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Mitigating Oxidative Stress in Streptozotoc in-Induced Diabetic Rats on a High-Fat Diet: Therapeutic Potential of Hesperet in-Loaded Chitosan Nanoparticles

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Abstract: This study investigated the therapeutic potential of Hesperetin-loaded chitosan nanoparticles (HSPCNP) in mitigating oxidative stress in streptozotocin (STZ)-induced diabetic rats fed a high-fat diet (HFD).Groups I and II received the standard pellet diet. Group III, IV, and V rats were initially fed an HFD for 4 weeks, followed by intraperitoneal injection of STZ (35 mg/kg b.w) to induce diabetes. After inducing diabetes, Group IV rats were administered a daily oral dose of 40 mg/kgb.w. HSPCNP for 4 weeks. Biochemical analysis of liver homogenates and plasma revealed significant decreases ($p < 0.05$) in the activities of SOD, CAT, GPx, GST, GR, vitamin E, vitamin C, and GSH in diabetic rats. Conversely, markers of lipid peroxidation, such as TBARS and LHP, were significantly increased ($p < 0.05$). Treatment with HSPCNP effectively reversed these alterations, significantly improving antioxidant status and reducing oxidative stress in diabetic rats, comparable to the standard drug metformin. These findings demonstrate that HSPCNP can alleviate oxidative stress in patients with diabetes and is a promising therapeutic approach for diabetes treatment.

Keywords: Hesperetin-loaded chitosan nanoparticles, Oxidative stress, Antioxidant, Lipid peroxidation, Type 2 diabetes mellitus

Introduction

Type 2 diabetes mellitus (T2DM) is a pervasive metabolic disorder characterized by chronic hyperglycemia resulting from impaired insulin secretion, insulin resistance, or both. The global prevalence of diabetes has reached alarming levels, which are primarily driven by factors such as obesity, sedentary lifestyle, and HFD.(Miguel A., et al., 2020) Among the numerous complications of diabetes, oxidative stress plays a critical role, contributing to its pathogenesis and progression. (Fatmah A., et al., 2012)Oxidative stress is defined as an imbalance between reactive oxygen species (ROS) production and antioxidant defenses, leading to cellular damage and exacerbated diabetic complications, particularly in

vital organs such as the liver, kidney, and pancreas. (Maria Angela, et al., 2018) (Helmut, et al., 2022)

Emerging therapeutic strategies for mitigating oxidative stress and its harmful consequences in patients with diabetes include enhancing endogenous defense mechanisms through the use of natural antioxidants. (Jasvinder Singh, et al., 2022)Hesperetin, a bioactive flavonoid found in citrus fruits, has garnered attention for its potent anti-inflammatoryandantioxidant qualities. However, the clinical application of hesperetin is limited because of its poor water solubility and bioavailability. (Bahare, et al., 2022)

To overcome these limitations, recent advancements in nanotechnology have facilitated the development of HSPCNP. Chitosan, a biocompatible and biodegradable polymer, enhances the stability and bioavailability of encapsulated compounds, making it an ideal carrier for drug delivery. HSPCNP can improve the therapeutic efficacy of hesperetin by facilitating targeted delivery and sustained release. (Lizha Mary, et al., 2018) (Shailendra, et al.,2022) (Anand B., et al., 2018)

This study investigated the therapeutic potential of HSPCNP in mitigating oxidative stress in STZ-induced diabetic rats fed a high-fat diet. By evaluating oxidative stress markers in the liver, kidney, and pancreas, this study elucidated the efficacy of HSPCNP against diabetes-induced oxidative damage. This study's findings could provide a foundation for developing novel antioxidant-based therapies for managing T2DM and its associated complications.

Materials and Methods

Chemicals

Streptozotocin (STZ), hesperetin, and chitosan nanoparticles were purchased from Sigma-Aldrich (St. Louis, USA). All other chemicals were sourced from Hi-Media Laboratories Private Limited, Mumbai, India.

Ethical Considerations

We purchased 42 healthy adult male Wistar rats from Mass Biotech (CPCSEA Reg. No: 2084/ PO /Bt/S/19/CPCSEA), Kanthalur, Tamil Nadu, when they were 8 weeks old and weighed between 180-200 g. The rats were kept in a temperature-controlled room (25 10°C) with a 12-h light and 12-h dark cycle. They were given a week to acclimate to the laboratory environment before the experiments began. The experimental procedure was approved by the Ethics Committee of the University of Annamalai (AU-IAEC /1320/6/22) and was conducted according to the institutional guidelines.

Design experimentation

The study involved five groups of animals, each consisting of six individuals. The experimental treatment was administered orally via gavage for 56 days.

Group I: The experimental rats were the control group and were given a regular pellet diet for 56 days.

Group II: The experimental group received oral HSPCNP at a dose of40 mg/kg b.w. for 28 days.

Group III: For 56 days, diabetic rats were given an HFD.

Group IV: HSPCNP (40 mg/kg b.w.) was administered orally to experimental diabetic rats for 28 days.

Group V: Rats with diabetes treated with oral metformin 100 mg/kg b.w. for 28 days.

