

***Tenebrio molitor* larvae extract prevents lipopolysaccharide-induced implantation failure
in pigs**

Running title: Mealworm extract prevents LPS-induced implantation failure in pigs

Juhyeong Seo^{1,†}, Hyunjin Kyoung^{1,†}, Minha Jeong¹, Hyun Uk Cho¹, Jumin Kim¹, Jiyeon Ham^{1,§}

¹Department of Animal Science & Biotechnology, College of Agriculture and Life Sciences,
Chungnam National University, Daejeon, 34134, Republic of Korea

[§]Corresponding author: Jiyeon Ham, Ph.D., Department of Animal Science & Biotechnology,
Chungnam National University, Daejeon 34134, Republic of Korea. Phone: +82-42-821-5778;
E-mail: jyham@cnu.ac.kr

[†]These authors contributed equally to this work.

Abstract

As an edible insect, the mealworm (*Tenebrio molitor*, TM) is recognized for its high protein content and various bioactive compounds that are expected to have antioxidant and anti-inflammatory properties. In swine production, enhancing endometrial receptivity and promoting successful implantation are critical for increasing litter size. However, factors such as inflammation caused by infection can sometimes hinder this process. The protective effects of mealworm-defatted extract (TM-DF) on lipopolysaccharide (LPS)-induced endometrial receptivity and implantation failure in swine have not yet been investigated. Therefore, we established an LPS-induced inflammation model using porcine luminal epithelial (pLE) cells obtained from early stage pregnant pigs, and investigated the protective effects of TM-DF against implantation failure. TM-DF pre-treatment significantly restored pLE cell viability, which was compromised by LPS exposure. TM-DF exhibited a strong protective effect by mitigating the excessive production of reactive oxygen species (ROS) and preventing the loss of mitochondrial membrane potential (MMP), maintaining mitochondrial integrity. In addition to these antioxidant effects, TM-DF pre-treatment effectively attenuated the LPS-induced upregulation of heat stress markers (HSP60, HSP70, and HSP90) and endoplasmic reticulum (ER) stress proteins (GRP78 and PERK) and attenuated their expression to normal physiological levels. TM-DF preserves the proliferative capacity of pLE cells by buffering cellular stress responses and protecting mitochondrial function through its bioactive activity. This study suggests that TM-DF is a promising therapeutic candidate for preventing LPS-induced endometrial dysfunction and implantation failure in pigs.

Keywords: *Edible insect, Tenebrio molitor, lipopolysaccharide (LPS), implantation, luminal epithelial cell*

1. Introduction

Implantation is a critical process that determines reproductive efficiency and litter size in pigs. Recent research using omics data has also proposed strategies to increase litter size in pigs. This includes increasing litter size through cytokine supplementation (1) and studies elucidating the mechanisms of receptivity formation through the analysis of endometrial proteins during the implantation period (2). Endometrial receptivity is key to successful implantation. In an omics study comparing embryo loss and successful pregnancies in pigs using single-transcriptome profiling, implantation markers such as *homeobox A10 (HOXA10)*, *insulin-like growth factor-binding protein 2 (IGFBP2)*, and *leukemia inhibitory factor (LIF)* were reduced in cases of embryo loss. Immune homeostasis was disrupted, forming IL-1 α dominated pro-inflammatory microenvironments, and high levels of reactive oxygen species (ROS) were observed (3). In farm environments, sows are exposed to various infectious and inflammatory stimuli. This includes the bacterial endotoxin lipopolysaccharide (LPS), which leads to endometrial inflammation, excessive oxidative stress, and structural damage. This causes implantation failure and induces inflammatory preterm birth and miscarriage, substantially reducing reproductive efficiency (4-6).

Similarly, research has been conducted on the use of feed additives containing bioactive compounds that can enhance antioxidant and anti-inflammatory properties, and receptivity. Melatonin prevents lipid accumulation in the uterus of sows at the cellular level and increases endometrial receptivity by regulating the PI3K/AKT signaling pathway (7). Melatonin improved endometrial receptivity and increases litter size in a mouse model (8). Fucoidan has been proven effective in downregulating the iNOS/AP-1 signaling pathway, which, in turn, diminishes NO levels in porcine peripheral blood mononuclear cells (9). These results provide a strong rationale for ongoing research aimed at improving inflammatory preterm birth outcomes using natural bioactive products.

Edible insects (EI) have been studied for their potential use in the food, feed, and biomedical sectors owing to their low nutritional value and high protein production efficiency (10, 11). EI can provide high protein content (35–65%), unsaturated fatty acids, essential micronutrients, and bioactive compounds (12). Owing to this potential, the global EI market is expected to grow from approximately USD 3.2 billion in 2023 to USD 9.8 billion by 2030 (10). Among EI, *Tenebrio molitor* (TM) is widely used because of its suitability for large-scale production and industrial processing (13). Recent studies have focused on the biological activity of TM. Yu et al. (2016) found that TM extracts exert anti-inflammatory and antioxidant effects by suppressing inflammatory mediators and scavenging free radicals (14).

In this study, we focused on the bioactive properties of TM-DF, a defatted extract of TM larvae. We conducted experiments using porcine uterine endometrial luminal epithelial (pLE) cells to investigate its preventive effects against LPS-induced inflammatory endometritis. TM-DF restored LPS-induced decreases in cell viability, increased the levels of reactive oxygen species (ROS), and alleviated the impaired mitochondrial membrane potential (MMP). TM-DF reduced the expression of heat shock proteins (HSPs), which are known intracellular stress markers, and the expression of endoplasmic reticulum (ER) stress proteins. This demonstrated that TM-DF has a protective effect against the LPS-mediated inflammatory environment.

2. Materials and Methods

2.1. Chemicals and Reagents

For defatting, n-hexane (96.0%; Cat. No. 4081-4110) were purchased from Daejung Chemicals and Metals Co., Ltd. (Siheung, Gyeonggi-do, Korea). For the DPPH radical scavenging assay, DPPH (2,2-diphenyl-1-picrylhydrazyl) (95.0%; Cat. No. 044150.MD) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). For reducing power assay iron (III) chloride anhydrous (98.0%; Cat. No. 5022-1405) was purchased from Samchun

Chemicals Co., Ltd. (Seoul, Korea). Potassium ferricyanide (Cat. No. 6574-4405) from Daejung Chemicals & Metals Co., Ltd. (Siheung, Gyeonggi-do, Korea), and trichloroacetic acid from Duksan Pure Chemicals Co., Ltd. (Ansan, Gyeonggi-do, Korea). Lipopolysaccharides from *Escherichia coli* O55:B5 (Cat. No. L2880) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of edible insect extract

Tenebrio molitor (TM) larvae was purchased from a commercial supplier (Cheongnyeon mealworm, Daegu, Korea). The defatting process for edible insects was slightly modified from the method described by Kim et al. (2022) (15). As described by Figure 1, *Tenebrio molitor* (TM) larvae (50 g) were mixed with n-hexane (96%) at a ratio of 1:5 (w/v). The mixture was stirred at 20 °C for 1 h to extract lipids. After extraction, the solvent phase was removed and the extraction was repeated five times under the same conditions. The residual n-hexane was evaporated overnight in a fume hood. The defatted TM larvae (TM-DF) were mixed with distilled water at a ratio of 1:10 (w/v) and stirred at 60 °C for 24 h for aqueous extraction. This step was repeated twice and the extracts were pooled. The combined extracts were filtered through a filter paper, and the filtrate was concentrated using a rotary vacuum concentrator (EYELA, Tokyo, Japan). The concentrate was freeze-dried (Bondiro, Ilshin, Seoul, Korea) to obtain the TM-DF, which was stored in a desiccator until further analysis.

