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Original Article

In vitro Evaluation of the Reno-Protective Effects of Captopril and Spironolactone in Streptozotocin-Induced Diabetic Nephropathy: Synergistic Antioxidant and Anti-Inflammatory Mechanisms

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ABSTRACT

Background: Diabetic Nephropathy (DN) is a major complication of diabetes characterized by oxidative stress and inflammation, leading to progressive renal damage. This study aimed to investigate the reno-protective effects of captopril and spironolactone, individually and in combination, against Streptozotocin (STZ)-induced nephrotoxicity *in vitro*. **Materials and Methods:** Normal Rat Kidney Epithelial (NRK-52E) cells were exposed to STZ to mimic oxidative and inflammatory injury. Cells were treated with captopril, spironolactone, or their combination. Cell viability and morphology were evaluated by MTT assay and microscopy. Antioxidant potential was assessed using Total Antioxidant Capacity (TAC) and Ferric Reducing Antioxidant Power (FRAP) assays, while anti-inflammatory activity was examined via protein denaturation assay and western blotting for IL-6, TNF-α, and TGF-β expression. **Results:** STZ exposure induced marked oxidative stress, cell death, and inflammation in NRK-52E cells. Captopril and spironolactone treatment significantly improved cell viability, preserved normal morphology, and reduced oxidative and inflammatory responses. Spironolactone exhibited stronger antioxidant activity than captopril, while the combination therapy produced a synergistic effect, restoring antioxidant balance and suppressing inflammatory cytokines more effectively than monotherapy. **Conclusion:** Captopril and spironolactone demonstrate significant antioxidant and anti-inflammatory properties, attenuating STZ-induced renal injury. Their combination enhances reno-protection, supporting the potential role of dual therapy in managing diabetic nephropathy. Further studies are needed to validate these findings and assess clinical translation.

Keywords: Spironolactone, Captopril, Diabetic nephropathy, Oxidative stress, Inflammation, NRK-52E cells.

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INTRODUCTION

Diabetes is a chronic metabolic condition characterized by elevated blood sugar levels due to either inadequate insulin secretion or impaired insulin utilization by the body. It stands as one of the most widespread health issues worldwide, impacting millions of individuals each year. One of its most serious side effects is Diabetic Nephropathy (DN), which frequently results in irreparable kidney damage (Xu *et al*., 1563). Diabetic Nephropathy (DN) remains a leading cause of chronic kidney disease and end-stage renal failure, contributing substantially to global morbidity and mortality. The incidence of DN continues to rise alongside increasing cases of diabetes and associated metabolic disorders. Poor glycemic control is a predominant risk factor, with sustained hyperglycemia driving oxidative stress and inflammation-key mechanisms in kidney damage. Although advancements in blood glucose regulation and the use of Renin-Angiotensin-Aldosterone System (RAAS) inhibitors have improved disease management, DN persists as a major indication for dialysis and renal transplantation. These therapeutic gaps underscore the pressing need for more targeted and effective treatment strategie (Li *et al*., 1564).

Excessive Reactive Oxygen Species (ROS) buildup is a result of oxidative stress, which is made worse by persistently elevated blood sugar. This advances DN by encouraging lipid peroxidation, cell damage, and the breakdown of vital biological structures. ROS also contribute to DNA damage, mitochondrial dysfunction, and protein oxidation, promoting kidney cell death and fibrosis (Wang *et al*., 2025). Inflammation, alongside

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oxidative stress, significantly contributes to Diabetic Nephropathy (DN). Cytokines like TNF-α and IL-6 trigger inflammatory pathways, leading to cell death, glomerular barrier disruption, and abnormal renal cell growth. These responses worsen kidney damage by promoting podocyte loss, endothelial dysfunction, and fibrosis. TGF-β further accelerates renal fibrosis by driving extracellular matrix buildup, tubular atrophy, and glomerular basement membrane thickening. Due to their central role in DN progression, both oxidative stress and inflammation have made antioxidants and anti-inflammatory agents promising targets for therapeutic intervention (Obeidat *et al.*, 2025).

Among these therapeutic agents are spironolactone, a mineral corticoid receptor antagonist, and captopril, an Angiotensin-Converting Enzyme (ACE) inhibitor, both demonstrating renoprotective benefits. Spironolactone exerts anti-fibrotic and anti-inflammatory effects by reducing oxidative stress, inhibiting macrophage infiltration, and decreasing TGF-β expression (Xie *et al.*, 0003). It has demonstrated the ability to reduce proteinuria, enhance endothelial function, and diminish renal fibrosis, indicating its potential as a therapeutic option for Diabetic Nephropathy (DN). Meanwhile, captopril mitigates DN progression by blocking angiotensin II-mediated vasoconstriction, lowering blood pressure, decreasing albuminuria, and preventing glomerular hypertension. In addition, it exhibits antioxidant activity by scavenging free radicals and minimizing cellular damage caused by oxidative stress.

Although both spironolactone and captopril offer renoprotective advantages, their combined effects in treating DN remain unclear. Research suggests that ACE inhibitors and mineral corticoid receptor antagonists may work synergistically to target multiple pathways involved in DN pathophysiology (Pastena *et al.*, 2025). This combination therapy could enhance kidney protection by regulating RAAS function, reducing oxidative stress, and suppressing inflammation. However, concerns regarding hyperkalemia and other adverse effects have limited its widespread clinical adoption (Wasehuus *et al.*, 2025). Further research is needed to assess the safety and efficacy of dual therapy for Diabetic Nephropathy (DN). This study investigates the renoprotective effects of captopril and spironolactone—alone and in combination—using an in vitro DN model. It focuses on their influence on oxidative stress and inflammatory markers such as TGF-β IL-6, and TNF-α. By examining the interaction between oxidative stress, inflammation, and RAAS dysregulation, the study aims to uncover therapeutic mechanisms and support the development of improved treatments to enhance kidney function and patient outcomes in diabetes.

MATERIALS AND METHODS

MTT analysis of renal cells

Cell viability was evaluated using the MTT assay. NRK-52E cells were exposed to varying concentrations (6.25-100 µg/mL) of streptozotocin, spironolactone, or captopril for 24 hr. For the combination group, both CAP and SPIRO were applied at 50 µg/mL each, or their combination for 24 hr. This methodology is consistent with previous antioxidant studies by Zhang, Z. T., *et al.*, (2024).