Biochemical estimation

The concentrations of biochemical parameters were examined in plasma and tissue samples using precise and sensitive colorimetric techniques. To measure superoxide dismutase activity in tissues, we usedKakkar et al.,'s method.(1984) SOD activity units are the enzyme amounts required to inhibit the reduction of NBT by 50%.Catalase activity was measured using Sinha's method (1972). The color produced during the enzyme's H_2O_2 usage was read at 620 nm. The method developed by Rotruck et al.,(1973) was used tomeasure GPx levelsin tissues. Using the technique outlined by Habig et al. (1974), the GST activity was calculated.The Ellman method (1959) was used to measurereduced glutathione (GSH) levels in the samples. The method involves reacting the sample with 5,5-dithiobis-2-nitrobenzoic acid to produce a yellow derivative, which is measured at 412 nm.The activity of GR was assayed by the method described by Carlberg I and Mannerviek B. et al., (1975).In addition, we measured plasma Vitamin C levels using the RoeKuether method (1943) and recorded absorbance at 540 nm.Determination of vitamin E in plasma and tissues: Vitamin E was assayed in plasma and tissues as described by Baker et al., (1981).

Moreover, the concentrations of TBARS and LHP in both plasma and tissue were measured using the methods described by Niehaus and Samuelsson(1968), and Jiang ZY and colleagues, respectively.(1992)

HSPCNP synthesis and characterization

In our recent study, we extensively discussed the synthesis and characterization of HSPCNP(Sivamathi Rathna Priya et al., 2024). In summary, the nanoparticles were synthesized using the ionic gelation technique, as described by Calvo et al., (1997). The characterization of the nanoparticles was conducted using Fourier-Transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM). The nanoparticles exhibited an average diameter of 145.0 nm and a zeta potential of 35.5

mV. SEM analysis demonstrated that HSPCNP possessed a spherical morphology with a smooth surface.

Histopathology

Kidney and liver tissue samples were harvested from the killed animals and fixed in a 10% neutral buffered formalin solution (NBF). The samples underwent standard paraffin-embedded, after which 5-mm-thick sections were prepared and stained with hematoxylin and eosin dye (H&E) at a magnification of 40x.

Analyzing statistics

Mean values with standard deviations for control and experimental animals were used to express quantitative measurements. Statistical analysis was performed using the Social Science Statistical Package (SPSS) version 20.0 from Chicago, IL, USA. A one-way analysis of variance (ANOVA) was conducted, followed by Duncan's multiple range test (DMRT). Statistical significance was determined when the p-value was less than 0.05.

Results and Discussion

Effects of HSPCNP on the antioxidant status of diabetic rats

Figures 1, 2, and 3 show the amounts of nonenzymatic antioxidants and the activity of enzymatic antioxidants in the liver, kidney, and pancreatic tissues of normal and diabetic rats. Diabetic rats showed significant ($p < 0.05$) reductions in the activity of SOD, CAT, GR, GPx, and GST, as well as in the levels of GSH, E, and C vitamins.

Oxidative stress plays a significant role in the development and complications of diabetes mellitus. (Caroline Pereira et al.,2016) This occurs because of the overproduction of free radicals that exceeds the body's antioxidant defenses, resulting in cell damage.(Giovanni et al.,2022)In patients with diabetes mellitus, high blood sugar levels lead to the production of free radicals that exhaust antioxidant defenses, disruptcellular functions and damage membranes and tissues. Strong evidence supports the link between hyperglycemia-induced oxidative stress and various health complications. (Svetlana et al.,2022)

Consuming a HFD increases the generation of ROS in the mitochondria, fatty acid oxidation, mitochondrial overload and malfunction, and poor electron transport. (Yue et al.,2020) Oxidative stress is particularly detrimental to diabetes and exacerbates hyperglycemia. (Patricia et al.,2023) HSPCNPs are used in diabetes treatment because they enhance antioxidant defenses, reduce oxidative stress, and improve the activity of endogenous antioxidant enzymes, thereby protecting pancreatic beta cells and improving insulin sensitivity.

SODs catalyze the dismutation of the superoxide radical (O_2^-) into oxygen (O_2) and hydrogen peroxide (H_2O_2) (Mishra P., & Sharma P. 2019). Catalase is an essential antioxidant enzyme. Catalase primarily functions in peroxisomes to detoxify hydrogen peroxide, preventing its conversion into reactive free radicals.(Ankita et al.,2019)The results indicated that the presence of enhanced lipid peroxidation was related to decreased activities of SOD and CAT in the hepatic, kidney, and pancreatic tissues of diabetic rats. Accordingly, activity increases susceptibility to oxidative damage in these cells, impairing their function and accelerating beta-cell dysfunction.

GPx is an essential enzyme in cellular defense against oxidative stress. It catalyzes the reduction of H_2O_2 to H_2O and O_2, using GSH as a substrate. This reaction is critical for detoxifying peroxides and protecting cells against oxidative damage.(Maria do et al.,2022) GR catalyzes the reduction of oxidized glutathione (GSSG) to its reduced form (GSH) using NADPH as a reducing agent. This reaction is crucial for maintaining the cellular pool of GSH, which is necessary for continuous antioxidant protection. (Jolán et al.,2016) GST plays a crucial role in cellular detoxification by catalyzing the conjugation of GSH to various endogenous and exogenous electrophilic compounds, facilitating their excretion from the body.(Ross and Thomas W. Moon.2012) Previous studies have confirmed that GPx, GR, and GST activity levels are lower in patients with diabetes. Treatment with40 mg/kg b.w. of HSPCNP increased the activity of these enzymatic antioxidants.

