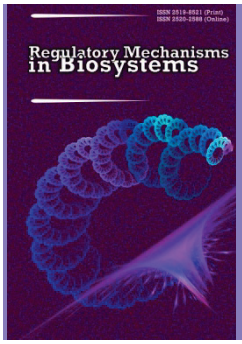

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Anti-apoptotic effect of umbilical cord cryoextract and its lyophilized form on L929 cells

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The umbilical cord cryoextract (UCC) and its lyophilized form (LUCC) contain numerous biologically active components, including growth factors, cytokines, and other regulatory molecules, which may exert an anti-apoptotic effect on cells. This study aimed to determine whether these samples can reduce apoptosis levels in healthy cells, which is particularly relevant in scenarios where apoptosis may be induced by chemotherapeutic agents such as doxorubicin. To evaluate the potential protective effects of umbilical cord cryoextracts, the L929 cell line treated with doxorubicin, a known apoptosis inducer, was selected as a model for assessing the anti-apoptotic properties of UCC and LUCC. In the experiment, L929 cells were cultured in standard Dulbecco's Modified Eagle Medium (DMEM) with the addition of doxorubicin. Experimental results demonstrated that the addition of fetal bovine serum (FBS), UCC, and LUCC significantly reduced apoptosis levels in L929 cells compared to the group treated with doxorubicin alone. In samples with UCC and LUCC, apoptosis levels were comparable to those in the FBS group, indicating a potential supportive and anti-apoptotic effect of both forms of umbilical cord cryoextracts. These findings suggest that both the cryoextract and its lyophilized form may serve as effective agents for reducing apoptosis, maintaining the stability of their active components even post-lyophilization. The results support the potential use of umbilical cord cryoextracts in chemotherapy involving agents such as doxorubicin to mitigate apoptosis in healthy cells and reduce toxic side effects. Further research is needed to elucidate the mechanisms of action of these extracts and to assess their efficacy *in vivo*, which may open new opportunities for their application in oncology therapy and other medical fields.

Keywords: cell culture; cryoextract; cryopreservation; lyophilization; umbilical cord; apoptosis.

Introduction

Apoptosis is a programmed cell death mechanism that plays a crucial role in maintaining the balance between cell growth and death and is an essential component of homeostasis (Elmore, 2007; Ketelut-Carneiro & Fitzgerald, 2022). Control of apoptosis is critical in cancer treatment, particularly during chemotherapy (Morana et al., 2022). Doxorubicin, a common chemotherapeutic agent, induces apoptosis in tumor cells; however, its high toxicity also affects healthy cells, limiting its therapeutic application (Minotti et al., 2004; Chatterjee et al., 2010; Almuqbil et al., 2020). Consequently, modern research emphasizes the need for adjunctive agents that can reduce the apoptotic impact of doxorubicin on healthy cells without compromising its antitumor efficacy.

Derivatives of the umbilical cord are widely used in regenerative medicine for treating various conditions, including diabetes (Sun et al., 2017; Alatyyat et al., 2020), cardiovascular disorders (Alatyyat et al., 2020), neurodegenerative diseases (Galieva et al., 2017; Chez et al., 2018), bone tissue regeneration (Hong et al., 2018), and wound healing (Alatyyat et al., 2020). However, the potential of umbilical cord extract as a source of biologically active compounds remains insufficiently explored, particularly with regard to cryoextracts or lyophilized forms, which can be stored long-term and transported conveniently for subsequent use.

The umbilical cord cryoextract (UCC) and lyophilized umbilical cord cryoextract (LUCC) contain numerous biologically active components, including growth factors, cytokines, and other regulatory molecules, which may exhibit anti-apoptotic effects, support cell viability, and reduce apoptosis levels (Ding et al., 2011). Owing to these bioactive compounds, umbilical cord cryoextracts may mitigate the adverse effects of chemotherapy on healthy cells. The lyophilization of cryoextracts preserves their properties over extended periods and enhances their usability in therapeutic applications, making them relevant as adjuvants in chemotherapy (Bullard et al., 2019; Prado et al., 2023).