2.3. Measurement of 2,2-Diphenyl-1-Picryl-Hydrazyl (DPPH) radical scavenging activity

The DPPH radical scavenging assay was performed according to the method described by Yong et al. (2023) (11). Each TM-DF sample was prepared at six concentrations (0.005, 0.05, 0.1, 0.2, 0.3, 0.5, and 1 mg/mL). The sample was then mixed with DPPH radicals at a 1:1 ratio. The solution was allowed to react at room temperature in the dark for 30 min. The absorbance

of each supernatant was measured at 517 nm using a spectrophotometer (Varioskan Lux; Thermo Fisher Scientific). Radical scavenging activity was calculated as a percentage using the following equation:

$$DPPH \text{ radical scavenging activity (\%)} = (1 - [A_s/A_c]) \times 100$$

Where A_c is the absorbance of the control and A_s is the absorbance of the sample.

2.4. Measurement of reducing power of edible insect extract

Each TM-DF sample was prepared at six concentrations (0.005, 0.05, 0.1, 0.2, 0.3, 0.5, and 1 mg/ml). Then, each sample (200 μ L) was mixed with 0.05M phosphate buffer (500 μ L; pH 6.6) and 1% potassium ferricyanide (500 μ L), followed by incubation at 50 °C water bath for 20 min. After cooling, 10% trichloroacetic acid solution (500 μ L) was added, and the mixture was centrifuged at $10,000 \times g$ for 10 min (MICRO 17TR, Hanil Science Industrial Co., Incheon, Korea). The supernatants were mixed with distilled water (500 μ L) and 0.1% ferric chloride (100 μ L), and the absorbance recorded at 700 nm using a spectrophotometer (Varioskan Lux). Reducing power was calculated using the following equation:

$$Reducing \text{ power (\%)} = ([A_s/A_c] - 1) \times 100$$

Where A_c is the absorbance of the control and A_s is the absorbance of the sample.

2.5. Cell culture methods

Porcine uterine endometrial luminal epithelial (pLE) cells were obtained from a day 12 pregnant gilt (16). pLE cells were cultured in DMEF/F-12 (1:1) medium (Cat No. SH30023.01; Cytiva, USA) supplemented with 10% fetal bovine serum (FBS) (Cat No. SH30919.03; Cytiva), 1% Zell Shield (Cat No. 13-0050, Minerva, USA), and 0.1% insulin (Cat No. I5500, Sigma-Aldrich). The cells were cultured in a 37 °C, 5% CO₂ incubator.

2.6. MTT and BrdU assay for measurement of cell viability

To evaluate changes in cell viability and proliferation in response to TM-DF, or LPS treatment, an MTT cell proliferation kit (Cat No. 11465007001, Roche, Indianapolis, IN, USA) and BrdU cell proliferation kit (Cat No. 11647229001, Roche) were used. MTT assay defined the proportion of metabolically active and live cells and BrdU assay quantified active DNA synthesis and cell division over time. pLE cells were seeded into 96-well plates at a density of 3×10^3 cells/well. Upon reaching appropriate confluence, the cells were incubated in serum-free medium for 24 h. The cells were treated with various concentrations of TM-DF (0, 1, 2, 5, 7.5 and 10 $\mu\text{g/mL}$) or LPS (0, 5, 10, 50, and 100 $\mu\text{g/mL}$) for 24–48 h. Proper reagents were added following the manufacturer's instructions, and the final optical density (OD) values were measured.

2.7. Determination of cellular reactive oxygen species (ROS)

Intracellular reactive oxygen species (ROS) levels were measured by staining with 2',7'-dichlorofluorescein diacetate (DCF-DA, Sigma-Aldrich) for 30 min in a 37 °C incubator. This was followed by treatment with TM-DF (5 $\mu\text{g/mL}$) and LPS (50 $\mu\text{g/mL}$) for 1 h. After treatment, the cells were washed with PBS, resuspended, and their fluorescence was measured using a flow cytometer. The results were evaluated using CytoExpert software.

2.8. Measurement of mitochondrial membrane potential (MMP)

Mitochondrial membrane potential (MMP) was determined using a JC-1 mitochondrial membrane potential detection kit (Cat No. 30001, Biotium). After seeding pLE cells in 6-well plates, the cells were pre-treated with TM-DF (5 $\mu\text{g/mL}$) for 2 h, followed by treatment with LPS (50 $\mu\text{g/mL}$) for 48 h. The cells were harvested, washed with PBS, and resuspended for

analysis. MMP was measured via flow cytometry, and evaluation was performed using CytoExpert software.

2.9. Western blot analysis

Proteins were extracted from cells pretreated with TM-DF (5 µg/mL) for 2 h and then treated with LPS (50 µg/mL) for 24 h. The concentrations of the extracted proteins were determined using the Bradford assay with bovine serum albumin (BSA) as the standard. Equal amounts of protein were separated using SDS-PAGE and transferred onto a nitrocellulose membrane. Each membrane was blocked with 5% BSA-TBST and incubated overnight at 4 °C with primary antibodies against HSP60 (Cat. No. 12165) and HSP70 (Cat. No. 4872) and HSP90 (Cat. No. 4877) and PERK (Cat. No. 3192) were purchased from Cell Signaling Technology (CST) (Danvers, MA, USA), and GRP78 (Cat. No. sc-166490) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The membranes were then washed thrice with TBST and incubated with peroxidase-labeled goat anti-rabbit (Cat. No. 474-1506) and goat anti-mouse (Cat. No. 474-1806) as secondary antibodies (KPL; Sera Care) at room temperature for 1 h. Enhanced chemiluminescence (ECL) was applied to the membranes, and the bands were visualized using an imaging system. GAPDH (Cat. No. 2118) from CST were used as internal controls for protein quantification, and band intensities were measured using the Image Lab software.

2.10. Statistical analysis

All the data were subjected to analysis of variance (ANOVA) using the general linear model (PROC-GLM) of SAS software (SAS Institute, Cary, NC, USA) to determine whether there were significant differences in cell viability, proliferation, intracellular ROS levels, MMP, and protein expression in pLE cells in response to treatment. Statistically significant *p*-value are

represented as asterisks (*) between the control and the LPS- or TM-DF groups (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). Crosshatches (#) indicate differences between the LPS-sole and LPS with TM-DF pre-treatment groups (# $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$).

3. Results

3.1. Antioxidant activities of TM-DF (*Tenebrio molitor* defatted extracts)

The DPPH radical scavenging activity and reducing power of TM-DF increased progressively with increasing concentration (0–1 mg/mL) (Figure 2A–B). A strong linear relationship was observed between the TM-DF concentration and DPPH scavenging activity ($R^2 = 0.9978$) and reducing power ($R^2 = 0.9991$), indicating a concentration-dependent antioxidant response. At 1 mg/mL of TM-DF, the DPPH scavenging activity and reducing power were $70.55 \pm 0.23\%$ and $93.96 \pm 2.21\%$, respectively.

3.2. Impact of TM-DF and LPS on viability of porcine luminal epithelial cells (pLE)

To evaluate the effect of TM-DF on cellular viability of pLE cells, we treated them with TM-DF at various concentrations of 0, 1, 2, 5, 7.5, and 10 $\mu\text{g/mL}$ for 24 h. The viability of pLE cells were significantly increased to 122.2% and 115.1% compared to control by 5 and 7.5 $\mu\text{g/mL}$ of TM-DF extract, respectively. Meanwhile, 10 $\mu\text{g/mL}$ of TM-DF extract rather decreased viability of pLE cells to 84.9% (Figure 3A). Among these, 5 $\mu\text{g/mL}$ of TM-DF, which increased cell viability by approximately 22.2%, was selected as the optimal treatment condition for further study. Subsequently, pLE cells were challenged with various concentrations of LPS (0, 5, 10, 50, and 100 $\mu\text{g/mL}$) for 48 h to determine whether it affected cellular proliferation. From 10 to 100 $\mu\text{g/mL}$ of LPS critically reduced cellular proliferation to 82.4% to 88.3% in pLE cells (Figure 3B). 50 $\mu\text{g/mL}$ of LPS were selected as inflammation-

induced cell model for subsequent studies aimed at elucidating the underlying mechanisms. This appears to reliably induce short-term inflammatory responses and cytotoxicity.