Figure 6 shows the levels of nonenzymatic antioxidants in the plasma of rats with normal and diabetic conditions.The levels of vitamins E and C and GSH were lower in the diabetic rats compared to the control rats.However, the administration of HSPCNP to diabetic rats significantly increased these nonenzymatic antioxidant levels, bringing them closer to normal values.

By directly scavenging ROS and serving as a substrate for GPx to lower peroxide production, GSH performs as a significant antioxidant.(Sofia K and Asterios S2023) Additionally, GSH is involved in detoxification and maintainingthe cellular redox state. Reduced GSH levels and increased oxidative stress contribute to the development of microvascular complications, such as retinopathy, nephropathy, and neuropathy. (Okezie I. et al.,2007)

A systematic review and meta-analysis showed that vitamin C and E supplementationimproves glycemic control and cardiovascular risk. Vitamin E is a lipophilic component of the antioxidant defense system; it protects cell membranes against oxidative damage.The reduced vitamin E activity in diabetic rats supports the hypothesis that excess plasma vitamin E plays a protective role against increased peroxidation in diabetes. (Pavithra, D. et al.,2018)

One significant physiological antioxidant is vitamin C. Research is ongoing to determine whether vitamin C can help prevent or delay the development of certain cancers, cardiovascular illnesses, and other diseases where oxidative stress is a contributing factor. This is because vitamin C limits the harmful effects of free radicals through its antioxidant action. (John C. and Mario Siervo, 2016) In diabetic rats, there was a noticeable drop in vitamin C concentration. This fall in nonenzymatic antioxidants could be because of their reduced synthesis or increased consumption for scavenging ROS. (G. Pushpanjali and colleagues, 2019)

Antioxidants capable of maintaining GSH levels enhance cellular defense mechanisms, inhibit lipid peroxidation, and protect tissues from oxidative damage.In the present study, HSPCNP treatment at the dosage of 40 mg/kg b.w. caused elevation in GSH and vitamins E and C levels and enhanced the activity of antioxidant enzymes, which improved Oxidative Stress in diabetic rats.These results are consistent with previous research demonstrating thatAlshehri, A. S., et al.,(2021), a flavonoid, reduced oxidative stress in STZ-induced diabetic rats.

HSPCNP as a Lipid Peroxidation Indicator in Tissues and Plasma

Lipid peroxidation is a process of oxidative degradation of lipids, mainly unsaturated fatty acids, by ROS.This process leads to cell damage and is a significant contributor to the pathogenesis of diabetes and its complications. TBARS and LHP are lipid peroxidation byproducts widely employed as oxidative stress indicators.(Samukelisiwe C. et al., 2022)

In this study, increased levels of TBARS and LHP were observed in the tissues of HFD/STZ-induced diabetic rats, indicating elevated oxidative stress. However, treatment with The HSPCNP at the dosage of 40 mg/kg body weight showed a significant decrease in TBARS and LHP levels, demonstrating reduced lipid peroxidation.This reduction highlights the potential of HSPCNP to attenuate oxidative damage to lipids, a common issue associated with elevated oxidative stress in diabetes.

Compared with normal controls, both lipid peroxidative markers, LHP and TBARS, showed increased levels in the tissues and plasma of diabetic rats.However, treatment with HSPCNP and metformin significantly decreased these markers in diabetic rats, as evidenced by reduced lipid peroxidation in plasma and tissues (Figures 4 and 5). These results are consistent with previous research demonstrating thatTowseef, et al., (2019) can scavenge free radicals.

