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Research Article

Keywords: biochanin A, diabetic cardiomyopathy, myocardial injury, oxidative stress, rat model

Posted Date: November 13th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11566445/v1

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Additional Declarations:
No competing interests reported.

Table 1 is available in the Supplementary Files section.

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Running title: Biochanin A protects diabetic myocardial injury

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FUNDING

This study was supported by Erciyes University Scientific Research Projects Unit (Project no. FDK-2022-12072).

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All experimental procedures involving animals were conducted in compliance with the ethical standards of the Institutional Animal Care and Use Committee (IACUC) of Erciyes University and were approved by the Local Animal Research Ethics Committee of Erciyes University (Approval no. 22/030/2022; Kayseri, Turkey).

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

DATA AVAILABILITY

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

Mohamed Lemine El Bechir: conceptualization, experimental design, data acquisition, analysis, and interpretation; drafting and critically revising the manuscript; Ali Tuğrul Akin: assisted in experimental design, provided intellectual input, and contributed to critical revision of the manuscript. Tayfun Ceylan: assisted in histopathological analyses and sample processing; Emin Kaymak: performed enzyme-linked immunosorbent assay (ELISA) procedures; Şaban Varinli: assisted with animal injections and experimental procedures; Ayşe Toluk: supervised the study, provided intellectual guidance, and critically revised the manuscript.

Abstract: Objective: Diabetic cardiomyopathy (DCM) is a diabetes-associated cardiovascular complication characterized by progressive myocardial structural remodeling and functional deterioration, representing a leading cause of diabetes-related morbidity and mortality. Biochanin A (BCA), a phytoestrogenic isoflavone derived from *Trifolium pratense*, exhibits pleiotropic biological activities including antidiabetic, anti-inflammatory, and antioxidant effects. This study evaluated the therapeutic effects of BCA in a streptozotocin (STZ)-induced rat model of diabetic myocardial injury. **Methods:** Forty male Wistar albino rats were randomized into four groups: Control, BCA (20 mg/kg/day), STZ (45 mg/kg), and STZ+BCA, with BCA administered i.p. for 30 days post-induction. Cardiac stress markers (ANP, NT-proBNP), endoplasmic reticulum stress (GRP78), and apoptosis (caspase-3) were assessed via immunohistochemistry, alongside serum myocardial injury markers (CK-MB, hs-cTnT, LDH), oxidative stress parameters (SOD, CAT, MDA), and pro-inflammatory mediators (TNF-α, IL-6, NF-κB). **Results:** BCA treatment significantly reduced hyperglycemia, increased SOD and CAT levels, decreased MDA, reduced circulating TNF-α, IL-6, and NF-κB, and lowered serum myocardial injury markers. Immunohistochemistry showed reduced ANP, NT-proBNP, GRP78, and caspase-3 immunoreactivity, accompanied by improved myocardial architecture on histopathology. **Conclusion:** These findings suggest that BCA exerts multitarget therapeutic effects on diabetic myocardial injury, through modulation of glycemic, oxidative, inflammatory, and ER stress-related apoptotic pathways, highlighting it as a promising candidate for the management of diabetic cardiac complications.

Key words: biochanin A, diabetic cardiomyopathy, myocardial injury, oxidative stress, rat model.

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterized by persistent hyperglycemia and remains one of the most significant global public health challenges. Type 1 diabetes mellitus (T1DM), which results from the destruction of pancreatic β-cells and absolute insulin deficiency, affects an estimated 9.5 million people worldwide in 2025, a 13% increase since 2021, and this number is projected to reach 14.7 million by 2040 (Ogle et al., 2025). Among the various complications associated with diabetes, diabetic cardiomyopathy (DCM) is particularly concerning, as it progressively impairs cardiac function and elevates mortality risk (Abdullah et al., 2025). Unlike other cardiovascular complications, DCM develops independently of classical risk factors such as hypertension and coronary artery disease, and is primarily characterized by myocardial structural remodeling, increased fibrosis, and deteriorating cardiac performance (Nakamura et al., 2022).

Cardiac dysfunction in diabetes largely stems from an imbalance between the excessive generation of reactive oxygen species (ROS) and the body's capacity to neutralize them through antioxidant defenses, leading to a state known as oxidative stress (Wilson et al., 2018). This imbalance overwhelms key protective enzymes, including catalase (CAT) and superoxide dismutase (SOD), leading to mitochondrial dysfunction and progressive oxidative damage to the myocardium (Abukhalil et al., 2021). Concurrently, lipid peroxidation is intensified, as reflected by elevated malondialdehyde (MDA) levels, which further promotes myocardial injury, fibrosis, and diastolic dysfunction (Aju et al., 2019). Hyperglycemia exacerbates oxidative stress by triggering excessive ROS formation while simultaneously depleting endogenous antioxidant reserves, further aggravating myocardial damage (Gu et al., 2020).

In addition to oxidative stress, disruption of endoplasmic reticulum (ER) homeostasis plays a significant role in DCM (Chistiakov et al., 2014). The accumulation of misfolded proteins within

the ER triggers the unfolded protein response (UPR), an adaptive mechanism aimed at restoring normal ER function. However, sustained ER disruption prolongs UPR activation, eventually causing cellular impairment, apoptosis, and intensified cardiac injury, thereby promoting further progression of DCM (Ren et al., 2021). Glucose-regulated protein 78 (GRP78) serves as a key upstream regulator of this pathway, persistent ER stress leads to caspase-3 activation, initiating apoptotic cell death through DNA fragmentation and irreversible cardiomyocyte loss (Gorman et al., 2012; Pandey et al., 2019).

Inflammatory signaling further amplifies myocardial damage in the diabetic setting and is closely intertwined with both oxidative and ER stress pathways (Frangogiannis, 2014). ROS-driven activation of nuclear factor kappa B (NF-κB) upregulates the production of pro-inflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), which collectively promote myocardial inflammation, fibrosis, and functional decline (Guo et al., 2018; Rolski and Blyszczuk, 2020; Uciechowski Dempke, 2020). As DCM progresses, circulating levels of cardiac stress biomarkers, including atrial natriuretic peptide (ANP) and N-terminal pro-brain natriuretic peptide (NT-proBNP), become markedly elevated, reflecting myocardial enlargement, increased wall stress, and deteriorating cardiac function. These biomarkers serve as clinically relevant indicators of disease severity and prognosis in diabetic patients (Rubattu et al., 2008; McKie and Burnett, 2016; Kuwahara, 2021).

Despite significant advances in diabetes pharmacotherapy, no targeted treatment currently exists to prevent or reverse DCM. Conventional strategies focused primarily on glycemic control, including prolonged insulin therapy, have shown limited efficacy in halting cardiomyopathic progression (Borghetti et al., 2018; Kennyand Abel, 2019). This therapeutic gap has fueled growing interest in naturally derived bioactive compounds that may target multiple pathogenic pathways simultaneously (Bontempo et al., 2024).

Biochanin A (BCA) is an O-methylated isoflavone naturally present in red clover, chickpea, and alfalfa, recognized for its well-documented antioxidant, anti-inflammatory, and cytoprotective properties (Yu et al., 2019). Mechanistically, BCA has been shown to suppress NF-κB signaling, reduce pro-inflammatory cytokine release, and scavenge ROS, positioning it as a promising multi-target agent in metabolic and inflammatory disorders. Emerging evidence further suggests that BCA can modulate glucose metabolism and enhance insulin sensitivity, reinforcing its potential relevance to diabetic pathophysiology (Harini et al., 2012; Oza and Kulkarni, 2018). Previous studies reported that BCA mitigated oxidative stress and inflammation in STZ plus isoproterenol-induced myocardial injury and attenuated cardiomyopathy in type 2 diabetic rats by increasing SIRT1 expression and reducing oxidative stress (Oza and Kulkarni, 2022; Mahajan and Goyal, 2025). However, whether BCA affects ER stress and ER stress-mediated apoptosis in the diabetic heart has not been addressed, and its therapeutic effect in STZ-induced type 1 diabetic cardiac injury has received little attention. Therefore, the present study aimed to evaluate the therapeutic effect of BCA (20 mg/kg) on myocardial injury in STZ-induced diabetic rats and to determine whether this effect is associated with modulation of oxidative stress, inflammation, and ER stress-related apoptosis (GRP78 and caspase-3).

MATERIALS AND METHODS

Animals

Forty male Wistar albino rats aged 8 weeks and weighing between 200 and 300 g were supplied by the Hakan Çetinsaya Experimental and Clinical Research Center, Erciyes University, Kayseri, Turkey. Rats were housed in cages, each accommodating ten animals, under strictly controlled environmental conditions, including temperature (20–24°C), humidity (40–60%), and a 12-hour cycle of alternating light and darkness. Animals had continuous, unrestricted access to standard rodent chow and water.

Drugs and Chemicals

Biochanin A (BCA, 5 g) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Streptozotocin (STZ) (Catalog No: BSM-817945) was obtained from Bostontchem (Cambridge, MA, USA). All other reagents and chemicals utilized during the experiments were commercially acquired and of analytical quality.

