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Investigating the protective effect of zinc supplementation on streptozotocin-induced pancreatic beta-cell damage via the HMGB1/TLR2/MAPK pathway

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ABSTRACT

Objective(s): This study investigated the protective effect of zinc supplementation on insulin release via modulation of the High Mobility Group Box 1 (HMGB1)/Toll-like receptor 2 (TLR2)/mitogen-activated protein kinase (MAPK) signaling pathway in streptozotocin (STZ)-induced pancreatic β-cell injury, a commonly used model of type 1 diabetes.

Materials and Methods: Human pancreatic β-cells (1.1B4) were divided into five groups: Control, Diabetes, Diabetes + Zinc, Diabetes + Box A, and Diabetes + Zinc + Box A. Diabetes was induced with STZ (20 mM, four hours, type-1), followed by treatment with zinc (30 μM) and/or Box A (100 ng/l). Cellular oxidative stress, apoptosis, mitochondrial membrane potential (MMP), cell viability, and insulin secretion were measured. Protein expressions of HMGB1, TLR2/4, extracellular signal-regulated kinases 1/2 (ERK1/2), and c-Jun N-terminal kinase (JNK) were analyzed using western blotting.

Results: Zinc supplementation restored MMP and total antioxidant status (TAS), reduced apoptotic cell death, and enhanced TLR2 and ERK1/2 expression suppressed by STZ. Combined zinc and Box A treatment markedly increased insulin secretion under both hypo- and hyperglycemic conditions.

Conclusion: Zinc exerts protective effects against STZ-induced β-cell damage, likely through modulation of the HMGB1/TLR2/MAPK axis. These findings highlight the therapeutic potential of zinc supplementation in protecting pancreatic β-cell function in diabetes mellitus.

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Introduction

Diabetes mellitus (DM) is a complex metabolic disorder characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Persistent hyperglycemia contributes to oxidative stress, inflammation, and the progressive loss of pancreatic β-cells, leading to severe microvascular and macrovascular complications (1). Diabetes is a significant public health problem with its increasing global prevalence and the complications it causes (2, 3). Both type 1 and type 2 diabetes share β-cell dysfunction as a central feature; therefore, understanding the molecular mechanisms of β-cell failure is critical for developing targeted therapies (4).

Zinc plays a crucial role in pancreatic β-cell physiology, including insulin synthesis, storage, and secretion. In addition, zinc regulates redox signaling and mitochondrial homeostasis, processes that are essential for maintaining β-cell function and survival (5). Accumulating evidence suggests that zinc deficiency may impair β-cell function and contribute to metabolic dysregulation associated with diabetes (6). Moreover, hyperglycemia-induced zinc loss through urinary excretion can lead to reduced serum zinc levels and impaired insulin secretion (7, 8).

Because pancreatic islets' inherently low anti-oxidant capacity renders them particularly susceptible to oxidative damage by reactive oxygen species (ROS), triggering apoptotic pathways in β-cells (9, 10), zinc supplementation has been reported to enhance anti-oxidant capacity, reduce oxidative stress, and improve glucose metabolism (11, 12). There it should be, however, how clarified zinc supplementation could reverse diabetic effect on pancreatic beta cell based on molecular pathways.

High Mobility Group Box 1 (HMGB1) is a redox-sensitive, non-histone nuclear protein that plays a key role in chromatin stability, DNA repair, and transcription regulation. Under conditions of cellular stress, injury, or hyperglycemia, HMGB1 translocates from the nucleus to the cytoplasm and is actively released into the extracellular

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space, where it functions as a damage-associated molecular pattern (DAMP) molecule (13). Extracellular HMGB1 engages pattern recognition receptors, including Toll-like receptors (TLR2 and TLR4) and the receptor for advanced glycation end products (RAGE), which can activate downstream signaling pathways such as NF-κB and MAPK, leading to increased production of pro-inflammatory cytokines (14-17). HMGB1 promotes pancreatic β-cell inflammation and apoptosis, primarily through TLR4-mediated signaling linked to the MAPK cascade (18). Recent evidence indicates that zinc deficiency exacerbates HMGB1 secretion and inflammatory signaling, whereas zinc supplementation inhibits NF-κB and MAPK activation (19-22).