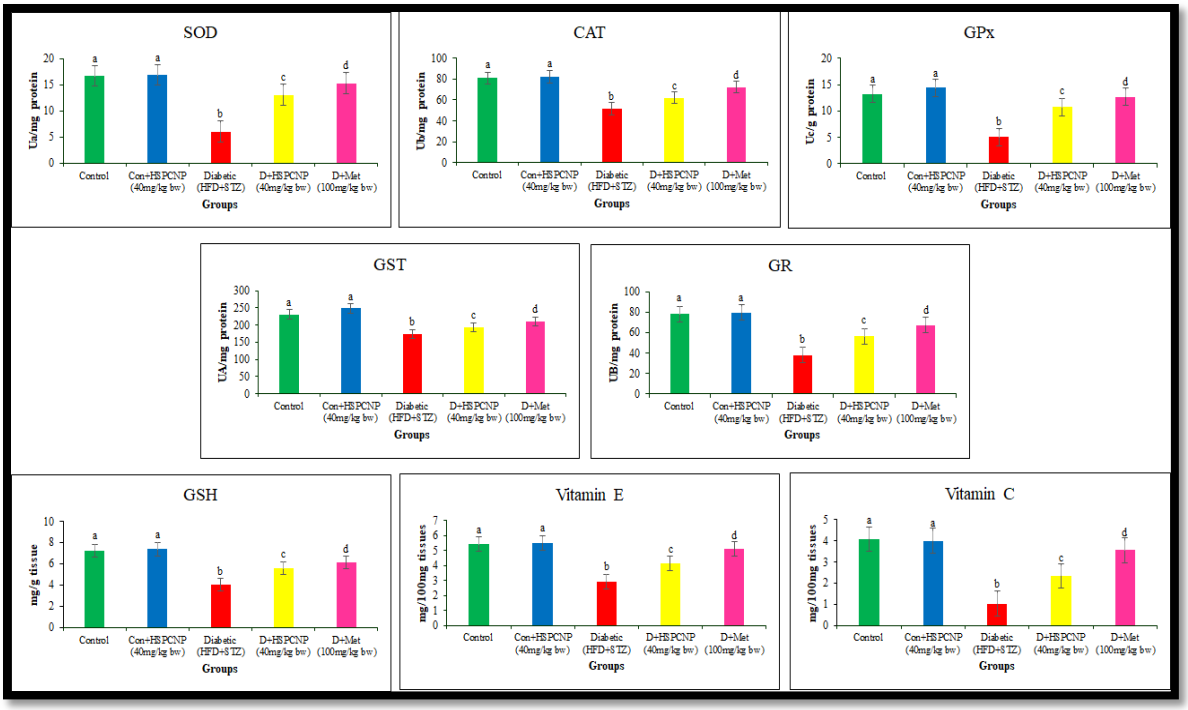

Figure 1. Impact of HSPCNP on the hepatic antioxidant changes in experimental and control rats.

The values, presented as mean ± SD for six rats, are considered to be significantly different ($p < 0.05$, DMRT) if they do not share the same superscript (a-d).

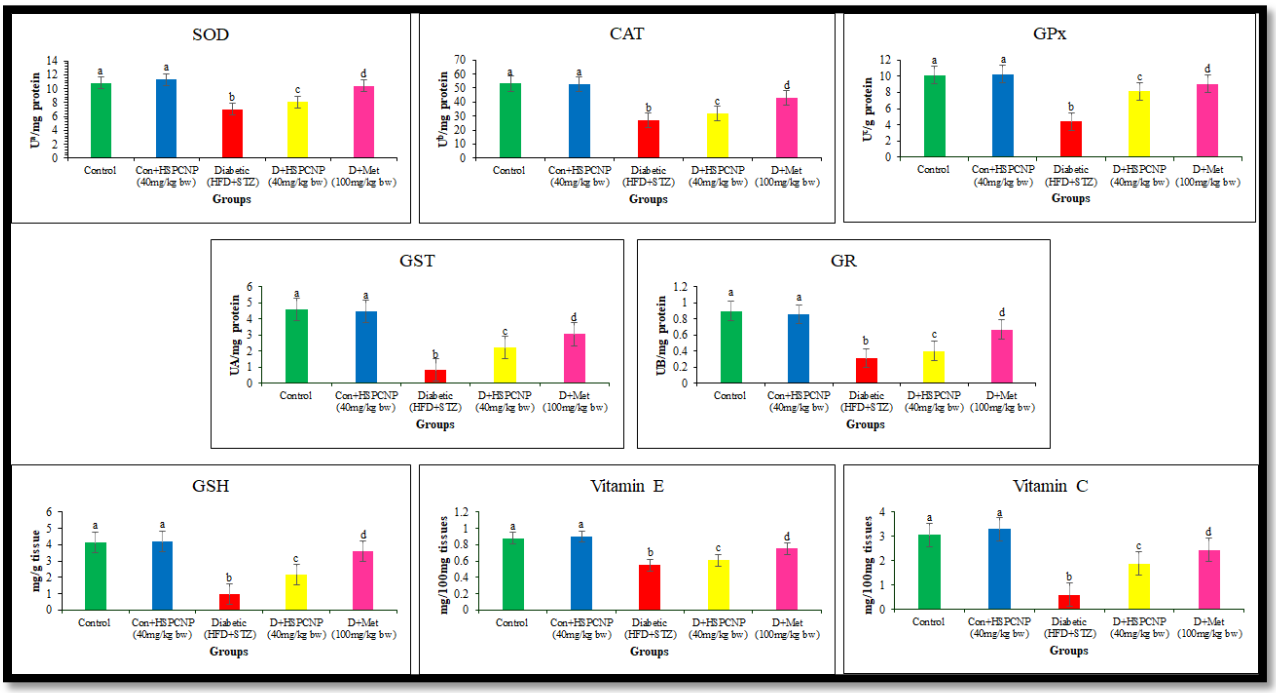

Figure 2. Effect of HSPCNP on enzymatic and nonenzymatic antioxidantconcentrations in the kidneys of experimental and control rats. The values, presented as mean ± SD for six rats, are considered to be significantly different ($p < 0.05$, DMRT) if they do not share the same superscript (a-d).

