

# Cytoprotective Effects of Tauroursodeoxycholic Acid in Heat Stress-Induced Endoplasmic Reticulum Stress in Male Germ Cells

(cell viability / endoplasmic reticulum stress / tauroursodeoxycholic acid / heat stress / male germ cells)

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**Abstract.** Heat stress disrupts cellular homeostasis in cells by inducing protein misfolding, activating endoplasmic reticulum stress pathways and impairing cell

survival mechanisms. The present study aimed to evaluate the cytoprotective effects of tauroursodeoxycholic acid (TUDCA) against heat stress-induced cellular damage in an *in vitro* spermatogonial GC1 cell model. The IC<sub>50</sub> concentration of TUDCA was determined by the MTT assay. GC1 cells were divided into four groups: control (GC1C), TUDCA-treated (GC1TUDCA), heat stress model (HSM; GC1HSM) and TUDCA-treated HSM (GC1HSMTUDCA). Standard culture conditions were used for GC1C. HSM group cells were incubated for 60 minutes at 43 °C. TUDCA groups of cells were treated with 200 µM TUDCA in culture media for 24 hours. Following the experiments, all groups were subjected to immunocytochemical staining with phosphorylated PERK (p-PERK) and C/EBP homologous protein (CHOP) antibodies, and immunoreactivity was evaluated using the H-Score system. The MTT assay determined the IC<sub>50</sub> value of TUDCA as 558 µM for GC1 cells, with 200 µM selected for subsequent experiments due to minimal cytotoxicity. Heat stress exposure was associated with a reduction in GC1 cell viability and an increase in p-PERK and CHOP expression compared with the GC1C group. In heat-stressed cells, TUDCA treatment was associated with a significant decrease in p-PERK and CHOP expression relative to the GC1HSM group. These results suggest that TUDCA might reduce the cellular reactions related to endoplasmic reticulum stress in heat-stressed GC1 spermatogonial cells under *in vitro* conditions.

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Abbreviations: ANOVA – analysis of variance, ATCC – American Type Culture Collection, ATF6 – activating transcription factor 6, CHOP – C/EBP homologous protein, DAB – 3,3'-diaminobenzidine, DMEM – Dulbecco's modified Eagle's medium, DMSO – dimethyl sulphoxide, ER – endoplasmic reticulum, GRP78 – glucose-regulated protein 78, H-Score – histological scoring system, HSM – heat stress model, IC<sub>50</sub> – half maximal inhibitory concentration, ICC – immunocytochemistry, IRE1α – inositol-requiring enzyme 1 alpha, MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PBS – phosphate-buffered saline, PERK – protein kinase RNA-like endoplasmic reticulum kinase, p-PERK – phosphorylated PERK, TUDCA – tauroursodeoxycholic acid, UPR – unfolded protein response.

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## Introduction

Spermatogenesis is a highly regulated process of male germ cell development that begins with mitotic proliferation, followed by meiosis and spermiogenesis. The spermatogenesis process undergoes modifications from both endogenous and exogenous elements, including radiation, poor nutrition, heat stress, and exposure to toxins such as pesticides or heavy metals (Dalia et al., 2019). Spermatogenesis occurs at a temperature 2–4 °C lower than the body temperature (Sharma and Agarwal, 2011). In most mammals, including humans, the optimal temperature for spermatogenesis is around 34–35 °C, whereas the normal body temperature of mammals is approximately 37–39 °C, depending on the species (Setchell, 2018). Due to this reason, in males, exposure to high temperatures has a serious detrimental effect on fertility by disrupting spermatogenesis; thus, heat stress is identified as an important risk factor for infertility (Kim et al., 2013; Hoang-Thi et al., 2022). Although increased DNA damage and apoptosis are observed in spermatogenic cells exposed to an abnormal temperature increase, the chain of molecular and cellular events underlying these processes has not yet been fully elucidated (Thonneau et al., 1998).

