Shiraz University

IJVR

ISSN: 1728-1997 (Print)
ISSN: 2252-0589 (Online)

Vol.25 No.4 Ser. No.89 2024

IRANIAN JOURNAL OF VETERINARY RESEARCH

Original Article

Impact of carboxymethyl dextran-asparaginase in NALM-6 cell apoptosis and autophagy

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10.22099/ijvr.2024.49166.7208

(Received 13 Jan 2024; revised version 24 Sept 2024; accepted 6 Nov 2024)

Abstract

Background: Acute lymphoblastic leukemia (ALL) is a malignant disorder in both humans and animals. L-asparaginase (L-ASNase) has limitations as a chemotherapy agent due to adverse effects and low serum stability. In a previous study, L-ASNase was chemically modified with carboxymethyl dextran to enhance its properties. **Aims:** This study aimed to validate the potential of these modifications using the NALM-6 cell line. **Methods:** NALM-6 cells were cultured and treated with various concentrations, including 0 IU/ml as negative control, 0.5, 1, 1.5, and 2 IU/ml of modified L-ASNase and L-ASNase. The optimal concentration was determined at specific intervals, and viability and metabolic activity were assessed through Trypan blue and MTT tests. Flow cytometry, using Annexin V/PI staining, was employed to evaluate apoptosis. Real-time RT-PCR techniques were used to determine changes in the expression of the *ATG2B* and *LC3-II* genes (important genes in autophagy), with data analysis conducted using PRISM software. **Results:** The modified L-ASNase reduced the viability of NALM-6 cells and induced higher levels of apoptosis (P=0.001). Interestingly, the modified enzyme had a lesser impact on autophagy, which is important for avoiding treatment resistance. **Conclusion:** The modified L-ASNase showed enhanced effectiveness in reducing the viability of NALM-6 cells and induced higher levels of apoptosis. Interestingly, the modified enzyme had a lesser effect on autophagy, which is important as excessive autophagy can lead to treatment resistance. These findings suggest that the modified L-ASNase may have the potential to be a more effective chemotherapeutic agent for ALL treatments.

Key words: Acute lymphoblastic leukemia, Apoptosis, Autophagy, L-asparaginase, NALM-6 cell line

Introduction

Acute lymphoblastic leukemia (ALL) is a malignant disorder that accounts for 34% of pediatric cancers (Shafique and Tehsin, 2018) and is also seen in dogs (Tufekci *et al*., 2022). FMS-like tyrosine kinase 3 (FLT3) is a commonly mutated protein in a variety of human acute leukemias canine lymphoproliferative diseases, including lymphoma and acute leukemias, exhibit evolutionarily conserved chromosomal aberrations and mutations within key oncogenes that are similar to their human counterparts (Suter *et al*., 2011). ALL can originate from the B-cell precursor (BCP) lineage, known as BCP-ALL, or though less frequently, from the early T-cell precursor lineage, referred to as ETP-ALL. While cure rates in children surpass 90%, ALL continues to be a major contributor to morbidity and mortality in both pediatric and adult populations. (Iacobucci and Mullighan, 2017). L-asparaginase (L-ASNase) is a highly potent chemotherapy agent to cure ALL. This enzyme converts L-asparagine into aspartic acid and ammonia (Batool *et al*., 2016). By depleting L-asparagine from the serum, the L-ASNase enzyme inhibits cell cycle progression and induces apoptosis, as L-asparagine is essential for the cell cycle (Piatkowska-Jakubas *et al*., 2008).

L-ASNase products, specifically the NH4+ ion, enter the cytosol and modify the pH, triggering the signal transduction pathway related to substrate phosphorylation and apoptosis. Furthermore, this enzyme inhibits protein synthesis by hydrolyzing L-asparagine (Tabandeh and Aminlari, 2009). Autophagy is a

fundamental cellular process that involves breaking down cytoplasmic components to help maintain energy balance (Yang and Klionsky, 2020). Autophagy, a cellular process for degrading and recycling cellular components, occurs at a low level under normal conditions but increases during stress to produce energy and help cells survive (Yorimitsu and Klionsky, 2005). However, excessive autophagy can be harmful and has been linked to various diseases, including cancer (Parzych and Klionsky, 2014). Autophagy involves a group of proteins called autophagy-related (ATG) proteins, which form functional complexes that activate and move to membranes to start the autophagy process (Yu *et al*., 2018). One type of autophagy, macro-autophagy, uses specialized vacuoles called autophagosomes to transport cellular cargo. These autophagosomes are newly formed by synthesizing autophagic membranes (Wirawan *et al*., 2012). It is thought that their origin lies in the endoplasmic reticulum, where they play a role in regulating the functions of ATG proteins. The autophagosome membrane elongation, formation, and closure involve two ubiquitin-like conjugation systems. The first system consists of the ATG16L1 complex, while the second involves the lipidation of MAP1LC3 (microtubule-associated protein 1 light chain 3).