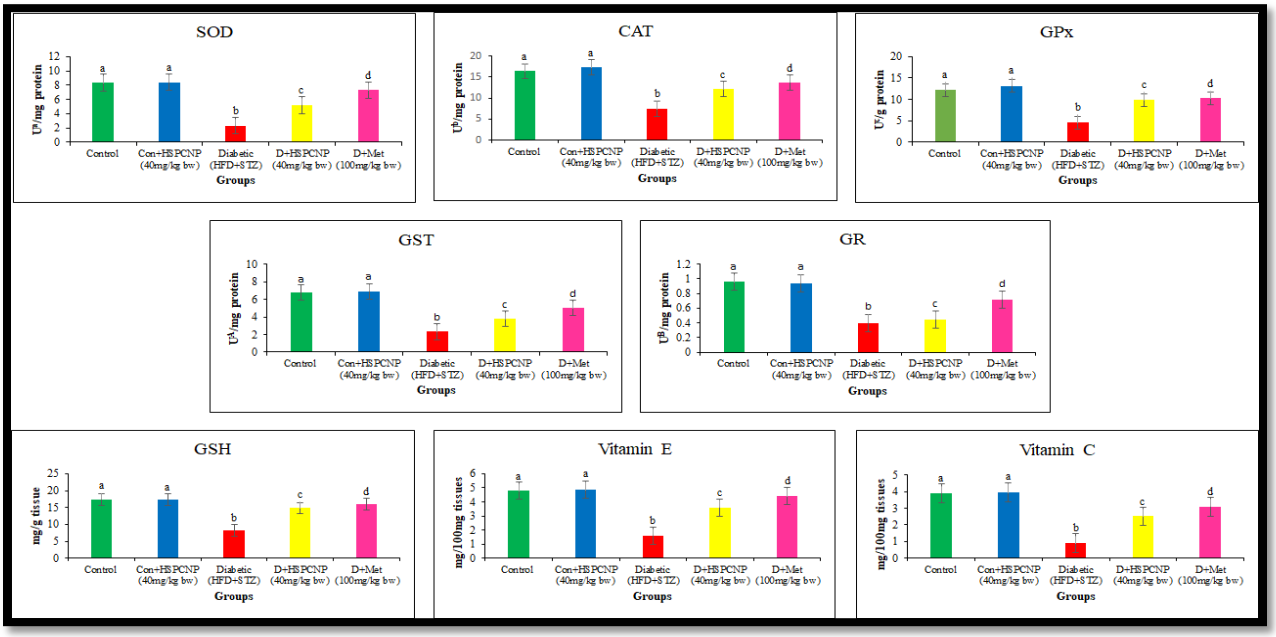

Figure 3. Effect of HSPCNP on enzymatic and non-enzymatic antioxidant concentrations in the pancreas of experimental and control rats. The values, presented as mean ± SD for six rats, are considered to be significantly different ($p < 0.05$, DMRT) if they do not share the same superscript (a-d).

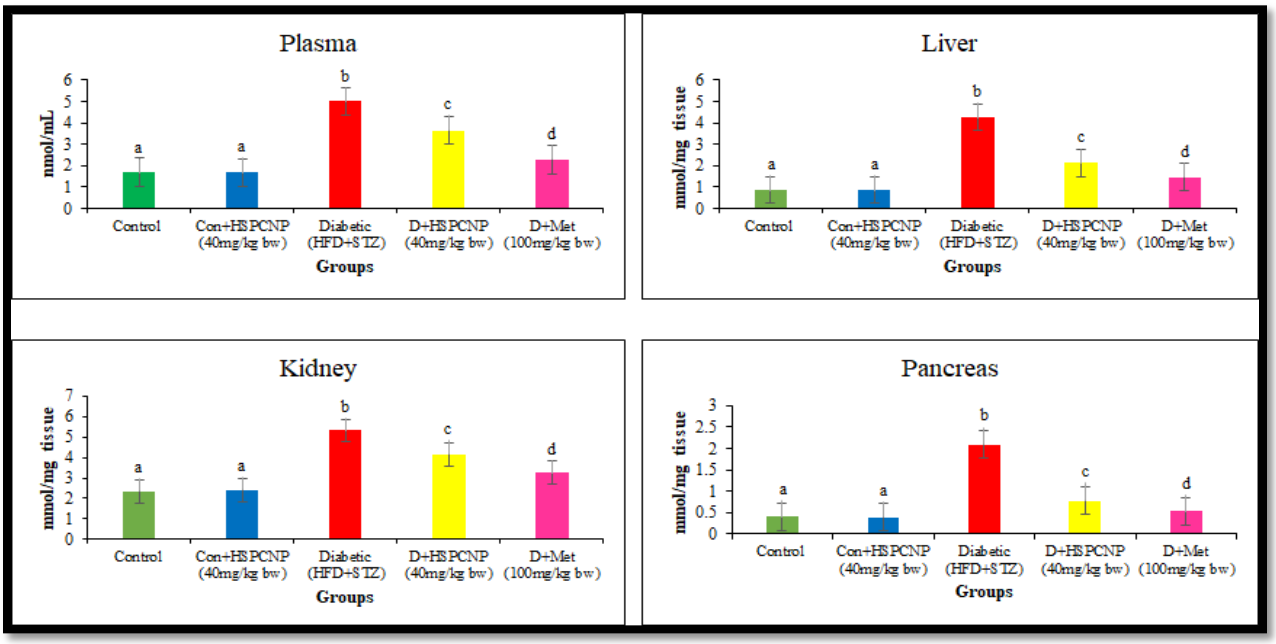

Figure 4. Effect of HSPCNP on the TBARS levels in plasma, liver, kidney and the pancreas of experimental and control rats. Values are mean ± SD for six rats; values are considered to be significantly different ($p < 0.05$, DMRT) if they do not share a common superscript (a–d).