3.3 TM-DF extracts prevent LPS-induced cell death in pLE cells

We next investigated whether the TM-DF extract had a cytoprotective effect on LPS-induced proliferation of pLE cells. For this, pLE cells was pre-treated with TM-DF (5 µg/mL) for 2 h, followed by exposure to LPS (50 µg/mL) for 48 h. We confirmed that pre-treatment with TM-DF significantly restored cellular proliferation up to 112.4% compared to the control, which had been reduced to 82.3% by LPS (Figure 3C). These findings confirm that TM-DF has a cytoprotective effect against LPS in pLE cells.

3.4. TM-DF extracts alleviate LPS-induced oxidative stress and mitochondrial dysfunction in pLE cells

To investigate whether the preventive cytoprotective effect of TM-DF was associated with the maintenance of intracellular homeostasis, changes in intracellular ROS levels and mitochondrial membrane permeability were evaluated. LPS-sole treatment increased ROS generation to 125.4% of the control group in pLE cells. Meanwhile, TM-DF sole treatment rather decreased ROS levels to 83.6% (Figure 4A). Following TM-DF pre-treatment, the LPS-treated group exhibited a protective effect against oxidative stress similar to normal levels. This resulted in an approximately 35.8% decrease in ROS production compared to the LPS-only treatment group in the pLE cells (Figure 4A).

The relative loss ratio of MMP was elevated by more than 2-fold in LPS-treated cells compared to that in vehicle-treated cells (Figure 4B). LPS-induced mitochondrial membrane permeabilization in pLE cells was restored to normal levels by pre-treatment with TM-DF (Figure 4B). These findings suggest that TM-DF exerts cytoprotective effects on the porcine

luminal endometrium by preventing LPS-induced ROS accumulation and MMP loss.

3.5. TM-DF extracts regulate LPS-induced heat-stress and ER-stress signaling pathway in pLE cells

To evaluate the cytoprotective effect of TM-DF at the protein level, the expression of heat shock proteins (HSP) and ER stress markers was assessed using western blot analysis. In pLE cells, LPS significantly upregulated the expression of HSP60, HSP70, and HSP90 by approximately 1.46-fold, 1.52-fold, and 1.43-fold, respectively (Figure 5A–C). However, TM-DF pre-treatment did not result in any change compared with the control group (Figure 5A–C). Rather, pre-treatment with TM-DF alleviated upregulated-HSPs proteins to the normal levels, which were induced by LPS (Figure 5A–C). The expression of the ER stress marker proteins GRP78 and PERK also increased 1.62- and 1.41-fold, respectively, in the LPS-only group (Figure 5D–E). TM-DF pre-treatment restored these upregulated GRP78 and PERK expressions to normal levels in pLE cells (Figure 5D–E). These results demonstrate that TM-DF suppresses the LPS-induced overexpression of HSPs and ER stress-related proteins.

4. Discussion

Recent studies have highlighted TM larvae as an alternative protein source and a functional feed ingredient with nutritional and metabolic regulatory properties. TM larvae contain high-quality crude protein with a well-balanced, indispensable amino acid profile and high digestibility. This supports efficient protein use in monogastric animals (17-19). Mealworms are rich in unsaturated fatty acids, chitin-derived polysaccharides, and bioactive compounds, including antimicrobial peptides (AMPs), polyphenols, and bioactive peptides. These are associated with immune modulation, metabolic regulation, and antioxidant activity (20-23). Dietary supplementation of TM larvae or their processed products, for example,

defatted or hydrolyzed forms, improve growth performance, nutrient digestibility, and nitrogen use in monogastric animals (21, 24-26). Improved crude protein digestibility, reduced blood urea nitrogen, and increased insulin-like growth factor-1 (IGF-1) have been reported in weaning pigs. This indicates enhanced amino acid utilization and systemic metabolic responses (27). Hydrolyzed mealworm products enhance the digestibility of crude proteins and are indispensable amino acids in growing pigs (28). Insect-derived AMPs exhibit antimicrobial activity by disrupting bacterial membranes and interacting with LPS. This may contribute to modulation of immune responses and alterations of intestinal microbial populations (29, 30). These properties suggest a potential role for insect-derived components in the regulation of endotoxin-induced inflammatory responses. Processing strategies such as defatting and hydrolysis may enhance the bioavailability of functional peptides and improve their antioxidant and anti-inflammatory activities (22, 31).

DPPH is a chemically stable radical widely used to assess radical-scavenging activity and antioxidant capacity. Quenching of DPPH radicals occurs primarily through hydrogen atom donation ($H\bullet$) from antioxidant compounds, resulting in a measurable decrease in radical intensity. Reducing power is commonly used to assess antioxidant potential, as it reflects the electron-donating ability of a sample. Although not all reductants function as antioxidants, compounds with strong reducing capacities are generally associated with enhanced antioxidant activities (32). Therefore, higher DPPH radical scavenging activity and reducing power indicate a greater antioxidant capacity. The results shown in Figure 2 are consistent with those of previous reports, indicating that edible insects possess various antioxidant activities. According to Yong et al. (2023), ethanolic extracts from TM larvae exhibited DPPH and 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate) (ABTS) radical-scavenging activity, reducing power, and metal-chelating ability (11). They also proposed that the antioxidant potential of

TM larval ethanol extracts could be attributed to their phenolic compounds, which were measured at 16.38 ± 0.50 mg/mL. In a similar context, the 20% ethanol-treated TM larvae protein showed ABTS radical-scavenging activity (approximately 38%) and provided the strongest protection against H₂O₂-induced oxidative stress in Vero cells relative to the untreated protein (approximately 34%) (33). Therefore, *T. molitor* may be a promising source of functional compounds with antioxidant properties. TM larva-derived extracts function as bioactive regulators under metabolic and inflammatory stress conditions. Our findings demonstrate that the attenuation of LPS-induced stress responses and the modulation of cell proliferation in pLE cells are consistent with the functional characteristics of TM larva-derived extracts.

LPS is a component of the outer membrane of the cell wall in *Escherichia coli*. It is released during bacterial death or proliferation, and is a highly toxic bacterial endotoxin. LPS causes a decline in reproductive performance in livestock. LPS exposure in lactating dairy cows reduced the conception rate by approximately 30% (34). In swine, repeated administration of LPS to sows significantly decreased daily feed intake and milk yield, resulting in a 0.32 kg reduction in the daily weight gain of their piglets (35). Co-infections with various pathogens, including chlamydiosis, PRRS, and PCV2, along with associated inflammatory damage, are the major causes of reproductive disorders in sows (36). To elucidate the molecular mechanisms underlying LPS-induced reproductive failure and to explore preventive strategies, we used an in vitro model by challenging pLE cells with LPS to induce an inflammatory and oxidative stress environment within the reproductive tract.

