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Mechanism of streptozotocin to induce cardiac fibrosis through TNFa and Bcl2 pathways in *in silico* and *in vivo* study

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Abstract

Background: Cardiac fibrosis is often associated with various heart-related problems such as heart failure, atrial arrhythmia, and sudden cardiac death, making it a leading cause of death globally. Diabetes-associated fibrosis, on the other hand, is influenced by activated cardiac fibroblasts and potentially involves fibrosis-inducing activity of macrophages, cardiomyocytes, and vascular cells. Streptozotocin (STZ) is a known diabetogenic agent, but inadequate preclinical data in animal models hinders its clinical success.

Aim: This study aims to provide practical guidelines for STZ utilization in inducing diabetes-associated cardiac fibrosis.

Methods: The research was conducted *in vivo* using white rats (*Rattus norvegicus*) of the Wistar strain, induced with STZ at doses of 30 mg/KgBW and 50 mg/KgBW per injection. Observations were carried out in the 4th and 8th weeks, consisting of the measurement of blood sugar levels and the examination of heart muscle cell fibrosis. Subsequently, *in silico* validation of STZ's affinity with inflammatory receptors causing diabetes pathology, such as TNFα and Bcl2, was performed.

Results: The study results indicated that the administration of STZ led to an increase in random blood sugar levels and extensive fibrosis of heart muscle cells in mice. The optimal dose for the diabetes model experimented in this study was 50 mg/KgBW for 8 weeks. *In silico* tests revealed an affinity for TNFα (PDB ID 2AZ5) and Bcl2 (PDB ID 6QGH).

Conclusion: Consequently, it can be concluded that administering STZ to mice at a dose of 50 mg/KgBW for 8 weeks is an effective inducer of a diabetes-associated cardiac fibrosis model.

Keywords: Cardiovascular risk factors, Diabetes, *In silico*, *In vivo*, Streptozotocin.

Introduction

Cardiovascular disease (CVD), a category of illnesses affecting the heart or circulatory system, accounts for 31% of all deaths globally and remains a leading cause of death worldwide (Hinderer and Schenke-Layland, 2019). It is also the top cause of global mortality in both developed and developing nations, estimated to affect 17.9 million people (44% of non-communicable disease-related deaths), according to the World Health Organization (Baeradeh *et al*., 2022; Nurwahyuni *et al*., 2023). In Indonesia, CVD remains the primary cause of death, contributing to 48% of all non-communicable disease-related deaths in the country. According to Indonesia's Basic Health Research (Riskesdas) in 2013 and 2018, the prevalence of CVD continued to rise from 0.5% in 2013 to 1.5% in 2018 (Nurwahyuni *et al*., 2023).

Cardiac fibrosis is often associated with various cardiovascular disorders such as cardiac failure (González *et al*., 2018), atrial arrhythmia (Ma *et al*., 2021), and sudden cardiac death (Disertori *et al*., 2017). Its pathophysiological process typically involves excessive extracellular matrix (ECM) production and deposition in the myocardial interstitium (Mao *et al*., 2023). Generally, cardiac fibrosis can be categorized into two main types based on its underlying causes and histological characteristics: reparative fibrosis and reactive interstitial fibrosis (Frangogiannis, 2021). Myocardial fibrosis has been documented in patients with both type 1 and type 2 diabetes, which can decrease myocardial compliance, affect cardiac failure pathogenesis, and trigger arrhythmias. Diabetes-related fibrosis is mediated by activated cardiac fibroblasts and may also involve the fibrogenic actions of macrophages, cardiomyocytes, and vascular cells (Russo and Frangogiannis, 2016).