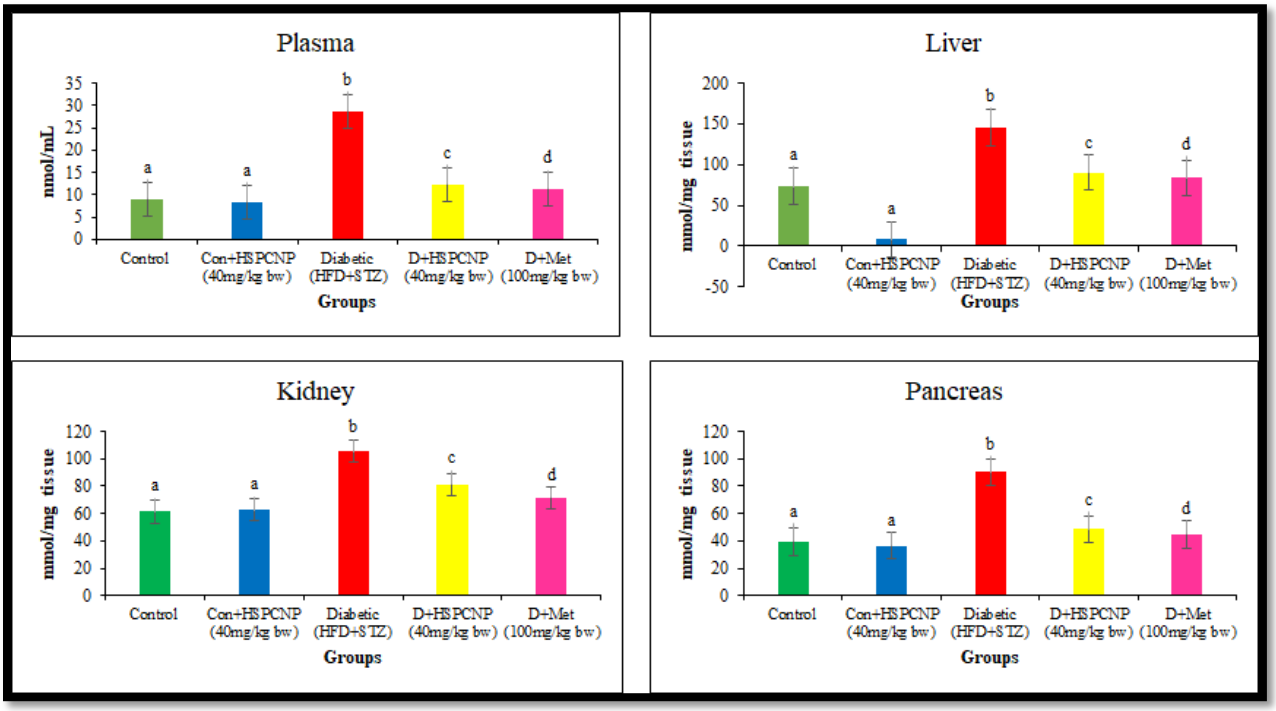

Figure 5. Effect of HSPCNP on the levels of LHP in plasma, liver, kidney and pancreas in experimental and control rats. Values are mean ± SD for six rats and are considered significantly different ($p<0.05$, DMRT) if they do not share a common superscript (a-d).