A necessary step in this research is to identify the anti-apoptotic properties of UCC and LUCC in an in vitro model using doxorubicin-treated L929 cells, which provides a suitable system to examine the effects of umbilical cord cryoextracts on apoptosis levels. The L929 cell line is widely used to test the cytotoxicity and regenerative potential of bioactive substances, making it a convenient model for studying anti-apoptotic effects in the context of doxorubicin toxicity (Wiegand & Hipler, 2008; Heydari et al., 2024).

Therefore, the aim of this study is to investigate the anti-apoptotic properties of umbilical cord cryoextract and its lyophilized form on doxorubicin-treated L929 cells to assess their potential for minimizing apoptosis in healthy cells and enhancing cell viability during therapy.

Materials and methods

The study was conducted using the L929 cell culture, obtained from the cryopreserved biological sample collection of the Institute for Problems of Cryobiology and Cryomedicine, National Academy of Sciences of Ukraine. Cells stored in liquid nitrogen at –196 °C were thawed in a 37 °C water bath until the ice crystals were fully dissolved. L929 cells

were suspended in Dulbecco's Modified Eagle Medium (DMEM) (Biowest, France), centrifuged at 1500 rpm using a laboratory centrifuge (LMSC-P10-01-ELEKON, Ukraine), and the supernatant was removed for further use. The L929 cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2 in culture vessels (SPL, South Korea) using DMEM medium (Biowest, France) supplemented with 10% fetal bovine serum (FBS) (Lonza, Germany) and 1% antibiotic-antimycotic (Biowest, France). Umbilical cord was obtained during delivery under conditional sterility and with the mother's informed consent. To remove erythrocytes, the cord was rinsed with sterile Phosphate-Buffered Saline (PBS) (Biowest, France). The rinsed and fragmented umbilical cord was centrifuged for 10 minutes at 1500 rpm (LMSC-P10-01-ELEKON, Ukraine) to remove residual erythrocytes, homogenized in PBS at a 1:2 ratio, and frozen at –80 °C in 15 mL polypropylene tubes (SPL, South Korea) for cell lysis. The umbilical cord suspension was thawed in a 37 °C water bath, centrifuged for 10 minutes at 1500 rpm, and the supernatant was collected in sterile tubes, yielding the umbilical cord cryoextract. Lyophilization of the umbilical cord cryoextract was performed following pre-cooling to –80 °C in an ultra-low temperature freezer DW-86W100J (Haier, China) and freeze-drying on Alpha 1-2 LDplus (Martin Christ Gefriertrocknungsanlagen GmbH, Germany). The lyophilized umbilical cord cryoextract was stored at +8 °C in sealed tubes for up to 3 months.

The anti-apoptotic properties of the cryoextract and lyophilized cryoextract of the umbilical cord were evaluated in a doxorubicin-induced apoptosis model. On the first day, the L929 cell line was seeded at 200,000 cells per well in a 24-well plate. On the second day, umbilical cord cryoextract or its lyophilized form was added, and on the following day, the doxorubicin-induced injury model was established by adding 20 μM doxorubicin for 24 hours of incubation. Following this, the anti-apoptotic properties of the umbilical cord extracts were assessed. Cells were detached, washed with Annexin binding buffer (BD Pharmygen, USA), and a suspension was prepared with 5 μL AnnexinV-FITC (BD Pharmygen, USA) and 5 μL 7-AAD (BD Pharmygen, USA), followed by a 30-minute incubation in the dark. Fluorescence intensity was measured using a BD FACS Canto II flow cytometer (Becton Dickinson, USA) with excitation at 488 nm and emission at 525 nm for AnnexinV-FITC, and excitation at 488 nm and emission at 633 nm for 7-AAD.

The experiment included five groups: group 1, control – cells cultured in DMEM without doxorubicin; four additional groups with doxorubicin: group 2 – DMEM, group 3 – DMEM+FBS, group 4 – DMEM with UCC, and group 5 – DMEM with LUCC. Standardization of UCC and LUCC was conducted based on protein concentration (0.1 mg/mL) (Kim et al., 2013). FBS was added at the standard concentration used in cell culture protocols, 10%. The choice of 0.1 mg/mL concentration was based on previous experiments with L929 cells, considering cell morphology, monolayer confluency, cell adhesion, neutral red uptake, cell doubling time, MTT assay, and scratch test results.