The endoplasmic reticulum (ER) functions as one of the vital organelles for metabolic cell processes and developmental survival functions (Kim et al., 2008). The organelle facilitates protein membrane synthesis and corrects protein folds for hormones and growth factors while conducting quality control of protein structures (Burman et al., 2018). ER stress arises from intracellular or extracellular disturbances of ER function (Mori, 2000), and heat exposure is recognized as an important environmental trigger of this response (Liu et al., 2012; Chen et al., 2021). ER stress plays a crucial role in male fertility, as proper protein folding and quality control in spermatogenic cells are essential for normal sperm development (Santiago et al., 2020). When ER stress occurs, proteins accumulate as unfolded or misfolded particles throughout the endoplasmic reticulum, which mitigates proper cell activity (Rahmani et al., 2023). ER stress leads to the accumulation of misfolded proteins, which can disrupt germ cell differentiation during spermatogenesis and result in defective sperm production and impaired fertility (Hetz, 2012; Tatar, 2024).

In response to prolonged ER stress, cells activate the unfolded protein response (UPR) to restore homeostasis; however, if the stress persists, this response may become insufficient to maintain cell survival (Malhotra and Kaufman, 2007). In mammals, UPR signalling is initiated by three ER transmembrane sensor proteins: inositol-requiring enzyme 1 alpha (IRE1 $\alpha$ ), protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (Schröder, 2008; Rana, 2020). In normal state, the sensor proteins bind their luminal sections to ER chaperone protein GRP78, yet stress-induced dissociation occurs during ER stress conditions (Walter and Ron, 2011). In addition to PERK activation, prolonged

ER stress is associated with induction of C/EBP homologous protein (CHOP), a stress-responsive transcription factor that plays a central role in ER stress-mediated apoptotic signalling. The disruption of ER homeostasis during an extensive UPR response leads to stress-intolerant cells, which ultimately activates death mechanisms (Tabas and Ron, 2011). The prolonged activation of the UPR pathway due to persistent ER stress can compromise the survival of spermatogenic cells, ultimately affecting sperm count and motility (Rahmani et al., 2023).

TUDCA is a highly hydrophilic bile acid that has been widely studied for its cytoprotective effects, particularly in alleviating ER stress (Hanafi et al., 2018). It is a chemical compound known as a stress suppressor (Gorman et al., 2012; Kusaczuk, 2019). During spermatogenesis, the stress that affects ER functions causes protein malformation, which then initiates germ cell death and results in inhibited sperm production. TUDCA maintains normal ER homeostasis while preventing germ cells from exposure to stress-induced damage associated with the activation of UPR pathways (Pioltine et al., 2021). The effect of TUDCA, specifically via the PERK pathway, on heat stress-induced ER stress signalling in spermatogonial cells remains inadequately understood.

The present study aimed to examine the effect of heat stress on the expression of ER stress markers (p-PERK and CHOP) in spermatogonial cells and to assess the potential regulatory impact of TUDCA on these factors.

## Material and Methods

### *Cell culture*

Mouse GC1 spermatogonial cells (ATCC<sup>®</sup> CRL-2053<sup>™</sup>) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % foetal bovine serum (FBS) (Guan et al., 2009). The culture medium was sterilized by filtration via a 0.2  $\mu$ m membrane filter before use. Cells were kept under sterile conditions within a humidified incubator at 37 °C with 5 % CO<sub>2</sub> (Onen et al., 2022). The study received the ethical approval from the Non-Invasive Clinical Research Ethics Committee of Kutahya Health Sciences University Faculty of Medicine (Approval No. 2020/06-05).

### *Dose modulation of TUDCA*

Cell viability and the potential cytotoxic effects of TUDCA were evaluated using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Liu et al., 1997). GC1 cells were seeded at a density of  $1 \times 10^4$  cells per well in 96-well plates and incubated with TUDCA at concentrations ranging from 1  $\mu$ M to 2000  $\mu$ M for 24 hours. Following treatment, cells were washed with phosphate-buffered saline (PBS). Subsequently, 100  $\mu$ l of fresh culture medium supplemented with 10  $\mu$ l of MTT stock solution was added to each well, and plates were incubated at 37 °C for 4 hours in the dark. After

incubation, the MTT-containing medium was removed, and 100  $\mu$ l of dimethyl sulphoxide (DMSO) was added to each well to solubilize the formazan crystals. Absorbance was measured at 570 nm using a microplate reader. All experiments were performed in triplicate.