Diabetic cardiomyopathy is a complication of diabetes characterized by structural and functional changes in the

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myocardium, including cardiac fibrosis. This fibrosis can increase the risk of ventricular stiffness and impair cardiac contractility (Salsabila *et al*., 2023). Following cardiovascular disorders, individuals with diabetes have a worse prognosis compared to those without diabetes. A significant impact of diabetes mellitus (DM) on the cardiovascular system is the development of diabetic cardiomyopathy, a potentially fatal complication that can lead to cardiac failure (Kobayashi and Liang, 2015). Streptozotocin [2-deoxy-2-(3-methyl-3-nitrosurea) 1-D-glucopyranose] (STZ), synthesized by Streptomyces achromogenes and categorized as a nitrosourea analog (Yulianti *et al*., 2018), is a diabetogenic chemical (Ghasemi *et al*., 2014), widely utilized in creating animal models of both type 1 and type 2 diabetes (Samuel *et al*., 2014). Its high affinity for the membrane of pancreatic β cells induces selective toxic effects on these cells. The induction effect occurs via the Glut-2 transporter, causing DNA alkylation. This, in turn, activates PARP, leading to NAD+ depletion, decreased cellular ATP, and inhibition of insulin production. Macrophages, being the first cells to infiltrate pancreatic β cells, influence diabetes development through cytokine production (Yulianti *et al*., 2018).

To obtain valid data from STZ-based animal models of diabetes, it is crucial to properly prepare and use STZ. Despite its extensive history in diabetes research, overlooked aspects such as correct preparation, appropriate dosage, and anomeric composition can hinder accurate comparisons between studies, potentially leading to a loss of translational relevance from animals to humans. The lack of optimal preclinical data in animal models contributes to the limited success rate of drugs during the clinical investigation phase (Singh and Seed, 2021; Ghasemi and Jeddi, 2023).

This study provides practical guidelines for the research use of STZ as a diabetes-associated cardiac fibrosis induction agent, aiming to enhance the quality of *in vivo* studies involving mice and *in silico* investigations related to TNFα and Bcl2 receptors. DM is characterized by increased secretion of proinflammatory cytokines, playing a significant role in the development of cardiovascular complications in DM (Shen *et al*., 2015). TNFα is pivotal in diabetes pathogenesis, including the induction of cardiomyocyte apoptosis (Singh *et al*., 2019), while Bcl2 influences beta-cell apoptosis and mitochondrial dysfunction (Sharifi Rad *et al*., 2020).

Materials and Methods

Material

The substance used in this research was STZ which was obtained from ®santacruz. Meanwhile, the tools used included micropipettes, pipette tips, stir bars, polyprolene microcentrifuge tubes, syringes, microscopes, cover glasses, object glass objects, and minor surgery.

Methods

Experimental and treatment of experimental animals

Adult male Wistar rats (*Rattus norvegicus*) weighing 160-180 g and aged 16-18 weeks were selected to minimize hormonal influences. Following a 2-week acclimatization period, rats were intraperitoneally injected with STZ at doses of 30 mg/KgBW and 50 mg/KgBW, injection once at the beginning of treatment. To prevent post-injection hypoglycemia, rats were provided with a 10% sucrose solution overnight. Blood glucose levels and cardiac muscle cell fibrosis were evaluated 2 days post-induction. Blood sugar levels were monitored via tail blood sampling using @Accu-chek glucostick. Cardiac extraction, washing with PZ, and histopathological preparations with Malory Azan staining under 400x magnification were conducted to evaluate fibrosis. Experimental animals were divided into five groups: control (P1), STZ 50 mg for 4 weeks (P2), STZ 50 mg for 8 weeks (P3), STZ 30 mg for 4 weeks (P4), and STZ 30 mg for 8 weeks (P5).

Collection of compound and protein data

STZ data were acquired from the PubChem database, and the 3D structure of each compound was downloaded in ".sdf" format. Furthermore, the 3D structures of inflammatory mediator proteins, specifically TNFα (PDB ID 2AZ5) and Bcl2 (PDB ID 6QGH), were obtained from the PDB database, with each protein structure downloaded in PDB format.

Molecular docking analysis

Molecular docking analysis was conducted to assess the interaction strength between the compound of interest (ligand) and the TNFα and BCL-2 proteins. The analysis was performed using PyRx 0.8 software, utilizing specific coordinates corresponding to the active site of each protein. The binding affinity score was employed to measure the strength of the bond between the ligand and the protein. A more negative binding affinity score indicates a stronger bond between the ligand and the protein in the molecular docking analysis (Fatimah *et al*., 2024).