Cell Morphology Assay

NRK-52E cells were cultured in 6-well plates at a density of 2×10^5 cells per well and incubated overnight for adherence. The following day, cells were treated with streptozotocin and spironolactone, either individually or in combination, at a concentration of 50 µg/mL for 24 hours. Morphological changes were examined under a phase-contrast microscope, following methods aligned with those used by Xi, Y., *et al.*, (2025).

Fluorescence Microscopy Assessment

Apoptotic changes in NRK-52E cells were evaluated using dual staining with acridine orange and ethidium bromide. Following treatment with streptozotocin, captopril, spironolactone, or their combination, cells were examined under a fluorescence microscope to assess cell viability and apoptosis. This method is consistent with previous oxidative stress studies reported by Padhye-Pendse, A., *et al.*, (2024).

DPPH Assay For Antioxidant Activity

The antioxidant activity of captopril, spironolactone, and their combination was evaluated using the DPPH assay at concentrations of 5-25 µg/mL. Absorbance was recorded at 517 nm after 30 min of incubation in the dark. This method aligns with antioxidant evaluation approaches previously described by Fajrin *et al.* The DPPH radical scavenging activity (%) was calculated using the following formula: % Scavenging Activity = [(Absorbance of Blank - Absorbance of Sample) / Absorbance of Blank] * 100 Fajrin, F. A., *et al.*, (2024).

Evaluation of Antioxidant Potential via TAC

Total Antioxidant Capacity (TAC) of captopril, spironolactone, and their combination was measured using the phosphomolybdenum method at concentrations of 5-30 µg/mL. Absorbance was recorded at 695 nm after incubation at 95°C for 90 min, and results were expressed in ascorbic acid equivalents. This approach is consistent with oxidative stress evaluation methods reported by El Tabaa, M. M., *et al.*, (2024).

FRAP Assay

The FRAP assay was used to evaluate the antioxidant potential of captopril, spironolactone, and their combination at 5-30 µg/mL by measuring Fe^{3+} to Fe^{2+} reduction. Absorbance was recorded at 700 nm after reaction with ferric chloride. This method aligns with antioxidant assessments previously described by Yeung, M. H., *et al.*, (2024).

Albumin Denaturation Assay for Assessing Anti-Inflammatory Activity

An *in vitro* anti-inflammatory study evaluated the effects of captopril, spironolactone, and their combination (5-20 µg/mL) based on protein denaturation inhibition. Turbidity was measured at 660 nm following incubation with bovine serum albumin. This assay approach is comparable to that used by Dh *et al.*, in evaluating anti-inflammatory potential in diabetic nephropathy. Dh, H. S., *et al.*, (2024).

Assessment Of Inflammatory and Oxidative Stress Biomarkers By Western Blotting

Inflammatory markers (TNF-α, TGF-β, IL-6) were analyzed in NRK-52E cells via western blot, following protein extraction and quantification using the Bradford assay. Expression levels were normalized to β-actin, revealing that captopril and spironolactone reduced inflammation. These findings align with previous anti-inflammatory and reno-protective outcomes reported by Shan, X. M., *et al.*, (2025).