Based on existing evidence, we hypothesize that HMGB1 contributes significantly to β-cell apoptosis in diabetes and that zinc supplementation attenuates β-cell damage through modulation of the HMGB1/TLR2/MAPK signaling pathway. Type 1 diabetes was induced using streptozotocin (STZ), which selectively destroys pancreatic β-cells, mimicking the pathophysiology of type 1 diabetes (23). This study aims to elucidate the molecular mechanisms underlying zinc's protective effect against STZ-induced β-cell injury.

Materials and Methods

Cell culture

The human pancreatic β-cell line (1.1B4) was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% bovine serum albumin, 1% pyruvate, 2 mM glutamine, and antibiotics (100 IU/mL penicillin and 0.1 mg/mL streptomycin). Cultures were maintained at 37 °C in a humidified incubator containing 5% CO_2. Experiments were initiated when cells reached approximately 80% confluence.

Experimental design

Five experimental groups were established:

1. Control (C)
2. Diabetes (D)
3. Diabetes + Zinc (DZ)
4. Diabetes + Box A (DB)
5. Diabetes + Zinc + Box A (DZB)

Diabetes was induced by treatment with 20 mM streptozotocin (STZ; Santa Cruz Biotechnology, SC-200719) for 4 hr (24, 25). Subsequently, cells were treated with 30 μM zinc (Thermo Scientific, 10316143) (26) and/or 100 ng/L Box A (HMG Biotech, HM-012; Milan, Italy) for 24 hr (27). In the DZB group, both zinc and Box A were administered simultaneously.

Cell viability measurement

Cell viability was assessed using the MTT assay (Serva, 20395.02; Heidelberg, Germany) as previously described (28). Cells were incubated with MTT reagent for 4 hr, followed by DMSO solubilization. Absorbance was measured at 570 nm. Viability was calculated as a percentage of control values using the formula:

$$CV = 100 \times (OD_s - OD_b) / (OD_c - OD_b) \quad (28)$$

Apoptotic detection using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Pancreatic β-cells were seeded on coverslips and assigned to experimental groups. After the incubation period, cells were fixed in 4% paraformaldehyde for one hour at room temperature. Cells were then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate, and apoptotic nuclei were labeled using a TUNEL kit (Roche Diagnostics, 1684795; Mannheim, Germany) according to the manufacturer's instructions. Following staining, coverslips were mounted on slides and images were captured using an Olympus BX51 microscope equipped with a DP72 camera and controlled with Olympus DP2-TWAIN software. TUNEL-positive cells were quantified using ImageJ, and apoptotic cells were expressed as a percentage of total cells (29).

Determination of oxidative stress

Total oxidant level

TOS was measured using a commercial kit (Rel Assay Diagnostics, RL0024; Gaziantep, Türkiye). The assay is based on the oxidation of ferrous to ferric ions by oxidants present in the sample, forming a colored complex with xylenol orange. Results were expressed as μmol H_2O_2 equivalents per liter (μmol H_2O_2 equivalent/L).

Total anti-oxidant level

TAS was measured using a Rel Assay kit (RL0017). The method relies on the reduction of the blue-green ABTS radical cation by anti-oxidants to its colorless form, with absorbance measured at 530 nm. Results were expressed as mmol Trolox equivalents per liter (mmol Trolox equivalent/l).

Oxidative stress index

OSI was calculated as:

$$OSI = [TOS\ (\mu mol\ H_2O_2\ Eq/l) / TAS\ (\mu mol\ Trolox\ Eq/L)] \times 100 \quad (29)$$

Measurements were performed in six replicates per group.

Mitochondrial membrane potential (MMP)

MMP was measured using a JC-10 dye assay kit (Abcam, ab112134; Cambridge, UK). Cells were incubated with JC-10 for 60 min at 37 °C. Red fluorescence represented intact mitochondrial potential, while green fluorescence indicated depolarization. Fluorescence was quantified using a Varioskan Lux Multimode Microplate Reader (Thermo Fisher Scientific) at excitation/emission wavelengths of 490/525 nm and 540/590 nm (29).

