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Ursodeoxycholic Acid Alleviates Palmitic Acid–Induced Apoptosis in Bovine Mammary Epithelial Cells

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ABSTRACT

This study investigated the protective effects of ursodeoxycholic acid (UDCA) against PA-induced apoptosis in the MAC-T bovine mammary epithelial cell (bMEC) line by assessing the level of cell viability, oxidative stress indicators, and expression of endoplasmic reticulum (ER) stress markers. MAC-T cells were pretreated with UDCA at 25, 50, and 100 μM before exposure to PA at 300 μM. UDCA was noncytotoxic at these concentrations and significantly improved cell viability, which was reduced by PA. UDCA pretreatment notably decreased PA-induced expression of GRP78, XBP1s, ATF4, and CHOP mRNA, indicating reduced ER stress. Moreover, UDCA substantially lowered PA-induced reactive oxygen species production and dichlorofluorescein fluorescence intensities. Although PA treatment elevated GSSG levels and disrupted redox balance by decreasing both the total GSH/GSSG and reduced GSH/GSSG ratios, UDCA effectively counteracted these effects. Specifically, UDCA reduced PA-induced GSSG levels and restored redox balance by increasing both total and reduced GSH/GSSG ratios. These findings suggest that UDCA mitigates PA-induced apoptosis in MAC-T cells by enhancing redox homeostasis. Therefore, incorporating UDCA into dairy cow feed could help reduce PA-induced cellular stress and improve milk production during periods of negative energy balance.

1 | Introduction

The bovine mammary gland is a vital exocrine organ that produces large quantities of milk during lactation. Milk production is significantly influenced by the number and viability of bovine mammary epithelial cells (bMECs), which are responsible for milk synthesis (Singh et al. 2010). In the lactation cycle of dairy cows, the transition period (the last 3 weeks of pregnancy and the first 3 weeks of lactation) is critical because of the pronounced negative energy balance (NEB) that occurs during this time. To compensate for the energy deficit in early lactation, cows mobilize body fat, producing elevated levels of nonesterified fatty acids (NEFAs) in the bloodstream (Chen et al. 2021). Palmitic acid (PA) is the most abundant saturated NEFA and is used by bMECs as an energy substrate and for milk lipid synthesis (Li et al. 2019).

Although PA serves as an energy substrate, this can also exert detrimental effects on various cell types, including bMECs (Sharmin et al. 2020). Our studies have previously demonstrated that PA induces endoplasmic reticulum (ER) stress–mediated apoptosis in bMECs (Habib et al. 2025), which occurs with misfolding or improper folding of proteins in the ER. In response to mild ER stress, the PKR-like ER kinase (PERK) protein is activated, promoting the translation of activating transcription factor 4 (ATF4) via the phosphorylation of eukaryotic initiation factor 2α (Harding et al. 2000). Under severe or prolonged ER stress, ATF4 enhances the transcription

of C/EBP homologous protein (CHOP), consequently inducing apoptosis (Zinszner et al. 1998).

Oxidative stress occurs when the redox homeostasis becomes imbalanced because of an excess of oxidants that surpass the antioxidant capacity of the cell (Sies 2015) and subsequently disrupt normal redox signaling and cause molecular damage, potentially leading to cell death if unresolved (Wang et al. 2019). Our recent results indicated that both ER and oxidative stresses are key contributors to PA-induced cell death in bMECs (Habib et al. 2025). Consequently, PA may significantly contribute to the reduction in bMECs viability and subsequent milk yield during early lactation. Therefore, therapeutic strategies that mitigate PA-induced apoptosis in bMECs could potentially help preserve bovine mammary gland health and sustain milk production.

Ursodeoxycholic acid (UDCA) is a primary bile acid found in bears (*Ursus arctos* and *Ursus thibetanus*). UDCA is a major component of the traditional oriental medicine Fel Ursi (Yutan), derived from dried bear gallbladder, and has been used to treat conditions such as stomach pains, diarrhea, liver disease, and jaundice (Bachrach and Hofmann 1982; Wang and Wu 2017). UDCA has demonstrated significant antioxidative properties, including reducing oxidative stress in diabetic mice and podocytes (Cao et al. 2016a) and in aortas stimulated by Ang II (Liu et al. 2016). Mitsuyoshi et al. (1999) reported that UDCA boosts glutathione (GSH) levels, protecting hepatocytes from hydroperoxide-induced oxidative damage. Additionally, UDCA can prevent high glucose-induced ER stress and reactive oxygen species (ROS) production in endothelial cells (Chung et al. 2016) and mitigate apoptosis in podocytes (Cao et al. 2016b). However, its effects on PA-induced cell death in bMECs have yet to be explored.

Therefore, this study investigated the protective effects of UDCA against PA-induced apoptosis in MAC-T cells (an established cell line of bMECs). We assessed bMEC cell viability after pretreatment with UDCA followed by PA exposure. Additionally, we analyzed the expression of ER-related and oxidative stress–related genes and measured intracellular ROS and redox homeostasis in PA-treated MAC-T cells with and without UDCA pretreatment.

2 | Materials and Methods

2.1 | Reagents

The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO, United States): Dulbecco's modified Eagle medium (DMEM), bovine hydrocortisone, insulin solution from bovine pancreas, and UDCA. Fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, Utah, United States). Penicillin–streptomycin mixed solution, L-trypsin, sodium hydrogen carbonate, and PA were sourced from Nacalai Tesque (Kyoto, Japan). Albumin solution (30% w/v) from bovine serum (BSA) and dimethyl sulfoxide (DMSO) were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). CM-H_2DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester) dye was obtained from Invitrogen (Thermo Fisher Scientific, Waltham, MA, United States).