Here, pre-treatment with TM-DF successfully restored the LPS-induced inhibition of cell proliferation in pLE cells. Reduced cell viability caused by LPS (50 µg/mL) in porcine mammary epithelial cells was recovered by selenium yeast (37). In porcine intestinal epithelial

cells, LPS treatment reduced cell viability and induced ROS accumulation; however, these effects were alleviated by luteolin, a natural flavonoid (38). Similarly, there are numerous previous studies showing that LPS-induced inflammatory conditions are alleviated when treated with various natural products or extracts (39, 40). We confirmed that TM-DF significantly mitigated LPS-induced elevation of intracellular ROS and loss of MMP in pLE cells. Excessive intracellular ROS accumulation induces lipid peroxidation of the mitochondrial membrane, leading to the collapse of MMP and, ultimately, a decline in cell proliferation (41). Importantly, such oxidative stress and mitochondrial dysfunction are known to severely compromise endometrial receptivity by disrupting cellular energy metabolism and normal physiological signaling required for successful embryo implantation in humans (42). These findings suggest that TM-DF not only protects cellular viability but also has the potential to preserve the functional receptivity of pLE cells against LPS-induced ROS accumulation and MMP depolarization.

To further elucidate the cytoprotective mechanisms of TM-DF, the expression levels of HSPs and ER stress-related proteins were evaluated. HSPs are essential molecular chaperones that respond to heat stress and prevent protein denaturation and facilitate proper folding under external stimuli, such as oxidative stress. When LPS binds to the TLR4 receptor on the cell surface, it elevates ROS levels and triggers an inflammatory response, leading to oxidative damage and the misfolding of intracellular proteins, which in turn upregulates the expression of chaperone proteins such as HSP70 and HSP90 (43). This prevents cell damage and promoting survival (44, 45). ER stress occurs when the accumulation of unfolded proteins within the cell reaches toxic levels (46). The UPR is activated to ensure cell survival during ER stress, the unfolded protein response (UPR) is activated, with GRP78 and PERK as key initiators (47). In resting cells, GRP78 resides in the ER lumen and inhibits UPR sensors such

as PERK, IRE1 and ATF6, by binding to them. However, when unfolded proteins increase owing to ER stress, GRP78 preferentially binds to these proteins. This releases sensors such as PERK to initiate the UPR (48). PERK is activated via autophosphorylation, inducing eIF2 α activation and ATF4 transcription to transmit survival signals (49). Under cellular stress, HSP70, an ER-resident chaperone, collaborates with the ER stress marker, GRP78, to bind to unfolded proteins, releasing PERK/IRE1/ATF6 sensors and promoting early UPR activation (50). HSP90 functions in tandem with HSP70 in the cytosol to alleviate ER stress (51). HSP60 is primarily localized within the mitochondria. Here, it protects mitochondrial proteins under oxidative stress, defends against MMP depolarization, and blocks apoptotic pathways (52). Thus, the HSP and ER stress defense mechanisms act as an integrated cellular protection system rather than separate pathways. This is supported by a previous report in porcine granulosa cells, where LPS was shown to increase HSP70 expression (53). Our results demonstrated that TM-DF pre-treatment effectively suppressed the expression of HSP and ER stress-related proteins induced by LPS. It is well established that an increased expression of HSP and ER stress-related proteins—which are involved in maintaining protein homeostasis—when induced by stressors such as LPS, leads to cell death and disrupts the normal embryo-receptive environment (54). In particular, it is well established that the excessive expression of HSPs and severe ER stress can induce fibrosis in various tissues, including the lungs and liver (55, 56). The accumulation of cellular stress and fibrosis within the endometrial tissue can disrupt decidualization—a process essential for implantation—and significantly impair endometrial receptivity (57). Taken together, TM-DF protects porcine endometrial cells by preventing LPS-triggered heat shock and ER stress signaling cascades, thereby potentially contributing to enhanced endometrial receptivity.

5. Conclusion

In this study, we elucidated the protective effects of defatted *Tenebrio molitor* (TM-DF) larval extract against LPS-induced cellular stress in porcine luminal epithelial (pLE) cells. Our findings validated the intrinsic antioxidant capacity and reducing power of TM-DF. We confirmed pre-treatment of TM-DF effectively restored pLE cell proliferation, which was suppressed by LPS. LPS maintains mitochondrial function and prevents excessive ROS production at the cellular level. These robust protective effects were also verified in intracellular signaling pathways: suppressed activation of heat stress and ER stress following TM-DF pre-treatment.

Although the current in vitro model has limitations in demonstrating the complexities of the implantation process, this study is important because it preemptively verifies the molecular efficacy of TM-DF, supporting its potential for industrial applications as a functional resource. These results provide a fundamental molecular basis for the use of TM-DF as a potential therapeutic agent to alleviate inflammation-induced reproductive challenges.

Acknowledgments

The authors gratefully acknowledge Professor Fuller W. Bazer at Texas A&M University for kindly providing the pLE cell line.

Funding

This work was supported by BK21 FOUR Program by Chungnam National University Research Grant, 2025. Also, this research was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (RS-2026-25479407).

CRedit author statement

Juhyeong Seo: data curation, investigation, visualization, writing-original draft, writing-review & editing; Hyunjin Kyoung: funding acquisition, writing-original draft, writing-review & editing; Minha Jeong: data curation, investigation, writing-review & editing; Hyun Uk Cho:

investigation, visualization, writing-review & editing; Jumin Kim: conceptualization, validation, writing-review & editing; Jiyeon Ham: conceptualization, validation, writing-review & editing, supervision, project administration, funding acquisition

Conflicts of interest

Juhyeong Seo declares that he has no conflict of interest. Hyunjin Kyoung declares that he has no conflict of interest. Minha Jeong declares that she has no conflict of interest. Hyun Uk Cho declares that he has no conflict of interest. Jumin Kim declares that she has no conflict of interest. Jiyeon Ham declares that she has no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Data availability statement

All datasets presented in this study are available from the corresponding author on reasonable request.

References

1. Lucas CG, Chen PR, Seixas FK, Prather RS, Collares T. Applications of omics and nanotechnology to improve pig embryo production in vitro. *Mol Reprod Dev.* 2019;86(11):1531-47.
2. Wang Y, Xue S, Liu Q, Gao D, Hua R, Lei M. Proteomic profiles and the function of RBP4 in endometrium during embryo implantation phases in pigs. *BMC Genomics.* 2023;24(1):200.
3. Chu T, Jin Y, Wu G, Liu J, Sun S, Song Y, et al. Insights into the single-cell transcriptome characteristics of porcine endometrium with embryo loss. *FASEB J.* 2025;39(6):e70395.
4. Romero R, Espinoza J, Goncalves LF, Kusanovic JP, Friel L, Hassan S. The role of inflammation and infection in preterm birth. *Semin Reprod Med.* 2007;25(1):21-39.
5. Kim M, Lim W, Song G, Park SJM, Toxicology C. Flutriafol disrupts trophoblast function: mitochondrial dysfunction mediated cell cycle arrest and apoptosis. 2025;21(2):429-42.
6. Kim M, Song G, Park SJM, Toxicology C. Adverse effects of halogenated organic compounds on implantation and placental development. 2026;22(2):239-46.