The conjugation of MAP1LC3 to a phosphatidylethanolamine protein, known as LC3-PE or LC3-II, is considered a specific marker for autophagy (Ravikumar *et al*., 2010; Velikkakath *et al*., 2012; Wirawan *et al*., 2012; Parzych and Klionsky, 2014; Noda and Inagaki, 2015; Galluzzi *et al*., 2017; Lin *et al*., 2018). As previously mentioned, L-ASNase has been used to treat ALL and is also commonly used for treating canine lymphoma (Lee *et al*., 2021). The main limitation of its therapeutic use is allergic side effects due to its high immunogenicity (Cecconello *et al*., 2020). Additionally, the enzyme exhibits low serum stability and is quickly cleared from the plasma due to the effects of native protease or specific antibodies (Soares *et al*., 2002). Other side effects include pancreatitis, liver dysfunction, coagulopathy, hyperglycemia, and central nervous system dysfunction (Wang *et al*., 2022). On the other hand, several mechanisms of resistance to L-ASNase treatment have been recently proposed (Zhou *et al*., 2023). In dogs, L-ASNase often causes hypersensitivity reactions with symptoms such as itching, vomiting, and collapse, as well as decreased protein synthesis. Pancreatitis and other gastrointestinal issues can also occur, linked to the enzyme's impact on protein synthesis (Lee *et al*., 2021). To enhance the enzyme's anticancer activity, various methods have been developed. One approach is to chemically modify L-ASNase with different polymers. For instance, N-bromosuccinimide and mono-methoxy polyethylene glycol (mPEG) are used for this purpose, similar to how bovine serum albumin is modified (Mohan Kumar *et al*., 2014). Another modification involves using 2, 4-bis (O-methoxy polyethylene glycol)-6-chloro-S-triazine (mPEG2) to increase the half-life of L-ASNase in the bloodstream (Zhang *et al*., 2004). Since 1994, PEG-ASNase has been commercially available as a bioconjugate form of L-ASNase, offering improvements in immunity, toxicity, and resistance to proteolysis, although it may increase the risk of pancreatitis (Rizzari *et al*., 2006). Our previous work involved chemically modifying L-ASNase with carboxymethyl dextran to improve its pharmacokinetic properties. This modification enhanced the enzyme's biochemical properties and significantly increased its half-life in rat serum and phosphate buffers compared to native ASNase (Chahardahcherik *et al*., 2020). Nonetheless, evidence of increased stability in human serum is lacking. In this study, we assessed the effects of carboxymethyl dextran-conjugated L-asparaginase on the NALM-6 cell line. NALM-6 is a leukemia cell line obtained from adolescent male B cells and serves as a xenograft model for acute lymphoblastic leukemia, characterized by CD24 positivity (Belviso *et al*., 2017).

We evaluated the viability and apoptosis of the NALM-6 cell line in the presence of both native and conjugated enzymes using the MTT assay and flow cytometry. Additionally, we analyzed the expression of *LC3-II*, a critical autophagy marker, and *ATG2B*, a gene associated with autophagy, through quantitative real-time RT-PCR (qRT-PCR) analysis. The goal of this study was to validate the potential application of modified L-ASNase in ALL treatment using the NALM-6 cell line, based on previous research.

Materials and Methods

Ethics statement

All experiments were carried out based on the principles of the Ethics Committee at Shiraz University, Shiraz, Iran. Also, the study has been approved at the School of Veterinary Medicine of Shiraz University.

Preparation of L-asparaginase modified with carboxymethyl dextran

In this experimental study, L-ASNase from a HAP-modified strain of *E. coli* with 10,000 IU activity was obtained from Leunase® (Kyowa Hakko Kirin, Japan). Carboxymethyl dextran (CMD), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), and other analytical-grade reagents were purchased from Merck (Darmstadt, Germany) and Sigma (St. Louis, MO, USA). L-ASNase was modified with carboxymethyl dextran as described previously (Chahardahcherik *et al*., 2020). In brief, to activate CMD, 100 µL of 0.02 M EDC was slowly added to 800 µL of 2.5 mM CMD, followed by 100 µL of 0.05 M NHS, and mixed, allowing the reaction to proceed at room temperature on a shaker incubator for 40 min. Next, 1 ml of L-ASNase solution (containing 15 mg of total protein in 0.1 M phosphate buffer, pH 7.2, and 5 mg of L-asparagine) was added gradually to 1 ml of the activated CMD solution, mixed gently, and incubated at room temperature on a shaker incubator for 2 h. The

product was then dialyzed overnight against distilled water at 4°C using a membrane with a molecular weight cut-off of 6-8 kDa. A control L-ASNase sample was treated under the same conditions without CMD, EDC, and NHS. CMD-modified L-ASNase was separated from unreacted compounds using Sephadex G-100 column chromatography, with the column equilibrated and eluted using 0.025 M ammonium acetate buffer. Protein content in each fraction was detected by measuring absorbance at 280 nm. Structural analysis confirmed the modification of the enzyme.