2.2 | Preparation of PA and UDCA Solutions

Stock solutions of PA and UDCA were prepared by dissolving the required amounts of PA and UDCA into DMSO, followed by warming at 55°C with continuous shaking. Thereafter, 10-mM solutions of PA and UDCA were each prepared by mixing with 10% (wt/vol) BSA–phosphates buffered saline (PBS; fatty acid–free) and heated to 55°C for 15–20 min. Solutions were then 0.20-μm sterile filtered and stored at −20°C.

Following parturition, healthy cows typically exhibit blood PA concentrations around 100 to 150 μM (Contreras et al. 2010), whereas dairy cows with fatty liver can have levels up to 400 μM (Rukkwamsuk, Wensing, and Kruip 1999). Our previous study showed that 300-μM PA induces severe ER stress-mediated apoptosis in bMECs (Sharmin et al. 2020). Consequently, the working solution concentration of PA was set at 300 μM, and UDCA was tested at 25, 50, and 100 μM by diluting them in cell culture media (DMEM with 10% FBS) before cell treatment. The DMSO concentrations in the final working solutions for treatment groups ranged from 0.06% to 0.075%.

2.3 | Cell Culture and Treatment

MAC-T cells (a bMEC line stably transfected with the large T-antigen of simian vacuolating virus 40) were a generous gift from Dr. Sangun Roh (Tohoku University, Sendai, Japan). Cells were cultured in DMEM supplemented with 10% FBS, bovine insulin (5 μg/mL), streptomycin and penicillin (1%), and hydrocortisone (1 μg/mL). UDCA was added to MAC-T cells 48 h before PA treatment to assess its effectiveness in preventing PA-induced damage. Cells were maintained at 37°C with 5% CO_2.

2.4 | Cell Viability and UDCA Toxicity Determination

Cell viability and UDCA toxicity evaluations were performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay kit (Biotium, Fremont, CA, United States). Briefly, MAC-T cells were seeded (0.5×10^4 cells/well) in a 96-well plate and incubated at 37°C and 5% CO_2 for 48 h to achieve 90%–100% confluency. Cells were then treated with UDCA (0, 25, 50, and 100 μM) for 48 h to assess the toxicity of UDCA. MAC-T cells were also pretreated with UDCA (25, 50, and 100 μM) for 48 h, followed by a 24-h PA challenge to examine the protective effects of UDCA against PA-induced cellular damage. Following the incubation period, 10 μL of MTT solution was added to each well, which contained 100 μL of cell culture medium, and then incubated at 37°C for 4 h, after which 200 μL of DMSO was added per well. The absorbance was then measured at 570 nm with a reference wavelength of 630 nm using a Multiskan SkyHigh absorbance microplate reader (Thermo Fisher Scientific).

2.5 | RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from MAC-T cells using Sepasol-RNA I Super G (Nacalai Tesque Inc.). The concentration and

purity of extracted RNA were assessed by using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific) to measure the optical density at 260 nm and the 260/280 nm ratio. The cDNA was synthesized from total RNA using the qPCR-RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). RT-qPCR assays were performed using the SYBR Premix Ex Taq II (TaKaRa Bio Inc., Japan) on the StepOne Real-Time PCR System (Applied Biosystems). RT-qPCR primers are listed in Table 1. The relative expression of each target gene was normalized to ACTB (β-actin) and calculated using the $2^{-\triangle\triangle Ct}$ method (Pfaffl 2001).

2.6 | Analysis of Intracellular ROS Production

MAC-T cells were cultured in a 24-well plate and pretreated with UDCA for 48 h. Confluent (90%–100%) cells were treated with 25, 50, and 100 μM of UDCA and subsequently exposed to PA for 24 h. Intracellular ROS levels were assessed as described previously (Habib et al. 2025). Briefly, cells were washed with PBS and incubated with 5-μM CM-H_2DCFDA diluted in PBS for 30 min at 37°C in the dark. After incubation, the ROS dye was discarded, and cells were washed three times with PBS to remove any excess dye. Prewarmed growth medium was then added to each well, and green-fluorescent images were captured using an EVOS® FL autoimaging system (Life Technologies; Carlsbad, CA, United States). Dichlorofluorescein (DCF) fluorescence intensities were background-subtracted and normalized to the background-subtracted control value using Image J software (National Institutes of Health, MD, United States). For each experiment, fluorescence intensity was measured in 10 different views. Data from triplicate experiments were combined, analyzed, and presented as a fold change relative to the control.

2.7 | Determination of GSSG Levels, Total GSH/GSSG Ratio, and Reduced GSH/GSSG Ratio in MAC-T Cells

MAC-T cells were cultured in 9-cm^2 dishes and pretreated with varying concentrations of UDCA (25, 50, and 100 μM) for 48 h. Once cells reached 90%–100% confluence, they were treated with UDCA (25, 50, and 100 μM) alone or followed by exposure to PA (300 μM) for 24 h. The oxidized glutathione (GSSG) levels, total GSH/GSSG ratio, and reduced GSH/GSSG ratio were then assessed using a GSSG/GSH Quantification Kit (#G257; Dojindo Laboratories). Briefly, cells were washed with PBS and centrifuged at 200×g for 10 min at 4°C. The supernatant was discarded, and 10 mmol/L HCl was added to the cell pellet. The cells were lysed by freezing and thawing twice. Subsequently, 5% (w/v) 5-sulfosalicylic acid dihydrate (SSA, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was added to the lysed cells, followed by centrifugation at 8000×g for 10 min at 4°C. The supernatant was collected, and ddH_2O was added to dilute the SSA concentration to 0.5% for the assay. Two sets of sample and standard solutions were prepared for both GSSG and total GSH, and absorbance was measured at 405 nm using a microplate reader (Multiskan SkyHigh, Thermo Fisher Scientific). The concentration of reduced GSH was calculated by subtracting twice the GSSG concentration from the total GSH concentration. Finally, the total GSH/GSSG ratio and the reduced GSH/GSSG ratio were determined and expressed as percentages relative to the control.