Ligand-protein interaction analysis

The chemical bond interactions formed in the ligand-protein complex were subsequently analyzed using Discovery Studio software. This analysis aimed to ascertain the position and binding pose of the ligand within the active site of the protein.

Molecular visualization

The 3D structure of the complex obtained from the results of the molecular docking analysis was then visualized using PyMOL software. The visualization was essential to confirm the binding position of the ligand to the target protein.

Data analysis

The analysis of data obtained from *in vivo* tests was conducted using SPSS software to examine the relationships between variables and differences between groups.

Ethical approval

Ethical approval was obtained from the Health Research Ethics Committee, Faculty of Medicine, Airlangga University (letter number: 257/EC/KEPK/FKUA/2023).

Results

The research findings indicated changes in random blood sugar levels among groups before and after STZ administration. Additionally, measurements of cardiac muscle fibrosis post-STZ revealed distinctions compared to the control group (P1).

The administration of STZ resulted in significantly elevated random sugar levels in mice (P2-P5), surpassing 200 mg/dl, indicating diabetes diagnosis based on internationally recognized criteria, including fasting blood sugar (>126 mg/dl), 2-hour blood sugar (>200 mg/dl), and random blood sugar (>200 mg/dl) (Fajarwati *et al*., 2023). Fajardo *et al*., (2014), classified normal glucose levels in rodents as <200 mg/dl, prediabetes as 200–249 mg/dl, and diabetes as >250 mg/dl, with some research considering mice with levels between 150–200 mg/dl within the diabetes category (Ghasemi *et al*., 2014). Additionally, all treatment groups (P2-P5) showed alterations in cardiac muscle fibrosis compared to the control group (P1), alongside random blood sugar levels.

Figure (1) shows increased random blood sugar levels in mice compared to the normal group (P1). Additionally, Figure (2) suggests that treatment group 3, given STZ 50 mg for 8 weeks, serves as a viable diabetes-associated cardiac fibrosis model in mice. Significant differences (*), compared to the normal group (P1), are noted in fibrosis extent.

Cardiac fibrosis, marked by excessive ECM buildup, results from remodeling involving profibrotic cells, growth factors, and inflammatory cytokines (Ridwan *et al*., 2023). Associated mediators encompass inflammatory cytokines, chemokines, reactive oxygen species, mast cell-derived proteases, endothelin-1, renin–angiotensin–aldosterone-system, cellular components, and growth factors like transforming growth factor β (Ridwan *et al*., 2023). This complication is well-documented in chronic DM patients (Pan *et al*., 2023). Elevated blood glucose levels significantly contribute to DM-associated cardiac fibrosis *in vitro* and animal studies. In a high glucose environment, cardiac fibroblasts produce excessive ECM proteins, including collagen, fibronectin, and matrix macromolecules. Antihyperglycemic drug administration in animal models reduces myocardial fibrotic changes (Aroor *et al*., 2018). This is in accordance with research conducted by Liu *et al* (2020), STZ injection in mice also facilitated the development of cardiac fibrosis and increased oxidative stress.

