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Research Article
Effect of Choline and CDP-Choline on Inflammation and Oxidative Stress in Burkitt's Lymphoma Cells

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Abstract

Background and Objective: Burkitt's lymphoma (BL) is a specific type of non-Hodgkin lymphoma. The BL is characterized by rapid progression and a tendency to metastasize the bone marrow and central nervous system. This study aims to evaluate the anticancer potential of choline and CDP-choline on BL cells (Ramos cells), *in vitro*. **Materials and Methods:** Ramos cells were treated with increasing concentrations of doxorubicin, choline and CDP-choline for 24 hrs after which cell viability was assessed using the MTT assay. Cytokine levels (IL-6 and TNF-α) and reactive oxygen species (ROS) production were measured using ELISA and fluorometric kits, respectively. One-way Analysis of Variance (ANOVA) with *post hoc* Tukey-Kramer multiple comparison tests were used for the statistical analysis, $p<0.05$ was accepted as a statistically significant level. **Results:** Choline and CDP-choline treatment for 24 hrs decreased Ramos cell viability, with IC_{50} values of 100, 02 and 5.45 μM, respectively. Both treatments increased ROS levels, indicating induction of oxidative stress. However, treatment of Ramos cells with these agents for 24 hrs did not induce cytokines (IL-6 and TNF-α) production. Choline treatment increased supernatant choline levels, whereas CDP-choline had no significant effect on intracellular choline in Ramos cells. **Conclusion:** Choline and CDP-choline reduced cell viability of Ramos cells probably via ROS dependent mechanism, but did not induce inflammatory responses at 24 hrs post-treatment. These findings suggested the possible anticancer potential of choline and CDP-choline against BL. This warrants further investigation into their potential therapeutic implications.

Key words: Burkitt's lymphoma, choline, CDP-choline, doxorubicin, reactive oxygen species, cytotoxicity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Lymphomas represent approximately 10% of the total cases of malignant illnesses in children who are under the age of 15. Burkitt's lymphoma (BL) is a specific type of non-Hodgkin lymphoma that is strongly associated with the Epstein-Barr Virus (EBV)[1]. The key clinical indicators for diagnosing BL are some manifestations in the jaws, which are caused by the infiltration of tumor cells or the lymphoid cells releasing substances activating the osteoclasts[2].

The BL is defined as extremely aggressive, monoclonal B-cell neoplasms exhibiting distinctive pathological characteristics at the cellular level. Morphologically, BL cells appear as medium-sized lymphocytes, with numerous spherical nuclei and an elevated mitotic rate, creating a distinctive "starry sky" pattern visible under the microscope. The cells demonstrated a significantly high proliferation index characterized by increased Ki-67 expression, frequently approaching 100% positivity. At the molecular level, BL cells are homogeneous and display surface markers such as CD20, CD10 and BCL-6, while exhibiting negativity for BCL-2, allowing the differentiation of BL from other B-cell lymphomas. The distinctive cellular structure and immunophenotype, along with MYC (Myelocytomatosis viral oncogene homolog) dysregulation, are pivotal to the aggressive clinical trajectory and fast advancement of BL[3,4].

The doxorubicin is a potent chemotherapeutic drug adopted in cancer treatment as a key component in combination regimens. It targets the rapidly growing B-cells typical of this aggressive malignancy and exerts its cytotoxic effects by intercalating into DNA and blocking topoisomerase II activity, resulting in double-strand DNA breaks and ultimately inducing cell death[5].

The choline is an essential nutrient that is found in many different types of food and multivitamin supplements. It contributes to the creation of the phospholipid membrane by facilitating the synthesis of its principal element, phosphatidylcholine, through the CDP-choline pathway. This is essential for preserving cell membrane integrity, fluidity and signaling functions. Moreover, choline serves as a precursor for the synthesis of Acetylcholine (ACh)[6]. In cancer, cells frequently have modified choline metabolism, characterized by increased choline absorption and synthesis rates to satisfy the requirements of membrane biosynthesis for their rapid proliferation[7]. Aberrant choline metabolism is frequently linked to cancer advancement and unfavorable prognosis since it supplies cancer cells with essential components for cellular division and influences critical pathways related to cell signaling and resistance to apoptosis[8].