Insulin secretion assay

Following 24 hr incubation, cells were washed and incubated in Krebs-Ringer bicarbonate buffer containing 0.5% fetal serum albumin and glucose concentrations of 1.1, 8.4, or 16.7 mM. After 60 min, supernatants were collected and stored at –40 °C. Insulin concentrations were quantified using a sandwich ELISA kit (Shanghai Coon Koon Biotech, CK-bio-14821, China). The reagents, samples, and standards were prepared in accordance with the instructions provided in the ELISA kit protocol. Briefly, 50 µl standards and 10 µl samples, 40 µl dilution solution were added to the wells, and the plates were incubated at 37 °C for 60 min. After washing the plates with wash buffer 5 times in the dark, 50 µl Chromogen A + 50 µl Chromogen B solution was added, and the plates were incubated at 37 °C for 15 min to develop color. According to the protocol, 50 µl of stop solution was added and the optical density was read in a microplate spectrophotometer (BioTek Eon) at 450 nm (28).

Determination of calcium and zinc content

Calcium levels were measured using a colorimetric calcium assay kit (Otto Scientific, OttoBC133; Ankara,

Türkiye) (30, 31). Zinc levels were determined using a colorimetric zinc assay kit (Rel Assay, RL0901; Gaziantep, Türkiye) (32, 33). Absorbance was recorded at 650 nm (calcium) and 548 nm (zinc) using a Thermo Scientific Varioskan Flash reader.

Determination of HMGB1 levels

HMGB1 content was quantified using a sandwich ELISA kit (Elabscience, E-EL-R0505; Wuhan, China). Six samples were analyzed per group. Plates were read at 450 nm using a BioTek Eon microplate spectrophotometer (34).

Protein extraction and western blot analysis

Cells were lysed using RIPA buffer (Santa Cruz Biotechnology, sc-24948) and centrifuged at 3,000 rpm for 5 min at 4 °C. Supernatants were collected, and protein concentration was determined using the Bradford assay. Equal amounts of protein (100 μg) were separated on 4–12% Bis-Tris gels and transferred to PVDF membranes. Membranes were probed with primary antibodies against HMGB1 (Abcam, ab18256), SAPK/JNK (CST 9258), ERK1/2 (CST 4695), TLR4 (Abcam, ab22048), and TLR2 (Abcam, ab191458). Alkaline phosphatase–conjugated secondary antibodies were used, and signals were visualized using an NCIP/BNP kit (Millipore, ES0006). Band densities were quantified using ImageJ software (NIH, USA).

Statistical analysis

Normality and homogeneity were verified using Kolmogorov–Smirnov and Levene's tests. Data are expressed as mean ± SEM. Statistical comparisons were performed using one-way ANOVA followed by Tukey's *post hoc* test. Statistically significant were consider when $P<0.05$, and exact P-values are reported where appropriate. Analyses were conducted using SPSS v25.0 (IBM Corp., Chicago, IL, USA).

Results

Cell viability

MTT assays revealed that STZ significantly reduced β-cell viability compared to control. Zinc supplementation improved cell viability relative to both diabetic and Box A–only groups (Figure 1).

Apoptotic cell counts

STZ treatment significantly increased the number of apoptotic β-cells compared to the control group (Figures 2b, 3). Zinc or Box A supplementation markedly reduced apoptosis (Figures 2Cc, Dd, 3), while co-supplementation of zinc and Box A exhibited the most pronounced anti-apoptotic effect (Figures 2Ee and 3).

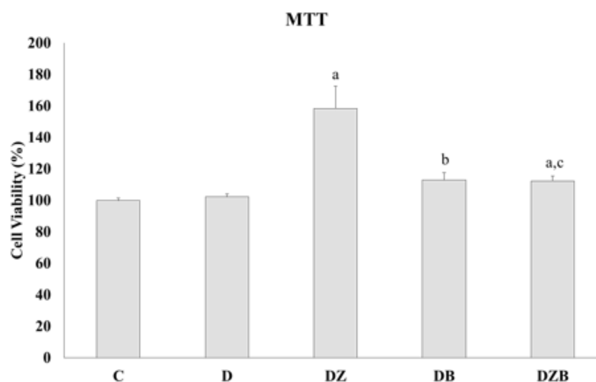

Figure 1. Effects of zinc and Box A supplementation on β-cell viability
C: Control; D: Diabetes; DZ: Diabetes + Zinc; DB: Diabetes + Box A; DZB: Diabetes + Zinc + Box A.
Data are presented as mean ± SEM (n = 12).
Statistical significance: a $P<0.01$ vs D; b $P<0.01$ vs DZ; c $P<0.05$ vs DZ.