2.8 | Statistical Analysis

Data are presented as means ± standard error of the mean (SEM) from a representative experiment conducted in triplicate dishes. Statistical analysis was performed using SPSS software

TABLE 1 | List of primers used for real-time quantitative polymerase chain reaction analysis.

Gene	Orientation	Primer sequence (5′ to 3′)	GenBank Accession No.	Reference
ACTB	Forward	CATCGCGGACAGGATGCAGAAA	NM_173979.3	Yonekura et al. (2018)
	Reverse	CCTGCTTGCTGATCCACATCTGCT		
CHOP	Forward	CTGAAAGCAGAGCCTGATCC	NM_001078163.1	Yonekura et al. (2018)
	Reverse	GTCCTCATACCAGGCTTCCA		
ATF4	Forward	CCGAGATGAGCTTTCTGAGC	NM_001034342	Yonekura et al. (2018)
	Reverse	AGCATCCTCCTTGCTGTTGT		
GRP78	Forward	GATTGAAGTCACCTTTGAGATAGATGTG	XM_024998380.2	Islam et al. (2021)
	Reverse	GATCTTATTTTTGTTGCCTGTACCTTT		
XBP1s	Forward	TGCTGAGTCCGCAGCAGGTG	NM_001271737.1	Islam et al. (2021)
	Reverse	GCTGGCAGACTCTGGGGAAG		
NRF2	Forward	CCAGCACAACACATACCA	AB162435.1	Islam et al. (2021)
	Reverse	TAGCCGAAGAAACCTCATT		
NQO1	Forward	CAACAGACCAGCCAATCA	NM_001034535.1	Islam et al. (2021)
	Reverse	ACCTCCCATCCTTTCCTC		

Abbreviations: ACTB, β-actin; ATF4, activating transcription factor 4; CHOP, C/EBP homologous protein; GRP78, glucose-regulated protein 78; NQO1, NAD(P)H quinone oxidoreductase 1; NRF2, nuclear factor (erythroid-derived 2)-like 2; XBP1s, X-box binding protein 1 splicing form.

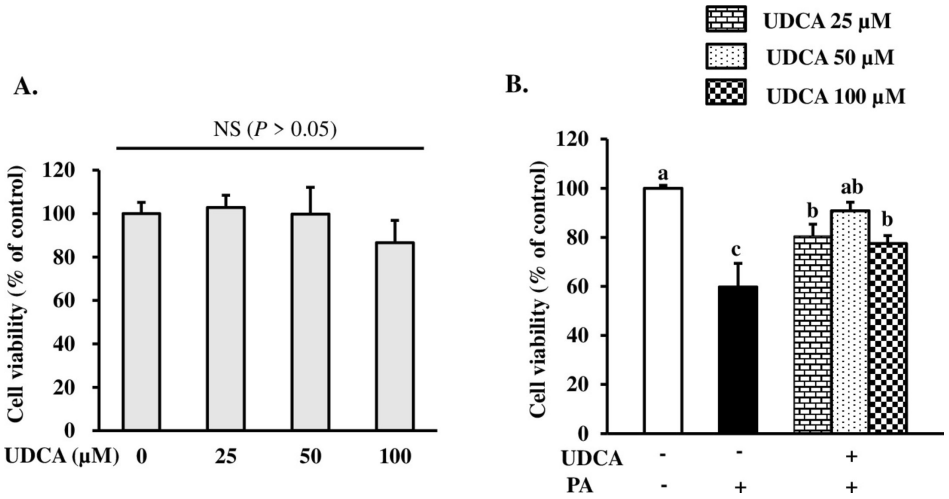

FIGURE 1 | UDCA treatment counters PA-induced cell viability reduction in MAC-T cells. (A) Effect of different concentrations of UDCA on the viability of MAC-T cells. Confluent cells (90%–100%) were treated with 25, 50, and 100 μM of UDCA for 48 h, and cell viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. (B) Confluent cells (90%–100%) were treated with 300 μM PA, either alone or with UDCA pretreatment at 25, 50, and 100 μM for 24 h. Cell viability was measured using the MTT assay. Data are presented as mean ± SEM (standard error of the mean) and are based on three independent experiments. A Tukey's honestly significant difference test was performed to compare treatment means. Means with different letters (a, b, c) differed significantly ($p < 0.05$). NS, no significant; PA, palmitic acid; UDCA, ursodeoxycholic acid.

(Version 20.0, IBM Corp., Armonk, NY, United States). One-way ANOVA followed by Tukey's honestly significant difference test was used for multiple comparisons. Differences were considered significant if the p value was < 0.05.

3 | Results

3.1 | UDCA Diminished PA-Induced Cell Viability Loss

To investigate the effect of UDCA on MAC-T cell viability, we exposed cells to 25, 50, and 100 μM of UDCA and used the MTT assay to measure cell viability. No significant effect on cell viability was found between the UDCA and control cells ($p > 0.05$, Figure 1A), suggesting that these concentrations of UDCA are noncytotoxic to MAC-T cells. We then investigated the effect of UDCA pretreatment on cell viability following 24 h of PA treatment (300 μM). The viability of MAC-T cells was significantly higher following pretreatment with all concentrations of UDCA (25, 50, and 100 μM) than that of the PA-treated group (Figure 1B).

