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Original Article

Protective Effects of Captopril and Spironolactone against Hyperglycemia-Induced Oxidative Stress in KGN Cells: Evidence from Viability and Mitochondrial Assays

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ABSTRACT

Background: Diabetes-induced oxidative stress adversely impacts ovarian granulosa cells, affecting fertility in women. This study explores the protective effects of captopril and spironolactone, two RAAS inhibitors, against STZ-induced damage in KGN cells. **Materials and Methods:** KGN cells were exposed to STZ (5 mM) to induce oxidative stress, followed by treatment with captopril (10-50 µM), spironolactone (5-25 µM), or their combination. MTT, DCFDA, and JC-1 assays were conducted to assess cell viability, ROS generation, and mitochondrial membrane potential. **Results:** STZ significantly increased ROS levels, reduced mitochondrial membrane potential (ΔΨm), and decreased cell viability. Both captopril and spironolactone improved these outcomes in a dose-dependent manner, with combination treatment showing the greatest protective effect. **Conclusion:** Captopril and spironolactone confer cytoprotection to granulosa cells under hyperglycemic stress by reducing oxidative damage and preserving mitochondrial function. These findings support their potential repurposing in diabetic reproductive dysfunction therapy.

Keywords: Captopril, Spironolactone, Hyperglycemia, Granulosa Cells, Oxidative Stress, Mitochondrial Dysfunction, Reproductive Health.

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INTRODUCTION

Polycystic Ovarian Syndrome (PCOS) is a prevalent endocrine disorder in women of reproductive age, marked by polycystic ovaries, hyperandrogenism, and chronic anovulation. It is frequently associated with metabolic problems such Type 2 Diabetes Mellitus (T2DM), obesity, and insulin resistance, which can exacerbate reproductive symptoms and raise the risk of endometrial cancer and cardiovascular disease (Athar *et al*., 2024). A key pathological factor in these conditions is hyperglycemia, which induces oxidative stress and disrupts cellular homeostasis, particularly affecting ovarian Granulosa Cells (GCs). Granulosa cells are crucial for hormone synthesis, oocyte development, and follicular growth. Under hyperglycemic conditions, GCs generate excessive Reactive Oxygen Species (ROS), Mitochondrial apoptosis and mitochondrial malfunction, including a decrease in mitochondrial membrane potential (ΔΨm). These oxidative insults impair GC viability, contributing to follicular atresia and infertility (Cavalcanti *et al*., 2023). Thus, therapies targeting oxidative stress and preserving mitochondrial function have gained research interest. Captopril, an Angiotensin-Converting Enzyme (ACE) inhibitor, and spironolactone, a Mineralocorticoid Receptor (MR) antagonist, are known for their cardiovascular and endocrine benefits. Both have demonstrated antioxidant effects: captopril by reducing ROS and enhancing mitochondrial stability, and spironolactone by modulating MR activity and reducing oxidative damage (Chang *et al*., 2024). Their protective roles in non-ovarian tissues are well-documented, but their potential to safeguard ovarian cells under hyperglycemic stress remains underexplored. This study utilizes KGN cells, a steroidogenic human granulosa-like cell line, to evaluate the protective effects of captopril and spironolactone against hyperglycemia-induced oxidative damage. Three assays were employed: the MTT assay to assess cell viability, the ROS assay to measure intracellular oxidative stress, and the JC-1 assay to evaluate mitochondrial membrane potential. These provide a comprehensive understanding of cell health and mitochondrial function. Reduced MTT conversion indicates compromised viability, while elevated ROS signals oxidative damage. A decline in ΔΨm, observed via JC-1 fluorescence shift, reflects mitochondrial dysfunction. Restoration of these parameters following treatment suggests cytoprotective activity. This research aims

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to bridge knowledge gaps in ovarian protection under diabetic conditions and explores the repurposing potential of captopril and spironolactone in reproductive medicine. Demonstrating their effectiveness could support future therapeutic strategies addressing both metabolic and reproductive dysfunctions in PCOS and diabetes.

MATERIALS AND METHODS

Cell Culture

The National Center for Cell Sciences (NCCS), located in Pune, India, provided the human granulosa-like tumor cell line (KGN). The cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM), which was enhanced with 100 mg/L streptomycin, 100 mg/L penicillin, 2 mM L-glutamine, and 10% Fetal Bovine Serum (FBS). The cultures were kept at 37°C with 5% CO_2 and 95% air in a humidified incubator. Cells were passaged once a week, and media was replaced every two to three days. All cultures were examined and found to be free of mycoplasma contamination, and the legitimacy of the cell lines was verified using Short Tandem Repeat (STR) profiling (Chen *et al*., 2024).

