential for targeted inhibition of DLBCL cell proliferation.

The results of our investigation revealed significant inhibitory effects of EP on DLBCL cell proliferation *in vitro*. This was evidenced by decreased cell viability and reduced colony formation capacity upon EP treatment in OCI-Ly3 and OCI-Ly7 cells. Furthermore, cell cycle analysis indicated that EP treatment led to G1 phase arrest, accompanied by a decrease in the proportion of cells in the S phase. These findings collectively suggest that EP exerts anti-proliferative effects on DLBCL cells, highlighting its potential as a therapeutic agent for DLBCL. Similarly, EP represses glioblastoma cell invasion and migration by the regulation of ERK and NF-κB-mediated EMT (Huang et al., 2018).

In the context of DLBCL, our findings provide novel insights into the potential therapeutic role of EP. DLBCL is characterized by dysregulated cell proliferation, and targeting this process is a key therapeutic strategy. The ability of EP to induce G1 phase arrest and reduce the proportion of cells in the S phase suggests its potential to interfere with cell cycle progression, thereby inhibiting tumor growth. Similarly, Cheng et al. (2014) reported that EP causes cell cycle arrest in hepatocellular carcinoma.

Treatment with EP induced apoptosis in DLBCL cells, as evidenced by an increase in TUNEL-positive cells. Moreover, EP treatment led to a suppression of Bcl-2 levels while promoting the expression of cleaved caspase-3 and Bax proteins in DLBCL cells. These changes suggest a shift towards apoptotic cell signaling upon EP treatment. Additionally, EP significantly hindered the adhesion ability of DLBCL cells *in vitro*, indicating its potential to impede tumor spread and metastasis. Similarly, EP attenuates the invasion, migration, and growth, and stimulates the lung cancer cell apoptosis by NF κB/STAT3 and the HMGB1/RAGE signaling (Liu et al., 2019).

In vivo experiments further supported the anti-tumor effects of EP, with significant attenuation of xenograft DLBCL tumor growth observed in mice treated with EP. Notably, EP treatment also suppressed DLBCL cell adhesion to bone marrow stromal cells in the model, underscoring its ability to disrupt tumor-stromal interactions critical for disease progression. Similarly, Liang et al. (2009) reported that EP treatment significantly inhibits hepatic tumor growth *in vivo*.

Overexpression of c-Jun has been observed in several types of lymphoma, suggesting its oncogenic potential. It contributes to tumor growth and survival by promoting cell proliferation, inhibiting apoptosis, and enhancing invasiveness. c-Jun plays critical roles during DLBCL development. For example, rafoxanide inhibits DLBCL progression by the JNK/c-Jun and PTEN/PI3K/Akt signaling (He et al., 2020). A novel MEF2C mutation promotes tumorigenesis by inducing c-JUN expression in DLBCL (Jingjing et al., 2020). We hypothesized that EP modulates DLBCL cells by targeting c-Jun, a key player in DLBCL development. Interestingly, treatment with EP led to a reduction in c-Jun overexpression in DLBCL cells (Fig. 4A–D). Furthermore, EP treatment induced apoptosis in these cells, accompanied by increased levels of Bax and cleaved caspase-3, while suppressing Bcl-2 expression. Moreover, it has been reported that the EP can inhibit HMGB1 signaling to repress the progression of DLBCL (Zhang et al., 2019a). Similar to this study, EP inhibits the invasion and growth of gallbladder cancer cells by downregulating HMGB1/RAGE signaling (Li et al., 2012).

Collectively, these findings highlight the therapeutic potential of EP in inhibiting DLBCL progression, both *in vitro* and *in vivo*. Further exploration of EP's clinical significance in cancer treatment is warranted, offering promising avenues for future research and therapeutic development.

Conclusion

Our study presents strong evidence supporting the anti-proliferative properties of EP in DLBCL cells. We observed that EP treatment markedly decreased cell viability, inhibited cell adhesion, and disrupted the cell cycle, ultimately reducing DLBCL cell proliferation. Additionally, EP induced DNA damage and triggered caspase-dependent apoptosis in DLBCL cells. Moreover, in DLBCL tumor-bearing mice, EP effectively suppressed tumor growth and hindered adherence to bone marrow stromal cells. We have also identified a role for EP in regulating c-Jun, although further investigation is needed to fully understand its underlying mechanisms in therapeutic intervention.

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Ethical aspects and conflict of interest
The authors have no conflict of interest to declare.

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