3.2 | UDCA Alleviated ER Stress Induced by PA

To determine whether UDCA pretreatment can reduce PA-induced ER stress in MAC-T cells, we assessed the mRNA expression of the following ER stress markers: glucose-regulated protein 78 (GRP78), X-box binding protein 1 splicing form (XBP1s), ATF4, and CHOP. The cotreatment of UDCA and PA produced significantly lower levels of GRP78, XBP1s, ATF4, and CHOP mRNA than those in the PA group alone (Figure 2), indicating that UDCA alleviated PA-induced ER stress in MAC-T cells.

3.3 | UDCA Reduced PA-Induced Oxidative Stress in MAC-T Cells

We measured intracellular ROS levels following PA treatment to investigate whether UDCA pretreatment can reduce PA-induced ROS accumulation. UDCA pretreatment significantly decreased PA-induced ROS production (Figure 3A). The DCF fluorescence intensities in cells cotreated with 25, 50, and 100 μM UDCA and PA were 0.80 ± 0.29-, 1.05 ± 0.26-, and 0.87 ± 0.23-fold that of the control, respectively, compared with 6.61 ± 0.23-fold in PA-treated cells alone (Figure 3B).

Because ROS mediates oxidative stress, we also examined the mRNA expression of nuclear factor (erythroid-derived 2)-like 2 (NRF2) and its downstream antioxidant gene, NAD(P)H quinone oxidoreductase 1 (NQO1), as markers of oxidative stress. NRF2 mRNA expression remained similar between cells treated with PA alone and those cotreated with UDCA and PA. However, the mRNA expression of NQO1 was significantly lower in cells cotreated with 25-μM UDCA and PA than in PA-treated cells (Figure 3C). These findings indicate that UDCA alleviated PA-induced oxidative stress primarily by reducing ROS accumulation, potentially through mechanisms independent of the NRF2–NQO1 axis.

3.4 | UDCA Reduced GSSG Levels and Increased Both Total and Reduced GSH/GSSG Ratios in MAC-T Cells, Counteracting PA-Induced Redox Imbalance

To assess the impact of UDCA on redox balance in MAC-T cells, we measured GSSG levels, the total GSH/GSSG ratio, and the reduced GSH/GSSG ratio following UDCA treatment. Our results showed that UDCA at concentrations of 25, 50, and 100 μM significantly reduced GSSG levels compared to