The CDP-choline, also known as citicoline, is a compound that is hydrolyzed into cytidine and choline, supports the synthesis of phosphatidylcholine and enhances cognitive function and neuroprotection[9]. The conversion of CDP-choline to choline is facilitated by designated enzymes, predominantly occurring intracellularly as part of the Kennedy route, which is crucial for the production of phospholipids. Hydrolysis of exogenously administered CDP-choline into choline and cytidine is primarily facilitated by phosphodiesterases (PDEs) and nucleotide pyrophosphatases (NPPs), by cleaving the cytidine-choline link, hence liberating free choline. In addition, the phospholipase D (PLD) pathway catalyzes the breakdown of phosphatidylcholine, a by-product of CDP-choline, to produce choline[10].

On the other hand, choline consumption was suggested to reduce the incidence of breast, colorectal and liver cancers[11]. Research investigating the impact of choline on cancer cell lines yielded promising results. Treatment of HepG2 liver cancer cells and MCF-7 breast cancer cells with choline resulted in a dose-dependent reduction in cell viability possibly by interfering with their altered metabolic pathways[12]. In normal cells, treatment with choline reduced oxidative stress in non-cancerous mouse fibroblast NIH/3T3 allowing to preserve cellular integrity and functionality. This antioxidant effect may alter the oxidative environment that tumors utilize for growth, hence possibly contributing to the reported antiproliferative effects[13]. Collectively, these findings suggest that choline may play a dual role, supporting healthy cell function while inhibiting cancer cell viability. To the best of our knowledge, there are no *in vitro* or *in vivo* studies that investigated the anticancer effect of choline and choline-containing compounds against Burkitt's lymphoma. This study examined the anticancer effect of choline/CDP-choline on EBV-negative B-cell lymphoma Ramos cells and compared its anticancer potential to that of the standard chemotherapeutic doxorubicin against BL.

MATERIALS AND METHODS

Study area: The study was conducted from June, 2023 to August, 2024 in the Laboratories of İzmir University of Economics and Eastern Mediterranean University, Famagusta, North Cyprus, Türkiye.

Cell culture: The EBV-Nuclear Antigen (EBNA)-negative human lymphoma cell line Ramos cells (ATCC, RA 1) were used for this study at passages # 6-9. They were maintained in RPMI medium (Sigma Aldrich D6429), supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and

100 µg/mL streptomycin (Gibco, Carlsbad, California) at 37°C in a 5% CO_2 incubator. Regular mycoplasma contamination checks were performed using a mycoplasma detection kit (Biowest, Riverside, Missouri).

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] assay: The MTT assay was performed to assess cell viability. A density of 5.000 cells/well was seeded in 96-well plates and treated with increasing concentrations of doxorubicin (10, 50, 100 and 500 nM), choline (1, 10 and 100 µM) or CDP-choline (0.5, 1, 5 and 10 µM) for 24 hrs after which MTT assay was done (Elabscience, E-CK-A341). Additionally, half-growth inhibitor concentration (IC_{50}, defined as 50% inhibitory concentration) values were calculated for choline, CDP-choline and doxorubicin and IC_{50} doses were used in subsequent experiments.

Determination of choline/ACh levels: Cells were seeded in 24-well plates at a density of 200.000 cells/well and treated with IC_{50} concentrations of choline and CDP-choline. The total choline/ACh levels in cell lysate and supernatant were detected with a commercial kit according to the manufacturer's instructions (Abcam, ab65345) after treating Ramos cells with choline or CDP-choline for 24 hrs.

Measurement of cytokines levels: Cells were seeded in 24-well plates at a density of 200.000 cells/well and treated with choline, CDP-choline or doxorubicin at IC_{50} concentrations for 24 hrs. This was followed by the determination of the levels of IL-6 and TNF-α released into the culture media by Enzyme-Linked Immunosorbent Assay (ELISA) according to the manufacturer's instructions (Invitrogen KHC0061 and KHC3011).

Measurement of ROS levels: Cells were seeded in 24-well plates at a density of 200.000 cells/well and treated with IC_{50} concentrations of choline, CDP-choline or doxorubicin for 24 hrs. This was followed by the measurement of reactive oxygen species (ROS) level via a fluorometric ROS kit (Elabscience, E-BC-K138-F, USA) according to the manufacturer's guidelines.

Statistical analysis: One-way Analysis of Variance (ANOVA) with *post hoc* Tukey-Kramer multiple comparison tests were employed. Half-growth inhibitor concentration (IC_{50}, defined as 50% inhibitory concentration) was calculated with nonlinear regression analysis (GraphPad Prism 5, La Jolla, California). Data were expressed as Mean±SEM and $p<0.05$ was accepted as statistically significant.