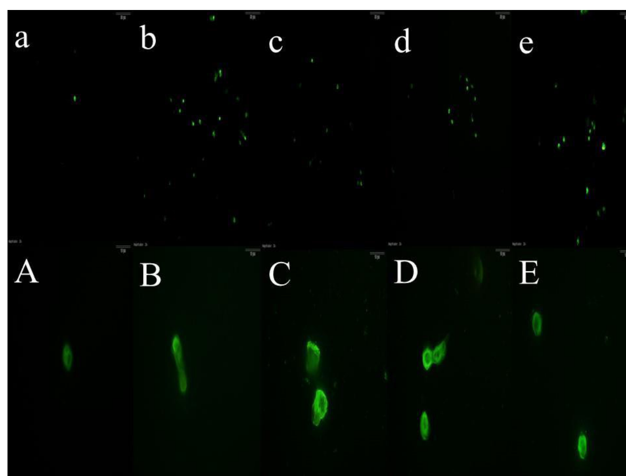

Figure 2. Effect of zinc and Box A supplementation on apoptosis in pancreatic β-cells
A, a: Control; B, b: Diabetes; C, c: Diabetes + Zinc; D, d: Diabetes + Box A; E, e: Diabetes + Zinc + Box A.
Magnifications: Capital letters = 100×; lowercase letters = 20×.

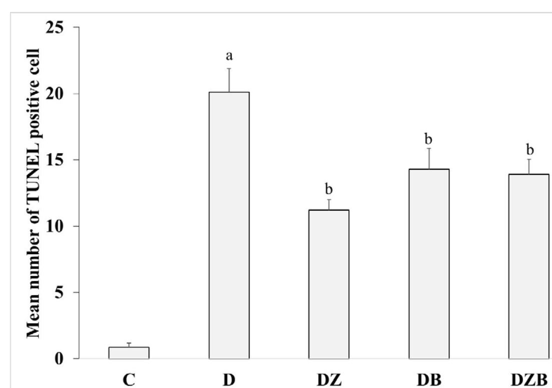

Figure 3. Quantification of apoptotic β-cells in STZ-treated cultures.
C: Control; D: Diabetes; DZ: Diabetes + Zinc; DB: Diabetes + Box A; DZB: Diabetes + Zinc + Box A.
Data are presented as mean ± SEM (n=12).
Statistical significance: a $P<0.05$ vs C; b $P<0.05$ vs D

Oxidative stress parameters

STZ exposure significantly decreased TAS compared with controls (Figure 4A). Zinc supplementation restored TAS levels, while TOS and OSI values showed no significant intergroup differences (Figure 4B–C).

Mitochondrial membrane potential

Healthy mitochondria exhibited intense red/green fluorescence, whereas depolarized mitochondria displayed reduced red and increased green fluorescence intensity.

STZ exposure significantly reduced mitochondrial membrane potential (MMP) in β-cells, as indicated by a decreased red/green fluorescence ratio compared to the control group. Zinc supplementation markedly restored MMP, as reflected by an increased fluorescence ratio relative to the diabetic group (Figure 5).

