

Calcium Carbonate-Stabilized Nano-Caffeine Emulsion Attenuates Diabetic Cardiomyopathy via Antioxidant, Anti-Inflammatory, and Anti-Fibrotic Pathways in Type 2 Diabetic Rats with HPLC-Quantified Cardiac Caffeine Levels

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Introduction: Type 2 diabetes mellitus (T2DM) is one of the most commonly diagnosed metabolic diseases. Notably, two-thirds of diabetic patients may develop diabetic cardiomyopathy (DCM), a life-threatening condition for which no curative treatment currently exists.

Methods: This study aimed to investigate the potential ameliorative effects of caffeine against DCM development, utilizing a novel oral sustained-release caffeine-loaded Pickering emulsion formula stabilized by calcium carbonate nanoparticles to enhance its pharmaceutical and pharmacological properties. Eighty-four rats were divided into seven groups: control, caffeine, nano-caffeine, diabetic, diabetic + rosuvastatin, diabetic + caffeine, and diabetic + nano-caffeine.

Results: Our findings demonstrated that the newly developed nano-caffeine formulation significantly downregulated myocardial injury markers (CK-MB, cTnI, ALT, AST, and LDH) and markedly ameliorated myocardial tissue injury and fibrosis, as confirmed by histopathological examination and desmin/ α -SMA expression analysis. Additionally, the nano-caffeine treatment reduced inflammatory cytokines (TNF- α and IL-1 β), attenuated hyperlipidemia, decreased iNOS and NO myocardial concentrations, and upregulated protective antioxidants (Nrf2, GSH, GSH-Px, SOD, and catalase) compared to the control group. Importantly, the cardioprotective effects of nano-caffeine were more pronounced than those observed in caffeine-treated diabetic rats. Furthermore, a novel, simple, and validated HPLC method was employed to quantify caffeine levels in cardiac tissues in all groups. The analysis revealed significantly higher caffeine concentrations in the nano-caffeine group compared to other groups, indicating improved tissue delivery.

Conclusion: The formulation significantly enhances the cardioprotective effects of caffeine against myocardial injury in T2DM rats by optimizing its pharmacodynamic and pharmacokinetic properties.

Keywords: type 2 diabetes mellitus, HPLC-quantification, diabetic cardiomyopathy, nano-caffeine, Pickering emulsion, cardioprotection, nanotechnology

Introduction

The incidence of diabetes mellitus (DM) has been consistently rising globally, and its correlation with cardiovascular diseases (CVD) is well-documented. Diabetes mellitus impacts almost 10% of the adult population worldwide.^{1,2} The likelihood of developing diabetic cardiomyopathy (DCM) is markedly elevated in persons with chronic DM. DCM has been

documented in up to 30% of diabetic patients, with its prevalence escalating alongside the length of diabetes mellitus, impacting around 12–22% of individuals after a decade of disease progression.³ The main pathological characteristics of diabetic cardiomyopathy are cardiomyocyte enlargement, myocardial interstitial and perivascular fibrosis, inflammation, apoptosis, and elevated indicators of oxidative stress.⁴ Oxidative stress is a critical pathophysiological factor in the onset and progression of pathological hypertrophy and remodeling, as well as in the onset and progression of heart failure in diabetic individuals.⁵ Moreover, oxidative stress elevates the expression of transforming growth factor- β (TGF- β), induces the transition of fibroblasts into myofibroblasts, and enhances collagen synthesis, resulting in myocardial fibrosis.⁶

Hyperglycemia is a critical antecedent for the activation of chemokines, cytokines, and leukocyte adhesion molecules, subsequently leading to myocardial inflammation. Chronic low-grade inflammation partially facilitates structural and metabolic alterations in the diabetic heart, including left ventricular hypertrophy, myocardial fibrosis, and calcium processing irregularities.⁷ The release of cytokines and chemokines stimulates a shared signaling pathway that involves NF- κ B. The activation of NF- κ B through toll-like receptor (TLR) 4 leads to the production of the proinflammatory cytokines, including TNF- α , IL-6, IL-1 β , and IL-8.⁸

Natural products have demonstrated efficacy in treating several diseases, exhibiting multiple therapeutic effects and an enhanced safety profile.⁹ Nonetheless, obstacles such as inadequate solubility and diminished bioavailability impede their absorption and restrict overall efficacy.¹⁰ Furthermore, the limited amounts reaching the target tissues *in vivo* lead to variable treatment outcomes.¹¹

We previously established that capsaicin, a naturally occurring compound, significantly ameliorates the development of myocardial structural alterations in diabetic rats.¹² That study highlighted capsaicin's effectiveness in attenuating myocardial damage through its hypoglycemic, hypolipidemic, antioxidant, anti-inflammatory, and anti-apoptotic effects. In accordance with these findings, we examined other natural compounds for their potential protective activity against myocardial injury in diabetics and tried to improve their formulation to enhance their pharmacokinetic properties. Consequently, we examined caffeine for its potential cardioprotective activity in case of diabetic cardiomyopathy.

Caffeine is the most widely consumed stimulant and psychoactive agent globally.¹³ Over 60 distinct plant species, such as cacao beans and tea leaves, inherently contain it in varied concentrations.¹⁴ Recent data over the past decade suggests that caffeine and its methylxanthine metabolites may influence redox and inflammation-related pathways.¹⁵ Moreover, it was found that the chronic use of caffeine has been demonstrated to decrease proinflammatory cytokine levels, including tumor necrosis factor-alpha (TNF- α) and interleukin 1beta (IL-1 β).¹⁶

Caffeine has rapid metabolism and systemic clearance ($t_{1/2}$ = 4–6 hours), so frequent daily dosing is necessary to sustain therapeutic plasma concentrations. However, this often leads to fluctuating plasma levels that can compromise efficacy.¹⁴ Furthermore, excessive caffeine intake is linked to adverse neurological and cardiovascular effects, such as anxiety, headaches, insomnia, hypertension, and tachycardia.¹⁷

Innovative drug delivery technologies offer potential solutions to the physicochemical constraints of natural products. A Novel Nano-Drug Delivery System (NDDS) denotes an innovative strategy in the pharmaceutical domain, utilizing the capabilities of nanotechnology for drug administration.^{18,19} Based on the utilized carrier materials and topologies, these nano-drug delivery systems proficiently overcome traditional challenges such as inadequate solubility and enhance drug targeting properties.²⁰

Pickering emulsions are a type of emulsion stabilized by solid particles rather than molecular surfactants. Owing to their high stability and loading capacity, they have attracted growing interest in various industries, including food, cosmetics, and biomedicine.²¹ Subsequent research has explored a wide range of solid stabilizers for these emulsions, such as silica, metal oxides, clay minerals, and polymer particles.^{22–25}

In this study, calcium carbonate (CaCO₃) particles were employed as stabilizers for the preparation of Pickering emulsions. Calcium carbonate was selected due to its cost-effectiveness, widespread availability, and established use as a food additive and pharmaceutical excipient. One of its key advantages is its acid-soluble nature, which enables pH-responsive destabilization of emulsions under acidic conditions. This property makes CaCO₃ particularly suitable for applications where controlled release or stimulus-responsive behavior is desired. Additionally, CaCO₃ particles offer functional benefits such as calcium fortification and acid neutralization. Upon exposure to gastric acid, CaCO₃ converts into absorbable calcium ions, enhancing its nutritional and therapeutic potential in oral delivery systems.²¹

To obtain stable Pickering emulsions, particle wettability is a critical factor. Emulsions stabilized by particles with intermediate wettability tend to exhibit the highest stability. In addition, particle size significantly influences both desorption energy and emulsion stability.²⁶ Because of that calcium carbonate nanoparticles (CaCO₃ NPs) were used as the stabilizing agent instead of normal CaCO₃ particles. DCM is a distinctive cardiovascular complication that necessitates a thorough therapeutic strategy, and there is no targeted treatment for this disease.

The current study primarily aimed to construct an oral, effective, stable, and sustained-release Pickering emulsion, stabilized by calcium carbonate nanoparticles, for the encapsulation of caffeine to enhance its therapeutic benefits in DCM circumstances. This paper presents the first creation and validation of a novel high-performance liquid chromatography (HPLC) technology that reliably quantifies caffeine contents in Pickering emulsion formulations and heart tissue samples. This dual analytical capacity is vital, as it allows for full analysis of caffeine's cardiac distribution, offering essential insights into its probable role in DCM pathogenesis. These methodological advancements signify a crucial progression in clarifying caffeine's extensive influence on cardiovascular health.

Materials and Methods

Materials

Caffeine monohydrate was obtained from ICN Biomedicals, Inc., Germany. Rosuvastatin calcium was gifted from Egyptian International Pharmaceutical Industries Co. (10th of Ramadan, Egypt). Sodium carbonate, calcium chloride, polyethylene glycol 2000 (PEG2000), and KH₂PO₄ were purchased from United Company for Chem. and Med. Prep., Egypt. Acetonitrile and methanol were purchased from Fisher Scientific. A Millipore purification system (Bedford, MA, USA) was used to obtain the Milli-Q[®] water used to prepare buffer solutions. Sunflower oil is a product of Alexandria Oil Company, Egypt.

Preparation of Calcium Carbonate Nanoparticles

CaCO₃ NPs were synthesized following a previously established procedure.²⁶ Briefly, 0.3 g of PEG2000 was dissolved in 120 mL of 0.1 mol/L calcium chloride solution. Next, 35 mL of 0.33 mol/L sodium carbonate solution was rapidly added to this stirred solution. Following a 24-hour reaction at room temperature, the suspension was centrifuged (5,000 g, 20 min). The resulting precipitate was washed three times with ultrapure water and then vacuum-dried for 12 hours.

Preparation of Pickering Emulsion Formulation Incorporating Caffeine

Caffeine was dissolved in deionized water, which served as the aqueous phase, while liquid paraffin was used as the oil phase of the emulsion. The emulsion was prepared at a fixed aqueous-to-oily phase ratio of 4:6, as described previously.²⁷ CaCO₃ NPs were suspended in the oil phase at selected concentrations. The aqueous phase containing caffeine was then added dropwise to the oily phase using a syringe. Homogenization was carried out using a high-speed homogenizer at 18,000 rpm for 3 minutes.

Characterization of CaCO₃ NPs and the Prepared Pickering Emulsion

Dilution method²⁸ was used to determine the type of emulsion. The method involved adding an emulsion drop to oil or water and observing its behavior. O/W emulsions dispersed in water but maintained their droplet structure in oil, while W/O emulsions dispersed in oil but retained droplet integrity in water.

Particle size distribution (expressed as average volume diameters) and polydispersity index (PDI) were measured via dynamic light scattering (DLS) using a Zetasizer Nano ZN instrument (Malvern Panalytical Ltd, UK). Measurements were performed at a fixed scattering angle of 173° and 25°C, with samples analyzed in triplicate.²⁹

Encapsulation Efficiency and Loading Capacity

The encapsulation efficiency (EE%) of caffeine in the selected formulation was evaluated indirectly by quantifying the unencapsulated (free) caffeine present in the supernatant. This analysis employed a validated HPLC method. Samples at 4 °C were subjected to centrifugation at 14,000 rpm for 15 minutes prior to measurement. EE% was calculated using

Equation 1, where T corresponds to the total amount of caffeine initially incorporated into the formulation and C represents the concentration of free caffeine remaining in the supernatant. All determinations were performed in triplicate to ensure reproducibility and statistical reliability.

$$EE(\%) = \frac{(T - C)}{C} \quad (1)$$

Furthermore, to evaluate drug loading capacity (LC%), the caffeine-loaded emulsion was dried and its mass recorded. LC% was derived by dividing the mass of the entrapped caffeine ($T - C$) by the total weight of the dried formulation, as detailed in Equation 2.

$$LC(\%) = \frac{(T - C)}{\text{Total Material weight}} \quad (2)$$

In vitro Drug Release Study

The in vitro release behavior of caffeine from the developed Pickering emulsion was investigated using the dialysis membrane diffusion method, with a pure caffeine solution serving as the control. In summary, 1 mL of the test formulation was introduced into a pre-soaked cellulose dialysis membrane (molecular weight cutoff: 12–14 kDa), tightly sealed, and placed in a well-closed glass bottle containing 50 mL of the release medium, which consisted of 0.1 M HCl pH 1.2 for 2 h followed by phosphate-buffered saline pH 6.8. The experimental setup was kept at $37 \pm 0.5^\circ\text{C}$ under constant stirring at 100 rpm within a thermostatic shaking water bath. Caffeine content was analyzed by HPLC at specified intervals throughout a 24-hour duration. Each release study was conducted three times. Different mathematical models were applied to measure the kinetics and mechanism of caffeine release from the prepared emulsion.

Storage and Temperature Stability

The long-term stability of the prepared Pickering emulsion was evaluated under two storage conditions, 4°C and 25°C over one month. The particle size, polydispersity index (PDI), and zeta potential were measured after 30 days to monitor any changes in emulsion characteristics.

Chromatographic Analysis

Instruments and Conditions

An Agilent 1260 Infinity II[®] HPLC System (Agilent Technologies, Germany) with a Pursuit-3 C18 column (150×4.6 mm, $3 \mu\text{m}$) set at 30°C was used for method development. ChemStation[®] software processed the output signals. The mobile phase comprises phosphate buffer (10 mM, pH 3.0), SDS (0.15%), and acetonitrile (97:3, v/v). Isocratic chromatographic runs were conducted at a flow rate of 0.5 mL/min, with a 20 μL injection volume and 278 nm detection wavelength.