RESULTS

Inhibitory effects of choline and CDP-choline on Ramos cell viability: Ramos cells were treated with each of doxorubicin (10-500 nM), choline (1-100 µM) and CDP-choline (0.5-10 µM) for 24 hrs after which cell viability was assessed by MTT assay (Fig. 1). Each of these agents induced a dose-dependent reduction of the cell viability of Ramos cells. A dose of 100 µM of choline decreased cell viability by 50.8% while a dose of 10 µM of CDP-choline reduced cell viability by 56.2%. In addition, a dose of 100 nM doxorubicin reduced it by 25.7%. The IC_{50} value of each doxorubicin, choline and CDP-choline at 24 hrs was 0.276, 100 and 5.45 µM, respectively in Fig. 1a-c.

Effect of choline and CDP-choline on total choline/ACh levels in cell lysate and supernatant: Next, the total choline/Ach levels in cells lysates and supernatant after treating Ramos cells with the IC_{50} doses of each of choline and CDP-choline measured for 24 hrs.

Results showed that treatment with choline induced a significant increase in both the cell lysates and supernatant compared with the untreated control. However, treatment with CDP-choline induced a significant increase in the supernatant but not in the cell lysate (Fig. 2a-b).

Effect of choline and CDP-choline on ROS production in ramos cells: Ramos cells were treated with 0.276 µM doxorubicin, 100 µM choline or 5.45 µM CDP-choline for 24 hrs after which ROS production was evaluated (Fig. 3). Compared with the untreated control, these three agents induced an increase in ROS production in Ramos cells. However, this increase was statistically significant only in choline and CDP-choline-treated cells and was more pronounced in CDP-choline-treated cells compared to choline-treated ones.

Effect of choline and CDP-choline on the levels of inflammatory cytokines in ramos cells: To evaluate the potential of choline and CDP-choline to induce the levels of inflammatory cytokines, Ramos cells were treated with 0.276 µM doxorubicin, 100 µM choline or 5.45 µM CDP-choline for 24 hrs and the level of the inflammatory cytokines TNF-α and IL-6 was assessed by ELISA. Results showed that these three agents had no significant effect on the levels of these cytokines (Fig. 4a-b).

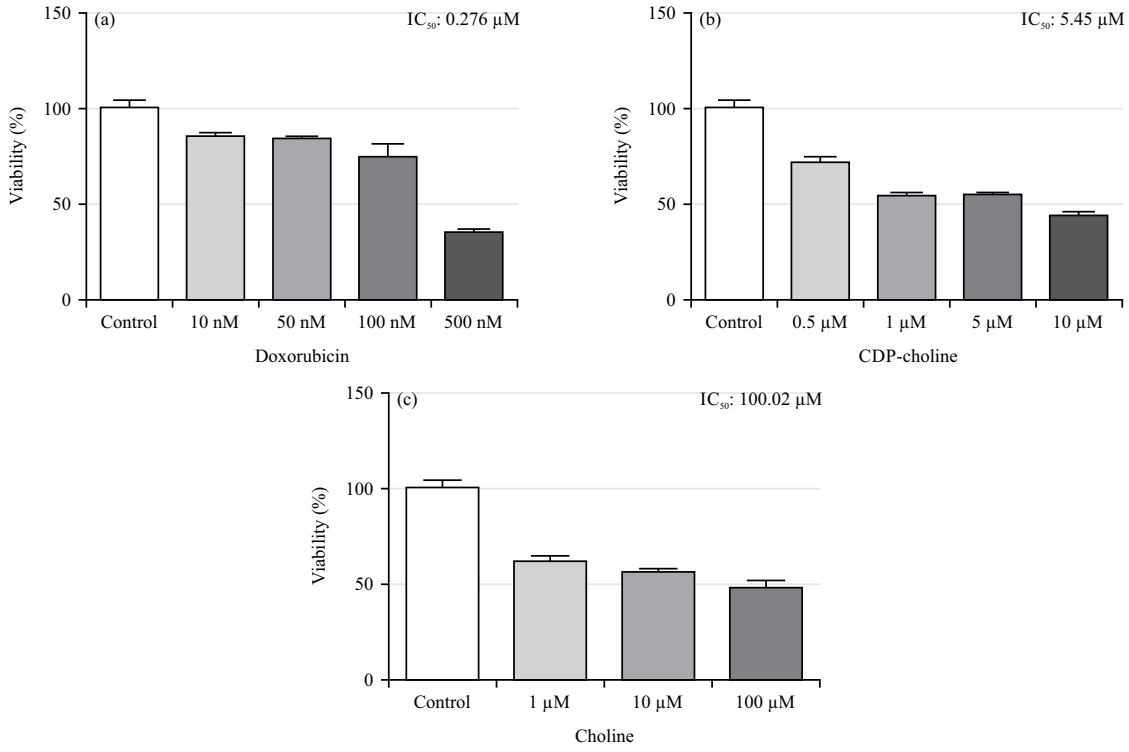

Fig. 1(a-c): Effect of (a) Doxorubicin, (b) Choline and (c) CDP-choline on Ramos cells viability