Microscopic images were processed using ToupView V 3.7 (Hangzhou ToupTek Photonics Co. Ltd, China) and ImageJ V.1.48 (National Institutes of Health, USA) to evaluate cell morphological changes. Flow cytometry data analysis was performed using FlowJo™ software (v10, BD Biosciences, USA) to determine the number of apoptotic cells and median fluorescence intensity cha-racterizing the extent of changes. The Mann-Whitney test was used to determine statistical significance. Statistical calculations and data processing were performed using Past V. 3.15 software (University of Oslo, Norway).

Results

The results of the L929 cell line cultivation are presented in microphotographs obtained using phase contrast microscopy (Fig. 1), illustrating morphological changes depending on cultivation conditions and the impact of doxorubicin. Cells cultured in DMEM medium without the addition of doxorubicin (Fig. 1a) exhibited an irregular elongated shape, characteristic of cells cultured without growth factors. Cells cultured with the addition of FBS and doxorubicin (Fig. 1b) predominantly exhibited an irregular elongated shape, which may result from the stressful conditions induced by doxorubicin, a known apoptosis inducer. Some cells detached from the plastic surface and were freely floating. Doxorubicin significantly reduced cell viability, as shown in Figure 1c, where cells exposed to it displayed altered morphology indicative of cellular damage and progression toward apoptosis; all cells detached from the culture substrate and were freely suspended in the culture medium solution. When using the UCC with doxorubicin (Fig. 1d) or LUCC with doxorubicin (Fig. 1e), some of the cells retained their adhesive properties, while others separated from the surface.

The histograms (Fig. 2) represent the distribution of the L929 cell population based on AnnexinV-FITC fluorescence intensity, allowing assessment of the apoptosis level in cells exposed to different samples. The histogram (Fig. 2a) corresponding to the control group, where cells were cultured in DMEM without doxorubicin, shows a low fluorescence level. This indicates a minimal number of apoptotic cells, with the main fluorescence peak shifted to the left, characteristic of low apoptotic activity. In the group (Fig. 2b), where cells were cultured in DMEM with the addition of doxorubicin, there is a substantial increase in fluorescence intensity. The peak shifts to the right, indicating a high number of apoptotic cells due to the toxic effect of doxorubicin. The histogram (Fig. 2c), representing cells in DMEM+FBS with doxorubicin, displays an intermediate fluorescence level between the control group and the DMEM with doxorubicin group. This suggests that the addition of FBS supports cell survival, although a slight level of apoptosis is still present.

Histograms (Fig. 2d, 2e) illustrate the effect of umbilical cord cryoextract and lyophilized umbilical cord cryoextract in DMEM medium with doxorubicin. In these groups, fluorescence intensity is markedly reduced compared to the group in Figure 2b, indicating a decrease in apoptosis levels. This suggests a protective effect of both forms of umbilical cord extract, which reduce the apoptotic impact of doxorubicin on the cells. Thus, the histograms illustrate differences in apoptosis levels in the L929 cell line depending on doxorubicin exposure and the application of umbilical cord cryoextracts, highlighting the potential anti-apoptotic properties of the latter.

Figure 3 presents a comparison of the median fluorescence intensity of cells under various cultivation conditions and doxorubicin treatment. The highest fluorescence intensity was observed in the DMEM with doxorubicin group, indicating substantial accumulation of the fluorescent signal in these cells. In other groups, including DMEM+FBS with doxorubicin, UCC with doxorubicin, and LUCC with doxorubicin, the fluorescence level was significantly lower compared to DMEM with doxorubicin ($P < 0.05$). In the control group without doxorubicin, a low fluorescence level was also observed, which differed significantly from all doxorubicin-treated groups. These results indicate varying degrees of doxorubicin impact on cell median fluorescence intensity depending on the added samples.