Analysis of Caffeine in Pickering Emulsion

The emulsion samples were broken using ethanol to recover the encapsulated caffeine. In total, 1 mL of emulsion was added into 9 mL of ethanol, and then the emulsion was broken with an ultrasonic probe. After the emulsion was broken, the solution was centrifuged for 10 min to separate the CaCO_3 NP precipitate from the clarified solution. After the solution was centrifuged, the supernatant was taken, and the peak area at 273 nm was examined with HPLC.

Preparation of Stock and Working Solutions

A caffeine stock solution was prepared at 10.0 mg/mL in the mobile phase and stored at -20°C for future analysis. Working solutions, at a concentration of 0.5 $\mu\text{g/mL}$, were freshly prepared by diluting the stock solution with the mobile phase.

Calibration of Caffeine in Tissue Homogenates

To a 0.5 mL aliquot of cardiac tissue homogenate, different volumes of a caffeine working solution were added to obtain final concentrations ranging from 2.5 to 78.0 $\mu\text{g/mL}$. The resulting mixtures were vortexed for 5 minutes, then

centrifuged at 12,000 rpm for 3 minutes at room temperature. Subsequently, a 20 μ L portion of the clear supernatant was injected into the HPLC system for analysis.

Animals

All animal procedures were approved by the ethical committee of the Faculty of Pharmacy, South Valley University (approval number P.S.V.U 225). All experimental procedures and animal care practices were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (8th edition, 2011) and we also adhered to the ARRIVE guidelines and its updates (Percie du Sert et al, 2020). The animals were obtained from the Egyptian Organization for Biological Products and Vaccines, located in Giza, Egypt. Adult male Sprague-Dawley rats, weighing 120–130 g, were accommodated in a well-ventilated and illuminated top-mash cage. The rodents were provided with unrestricted access to water and normal rodents chow and were maintained under controlled conditions of $25 \pm 2^\circ\text{C}$ with a 12-hour light and 12-hour dark cycle. Following a 10-day acclimatization period, the animals underwent overnight fasting and received a single intraperitoneal injection of streptozotocin (STZ) at a dosage of 35 mg/kg, dissolved in 0.1 mM citrate–phosphate buffer (pH 4.5). The STZ solution was administered promptly following its preparation before its degradation. The control group received an injection of citrate-phosphate buffer. All animals received a standardized diet for two weeks post-injection.

The onset of hyperglycemia in the experimental subjects was validated through an oral glucose tolerance test (OGTT) utilizing ACCU-Chek Active (Roche Diagnostics). The OGTT was conducted on two separate days following two weeks of STZ injection. Blood glucose levels were measured in a fasting state and at 30, 60, 90, and 120 minutes following the oral administration of a 40% glucose solution (3 g/kg of body weight). Rats exhibiting blood glucose levels over 11.1 mmol/L after 120 minutes from the commencement of the test were considered diabetic, and only those consistently diabetic rats were recruited for subsequent investigations.³⁰ The diabetic mice were administered a high-fat diet (HFD) containing the following percentages: 17% carbohydrates, 25% proteins, and 58% fats. The dietary content per kilogram was as follows: Powdered standard pellet diet: 365 g, Lard: 310 g, Casein: 250 g, Cholesterol: 10 g. Vitamin and mineral mixture: 60 g, dl-Methionine: 3 g, yeast powder: 1 g, sodium chloride: 1 g. The controlled animals were provided with a regular diet. The animals were categorized into the following groups (n = 12) as detailed below:

1. Control normal group; in which normal animals were fed a normal diet and received a daily dose of distilled water (1 mL/kg, P.O.) for 12 weeks.
2. Caffeine group; in which normal animals fed a normal diet and received a daily dose of caffeine (25 mg/kg, P.O.) for 12 weeks.
3. Nano-caffeine; in which normal animals fed a normal diet and received a daily dose of nano-caffeine (25 mg/kg, P.O.) for 12 weeks.
4. 4- Diabetic group; in which diabetic animals were fed an HFD for 12 weeks.
5. Diabetic + rosuvastatin (rosu); in which diabetic animals fed an HFD plus a daily dose of rosu (20 mg/kg) for 12 weeks.³¹
6. Diabetic + caffeine; in which diabetic animals were fed an HFD plus a daily dose of caffeine (25 mg/kg) for 12 weeks.³²
7. Diabetic + nano-caffeine; in which diabetic animals were fed an HFD plus a daily dose of nano-caffeine (25 mg/kg).

Animal weights were determined weekly during the experiment, and treatment dosages were modified correspondingly. One week before the end of the trial, all animals underwent a 12-hour fasting period, followed by an OGTT test in triplicate. Upon completion of the experiment, the animals underwent a 12-hour fast and were euthanized at 8:00 a.m. using ketamine anesthesia (50 mg/kg, i.p). Blood samples were collected from the inferior vena cava and centrifuged at 3000 rpm for 15 minutes. The blood serum was taken and frozen at -20°C for further biochemical investigation. The cardiac tissues were gently excised from the animals and rinsed with cold saline (4°C). Myocardial samples were homogenized with a cold solution of potassium phosphate buffer (100 mM), followed by centrifugation at 3000 rpm for

20 minutes to provide a 10% homogenate. The supernatants were collected and preserved at -80°C for subsequent biochemical investigations.

Histopathological Examination

H&E Staining

The rat's myocardium was kept in 10% formalin at 28°C for 24 hours, subsequently embedded in wax, and sectioned to $5\text{ }\mu\text{m}$ thickness. 0.8% hematoxylin was added for 5 minutes, followed by 0.35% eosin stain for 3 minutes at 28°C . The cardiac tissue architectures were examined using an Olympus BX53 light microscope at a magnification of $\times 200$. Two independent pathologists evaluated the myocardial injury score using a 0–4-point scoring system: 0 indicates no tissue damage; 1 signifies an irregular arrangement of myocardial cells with mild necrosis and edema; 2 reflects slight disarray in myocardial cells with moderate local necrosis and swelling; 3 denotes significant distortion in cell arrangement with severe necrosis and infiltration of inflammatory cells; and 4 represents extreme disarray of cells with very severe diffuse necrosis and inflammatory infiltration. A total of six randomly selected fields per slide were analyzed.³³

Masson's Trichrome Staining

Blue aniline Masson's trichrome stain was used to stain serial sections ($5\text{ }\mu\text{m}$) of paraffin-embedded heart tissues. Following previously established protocols, the sections were examined using optical microscopy (magnification 400x) to assess the size fraction of interstitial and perivascular fibrosis. Six randomly chosen fields per slide were examined.³⁴

Immunohistochemistry

The processes were executed as initially outlined. Rat heart tissues were dried, preserved in paraffin, and cut into $3\text{ }\mu\text{m}$ thick slices using a rotary microtome. The tissues were deparaffinized, and then antigen retrieval was performed by heating the slides in boiling water for 20 minutes at 80°C . Washing cycles of 5 minutes using 0.1 M PBS at pH 7.4 were conducted between steps. Cardiac tissue slices were treated for 16 hours with diluted rabbit polyclonal primary antibodies: anti-SMA (Cat No.: ab300164, Abcam, Cambridge, UK), anti-Nrf-2 (Cat No.: ab313825, Abcam, Cambridge, UK), anti-Desmin (Cat No.: ab32362, Abcam, Cambridge, UK), and anti-iNOS (Cat No.: ab178945, Abcam, Cambridge, UK), in accordance with the manufacturer's instructions. Cardiac tissue sections were viewed using a light microscope (Leica DM2000 LED Ergonomic System Microscopes). Six randomly selected fields per slide were evaluated and quantified using Image-Pro Plus 5.0 image analysis software (National Institutes of Health, Bethesda, MD, USA). Two independent pathologists, unaware of the pathological results, assessed histology and immunohistochemical staining scoring, with the overall protein expression calculated as the mean of histoscores.³⁵

Tissue Total Protein Determination

The protein level of cardiac tissue homogenates was assessed using the Bradford method outlined in the Bio-Rad protein assay kit (Cat No: 5000201EDU).

Measurement of AST, ALT, and LDH

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) are enzymes that are extensively distributed in the body, predominantly in the heart and liver. Their levels are markedly elevated in instances of heart damage. The activities of AST, ALT, and LDH were assessed using a commercially available kit purchased from Abcam (Waltham, MA, USA) with Cat Nos: ab263883, ab234579, and ab222910, respectively.

Determination of Serum Creatine Kinase-MB (CK-MB)

The activity of creatine kinase (CK) in rat blood serum was measured colorimetrically using a CK test kit from Abcam (Cat No: ab285275, Waltham, MA, USA). The kit utilizes a reaction between phosphocreatine and adenosine diphosphate, which interacts with the CK enzyme mixture to produce an intermediate that subsequently generates a colored product with an absorbance at a wavelength of 450 nm.³⁶

Determination of Cardiac Troponin I Levels

Cardiac troponin I (cTnI) is a frequently used confirmatory biomarker for myocardial injury. In this test, cTnI concentrations were measured utilizing an enzyme-linked immunosorbent assay (ELISA) kit (Cat No: SEB820Ra, Cloud-Clone Corp, Texas, USA). The kit is a sandwich enzyme immunoassay intended for the quantitative measurement of cTnI in rat serum. Freshly withdrawn serum was allowed to coagulate for 2 hours at ambient temperatures. The serum was subsequently centrifuged for 20 minutes at about 1000 g. The supernatant was aliquoted into a 96-well plate, followed by the addition of 100 μ L of detection reagent A. After one hour, reagent B was introduced, and the plate was incubated at 37°C for thirty minutes. The plate underwent three wash cycles to eliminate unbound antibodies. The substrate solution was thereafter introduced and incubated for 30 minutes. A stop solution was introduced, and the absorbance was quantified at 450 nm.³⁷

Determination of Cardiac Catalase Activity

This test measures cardiac catalase antioxidant activity, which protects the cardiac tissue from oxidative damage by turning hydrogen peroxide into water and oxygen. The assay quantifies the residual hydrogen peroxide following the addition of cardiac tissue homogenate samples that contain the catalase enzyme using a commercial kit purchased from Biodiagnostics (Cat No: CA 25 17, Cairo, Egypt).^{38,39}

Determination of Superoxide Dismutase (SOD) Activity

The SOD enzyme activity was assessed with a standard commercial kit from Biodiagnostics (Cat No: SD 25 21, Cairo, Egypt). The kit utilizes tetrazolium salts that yield a water-soluble formazan color when reduced by superoxide anions. The absorbance of the formazan dye was measured at 450 nm.⁴⁰

Determination of Reduced Glutathione (GSH) Concentration

The levels of glutathione (GSH) in cardiac tissue homogenates were determined with a commercial kit from Biodiagnostics (Cat No: GR 25 11, Cairo, Egypt) following the manufacturer's guidelines. The technique relies on the reduction of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) by glutathione (GSH), resulting in a yellow chromophore that is determined colorimetrically at 412 nm.⁴¹

Determination of Glutathione Peroxidase (GSH-Px) Activity

The glutathione peroxidase (GSH-Px) activity in the myocardial homogenate was assessed utilizing a commercial kit from Biodiagnostics (Cat No: GP 2524, Cairo, Egypt). The GSH-Px activity was assessed at a wavelength of 412 nm, aligning with the absorption maximum of the enzyme-catalyzed reaction product.⁴²

Determination of Serum Lipids

Serum lipid profiles, comprising high-density lipoprotein (HDL), triglyceride (TG), and total cholesterol (TC) levels, were assessed enzymatically utilizing commercial assay kits from Elabscience (Cat NO: E-EL-R0504, E-BC-K238, and E-BC-K109-S, respectively, Houston, Texas, USA) under the manufacturer's guidelines. Low-density lipoprotein (LDL) levels were determined using the formula $LDL = TC - (HDL + VLDL)$, where very-low-density lipoprotein (VLDL) is calculated as $VLDL = TG/5$.

Determination of Cardiac Malondialdehyde (MDA) Content

Cardiac lipid peroxidation was evaluated in the tissue by determining malondialdehyde (MDA) levels. The technique relies on the interaction between MDA and thiobarbituric acid in an acidic medium at 95°C, resulting in a pink color measurable at a wavelength of 532 nm.^{43,44}

Nitric Oxide Determination (NO)

Nitric oxide (NO) concentrations in cardiac tissue homogenate were assessed with the Griess reagent test. The Griess reagent assay is a colorimetric method that quantifies nitric oxide generation. The assay involves the addition of Griess reagent to a sample, resulting in a deep purple color determined at a wavelength of 540 nm.⁴⁵

Inflammatory Mediators' Determination

Plasma concentrations of the inflammatory mediators interleukin-1 beta (IL1 β) and tumor necrosis factor-alpha (TNF- α) were determined using the ELISA technique, following the manufacturer's guidelines from R&D Systems (Cat No: RLB00 and RTA00, respectively, Minneapolis, MN, USA).

Statistical Analysis

All parameters in this study were presented as the mean $\pm$ SD, and the data were statistically analyzed using a one-way ANOVA test, followed by Tukey's multiple comparison test. The statistical significance was established at $p < 0.05$. A statistical analysis was performed utilizing the GraphPad Prism software tool (GraphPad Software, San Diego, CA 92108, United States).