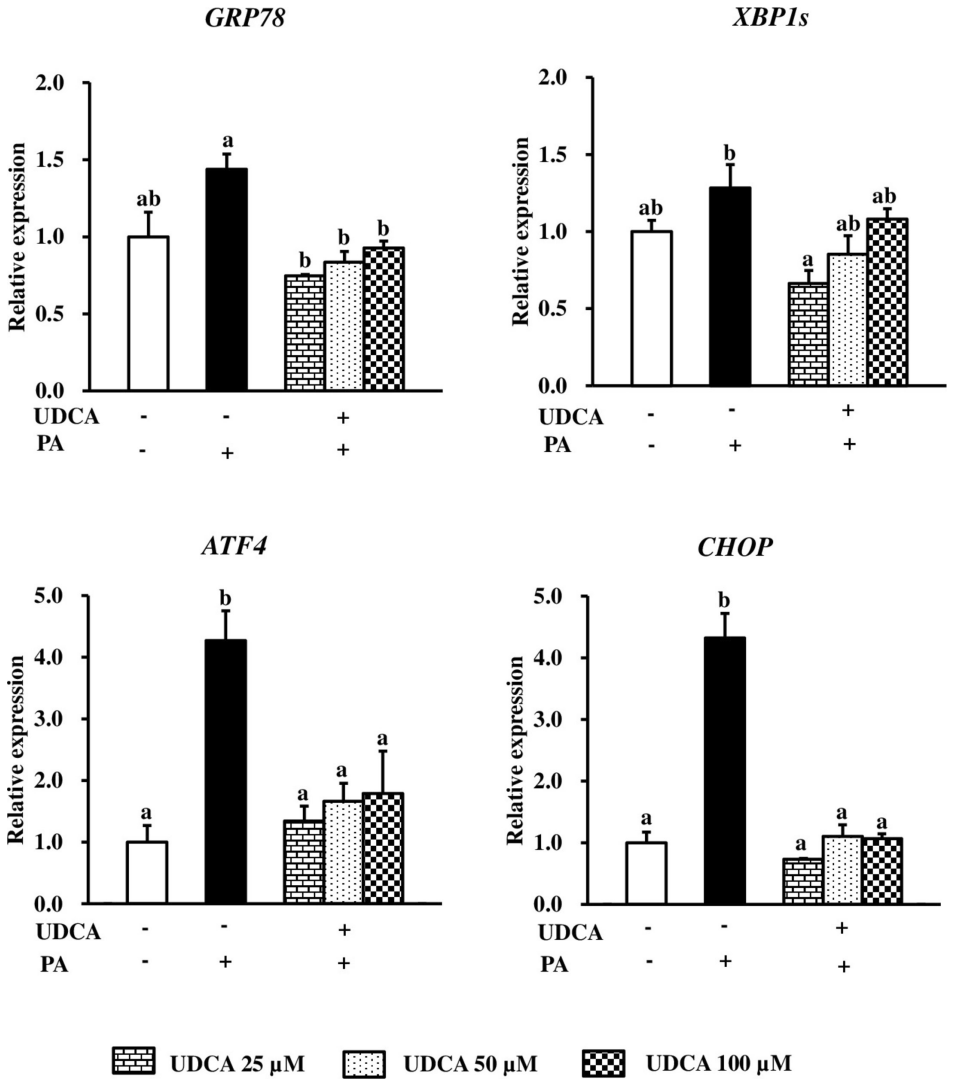

FIGURE 2 | UDCA reduced PA-induced ER stress in MAC-T cells. (A) Confluent cells (90%–100%) were treated with 300 μM PA, either alone or with UDCA pretreatment at 25, 50, and 100 μM for 24 h. The mRNA expressions of ER stress markers GRP78, XBP1s, ATF4, and CHOP were determined via RT-qPCR and normalized to that of ACTB. The relative transcript expression was calculated by the $2^{-\Delta\Delta Ct}$ method and is presented as values relative to the control cells (received no UDCA and PA). Data are presented as mean ± SEM (standard error of the mean) for three independent experiments. Tukey's honestly significant difference test was performed to compare treatment means; means with different letters (a, b) indicate a significant difference ($p < 0.05$). ACTB, β-actin; ATF4, activating transcription factor 4; CHOP, C/EBP homologous protein; GRP78, glucose-regulated protein 78; PA, palmitic acid; RT-qPCR, real-time quantitative polymerase chain reaction; UDCA, ursodeoxycholic acid; XBP1s, X-box binding protein 1 splicing form.

control cells. Notably, cells treated with 25 μM UDCA exhibited the highest total GSH/GSSG and reduced GSH/GSSG ratios (Figure 4A).

Given UDCA's significant role in modulating redox balance in MAC-T cells, we hypothesized that UDCA mitigates PA-induced damage by improving redox homeostasis through modulation of the GSH/GSSG ratio. To investigate this, we measured GSSG levels, total and reduced GSH/GSSG ratios in MAC-T cells treated with PA, either alone or in combination with 25, 50, and 100 μM UDCA. Results showed that PA treatment significantly increased GSSG levels while decreasing both the total GSH/GSSG and reduced GSH/GSSG ratios. In contrast, cotreatment with UDCA and PA reversed these effects, significantly lowering GSSG levels and increasing both the total and reduced GSH/GSSG ratios in MAC-T cells (Figure 4B).

4 | Discussion

Dairy cows in the transition period commonly exhibit NEB, which induces high blood concentrations of NEFAs (Zurek, Foxcroft, and Kennelly 1995; Holtenius 1989), of which PA is the most abundant saturated NEFA (Li et al. 2019). Despite serving as an energy substrate for bMECs, PA can also cause cellular dysfunction and apoptosis. Our recent study demonstrated that PA induces ER and oxidative stress–mediated cell death in MAC-T