7. Qin X, Yang M, Yu Y, Wang X, Zheng Y, Cai R, et al. Melatonin improves endometrial receptivity and embryo implantation via MT2/PI3K/LIF signaling pathway in sows. 2025;16(1):4.
8. Qin X, Yang M, Yu Y, Wang X, Zheng Y, Cai R, et al. Melatonin improves endometrial receptivity and embryo implantation via MT2/PI3K/LIF signaling pathway in sows. J Anim Sci Biotechnol. 2025;16(1):4.
9. Park J, Ahn C, Kang B-T, Kang J-H, Jeung E-B, Yang M-PJovc. Effects of fucoidan on nitric oxide production and activator protein-1 activation in lipopolysaccharide-stimulated porcine peripheral blood mononuclear cells. 2015;32(4):289-94.
10. Abbasi EJA, Security F. Edible insects in human nutrition: nutritional value, economic potential, and environmental implications for sustainable food production. 2026.
11. Yong HI, Kim T-K, Cha JY, Lee JH, Kang M-C, Jung S, et al. Effects of edible insect extracts on the antioxidant, physiochemical, and microbial properties of Tteokgalbi during refrigerated storage. 2023;52:102377.
12. Lee JH, Kim TK, Cha JY, Jang HW, Yong HI, Choi YS. How to develop strategies to use insects as animal feed: digestibility, functionality, safety, and regulation. J Anim Sci Technol. 2022;64(3):409-31.
13. Efsa Panel on Nutrition NF, Food A, Turck D, Bohn T, Castenmiller J, De Henauw S, et al. Safety of frozen and dried formulations from whole yellow mealworm (*Tenebrio molitor* larva) as a novel food pursuant to Regulation (EU) 2015/2283. EFSA J. 2021;19(8):e06778.
14. Yu J-M, Jang J-Y, Kim H-J, Cho Y-H, Kim D-i, Kwon O-j, et al. Antioxidant capacity and Raw 264.7 macrophage anti-inflammatory effect of the *Tenebrio molitor*. 2016;23(6):890-8.
15. Kim TK, Lee JH, Yong HI, Kang MC, Cha JY, Chun JY, et al. Effects of Defatting Methods on the Physicochemical Properties of Proteins Extracted from *Hermetia illucens* Larvae. Foods. 2022;11(10).
16. Wang G, Johnson GA, Spencer TE, Bazer FW. Isolation, immortalization, and initial characterization of uterine cell lines: an in vitro model system for the porcine uterus. In Vitro Cell Dev Biol Anim. 2000;36(10):650-6.
17. Ghosh S, Lee S-M, Jung C, Meyer-Rochow VBjJoA-PE. Nutritional composition of five commercial edible insects in South Korea. 2017;20(2):686-94.
18. Hong J, Han T, Kim YY. Mealworm (*Tenebrio molitor* Larvae) as an Alternative Protein Source for Monogastric Animal: A Review. Animals (Basel). 2020;10(11).
19. Makkar HP, Tran G, Heuzé V, Ankers PJAfs, technology. State-of-the-art on use of insects as animal feed. 2014;197:1-33.
20. Lawal KG, Kavle RR, Akanbi TO, Miroso M, Agyei DJFF. Enrichment in specific fatty acids profile of *Tenebrio molitor* and *Hermetia illucens* larvae through feeding. 2021;3:100016.
21. Bovera F, Loponte R, Marono S, Piccolo G, Parisi G, Iaconisi V, et al. Use of larvae meal as protein source in broiler diet: Effect on growth performance, nutrient digestibility, and carcass and meat traits. J Anim Sci. 2016;94(2):639-47.
22. Syahrulawal L, Torske MO, Sapkota R, Naess G, Khanal P. Improving the nutritional values

- of yellow mealworm *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae as an animal feed ingredient: a review. *J Anim Sci Biotechnol.* 2023;14(1):146.
23. Kim T-K, Yong HI, Kim Y-B, Kim H-W, Choi Y-SJFsoar. Edible insects as a protein source: a review of public perception, processing technology, and research trends. 2019;39(4):521.
 24. De Marco M, Martínez S, Hernandez F, Madrid J, Gai F, Rotolo L, et al. Nutritional value of two insect larval meals (*Tenebrio molitor* and *Hermetia illucens*) for broiler chickens: Apparent nutrient digestibility, apparent ileal amino acid digestibility and apparent metabolizable energy. 2015;209:211-8.
 25. Yoo JS, Cho KH, Hong JS, Jang HS, Chung YH, Kwon GT, et al. Nutrient ileal digestibility evaluation of dried mealworm (*Tenebrio molitor*) larvae compared to three animal protein by-products in growing pigs. *Asian-Australas J Anim Sci.* 2019;32(3):387-94.
 26. Ko H, Kim Y, Kim J. The produced mealworm meal through organic wastes as a sustainable protein source for weanling pigs. *J Anim Sci Technol.* 2020;62(3):365-73.
 27. Jin XH, Heo PS, Hong JS, Kim NJ, Kim YY. Supplementation of Dried Mealworm (*Tenebrio molitor* larva) on Growth Performance, Nutrient Digestibility and Blood Profiles in Weaning Pigs. *Asian-Australas J Anim Sci.* 2016;29(7):979-86.
 28. Cho KH, Kang SW, Yoo JS, Song DK, Chung YH, Kwon GT, et al. Effects of mealworm (*Tenebrio molitor*) larvae hydrolysate on nutrient ileal digestibility in growing pigs compared to those of defatted mealworm larvae meal, fermented poultry by-product, and hydrolyzed fish soluble. *Asian-Australas J Anim Sci.* 2020;33(3):490-500.
 29. Jozefiak A, Engberg RMJJoA, Sciences F. Insect proteins as a potential source of antimicrobial peptides in livestock production. A review. 2017;26(2):87-99.
 30. Veldkamp T, Dong L, Paul A, Govers CJJolaF, Feed. Bioactive properties of insect products for monogastric animals—a review. 2022;8(9):1027-40.
 31. Zielinska E, Baraniak B, Karas M. Antioxidant and Anti-Inflammatory Activities of Hydrolysates and Peptide Fractions Obtained by Enzymatic Hydrolysis of Selected Heat-Treated Edible Insects. *Nutrients.* 2017;9(9).
 32. Lee JH, Kim T-K, Jeong CH, Yong HI, Cha JY, Kim B-K, et al. Biological activity and processing technologies of edible insects: a review. 2021;30(8):1003-23.
 33. Lee JH, Kim Y-J, Kim T-K, Song K-M, Choi Y-SJFC. Effect of ethanol treatment on the structural, techno-functional, and antioxidant properties of edible insect protein obtained from *Tenebrio molitor* larvae. 2024;437:137852.
 34. Baek K, Park S, Park S, Kim H, Lee W, Jeong G, et al. Effect of Treatment of Lipopolysaccharides on Reproductive Performance in Lactating Dairy Cows. 2006;21(3).
 35. Sauber TE, Stahly TS, Nonnecke BJ. Effect of level of chronic immune system activation on the lactational performance of sows. *J Anim Sci.* 1999;77(8):1985-93.
 36. Wang Y, Jin Y, Wang Y, Li Y, Wang X, Li Z, et al. Sow reproductive disorders: a key issue affecting the pig industry. *Front Vet Sci.* 2025;12:1535719.
 37. He Z, Su S, Zhang B, Chen D, Yuan S, Guan W, et al. Selenium yeast attenuated