Results

Characterization of Calcium Carbonate Particles and Pickering Emulsion

Scanning electron microscopy (SEM) enables detailed observation of the surface morphology of materials. The SEM image of the synthesized CaCO_3 particles is shown in Figure 1A. As illustrated, the CaCO_3 particles exhibited a rod-like shape, with diameters ranging from approximately 0.11 to 0.13 μm and lengths between 0.154 and 0.757 μm . The droplet size of the Pickering emulsion prepared using these CaCO_3 particles (Figure 1B) was measured by dynamic light scattering (DLS). Results indicated a mean droplet diameter of 612.1 ± 58.79 nm, with a polydispersity index (PDI) of 0.534. However, the TEM analysis (Figure 1C) enables precise characterization of the CaCO_3 solid particles acting as Pickering stabilizers. These particles exhibit a mean diameter of 49.3 ± 3.7 nm and a polydispersity index (PDI) of 0.37. This dual-scale analysis confirms that the sub-50 nm CaCO_3 particles are appropriately sized to stabilize the larger emulsion droplets. The stability of the nano-caffeine emulsion system is further supported by a zeta potential of -29.6 ± 3.9 mV. This negative surface charge generates sufficient electrostatic repulsion to prevent droplet coalescence,

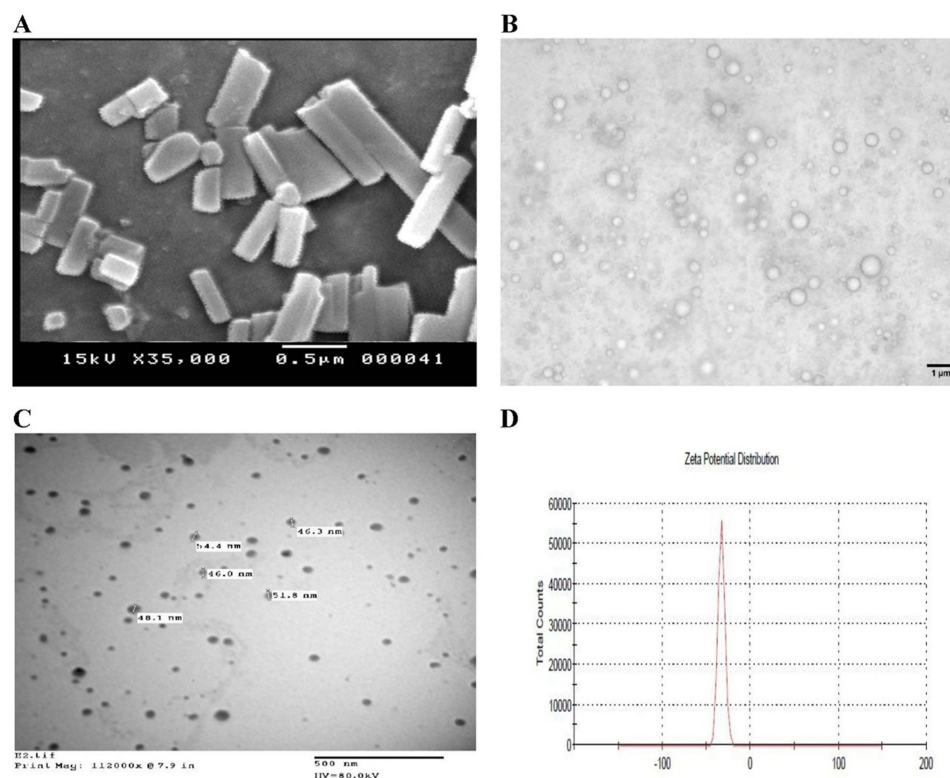


Figure 1 (A) SEM of the formed CaCO_3 particles; (B) The optical microscopy and appearance inspection of Pickering emulsion; (C) The Transmission Electron Microscopy (TEM) of Pickering emulsion; (D) The Zeta Potential Distribution of nanoemulsion emulsion.

effectively complementing the robust mechanical barrier formed by the CaCO_3 “shell” at the oil–water interface (Figure 1D).

Additionally, a drop taken from the Pickering emulsion was miscible with the oily phase, confirming that the emulsion was of the water-in-oil (w/o) type.

In vitro Release Profile of Caffeine

The in vitro release profiles of caffeine from the control solution reached a complete release within the first 2 hours, the Pickering emulsion exhibited a protracted release, reaching a cumulative value of approximately 45% at the 24-hour mark. This suggests that the CaCO_3 nanoparticle layer serves as a robust physical barrier, effectively sequestering the caffeine within the core and significantly increasing the diffusion path length required for the drug to reach the external medium. To quantitatively evaluate the release mechanism, the cumulative release data were fitted to several mathematical models. The Zero-order kinetic model provided the highest degree of correlation, yielding a regression coefficient R^2 of 0.9391. This indicates that the system approaches an ideal sustained-release profile, where the rate of drug displacement remains constant over time and is independent of the concentration remaining within the carrier. Such behavior is highly advantageous for oral delivery systems, as it minimizes fluctuations in drug concentration and potentially reduces the frequency of dosing.

The relatively low release observed during the first 2 hours at pH 1.2 (<10%) implies that even under acidic conditions that typically promote CaCO_3 dissolution, the oily phase and the specific arrangement of the nanoparticles sufficiently delayed the entrance of the medium and the subsequent leakage of caffeine. Furthermore, the linear progression of the release after the medium was switched to pH 6.8 indicates that the formulation is stable in intestinal conditions, allowing for a steady, controlled liberation of the emulsion.

Storage and Temperature Stability

The long-term physical stability of the CaCO_3 -stabilized Pickering emulsion was monitored over a 30-day period at both refrigerated 4 °C and ambient 25 °C temperatures to ensure the structural integrity of the delivery system. The experimental results, detailed in Table 1, revealed no significant variations in the physical parameters after one month of storage, with the mean droplet size remaining relatively constant at 605.3 ± 10.25 nm (4 °C) and 617.12 ± 12.22 nm (25 °C). This resistance to droplet growth indicates that the CaCO_3 nanoparticles formed a rigid, protective shell at the oil-water interface, effectively preventing coalescence through steric stabilization. The stability observed at 4 °C further suggests that lower temperatures effectively reduced the kinetic energy and collision frequency of the droplets. Throughout the study, the polydispersity index (PDI) remained consistently low (0.531 to 0.579), reflecting a narrow size distribution and a homogeneous system. Moreover, the zeta potential maintained high absolute values of approximately -29.6 ± 0.09 mV and -27.6 ± 0.34 mV, respectively, indicating strong electrostatic repulsion that prevents droplet aggregation. Collectively,

Table 1 Particle Size, Zeta Potential, Polydispersity Index (PDI) of Perking Emulsion Stored at 4 °C and at 25 °C for One Month

Storage Temperature (°C)	4	25
Size (nm)	605.3 ± 10.25	617.12 ± 12.22
PDI	0.531 ± 0.14	0.579 ± 0.6
Zeta potential (mV)	-29.6 ± 0.09	-27.6 ± 0.34

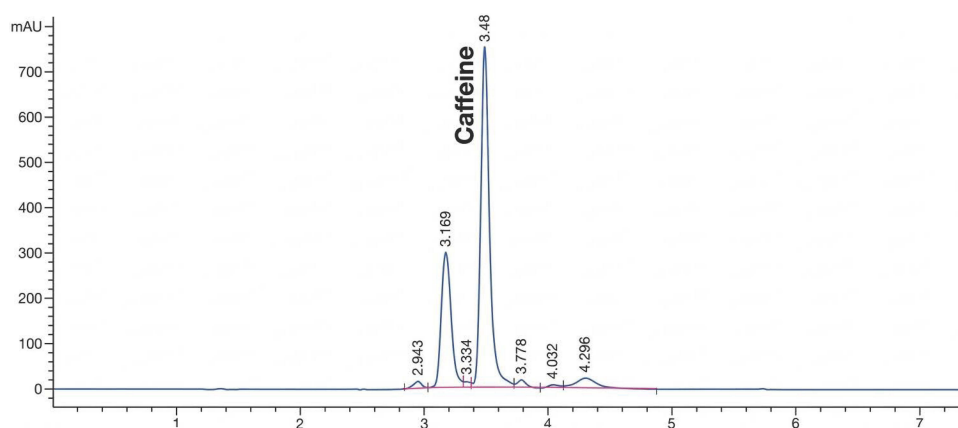


Figure 2 HPLC chromatogram of the analysis of tissue homogenate containing standard Caffeine taken under optimized conditions.

HPLC Method

The optimal mobile phase was a mixture of 3% acetonitrile and 97% phosphate buffer (10 mM, pH 3.0, SDS 0.08%). The column temperature was maintained at 30°C. Under these conditions, the retention times were observed to be 3.48, as shown in Figure 2, with detection performed at 274 nm.

Subsequently, the analytical method was validated for several parameters, including linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ). Table 2 presents the results of this validation process, while Table 3 outlines the system suitability parameters for the proposed analytical method. The concentrations of caffeine in cardiac homogenates were measured using HPLC. The results showed markedly higher levels of caffeine ($6.51 \pm 0.42 \mu\text{g/mL}$) in the diabetic nano-caffeine group than in the diabetic caffeine group ($2.72 \pm 0.01 \mu\text{g/mL}$).

Table 2 Summary of HPLC Method Development and Validation

Parameter	Value
Retention time (min.)	3.48
Linearity range ($\mu\text{g/mL}$)	1.0–22.0
Regression equation	$Y = 2773.4x + 174.81$
Correlation coefficient	0.999
LOD ($\mu\text{g/mL}$)	0.169
LOQ ($\mu\text{g/mL}$)	0.514
Accuracy (n=3)	
Mean recovery (%)	98.02–101.03
%RSD	0.504–1.02
Precision (n=3)	
Intra-day (%RDS)	0.822
Inter-day (%RDS)	1.411
Mean recovery %	99.5 ± 0.821
%RSD	0.83

Table 3 System Suitability Parameters for the HPLC Method

HPLC Parameter	Caffeine	Acceptable Limits
Asymmetry factor	0.720	> 1.5
Theoretical plates/m	12798	< 200
Tailing factor	1.27	> 2.0
HETP (cm)	0.00117	

Histopathological Examination

H&E Staining

The control, caffeine, and nano-caffeine groups showed normal cardiac fiber architecture with regular striations. On the other hand, the myocardium of the diabetic group had cardiac fiber separations, vacuolated cells, and edema associated with remarkable infiltration of inflammatory cells around blood vessels, and the cardiac muscle was congested and moderately degenerated. The diabetic group treated with rosuvastatin had a noticeable alteration in heart muscle striations and mild vacuolization in cardiac muscle cells, associated with noticeable inflammatory cell infiltration. However, in the diabetic + caffeine group, the cardiac smooth muscle bundles had mild degeneration, and the cytoplasm appeared pale and eosinophilic. The diabetic + nano-caffeine group exhibited mild disrupted cardiac muscle striation without vacuolization, few inflammatory cell infiltrations, and nearly an absence of myocardial degeneration, as shown in [Figure 3](#).

Masson's Trichrome Staining

The histopathological analysis of the myocardial sections utilizing Masson's trichrome staining showed that control, caffeine, and nano-caffeine groups had minimal collagenous fiber deposition around blood vessels ($\times 200$). The diabetic group exhibited significant and dense collagen accumulation encircling almost all blood vessels in the myocardial sections, whereas fibrosis was identified in both perivascular and interstitial myocardium areas. However, the diabetic rats treated with rosuvastatin exhibited moderate collagen deposition accompanied by extensive perivascular fibrosis. The caffeine-treated group had less cardiac fibrosis with little collagen accumulation around cardiac blood vessels, while the diabetic group treated with nano-caffeine showed the lowest observed collagenous and fibrotic tissues among all diabetic-treated groups, as shown in [Figure 4](#).

Immunohistochemistry

Effects of Treatments on iNOS Protein Distribution

The myocardium sections of the control group exhibited moderate immunostaining for iNOS, while both caffeine and nano-caffeine-treated groups showed more intense iNOS staining. The cardiac sections of the diabetic group exhibited a significant upregulation of iNOS protein distribution in the myocardium compared to the control group, while rosuvastatin administration did not produce any effect on iNOS myocardial distribution. The myocardial sections of the caffeine and nano-caffeine-treated groups exhibited a significant upregulation of positively iNOS-immunostained myocardial cells, which was more prominent in the nano-caffeine group, as shown in [Figure 5](#).