The chart (Fig. 4) illustrates the findings on the apoptotic properties of the L929 cell line under various experimental conditions using a doxorubicin-induced injury model. The control group without doxorubicin exhibits the lowest level of apoptosis, with a value close to 15%. In group DMEM with doxorubicin, there is a substantial increase in apoptotic cells, reaching a maximum of approx-imately 100%, indicating a strong apoptotic effect of doxorubicin. The addition of fetal bovine serum in the DMEM+FBS with dox-orubicin group results in a reduction of apoptosis compared to the DMEM with doxorubicin group, as FBS promotes cell growth and enhances cell viability, partially protecting cells from doxorubicin-induced apoptosis and mitigating its toxic effect. The chart shows that apoptosis levels in the groups with umbilical cord cryoextract (UCC with doxorubicin) and lyophilized umbilical cord cryoextract (LUCC with doxorubicin) are similar to the DMEM+FBS with doxorubicin group but not necessarily lower. These groups display apoptosis levels significantly lower than in the group DMEM with doxorubicin but do not differ substantially from the DMEM+FBS with doxorubicin group.

Discussion

Apoptosis is a key mechanism in regulating cell viability, maintaining homeostasis, and balancing cell growth and death (Elmore, 2007; Ketelut-Carneiro & Fitzgerald, 2022). Control over apoptotic processes is particularly important in chemotherapy, as the induction of apoptosis is essential for eliminating tumor cells. However, the toxic impact of chemotherapeu-

tic agents, such as doxorubicin, on healthy cells limits their application (Morana et al., 2022). While doxorubicin is effective against malignant cells, it also induces significant apoptosis in healthy cells, leading to side effects and complications (Minotti et al., 2004; Chatterjee et al., 2010; Al-muqbil et al., 2020). Therefore, there is a critical need for additional agents that can reduce doxorubicin's apoptotic impact on healthy cells without compromising its antitumor efficacy.