Preparation of BCA and STZ

For the solubility assessment of BCA, 2 mg of the compound was dissolved in a mixture consisting of 1 mL ethanol and 1 mL physiological saline. The resulting solution was vigorously mixed at 1500 rpm for 5 minutes and subsequently sonicated for 10 minutes to ensure complete dissolution. The solution was prepared freshly each day, and the injected volume was calculated individually for each animal according to its body weight to deliver a dose of 20 mg/kg. Streptozotocin (STZ) was separately prepared by dissolving it thoroughly in citrate buffer immediately before use.

Experimental Design

The rats were randomly assigned to four experimental groups. The control group (n = 10) received no treatment, while the BCA group (n = 10) was administered intraperitoneal (i.p.) injections of BCA (20 mg/kg) once daily for 30 days. The diabetes group (n = 10) received a single intraperitoneal dose of STZ (45 mg/kg) to induce diabetes. Meanwhile, the Diabetes + BCA group (n = 10) received BCA (20 mg/kg i.p. per day for 30 days) following diabetes induction (Karganov et al., 2020; Karabulut et al., 2020; Amri et al., 2022). Blood glucose concentrations were assessed two days following STZ administration by collecting samples from the tail vein and analyzing them with a glucometer. Animals exhibiting glucose concentrations equal to or greater than 250 mg/dL were considered diabetic and selected for further experimental evaluation (Fig. 1).

Tissue and Serum Sample Collection

After completing the 30-day experimental period, the rats were anesthetized via intraperitoneal administration of ketamine (75 mg/kg) and xylazine (10 mg/kg). Blood samples were then collected from the abdominal aorta for serum isolation. Blood samples were centrifuged at 3000 rpm for 10 minutes to obtain serum, which was then collected and stored at -80°C until biochemical analyses were performed. Additionally, heart tissues were carefully excised and placed in sterile plastic vials, then preserved at -80°C for further experimental analyses. For histopathological and immunohistochemical assessments, selected portions of heart tissue were immediately transferred into designated fixative solutions.

Assessment of Body Weight and Glucose

Body weight was recorded before STZ injection for dose calculation. Blood glucose was first measured 48 h after STZ injection to confirm diabetes (day 0), and BCA treatment started on the same day. Body weight and blood glucose were then measured weekly (days 7, 14, 21, and 30),

using tail-vein samples and a Contour Plus glucometer, to follow the progression of hyperglycemia and the metabolic effects of diabetes (Fig. 2).

Histopathological Examination

At the end of the experiment, the rat hearts were promptly removed and fixed in 10% formaldehyde. Following fixation, the samples were washed extensively with water and subjected to progressive dehydration using an ascending ethanol series (50, 70, 80, 96%, and three consecutive changes in 100% ethanol). Subsequently, the tissue samples were cleared in xylene and embedded into paraffin wax blocks. Tissue sections measuring 5 μm were then prepared using a microtome, placed onto glass slides, and stained with hematoxylin and eosin (H&E) for routine histological evaluation and immunohistochemistry.

Hematoxylin and Eosin

After preparation, heart tissue sections were placed in an oven for a minimum of 2 hours to ensure thorough drying. Paraffin removal was carried out using xylene, followed by stepwise tissue rehydration through a graded ethanol series, consisting of three washes in 100% ethanol, then sequential washes in 96, 80, 70, and 50 ethanol. The sections were subsequently rinsed with distilled water before being submerged in a hematoxylin solution for nuclear staining. After an additional distilled water rinse, an eosin solution was applied to counterstain cytoplasmic components. Following a final rinse, the sections underwent dehydration through an ascending ethanol series, beginning with 50, 70, 80, 96%, and three final washes with 100% ethanol. They were subsequently cleared with xylene and mounted using Entellan mounting medium.

For histological analysis, a light microscope was used to examine ten randomly chosen fields from each section. The extent of myocardial damage was determined using a semi-

quantitative scoring system, where 0 indicated no damage, 1 represented mild damage, 2 denoted moderate damage, and 3 corresponded to severe damage (Gibson-Corley et al., 2013).

Immunohistochemistry

Slides were first cleared of paraffin using xylene and subsequently rehydrated using a graded ethanol series. Antigen retrieval was then performed by incubating the sections in 0.01 M citrate buffer. Finally, slides were rinsed thoroughly in phosphate-buffered saline (PBS) to remove residual buffer and prepare for immunostaining. To block endogenous peroxidase activity, sections were incubated in hydrogen peroxide solution for 10 minutes, and non-specific antibody binding was minimized by applying a serum blocking agent for an additional 10 minutes. Afterward, the sections were incubated separately with primary antibodies: atrial natriuretic peptide (ANP; 1 : 50, FNab09389; FineTest, China), N-terminal pro-B-type natriuretic peptide (NT-proBNP) (1 : 70, DF6902; Affinity, China), glucose-regulated protein 78 (GRP78; 1 : 50, FNab03662; FineTest, China), and caspase-3 (1:100, FNab03077; FineTest, China), overnight. Following incubation, the slides underwent PBS washes and were then treated with biotinylated secondary antibodies. Lastly, a serum blocking step was performed to minimize nonspecific binding. To enhance the immunoreaction, the avidin-biotin-peroxidase method was used, followed by 3,3'-diaminobenzidine (DAB) for visualizing immune reactivity (Kim et al., 2016; Karabulut et al., 2021). Next, the sections underwent counterstaining with Gill's hematoxylin, dehydration via a graded ethanol series, clearing in xylene, and final mounting using Entellan medium. Stained sections were later examined and imaged with a Leica DM IL LED FLUO microscope.

Semi-Quantitative Analysis

Semi-quantitative analysis of immunohistochemical staining for ANP, NT-proBNP, Caspase-3, and GRP78 was carried out with ImageJ software (version 1.53t bundled with Zulu

OpenJDK 13.0.6; National Institutes of Health, Bethesda, MD, USA) (Hartig, 2013). Fifty images were acquired per group (Control, BCA, STZ, and STZ+BCA) with five images taken per tissue section from each animal (n = 10 per group). Images of cardiac muscle tissue were obtained using a Leica DM IL LED microscope (Leica Microsystems, Germany) at a lens magnification 40×x. For each image, the mean optical density of the respective staining (e.g., brown staining for ANP) was measured in three randomly selected areas within the cardiac muscle. The region of interest (ROI) was outlined manually, and the "Measure" function in ImageJ was used to determine the mean optical density. To account for variations in background staining and illumination, the mean optical density of three additional randomly selected areas within the non-stained regions of the cardiac muscle was also measured for each image. These background measurements were then averaged and subtracted from the corresponding ROI measurements to obtain the corrected mean optical density value for each image.

Biochemical Analyses

Following euthanasia, blood samples were collected from the abdominal aorta and centrifuged at 3000 rpm to separate serum. The obtained serum samples were analyzed to assess myocardial injury markers, including high-sensitivity troponin T (hs-cTnT), creatine kinase-MB (CK-MB), and lactate dehydrogenase (LDH), using a cobas e 801 analyzer (Roche Diagnostics, Mannheim, Germany). These biochemical evaluations provided critical insights into the extent of cardiac tissue damage.

Assessment of Biomarkers via ELISA

Oxidative stress markers were measured in heart tissue homogenates, and inflammatory markers were measured in serum, using commercially available rat-specific ELISA kits (Bostonchem, USA). The kits used were: superoxide dismutase (SOD, Cat. No: BLS-8178Ra),

catalase (CAT, Cat. No: BLS-6986Ra), and malondialdehyde (MDA, Cat. No: BLS-8612Ra) in heart tissue, and tumor necrosis factor-alpha (TNF-α, Cat. No: BLS-1396Ra), interleukin-6 (IL-6, Cat. No: BLS-1158Ra), and nuclear factor kappa B p105 (NF-κB p105, Cat. No: BLS-5691Ra) in serum. Heart tissue samples stored at -80°C were homogenized on ice and centrifuged, and the supernatants were collected into Eppendorf tubes. Serum was obtained by centrifuging blood samples at 3000 rpm for 10 min and stored at -80°C until analysis. All assays were performed according to the manufacturer's instructions, and absorbance was read at 450 nm using an ELISA plate reader.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism software (version 10 for Mac; GraphPad Software, La Jolla, California, USA). Initially, data normality was assessed by employing the D'Agostino-Pearson test. Normally distributed data were analyzed using one-way ANOVA followed by Tukey's post-hoc comparison to identify significant group differences. When data showed abnormal distribution, Kruskal-Wallis test were performed to analyze variables. All values are presented as mean ± standard deviation (SD), and statistical significance was defined as $p < 0.05$.