MTT Assay (Cell Viability)

After being planted at a density of 5,000-10,000 cells/well in 96-well plates, KGN cells were left to adhere for the entire night. Cells were exposed to different concentrations of spironolactone, captopril, Streptozotocin (STZ), and their combinations the following day. Doxorubicin was utilized as a positive control, and untreated cells were utilized as negative controls. Each well received 10 µL of MTT (5 mg/mL) following a 24- to 72-hr drug exposure period, and the wells were then incubated for 4 hr. The resulting formazan crystals were dissolved in 100 µL of DMSO, and a microplate reader was used to measure absorbance at 570 nm (reference: 630 nm). In comparison to the control, the percentage of live cells was computed (Ghosh *et al*., 2024).

ROS Measurement by DCFDA Staining

Oxidative stress was induced using 5 mM STZ for 24 hr. Cells were subsequently treated with captopril (10 or 50 µM), spironolactone (5 or 25 µM), or combinations for 24 and 48 hr. Cells were treated with 10 µM DCFDA in serum-free DMEM at 37°C for 30 min in the dark to measure intracellular ROS levels. Fluorescence intensity was measured at 485 nm excitation and 535 nm emission following washing. Increased fluorescence indicated elevated ROS levels, while a decrease suggested antioxidant activity of the treatments (Hu *et al*., 2024).

Mitochondrial Membrane Potential by JC-1 and Rhodamine 123

To evaluate mitochondrial function, STZ (5 mM) was applied to KGN cells for 24 hr, and then they were treated for 24-48 hr with captopril, spironolactone, or both. To measure changes in mitochondrial membrane potential (ΔΨm), JC-1 labeling (5 µg/mL) was employed. Depolarization of the mitochondria was evidenced by a decreased red/green fluorescence ratio. Rhodamine 123 (5 µM) staining was also performed, with reduced fluorescence signifying mitochondrial dysfunction. Both assays helped confirm mitochondrial protective effects of the treatments (Li *et al*., 2024).

RESULTS

KGN cell viability was assessed using the MTT test. STZ (100 µg/mL) caused 75% cell death, but captopril and spironolactone (6.25-100 µg/mL) promoted cell proliferation without being harmful. They had no toxicity at 1054 µg/mL for captopril and 1062 µg/mL for spironolactone, as shown in Figures 1D-E. While Figure 1G demonstrates that combined treatment dramatically restored vitality in a dose-dependent manner, with 100 µg/mL restoring control levels, Figure 1F displays IC_{50} values. These findings indicate that captopril and spironolactone effectively protect KGN cells from STZ-induced cytotoxicity, demonstrating greater efficacy at higher doses (Lindsay and Rhodes, 2025).

DCFDA Staining for Measuring ROS Levels in KGN cells Treated with Streptozotocin, Captopril, and Spironolactone

KGN cells exposed to STZ (5 mM, 24 hr) showed elevated ROS levels, confirmed by strong DCFDA fluorescence (Figure 2). Captopril (50 µM) moderately reduced ROS, while spironolactone (25 µM) had a slightly stronger effect. Their combination significantly lowered ROS levels, demonstrating the most pronounced antioxidant activity. These findings suggest that captopril and spironolactone together offer enhanced protection against STZ-induced oxidative stress, likely due to a synergistic ROS-scavenging effect in KGN cells.

DCFDA Staining for KGN cells to measure ROS levels.