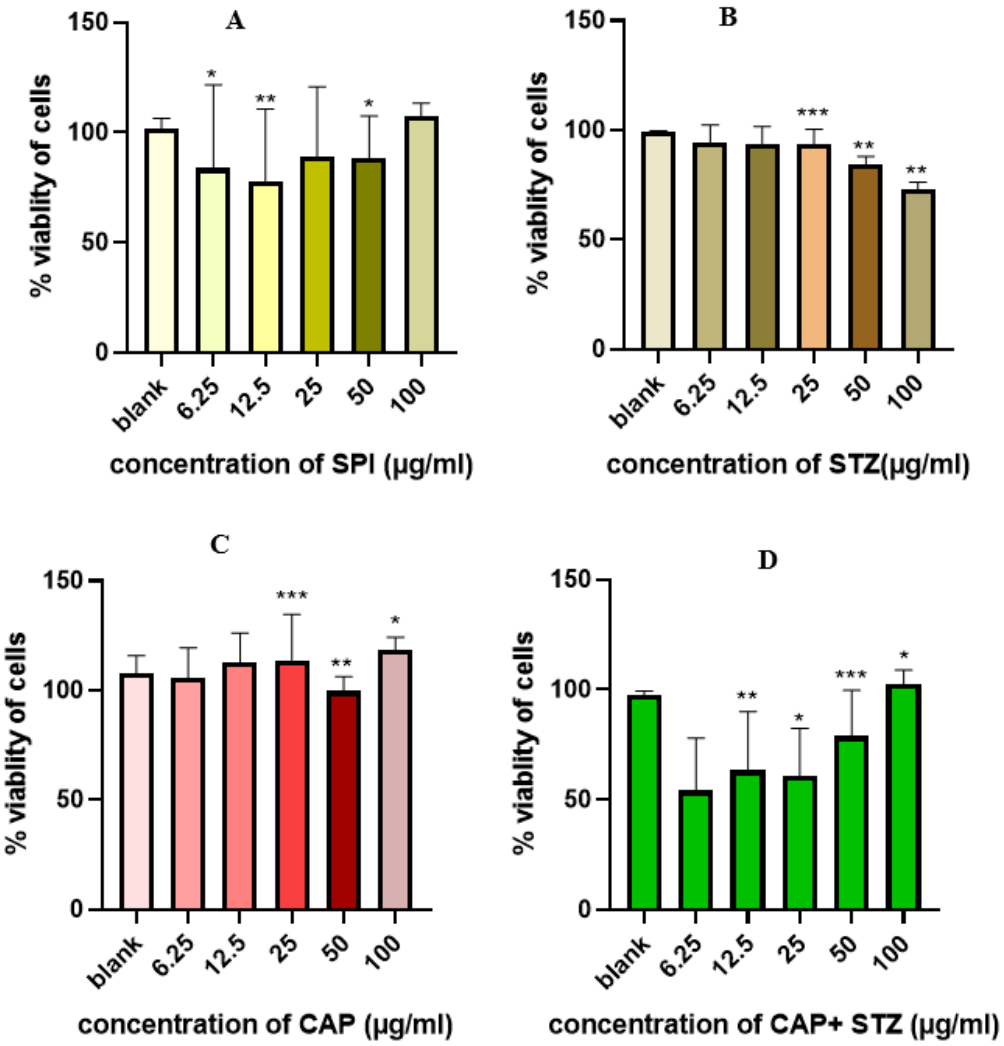
RESULTS

MTT Analysis of Renal Cells

Cell viability in NRK-52E cells following exposure to spironolactone, captopril, and Streptozotocin (STZ) was evaluated using the MTT colorimetric assay. STZ exhibited dose-dependent cytotoxicity, inducing approximately 75% cell death at 100 µg/mL. In contrast, captopril and spironolactone showed no toxicity across the 6.25-100 µg/mL range and supported cell proliferation (Figures 1A-C). Captopril and spironolactone alone displayed IC_{50} values of 1054 µg/mL and 1062 µg/mL, respectively (Figure 1F displays IC_{50} values for spironolactone, captopril, and STZ.), confirming their safety. Figures 1D and 1E illustrate their individual protective effects against STZ-induced damage, while co-treatment markedly restored viability close to control levels at 100 µg/mL (Figure 1G), highlighting the combination's synergistic efficacy.

Cell Morphology Assay

NRK-52E cells were exposed to STZ (5 mM), cap (30 µM), spi (30 µM), and their combination (10 µM + 20 µM) for 48 and 24 hr to



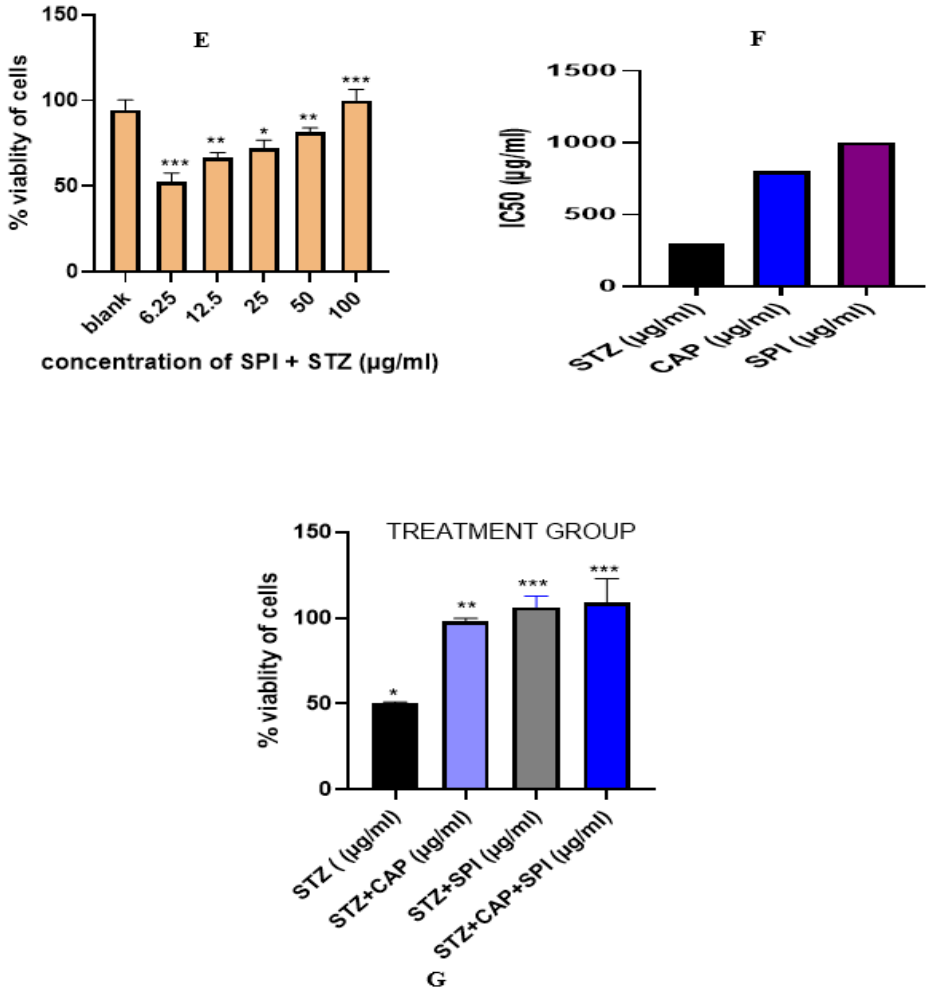


Figure 1: Evaluation of NRK-52E cell viability following treatment with captopril, spironolactone, and Streptozotocin (STZ). Panels (A-D) show the effects of captopril and STZ co-treatment, while panel (E) depicts the impact of spironolactone with STZ. Panel (F) presents the IC_{50} values for spironolactone, captopril, and STZ, and panel (G) illustrates overall changes in cell viability across treatment groups. Data are presented as mean ± SEM ($n = 3$). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. *$p < 0.05$ vs control group; #$p < 0.05$ vs STZ-treated group.

evaluate effects on cell morphology and viability. After treatment, cells were examined using an inverted light microscope. Control cells maintained typical morphology, characterized by clear plasma membranes, evenly distributed cytoplasm, and intact nuclei (Figure 2). In contrast, treated cells showed significant morphological changes such as cellular enlargement, irregular shapes, cytoplasmic vacuolization, membrane blebbing, and detachment from neighboring cells. These alterations were more evident with higher doses and longer treatment durations, suggesting that captopril and spironolactone, whether administered alone or in combination, can induce cellular stress and morphological disruption in NRK-52E cells.