RESULTS

Body Weight Assessment

At baseline (day 0), body weight differed among groups ($p = 0.0142$), being higher in the STZ group (Table 1). No significant differences were observed on days 7, 14, and 21. On day 30, STZ-treated rats had lost body weight (−68.6 g, about 25% of their initial weight), whereas the control, BCA, and STZ + BCA groups gained weight (overall $p = 0.0011$). Body weight in the STZ + BCA group was significantly higher than in the STZ group and did not differ from the control group (Table 1).

Influence of BCA on Blood Glucose Levels

Blood glucose levels were measured on days 0, 7, 14, 21, and 30 (Fig. 2). STZ-treated rats showed markedly elevated glucose levels compared with control rats at all time points ($p < 0.0001$). BCA treatment significantly reduced blood glucose in diabetic rats compared with the STZ group, starting from day 7 ($p < 0.05$) and becoming more pronounced from day 14 onward ($p < 0.0001$), although glucose levels in the STZ + BCA group remained higher than those of the control group. BCA alone did not significantly alter glucose levels in non-diabetic rats.

Histopathological Findings

As depicted in Fig. 3, histological examination of cardiac tissue revealed variations between the control and STZ-induced diabetes groups. Cardiac tissue from the control group displayed normal morphology, characterized by well-aligned myofibers and clear striations. In contrast, the STZ-treated group displayed significant myocardial injury, including widespread myofibrillar degradation, cytoplasmic eosinophilia in specific cardiomyocytes, and focal degeneration. These structural abnormalities indicate myocardial hypertrophy and cardiomyocyte necrosis, reinforcing the association between diabetes and cardiac injury.

However, treatment with BCA (20 mg/kg) led to a substantial reduction in these pathological changes. The BCA-treated diabetic group displayed less severe myofibril loss, reduced myocardial hypertrophy, and decreased cardiomyocyte necrosis compared to the untreated diabetic group, suggesting that BCA exerts a therapeutic effect against diabetes-induced myocardial injury.

Immunohistochemical Findings

Immunohistochemical analysis utilizing the avidin-biotin-peroxidase method was conducted to assess the levels of important indicators related to cardiac dysfunction (ANP, NT-proBNP), endoplasmic reticulum stress (GRP78), and apoptosis (caspase-3) in cardiac tissue sections. The results revealed significant differences in the expression levels of these markers across groups, highlighting the therapeutic effects of Biochanin A (BCA) in mitigating diabetes-induced myocardial injury (Fig. 4, 5).

Assessment of Cardiac Stress Markers (ANP and NT-Probnp)

Marked elevation in atrial natriuretic peptide (ANP) and N-terminal pro-brain natriuretic peptide (NT-proBNP) was observed, indicating increased myocardial stress and hypertrophic changes in the cardiac tissue of diabetic animals compared to controls. Quantitative analysis demonstrated a statistically significant rise in both ANP and NT-proBNP levels in the STZ-treated group compared to the control and BCA-treated groups ($p < 0.0001$), suggesting that diabetes imposes substantial structural and hemodynamic strain on the heart.

Interestingly, diabetic rats receiving biochanin A (BCA) treatment demonstrated a substantial decrease in the levels of cardiac stress markers ANP and NT-proBNP compared to untreated diabetic counterparts. This improvement suggests a therapeutic effects by BCA against myocardial damage, possibly attributable to its significant antioxidant and anti-inflammatory properties.

Assessment of ER stress

To assess endoplasmic reticulum (ER) stress, the expression of GRP78, a critical marker of ER stress activation, was measured. GRP78 expression was significantly increased in the cardiac tissue of untreated diabetic rats, indicating substantial impairment in protein folding mechanisms and elevated endoplasmic reticulum stress relative to control and BCA-treated groups. Persistent ER stress is known to compromise cellular function and trigger apoptosis, thereby exacerbating myocardial damage.

Notably, the STZ + BCA group exhibited a marked decrease in GRP78 expression compared to the STZ group ($p < 0.0001$), indicating that BCA helps mitigate diabetes-induced ER stress. This finding suggests that modulation of ER stress contributes to the therapeutic effect of BCA on diabetic myocardial injury.

Assessment of Apoptosis

Caspase-3 immunoreactivity was significantly higher in the STZ group than in the control and BCA-only groups ($p < 0.0001$), consistent with increased apoptosis in diabetic cardiac tissue, and this increase was accompanied by myocardial degeneration on histology. In the STZ + BCA group, caspase-3 immunoreactivity was significantly lower than in the STZ group ($p < 0.0001$), suggesting an anti-apoptotic effect of BCA.

Effect of BCA on Cardiac Oxidative Stress Markers

ELISA results revealed that diabetic rats induced by STZ exhibited a notable rise in malondialdehyde (MDA) concentrations ($p < 0.05$), reflecting enhanced lipid peroxidation. Conversely, the enzymatic activity of key antioxidants, such as SOD and CAT, was significantly reduced in diabetic animals compared to controls.

A comparative analysis between the STZ and STZ+BCA groups demonstrated a substantial variation in oxidative stress markers. Diabetic rats treated with BCA showed a significant decline in MDA levels, along with the restoration of SOD and CAT activity, reaching values close to those in the control and BCA-only groups. These findings strongly suggest that BCA exerts a therapeutic effect by alleviating diabetes-induced oxidative stress in cardiac tissue.

BCA Attenuates Cardiac Inflammationin Diabetic Rats

ELISA results indicated a substantial increase in inflammatory markers, including TNF-α, IL-6, and NF-κB, in diabetic rats compared to controls, confirming heightened inflammation following STZ administration. However, treatment with BCA effectively reversed these elevations, significantly lowering the concentrations of TNF-α, IL-6, and NF-κB. These findings emphasize the robust anti-inflammatory capacity of BCA in mitigating diabetes-induced cardiac inflammation.

These findings underscore the intricate link between diabetes, oxidative stress, and inflammation in the progression of DCM. While diabetes disrupts cardiomyocyte homeostasis, BCA treatment attenuated these pathological changes, as reflected by improved oxidative and inflammatory markers (Fig. 6).

Effects of BCA on Serum Myocardial Injury Markers

To determine how diabetes affects cardiac function, serum concentrations of high-sensitivity cardiac troponin T (hs-cTnT), creatine kinase-MB (CK-MB), and lactate dehydrogenase (LDH) were measured. Compared to the healthy control animals, diabetic rats (STZ group) displayed significantly elevated levels of these biomarkers ($p < 0.05$), clearly indicating diabetes-induced cardiac dysfunction and myocardial injury. Conversely, diabetic rats treated with biochanin A (STZ+BCA group) exhibited a significant reduction in serum hs-cTnT, CK-MB, and

LDH levels, demonstrating the therapeutic effects of BCA in attenuating myocardial injury associated with diabetes (Fig. 6).

DISCUSSION

Diabetes mellitus is a chronic metabolic disorder characterized by numerous complications, significantly impacting global health due to its increasing prevalence and association with various systemic injuries, including cardiac dysfunction (Chandrasekaran and Weiskirchen, 2024). One of its most concerning complications is diabetic cardiomyopathy (DCM), which significantly contributes to both morbidity and mortality. DCM is characterized by progressive alterations in cardiac structure and function, which may eventually lead to heart failure (Gulsin et al., 2019). Despite significant advancements in diabetes treatment, DCM remains a prevalent issue, highlighting the necessity of gaining deeper insights into its underlying mechanisms and developing more effective therapeutic interventions (Fabila-de la Cruz et al., 2024). In this study, we explored the therapeutic effects of BCA using a rat model of diabetes-induced myocardial injury. Our study evaluated the therapeutic potential of BCA in diabetic myocardial injury by assessing key biomarkers associated with oxidative stress, inflammation, apoptosis, and endoplasmic reticulum (ER) stress in cardiac tissue. The findings suggest that BCA holds significant potential as a therapeutic agent for attenuating diabetes-related myocardial injury.

Extensive research has shown that streptozotocin (STZ)-induced diabetes leads to severe cardiac abnormalities in experimental models. These pathological changes, including myocyte hypertrophy, fibrosis, and inflammation, contribute to diastolic dysfunction and may progress to systolic impairment, ultimately increasing the risk of heart failure (Baraka and AbdelGawad, 2010; Kumar et al., 2013). Consistent with previous studies (Huo et al., 2021; Alharbi et al., 2025), our histopathological analysis revealed substantial myocardial damage in the STZ group. HandE staining demonstrated clear signs of cardiomyocyte hypertrophy, myofibril loss, cytoplasmic eosinophilia and necrosis, with a significantly higher myocardial damage score in the diabetic

group from the control and BCA-treated groups. These findings confirm the deleterious effects of STZ-induced diabetes on cardiac structure. Notably, administering BCA at a dose of 20 mg/kg significantly mitigated these pathological changes, indicating its potential in reducing diabetes-induced cardiac injury. BCA also significantly reduced blood glucose and prevented the body weight loss seen in diabetic rats, consistent with its reported effects on glucose metabolism (Mehrabadi et al., 2021). Therefore, part of the observed cardiac improvement may be secondary to improved glycemic control, and direct myocardial effects cannot be fully separated.