Effects of Treatments on Nrf-2 Protein Distribution

Immunohistochemistry was employed to determine Nrf-2 expression in rats' cardiac tissues. The control group demonstrated modest immunoexpression of Nrf-2 in the myocardium, which was slightly increased in caffeine and nano-caffeine-treated groups. On the other hand, the untreated diabetic group displayed a nearly complete absence of Nrf-2 protein immunoexpression. In this investigation, we observed that the diabetic groups treated by caffeine or nano-caffeine exhibited a noticeable upregulation in the positively immunostained Nrf-2 cardiac myocytes compared to the diabetic non-treated group, which was more prominent in nano-caffeine than caffeine-treated groups. Moreover, we

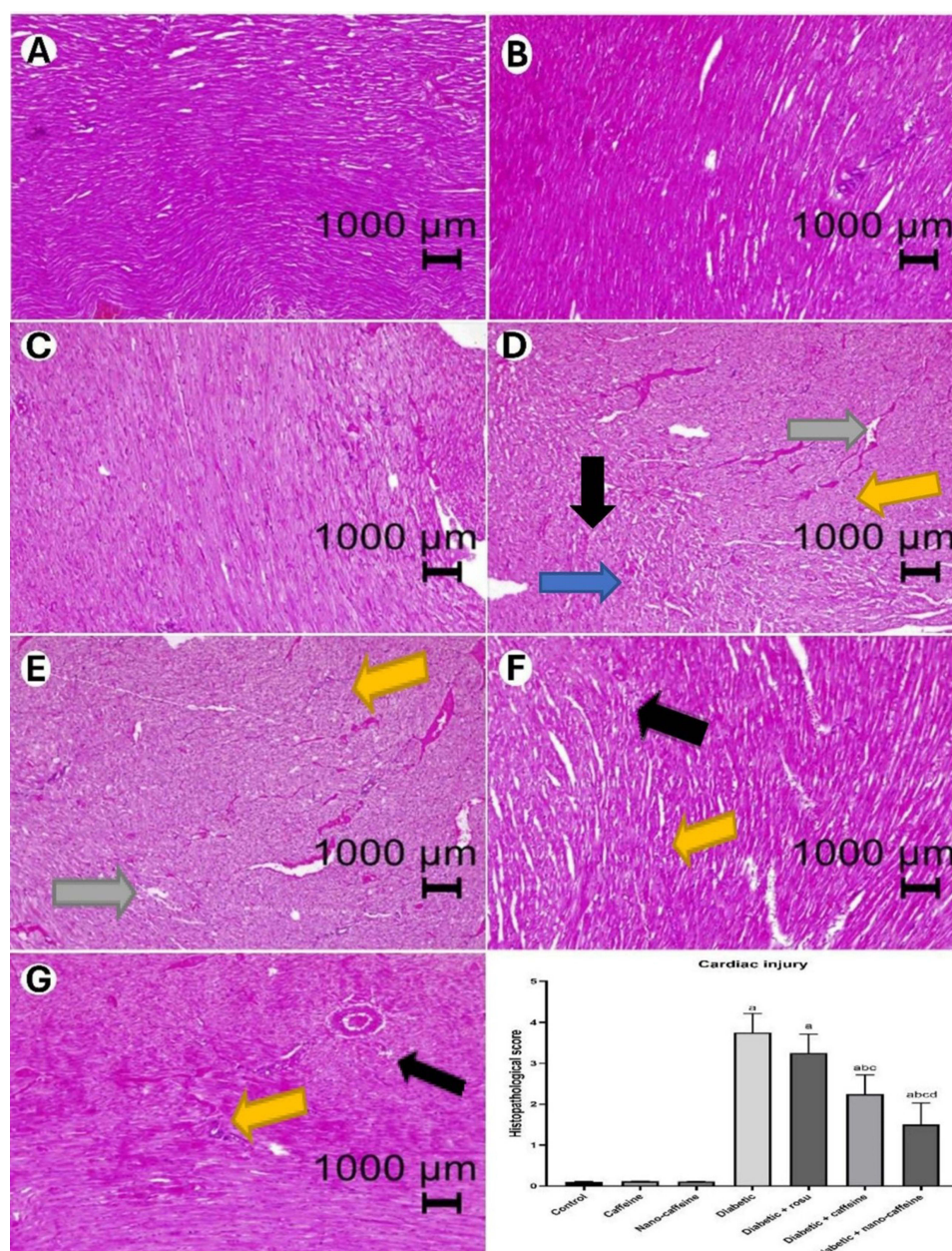


Figure 3 Histopathology of rats' heart (H&E stain) from: (A) normal Control group; (B) normal rats received caffeine; (C) normal rats received nano caffeine, (D) Diabetic rats, (E) Diabetic rats received rosuvastatin; (F) Diabetic rats received Caffeine and (G) Diabetic rats received nano caffeine. The black arrow point to tissue degeneration, the yellow arrow point to inflammatory cells infiltration, the gray arrow point to cardiac fiber separation, the blue arrow point to congested blood vessels. a = significantly different compared to the control group, b = significantly different compared to the diabetic group, c = significantly different compared to the Diabetic + Rosuvastatin group, and d significantly different compared to the Diabetic + caffeine.

found that rosuvastatin treatment did not significantly affect Nrf-2 myocardial concentration compared to the diabetic non-treated group, as shown in Figure 6.

Effects of Treatments on α -SMA Protein Distribution

Immunohistochemical staining of myocardial tissue for α -smooth muscle actin (α -SMA) demonstrated a significant upregulation of this protein in the diabetic group relative to the control one, which exhibited nearly undetectable levels of α -SMA protein. Both caffeine and nano-caffeine-treated groups exhibited weakly positive α -SMA protein staining, localized in certain perivascular regions. The group treated with rosuvastatin exhibited nonsignificant changes in the levels of α -SMA protein compared to the diabetic non-treated group, as shown in Figure 7.

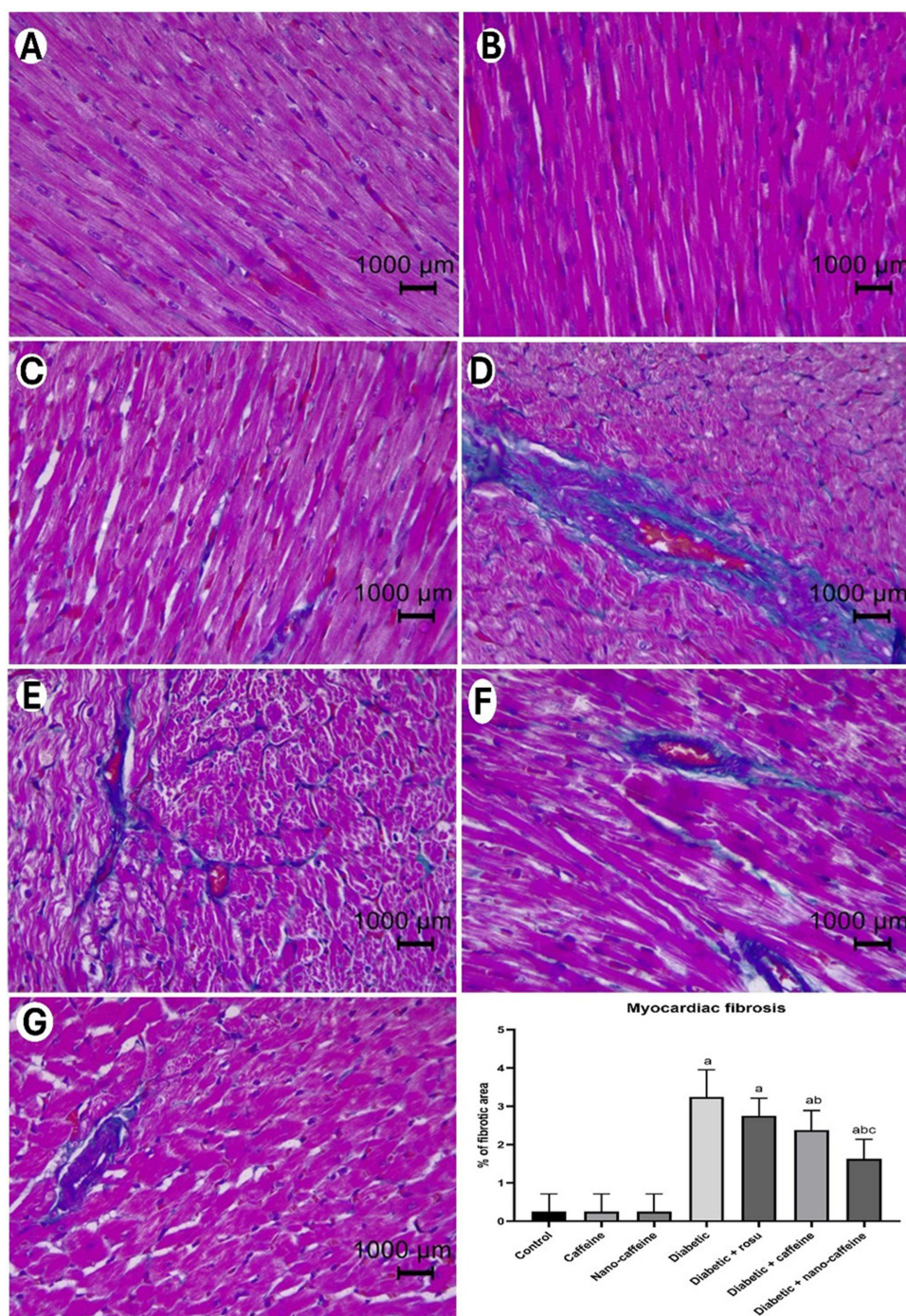


Figure 4 Histopathology of rats' heart (Masson trichrome stain) from: (A) normal Control group; (B) normal rats received caffeine; (C) normal rats received nano caffeine, (D) Diabetic rats, (E) Diabetic rats received rosuvastatin; (F) Diabetic rats received Caffeine and (G) Diabetic rats received nano caffeine. a = significantly different compared to the control group, b = significantly different compared to the diabetic group, c = significantly different compared to the Diabetic + rosuvastatin group, and d significantly different compared to the Diabetic + caffeine.

Effect of Treatments on Desmin Protein Distribution

The current study's immunohistopathological analysis of cardiac tissue showed that the control, caffeine, and nano-caffeine groups had intense positivity to desmin antibodies, with normal staining observed at Z-lines and intercalated disks. The diabetic rats exhibited anomalous faint desmin staining with an uneven pattern of cross-striations; desmin aggregation was also noticed in the perinuclear regions and intermyofibrillar gaps. The Rosuvastatin-treated group showed reduced desmin staining

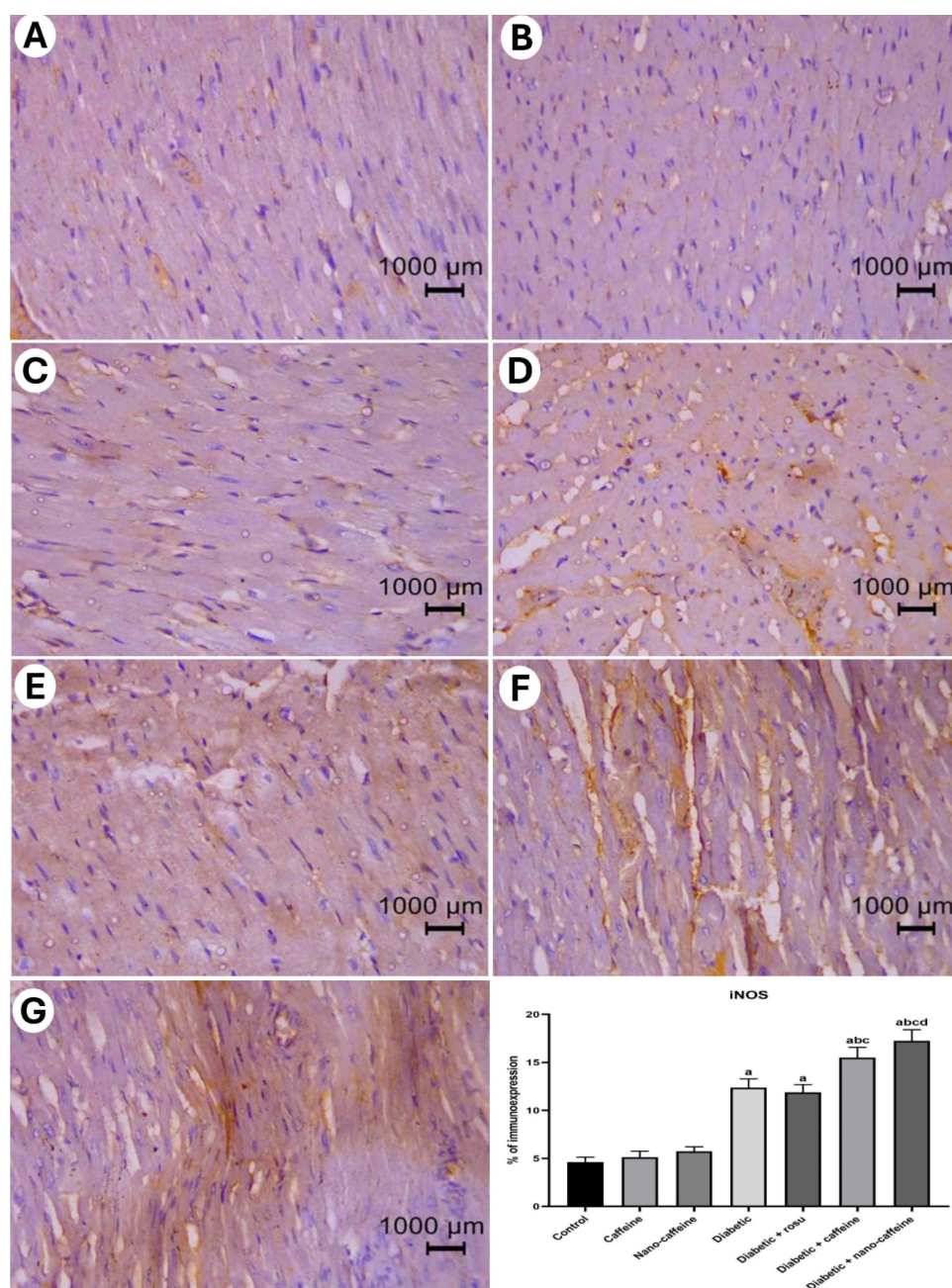


Figure 5 Immunohistochemistry results of iNOS in cardiac tissues from: (A) normal Control group; (B) normal rats received caffeine; (C) normal rats received nano caffeine; (D) Diabetic rats; (E) Diabetic rats received rosuvasatin; (F) Diabetic rats received Caffeine and (G) Diabetic rats received nano caffeine. a = significantly different compared to the control group, b = significantly different compared to the diabetic group, c = significantly different compared to the Diabetic + Rosuvastatin group, and d significantly different compared to the Diabetic + caffeine.

accompanied by considerable distortion in desmin alignment, and desmin aggregates were observed in the perinuclear regions. On the other hand, the caffeine-treated group exhibited nearly normal desmin staining without obvious distortion or aggregations, while the nano-caffeine-treated group showed normal desmin architecture characterized by regular cross-striation patterns and enhanced staining at Z-lines and intercalated disks, in addition to a nearly consistent cross-striation pattern, as shown in Figure 8.