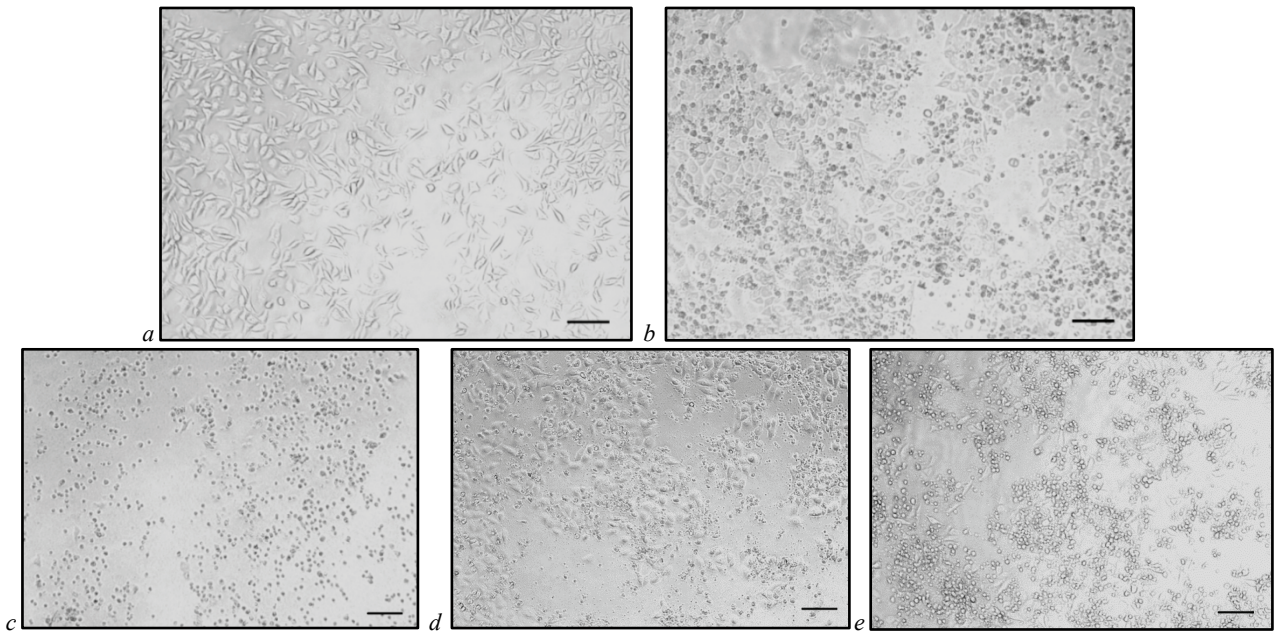

Fig. 1. Microphotographs of L929 cells: DMEM without doxorubicin (*a*), DMEM+FBS and with doxorubicin (*b*), DMEM with doxorubicin (*c*), UCC with doxorubicin (*d*), LUCC with doxorubicin (*e*); phase contrast, scale bar 100 μm