Cell culture and treatment

The NALM-6 cell line was obtained from the Pasteur Institute of Iran and cultured in RPMI 1640 medium (Cell Biotechnology Saba Arna, Iran) enriched with 10% fetal bovine serum, 100 U/ml penicillin-streptomycin (both from Gibco Life Technologies, Waltham, MA, USA), and 2 mM L-glutamine (Shellmax, China). Culturing occurred at 37°C in an environment with 5% CO_2 and 95% humidity. Both modified and native L-ASNase were dissolved in RPMI media, and the cells were treated with varying concentrations (0.5, 1, 1.5, 2 IU/ml) of native and modified L-ASNase (Parmentier *et al*., 2015). The cells were incubated for 24, 48, and 72 h at 37°C in a humidified atmosphere containing 5% CO_2.

MTT assay

To conduct the MTT assay, a total of 1×10^4 cells were seeded in each well of a 96-well plate using 100 µL of culture medium. The cells were subsequently exposed to various concentrations of both native and modified L-ASNase and were incubated for 24, 48, and 72 h as described. Cell viability was assessed by adding 10 µL of sterile 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) solution at a concentration of 5 mg/ml (From, Melford, Ipswich, UK). Following a 4 h incubation at 37°C, the MTT solution was discarded, and 100 µL of dimethyl sulfoxide (DMSO) (Shellmax, China) was added to dissolve the formazan crystals. This procedure relies on mitochondrial enzymes converting MTT to MTT-formazan. The absorbance was then measured at 545 nm using a Stat Fax 2100 Microplate photometer (Stat Fax 2100, SKU: 8036-10-0020).

RNA extraction, cDNA synthesis, and qRT-PCR

To investigate how native and modified L-ASNase influence the expression levels of *ATG2B* and *LC3-II*, 1.2×10^6 cells were cultured in a 6-well plate with 3.3 ml of RPMI 1640 medium. The cells were treated with both native and modified enzymes at a concentration of 2 IU/ml (as established through multiple MTT assays). Following incubation periods of 24, 48, and 72 h, the cells were collected by centrifugation and washed with PBS (Sigma-Aldrich, Germany), and lysed in 700 µL of TRIzol reagent. The lysate was mixed with chloroform, shaken vigorously for 15 s, and kept at 4°C for 2-3 min. The mixture was centrifuged at 12,000 × g for 15 min at 4°C. The supernatant was combined with 0.5 ml of isopropanol (per ml of TRI reagent) and placed on ice. After 20 min of incubation, the mixture underwent another centrifugation for 10 min at 12,000 × g and 4°C. The resulting pellet was washed with 75% ethanol and centrifuged again at 7,500 × g for 8 min at 4°C. Finally, the RNA pellet was reconstituted in 20-30 µL of DEPC-treated water and incubated at 57°C for 12 min. The purity of the extracted RNA was assessed using a NanoDrop instrument (Hellna, NY, China).

Complementary DNA (cDNA) was synthesized using the Prime TM RT reagent kit (Yektatajhiz Azma, Iran). First-strand cDNA was synthesized by mixing 1 µL RNA and 1 µL Random hexamer primer (50 µM) in a microtube containing 13.4 µL DEPC-treated water. The mixture was incubated for 5 min at 70°C and then cooled on ice. Subsequently, 4 µL of 5X first-strand buffer, 1 µL of dNTPs, 0.5 µL of RNase inhibitor, and 1 µL of M-MLV were added to form the cDNA synthesis mix, which was then incubated at 37°C for 60 min. The reverse transcription reaction was performed using a thermal cycler system (Astec, Japan).

Real-time PCR was conducted using the Rotor-Gene Q (Qiagen, USA) under the following conditions: 95°C for 1.5 min, 95°C for 5 s, 57°C for 30 s, and 72°C for 30 s. The B2m (β-2 microglobulin) gene was used as an internal control (Ullmannova and Haskovec, 2003). Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001). The primers were designed using the Allele ID v7.70 software (PREMIER Biosoft, USA) and are listed in Table 1. A real-time PCR standard curve was constructed using a serial dilution from cDNA. To determine the accuracy of the real-time PCR performance, the melting curve of the genes was drawn.