Natriuretic peptides (NPs) constitute a family of hormones essential for preserving cardiovascular balance, notably by controlling blood pressure and regulating fluid homeostasis. These hormones, primarily involved in the modulation of blood volume and vascular tone, include peptides ANP and BNP. ANP and BNP are secreted predominantly as a physiological response to elevated cardiac workload conditions, such as increased ventricular pressure, afterload stress, or atrial distension (Goetze et al., 2020; Della Corte et al., 2023). Their physiological effects include diuresis, natriuresis, and vasodilation, all of which help alleviate cardiac strain (Nishikimi and Kato, 2022).

In pathological conditions such as heart failure (HF), ischemic heart disease, arrhythmias, and cardiomyopathy, excessive myocardial stress leads to an upregulation of BNP and its inactive fragment, NT-proBNP, which serve as valuable diagnostic and prognostic biomarkers (Lemos et al., 2026; Mohammadi et al., 2026). Findings from the International Collaborative of NT-proBNP (ICON) study suggest that NT-proBNP may be a more reliable biomarker than BNP, given its longer half-life and sustained elevation (approximately 12 weeks on average) (Januzzi et al., 2018).

In our study, STZ-induced diabetes resulted in a marked imbalance between oxidant and antioxidant defenses, leading to both structural and functional impairments in cardiac tissue. The

observed increase in ANP and NT-proBNP levels reflected heightened myocardial stress and cardiomyocyte damage in diabetic rats. However, administration of BCA at 20 mg/kg effectively counteracted these detrimental effects by reducing ANP and NT-proBNP immunoreactivity, suggesting that BCA attenuates cardiac stress and diabetes-induced myocardial injury.

The endoplasmic reticulum (ER) stress response is increasingly recognized as a pivotal mechanism in the pathogenesis of DCM, playing a crucial role in disrupting cellular homeostasis (Xu et al., 2012). Research indicates that ER stress is activated in the cardiac tissue of both diabetic patients and experimental animal models (Li et al., 2020; Yuan et al., 2020)**.** Under hyperglycemic conditions, the accumulation of misfolded or unfolded proteins in the endoplasmic reticulum activates the unfolded protein response (UPR), a protective mechanism designed to restore ER homeostasis and ensure proper protein folding (Kitakata et al., 2021)**.** However, when ER stress is prolonged or reaches excessive levels, the UPR can become dysregulated, shifting from a protective mechanism to a pathological process. This disruption activates apoptotic pathways, ultimately leading to cardiomyocyte loss and a decline in cardiac function (Guo et al., 2024).

Findings from this study indicate that BCA could exert therapeutic effects through the modulation of ER stress and apoptosis. Our findings demonstrated that STZ-induced diabetes significantly increased GRP78 expression in cardiac tissue, a well-established marker of ER stress. However, administration of BCA effectively reduced GRP78 levels, indicating that BCA mitigates ER stress and may prevent diabetes-induced apoptosis. These findings highlight BCA as a promising therapeutic compound capable of reducing diabetic myocardial injury, potentially through its ability to regulate endoplasmic reticulum stress and inhibit apoptotic pathways.

When ER stress becomes excessive or prolonged, it can interfere with normal cellular function and trigger apoptotic pathways, ultimately leading to cell death (Li et al., 2020).

Apoptosis, or programmed cell death, is a carefully regulated process essential for development and tissue homeostasis (Li et al., 2025). However, dysregulated apoptosis is implicated in various pathological conditions, including DCM. One of the key executioner enzymes in the apoptotic cascade is caspase-3, whose activation leads to hallmark features of apoptosis, such as DNA fragmentation, cytoskeletal degradation, and the formation of apoptotic bodies (Luo et al., 2025).

To investigate apoptotic changes associated with diabetes-induced myocardial injury, caspase-3 expression was measured in cardiac tissues. The results demonstrated significantly elevated caspase-3 expression in the STZ-treated diabetic group, reflecting enhanced myocardial apoptosis. Conversely, treatment with BCA markedly decreased caspase-3 expression in diabetic rats (STZ + BCA group), indicating reduced apoptotic activity. These findings highlight the therapeutic ability of BCA to alleviate diabetic myocardial injury by mitigating apoptosis and modulating ER stress responses.

Prolonged hyperglycemia triggers a state of increased oxidative imbalance, significantly contributing to the pathogenesis of diabetic DCM and the resulting impairment in cardiac function. This process has been well-documented in clinical studies as well as experimental animal models. Mechanistically, sustained hyperglycemia disrupts the equilibrium between excessive production of ROS and antioxidant defense mechanisms, ultimately causing cellular damage. Consequently, elevated oxidative damage promotes myocardial remodeling and cellular dysfunction, advancing the progression of DCM (Iacobini et al., 2021; Tang et al., 2022).

Moreover, oxidative stress intensifies lipid peroxidation, resulting in structural impairment of cell membranes (Alsulaim et al., 2024). The body's antioxidant defense system relies on key enzymes such as superoxide dismutase (SOD) and catalase (CAT), which play a vital role in mitigating ROS and preserving cellular redox balance(Naghdi et al., 2022). However, previous

studies have demonstrated that STZ-induced diabetes weakens these antioxidant defenses, resulting in reduced SOD and CAT activity, accompanied by heightened lipid peroxidation, as evidenced by elevated malondialdehyde (MDA) levels in cardiac tissue (Ebrahimpour et al., 2026; Yıldızhan et al., 2026).

Our findings confirmed that STZ administration resulted in a significant elevation of MDA concentrations, an important indicator of oxidative injury and lipid peroxidation. This aligns with previous research associating increased oxidative stress with diabetic cardiac complications. Additionally, we observed a notable decline in antioxidant enzyme levels (SOD and CAT) within the cardiac tissue of diabetic rats, reinforcing the concept that hyperglycemia-induced oxidative stress contributes to myocardial injury.

Interestingly, treatment with BCA (20 mg/kg) significantly attenuated diabetes-induced oxidative stress in cardiac tissue. BCA treatment not only increased SOD and CAT levels, strengthening antioxidant defenses, but also reduced MDA concentrations, signifying a decrease in lipid peroxidation and oxidative damage. These findings suggest that BCA's therapeutic effects may be largely attributed to its ability to counteract oxidative stress and reinforce antioxidant mechanisms in the diabetic heart.

CK-MB is an enzyme predominantly localized in cardiac muscle and is widely recognized as a key biomarker for myocardial injury. Elevated CK-MB levels in the bloodstream strongly indicate cardiac cell damage and are commonly used to predict acute myocardial infarction (AMI) (Keceli et al., 2022). However, it is essential to note that CK-MB is not entirely cardiac-specific, as its release may also occur due to skeletal muscle injury, renal dysfunction, or even strenuous physical exertion (Moghadam-Kia et al., 2016).

Another key biomarker, LDH, is a metabolic enzyme involved in cellular energy production and is released into circulation following cardiomyocyte damage. It serves as a valuable indicator for assessing myocardial ischemia and cardiac injury (Yu et al., 2024). High-sensitivity cardiac troponin T (hs-cTnT) is a biomarker that is highly specific and sensitive for detecting myocardial injury. Elevated levels of hs-cTnT are indicative of heart muscle damage and are utilized in the diagnosis of conditions such as myocardial infarction (He et al., 2018).

To assess cardiac injury in our experimental model, we measured the serum concentrations of CK-MB, LDH, and hs-cTnT. As anticipated, the STZ group exhibited a significant increase in all three markers compared to the control group, confirming that STZ-induced diabetes leads to substantial myocardial damage and cardiac dysfunction. Notably, rats treated with BCA (20 mg/kg) showed significantly lower levels of hs-cTnT, CK-MB, and LDH than those in the STZ-only group, with values approaching those observed in healthy controls. These findings indicate that BCA treatment significantly attenuates diabetes-induced myocardial injury.

In DCM, oxidative stress resulting from chronic hyperglycemia disrupts the balance between pro-inflammatory and anti-inflammatory processes (Noutsias et al., 2009). This imbalance activates inflammatory pathways, notably the NF-κB signaling cascade, leading to increased production of pro-inflammatory cytokines such as TNF-α and IL-6 (Bellemare et al., 2025).

STZ administration has been shown to induce a pronounced inflammatory response in cardiac tissue, characterized by elevated levels of pro-inflammatory cytokines such as TNF-α and IL-6. Our findings support these observations, as ELISA analysis demonstrated significantly increased levels of TNF-α, IL-6, and NF-κB in the STZ group compared to the control. Additionally, diabetic rats exhibited heightened NF-κB expression, which indicates that this transcription factor is crucial in regulating the inflammatory process within the diabetic myocardium.

BCA administration resulted in a significant reduction in inflammatory markers, including NF-κB, TNF-α, and IL-6, in the STZ+BCA group compared to the STZ-only group. This suggests that BCA possesses anti-inflammatory properties, potentially by inhibiting NF-κB activity and reducing the production of pro-inflammatory cytokines, thereby modulating the inflammatory response associated with diabetes-induced myocardial injury.