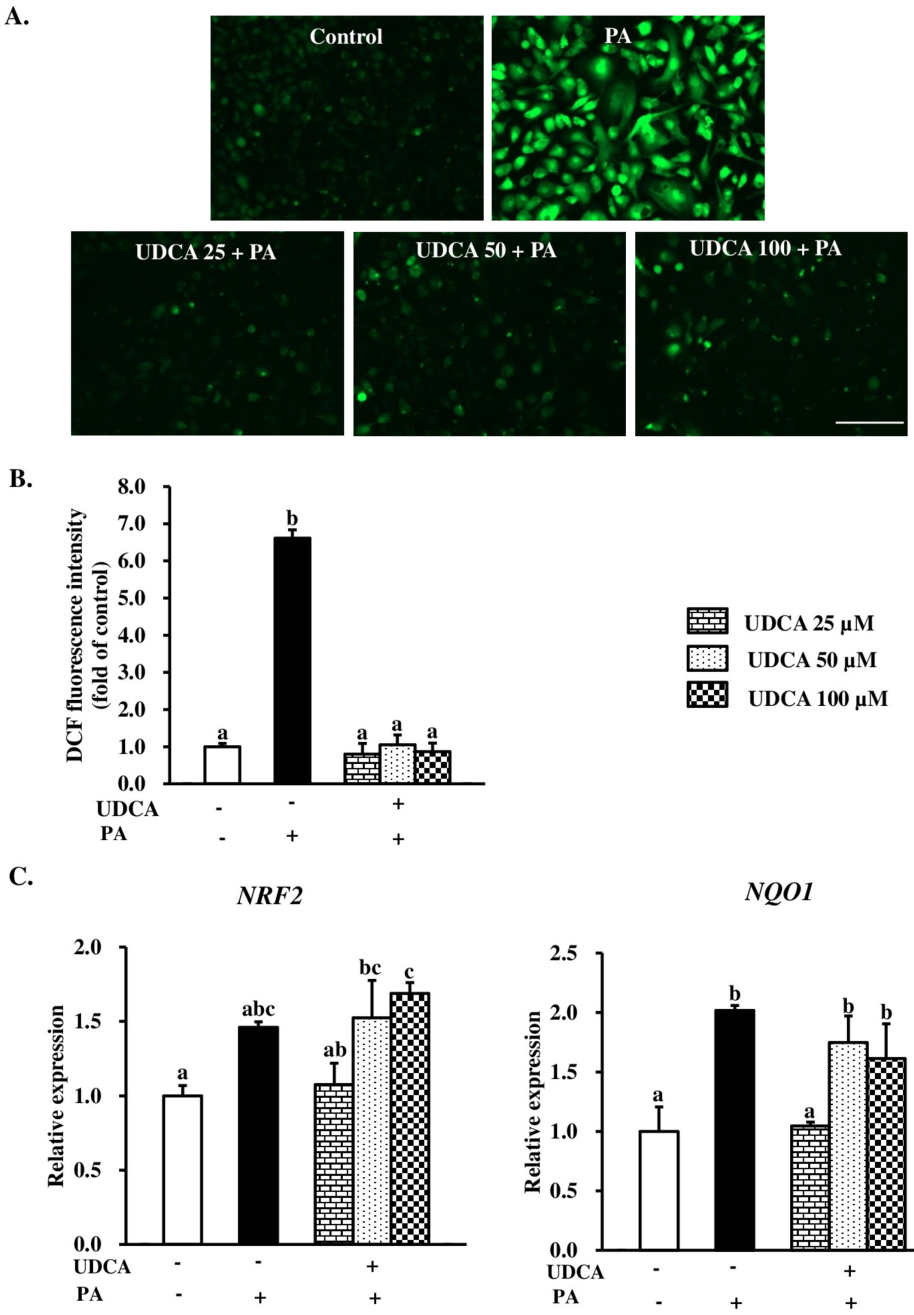

FIGURE 3 | Effect of UDCA on the intracellular reactive oxygen species (ROS) levels and oxidative stress–related gene expression in MAC-T cells following PA treatment. Confluent cells (90%–100%) were treated with 300 μM PA, either alone or with UDCA pretreatment at 25, 50, and 100 μM for 24 h. (A) Representative photomicrographs of DCF fluorescence in control cells, cells treated with PA alone, and cells cotreated with PA and different concentrations of UDCA. Scale bar = 200 μm. (B) Quantitative DCF fluorescence is based on images in Figure 3A. The average fluorescence intensity was obtained from 10 images per experiment; data from three independent experiments were combined, analyzed, and expressed as the fold change relative to control cells and presented as mean ± SEM. (C) The mRNA expression of NRF2 and NQO1 was determined by RT-qPCR and normalized to that of ACTB. The relative transcript expression was calculated by the $2^{-\Delta\Delta Ct}$ method and is presented as values relative to the control cells (received no UDCA and PA). Data are presented as mean ± SEM for three independent experiments. Tukey's honestly significant difference test was performed to compare treatment means; means with different letters (a, b, c) differed significantly ($p < 0.05$). ACTB, β-actin; DCF, dichlorofluorescein; NQO1, NAD(P)H quinone oxidoreductase 1; NRF2, nuclear factor (erythroid-derived 2)-like 2; PA, palmitic acid; RT-qPCR, real-time quantitative polymerase chain reaction; SEM, standard error of the mean; UDCA, ursodeoxycholic acid.

cells (Habib et al. 2025). Therefore, exploring therapeutic strategies to counteract the detrimental effects of PA in bMECs is crucial for maintaining bovine mammary gland health and sustaining milk production. UDCA is recognized for having potent antioxidative and ER stress–alleviating properties in hepatocytes (Mitsuyoshi et al. 1999), podocytes (Cao et al. 2016a, 2016b), and endothelial cells (Chung et al. 2016). Consequently, UDCA supplementation may help mitigate PA-induced cytotoxicity in