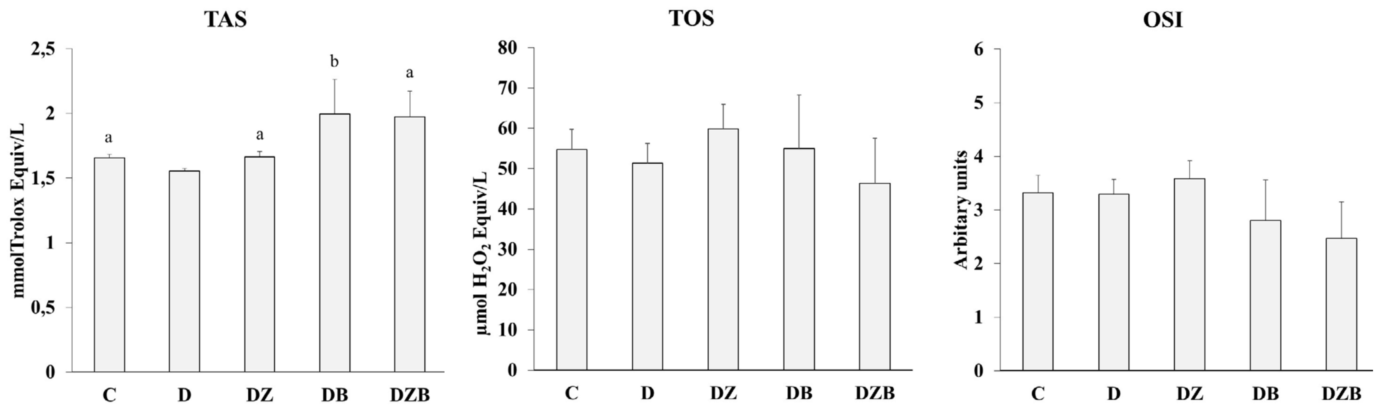

Figure 4. Effects of zinc and Box A supplementation on oxidative stress parameters
(A) Total Antioxidant Status (TAS), (B) Total Oxidant Status (TOS), and (C) Oxidative Stress Index (OSI)
C: Control; D: Diabetes; DZ: Diabetes + Zinc; DB: Diabetes + Box A; DZB: Diabetes + Zinc + Box A. Data are presented as mean ± SEM (n=7).
Statistical significance: a P<0.01 vs D; b P<0.05 vs D

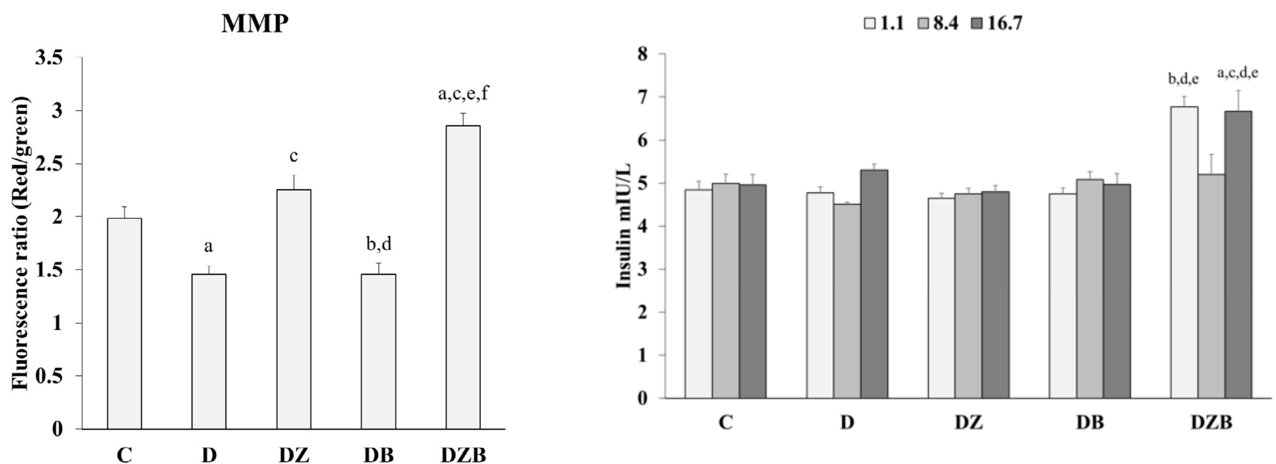

Figure 5. Effect of zinc supplementation on mitochondrial membrane potential (MMP) in pancreatic β-cells
MMP was assessed as the fluorescence ratio of red/green signals.
Data are presented as mean ± SEM (n = 12).
Statistical significance: a P<0.01 vs C; b P<0.05 vs C; c P<0.01 vs D; d P<0.01 vs DZ; e P<0.05 vs DZ; f P<0.01 vs DB.
C: Control group; D: Diabetes group; DZ: Diabetes + Zinc group; DB: Diabetes + Box A group; DZB: Diabetes + Zinc + Box A group.