Mitochondrial Membrane Potential (MMP) Assay in KGN cellsUsing JC-1 Staining

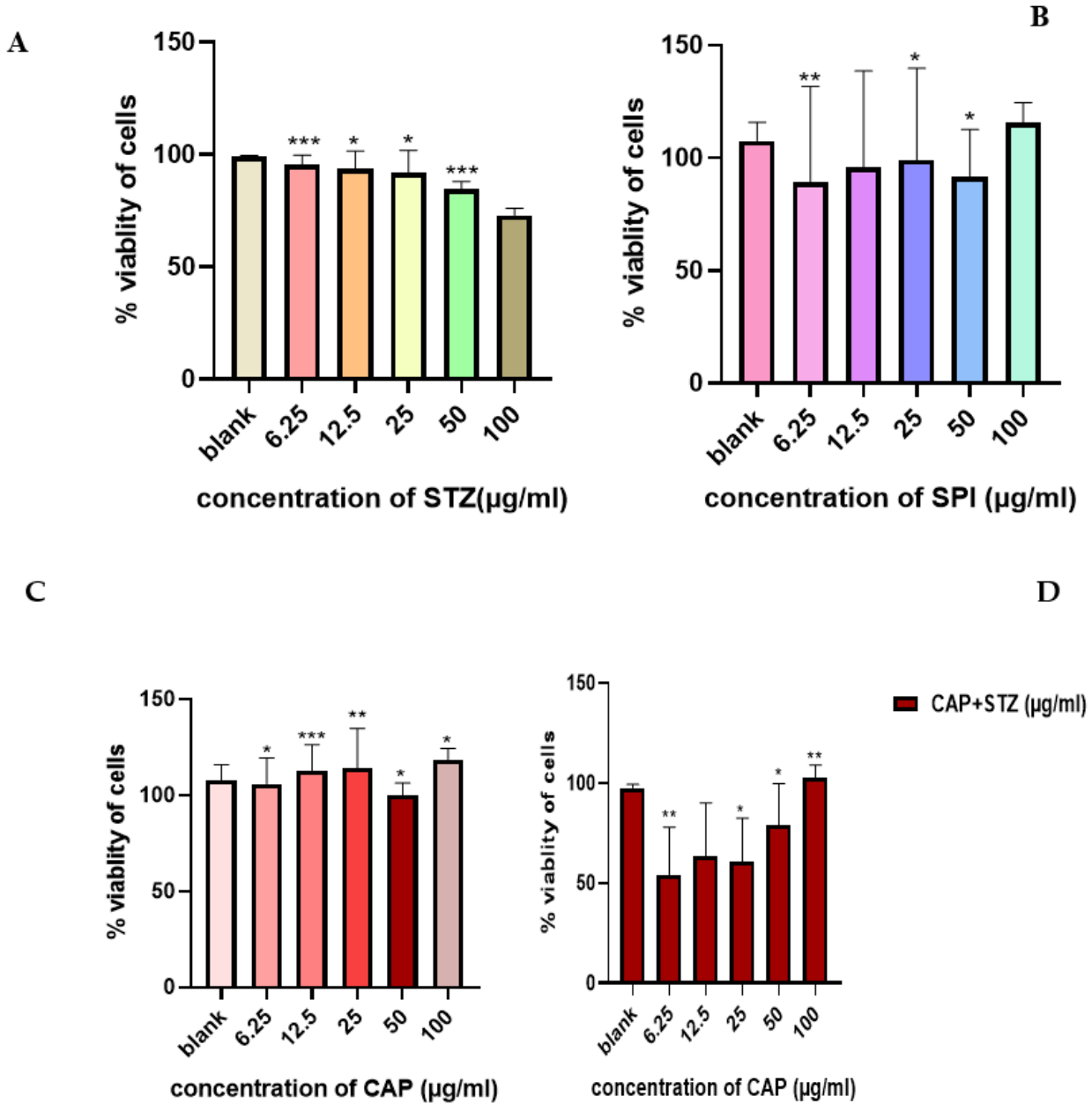
Significant mitochondrial dysfunction was demonstrated by KGN cells treated with STZ (5 mM, 24 hr), as seen by decreased red fluorescence (JC-1 aggregates) and increased green (JC-1 monomers) (Figure 3). Captopril (50 µM) partially restored Mitochondrial Membrane Potential (MMP), while spironolactone (25 µM) exhibited a stronger effect. Their combination provided the highest MMP restoration, with a significant shift toward red fluorescence, suggesting superior mitochondrial stability. These findings indicate that captopril and spironolactone together offer enhanced protection against STZ-induced mitochondrial dysfunction, likely due to a synergistic effect in maintaining mitochondrial integrity in KGN cells.

JC-1 Staining for the KGN Cell Mitochondrial Membrane Potential (MMP) Assay.

DISCUSSION

Reproductive dysfunction under diabetic conditions is a growing clinical and public health challenge, particularly among women with metabolic disorders such as Type 2 Diabetes mellitus (T2DM) and Polycystic Ovarian Syndrome (PCOS). These conditions not only disrupt metabolic homeostasis but also exert profound negative effects on ovarian function, leading to compromised oocyte quality, disrupted folliculogenesis, menstrual irregularities, and infertility. A critical mechanism underlying this dysfunction is hyperglycemia-induced oxidative stress, which overwhelms cellular antioxidant defenses, damages mitochondria, and triggers Granulosa Cell (GC) apoptosis (Liu, 2025).