Flow cytometry

For flow cytometry analysis, each well was inoculated with 1×10^5 cells and treated with either native or modified L-ASNase at a combined activity of 2 IU/ml, as established by repeated MTT assays. Some wells also received 30 µM of Chloroquine (CQ) (Temad,

Table 1: Primer sequences used in the present study

Gene name	Sequence	Product size (base pair)	T_m
LC3-II	F: 5´-GTGATAATAGAACGATACAAGG-3´ R: 5´-CACTCTCATACACCTCTG-3´	208 bp	53°C
ATG2B	F: 5´-CCAGAAACTAACAGATGAACAAAG-3´ R: 5´-TCAGCAGTTTCATCACAGTACAC-3´	210 bp	60°C
B2m	F: 5´-TATGCCTGCCGTGTGAAC-3´ R: 5´-CTACCTGTGGAGCAACCTG-3´	284 bp	57°C

Iran), which is an inhibitor of autophagy. The Annexin V-PE Apoptosis Detection Kit (BD Bioscience, San Jose, CA) was utilized to assess cell apoptosis after 24, 48, and 72 h of incubation. The cells were washed twice with phosphate-buffered saline (PBS) and then resuspended in 100 µL of 1X Annexin V binding buffer. Staining with Annexin V and 7-AAD was carried out in the dark for 15 min. Afterward, 400 µL of Annexin V binding buffer was added, and the samples were analyzed using a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA). Flow cytometry results were processed with Flow Jo V10 software.

Statistical analysis

All experiments were performed in triplicate. Data were analyzed using two-way analysis of variance (ANOVA) and the Kruskal-Wallis test. P-values of <0.05 were considered statistically significant. Statistical analysis was conducted using GraphPad Prism 9.

Results

Cell viability assessment

The NALM-6 cell line was exposed to varying concentrations of native and carboxymethyl-conjugated L-ASNase (0, 0.5, 1, 1.5, and 2 IU/ml), and cell viability was evaluated using the MTT assay. A minimum of three independent experiments were conducted in triplicate. The findings revealed that higher concentrations of both enzymes (1.5 and 2 IU/ml) resulted in a significant reduction in cell viability after 24 h (P=0.01). Notably, the native enzyme exhibited a slightly greater cytotoxic effect compared to the modified one. After 48 h, the modified enzyme demonstrated a marked increase in its capacity to reduce cell viability at elevated concentrations (P=0.01). By the 72-hour mark, significant decreases in cell viability were evident for both native and modified enzymes at the higher concentrations (P=0.01).

Real-time RT-PCR

Quantitative analysis of the effects of native and modified L-ASNase on the expression of autophagy-related genes showed a significant increase in *LC3-II* expression after 24 h of treatment with both enzymes (P<0.0001), as illustrated in Fig. 1. However, the increase in the native enzyme group was significantly higher than that of the modified enzyme (P=0.02). After 48 and 72 h of treatment with the modified enzyme, a substantial increase in *LC3-II* expression was noted compared to the control and native enzyme groups. Additionally, after 24 h of exposure to the native enzyme, *ATG2B* expression showed a statistically significant increase relative to the control and modified enzyme groups (P=0.04). Other observed changes were minimal and did not reach statistical significance.

Analysis of apoptosis induction in NALM-6 cells

Apoptosis in NALM-6 cells treated with native and modified L-ASNase, individually and in combination with CQ, was assessed using flow cytometry. The results indicated that a 24-hour treatment with the native enzyme led to a significant increase in apoptosis compared to the control group (P=0.01). The modified enzyme not only significantly raised apoptosis levels compared to the control but also surpassed the effect of the native enzyme (P=0.001). When CQ was administered alongside the enzymes, a reduction in apoptosis was observed for both native and modified samples, being statistically significant only in the modified enzyme group (P=0.01) (Figs. 2a-c). Following a 48-hour treatment period, both native and modified L-ASNase significantly elevated apoptosis compared to the control (P=0.01 and P=0.001, respectively), with the modified enzyme demonstrating a greater effect. The presence of CQ again resulted in decreased apoptosis for both enzyme conditions, although this change did not reach statistical significance (Figs. 3a-c). After 72 h of treatment, the modified enzyme led to a significant increase in apoptosis compared to the control (P=0.01), while the native enzyme group did not show a significant change. The combination of CQ with the native enzyme resulted in a slight reduction in apoptosis, whereas the effect was additive in the modified enzyme sample (Figs. 4a-c).