Although we observed a significant reduction in NF-κB levels using ELISA in the BCA-treated group, this alone does not conclusively confirm direct inhibition of the NF-κB signaling pathway. ELISA detects protein abundance but does not provide information about nuclear translocation or transcriptional activity. Therefore, future studies should employ more specific mechanistic approaches, such as Western blotting for phosphorylated IκBα, NF-κB nuclear translocation assays, or siRNA-mediated knockdown, to validate whether BCA directly modulates NF-κB signaling in diabetic cardiac tissue.

Collectively, our findings suggest that BCA exerts therapeutic effects through multiple mechanisms, including regulation of ER stress and apoptosis, reduction of oxidative stress, and suppression of inflammation. These multi-target actions identify BCA as a promising candidate for the management of diabetes-related myocardial injury. Further studies are required to determine the optimal dose, long-term safety, and the precise molecular pathways involved. A graphical summary of the study design and main outcomes is presented in Fig. 7.

CONCLUSION

In this rat model of STZ-induced diabetes, myocardial injury was accompanied by increased oxidative stress, systemic inflammation, ER stress (GRP78), and apoptosis (caspase-3), together with elevated cardiac injury markers and histopathological damage. Treatment with BCA (20 mg/kg for 30 days) reduced blood glucose and attenuated these alterations, suggesting a therapeutic potential of BCA in diabetic myocardial injury, possibly through modulation of oxidative stress, inflammation, and ER stress-related apoptosis. This study has some limitations. First, heart weight, the heart-to-body weight ratio, and functional assessments such as electrocardiography were not performed; myocardial injury was instead evaluated through biochemical markers and histopathological examination, so the functional effects of BCA on the diabetic heart remain to be established. Second, because BCA also lowered blood glucose, part of its cardiac effect may be secondary to improved glycemic control, and direct myocardial actions cannot be fully separated. Further functional and mechanistic studies are needed to confirm these findings.

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FIGURE LEGENDS

Fig. 1: Diagrammatic representation of experimental design

Fig. 2. STZ-induced hyperglycemia and the effect of biochanin A (BCA) in rats. Diabetes was induced by a single intraperitoneal dose of STZ (45 mg/kg). Day 0 corresponds to confirmation of diabetes 48 h after streptozotocin (STZ) injection (blood glucose ≥250 mg/dL) and the start of BCA treatment (20 mg/kg/day for 30 days). Blood glucose was measured on days 0, 7, 14, 21, and 30. Data are presented as mean ± standard deviation (n = 10 per group). Differences between groups at each time point were determined by one-way ANOVA followed by Tukey's post hoc test. (*) $p < 0.05$; (****) $p < 0.0001$

Fig. 3: (a) The morphology of cardiac tissue**.** The images were representatives the hematoxylin and eosin stained sections of heart from our experimental groups: control group showing normal cardiac structure with well-organized cardiomyocytes and no visible lesion, biochanin A (BCA)-treated group exhibiting normal morphology similar to the control, streptozotocin (STZ)-induced diabetic group (arrow, enlarged necrotic cells; star, muscle fiber damage), and BCA+STZ-treated group showing improved cardiac morphology with reduced muscle fiber damage, inflammation, and hypertrophy, resembling the control group; original magnification: 40×, scale bar: 100 μm. (b) Statistical analysis of the cardiac muscle injury score shows that the level of the cardiac damage in the STZ group was significantly higher than those in control and BCA groups ($p < 0.0001$). On the contrary, BCA administrations significantly reduced the cardiac damage in the STZ + BCA group ($p < 0.0001$). (*) $p < 0.05$, (**) $p < 0.01$, (****) $p < 0.0001$

Fig. 4. Representative images of heart tissue sections stained for caspase-3 (apoptosis marker), NT-proBNP, ANP (cardiac stress markers), and GRP78 (endoplasmic reticulum stress marker), using the avidin-biotin-peroxidase method (magnification 40×). Increased caspase-3 staining in the STZ

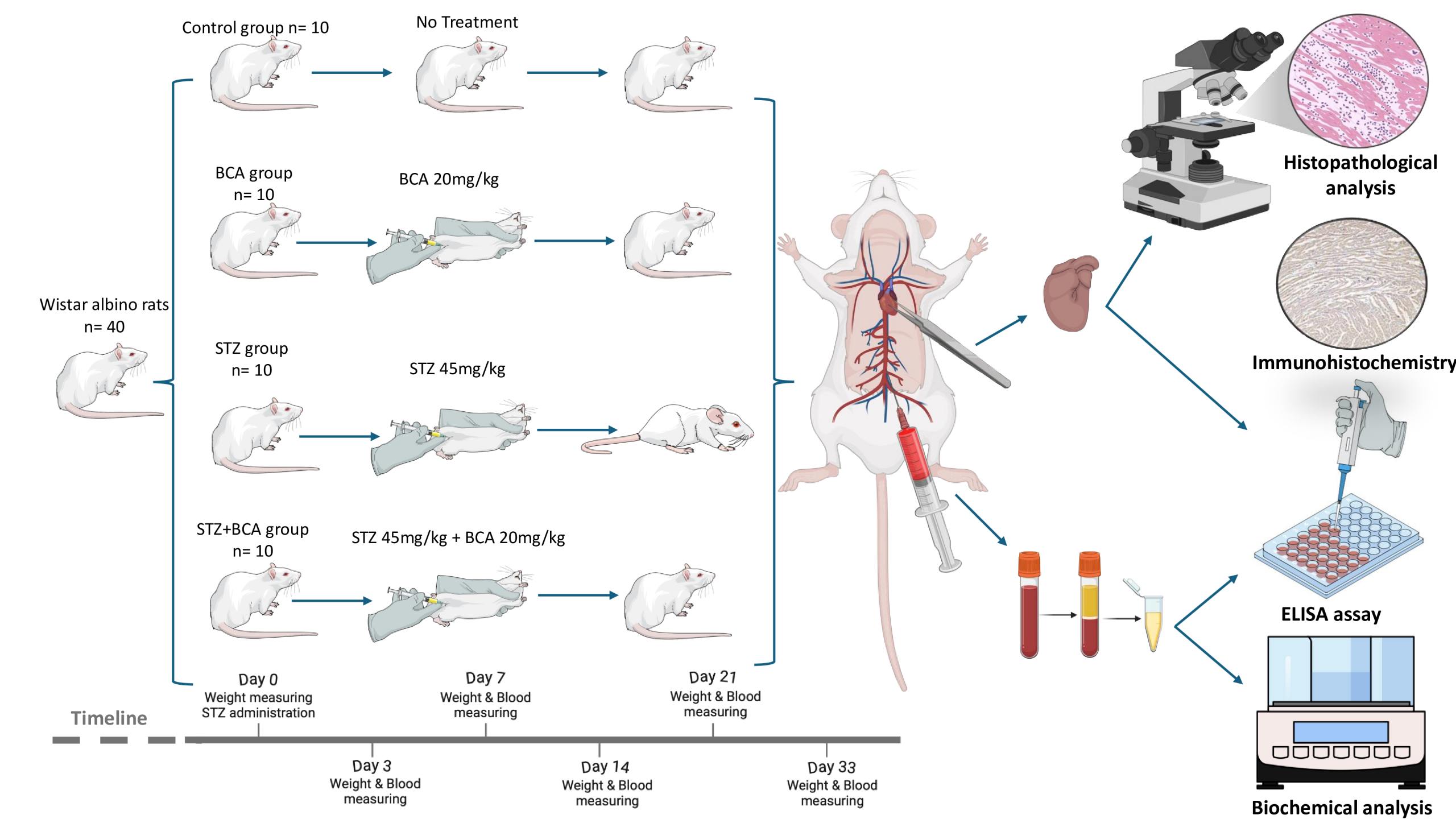
group (brown color) indicates elevated apoptosis, which was attenuated in the STZ+BCA group. Similarly, increased NT-proBNP and ANP expression in the STZ group suggests cardiac stress and hypertrophy, which were reduced with BCA treatment. GRP78 expression, a marker of endoplasmic reticulum stress, was also elevated in the STZ group and mitigated by BCA. Scale bar: 100 μm. Abbreviations: ANP, atrial natriuretic peptide; NTproBNP, N-terminal pro B-type natriuretic peptide; GRP78, glucose regulated protein 78