Fluorescence Microscopy Assessment

Cell viability and apoptosis in NRK-52E cells were assessed using dual staining with Acridine Orange (AO) and Ethidium Bromide (EB). This technique allows differentiation between live cells, which fluoresce green, and apoptotic cells, which exhibit yellowish-orange fluorescence. Cells exposed to Streptozotocin (STZ) displayed marked apoptotic characteristics, as evidenced by EB uptake and distinct nuclear staining. In contrast, cells treated with captopril, spironolactone, or their combination maintained green fluorescence, suggesting preserved membrane integrity and reduced apoptosis. These findings indicate the cytoprotective effects of both drugs against STZ-induced cell damage. Notably, the results were consistent with MTT assay data, reinforcing the role of the combination therapy in preserving cell viability and mitigating STZ toxicity (Figure 3).

DPPH Assay For Antioxidant Activity

Spironolactone showed concentration-dependent inhibition of the oxidative activity and exhibited a 75% inhibition of DPPH radicals at doses that ranged from 5 to 25 µg/mL. Captopril exhibited a reduction of 20% in free radicals with the maximum

dose of 25 µg/mL. The combined treatment of captopril and spironolactone offered a 95% reduction in free radicals, exceeding the effect of captopril alone and showing prominent antioxidant effects equivalent to high doses of ascorbic acid (Figure 4).

Evaluation of Antioxidant Potential via TAC

The TAC was evaluated for captopril, spironolactone, and their combination at doses ranging from 5 to 30 µg/mL using the phosphomolybdenum assay. The results showed how effective spironolactone is at scavenging free radicals, more effectively than the other treatments, and gave significant antioxidant activity, as shown by captopril. When the two drugs were combined, there was an intermediate level of TAC, suggesting that the combination did not enhance the antioxidant effect compared to spironolactone alone. These results indicated that the combination of the drugs showed different degrees of antioxidant potential compared to individual drugs, and spironolactone was found to be the most effective drug in reducing oxidative stress (Figure 5).

FRAP Assay

Demonstrated an enhanced ferric-reducing ability, with a cumulative effect that resulted in a greater reduction of ferric ions compared to each drug individually. The ferric-reducing power was 51 µg/mL for the combined treatment of captopril and spironolactone at a 30 µg/mL concentration, higher greater the effect of each drug alone. This would be suggestive of synergistic action since together, these two drugs amplify the ability of these drugs in the reduction of oxidative stress. Such an action enhances the conversion of ferric ions Fe^{3+} into ferrous ions Fe^{2+}, suggesting that the combination of drugs may show greater antioxidant capacity than monotherapy and, thereby, provide protection against oxidative damage (Figure 6).

Albumin Denaturation Assay for Assessing Anti-Inflammatory Activity

The albumin denaturation assay showed that the two drugs both possessed significant anti-inflammatory activity because

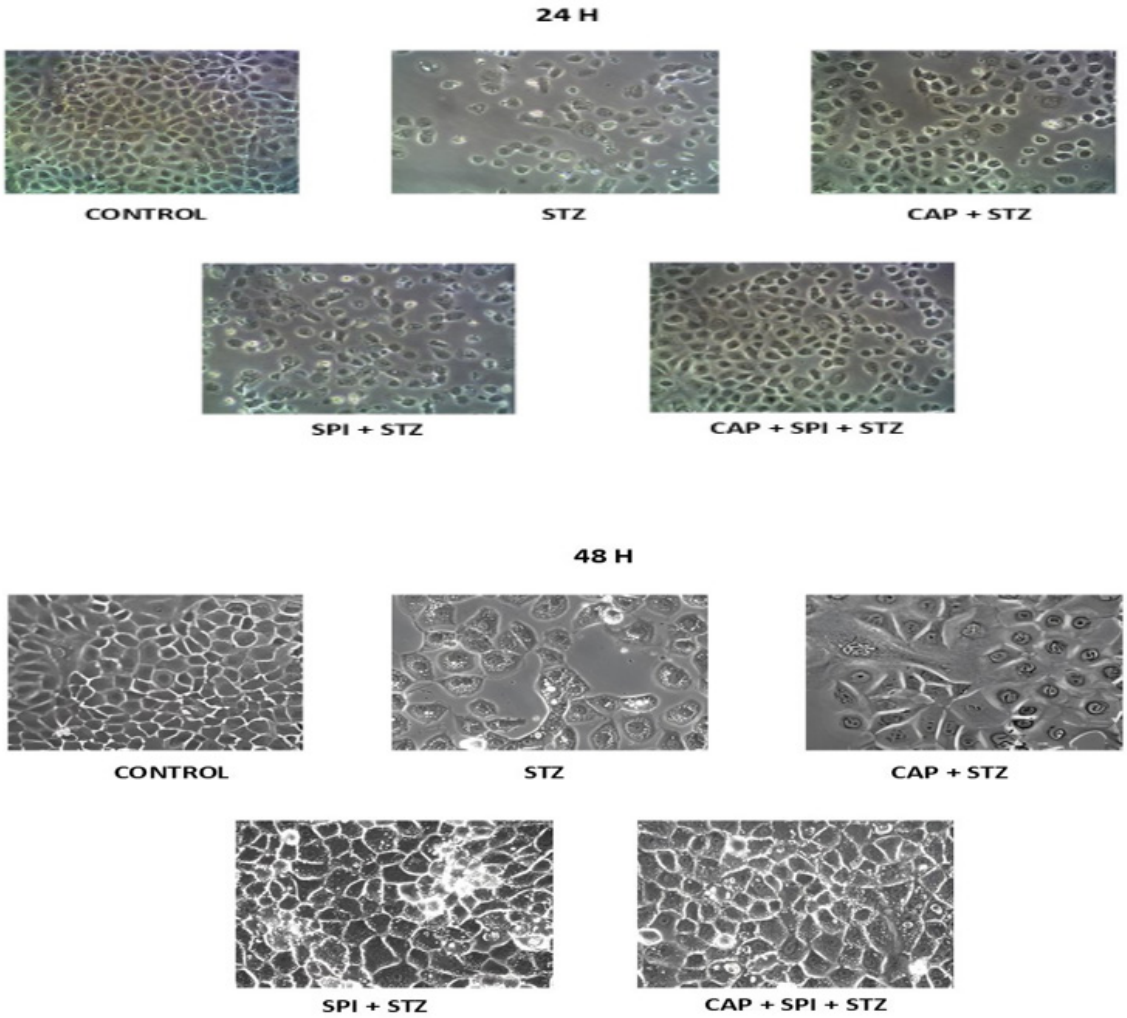


Figure 2: Morphological assay of NRK-52E cells under 100× phase contrast microscopy (24 hr, 48 hr) with spironolactone, captopril, and their combination.