Figure 6. Insulin secretion of β-cells under varying glucose concentrations
C: Control; D: Diabetes; DZ: Diabetes + Zinc; DB: Diabetes + Box A; DZB: Diabetes + Zinc + Box A.
Data are presented as mean ± SEM (n=7). Glucose concentrations: 1.1 mM (light gray), 8.4 mM (dark gray), 16.7 mM (black). Statistical significance: a P<0.01 vs C; b P<0.05 vs C; c P<0.05 vs D; d P<0.05 vs DZ; e P<0.05 vs DB.

Insulin secretion

Insulin release was evaluated under hypo- (1.1 mM), moderate- (8.4 mM), and hyperglycemic (16.7 mM) glucose concentrations. Co-supplementation of zinc and Box A significantly enhanced insulin secretion under both fasting and stimulated conditions compared to diabetic and single-supplement groups (Figure 6).

Cellular HMGB1, zinc, and calcium levels

STZ exposure led to increased extracellular HMGB1 release, while zinc and Box A supplementation reduced HMGB1 content compared with diabetic cells (Figure 7A). Intracellular zinc levels did not significantly differ among groups (Figure 7B). Calcium content was elevated in DB and DZB groups relative to controls (Figure 7C).

Protein expressions analysis

HMGB1 expression

HMGB1 protein levels were reduced in the diabetic group compared with controls. Zinc and Box A each down-regulated HMGB1 expression compared to the diabetic group. Co-treatment produced no further alteration compared to individual treatments (Figure 8a).

TLR2 and TLR4 expression

STZ treatment decreased TLR2 protein levels, while zinc supplementation restored expression. Co-treatment with zinc and Box A reduced TLR2 expression compared to the DZ group. TLR4 expression remained unchanged after STZ exposure but increased with zinc supplementation; however, combined treatment reduced TLR4 expression relative to zinc alone (Figure 8b, c).

ERK1/2 an JNK expression

STZ reduced ERK1/2 expression and increased JNK levels compared with control cells. Zinc and Box A individually up-regulated ERK1/2 while attenuating JNK expression. Combined supplementation yielded the highest ERK1/2 expression and the lowest JNK activation among all treated groups (Figures 8d and 8e).

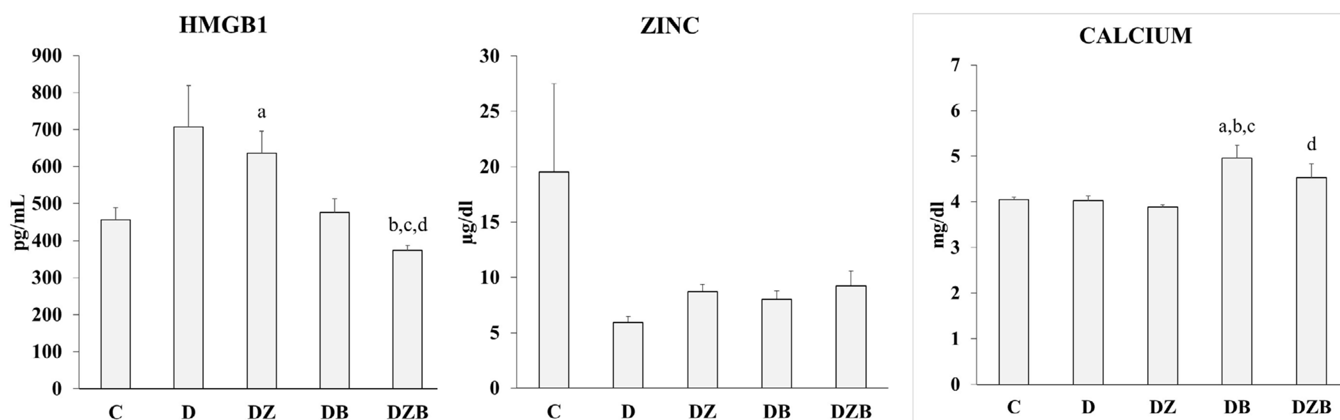

Figure 7. Cellular biomarker changes following zinc and Box A supplementation
(A) High mobility group box 1 (HMGB1) levels, (B) Zinc content, and (C) Calcium content.
C: Control; D: Diabetes; DZ: Diabetes + Zinc; DB: Diabetes + Box A; DZB: Diabetes + Zinc + Box A. Data are presented as mean ± SEM (n=7).
Statistical significance: (A) a $P<0.05$ vs C; b $P<0.01$ vs D; c $P<0.01$ vs DZ; d $P<0.05$ vs DB. (C) a $P<0.05$ vs C; b $P<0.05$ vs D; c $P<0.01$ vs DZ; d $P<0.05$ vs DZ.