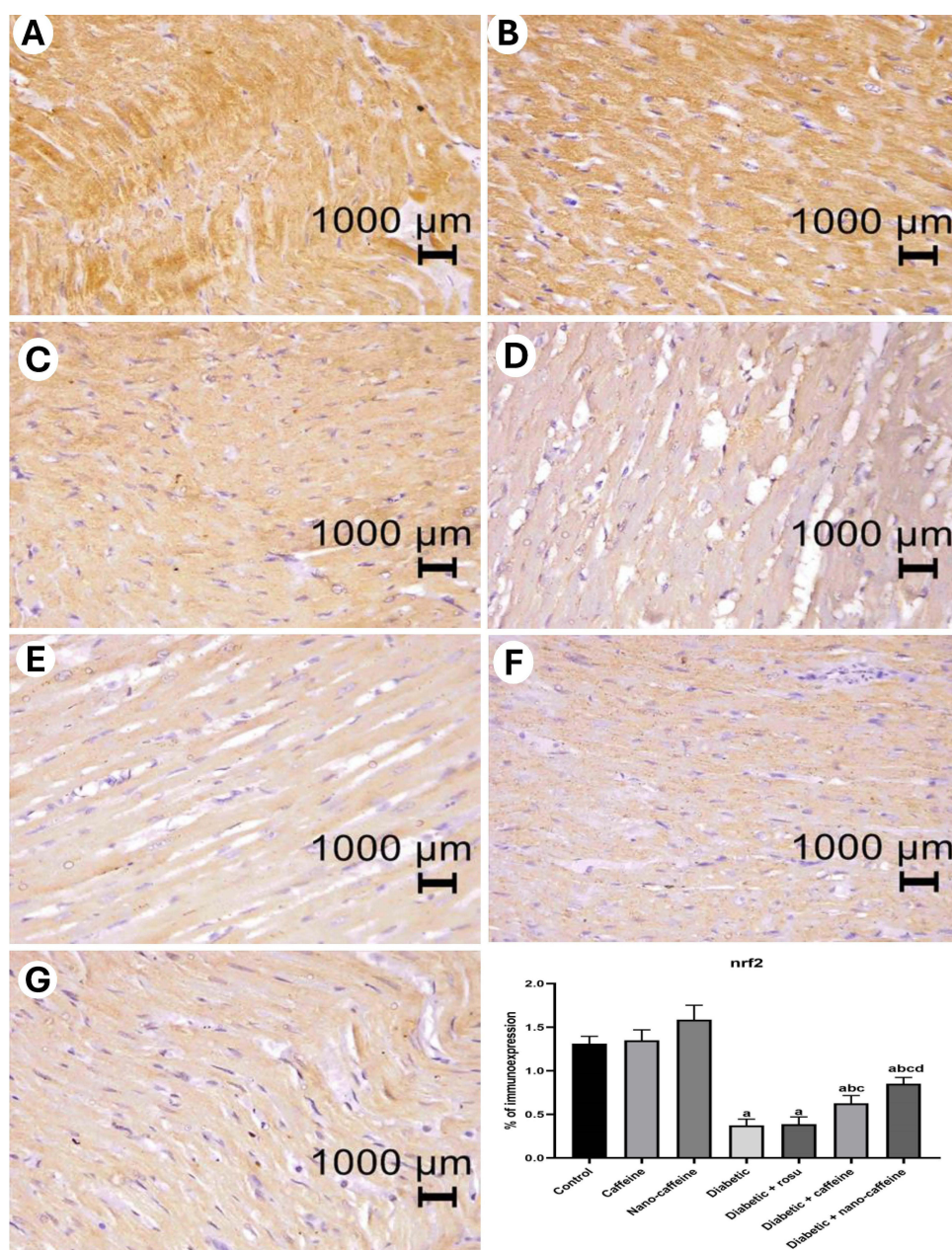


Figure 6 Immunohistochemistry results of Nrf2 in cardiac tissues from: (A) normal Control group; (B) normal rats received caffeine; (C) normal rats received nano caffeine, (D) Diabetic rats, (E) Diabetic rats received rosuvastatin; (F) Diabetic rats received Caffeine, and (G) Diabetic rats received nano caffeine. a = significantly different compared to the control group, b = significantly different compared to the diabetic group, c = significantly different compared to the Diabetic + Rosuvastatin group, and d significantly different compared to the Diabetic + caffeine.

Effect of Caffeine on Lipid Profile

Diabetic rats showed a significant increase in serum cholesterol (TC), triglycerides (TG), LDL, and VLDL levels compared to the normal control by 1.8, 1.4, 2.4, and 3 times, respectively. Moreover, diabetic rats showed a significant decrease in HDL levels by 35% compared to the normal control group. On the other hand, diabetic rats treated with nano-caffeine showed a significant decrease in serum cholesterol, TG, LDL, and VLDL levels compared to diabetic rats by 43%, 57%, 42%, and 49%, respectively, and by 22%, 16%, and 18%, respectively, compared to the caffeine-treated group. Additionally, nano caffeine increased serum HDL by 40% compared to the diabetic group and by 9% in comparison with caffeine-treated rats. These results were slightly lower than those of the rosuvastatin-treated

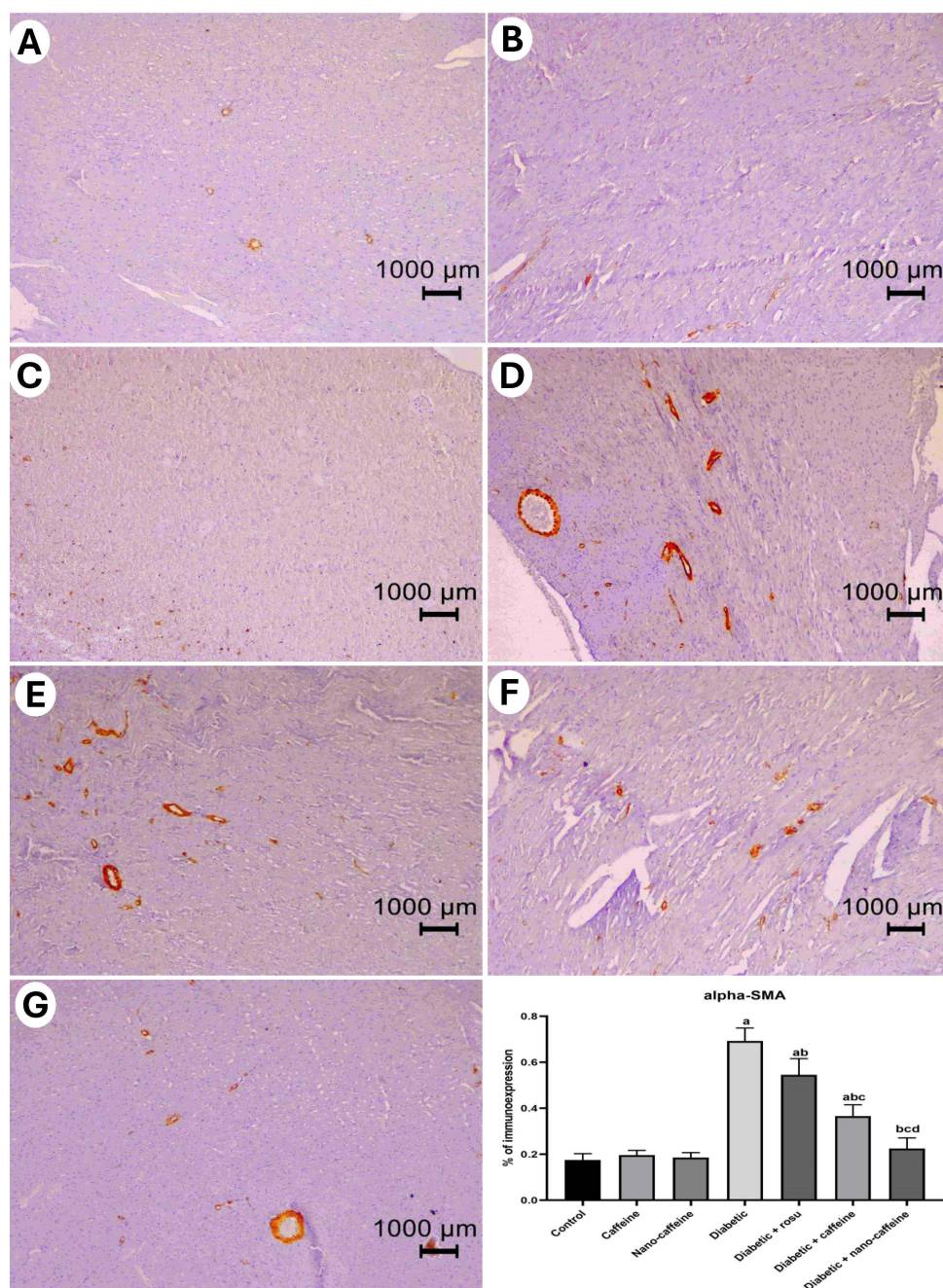


Figure 7 Immunohistochemistry results of α -SMA in cardiac tissues from: (A) normal Control group; (B) normal rats received caffeine; (C) normal rats received nano caffeine, (D) Diabetic rats, (E) Diabetic rats received rosuvastatin; (F) Diabetic rats received Caffeine, and (G) Diabetic rats received nano caffeine. a = significantly different compared to the control group, b = significantly different compared to the diabetic group, c = significantly different compared to the Diabetic + Rosuvastatin group, and d significantly different compared to the Diabetic + caffeine.

animals for cholesterol, TG, LDL, VLDL, and TC levels. However, there was no significant difference in HDL levels between the nano-caffeine and rosuvastatin-treated groups, as shown in Figure 9.

Effect of Drug Treatments on Oxidative Stress Biomarkers

As demonstrated in Figure 10, diabetic rats exhibited a significant depletion of antioxidant enzymes glutathione, glutathione peroxidase, catalase, and SOD compared to the normal control by 60%, 63%, 61%, and 43%, respectively. On the other hand, treatment with nano-caffeine significantly reserved these enzyme activities, compared to diabetic rats