- lipopolysaccharide-induced inflammation in porcine mammary epithelial cells by modulating MAPK and NF- κ B signaling pathways. 2025;14(3):334.
38. Yuan J, Zhang K, Yang L, Cheng X, Chen J, Guo X, et al. Luteolin attenuates LPS-induced damage in IPEC-J2 cells by enhancing mitophagy via AMPK signaling pathway activation. *Front Nutr*. 2025;12:1552890.
39. Yang YJ, Kim MJ, Heo JW, Choi HN, Lee D, Kim YH, et al. Angelica gigas Nakai hot water extracts alleviate IL-6-induced ulcerative colitis by restoring intestinal barrier function. 2025;21(3):639-49.
40. Kang DY, Park Y, Shin H, Kwon Y, Kim HT, Shin W, et al. Protocatechuic acid alkyl ester derivatives alleviate TNF- α and LPS-induced inflammation in HaCaT cells. 2026;22(1):117-26.
41. Su LJ, Zhang JH, Gomez H, Murugan R, Hong X, Xu D, et al. Reactive Oxygen Species-Induced Lipid Peroxidation in Apoptosis, Autophagy, and Ferroptosis. *Oxid Med Cell Longev*. 2019;2019:5080843.
42. Hou Z, Hua Y, Gao S, Si F, Wang S, Jin C, et al. Mechanisms and clinical implications of oxidative stress-mediated endometrial receptivity impairment in polycystic ovary syndrome. *J Ovarian Res*. 2026;19(1).
43. Liu PY, Shen HH, Kung CW, Chen SY, Lu CH, Lee YM. The Role of HSP70 in the Protective Effects of NVP-AUY922 on Multiple Organ Dysfunction Syndrome in Endotoxemic Rats. *Front Pharmacol*. 2021;12:724515.
44. Benjamin IJ, McMillan DR. Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. *Circ Res*. 1998;83(2):117-32.
45. Becker J, Craig EA. Heat-shock proteins as molecular chaperones. *Eur J Biochem*. 1994;219(1-2):11-23.
46. Corazzari M, Gagliardi M, Fimia GM, Piacentini M. Endoplasmic Reticulum Stress, Unfolded Protein Response, and Cancer Cell Fate. *Front Oncol*. 2017;7:78.
47. Li J, Ni M, Lee B, Barron E, Hinton DR, Lee AS. The unfolded protein response regulator GRP78/BiP is required for endoplasmic reticulum integrity and stress-induced autophagy in mammalian cells. *Cell Death Differ*. 2008;15(9):1460-71.
48. Ong G, Ragetli R, Mnich K, Doble BW, Kammouni W, Logue SE. IRE1 signaling increases PERK expression during chronic ER stress. *Cell Death Dis*. 2024;15(4):276.
49. Louessard M, Bardou I, Lemarchand E, Thiebaut AM, Parcq J, Leprince J, et al. Activation of cell surface GRP78 decreases endoplasmic reticulum stress and neuronal death. *Cell Death Differ*. 2017;24(9):1518-29.
50. Terrab L, Wipf P. Hsp70 and the Unfolded Protein Response as a Challenging Drug Target and an Inspiration for Probe Molecule Development. *ACS Med Chem Lett*. 2020;11(3):232-6.
51. Marcu MG, Doyle M, Bertolotti A, Ron D, Hendershot L, Neckers L. Heat shock protein 90 modulates the unfolded protein response by stabilizing IRE1 α . *Mol Cell Biol*. 2002;22(24):8506-13.
52. Zhou C, Sun H, Zheng C, Gao J, Fu Q, Hu N, et al. Oncogenic HSP60 regulates mitochondrial

oxidative phosphorylation to support Erk1/2 activation during pancreatic cancer cell growth. Cell Death Dis. 2018;9(2):161.

53. Li H, Guo S, Cai L, Ma W, Shi Z. Lipopolysaccharide and heat stress impair the estradiol biosynthesis in granulosa cells via increase of HSP70 and inhibition of smad3 phosphorylation and nuclear translocation. Cell Signal. 2017;30:130-41.

54. Wu JX, Lin S, Kong SB. Psychological Stress and Functional Endometrial Disorders: Update of Mechanism Insights. Front Endocrinol (Lausanne). 2021;12:690255.

55. He Y, Xu J, Hong S, Tao QJM, Toxicology C. HSP90AA1/P450s as a new target of nicotine metabolite cotinine induced liver fibrosis: a comprehensive approach. 2026;22(1):9-20.

56. Bao M, Feng Q, Zou L, Huang J, Zhu C, Xia WJR. Endoplasmic reticulum stress promotes endometrial fibrosis through the TGF- β /SMAD pathway. 2023;165(2):171-82.

57. Karaoğlu Ö, Tap Ö, Ürünsak iFJIJoMS. Redefining Endometrial Decidualization: The Central Role of the ER Stress–Immune–Metabolic Axis. 2026;27(10):4382.

Figure captions

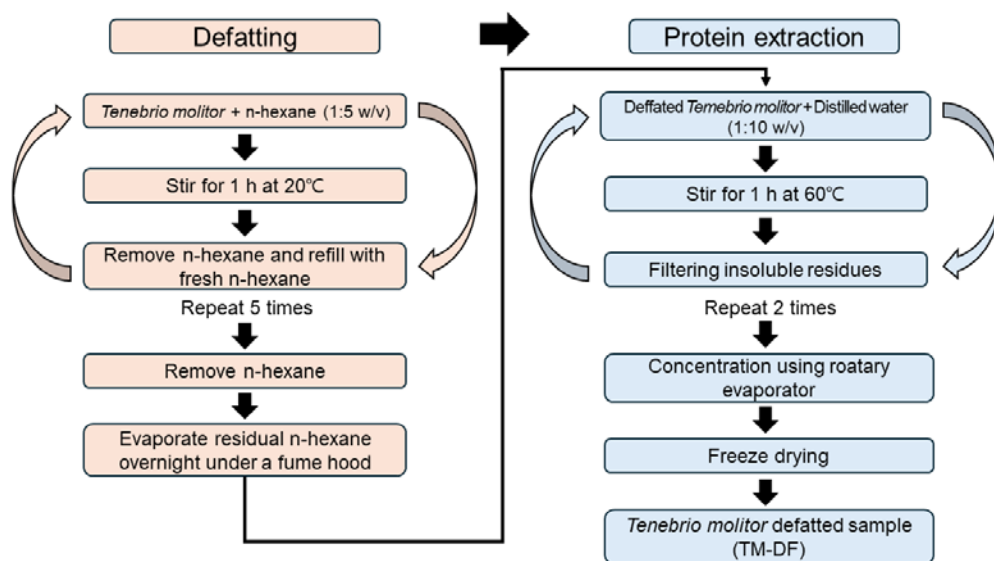


Figure 1. Schematic diagram of the preparation process of defatted *Tenebrio molitor* larvae extract (TM-DF).

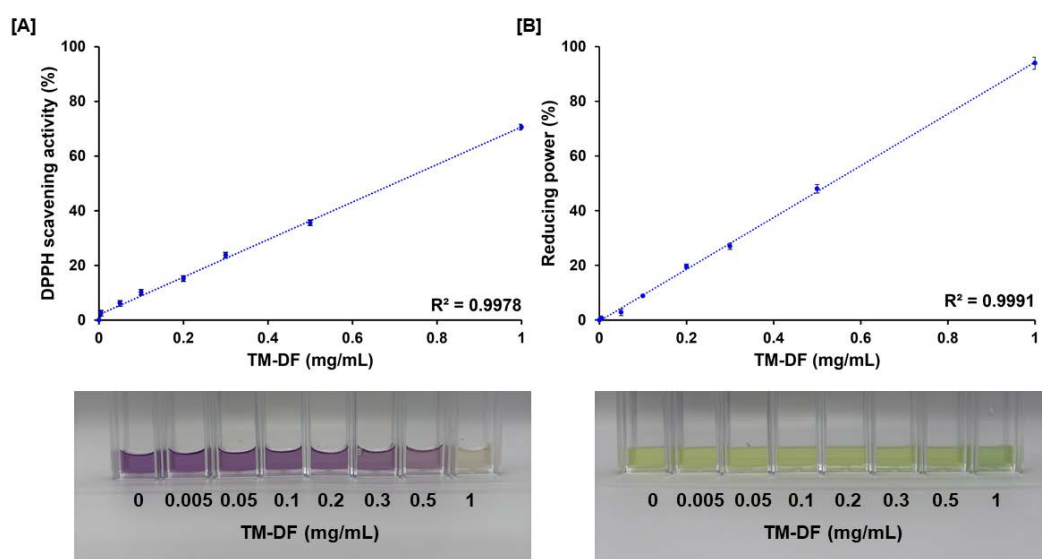


Figure 2. Antioxidant activity of TM-DF. [A] DPPH radical scavenging activity of TM-DF at different concentrations (0–1 mg/mL). These scavenging activities were quantified and presented as the absorbance values corresponding to the degree to which DPPH, a purple free radical, was reduced to a yellow color. The image below shows the color changes at different concentrations. [B] Reducing power of TM-DF at different concentrations (0–1 mg/mL). The ferrocyanide reduced by TM-DF reacted with FeCl_3 to form Prussian blue, and this was measured by absorbance.