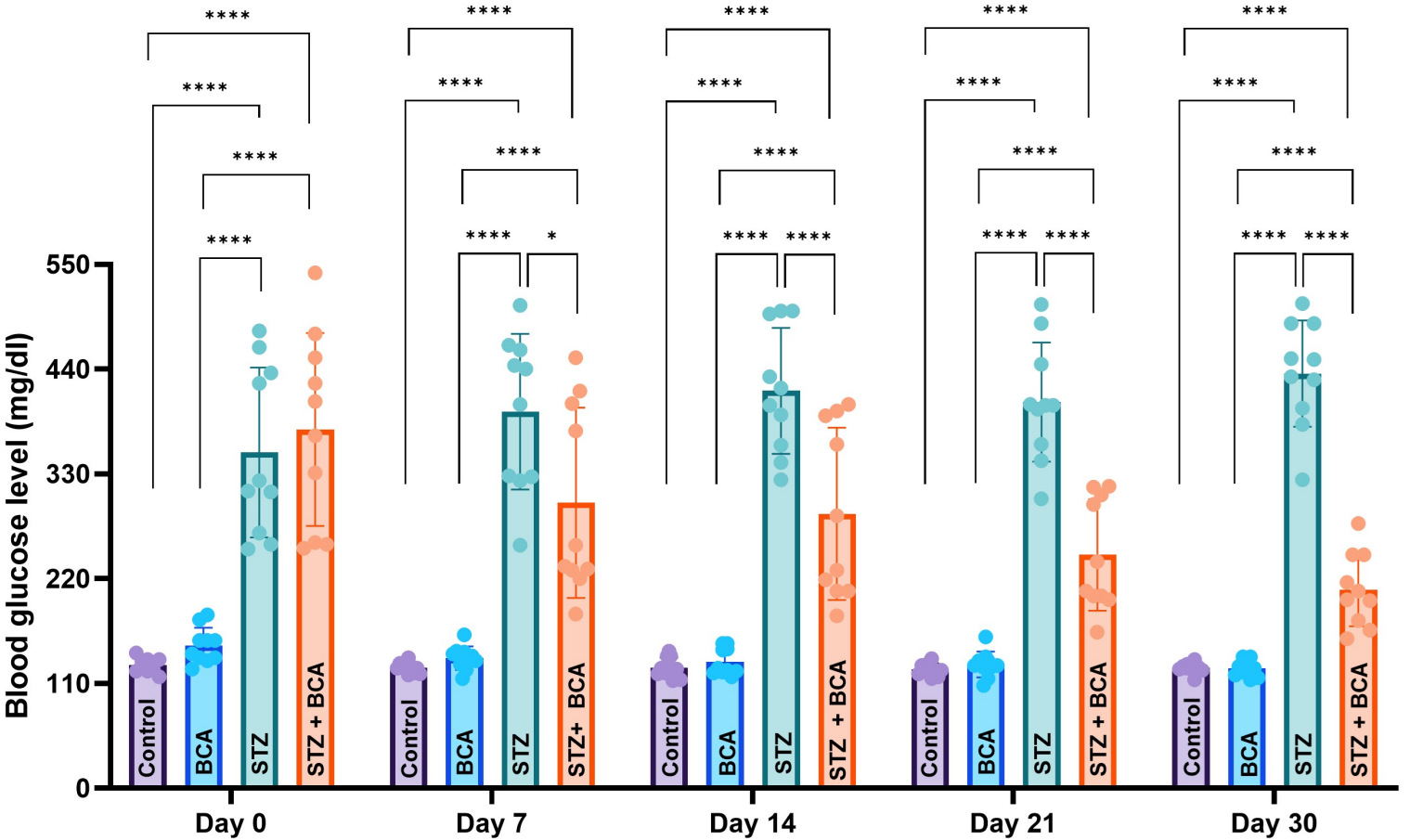
Fig. 5. Semi-quantitative analysis of caspase-3, ANP, NTproBNP and GRP78 immunoreactivities. Values are presented as means ± SD. Immunoreactivity of all four markers was significantly higher in the STZ group than in the control and BCA groups ($p < 0.0001$) and significantly lower in the STZ + BCA group than in the STZ group ($p < 0.0001$). (****) $p < 0.0001$. For abbreviations see **Fig. 1**

Fig. 6. (a—c) Effect of BCA on serum inflammatory biomarkers of diabetic cardiac damage. (a) TNF alpha, NF-kB and IL-6; the expression in the three groups was significantly higher in the STZ-induced cardiac injury group in comparison with the control and BCA treatment significantly reduced its level in comparison with the STZ-induced cardiac injury group. (d—f) Changes in antioxidant markers and statistical analysis of them in experimental groups; STZ group showed decreased tissue superoxide dismutase (SOD) and catalase (CAT) levels compared to control group and BCA group. Moreover, tissue malondialdehyde (MDA) level significantly increased in STZ group compared to control group and BCA group ($p < 0.0001$); however, increased tissue SOD and CAT levels and significantly decreased tissue MDA levels ($p < 0.0001$) were observed in STZ+BCA group compared to STZ group. (g—i) Diabetic animals (STZ) showed significantly elevated levels of the heart damage markers hs-cTnT, Ck-MB and LDH compared to the control group. Treatment with BCA (STZ+BCA) reduced these markers, suggesting a therapeutic effect

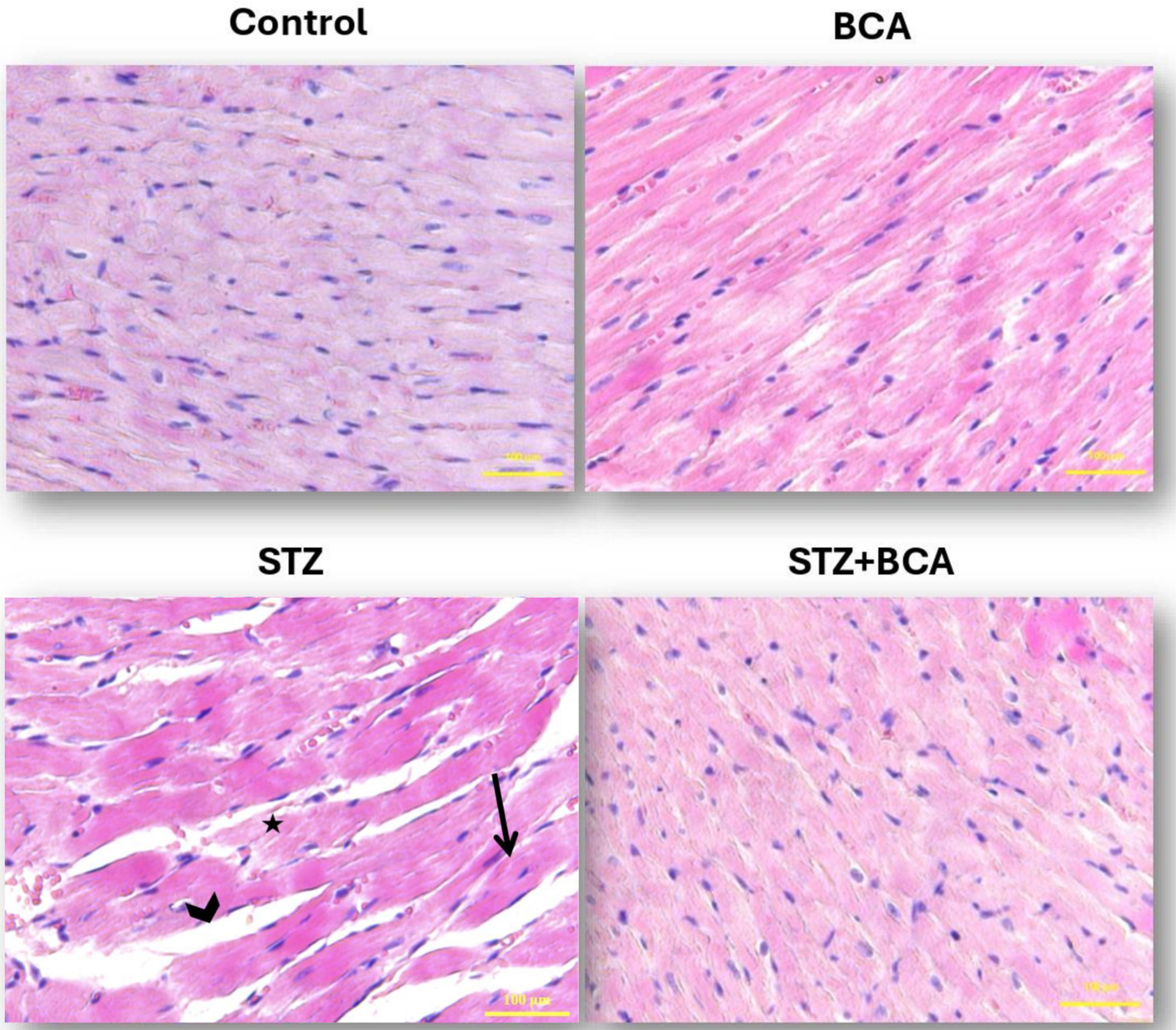
against diabetes-induced myocardial injury; this effect was most pronounced for hs-cTnT, a highly sensitive indicator of heart muscle damage. $p < 0.05$ was considered as significant: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$. Graphs were drawn by using GraphPad Prism version 10.2.0 for Mac, GraphPad Software, La Jolla, California, USA.