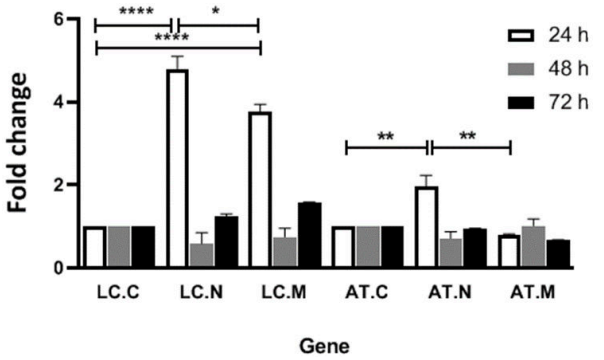

Fig. 1: Real-time RT-PCR analysis of *LC3-II* and *ATG2B* genes. Expression of *LC3-II* increased after 24 h of treatment with both modified (P<0.01) and native enzymes (P<0.01). A significant increase in *ATG2B* expression was observed only after 24 h of treatment with the native enzyme (P=0.04). Two-way analysis of variance (ANOVA) was used. P<0.05 were considered statistically significant. * P=0.02, ** P=0.004, and **** P=0.0001. LC: *LC3-II*, AT: *ATG2B*, C: Control, N: Native enzyme, and M: Modified enzyme

Discussion

This study investigates the effects of native and carboxymethyl-conjugated L-asparaginase (L-ASNase) on cell viability, apoptosis, and autophagy in the NALM-6 cell line, a well-established model for acute lymphoblastic leukemia (ALL). Despite L-ASNase's established use in chemotherapy, its adverse effects and serum stability limitations necessitated the exploration of modifications that enhance its efficacy. Our findings indicate that both the native and modified L-ASNase

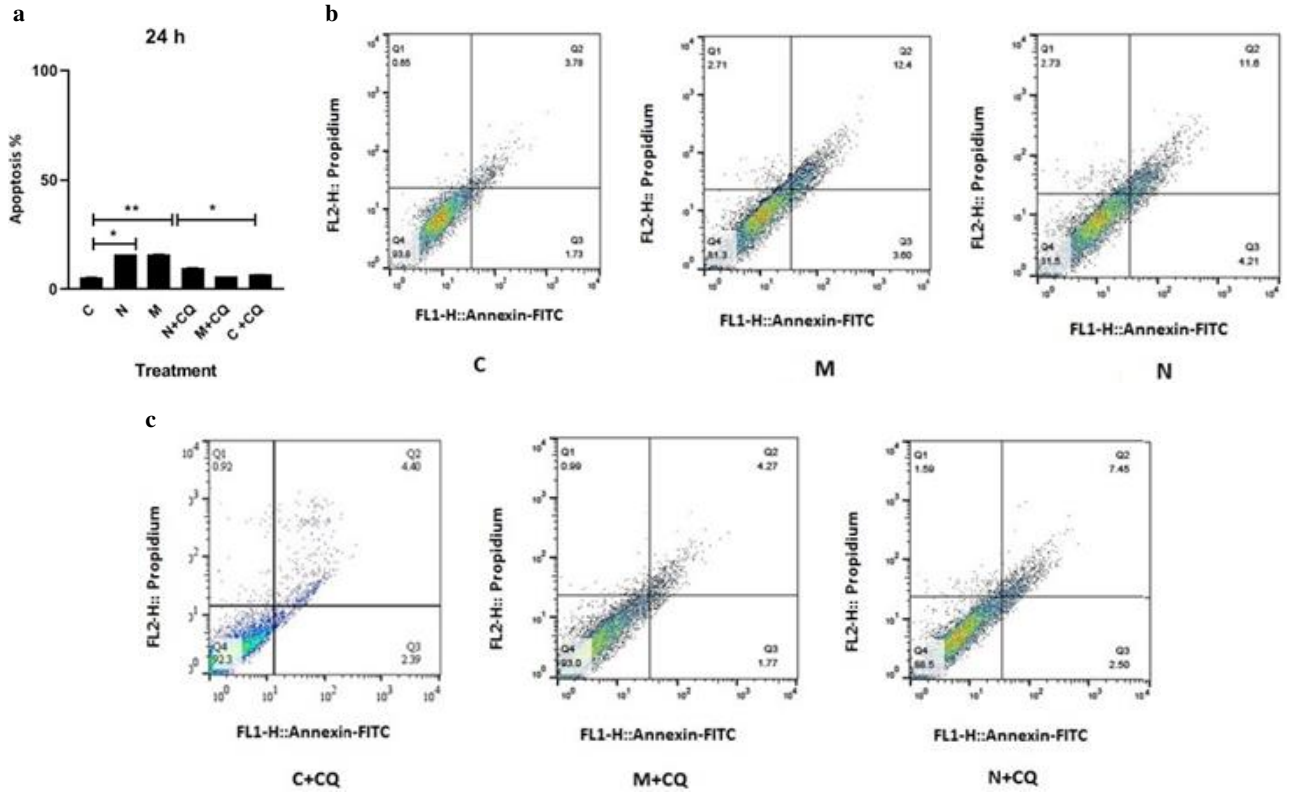

Fig. 2: Flow cytometry analysis after 24 h. (**a**) Comparative graph of apoptosis rate in samples after 24 h, (**b**) Both native and modified enzymes (without chloroquine) increased apoptosis compared to the control sample (P=0.01 and P=0.001), respectively, and (**c**) CQ combined with both native and modified enzymes decreased apoptosis (P=0.41 for N *vs*. N+CQ) and (P=0.01 for M *vs*. M+CQ). The Kruskal-Wallis test was used and P<0.05 was considered statistically significant. * P=0.01, and ** P=0.001. C: Control, N: Native enzyme, M: Modified enzyme, and CQ: Chloroquine