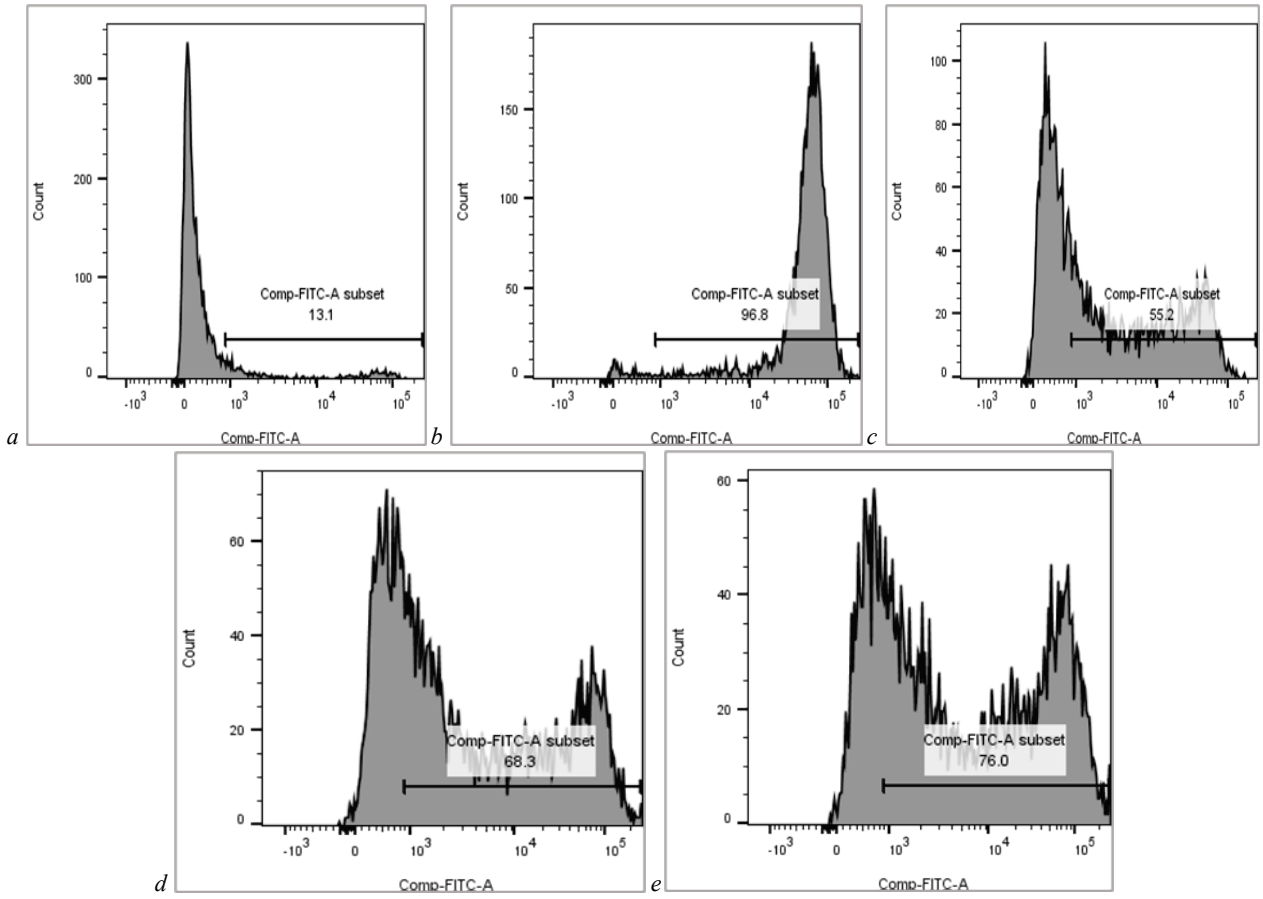

Fig. 2. Apoptosis histograms of the L929 cell line in a doxorubicin-induced injury model: analysis was conducted using AnnexinV to identify apoptotic cells; control group: cells cultured in DMEM without doxorubicin (*a*), DMEM with doxorubicin (*b*), DMEM+FBS with doxorubicin (*c*), DMEM with UCC and doxorubicin (*d*), DMEM with LUCC and doxorubicin (*e*)

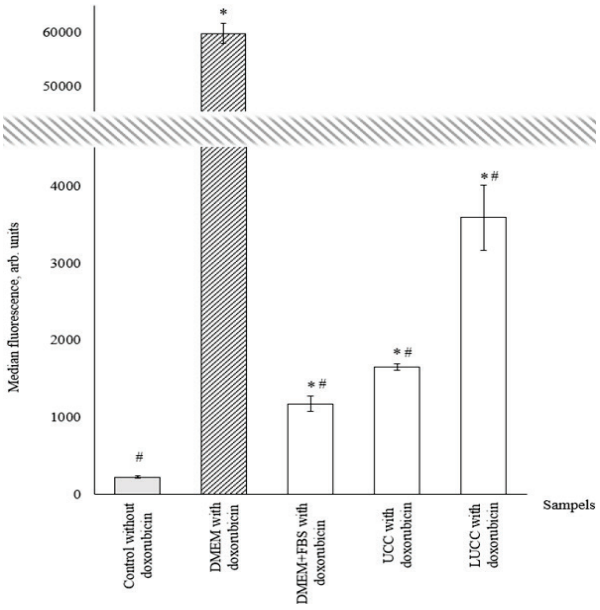

Fig. 3. Median fluorescence of Annexin-positive L929 cells under different experimental conditions in the doxorubicin-induced injury model (arbitrary units): * – statistically significant difference compared to the control without doxorubicin ($P < 0.05$); # – statistically significant difference compared to the DMEM with doxorubicin group ($P < 0.05$)

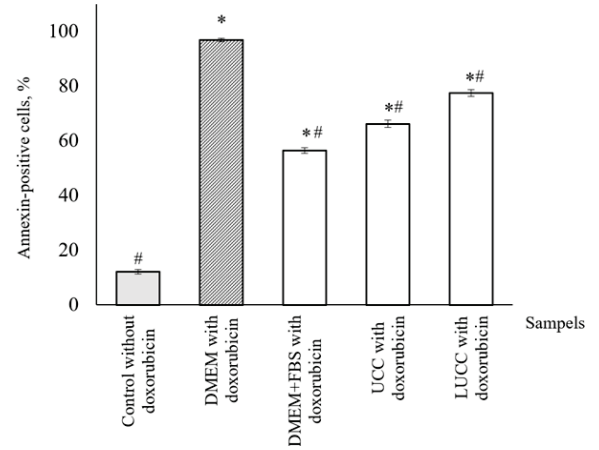

Fig. 4. Annexin-positive cells in the L929 cell line under various experimental conditions using the doxorubicin-induced injury model (%); * – statistically significant difference from the control group without doxorubicin ($P < 0.05$); # – statistically significant difference from the DMEM with doxorubicin group ($P < 0.05$)

The addition of UCC and LUCC to the culture medium with doxorubicin enabled a comparison of their effects on apoptosis levels with the influence of FBS, which is commonly used as a supportive medium. All samples were standardized to a protein concentration of 0.1 mg/mL, ensuring comparability across experimental groups. Apoptotic activity was assessed using AnnexinV and flow cytometry, providing accurate quantification of apoptosis in each experimental group. This design allowed a comprehensive evaluation of UCC and LUCC as potential anti-apoptotic agents and facilitated comparison with other known supportive substances.