Fig. 7. Graphical summary of the experimental design assessed parameters, and main outcomes of the study. (a) STZ group; (b) STZ + BCA group. Red arrows indicate changes in the STZ group compared with the control group; green arrows indicate changes in the STZ + BCA group compared with the STZ group. Blood glucose values are group means on day 30. ELISA, enzyme-linked immunosorbent assay; IHC, immunohistochemistry. Created with BioRender.com

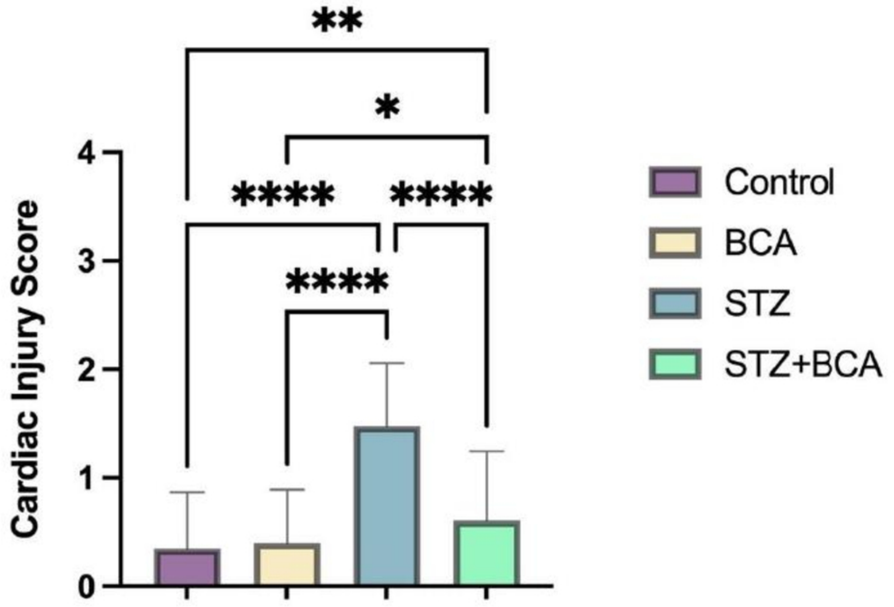

Control group n= 10
No Treatment
BCA group
n= 10
BCA 20mg/kg
Wistar albino rats
n= 40
STZ group
n= 10
STZ 45mg/kg
STZ+BCA group
n= 10
STZ 45mg/kg + BCA 20mg/kg
Timeline
Day 0
Weight measuring
STZ administration
Day 3
Weight & Blood
measuring
Day 7
Weight & Blood
measuring
Day 14
Weight & Blood
measuring
Day 21
Weight & Blood
measuring
Day 33
Weight & Blood
measuring
Histopathological
analysis
Immunohistochemistry
ELISA assay
Biochemical analysis

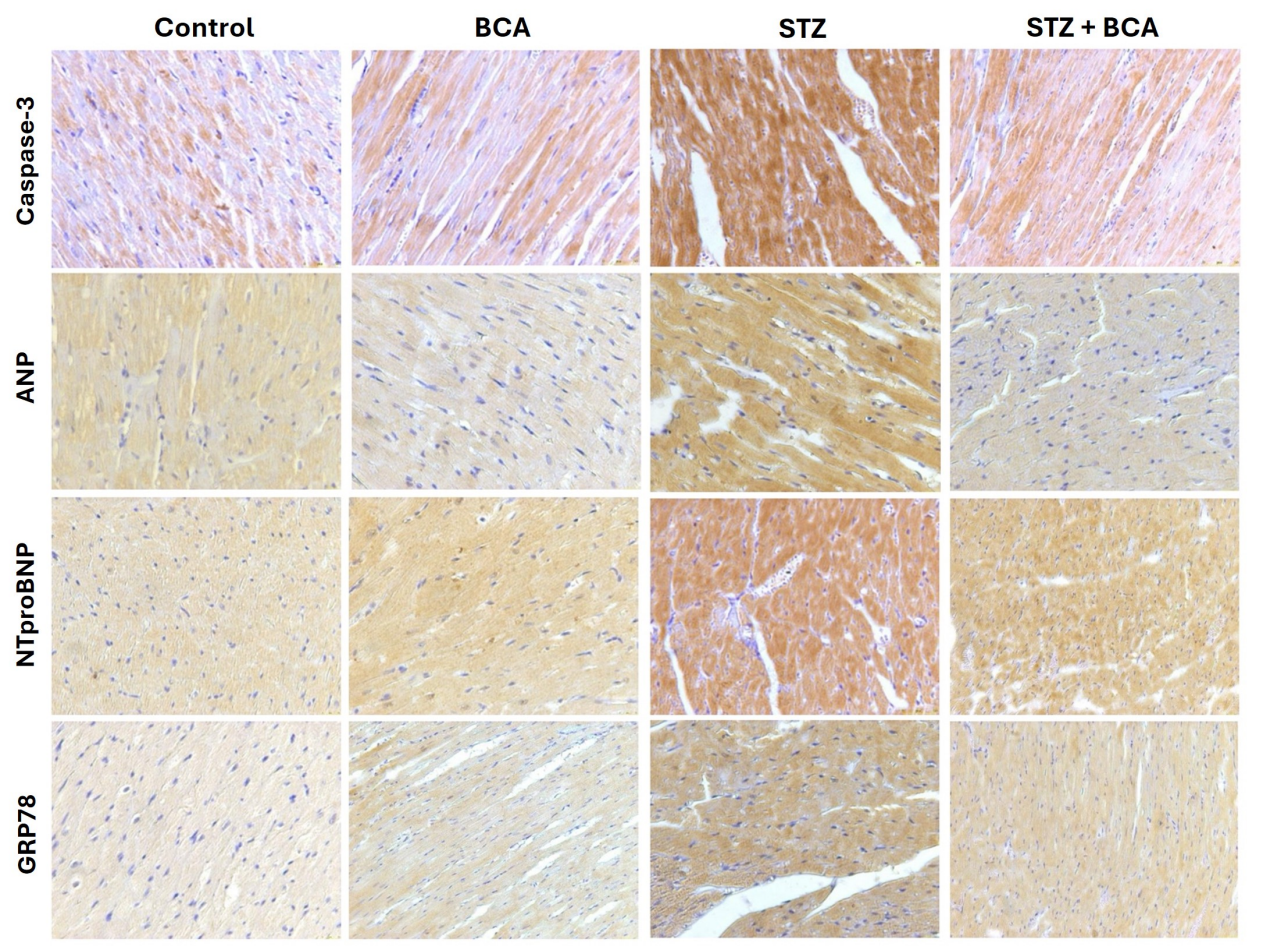

Blood glucose level (mg/dl)
550
440
330
220
110
0
Control
BCA
STZ
STZ + BCA
STZ+ BCA
Day 0
Day 7
Day 14
Day 21
Day 30
****
*