Our study explored the cytoprotective effects of captopril and spironolactone, two well-established RAAS inhibitors, in an *in vitro* model of STZ-induced hyperglycemic stress in KGN granulosa-like cells. Our findings showed that STZ exposure significantly compromised cell viability, increased ROS production, and reduced mitochondrial membrane potential ($\Delta\Psi m$). Treatment with captopril or spironolactone alone improved these parameters in a dose-dependent manner; however, their combination produced the most robust protective effects, fully restoring viability, reducing ROS to near-control



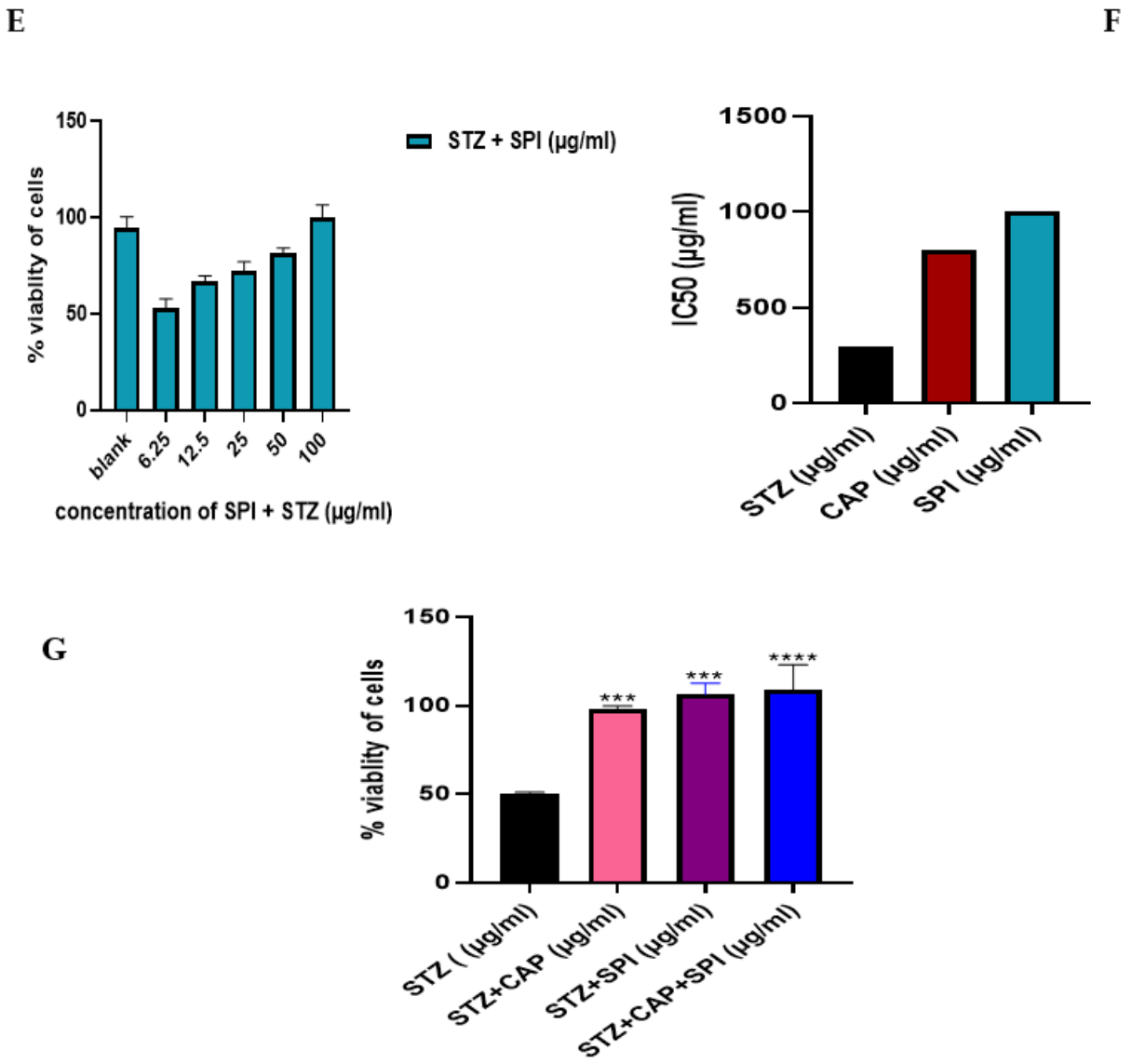


Figure 1: A, B, C:The effects of captopril, spironolactone, and STZ on KGN cell viability were analyzed. Statistical significance was determined using one-way ANOVA (GraphPad Prism 8), with *p<0.05, **p<0.01, ***p<0.001.

levels, and stabilizing mitochondrial function (Long *et al*., 2024; Pililis *et al*., 2024).