The results of our study indicate an anti-apoptotic effect of umbilical cord cryoextract and its lyophilized form on L929 cells treated with doxorubicin, consistent with previous studies demonstrating the protective role of umbilical cord derivatives under oxidative and chemotherapeutic stress (Ding et al., 2011; Alatyyat et al., 2020). The selection of the L929 cell line for modeling doxorubicin-induced apoptosis is justified, as this line responds well to toxic stimuli, allowing assessment of both cell death and the potential protective effects of exogenous agents (Heydari et al., 2024). The use of doxorubicin to induce apoptosis is typical in studies of anti-apoptotic properties, as its mechanism includes activation of reactive oxygen species (ROS) pathways and engagement of signaling cascades, such as p53 activation, which promotes caspase activation and apoptosis (Minotti et al., 2004).

The observed effect of UCC and LUCC is particularly significant, given that FBS, as a standard component in normal cell culture media, exhibits similar anti-apoptotic properties. This suggests that UCC and LUCC may serve as natural alternatives to FBS, supporting cell survival. Furthermore, the lyophilized form of UCC demonstrated comparable anti-apoptotic effects, making it promising for clinical applications due to its storage and transport advantages (Prado et al., 2023).

The anti-apoptotic effect of umbilical cord cryoextract and its lyophilized form on L929 cells treated with doxorubicin is likely associated with the activation of specific signaling pathways that promote cell survival and inhibit apoptosis. Bioactive molecules, such as growth factors and cytokines found in umbilical cord derivatives, may stimulate anti-apoptotic pathways, particularly phos-phatidylinositol 3-kinase (PI3K)/AKT/mammalian target of the rapamycin (mTOR) (Chen et al., 2022; Peng et al., 2022; He et al., 2024) and mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) (Sun et al., 2015; Wen et al., 2022). Activation of the PI3K/Akt pathway facilitates phosphorylation and inhibition of key proteins involved in apoptosis initiation, such as BCL2 associated agonist of cell death and Caspase 9, ultimately reducing apoptosis and maintaining cell viability (Martorana et al., 2021; Vanhaesebroeck et al., 2021; Yu et al., 2022). Therefore, it can be hypothesized that the bioactive molecules in UCC and LUCC activate mechanisms similar to those that confer cellular resistance to the toxic effects of chemotherapeutic agents.

Furthermore, doxorubicin induces the accumulation of reactive oxygen species (ROS), which damage DNA and trigger the apoptotic cascade through the activation of p53 and caspases (Tacar & Dass, 2013; Cao et al., 2021; Guo et al., 2023). Doxorubicin also causes doxorubicin-induced cardiomyopathy, which can lead to fatal outcomes (Minotti et al., 2004; Chatterjee et al., 2010). The molecules contained in UCC and LUCC may possess antioxidant properties that reduce ROS levels, thereby minimizing DNA damage and inhibiting the apoptotic cascade (Kim et al., 2006; Elmore, 2007). Growth factors have been shown to influence the expression of Bcl-2, an anti-apoptotic protein that stabilizes the mitochondrial membrane and prevents cytochrome c release – a critical step in apoptosis (Tacar & Dass, 2013). Thus, it can be hypothesized that the anti-apoptotic effect of UCC and LUCC is due to the combined action of active molecules that engage antioxidant mechanisms and activate survival signaling pathways, counteracting doxorubicin-induced apoptotic processes. UCC and LUCC hold potential as alternatives or adjuncts to current strategies for reducing chemotherapy toxicity, which could improve patient quality of life and decrease complication risks. Further studies could focus on elucidating these mechanisms and identifying key molecules within UCC and LUCC that provide protection against apoptosis.

Conclusion

The umbilical cord cryoextract and its lyophilized form demonstrate the ability to reduce doxorubicin-induced apoptosis in L929 cells. In both cases, the addition of cryoextracts to the doxorubicin-containing medium led to a decrease in apoptosis levels compared to the group treated with doxorubicin alone. These results highlight the potential anti-apoptotic properties of umbilical cord cryoextract and its lyophilized form, which may be beneficial for protecting cells from the toxic effects of chemotherapeutic agents, particularly doxorubicin.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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