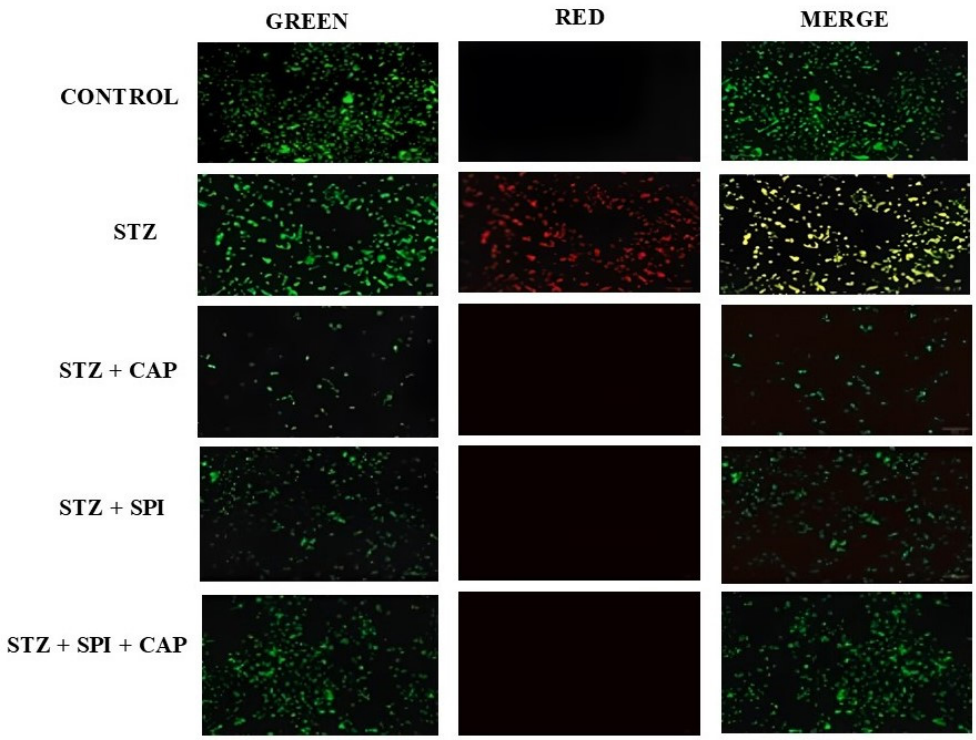


Figure 3: Fluorescence staining of NRK-52E cells with Captopril, Spironolactone and their co-treatment.

they reduced the protein denaturation. It was observed at concentrations of 5 to 20 µg/ml that the dose-dependent protein denaturation inhibition was established with a maximum of 50% inhibition by captopril at 25 µg/mL (Figure 7). Similar concentration of spironolactone demonstrated 42% inhibition of protein denaturation, while combination of captopril and spironolactone inhibited with a little greater value of 48%. In summary, this result demonstrates the synergistic action of captopril and spironolactone combination while the captopril alone exhibits slightly greater inhibitory anti-inflammatory effect than its combination. The data suggests that both drugs, individually and together, may be used as therapeutic agents to counteract inflammation through the inhibition of protein denaturation.

Assessment Of Inflammatory And Oxidative Stress Biomarkers By Western Blotting

Analysis was conducted to evaluate the impact of STZ, captopril, and spironolactone on the expression of pro-inflammatory cytokines TNF-α, TGF-β, and IL-6 in NRK-52E cells. Control cells exhibited baseline levels of these markers (Figure 8). Exposure to STZ significantly increased TGF-β expression by about 3.6 times, reflecting an inflammatory response triggered by STZ. However, simultaneous treatment with captopril and spironolactone effectively suppressed TGF-β expression, bringing it close to the levels observed in untreated control cells. Similarly, STZ significantly elevated TNF-α and IL-6 expression, both of which were attenuated by individual treatments with captopril or spironolactone. However, the combination treatment resulted in the most substantial reduction, suggesting a synergistic anti-inflammatory effect. These findings support the protective role of captopril and spironolactone against STZ-induced inflammation and fibrosis in renal epithelial cells. Statistical analysis confirmed significant differences in cytokine expression among treatment and control groups ($p < 0.05$) (Figures 8B-D), highlighting the promising therapeutic role of the combined treatment in reducing inflammation linked to diabetic nephropathy (Figure 8).

DISCUSSION

Diabetes Mellitus (DM) is a major cause of kidney impairment, often progressing to End-Stage Renal Disease (ESRD). Persistent hyperglycemia plays a central role in Diabetic Nephropathy (DN) by inducing mesangial cell hypertrophy, excessive Extracellular Matrix (ECM) deposition, and abnormal renal cell proliferation, leading to glomerular enlargement and functional decline (Hou *et al.*, 2024). Elevated glucose also stimulates mesangial proliferation and ECM protein synthesis, while ectopic fat accumulation in non-adipose tissues contributes to lipotoxicity, increasing triglycerides, fatty acids, and non-esterified fatty acids, thereby exacerbating renal injury (Ismail *et al.*, 2024).

Spironolactone improves lipid metabolism and attenuates early renal impairment in DN, while antihypertensive drugs such as captopril are effective in reducing inflammation and preventing glomerular filtration decline (Zhou *et al.*, 2024). In this study, NRK-52E cells were used to evaluate the cytotoxic effects of Streptozotocin (STZ) and the protective roles of

captopril and spironolactone. Antioxidant activity was measured using the DPPH assay, showing that both drugs significantly reduced oxidative stress, although the synergistic effect of their combination remains uncertain.

STZ exposure induced distinct morphological changes—cell enlargement, membrane blebbing, cytoplasmic vacuoles, and reduced cell-to-cell adhesion—indicating cellular stress and potential dysregulation of apoptosis and autophagy. Treatment with captopril, spironolactone, or both preserved renal cell morphology by reducing oxidative stress, inflammation, and fibrosis (Rosas-Martínez *et al.*, 2024).