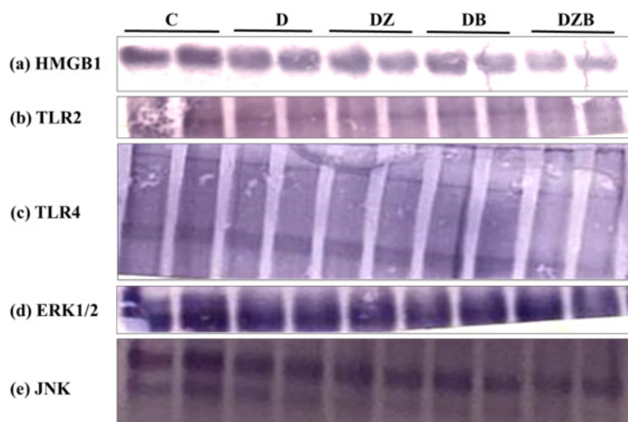

Figure 8. Western blot analyses of protein expressions in pancreatic β-cells
(a) High mobility group box 1 (HMGB1), (b) Toll-like receptor 2 (TLR2), (c) Toll-like receptor 4 (TLR4), (d) extracellular signal-regulated kinase 1/2 (ERK1/2), and (e) c-Jun N-terminal kinase (JNK). Each lane represents independent samples.
C: Control; D: Diabetes; DZ: Diabetes + Zinc; DB: Diabetes + Box A; DZB: Diabetes + Zinc + Box A.

Discussion

This study provides novel evidence regarding the protective mechanisms of zinc supplementation against STZ-induced pancreatic β-cell toxicity, emphasizing the involvement of the HMGB1/TLR2/MAPK signaling pathway.

The present study revealed that STZ exposure decreased TAS and MMP levels in pancreatic β-cells by elevation of HMGB1 secretion, whereas zinc supplementation restored these parameters. STZ also markedly increased apoptotic cell death; however, zinc treatment attenuated apoptosis and improved overall cell viability. Moreover, the combined administration of zinc and Box A enhanced insulin secretion under both hypoglycemic and hyperglycemic conditions. In addition, zinc modulated TLR2 and TLR4 expression and regulated ERK1/2–JNK signaling pathways, suggesting a potential involvement in β-cell survival mechanisms.

These results collectively indicate that zinc supplementation exerts cytoprotective effects through the modulation of oxidative stress and inflammatory signaling cascades, primarily mediated via HMGB1/TLR2/MAPK pathways.

The apparent discrepancy between increased secretion of HMGB1-related inflammatory signaling and reduced HMGB1 protein expression in western blot analysis can be explained by the different biological compartments assessed by these methods. In our study, western blot analysis was performed using the cell lysates and therefore primarily reflects intracellular retained HMGB1 levels. In contrast, diabetes-induced cellular stress, oxidative injury, and inflammatory activation may promote HMGB1 nuclear-to-cytoplasmic translocation and subsequent extracellular release. Moreover, type-2 diabetic patients reportedly had high levels of HMGB1 vs nondiabetic subjects in serum (35). Therefore, a reduction in intracellular HMGB1 detected using western blot does not necessarily indicate reduced HMGB1 activity; rather, it may reflect depletion of intracellular HMGB1 due to its release into the extracellular space. Extracellular HMGB1 acts as a damage-associated molecular pattern and can activate inflammatory signaling through receptors such as RAGE and TLR4/TLR2 (35). Thus, elevated extracellular HMGB1 activity may coexist with reduced intracellular HMGB1 protein levels in diabetic tissue. In this context, zinc may reduce extracellular HMGB1 release and/or HMGB1-mediated inflammatory signaling, even if intracellular HMGB1 levels measured using western blot appear reduced in the diabetic group.