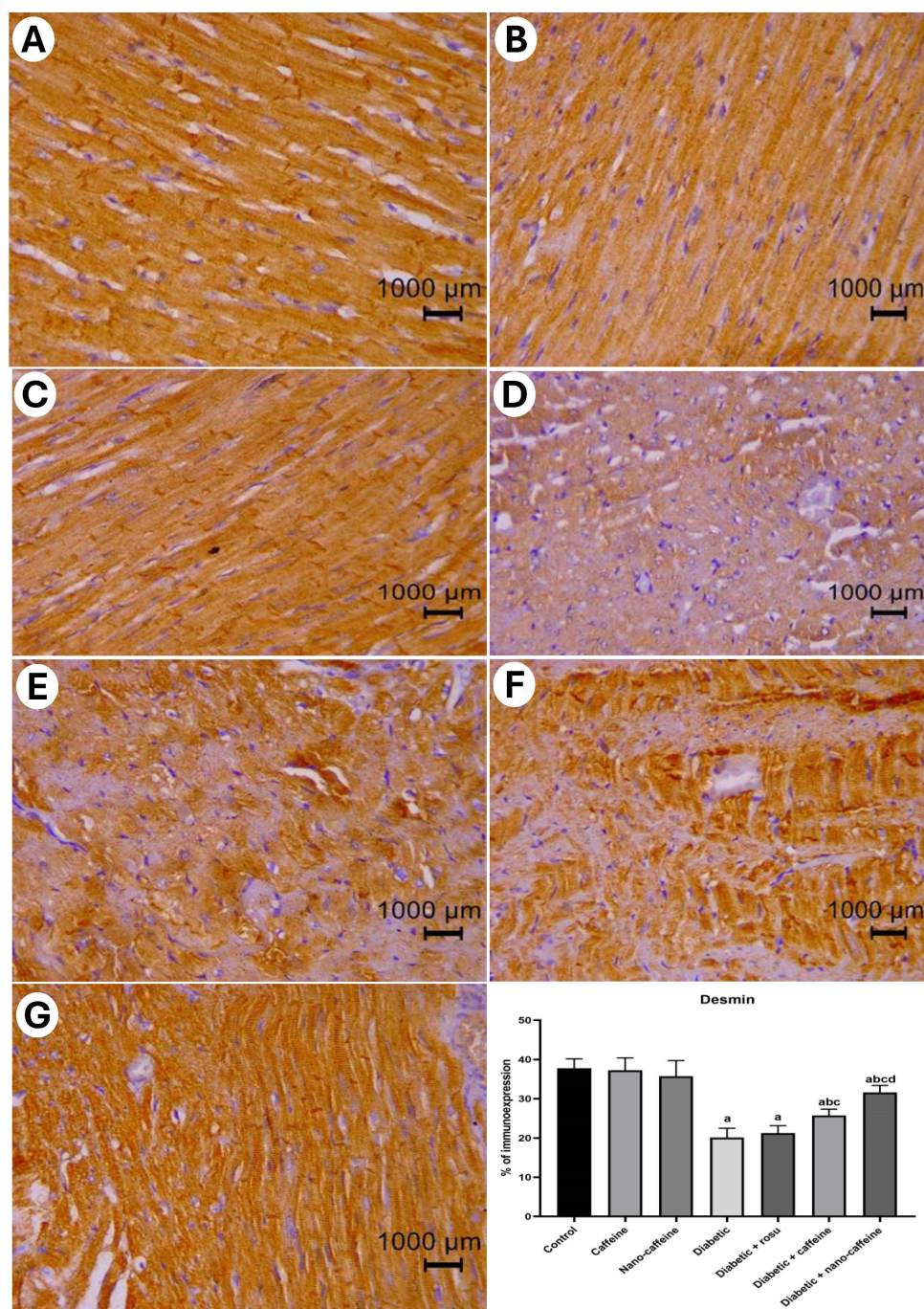


Figure 8 Immunohistochemistry results of desmin in cardiac tissues from: (A) normal Control group; (B) normal rats received caffeine; (C) normal rats received nano caffeine, (D) Diabetic rats, (E) Diabetic rats received rosuvasatin; (F) Diabetic rats received Caffeine, and (G) Diabetic rats received nano caffeine. a = significantly different compared to the control group, b = significantly different compared to the diabetic group, c = significantly different compared to the Diabetic + Rosuvastatin group, and d significantly different compared to the Diabetic + caffeine.

by 118%, 152%, 104%, and 55%, respectively, and by 47%, 37%, 25%, and 18%, compared to the caffeine-treated diabetic group. These results are presented in Figure 10.

Effect of Drug Treatments on Lipid Peroxidation

The concentration of malondialdehyde (MDA) is a frequently utilized biomarker to assess tissue lipid peroxidation. In this study, we found that diabetes significantly elevated the lipid peroxidation process as manifested by a high level of

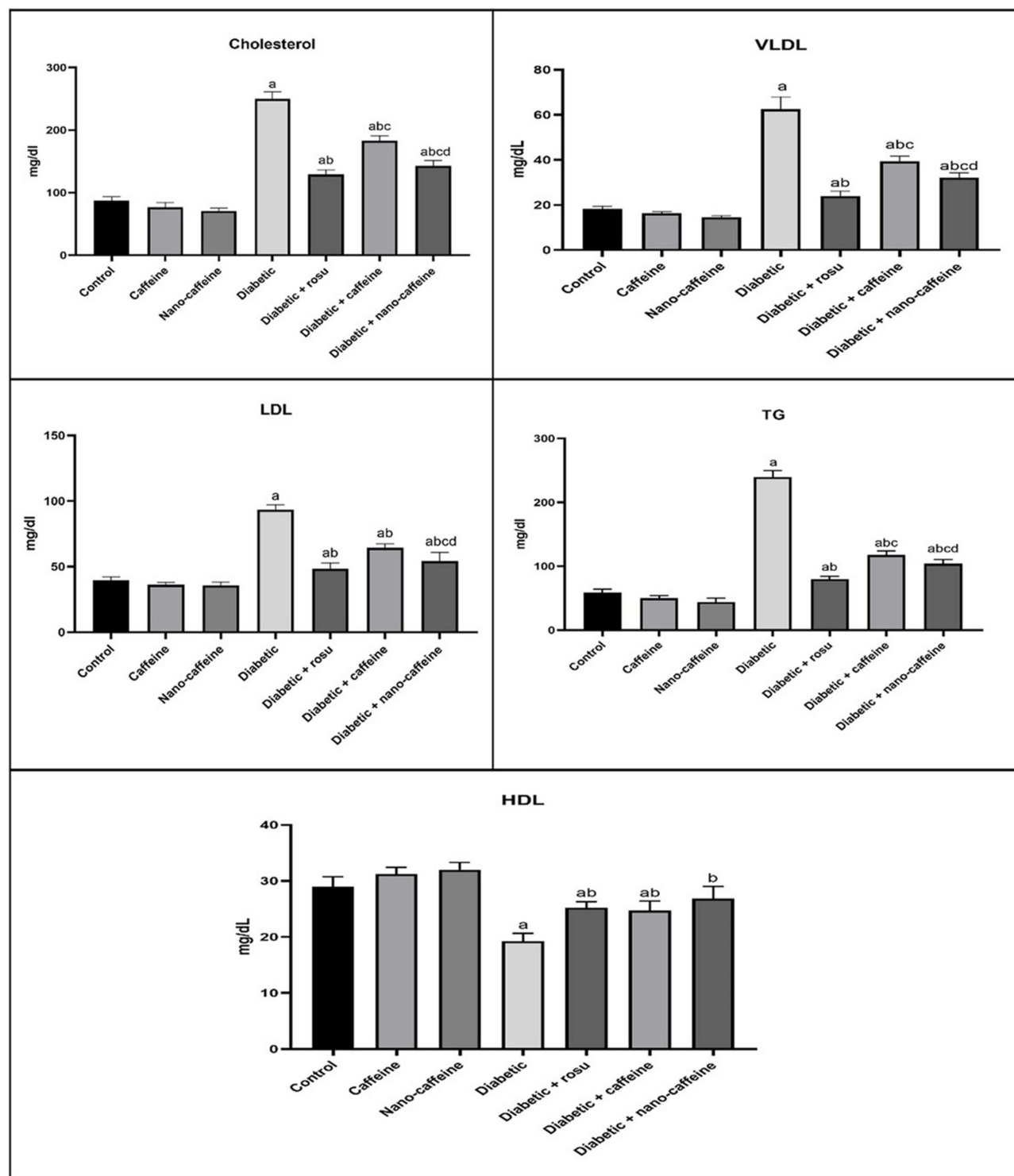


Figure 9 Effect of treatments on the serum lipid levels in each group: total cholesterol (TC) levels, very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), triglyceride (TG), and high-density lipoprotein (HDL). a = significantly different compared to the control group, b = significantly different compared to the diabetic group, c = significantly different compared to the Diabetic + Rosuvastatin group, and d significantly different compared to the Diabetic + caffeine. Results are presented as mean $\pm$ SEM (n = 12).

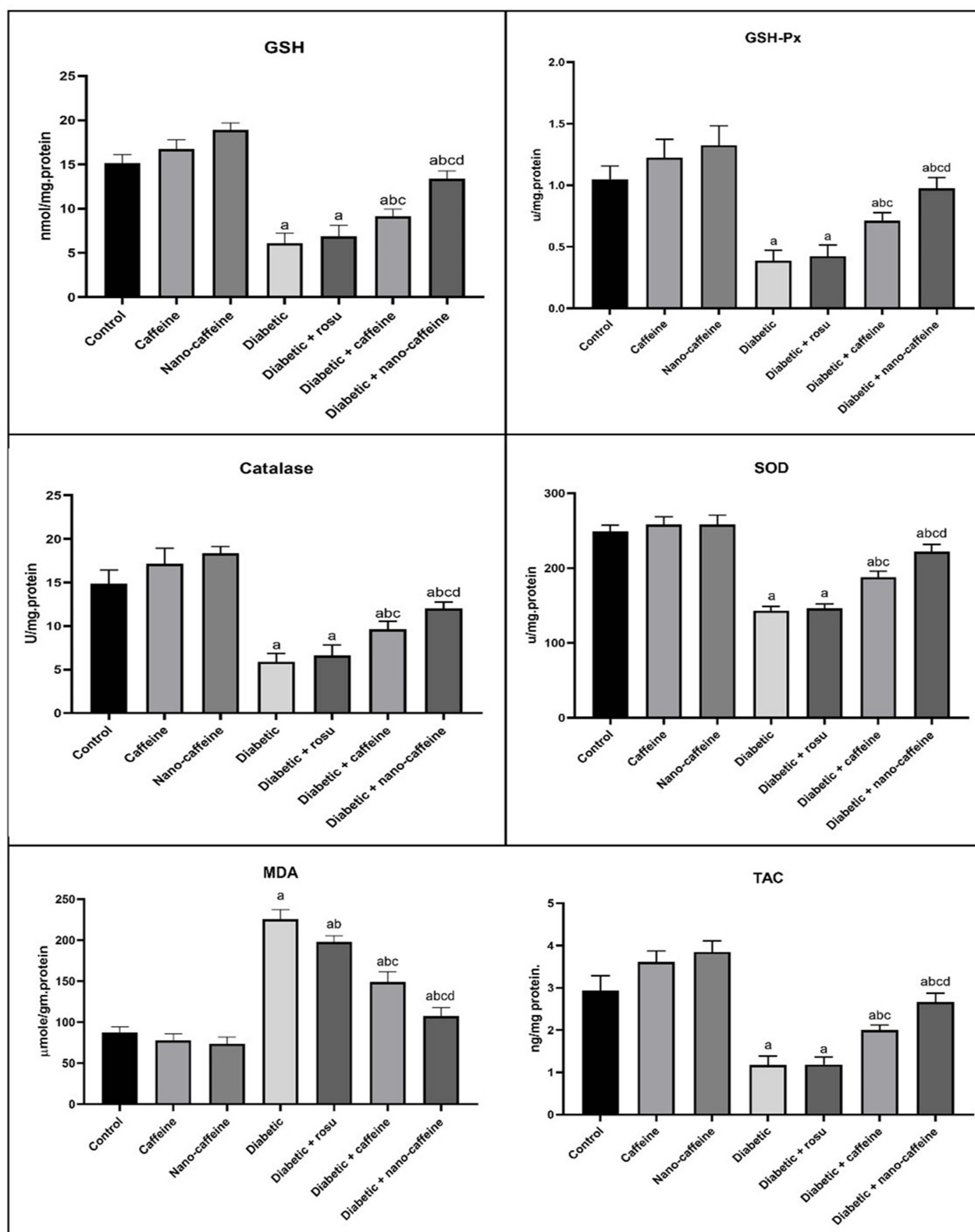


Figure 10 Effect of treatments on each group's antioxidant enzymes and lipid peroxidation products: glutathione (GSH), glutathione peroxidase (GSH-Px), Catalase, Superoxide dismutase, and malonaldehyde (MDA). a = significantly different compared to the control group, b = significantly different compared to the diabetic group, c = significantly different compared to the Diabetic + Rosuvastatin group, and d significantly different compared to the Diabetic + caffeine. Results are presented as mean $\pm$ SEM (n = 12).

MDA 1.57-fold compared to normal control rats. Moreover, the diabetic group exhibited a lower total antioxidant capacity TAC by 60% compared to the normal one. However, treatment with nano-caffeine significantly ($P < 0.05$) reduced the myocardial lipid peroxidation. The cardiac tissue MDA concentration in the nano-caffeine treated group was significantly ($P < 0.05$) lower than that observed in the diabetic group by 52%. At the same time, the rosuvastatin-treated group and the caffeine-treated group showed significantly ($P < 0.05$) higher MDA concentrations by 37% and 28%, respectively, compared to the nano-caffeine-treated group, as shown in [Figure 10](#).

Effect of Drug Treatments on Cardiac Enzymes

In our study, we found that the diabetic group had a significant ($P < 0.05$) elevation of cardiac enzymes creatine kinase (CK), lactate dehydrogenase (LDH), and cardiac troponin I (cTnI), compared to the normal control by 2, 2.8, and 2.2 folds, respectively. On the other hand, treatment with nano-caffeine significantly ($P < 0.05$) decreased these levels by 42%, 49%, and 48%, respectively, compared to the diabetic group and by 21%, 26%, and 14% relative to the caffeine-treated rats. Moreover, treatment with rosuvastatin could moderately decrease CK, LDH, and cTnI levels by 26%, 13%, and 30%, respectively, compared to the diabetic group, as shown in [Figure 11](#).

Effect of Drug Treatments on Inflammatory Mediators

As shown in [Figure 11](#), the diabetic group exhibited a significantly ($P < 0.05$) high level of either interleukin-1 beta (IL-1 β) and tumor necrosis factor-alpha (TNF- α) (4 and 2 folds, respectively) compared to the normal control group. However, treatment with nano-caffeine significantly ($P < 0.05$) downregulates both IL-1 β and TNF- α by 46% and 49%, respectively, compared to the untreated diabetic group. At the same time, nano-caffeine showed a significant decrease in these biomarkers by 21% and 23%, respectively, compared to the caffeine-treated group. The rosuvastatin-treated group showed a mild decrease in these inflammatory biomarkers by 4% and 10%, respectively, compared to the diabetic group, as is shown in [Figure 11](#).