Cell viability was then determined using an MTT assay. Values are expressed as a percentage of viable cells relative to untreated control. Each value represents the Mean±SEM of the n = 6 experiment performed in duplicates

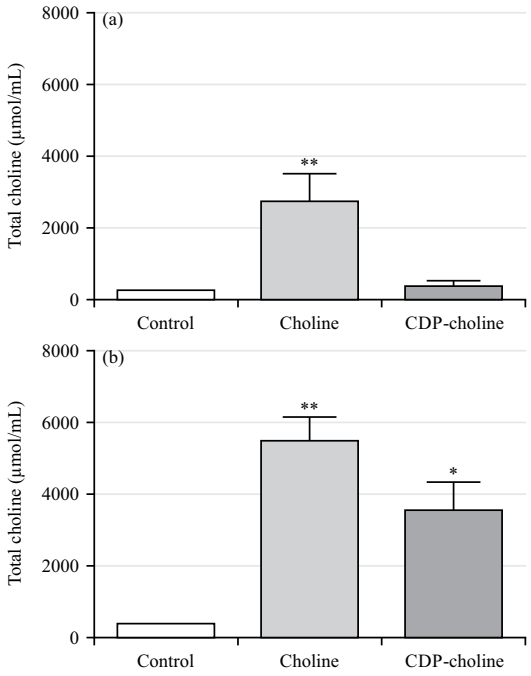

Fig. 2(a-b): Effect of choline and CDP-choline on the total choline/ACh amount in (a) Ramos cells lysate and (b) Supernatant of Ramos cells

Data represents the Mean±SEM of n = 6 experiments performed in duplicates. *p<0.05 and **p<0.01 indicate significant differences from control (ANOVA with Tukey-Kramer *post hoc* test)

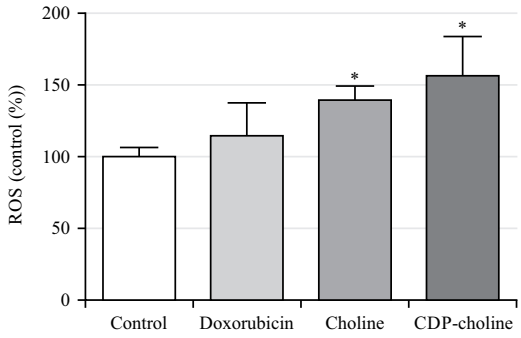

Fig. 3: Effects of doxorubicin, choline and CDP-choline on ROS production in Ramos cells

Ramos cells were treated with 0.276 µM doxorubicin, 100.02 µM choline or 5.45 µM CDP-choline for 24 hrs after which ROS production was evaluated using Fluorometric Assay Kit. Values are expressed as a percentage of ROS relative to untreated control. Each value represents the Mean±SEM of n = 6. *p<0.05 is significantly different from the control using ANOVA with *post hoc* Tukey-Kramer multiple comparison tests

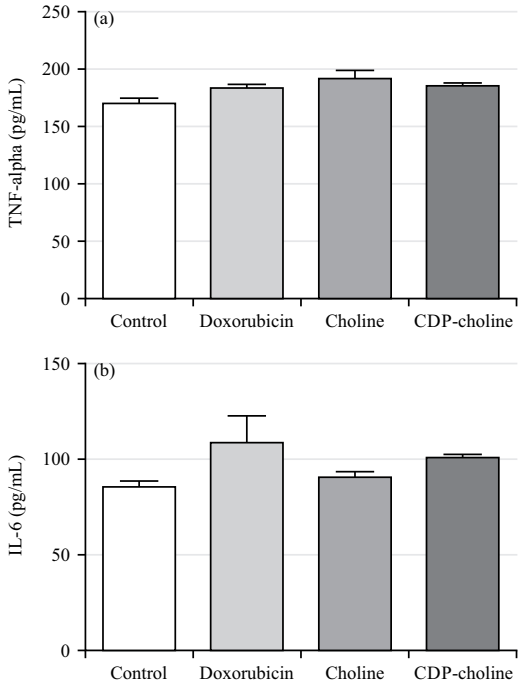

Fig. 4(a-b): Effects of doxorubicin, choline and CDP-choline on the levels of inflammatory cytokines in Ramos cells, (a) TNF-α and (b) IL-6