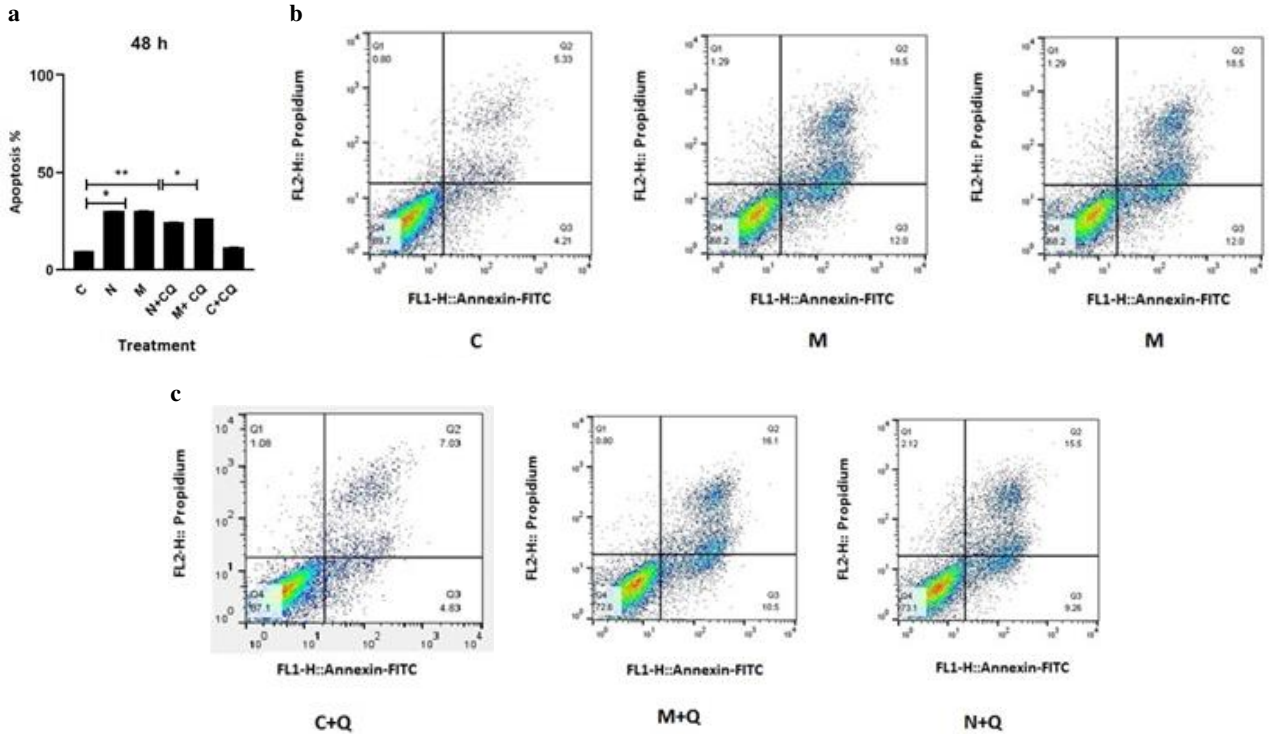

Fig. 3: Flow cytometry analysis after 48 h. (**a**) Comparative graph of apoptosis rate in samples after 48 h, (**b**) Treatment of cells with both native and modified enzymes (without chloroquine) increased apoptosis compared to the control group (P=0.01 and P=0.001, respectively), and (**c**) CQ combined with native and modified enzymes decreased their apoptosis (P=0.10 and P=0.10), respectively. Kruskal-Wallis test was used and P<0.05 was considered statistically significant. * P=0.01, and ** P=0.001. C: Control, N: Native enzyme, M: Modified enzyme, and CQ: Chloroquine