### Creation of the *in vitro* heat stress model

Cells were divided into four experimental groups: control (GC1C), TUDCA-treated (GC1TUDCA), heat stress model (GC1HSM) and TUDCA-treated heat stress model (GC1HSMTUDCA). Cells in the GC1C group were cultivated under standard conditions without treatment, while GC1TUDCA cells were cultured under identical conditions and treated with 200  $\mu$ M TUDCA. Heat stress was generated in the GC1HSM and GC1HSMTUDCA groups with exposure to 43  $^{\circ}$ C for 60 minutes, a procedure designed based on preliminary optimization and previously shown to induce similar cellular stress responses (Jacobs et al., 2020; Shahat et al., 2020; Gao et al., 2022). Following heat exposure, cells were returned to 37  $^{\circ}$ C and the medium replaced with a fresh medium to remove stress-related metabolites. In the GC1HSMTUDCA group, the fresh medium was added with 200  $\mu$ M TUDCA, while the GC1HSM group was administered the fresh medium with no addition of TUDCA. The selected TUDCA concentration was chosen from previous experimental research (Xie et al., 2002; Lee et al., 2010). All groups were incubated for 24 hours for further analyses.

### Immunocytochemical staining

Cells from all experimental groups were washed with PBS and afterward fixed with 4 % paraformaldehyde for 30 minutes (Katikireddy and O'Sullivan, 2011). After fixing, cells were subjected to two washes with PBS and permeabilized with 0.1 % Triton X-100 for 15 minutes on ice. Endogenous peroxidase activity was inhibited by a 10-minute incubation with hydrogen peroxide at room temperature, made by washes with PBS. Non-specific binding was inhibited by incubating with a blocking solution for one hour at room temperature.

Primary antibodies targeting phosphorylated PERK (p-PERK; Bioss, bs-3330R; 1 : 100) and CHOP (Biorbyt, orb157704; 1 : 100) were incubated overnight at 4  $^{\circ}$ C. Following washing, the cells were incubated with biotinylated secondary antibodies for 30 minutes, subsequently followed by streptavidin-horseradish peroxidase for an additional 30 minutes. Immunoreactivity was visualized using 3,3'-diaminobenzidine (DAB) for five minutes. Following that, the nuclei were counterstained with Mayer's haematoxylin for a duration of two to three minutes. Subsequently, the slides were prepared for microscopic analysis.

Immunocytochemical analyses were conducted using three independent biological replicates for each group. Each replicate involved assessment of ten randomly chosen microscopic fields at 20 $\times$  magnification. Two independent, blinded histologists evaluated the staining intensity. Immunoreactivity was quantified utilizing the

H-score method, defined as  $H\text{-score} = \sum (P_i \times i)$ , where  $i$  indicates staining intensity (0–3) and  $P_i$  signifies the percentage of cells at each intensity level. The H-score varied between 0 and 300 (Kendirci-Katirci et al., 2025).

### Statistical analysis

The experimental data were subjected to statistical analysis utilizing GraphPad Prism version 10.3 (GraphPad Software Inc., San Diego, CA). The distinctions between the four experimental groups (GC1C, GC1TUDCA, GC1HSM and GC1HSMTUDCA) were assessed utilizing one-way analysis of variance (ANOVA). After finding a significant overall difference, Tukey's post-hoc test was utilized for pairwise comparisons among groups. A P value of less than 0.05 was considered statistically significant (Henson, 2015).

## Results

The MTT assay was used to evaluate cell viability *in vitro*, investigating the effect of increasing doses of TUDCA (0, 50, 100, 250, 500, 1000 and 2000  $\mu$ M) on the viability of GC1 cells. A concentration-dependent decline in cell viability was noted with increasing TUDCA doses, with the  $IC_{50}$  value determined to be 558  $\mu$ M (Fig. 1).

GC1C cells showed typical epithelioid structure and preserved normal confluence under control conditions. Similar morphological characteristics were observed in the GC1TUDCA group after administration of 200  $\mu$ M TUDCA. A significant decrease in cell number, together with cytopathic alterations, was observed in the GC1HSM group. In the GC1HSMTUDCA group, morphological changes and increases in numbers of cells were alleviated compared to the GC1HSM group, but they were not completely returned to control levels (Fig. 2).