Previous studies have shown that mitochondrial dysfunction contributes to progressive β-cell loss and impaired insulin secretion (36, 37). The present study corroborates these findings, as zinc restored MMP integrity and improved anti-oxidant defenses. The restoration of TAS levels suggests that zinc enhances endogenous anti-oxidant mechanisms such as metallothionein and glutathione (11, 12, 38).

Interestingly, our results demonstrated that TLR2 expression, rather than TLR4, was significantly altered by STZ treatment and zinc supplementation. While earlier reports have emphasized TLR4-mediated β-cell damage (39, 40), our data highlight TLR2 and ERK1/2 down-regulation as a major contributor to STZ-induced cytotoxicity, possibly through oxidative stress-mediated inhibition of MAPK/ERK signaling, as reported previously (41-43). The restoration of TLR2 by zinc suggests a potential link between innate immune signaling and mechanisms involved in β-cell survival (44). This observation aligns with previous reports that underscore the involvement of TLR2 in the regulation of oxidative stress and facilitation of tissue

regeneration (45).

The most striking observation was the synergistic effect of zinc and Box A on insulin secretion under both hypoglycemic and hyperglycemic conditions. Physiological concentrations of zinc are known to stimulate insulin release, whereas zinc deficiency or chelation suppresses it (26). Interestingly, despite the reduction in intracellular calcium observed with zinc supplementation, insulin release increased—suggesting that zinc can promote insulin secretion through calcium-independent mechanisms, possibly by influencing vesicular zinc transporters or insulin granule stability (46, 47).

HMGB1 plays a dual role in cellular physiology and pathology. In diabetic conditions, extracellular HMGB1 acts as a proinflammatory mediator, promoting β-cell apoptosis through TLR4 and RAGE signaling (15, 48, 49). The observed decrease in extracellular HMGB1 after zinc and Box A treatment supports the hypothesis that zinc suppresses inflammation by inhibiting HMGB1 release (19) and downstream MAPK activation (50). The findings obtained may contribute to the design of future studies incorporating these pathways.

The observed up-regulation of ERK1/2 and down-regulation of JNK by zinc and Box A co-supplementation establishes a favorable balance between survival and stress-response pathways. JNK activation has been reported to suppress insulin gene expression and secretion under oxidative stress conditions (51, 52). Thus, the attenuation of JNK activity by zinc represents a key mechanism underlying its protective effects on β-cells.

The role of HMGB1 in diabetes remains complex, with studies demonstrating both protective and detrimental effects (53, 54).

Although our study provides valuable mechanistic insights, several limitations should be acknowledged. First, while the use of the HMGB1 inhibitor Box A suggests the involvement of HMGB1 signaling in zinc-mediated protection, the present study does not provide direct causal evidence for this mechanism. Second, our findings are based on an in vitro β-cell model, which may not fully reflect the complex physiological environment of pancreatic islets *in vivo*. Future *in vivo* studies should validate these findings using diabetic animal models to confirm zinc's therapeutic efficacy and its long-term metabolic effects. Moreover, assessing the interplay between zinc homeostasis, HMGB1 expression, and immune modulation could uncover novel targets for diabetes management.

Conclusion

This study demonstrates that zinc supplementation protects pancreatic β-cells from STZ-induced oxidative and apoptotic damage. The protective effect appears to be associated with modulation of the HMGB1/TLR2/MAPK signaling axis, accompanied by restoration of anti-oxidant capacity, preservation of mitochondrial function, and regulation of ERK1/2–JNK activity. These findings suggest that zinc may represent a promising adjunctive strategy for DM by promoting β-cell survival and functional recovery. Further studies, particularly those employing genetic modulation of HMGB1 signaling and *in vivo* models, are needed to clarify the mechanistic basis of these effects and to support their potential therapeutic relevance.

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Authors' Contributions

E T and Ş Y designed the study and performed the experimental work together with Ş Y. Both authors contributed to data collection and analysis. E T and EYA supervised the study and provided critical scientific guidance. All authors participated in the interpretation of results, and manuscript preparation, and approved the final version of the paper.

Conflicts of Interest

The authors have no conflicts of interest.

Declaration

We acknowledge the use of ChatGPT for language editing and minor text refinement. The authors take full responsibility for the content of the manuscript.

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