Spironolactone demonstrated the highest Total Antioxidant Capacity (TAC), followed by combination therapy, with captopril alone exhibiting the lowest TAC. FRAP assay results corroborated these findings, showing greater ferric ion-reducing ability for spironolactone (Okail *et al.*, 2024). STZ-induced nephrotoxicity is linked to Reactive Oxygen Species (ROS) overproduction, tubular hypertrophy, and interstitial damage, processes attenuated by both treatments (Zeng *et al.*, 2024).

Hyperglycemia disrupts mitophagy through the AMPK/mTOR pathway, contributing to mitochondrial dysfunction. MTT assay results indicated that spironolactone preserved cell viability, while

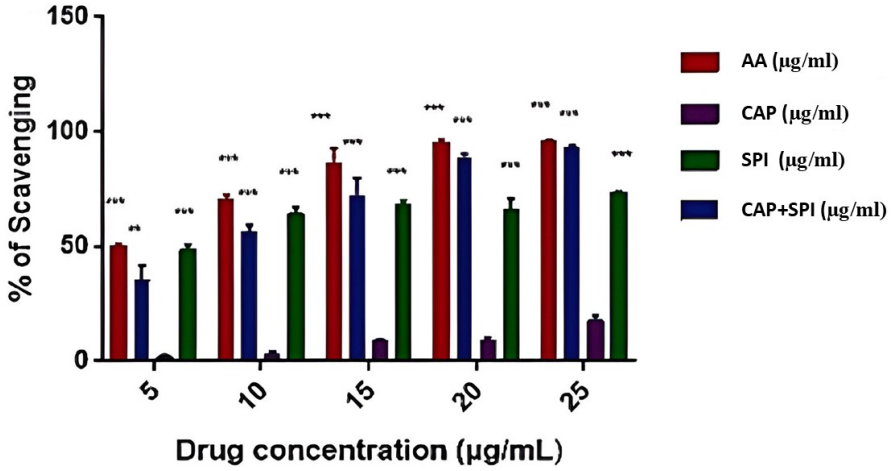


Figure 4: Spironolactone, captopril, and their combination's capacity to scavenge free radicals was contrasted with that of ascorbic acid Data are presented as mean ± SEM (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post-hoc* test. *p < 0.05 vs control group; #p < 0.05 vs STZ-treated group.

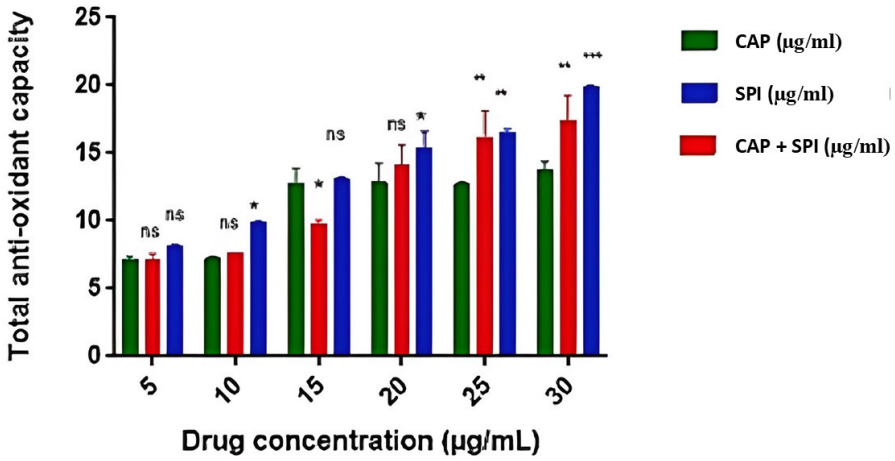


Figure 5: Captopril and spironolactone showed dose-dependent TAC (5-30 µg/mL), with spironolactone having the highest activity, followed by the combination and captopril alone. Data are expressed as mean ± SEM Data are presented as mean ± SEM (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post-hoc* test. *p < 0.05 vs control group; #p < 0.05 vs STZ-treated group.

both drugs reduced oxidative stress-related injury. Combined treatment showed enhanced protective effects, although further mechanistic studies are needed (Ye *et al.*, 2025).

ROS generation by STZ is a critical trigger for apoptosis in renal cells, including podocytes and proximal tubular epithelial cells. Captopril and spironolactone significantly improved cell survival, highlighting their renoprotective potential. Chronic hyperglycemia also promotes inflammation, tubular atrophy, glomerulosclerosis, and fibrosis (Wang *et al.*, 2025).

Immunoblotting revealed STZ-induced increases in IL-6, TNF-α, and TGF-β, indicating activation of pro-fibrotic and inflammatory pathways. Treatment with spironolactone and captopril markedly reduced TGF-β, a key mediator of fibrosis (Li *et al.*, 2024). Reduction in TNF-α levels was evident with spironolactone alone and in combination, while captopril significantly decreased IL-6 expression. One-way ANOVA followed by Tukey's post-hoc analysis confirmed statistically significant reductions compared with controls (Liu *et al.*, 2025).

In summary, captopril and spironolactone effectively attenuated oxidative stress, inflammation, and fibrosis in STZ-induced renal injury. Using NRK-52E cells as an in vitro DN model, this study supports their potential as adjunct therapies for DN. Further investigations are warranted to elucidate their molecular mechanisms and assess their translational value.

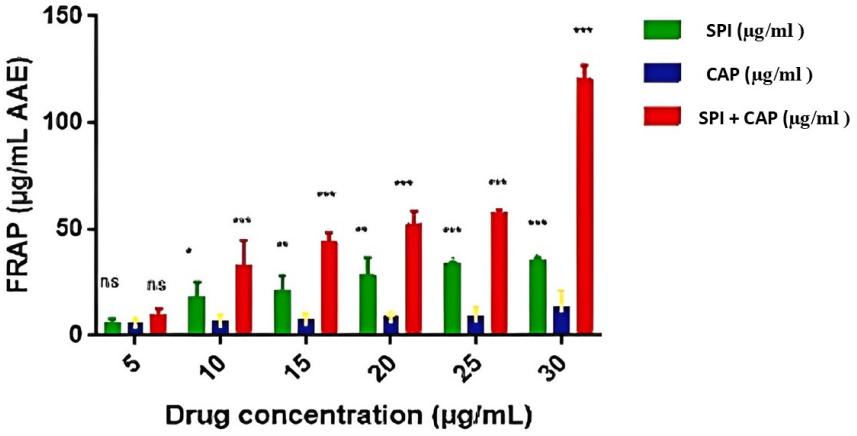


Figure 6: At 30 µg/mL, the combination of spironolactone and captopril showed higher ferric-reducing power than either drug alone, comparable to ascorbic acid Data are presented as mean ± SEM (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post-hoc* test. *p < 0.05 vs control group; #p < 0.05 vs STZ-treated group.