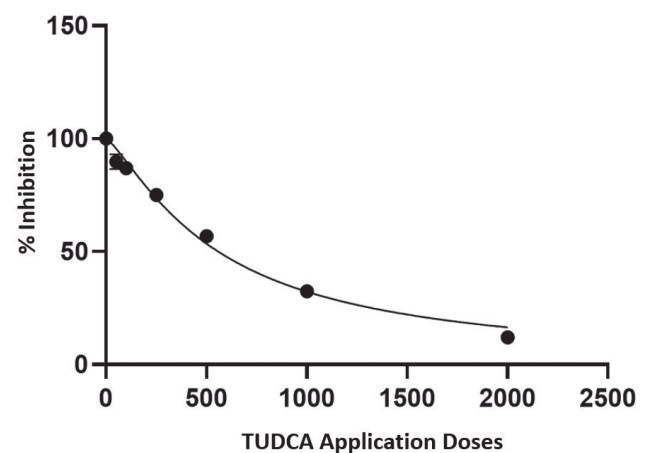
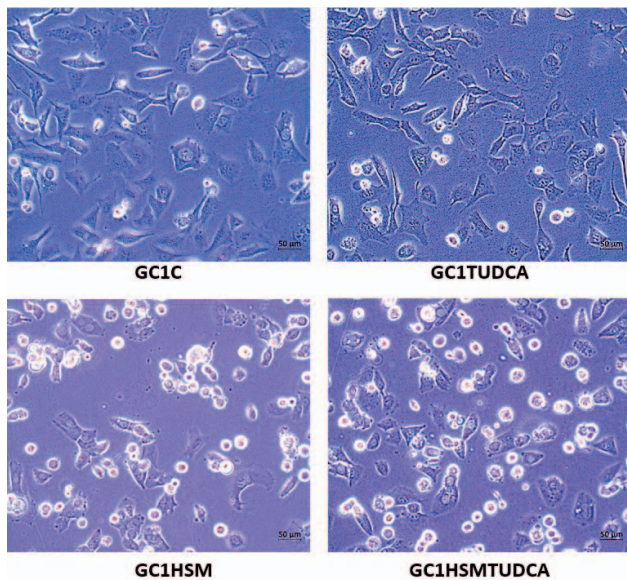


Fig. 1. Graph of GC1 TUDCA application concentration and percentage of inhibition

The dose-response curve of TUDCA on GC1 cell viability, measured through the MTT assay. The graph illustrates the percentage of inhibition at varying TUDCA concentrations, with the  $IC_{50}$  determined at 558  $\mu$ M.