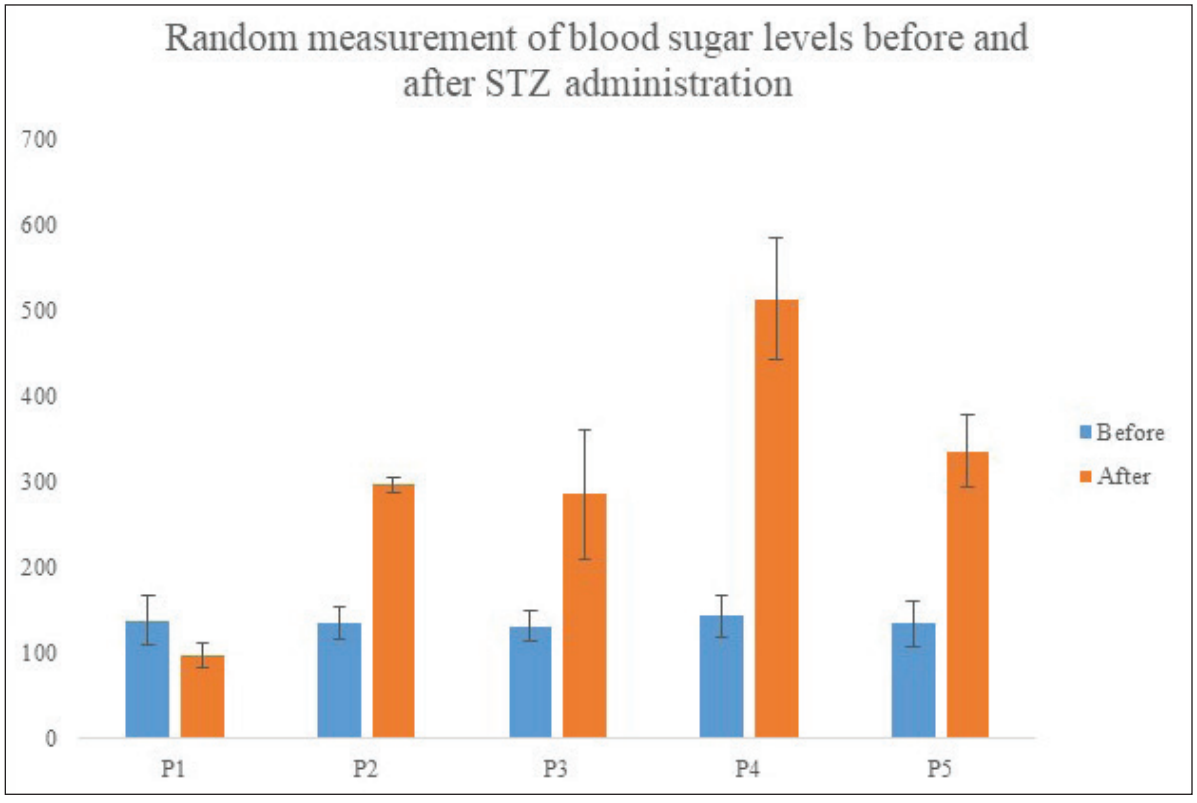

Fig. 1. Measurement of random blood sugar levels in mice before and after administration of STZ.