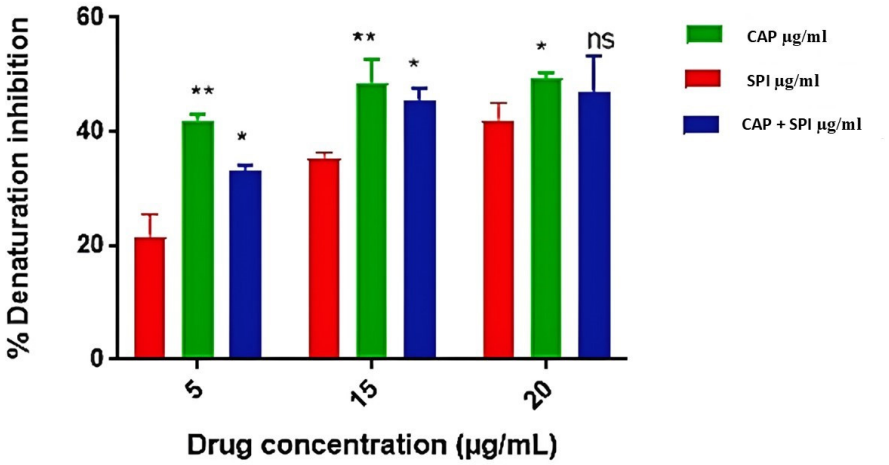
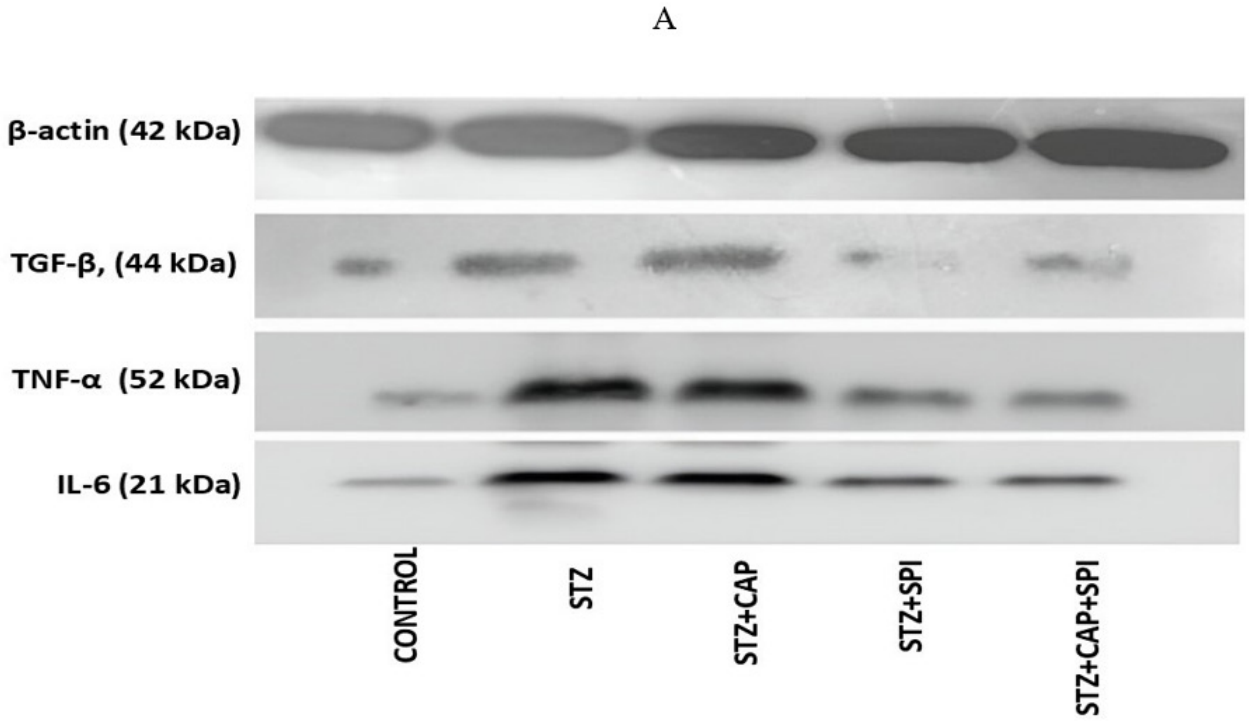


Figure 7: The anti-inflammatory effects of captopril, spironolactone, and their combination were evaluated by measuring their dose-dependent inhibition of protein denaturation, with ascorbic acid serving as reference. Data are presented as Mean ± SEM (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. *p < 0.05 vs control group; #p < 0.05 vs STZ-treated group.