Effect of Drug Treatments on ALT and AST Activities

As demonstrated in this study, diabetes significantly elevated ($P < 0.05$) both ALT and AST activities by 2 and 2.5 folds, respectively, in comparison with the normal control group. However, treatment with nano-caffeine significantly ($P < 0.05$) reduced both ALT and AST activities by 42% and 57% compared to the untreated diabetic group. At the same time, nano-caffeine showed a significant decrease in these activities by 24% and 34%, respectively, compared to the caffeine-treated. Furthermore, treatment with rosuvastatin could also decrease ALT and AST activities by 7% and 14%, respectively, compared to the diabetic group, as shown in [Figure 12](#).

Effect of Drug Treatments on NO Levels

As shown in [Figure 12](#), nitric oxide (NO) concentrations in diabetic rats were significantly higher ($P < 0.05$) than in the control group (69% increase). Treatment with either caffeine or nano-caffeine further amplifies this NO increase (22% and 12%, respectively). On the other hand, administration of rosuvastatin did not produce any significant change ($P < 0.05$) (6% increase) compared to the diabetic group.

Effect on Oral Glucose Tolerance Test (OGTT)

The OGTT profiles show distinct variations in how the experimental groups handle glucose. Compared to the control group, the diabetic group showed a marked hyperglycemic response after glucose loading, with noticeably higher glucose concentrations at every time point. Diabetic animals showed lower glucose disposal and poor glucose tolerance, with peak glucose levels occurring at 60 minutes and staying significantly higher throughout the test time.

Although the general pattern of reduced glucose tolerance persisted, caffeine administration to diabetic animals resulted in a moderate attenuation of hyperglycemia and somewhat lower glucose levels across most time points. There was only a minor improvement in glucose management; nevertheless, the drop was insufficient to approximate normoglycemic readings.

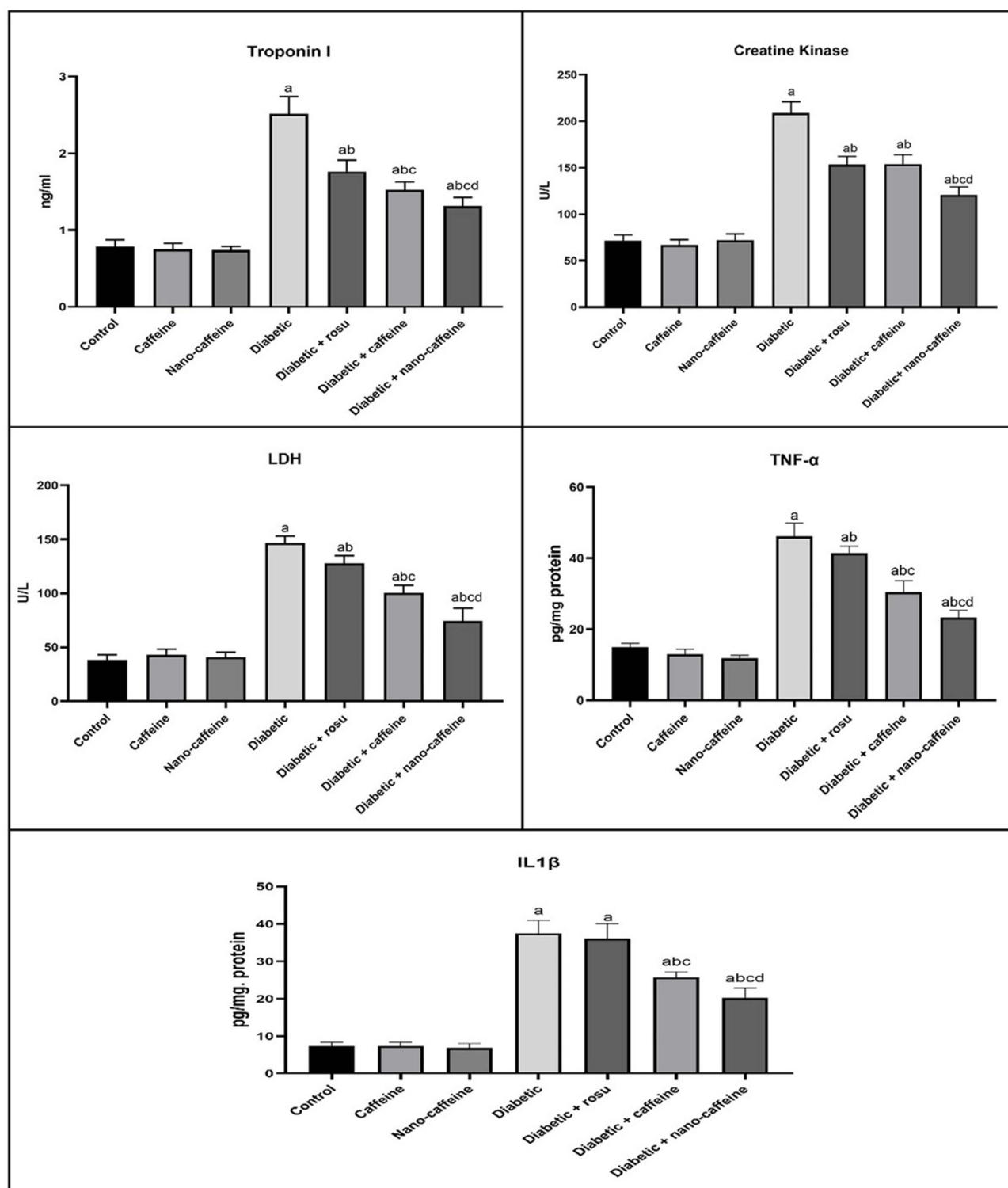


Figure 11 Effect of treatments on cardiac enzymes and inflammation biomarkers: Troponin I, creatine kinase, TNF- α , IL-1 β . a = significantly different compared to the control group, b = significantly different compared to the diabetic group, c = significantly different compared to the Diabetic + Rosuvastatin group, and d significantly different compared to the Diabetic + caffeine. Results are presented as mean $\pm$ SEM (n = 12).

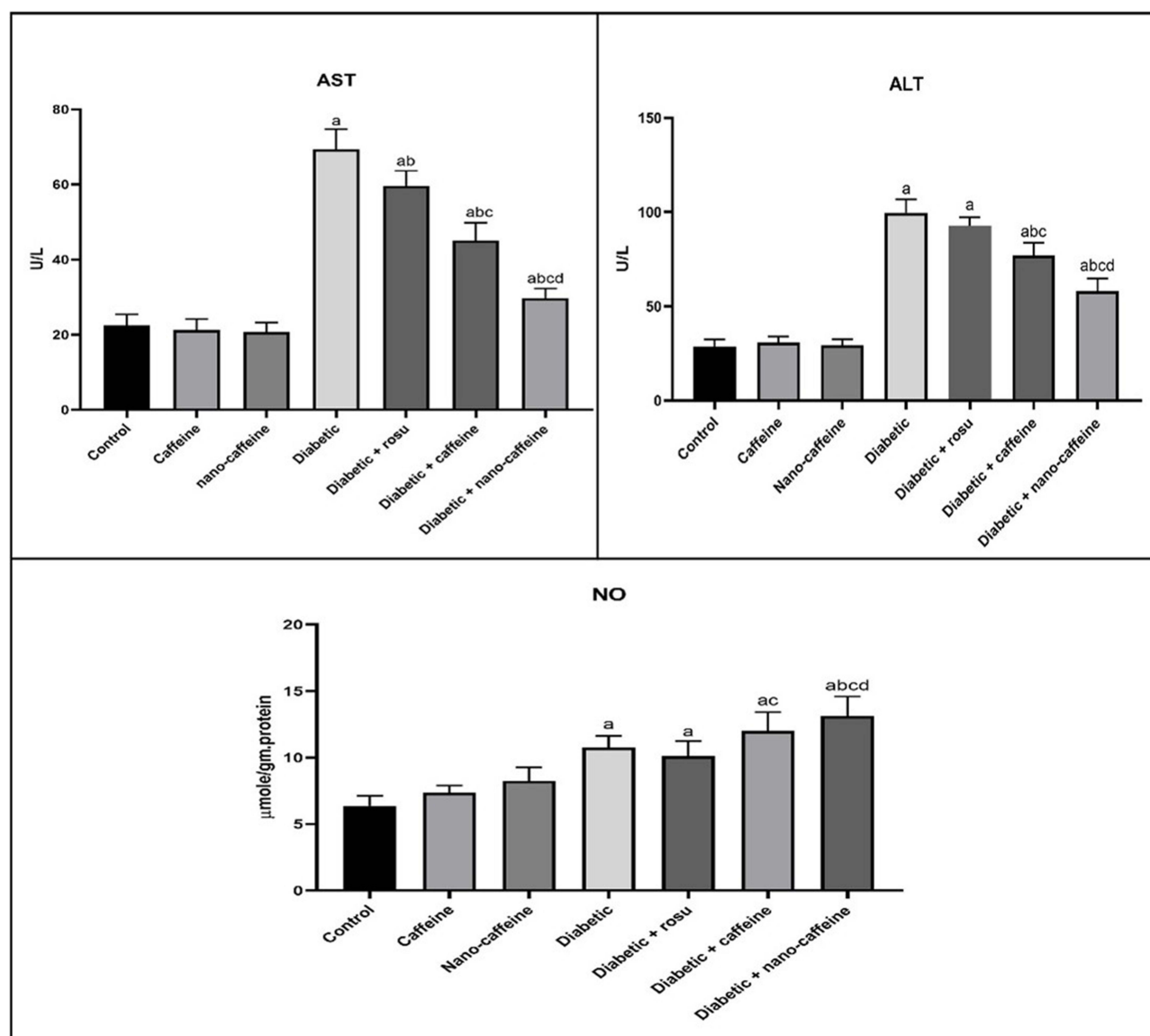


Figure 12 Effect of treatments on the activities of serum AST, ALT, and NO. a = significantly different compared to the control group, b = significantly different compared to the diabetic group, c = significantly different compared to the Diabetic + Rosuvastatin group, and d significantly different compared to Diabetic + caffeine. Results are presented as mean $\pm$ SEM (n = 12).

The diabetic group treated with nano-caffeine, on the other hand, had a more pronounced improvement. In comparison to the untreated diabetic and (diabetic + caffeine) groups, this group continuously showed lower glucose levels, especially at 60 and 90 minutes, indicating improved post-prandial glucose elimination and increased glucose tolerance, as shown in Figure 13.

Discussion

In recent decades, age-adjusted mortality, particularly from cardiovascular causes, has significantly decreased. On the other hand, mortality due to diabetes has paradoxically increased considerably, whereas scientists have found that cardiovascular diseases are the primary cause of death among diabetic patients.^{46,47} Consequently, individuals with diabetes have not benefited from the positive reduction in cardiovascular mortality observed in those without diabetes.⁴⁸ Therefore, DCM treatment has emerged as a focal point of inquiry for researchers. Pharmacological treatment is a crucial intervention alongside lifestyle change for treating DCM. The etiology of DCM encompasses metabolic disorder,

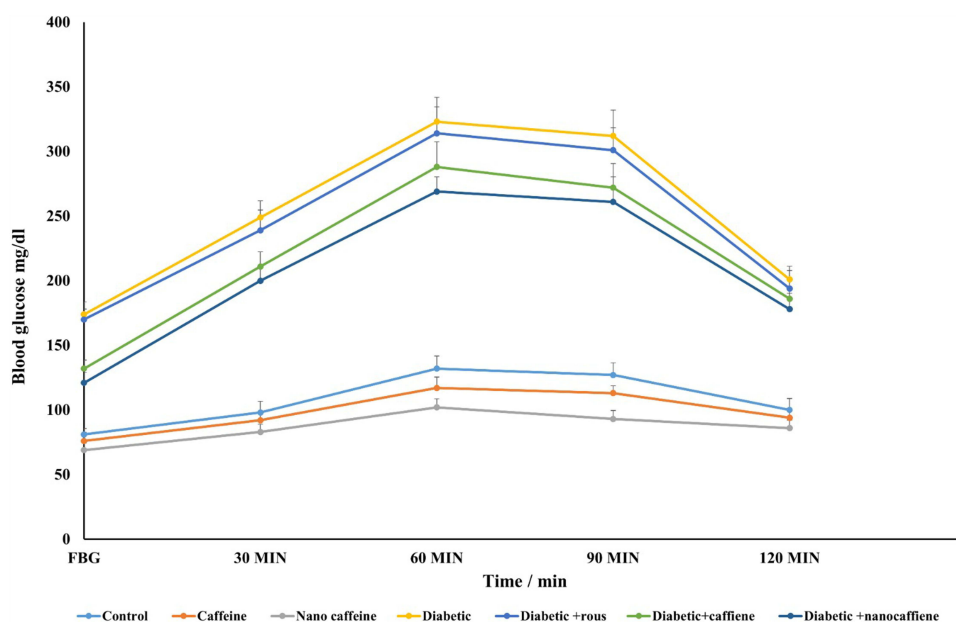


Figure 13 Effect of oral administration of treatments on rats' blood glucose concentrations at 0, 30, 60, 90, and 120 min of OGTT.

changes in subcellular components, oxidative stress, apoptosis and autophagy, and inflammatory response. Currently, available therapies for DCM do not offer curative treatment, and some of them have intolerable side effects in certain patients,^{49,50} therefore, there is a need to develop new therapies that can introduce higher efficacy with lower adverse events. Regarding our investigations in this field to find new compounds that could attenuate the development of cardiomyopathy in diabetics, we found that capsaicin could significantly attenuate the progression of this process via different mechanisms, including antioxidant and anti-inflammatory pathways. Hence, in this study we examined other naturally occurring molecule (caffeine) to protect the cardiac muscle against diabetic cardiomyopathy and examined a newly developed nano formula to improve its pharmacodynamic effects and overcome some of its pharmacokinetic hindrances.