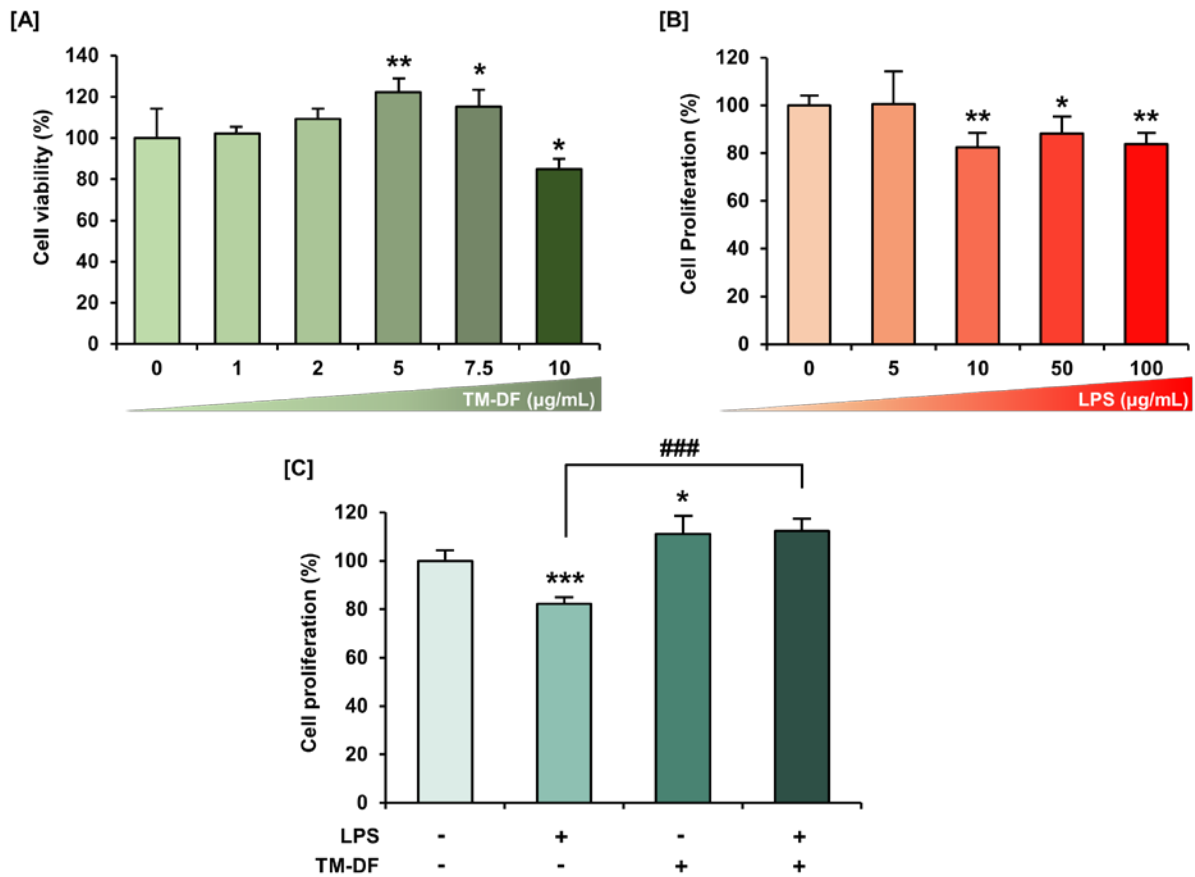


Figure 3. The viability and proliferation changes of pLE cells in response to TM-DF or LPS treatment in pLE cells. [A] Cellular viability of pLE cells in response to TM-DF in dose-dependent manners (0, 1, 2, 5, 7.5 and 10 μg/mL) for 24 h. [B] Proliferation of pLE cells in response to LPS (0, 5, 10, 50, and 100 μg/mL) for 48 h. [C] Proliferation of pLE cells in response to LPS (50 μg/mL) for 48 h with or without pre-treatment of TM-DF (5 μg/mL) for 2 h.

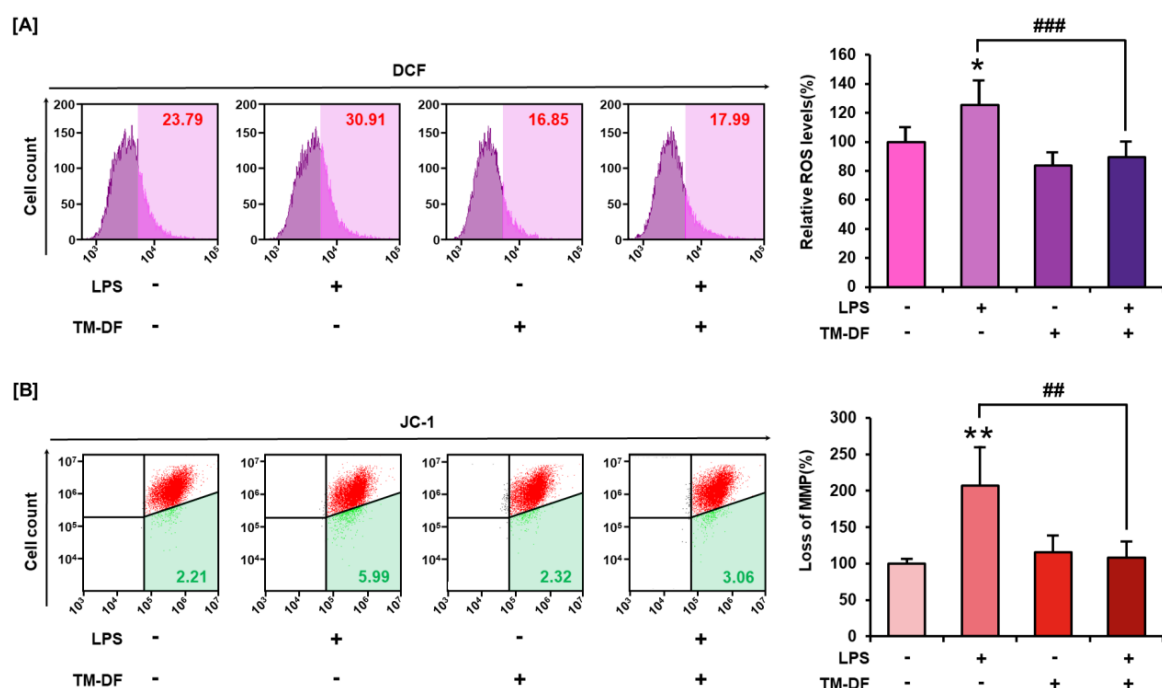


Figure 4. Protective effects of TM-DF on LPS-induced ROS and mitochondrial permeabilization in pLE cells. [A] DCFH-DA were used to detect relative ROS levels using flow cytometry (FACS). The positive DCF area, which are the colored area of histogram plots, indicates relative ROS values. This relative cell population were represented as percentages bar graphs relative to the control group. [B] JC-1 dye was used to detect the mitochondrial membrane potential (MMP). The lower right panel of the dot plot indicates the relative population of JC-1 monomer, which is the marker of loss of MMP. Relative population of this area were indicated as percentage bar graphs relative to the control group. For analysis, pLE cells were treated with LPS (50 $\mu\text{g/mL}$) for 48 h with or without pre-treatment of TM-DF (5 $\mu\text{g/mL}$) for 2 h.