These results are consistent with previous studies that have highlighted the antioxidant, anti-inflammatory, and anti-apoptotic roles of ACE inhibitors and mineralocorticoid receptor antagonists in various tissues, including cardiovascular and renal systems. Importantly, our data extend these findings to ovarian granulosa cells, an underexplored area. Prior work by Jin *et al*. (2016) demonstrated that hyperglycemia promotes ROS generation and mitochondrial dysfunction in ovarian cells, while Ding *et al*. (2020) showed that RAAS modulation improves ovarian morphology and function in diabetic rats. Our study provides mechanistic cellular evidence supporting these *in vivo* observations, suggesting that captopril and spironolactone act synergistically to preserve granulosa cell health by alleviating oxidative and mitochondrial stress (Stener-Victorin *et al*., 2024).

he observed reductions in ROS levels further highlight the antioxidant potential of these agents. Captopril's sulfhydryl group scavenges free radicals, while spironolactone inhibits aldosterone-mediated NADPH oxidase activation, reducing ROS production. Their combined use produced the strongest ROS reduction, suggesting a synergistic or additive antioxidant effect (Tang *et al*., 2024).

Our work contributes to the emerging understanding that the RAAS system is not only critical for cardiovascular and renal function but also plays a significant role in ovarian physiology. Dysregulation of RAAS under diabetic or hyperandrogenic conditions exacerbates ovarian oxidative stress, inflammation,

and fibrosis, leading to impaired folliculogenesis, anovulation, and infertility. By targeting both ACE and mineralocorticoid receptors, captopril and spironolactone may help restore ovarian homeostasis and preserve fertility potential in women with PCOS or diabetes (Wimalawansa *et al.*, 2024; Xu *et al.*, 2024).

Despite the promising findings, this study has several limitations. First, it was conducted solely *in vitro* using the KGN cell line, which, although widely used, may not fully recapitulate the complex ovarian microenvironment. Future research should validate these findings in primary human granulosa cells, animal models, and clinical settings. Second, while we focused on oxidative stress and mitochondrial function, additional endpoints such as hormone production, apoptosis markers, inflammatory mediators, and extracellular matrix remodeling should be explored. Third, long-term studies are necessary to determine whether the protective effects observed here translate into improved ovarian reserve, ovulation rates, and fertility outcomes over time (Zheng *et al.*, 2024).

Furthermore, investigating potential combinatorial effects of RAAS inhibitors with standard anti-diabetic drugs such as metformin or GLP-1 receptor agonists may reveal new strategies to synergistically target both metabolic and reproductive dysfunction in diabetic women.

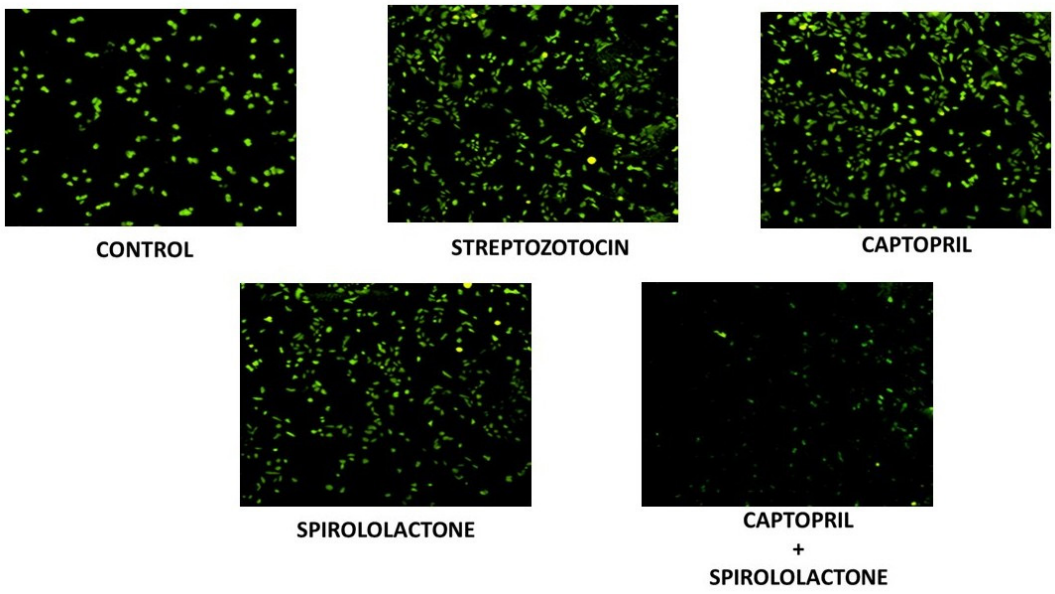


Figure 2: DCFDA Staining for KGN cells to measure ROS levels.