Extensive studies made by researchers have found that caffeine possesses antioxidant, anti-inflammatory, and anti-apoptotic properties.^{51,52} However, caffeine undergoes rapid distribution and elimination in the body, with an elimination half-life of approximately 3–5 hours. Due to this short elimination half-life, frequent administration is required to maintain therapeutic levels, which can negatively impact patient compliance. Therefore, the development of a controlled sustained-release formulation of caffeine is highly desirable. Such a formulation would reduce dosing frequency, ensure consistent therapeutic drug levels, and enhance overall patient adherence to treatment.

Pickering emulsion-based systems have been investigated as a promising approach to improve drug bioavailability and enable controlled release. In these emulsions, droplets are encapsulated by a protective shell formed by solid stabilizing particles, which act as micro- or nano-capsules. This structure facilitates effective drug entrapment and provides a means to modulate the release profile of the active ingredient.

CaCO₃ nanoparticles were synthesized in this study and used to stabilize the investigated Pickering emulsion. CaCO₃ NPs offer several biomedical and economic advantages, as they can be prepared from readily available and cost-effective precursors. Additionally, they are considered non-toxic, which makes them preferable over various metallic agents.²¹

The effectiveness of the synthesized CaCO₃ nanoparticles in producing emulsions with consistently small droplets is reflected in the overall droplet size of the emulsion. This improvement can be explained by the fact that smaller nanoparticles are better able to pack tightly and create a more uniform interfacial barrier. This barrier helps prevent droplets from merging, thereby enhancing emulsion stability and resulting in smaller, more uniform droplets.

The mobile phase conditions for HPLC separation were carefully optimized to achieve sharp, symmetrical, and well-resolved peaks. Through systematic evaluation of varying compositions and ratios, the best performance was attained

using a mixture of 3% acetonitrile and 97% phosphate buffer (pH 3) containing 0.15% SDS, with the column maintained at 30°C. The method demonstrated high recovery (>99%) and precision, with both intraday and interday relative standard deviations (RSD < 2%), meeting stringent validation criteria. Accuracy was further confirmed, with measured concentrations deviating by less than $\pm 3\%$ from the target values.

The caffeine content in both Pickering emulsion and cardiac tissue samples requires analysis using a rapid and straightforward analytical method. In this study, a new and validated HPLC method was developed for the determination of caffeine in these sample types. The method eliminates the need for extensive pretreatment of biological samples by employing a direct injection approach using a micellar liquid chromatographic technique. Following optimization of key experimental parameters, the method yielded well-resolved peaks, a short analysis time, and minimal interference from proteins. Method validation was performed in accordance with ICH guidelines, demonstrating excellent accuracy, precision, and linearity across the tested concentration range. The results demonstrated that the diabetic nano-caffeine group had higher caffeine concentrations compared to the normal caffeine group, suggesting that the Pickering emulsion formulation enhances caffeine delivery more effectively than conventional caffeine administration.

In the present study, T2DM was developed in rats by i.p. injection of streptozotocin in combination with a high-fat diet for 3 months, which resulted in a noticeable myocardial injury evidenced by histopathological examination, in addition to a dramatic upregulation in oxidative stress parameters and depletion of the antioxidant defensive mechanisms. Moreover, a significant upregulation in the myocardial content of the inflammatory biomarkers accompanied by significant hyperlipidemia and hyperglycemia was determined. On the other hand, the caffeine-treated group showed a moderate amelioration in the development of myocardial injury with noticeable antioxidant and anti-inflammatory effects. Interestingly, our newly developed formula could dramatically inhibit the development of myocardial injury, in addition to antioxidant and anti-inflammatory effects, which were significantly higher than those observed in both the caffeine- and rosuvastatin-treated groups.

Desmin is an intermediate filament protein crucial for the structural integrity and functionality of the cardiomyocytes.⁵³ It maintains the integrity of the myocardium and facilitates inter- and intracellular connections, and its disruption is commonly found in the different forms of myocardial injury.⁵⁴ The current investigation has found that oral treatment with nano-caffeine could noticeably maintain the normal desmin protein distribution in the myocardium of diabetic rats, which was more efficient than that observed in both caffeine- and rosuvastatin-treated groups. Conversely, the untreated diabetic group exhibited a significant disruption of desmin filaments in the myocardial muscle.

Transforming growth factor β (TGF- β) is a crucial cytokine/growth factor that activates fibroblasts and accelerates extracellular matrix (ECM) formation in different tissues, including cardiac muscle.^{55,56} Moreover, scientists have found that TGF- β plays a pivotal role in α -SMA tissue upregulation.⁵⁷ In normal myocardium, many fibroblast-like cells exist in a dormant state under normal conditions, but in the presence of injurious activities, these cells are transformed into activated α -SMA-positive myofibroblasts.⁵⁸ Myofibroblasts are essential for secreting extracellular matrix proteins, so they protect the myocardial muscle against rupture.^{58,59} The overproduction of activated fibroblasts may lead to myocardial fibrosis, resulting in increased stiffness and subsequent systolic and diastolic dysfunction.⁶⁰

In our study, we noticed a significant increase in the α -SMA distribution in the myocardium of the diabetic group relative to the control one, and it had a modest distribution among caffeine- and rosuvastatin-treated animals, as demonstrated by the immunostaining technique. However, the nano-caffeine-treated group exhibited a minor increase of α -SMA relative to the control group. The observed increase may be ascribed to the inhibitory effect of caffeine on TGF- β production, which was in agreement with former studies in this regard.⁶¹

In case of advanced myocardial damage, such as DCM, cardiac remodeling is frequently initiated by alterations in the extracellular matrix, fibrosis, and ultimately apoptosis; these alterations are frequently induced by oxidative stress.⁶² In DCM, excessive fatty acid oxidation is the primary source of ROS generation, which can interfere with several intracellular signal transduction pathways, including those associated with nuclear factor-kappa B (NF- κ B), activator protein-1 (AP-1), and mitogen-activated protein kinases, resulting in cardiac myopathy. Moreover, ROS upregulation has been demonstrated to induce the cleavage of sarcomeric proteins, including troponin I, resulting in contractile failure and myocyte death.⁶³

Various investigations have confirmed the potent antioxidant properties of caffeine, indicating that this molecule can effectively alleviate intracellular oxidative stress.⁶⁴ Caffeine has been shown to inhibit oxidative damage by regulating the expression of heat shock protein 1, enhancing the mRNA levels of SOD and hydroperoxide glutathione peroxidase, thereby reducing MDA upregulation and ameliorating the decline in GSH-Px, GSH, and SOD concentrations.^{65,66} In the present study, the diabetic group fed with a high-fat diet had a significant decrease in SOD activity, corroborating prior research findings;^{67,68} hence, SOD serves as the primary defense against reactive oxygen species (ROS)-induced cellular damage. It facilitates the transformation of superoxide anion free radicals into molecular oxygen and hydrogen peroxide, reducing oxygen free radical concentrations that can produce cellular injury.⁶⁹ Moreover, GPx, an intracellular antioxidant enzyme, protects cellular components from oxidative stress by reducing ROS or hydroperoxides into water or corresponding alcohols via GSH oxidation.⁷⁰

In this study, we found that the oral administration of caffeine could moderately restore SOD, GPx, and GSH myocardial concentrations, while the nano-caffeine treated group could nearly normalize their levels. Concurrently, rosuvastatin exhibited no significant impact on their tissue levels.

Coffee extracts, including caffeine, have been shown to reduce the activity of key enzymes responsible for lipogenesis: acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and/or stearoyl-CoA desaturase (SCD).^{71,72} ACC and FAS are responsible for the first two steps of de novo lipogenesis, while SCD is responsible for the synthesis of monounsaturated fatty acids for fat storage.⁷³ The enzymatic inhibitory effects of coffee and/or its bioactive compounds were in part via regulation of upstream transcription factors for lipogenesis: CCAAT/enhancer-binding proteins (C/EBP), peroxisome proliferator-activated receptors (PPAR, especially PPAR γ), and/or sterol regulatory element-binding proteins (SREBP).^{74–76} These transcription factors are well-known to regulate adipogenesis, including lipogenesis, resulting in reduced blood lipids (triglycerides, LDL, and VLDL).^{71,77} On the other hand, Shuwei Hu et al have found that caffeine administration can boost histone acetylation and the expression of genes involved in cholesterol synthesis by inhibiting the A2AR/cAMP/PKA pathway and down-regulating sirtuin1, resulting in increased hepatic cholesterol synthesis and hypercholesterolemia in male offspring rats.⁷⁸ In the present study, caffeine administration produced a reduction in blood cholesterol levels in T2DM rats, and the caffeine hypolipidemic effect was significantly improved by using our newly developed nano-formula.

Caffeine, one of the most commonly ingested psychoactive compounds, has demonstrated controversial results regarding its effects on the blood glucose levels, revealing both advantageous and detrimental effects. Keijzers et al have found that acute caffeine consumption has been linked to temporary insulin resistance and increased blood glucose levels, likely due to its antagonistic actions on adenosine receptors, which may disrupt insulin signaling.⁷⁹ In contrast, van Dam et al have found that prolonged coffee intake has been associated with enhanced insulin sensitivity and a diminished incidence of type 2 diabetes in certain epidemiological studies.⁸⁰ Nonetheless, in the present study, we did not find a significant effect of caffeine or nano-caffeine administration on diabetic rats' blood glucose levels compared to the non-treated diabetic group, which was in agreement with Luiz Augusto da Silva et al study.⁸¹

Regarding the cardiovascular effect of NO, researchers have demonstrated that NO has a vasodilating effect, especially on coronary arteries, via activating soluble guanylate cyclase (sGC), which increases cyclic guanosine monophosphate (cGMP) levels and coronary blood flow and myocardial oxygen supply.^{82,83} NO also reduces cardiomyocyte apoptosis and inhibits inflammatory responses during ischemia-reperfusion.⁸⁴ On the other hand, different studies have found that caffeine's effects on NO are context-dependent, influenced by dose, duration of intake, and individual vascular health.^{85,86} While acute doses may impair NO-mediated vasodilation,⁸⁶ chronic consumption often improves endothelial function and upregulates the cardiovascular concentrations of NO.⁸⁷ In the present study, we found that oral caffeine administration could moderately increase the cardiac muscle content of iNOS and NO, and this effect was significantly enhanced by using our newly developed nano-caffeine formula.

An interesting study involving 114 people showed that caffeine consumption correlates with reduced inflammation and inflammasome activation, leading to diminished production of the pro-inflammatory cytokine interleukin-1 beta (IL-1b),⁸⁸ while Li et al reported that caffeine-infused rats had lower levels of TNF- α than the placebo in a myocardial ischemia/reperfusion injury model.⁸⁹ In our study, we found a significant ameliorative effect produced by our nano-

caffeine formula on the inflammatory mediators IL-1 β and TNF- α compared to the non-treated group, and this effect was significantly higher than that observed in the caffeine-treated group.

Cardiac enzymes ALT, AST, LDH, and troponins play a crucial role in determining the extent of the myocardial injury, particularly in diagnosing myocardial infarction and myocardial damage,^{90,91} hence, troponin I is considered the most sensitive and specific biomarker for cardiac injury, as it is released into the bloodstream following myocardial necrosis and remains detectable for several days.⁹¹ In the present study, we used the blood serum levels of ALT, AST, LDH, and troponin I, in addition to the myocardial histopathological examination, as an indicator of the extent of the myocardial damage; hence, we found a significant upregulation in the serum level of these enzymes in the diabetic rats in addition to myocardial tissue inflammatory changes and necrosis. On the other hand, a significant reduction in the level of these enzymes was found in the caffeine-treated animals compared to the non-treated diabetic group. Also, we found that the nano-caffeine-treated group showed the lowest determined levels of these enzymes compared to other treated groups, indicating the significant improvement in the caffeine's myocardial protective properties when processed by our newly developed nano-formula.

It is important to note that this study assessed caffeine accumulation in cardiac tissue at a single steady-state time point following chronic administration. A detailed pharmacokinetic analysis involving serial sampling (eg., AUC, $t_{1/2}$, and Cmax determination) was not performed and remains a subject for future investigations to fully characterize the absorption and elimination kinetics of this nano-formulation.

Conclusion

In conclusion, the present study has found that caffeine administration produced significant protective activity against T2DM-induced myocardial injury (via antioxidant, anti-inflammatory, and hypolipidemic effects), and this effect was significantly enhanced by using our newly developed nano-formula that increased its cardiac tissue concentrations, which was determined using a new validated HPLC technique. These findings suggest that the nano-formulated caffeine could be a promising therapeutic agent for mitigating cardiovascular complications associated with type 2 diabetes mellitus. Further research is warranted to explore the long-term effects and potential clinical applications of this innovative approach.

Data Sharing Statement

Data generated and/or analyzed during this study are available from Dr. Adel M. Ahmed upon reasonable request.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflicts of interest.

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