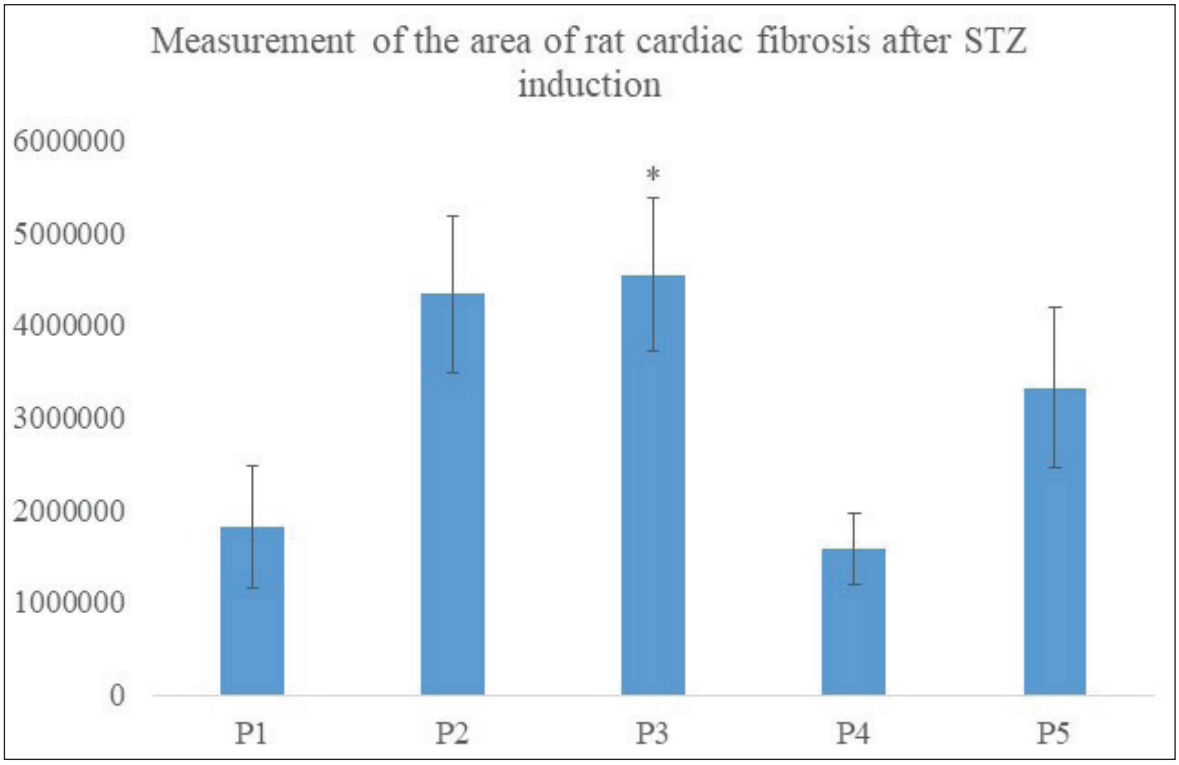

Fig. 2. Measurement of area of rat cardiac fibrosis after administration of STZ.

This study also confirmed the affinity of STZ for inflammatory mediators involved in diabetes-associated cardiac fibrosis pathology, specifically TNFα and Bcl2 (Mohammadi *et al*., 2021). The SMILE notation, a compound nomenclature readable by computing programs (Manjula *et al*., 2015), facilitated the collection of the 3D structure of each compound of interest for further analysis. Additionally, the inflammatory mediator proteins, TNFα (PDB ID 2AZ5), and Bcl2 (PDB ID 6QGH) were obtained from the PDB database. The proteins were then processed by removing water molecules and adding polar hydrogen (Hidayat *et al*., 2021).

Molecular docking analysis of the compound of interest with the Bcl2 protein was conducted using the 3D structure with the PDB ID code 6QGH, chosen for its good structural resolution of 2Å and origin from the Homo sapiens organism. The Bcl2 structure (PDB ID 6QGH) also included a native ligand, ABT-263, used as a control, while TNFα (PDB ID 2AZ5) features a native ligand, Ligand 307.

Molecular docking analysis of the Bcl2 protein was carried out using specific docking with Dimensions (Amstrong) X coordinate 37.667; Y: 38,136; Z: 41.904 and Center X: -7.175; Y: 2,805; Z: 9,857. Meanwhile, for TNFα, specific docking coordinates were used, namely Dimensions (Amstrong) X: 22,229; Y: 24,969; Z: 25.482 and Center X: - 19.102; Y: 74,814; Z: 33,523. Coordinates were adjusted to the active sites on Bcl2 and TNFα proteins. Analysis (Table 1) showed that STZ's binding affinity score was not more negative than the control, indicating no stronger binding to Bcl2 protein compared to the control. A more negative score suggests a stronger ligand-protein interaction (Muslikh *et al*., 2023; Fatimah *et al*., 2024).

Table 1. Binding affinity values of STZ and control for each receptor.

Compound	Binding affinity (kcal/mol)	
	TNFα (2AZ5)	Bcl2 (6QGH)
STZ	−8.1	−5.9
Controls	−8.4	−11.9

The interaction of each compound of interest with the Bcl2 and TNFα proteins was further analyzed using Discovery Studio software to identify the amino acid residues involved in the interaction (Figs. 3 and 4). The analysis aimed to ensure that each compound bound to the active site corresponding to the control, with different colors indicating the types of amino acid residue bonds.