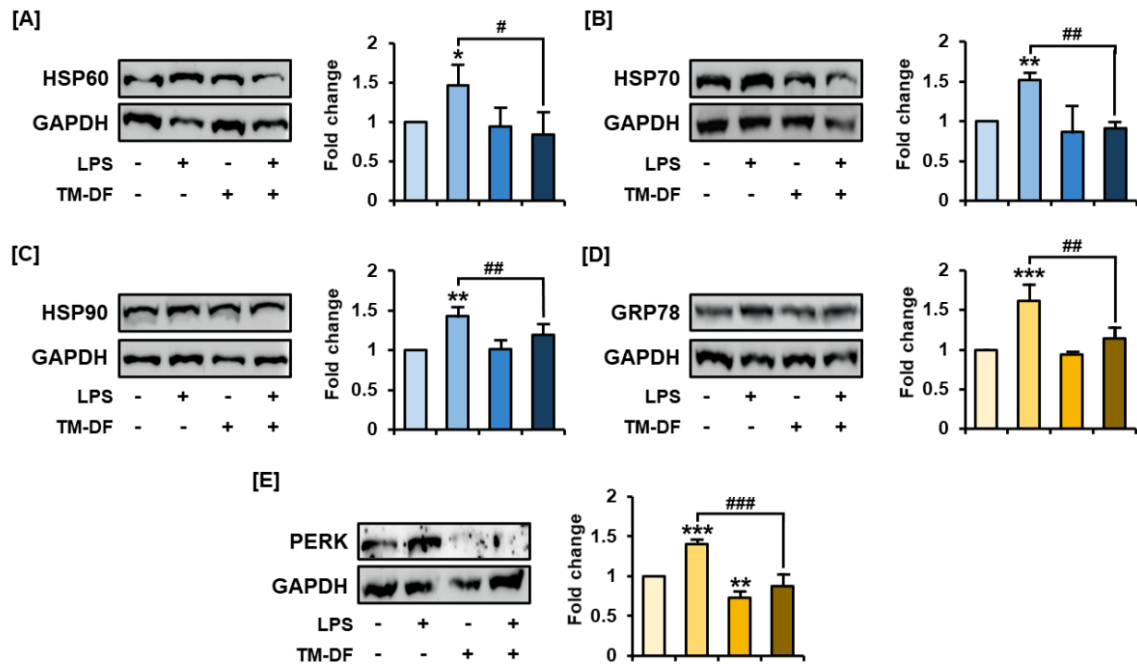


Figure 5. Alleviative effect of TM-DF on LPS-induced heat stress and ER stress on pLE cells. [A–E] The relative protein expressions of HSP60 [A], HSP70 [B], HSP90 [C], GRP78 [D], and PERK [E] were analyzed following treatment with LPS (50 µg/mL) for 24 h with or without pre-treatment of TM-DF (5 µg/mL) for 2 h. GAPDH protein were used to normalize the expressions of targeted proteins. The immunoblot intensity were analyzed using ImageLab Software.

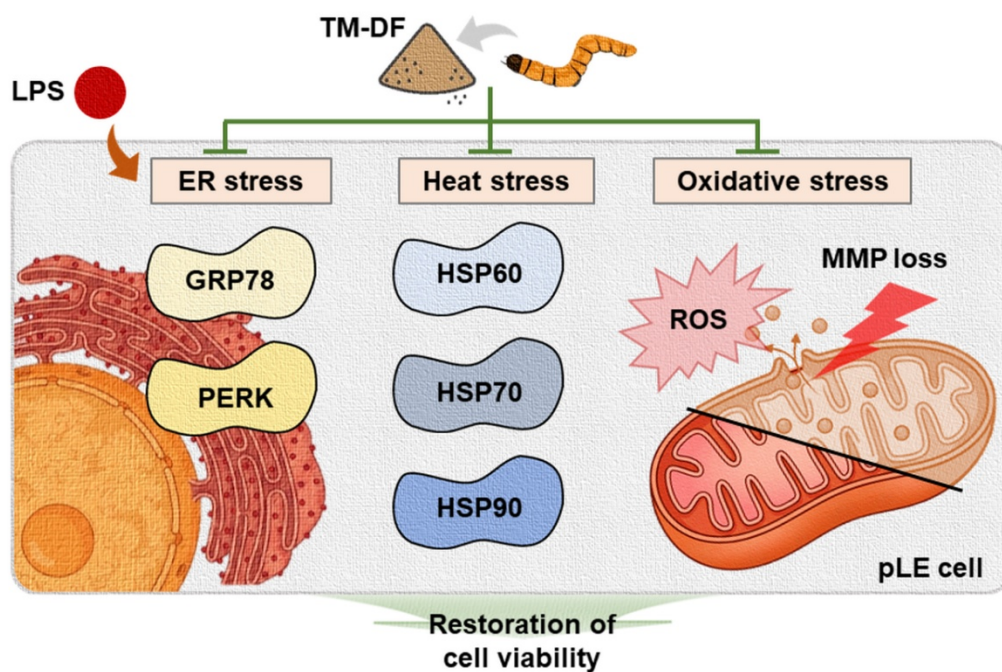
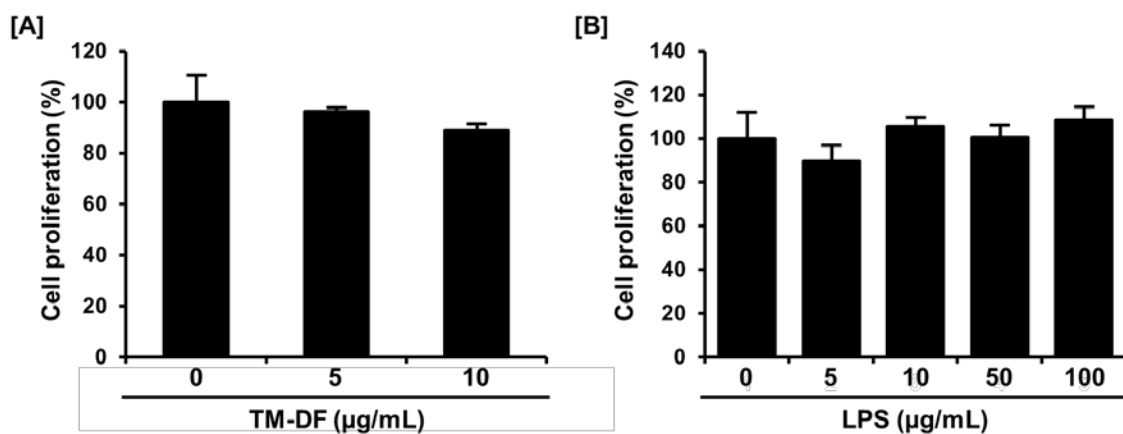


Figure 6. Schematic illustration of the mechanism underlying the alleviation of LPS-induced intracellular stress in pLE cells by TM-DF pre-treatment.



Supplementary Figure 1. Changes in pLE cell proliferation following treatment with TM-DF or LPS. [A] Proliferation of pLE cells in response to TM-DF (0, 5, 10 µg/mL) for 48 h. [B] Proliferation of pLE cells in response to LPS (0, 5, 10, 50, 100 µg/mL) for 24 h.