Ramos cells were treated with 0.276 µM doxorubicin, 100.02 µM choline or 5.45 µM CDP-choline for 24 hrs after which the levels of TNF-α and IL-6 were evaluated using a fluorometric assay kit. Each value represents the Mean±SEM of n = 6

DISCUSSION

Choline and CDP-choline were found to reduce cell viability, with IC_{50} values of 100.02 and 5.45 µM, respectively. The CDP-choline was approximately 20-fold more potent than choline. In addition, the IC_{50} values of both tested compounds were higher than that of doxorubicin. Previous research revealed that doxorubicin demonstrated a concentration-dependent response in MCF-7 breast and HepG2 liver cancer cell lines. The IC_{50} values ranged from 0.1 to 1 µM which was in good accordance with our findings[14,15]. In B cell lymphoma cell lines, doxorubicin exhibited low IC_{50} values, demonstrating high sensitivity to the drug. The IC_{50} values were previously reported to be 6.8 µM for CLB1 (B cell lymphoma) and 2.5 µM for non-Hodgkin B cell lymphoma cells[16,17].

This study showed that treating the Ramos cell line with 100 μM choline increased the total choline levels within the cell lysate. This indicates that choline can penetrate Ramos cells, potentially elevating the levels of free and/or bound choline (such as CDP-choline and phosphatidylcholine etc.). Additionally, the significant increase of choline levels in the supernatant suggests that not all of the administered choline penetrated the cells or that the choline may be released back into the supernatant following the induced cell death. In contrast to choline treatment, treating Ramos cells with CDP-choline did not significantly increase the total intracellular choline levels but led to a significant increase in choline levels in the supernatant. We hypothesize that the choline that can be generated in cells from CDP-choline may be released back into the supernatant following the induced cell death[18].

Choline uptake into a cell occurs mainly in 3 different pathways: Sodium-dependent uptake (high-affinity choline transport system), sodium-independent uptake (low-affinity choline transport system) and organic cation transporter-mediated mechanism[19-21]. Choline uptake has also been shown in specific leukemia cells (acute promyelocytic leukemia, HL-60 cell line)[21]. Additionally, the human leukemic T-cell line as the Jurkat cell line, exhibited a connection between Choline Transporter-Like Protein 1 (CTL1) and ACh production, indicating the presence of a non-neuronal cholinergic system in these leukemic cells. The transformation of choline into acetylcholine was verified solely in Jurkat cells as a hematological malignant cell line[22,23]. This study proposed that Choline Transporter-Like Protein 1 (CTL1) can be the transporter that may uptake choline into Ramos cells. However; we didn't investigate the presence of CTL1 and other choline transporters in our cell lines. Because there is not enough information regarding the choline transporters in Ramos cells, we have further planned to investigate the role of choline transporters following choline administration in Ramos cells as well as other lymphoma/leukemia cell lines.

On the other hand, previous studies showed that inhibition of choline entry into leukemic cells may induce apoptosis. The suppression of choline uptake in leukemia cells, especially via CTL1, may impair phospholipid metabolism and result in cell death due to the buildup of compounds such as ceramide. Inhibition of CTL1 resulted in diminished levels of phosphocholine (PCho) and phosphatidylcholine, both of which are critical for the proliferation of leukemic cells. This resulted in reduced cell viability and increased caspase-3/7 activity, indicating apoptosis. The uptake of choline is facilitated by these choline transporters, particularly CTL1[22,23]. Thus, Ramos cells could use CTL1 or other choline transporters for choline uptake. Although experimental evidence for the activity of choline transporters in Ramos cells is currently lacking, the metabolic demands of rapidly proliferating cancer cells, such as lymphoma cells, suggested the presence of analogous choline uptake mechanisms. However, further experimental studies are necessary to validate this hypothesis in this specific cell line.