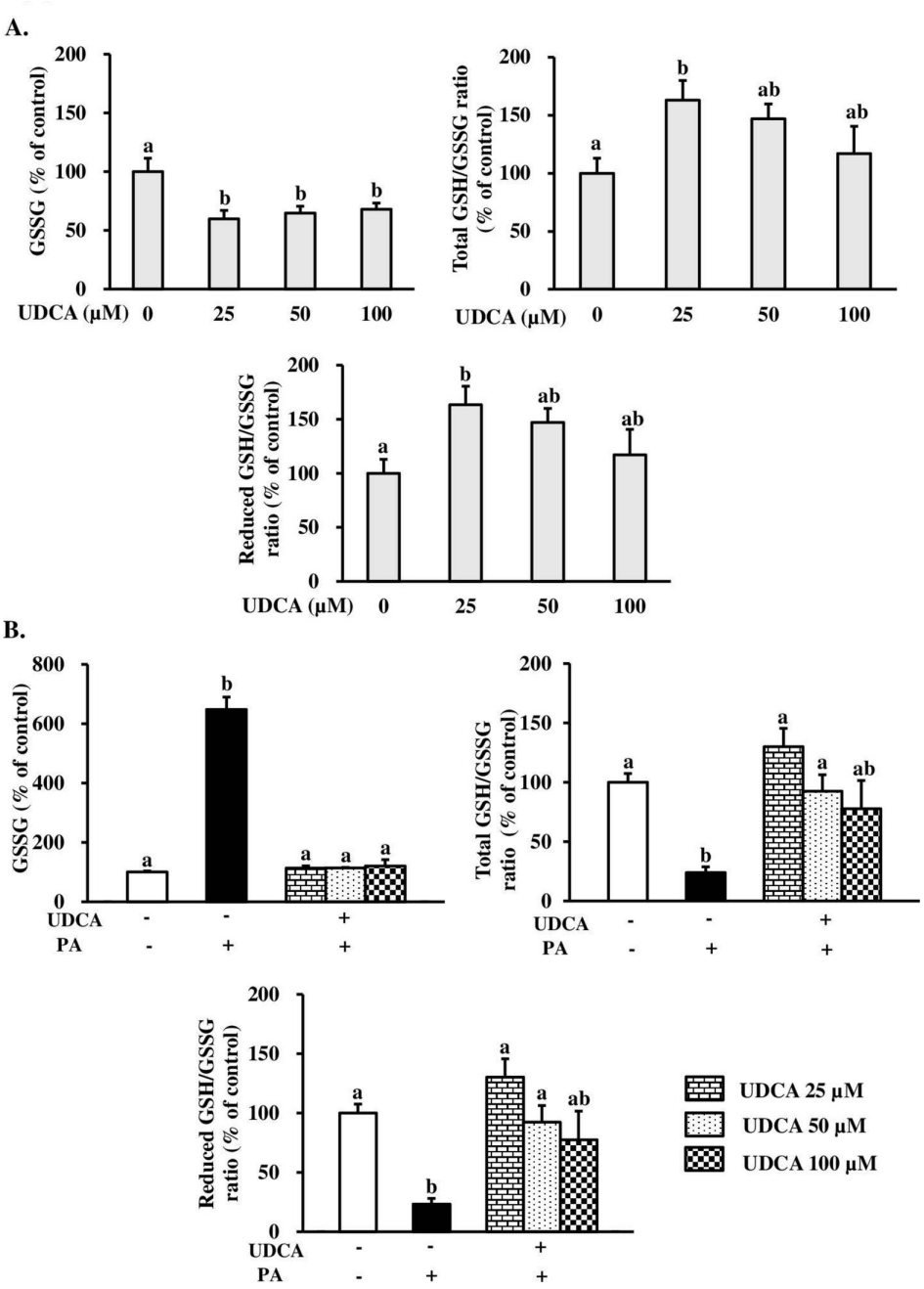

FIGURE 4 | UDCA reduced GSSG levels and increased both total and reduced GSH/GSSG ratios in MAC-T cells, counteracting PA-induced redox imbalance. (A) Effect of different concentrations of UDCA on GSSG levels, total GSH/GSSG ratio, and reduced GSH/GSSG ratio in MAC-T cells. Cells were pretreated with 25, 50, and 100 μM UDCA for 48 h, and then, confluent cells (90%–100%) were treated with the same concentrations of UDCA for an additional 24 h. (B) UDCA reduced PA-induced GSSG levels, increased the total GSH/GSSG ratio, and restored the GSH/GSSG balance in MAC-T cells. Cells were pretreated with 25, 50, and 100 μM UDCA for 48 h, and then, confluent cells (90%–100%) were treated with 300 μM PA, either alone or with UDCA (25, 50, and 100 μM) for 24 h. Data from three independent experiments were combined, analyzed, and expressed as a percentage of control cells. Results are presented as mean ± SEM (standard error of the mean). Means with different letters (a, b) differed significantly ($p < 0.05$). GSH, glutathione; GSSG, oxidized glutathione; PA, palmitic acid; UDCA, ursodeoxycholic acid.

MAC-T cells. Our results indicate that UDCA, at concentrations of 25, 50, and 100 μM, did not significantly affect MAC-T cell viability (Figure 1A), which aligns with results from previous studies where UDCA was reported as nontoxic at similar concentrations in various cell types (Li et al. 2020; Tsagarakis et al. 2010). The pretreatment of MAC-T cells with UDCA before PA exposure produced a significant increase in cell viability at all UDCA concentrations compared with that observed in the PA-treated group alone (Figure 1B), suggesting that UDCA effectively counteracts the PA-induced damage to MAC-T cells. These results are consistent with other studies showing UDCA's protective effects against cell damage induced by various stressors (Li et al. 2020; Wang et al. 2024) and extend this protective role to PA-induced damage in MAC-T cells.

ER stress is a tightly regulated cellular process that plays a vital role in various physiological activities and triggers complex adaptive or proapoptotic signals, known as the unfolded protein response (UPR). The UPR contains three major arms, which remain inactive under nonstress conditions because of their binding with GRP78. Upon ER stress, these transducers dissociate from GRP78 to activate PERK (Bertolotti et al. 2000), which subsequently increases the expression of the transcription factor CHOP, promoting cell apoptosis (Zinszner et al. 1998). We recently revealed that PA induces ER stress–mediated apoptosis in MAC-T cells by increasing the mRNA levels of XBP1s, ATF4, and CHOP (Sharmin et al. 2020). Herein, UDCA pretreatment significantly reduced the PA-induced increase in the mRNA levels of GRP78, XBP1s, ATF4, and CHOP (Figure 2). This suggests that UDCA can alleviate PA-induced ER stress mediated cell death in MAC-T cells. Similar effects have been observed in other studies where UDCA treatment improved ER stress responses and reduced podocyte apoptosis in diabetic nephropathy (Cao et al. 2016b). Specifically, UDCA has been shown to enhance cell viability and may contribute to reducing ER stress and apoptosis as suggested by its recognized effects on enhancing chaperone activity and modulating stress–signaling pathways.

Oxidative stress arises from an imbalance between ROS production and their elimination by antioxidative systems. Although ROS are naturally generated during cellular metabolism, excessive levels can cause cell death. Our previous study reported that PA generates extreme levels of ROS in MAC-T cells and trigger apoptosis in MAC-T cells (Habib et al. 2025). Herein, we observed that UDCA pretreatment significantly reduced PA-induced ROS levels in MAC-T cells (Figure 3A,B), indicating that UDCA effectively reduced PA-induced ROS accumulation in MAC-T cells. These findings are consistence with previous research showing that UDCA has antioxidant properties and can decrease ROS accumulation in various cell types (Liu et al. 2016; Chung et al. 2016). Thus, UDCA may alleviate PA-induced oxidative stress by either directly scavenging ROS or enhancing cellular antioxidant defenses.

NRF2 is a transcription factor essential for activating antioxidant genes and protecting cells from oxidative stress (Furfaro et al. 2016). The similar NRF2 mRNA expression levels found between the PA group and the UDCA + PA cotreated cells in this study suggest that NRF2 activation may not be the main mechanism whereby UDCA exerts its protective effects. Although NRF2 can regulate the antioxidant response (Zhang et al. 2010), our findings imply that UDCA may use alternative pathways. The significant reduction in NQO1 expression with 25 μM UDCA and PA cotreatment suggests concentration-dependent effects of UDCA on the cellular antioxidant response. NQO1 is essential for detoxifying quinones and protecting cells from oxidative damage. The decrease in NQO1 expression at lower UDCA concentrations may reflect a more efficient reduction in ROS levels, thereby lessening the need for upregulated expression of NQO1. At higher concentrations, UDCA may engage different protective mechanisms that do not involve modulating NQO1 expression. This implies that UDCA primarily mitigates PA-induced oxidative stress through direct ROS reduction rather than through modulating the expression of NRF2 or NQO1 at higher concentrations. The antioxidant properties of UDCA, as shown in previous studies (Liu et al. 2016; Chung et al. 2016), likely account for its protective effects, highlighting the potential of UDCA as a therapeutic agent for alleviating PA-induced oxidative stress in MAC-T cells.