A
β-actin (42 kDa)
TGF-β, (44 kDa)
TNF-α (52 kDa)
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CONTROL
STZ
STZ+CAP
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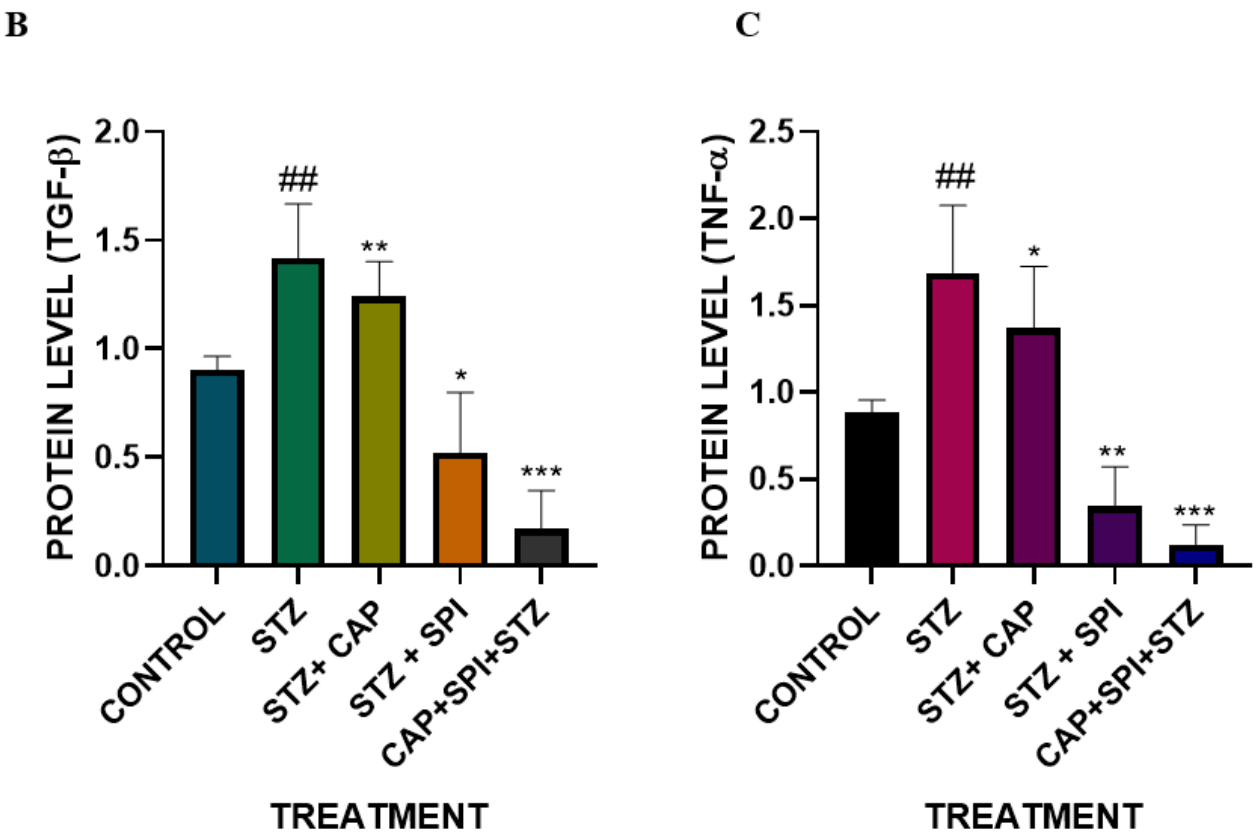
STZ+CAP+SPI


B
PROTEIN LEVEL (TGF-β)
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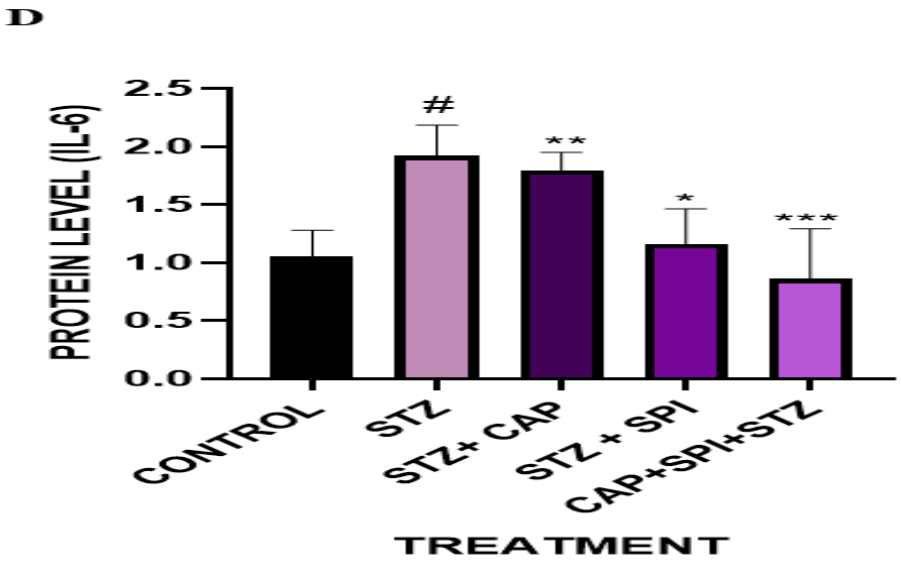

Figure 8: (A) Western blot analysis showing expression levels of TNF-α, IL-6, and TGF-β in STZ-treated NRK-52E cells, with β-actin as the loading control. (B-D) Quantitative graphs depict the synergistic anti-inflammatory effects of treatments. Data are presented as mean ± SEM (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. *p < 0.05 vs control group; #p < 0.05 vs STZ-treated group.

CONCLUSION

This study highlights the protective effects of captopril and spironolactone against STZ-induced nephrotoxicity in rat kidney cells, mitigating apoptosis, oxidative stress, and inflammation via suppression of IL-6, TNF-α, and TGF-β. These findings support their therapeutic potential in diabetic nephropathy, warranting further research to confirm mechanisms and explore clinical applications.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare no conflict of interest related to this study.

ABBREVIATIONS

AMPK: Adenosine monophosphate-activated protein kinase; **ANOVA:** Analysis of variance; **CKD:** Chronic kidney disease; **DN:** Diabetic nephropathy; **DPPH:** 2,2-Diphenyl-1-picrylhydrazyl; **ECM:** Extracellular matrix; **ESRD:** End-stage renal disease; **FRAP:** Ferric reducing antioxidant power; **IL-6:** Interleukin-6; **mTOR:** Mammalian target of rapamycin; **NRK-52E:** Normal rat kidney epithelial cells; **PCT:** Proximal convoluted tubule; **ROS:** Reactive oxygen species; **STZ:** Streptozotocin; **TAC:** Total antioxidant capacity; **TGF-β:** Transforming growth factor-beta; **TNF-α:** Tumor necrosis factor-alpha.

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.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study did not involve human or animal participants; therefore, ethical approval and consent were not required.

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