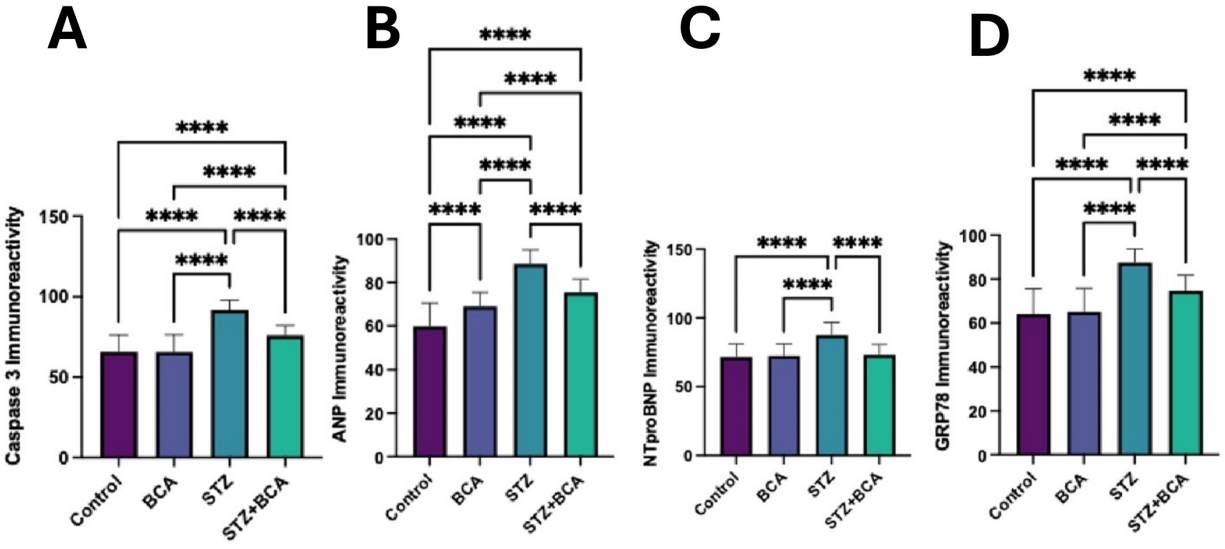
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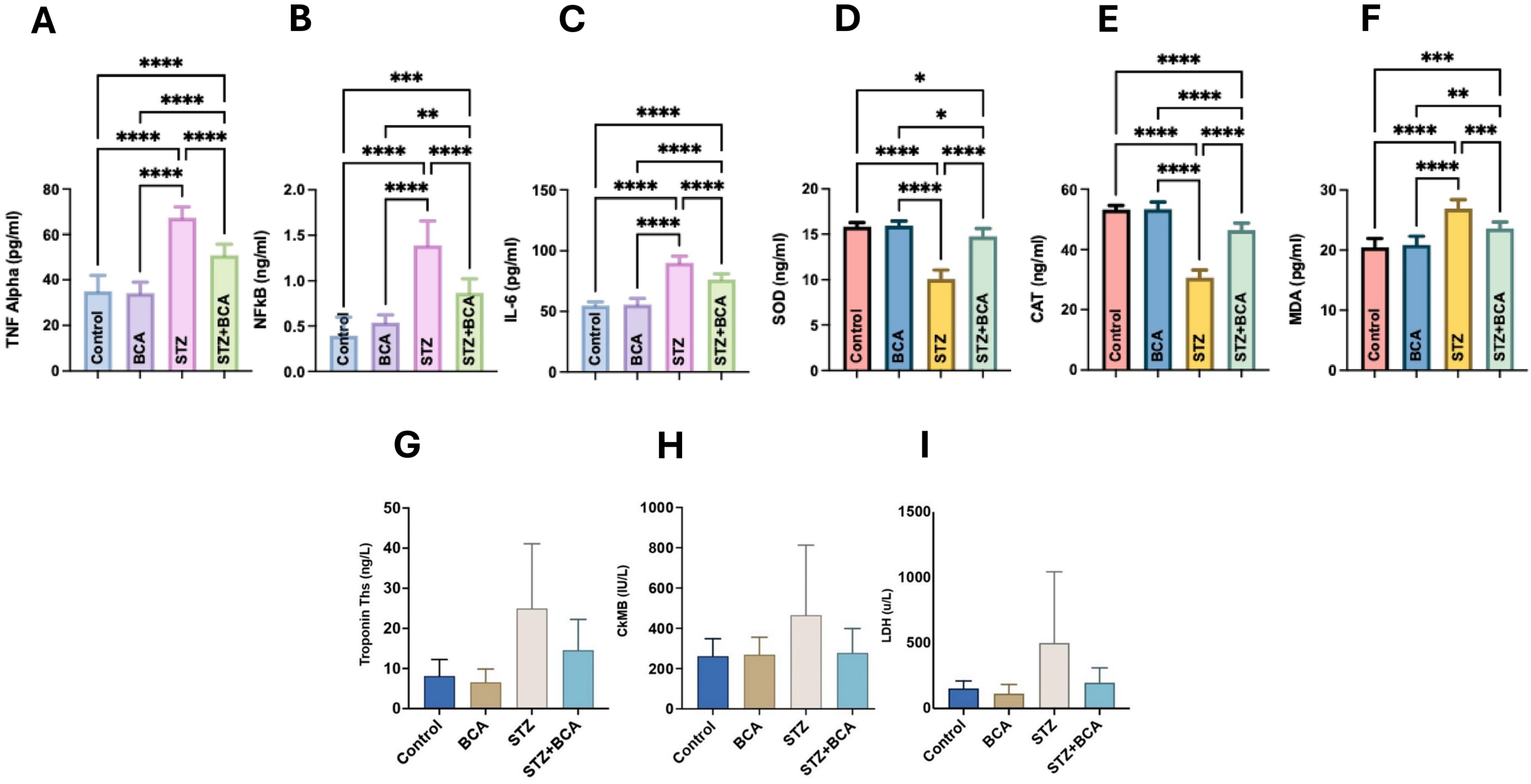

Control
BCA
STZ
STZ+BCA
100 µm

B


**
*
****
****
****
Cardiac Injury Score
0
1
2
3
4
Control
BCA
STZ
STZ+BCA


Control
BCA
STZ
STZ + BCA
Caspase-3
ANP
NTproBNP
GRP78


A
Caspase 3 Immunoreactivity
150
100
50
0
****
****
****
****
Control
BCA
STZ
STZ+BCA
B
ANP Immunoreactivity
100
80
60
40
20
0
****
****
****
****
****
Control
BCA
STZ
STZ+BCA
C
NTproBNP Immunoreactivity
150
100
50
0
****
****
****
Control
BCA
STZ
STZ+BCA
D
GRP78 Immunoreactivity
100
80
60
40
20
0
****
****
****
****
****
Control
BCA
STZ
STZ+BCA


A
TNF Alpha (pg/ml)
B
NFkB (ng/ml)
C
IL-6 (pg/ml)
D
SOD (ng/ml)
E
CAT (ng/ml)
F
MDA (pg/ml)
Control
BCA
STZ
STZ+BCA
G
Troponin Ths (ng/L)
H
CkMB (IU/L)
I
LDH (u/L)

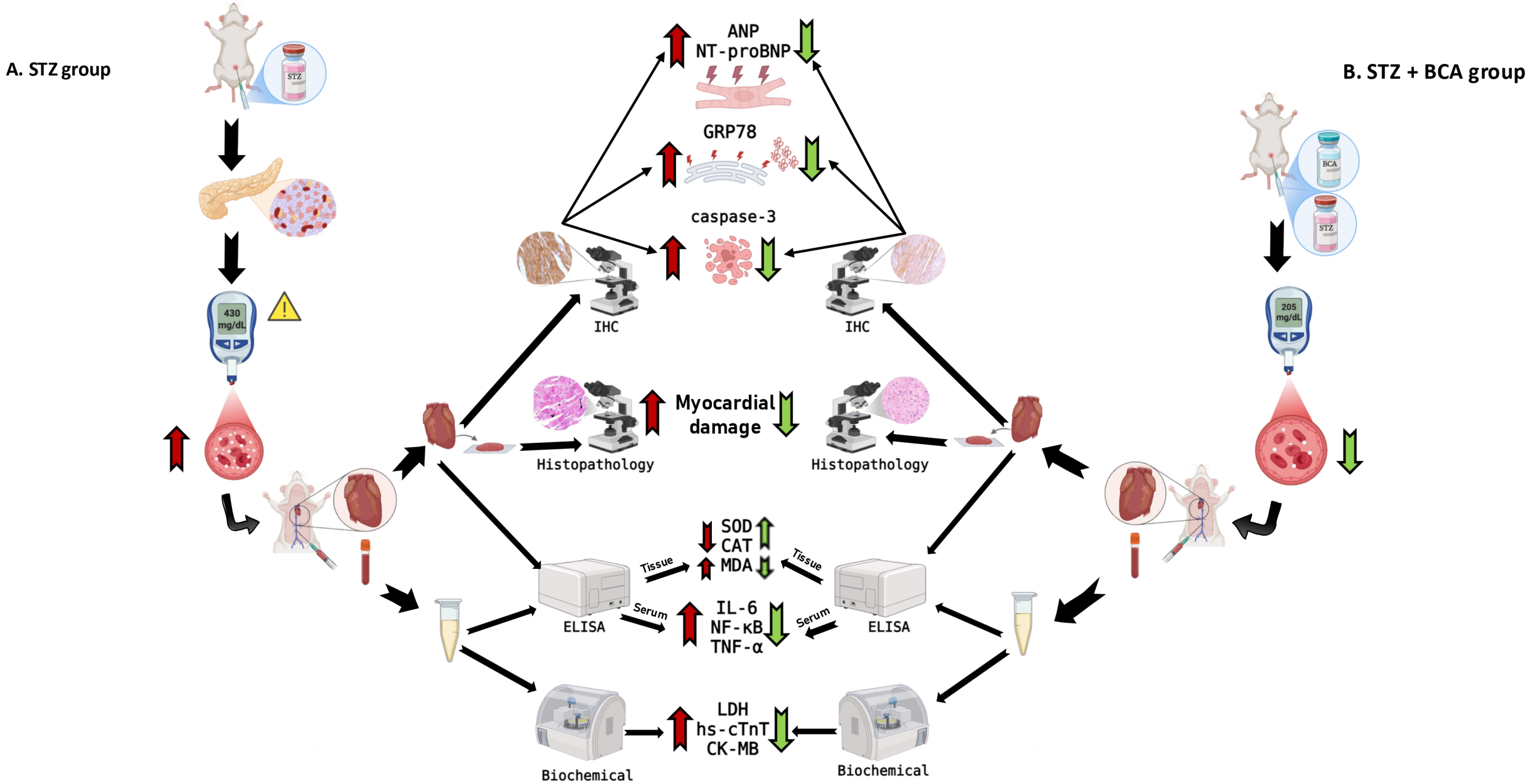


Figure 7: A Graphical summary of the experimental design, assessed parameters, and main outcomes of the study

Supplementary Files

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- Tables.docx