The ROS are highly reactive molecules that can cause significant cellular damage, including DNA damage, lipid peroxidation and protein oxidation. Initially, a moderate increase in ROS can promote apoptosis by activating pro-apoptotic pathways and causing mitochondrial damage, leading to the release of cytochrome-C (Cyt C) and the activation of caspases[24,25]. Elevated ROS levels in cancer cells render them more vulnerable to oxidative stress compared to normal cells. Choline's capacity to regulate reactive oxygen species and mitochondrial function may trigger apoptosis in cancer cells, which rely heavily on mitochondrial function for their survival and proliferation[26,27]. These results indicate that increased oxidative stress may play a role in the reduced viability of Ramos cells following the administration of choline and CDP-choline. However, the mechanism of cytotoxic action for doxorubicin at this concentration most likely does not involve ROS production. Despite current findings, it is important to acknowledge that other studies explored the effect of choline on ROS levels and reported different results in liver cancer. For example, choline deficiency in CWSW1 immortal rat hepatocytes was found to increase ROS levels in dose and time-dependently manner[28]. While choline administration to C3A hepatocellular carcinoma derived cancer cell line reduced significantly ROS level significantly[27]. These discrepancies could be attributed to the fact that different cell lines may respond differently to choline treatment. To better understand the underlying mechanism of action, we explored several possible pathways. Intracellularly, choline is converted to phosphatidylcholine and a poly-unsaturated fatty acid component of phosphatidylcholine can be easily oxidized by free radicals which may lead to hydroperoxide species production[23,29,30]. Additionally, it has been shown that mitochondrial Cyt C can interact with hydroperoxide species of non-anionic phospholipids leading to a mitochondrial-mediated apoptosis[31]. The presence of neuronal-type nicotinic acetylcholine receptors has been reported in the outer membrane of mitochondria and these receptors may regulate the release of ROS and pro-apoptotic substances like Cyt C[32]. It is known that choline can bind to neuronal ACh receptors which can activate or desynthetize these receptors[33,34]. As a result, the increased choline levels inside the cell following choline administration in Ramos cells may interact with mitochondrial neuronal acetylcholine receptors which may end up with the increase in ROS production and Cyt C release-induced apoptosis.

Cytokine networks between different types of immune cells are very complex. The B cells are capable of releasing several cytokines including TNF-α and IL-6 to communicate with other immune cells[35]. Oxidative stress might serve as an initial trigger for subsequent inflammatory responses. This early oxidative stress can trigger downstream signaling pathways that later result in the production of inflammatory cytokines such as TNF-α and IL-6[36]. Studies have shown that oxidative stress markers increase significantly before the increase in cytokine levels in conditions like acute inflammation and ischemia-reperfusion injury[37]. In this study, choline, CDP-choline and doxorubicin did not modulate the levels of studied proinflammatory cytokines; TNF-α and IL-6, at the administered IC_{50} doses 24 hrs post-treatment. This could be due to the short treatment period with choline/CDP-choline and production of these cytokines necessitates a longer period of exposure to choline/CDP-choline. Additionally, it should be kept in mind that Ramos cells are undifferentiated cells which may have an impact on cytokine synthesis and release.

The study investigated the effects of choline and CDP-choline on Ramos cells, yet several important limitations must be considered. One major limitation is the relatively short duration of the treatment protocol, which may not be sufficient to capture the full spectrum of cellular responses, particularly in terms of protein expression and the long-term effects on apoptosis and cytokine production. Additionally, the use of a single cell line restricts the generalizability of the findings to other lymphoma subtypes. Future research should involve extended treatment durations, a broader range of concentrations and the inclusion of multiple cell lines to validate and expand upon these results. It is important to note that this study serves as a preliminary investigation and further studies are planned to explore the underlying mechanisms of cell toxicity, including apoptosis and associated intracellular pathways. Additionally, future studies will include isobolographic analysis to assess the synergistic or antagonistic effects of combining doxorubicin (and other cytotoxic agents) with choline and CDP-choline.

CONCLUSION

The study revealed promising findings regarding the effect of choline and CDP-choline on Ramos cells derived from BL. Both choline and CDP-choline significantly reduced cell viability which might be due to increased ROS production. The IC50 values of CDP-choline were observed much lower than choline. However, choline and CDP-choline-induced oxidative stress did not significantly alter cytokine levels after 24 hrs in Ramos cells. To our knowledge, this is the first study that evaluated the effects of choline and CDP-choline in an undifferentiated hematological cancer cell line. Further investigations are crucial to elucidate their mechanism of action on BL cell lines.

SIGNIFICANCE STATEMENT

This study is the first to investigate the effects of choline and CDP-choline on Ramos cells derived from Burkitt's Lymphoma (BL). Both compounds significantly reduced cell viability, likely through increased ROS production, with CDP-choline showing greater efficacy. These findings highlight their potential as therapeutic agents for BL, warranting further research to understand their mechanisms of action.

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