GSH is a vital antioxidant that scavenges ROS, protecting cells from oxidative stress by reducing hydroperoxides and organic peroxides, thereby maintaining cell membrane integrity during redox reactions (Ursini and Maiorino 2020). In this study, UDCA significantly modulated the glutathione redox balance in MAC-T cells compared to the control group, as evidenced by reduced GSSG levels and increased total GSH/GSSG and reduced GSH/GSSG ratios. These findings underscore UDCA's role in enhancing antioxidant capacity and maintaining a reduced intracellular environment. Notably, the optimal effect was observed at 25-μM UDCA, suggesting a dose-dependent response, with higher concentrations potentially leading to saturation effects without additional benefits. This observation aligns with Arisawa et al. (2009), who demonstrated that UDCA increased GSH synthesis in HepG2 cells at specific concentrations.

PA is well documented to induce oxidative stress by elevating GSSG levels and disrupting intracellular redox balance, as reflected by significant reductions in both total and reduced GSH/GSSG ratios in this study. These disruptions lead to cellular dysfunction and apoptosis. UDCA cotreatment effectively counteracted these effects by lowering GSSG levels and restoring redox balance, particularly the reduced GSH/GSSG ratio, which is critical for cellular redox signaling and protection against oxidative damage. By mitigating PA-induced oxidative stress, UDCA preserved cell viability and strengthened the antioxidant defense system in MAC-T cells. These findings are consistent with previous studies, such as Mitsuyoshi et al. (1999), who reported UDCA protects hepatocytes from oxidative injury by inducing antioxidant mechanism, and Buko et al. (2002), who demonstrated that UDCA prevents reduced GSH depletion in the liver of rats subjected to oxidative stress caused by gamma-irradiation.

Because UDCA reduced PA-induced ROS levels in MAC-T cells (Figure 3A,B), this supports its role in combating oxidative stress. GSH is essential for maintaining oxidoreductase activity and buffering ROS generated in the ER (Chakravarthi, Jessop, and Bulleid 2006). Disruptions in the GSH redox state can impair ER function, activate the UPR, and trigger apoptosis (Frand and Kaiser 2000). McCullough et al. (2001) reported that ER stress induces CHOP expression, which promotes GSH depletion and oxidative injury in gadd153−/− MEF cells, linking oxidative stress and ER stress in apoptosis pathways. In this study, UDCA restored the PA-induced imbalance in the GSH/GSSG ratio in MAC-T cells, thereby protecting against oxidative and ER stress-mediated damage. Overall, UDCA's regulation of the GSH system appears to function as a buffering mechanism, mitigating PA-induced ROS levels and oxidative stress in MAC-T cells. These findings position UDCA as a promising therapeutic candidate for managing oxidative stress-related cellular damage and apoptosis.

Given that NEB is a prevalent challenge during the early lactation phase, resulting in elevated levels of NEFAs (of which PA constitutes 34% of saturated fatty acid) in the bloodstream, the increased NEFA levels, particularly PA, can induce cellular

stress and apoptosis in bMECs, thereby compromising milk yield. Incorporating UDCA into feeding strategies may mitigate the risk of PA-induced apoptosis in bMECs during this critical period. This approach has the potential to augment both milk yield and quality by preserving the viability of bMECs, particularly in high-producing herds that are more susceptible to NEB. Although direct evidence regarding the effects of dietary UDCA supplementation on lactation physiology in dairy cows is lacking, studies in other types of cattle have demonstrated its beneficial effects. For instance, dietary supplementation with UDCA (2.5 g/animal/day) improved marbling and carcass characteristics in Wagyu cattle without impairing growth (Irie, Kouda, and Matono 2011). Similarly, oral administration of UDCA (50 g/animal/day) in Japanese Black fattening cattle enhanced vitamin absorption, digestion, and liver cell protection while inhibiting inflammatory cytokines, potentially mitigating transportation-induced respiratory diseases (Matsuda, Maeda, and Takahashi 2018). Given the pharmacokinetics of UDCA, it has the potential to reach the mammary glands in its unchanged form. Consequently, the use of UDCA as a feed additive or therapeutic agent represents a promising strategy for enhancing dairy production efficiency and promoting animal welfare during crucial lactation stages.

In conclusion, our study demonstrates that UDCA effectively mitigates PA-induced loss of cell viability, ER stress, and oxidative stress in MAC-T bMECs. Pretreatment with UDCA at 25, 50, and 100 μM did not affect cell viability and significantly improved that reduced by PA. Moreover, UDCA alleviated PA-induced ER stress by reducing the mRNA levels of key ER stress markers and decreased PA-induced ROS production. UDCA treatment also significantly reduced PA-induced GSSG levels and enhanced both the reduced GSH/GSSG and total GSH/GSSG ratios, highlighting its efficacy as a protective agent against oxidative cellular damage. Therefore, UDCA may have potential as a therapeutic compound for protecting bMECs from PA-induced apoptosis, which could help maintain mammary gland health and support milk production in dairy cows.

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Conflicts of Interest

The authors declare no conflicts of interest.

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