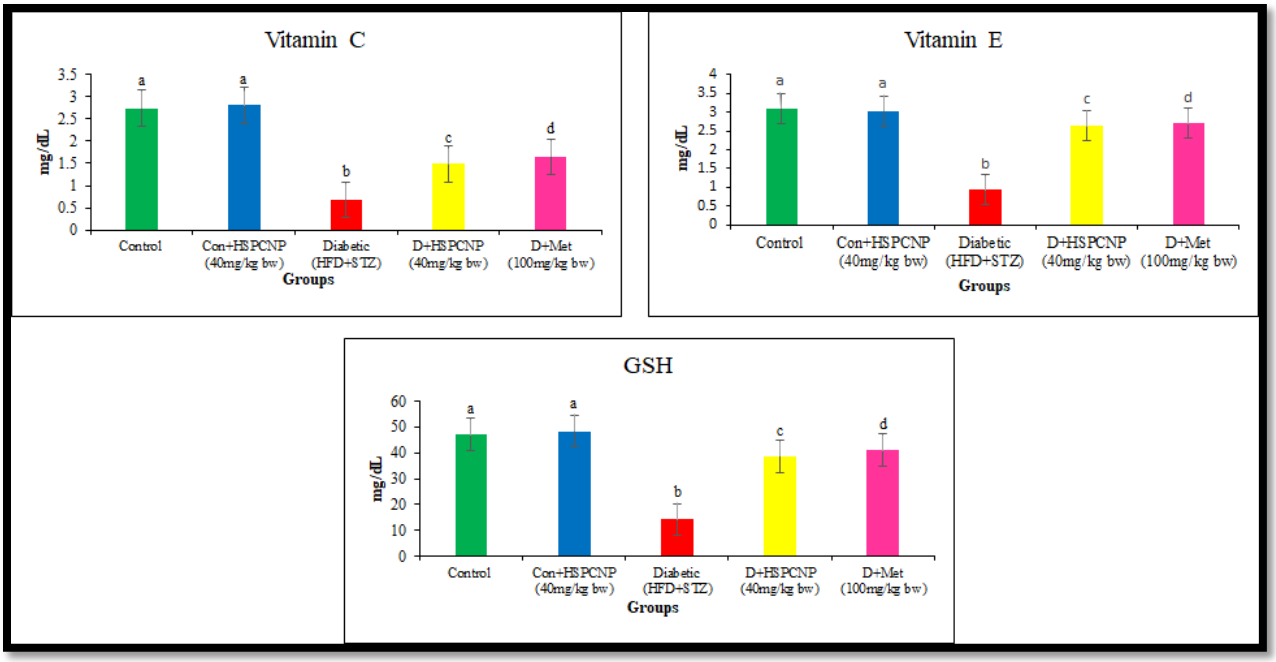

Figure 6. Effects of HSPCNP on plasma vitamin C, E, and GSH levels in normal and experimental rats. Values are mean ± SD for six rats and are considered to be significantly different ($p < 0.05$, DMRT) if they do not share a common superscript (a-d).

Histopathological Analysis of liver and kidney tissues

Histological observations of control and experimental rats' liver tissue stained with H&Eare presented in Figure 7. The control group exhibited normal liver histological architecture, characterized by well-preserved hepatocytes, clear sinusoidal spaces, and intact central veins, with no signs of steatosis, inflammation, or necrosis. In contrast, the diabetic group showed significant histopathological alterations, including hepatocyte ballooning, cytoplasmic vacuolization, fatty changes (steatosis), inflammatory cell infiltration, and necrosis, indicating severe liver damage due to HFD/STZ-induced diabetes. HSPCNP-treated groups exhibited reduced fatty changes, hepatocyte ballooning, and inflammatory cell infiltration compared with the diabetic control group. Histological analysis ofthe metformin-treated diabetes group showed similar improvements in liver tissue integrity.

Figure 8 presents the histopathological analysis of the control and experimental kidney tissues stained with H&E. The kidney tissue of control rats hada normal architecture with intact glomeruli and tubules, no signs of inflammation or degeneration, and normal interstitial spaces. In contrast, significant histopathological changes were observed in diabetic rats, including tubular degeneration, glomerular hypertrophy, interstitial inflammation, and necrosis,

indicating severe kidney damage due to diabetes. Both HSPCNP and metformin-treated diabetic rats showed marked improvements, with decreased tubular and interstitial damage and only modest tubule fat formation. Our findings are consistent with those reported in the study on isopulegol's effects, which demonstrated that it mitigates hyperglycemia-induced oxidative and endoplasmic reticulum stress in HFD/STZ-induced diabetic rats.Kalaivani, K. (2020)