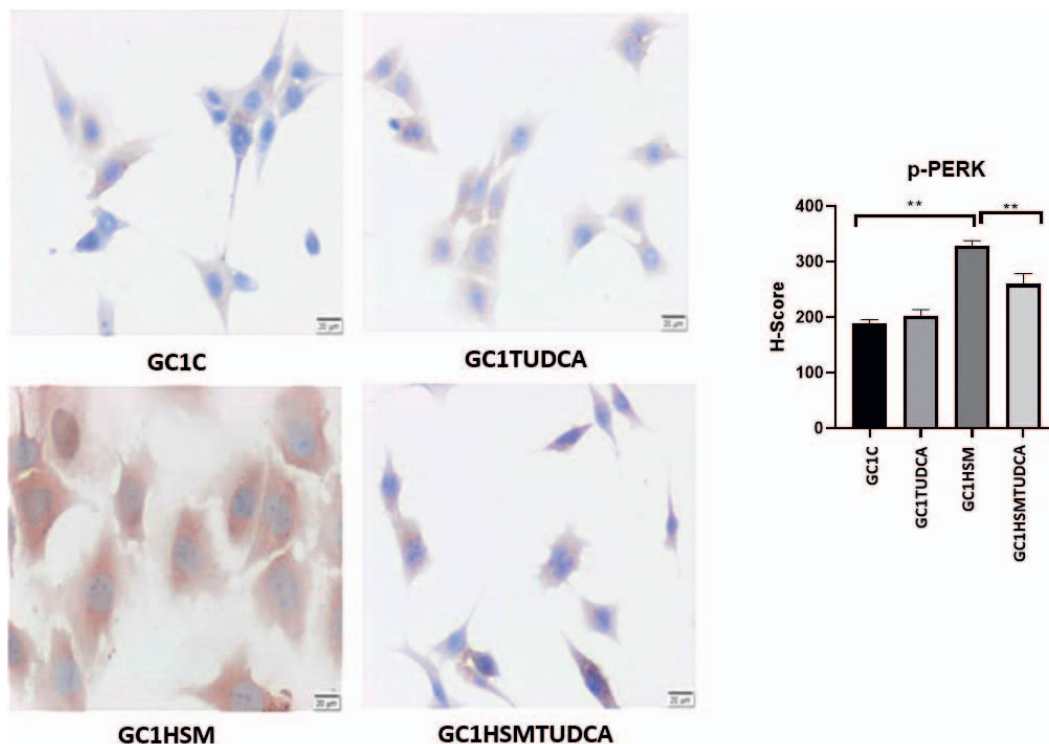


**Fig. 2.** Inverted microscope images of all experimental groups

Representative inverted microscopy images of GC1 spermatogonial cells under different experimental conditions. GC1C and GC1TUDCA groups exhibit preserved cellular morphology. In the GC1HSM group, reduced cell density and cell rounding are observed. In the GC1HSMTUDCA group, cellular morphology and cell density are partially preserved relative to the GC1HSM group. Scale bar = 50 µm.

After immunocytochemical staining for p-PERK, 10 selected at random microscopic areas from each experimental group were analysed at 20× magnification. In the GC1C and GC1TUDCA groups, p-PERK immunoreactivity was largely weak. In contrast, the GC1HSM group demonstrated significantly higher p-PERK immunoreactivity, characterized by strong to very strong staining intensity. In the GC1HSMTUDCA group, p-PERK immunoreactivity decreased compared to the GC1HSM group, showing mainly moderate intensity with rare strong staining. Statistical analysis showed no significant difference between the GC1C and GC1TUDCA groups ( $P > 0.05$ ). p-PERK immunoreactivity was significantly higher in the GC1HSM group compared to the GC1C group ( $P \leq 0.05$ ), while a statistically significant decrease was observed in the GC1HSMTUDCA group compared with the GC1HSM group ( $P < 0.05$ ) (Fig. 3).

Following immunocytochemical staining for CHOP, 10 selected at random microscopic areas from each experimental group were examined at 20× magnification. CHOP immunoreactivity was mainly mild in the GC1C and GC1TUDCA groups, while significant to very strong staining intensity was observed in the GC1HSM group. In the GC1HSMTUDCA group, CHOP immunoreactivity was mostly moderate. Statistical analysis showed no significant difference between the GC1C and GC1TUDCA groups ( $P > 0.05$ ). CHOP immunoreactivity was signifi-



**Fig. 3.** The p-PERK immunocytochemical staining images and statistical analysis results

Representative immunocytochemical staining images of p-PERK in GC1 spermatogonial cells. Differences in staining intensity indicate variations in p-PERK immunoreactivity among experimental groups. Quantitative analysis shows a significant increase in p-PERK immunoreactivity in the GC1HSM group compared with GC1C and a significant decrease in the GC1HSMTUDCA group relative to the GC1HSM group ( $**P \leq 0.01$ ). Scale bar = 20 µm.