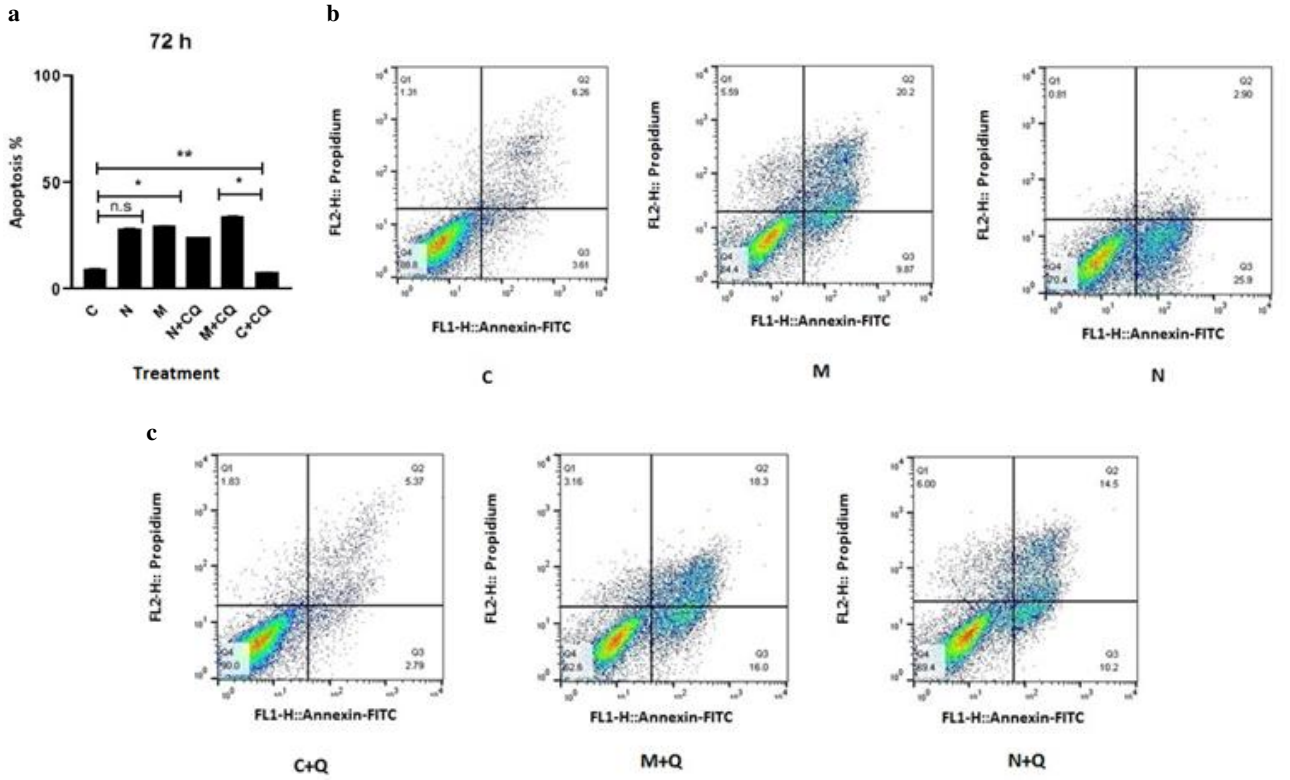

Fig. 4: Flow cytometry analysis after 72 h. (**a**) Comparative graph of apoptosis rate in samples after 72 h, (**b**) Without chloroquine. In the modified enzyme group, apoptosis increased significantly compared to the control group (P=0.01), but the increase in the apoptosis of the native enzyme group was not significant (P=0.10), and (**c**) An increase in apoptosis was seen after combining CQ with a modified enzyme compared to the control group (P=0.001) and the N+CQ group (P=0.01). Kruskal-Wallis test was used and P<0.05 was considered statistically significant. * P=0.01, and ** P=0.001. C: Control, N: Native enzyme, M: Modified enzyme, and CQ: Chloroquine

significantly impact cell viability and apoptosis induction, warranting further exploration of their potential in therapeutic applications.

The MTT assay results revealed that both native and modified L-ASNase significantly reduced the viability of NALM-6 cells at high concentrations (1.5 and 2 IU/ml) after 24 h (P=0.01). Interestingly, while the native enzyme exhibited slightly higher toxicity, the modified enzyme demonstrated enhanced efficacy at 48 h, highlighting its potential benefits when administered chronically (Gharehchahi *et al*., 2023). This observation suggests that the chemical modification with carboxymethyl dextran may improve the stability and prolonged efficacy of L-ASNase without significantly worsening its toxicity profile (Parmentier *et al*., 2015). The persistent reduction in cell viability observed across all time points underscores the therapeutic potential of modified L-ASNase in leukemia treatment (Song *et al*., 2015). The current study's findings on the antiproliferative effect of native and modified enzymes corroborate several previous studies (Belviso *et al*., 2017; Hlozkova *et al*., 2020). It should be noted that this cytotoxic effect depends on several factors, including the duration of the treatment and the enzyme concentration.