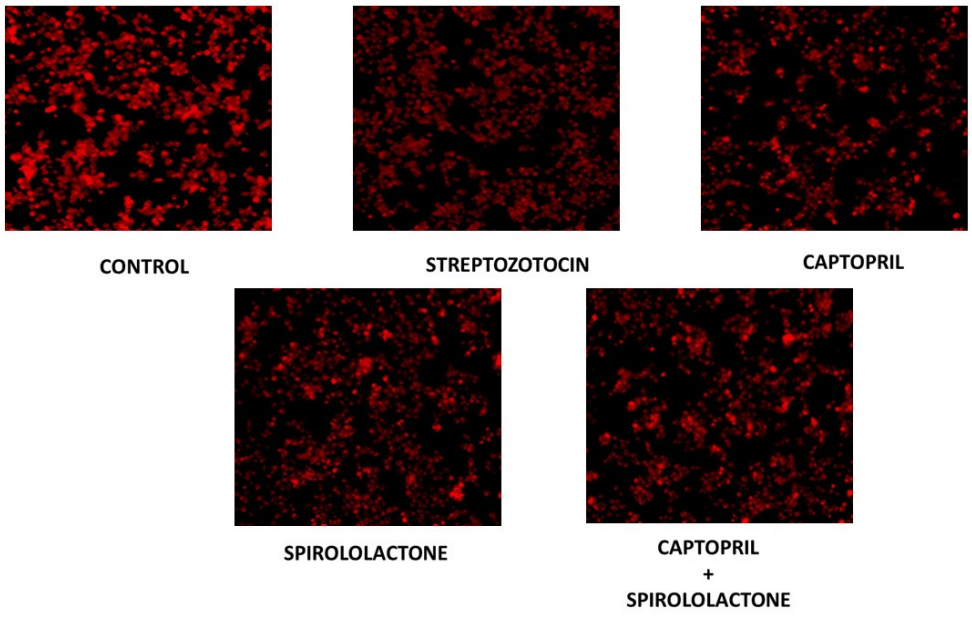


Figure 3: JC-1 Staining for the KGN Cell Mitochondrial Membrane Potential (MMP) Assay.

CONCLUSION

This study provides novel evidence that captopril and spironolactone, individually and in combination, protect human granulosa-like KGN cells from hyperglycemia-induced oxidative stress, mitochondrial dysfunction, and cell death. By reducing ROS levels, preserving mitochondrial membrane potential, and improving cell viability, these RAAS inhibitors show strong cytoprotective potential in an *in vitro* model of diabetic ovarian dysfunction. Our findings extend previous systemic observations to the cellular level, suggesting a promising therapeutic avenue for addressing infertility associated with PCOS and diabetes. Future *in vivo* studies and clinical trials are essential to validate these results and explore their long-term impacts on hormonal function, ovarian reserve, and fertility outcomes in women affected by metabolic reproductive disorders.

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ABBREVIATIONS

ACE: Angiotensin-Converting Enzyme; **ΔΨm:** Mitochondrial Membrane Potential; **DMEM:** Dulbecco's Modified Eagle Medium; **DMSO:** Dimethyl Sulfoxide; **FBS:** Fetal Bovine Serum; **GC:** Granulosa Cell; **JC-1:** 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; **KGN:** Human Granulosa-Like Tumor Cell Line; **MMP:** Mitochondrial Membrane Potential; **MR:** Mineralocorticoid Receptor; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; **RAAS:** Renin-Angiotensin-Aldosterone System; **ROS:** Reactive Oxygen Species; **STZ:** Streptozotocin; **T2DM:** Type 2 Diabetes Mellitus.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Chandana G and Bharathi D.R. formulated the research question(s), designing the study, carrying it out, analysing the data and writing the manuscript. Mohammad Ali and Bharathi D.R. reviewed the manuscript. All the authors provided approval for publishing the manuscript.

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