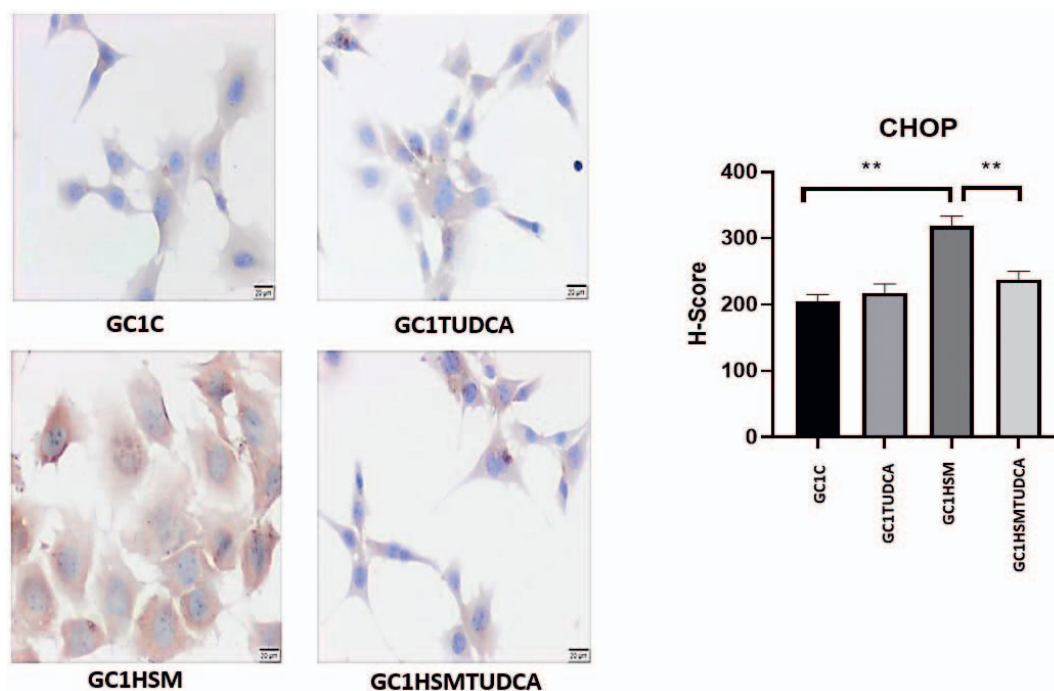


Fig. 4. CHOP immunocytochemical staining images and statistical analysis results

Representative immunocytochemical staining images of CHOP in GC1 spermatogonial cells. Differences in staining intensity reflect variations in CHOP immunoreactivity among experimental groups. Statistical analysis demonstrates a significant increase in CHOP immunoreactivity in the GC1HSM group compared with GC1C and a significant decrease in the GC1HSMTUDCA group relative to the GC1HSM group (\*\* $P \leq 0.05$ ). Scale bar = 20  $\mu\text{m}$ .

cantly higher in the GC1HSM group compared to the GC1C group ( $P < 0.05$ ) and was significantly decreased in the GC1HSMTUDCA group in comparison to the GC1HSM group ( $P \leq 0.05$ ). Compared group analysis and representative immunocytochemical images are shown (Fig. 4).

## Discussion

Heat stress has been described as an important environmental factor that hinders male reproductive function, primarily by negatively impacting spermatogenic cells (Durairajanayagam et al., 2015; Shahat et al., 2020). Numerous studies indicate that elevated temperatures can adversely affect male fertility by inducing DNA damage, cellular apoptosis and disruptions in normal cellular functions (Paul et al., 2008; Capela et al., 2022; Gao et al., 2022). The ER is very important for keeping cells stable because it controls how proteins are made, folded, and how cells stay alive. When cells are stressed, like when it's hot, the ER can stop working right and make the ER stress responses happen (Mori, 2000).

In this context, the present study investigated the effects of TUDCA on heat stress-induced ER stress utilizing an *in vitro* GC1 spermatogonial cell model. Our findings suggest that TUDCA may alleviate cellular responses associated with ER stress in experimental heat stress conditions. The MTT experiment demonstrated that elevated concentrations of TUDCA (0–2000  $\mu\text{M}$ )

correlated with a consistent decline in cell viability, yielding an  $\text{IC}_{50}$  value of 558  $\mu\text{M}$ . Based on these findings, a concentration of 200  $\mu\text{M}$  was selected for additional tests, as it exhibited minimal cytotoxicity and allowed for the assessment of cellular self-protection mechanisms.