Amino acid residues are specific amino acids in a protein that are bound to a particular ligand or compound, and the active site of protein binding varies for each amino acid. The percentage of similarity between the amino acid residues in the compound and the control (native

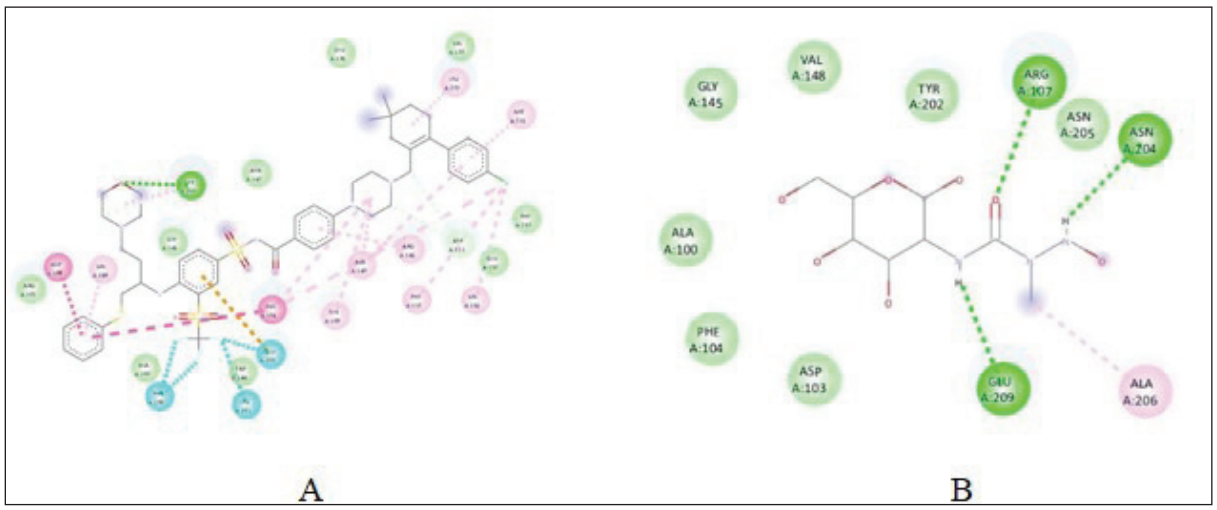

Fig. 3. Residue sour amino interaction between proteins Bcl2 with control (A), STZ (B).

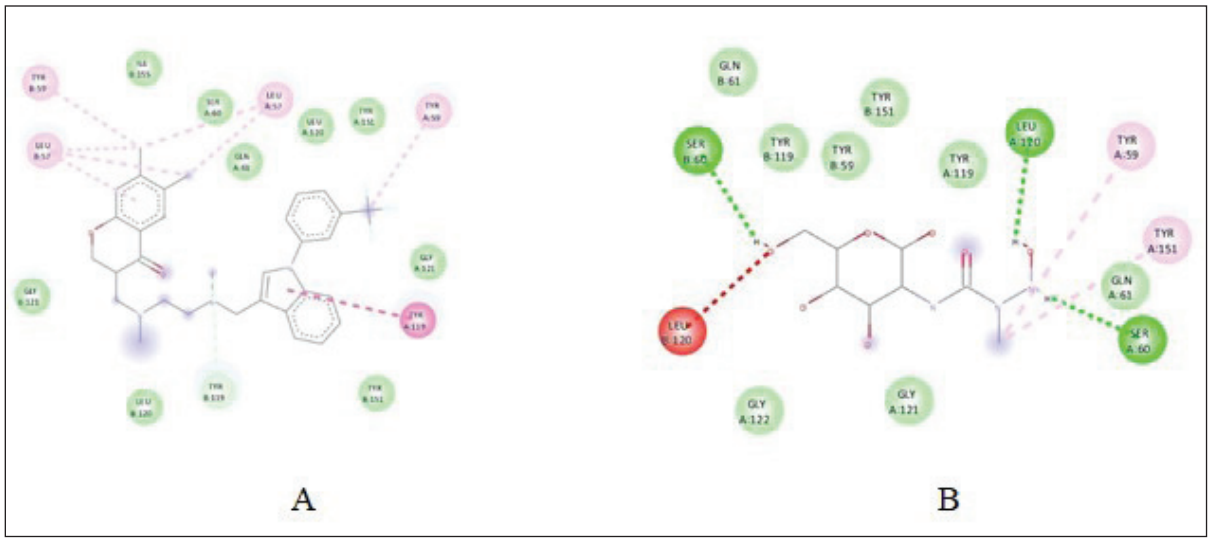

Fig. 4. Amino acid residues of interaction between TNFα protein and control (A), streptozotocin(B).