The results of the quantitative analysis on the effects of native and modified L-asparaginase (L-ASNase) on the expression of autophagy-related genes suggest that both enzymes influence the autophagy pathway in NALM-6 cells. Autophagy is vital for tumor development, treatment response, and cellular homeostasis. In contrast, cancer cells often accelerate this process to withstand metabolic and therapeutic stresses (Galluzzi *et al*., 2017). The role of autophagy in cancer is complex and somewhat controversial; it appears to act as a tumor suppressor during cancer development but may promote tumor cell survival during cancer progression. Additionally, tumor cells can exploit autophagy to resist anticancer therapies, and silencing genes that regulate the autophagy process can either increase or decrease cancer cell death (Tandel *et al*., 2022). Autophagy, also referred to as type II cell death, is linked to other forms of cell death, such as apoptosis. It can lead to cell death, promote cell survival by inhibiting apoptosis, or serve as a precursor to apoptosis (Denton *et al*., 2015; Sever and Demir, 2017). Understanding the relationship between autophagy and cell death is critical for developing effective cancer treatments. Chloroquine, an antimalarial drug, has been reported to possess anticancer properties (Cuomo *et al*., 2019). This compound inhibits autophagy by interfering with the fusion of autophagosomes and lysosomes (Mauthe *et al*., 2018).

The increase in LC3-II expression, a marker for autophagy, by both modified and native L-asparaginase (L-ASNase) suggests that both enzymes activate autophagy, but the modified enzyme induces apoptosis

more effectively (Chen *et al*., 2017; Jia *et al*., 2018).

Flow cytometry results demonstrate that both enzymes significantly induce apoptosis in NALM-6 cells. Specifically, the modified enzyme not only increased apoptosis more than the native enzyme (P=0.001 after 24 h) but also maintained significant effects over the extended treatment window, suggesting a sustained apoptotic signaling capability. The interaction with CQ revealed a complex relationship, where CQ diminished apoptosis in the presence of both enzymes, with significant reductions observed only in the modified group (P=0.01). This implies a potential defensive mechanism through autophagy when CQ is combined with modified L-ASNase that could be explored further in therapeutic contexts (Métayer *et al*., 2019). This difference can arise from several mechanisms including:

Differential effects on autophagy regulation

The modified enzyme may more efficiently activate autophagic pathways due to its interaction with cellular receptors, enhancing apoptotic signaling.

Increased binding affinity: The modified enzyme may bind better to specific receptors on NALM-6 cells (Zaffagnini and Martens, 2016).

Selective activation of pathways

The modified enzyme might preferentially activate apoptotic pathways even when autophagy is present (Wang and Wang, 2019).

Crosstalk between autophagy and apoptosis

The interplay between autophagy and apoptosis is complex and can support or inhibit each other.

Pro-apoptotic role of autophagy

Autophagy can prepare cells for apoptosis by eliminating survival-promoting components, making cells more susceptible to apoptotic signals (Bhat *et al*., 2018).

Balance of cellular stress

While both enzymes trigger autophagy, the modified enzyme may push cells toward apoptosis due to increased cytotoxicity.

Temporal dynamics of response

The timing of responses affects apoptosis rates; the modified enzyme may induce apoptosis more rapidly, leading to higher cell death despite similar autophagy levels. In contrast, the native enzyme's effects may initially serve as a survival mechanism.

Modified enzyme's reduced autophagic flux

Increased *LC3-II* levels do not always indicate effective autophagy. The modified enzyme may signal autophagy without promoting complete autophagic flux, causing stress accumulation that can enhance apoptosis (Huang *et al*., 2010).

In summary, although both the modified and native L-ASNase induces autophagy marked by increased *LC3-II* levels, the modified enzyme's enhanced apoptosis could stem from its unique interaction with apoptotic signaling pathways, the crosstalk dynamics between apoptosis and autophagy, and potentially altered temporal effects. These factors indicate that the mechanisms governing cell fate in response to treatment are multifaceted and merit further investigation to optimize therapeutic strategies against acute lymphoblastic leukemia. Understanding these pathways could lead to more targeted treatments that leverage apoptosis and autophagy in cancer therapy.

In conclusion, these findings establish that carboxymethyl-conjugated L-ASNase exhibits enhanced effectiveness in inducing apoptosis while remaining relatively stable and less toxic than its native counterpart. The differential modulation of autophagy markers as demonstrated through real-time RT-PCR analysis further emphasizes the potential of modified L-ASNase in overcoming treatment resistance commonly encountered in leukemia. The complexity of the apoptosis-autophagy interplay when modulating drug treatments highlights the necessity for further investigation into these mechanisms for improved therapeutic strategies in ALL. Future studies should focus on the underlying molecular mechanisms driving these responses and evaluate the clinical implications of these findings in the context of standard leukemia therapies.

Acknowledgement

This research was financially supported by grants No. 97-GCU2M235254 from Shiraz University Research Council.

Conflict of interest

The authors declare that they have no competing financial interests.

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