Previous experimental reports validate the concentration range employed in this study. For instance, TUDCA doses between 50 and 200  $\mu\text{M}$  have shown a decrease in cryopreservation-induced cytotoxicity in bovine oocytes (Khatun et al., 2020). Additionally, studies on *in vitro* oocyte maturation have demonstrated that TUDCA at concentrations of 50  $\mu\text{M}$  and 100  $\mu\text{M}$  does not adversely affect cell viability, although a 200  $\mu\text{M}$  concentration may reduce intracellular oxidative stress (Pioltine et al., 2021). These findings emphasize that  $\text{IC}_{50}$  values and cellular responses to pharmacological agents can vary markedly depending on cell type and experimental conditions (Ye et al., 2004). Consequently, the 200  $\mu\text{M}$  concentration employed in this study should be considered appropriate for the present *in vitro* GC1 cell type rather than as a universally optimal dosage.

Immunocytochemical analyses clarified the cellular mechanisms underlying these findings. Heat stress markedly increased p-PERK immunoreactivity in GC1 cells, signifying activation of the PERK pathway in the unfolded protein response. TUDCA treatment resulted in a reduction of p-PERK immunoreactivity, signifying modulation of ER stress signalling pathways. These findings validate previous studies demonstrating diminished

PERK activation following TUDCA treatment in stressed oocytes and spermatogenic cells (Uppala et al., 2017; Khatun et al., 2020; Zhao et al., 2022). This decrease may indicate that excessive ER stress activation is unattainable at elevated temperatures, given the critical role of PERK signalling in regulating both beneficial and detrimental responses to ER stress (Fang et al., 2022).

Morphological observations confirmed these molecular results. Under normal circumstances, GC1 cells maintained their typical epithelioid morphology, and exposure to 200  $\mu$ M TUDCA resulted in only minor structural modifications. Conversely, treatment at the IC<sub>50</sub> concentration induced distinct cytopathic alterations, including cell rounding and reduction in cell volume, indicative of heightened cytotoxic stress. These results suggest that lower concentrations of TUDCA may maintain cellular integrity, whereas higher doses may impose an elevated metabolic burden *in vitro*.

TUDCA therapy not only affected PERK signalling but also corresponded with reduced CHOP immunoreactivity in heat-stressed GC1 cells. CHOP is a major downstream mediator of apoptosis caused by ER stress, and its levels have been shown to rise in several cellular stress models, such as heat stress and paraquat-induced damage (Song et al., 2019). The reduction in CHOP expression following TUDCA treatment signifies a decrease in ER stress-related apoptotic signalling, consistent with previous research that has demonstrated TUDCA's anti-apoptotic properties in diverse experimental settings (Khatun et al., 2020; Pioltine et al., 2021).

The findings of this study suggest that TUDCA may modify heat-induced ER stress and apoptosis-related signalling pathways in spermatogonial cells by influencing PERK-dependent unfolded protein response mechanisms and CHOP activation *in vitro*.

Despite the findings presented, several limitations of the present study should be acknowledged. First, the results are derived exclusively from an *in vitro* GC1 spermatogonial cell model, and therefore may not fully reflect the complexity of *in vivo* testicular physiology. Second, the analysis focused specifically on the PERK branch of the ER stress response, without evaluating other major unfolded protein response pathways such as IRE1 and ATF6. Future studies incorporating *in vivo* models and broader mechanistic approaches will be necessary to further clarify the role of ER stress modulation and to better understand the contribution of additional signalling pathways in heat-induced germ cell stress.

## Conclusion

This study investigated the responses of cells to heat stress through an *in vitro* GC1 spermatogonial cell model and evaluated the modulatory effects of TUDCA on ER stress-related pathways. Heat stress induced decreased cell viability and activation of PERK-dependent stress signalling, while TUDCA therapy produced a relative reduction of these responses under experimental conditions. Our findings contribute to the expanding evidence

indicating the significance of ER stress modulation in experimental studies of heat-induced germ cell stress and provide suggestions for further biological and *in vivo* studies.

## Institutional review board statement

Ethical approval for this study was obtained from the Kutahya Health Sciences University Non-Invasive Clinical Research Ethics Committee under acceptance number 2020/06-05.

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## Conflicts of interest

The authors declare that there are no conflicts of interest related to this study.

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