ligand) indicates the potential similarity of the active site. This similarity can result in strong binding and similar biological activity to controls (Gondokesumo *et al*., 2023).

Discussion

The evaluation of STZ as a diabetes-associated cardiac fibrosis model in mice within this study involved assessing random blood sugar levels and the extent of fibrosis in the cardiac muscle. Random observations of blood sugar levels were conducted both before and after treatment to compare conditions preceding and following STZ injection. Simultaneously, assessments of cardiac muscle fibrosis were carried out post-STZ administration.

Successful DM management encompasses various approaches, including the regulation of blood glucose levels, engagement in physical activities, adherence to a balanced diet, weight reduction, and the control of blood pressure/lipids, among other measures. These actions collectively aim to prevent complications at both microvascular and macrovascular levels (Wasir *et al*., 2018). Effectively controlling glucose levels can significantly reduce the risk of complications associated with DM (Kotwal and Pandit, 2012; Arif, 2018).

STZ, commonly used alongside Aloxan, is a diabetogenic agent in diabetes models. Research shows that 30.3% of studies used alloxan, while 57.9% utilized STZ to induce diabetes in experimental animals (Fajarwati *et al*., 2023). Streptozotocin, an unusual aminoglycoside, contains a nitrosoamino group that allows its metabolite to act as a nitric oxide (NO) donor. NO serves as a crucial messenger

molecule in various physiological and pathological processes. Widely used to induce diabetes in rodent models by inhibiting β-cell O-GlcNAcase (Eleazu *et al*., 2013), STZ exhibits antibiotic, β-cell cytotoxic, oncolytic, and oncogenic effects. Its use in diabetes research is typically associated with specific toxicity to pancreatic β cells and inhibits DNA synthesis in mammalian and bacterial cells (Busineni *et al*., 2015).

Toxicity to β cells is induced by protein carbamoylation, DNA alkylation, release of free radicals (ROS and RNS), and inhibition of O-GlcNAcase. Insulin production by β cells is disrupted by DNA methylation through the formation of carbonium ions (CH3+), triggering the activation of the core enzyme poly ADP-ribose synthetase, leading to depletion of NAD+ and ATP. Free radicals generated during STZ decomposition and metabolism reduce mitochondrial enzyme activities and inhibit O-GlcNAcase, causing a decrease in cellular energy levels and suppressing the biological function of islet cell proteins (Busineni *et al*., 2015). The selection of STZ for inducing diabetes-associated cardiac fibrosis is attributed to its stability relative to other substances, rendering it suitable for prolonged experimental investigations (Hikmah *et al*., 2015; Liu *et al*., 2020).

Conclusion

STZ served as an inducer for diabetes-associated cardiac fibrosis models in experimental studies, and an effective dose for inducing diabetes in mice involved in administering 50 mg of STZ over 8 weeks. This dosage elevated blood sugar and fibrosis. *In silico* analysis showed STZ-bound inflammatory mediators in diabetes-associated cardiac fibrosis models.

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The authors have no conflicts of interest to declare.

Conflict of interest

The authors declare that there is no conflict of interest.

Data availability

All the data are presented within this article.

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This research received no specific grant.

Authors' contributions

NF: Contributed to conceptualization, and methodology, practical work, histopathological work, writing manuscript draft, editing, and revising the manuscript. AM: Contributed to conceptualization, methodology, and supervision, writing manuscript drafts, editing, and revising the manuscript. SAS: Contributed to conceptualization, methodology, and supervision, writing manuscript drafts, editing, and revising the manuscript. All authors revised and approved the manuscript for publication.

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