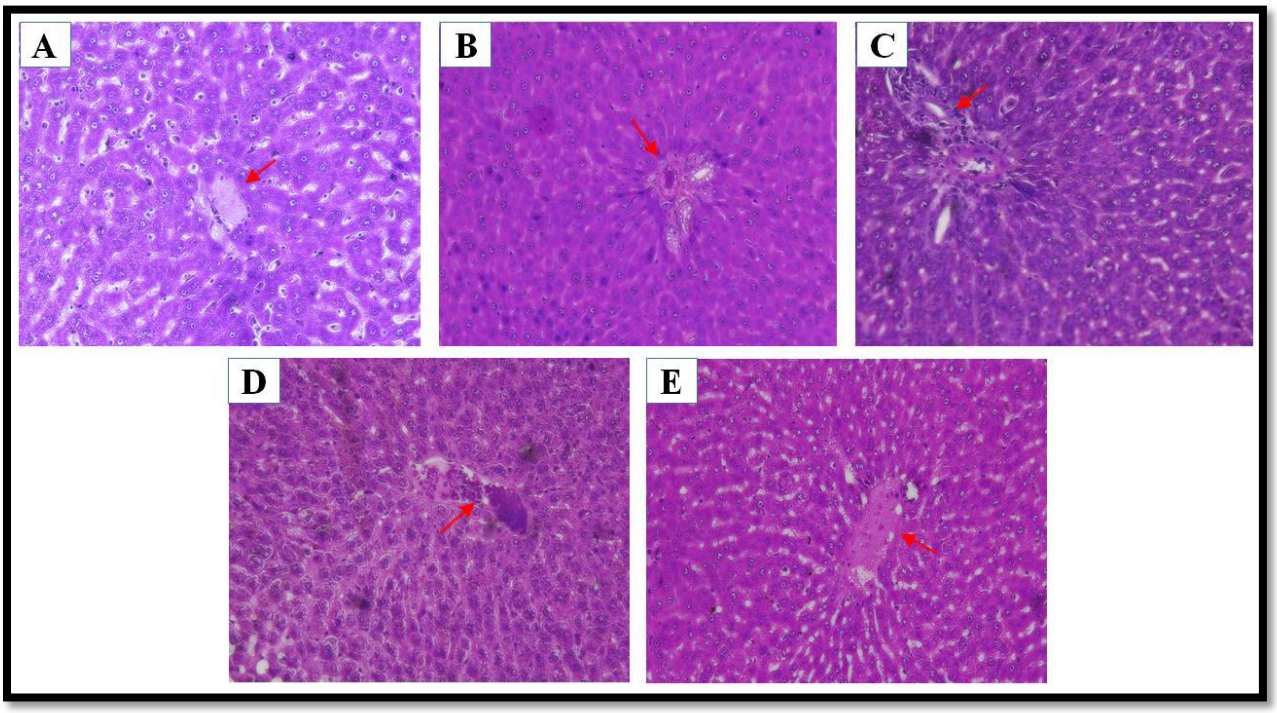

Figure 7. Histopathological changes were observed in the liver tissues ofthe experimental rats.

A. Control: Normal liver architecture with well-preserved hepatocytes.
B. Control + HSPCNPs (40mg/kg b.w): Hepatocytes with clear, round nuclei and intact sinusoidal spaces. The central vein is well preserved.
C. Diabetic (HFD + STZ): Hepatocyte ballooning, cytoplasmic vacuolization, fatty changes (steatosis), and mild inflammatory cell infiltration. Central vein shows.
D. Diabetic + HSPCNPs (40 mg/kg b.w): Reduced hepatocyte ballooning and steatosis, with some inflammatory cells still present.
E. Diabetic + Metformin (100 mg/kg b.w): Reduced steatosis and inflammatory cell infiltration. Hepatocytes are preserved, and the central vein is almost normal.

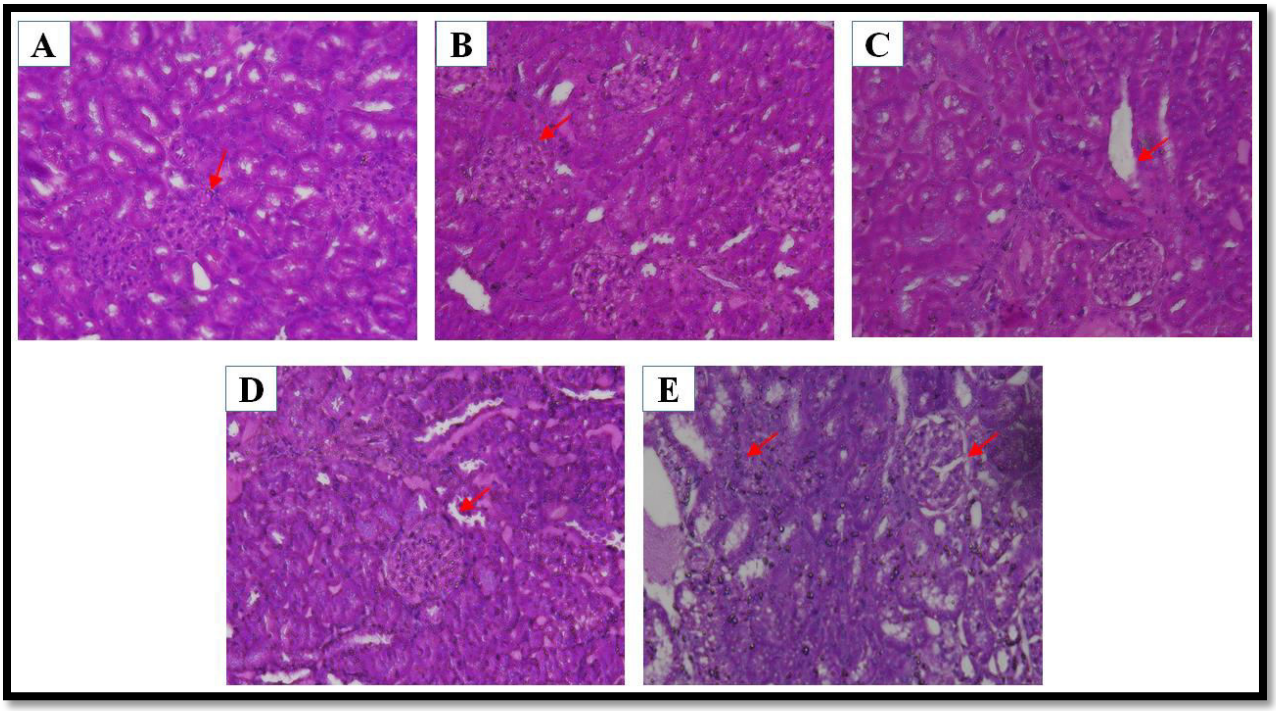

Figure 8. Histopathological changes were observed in the kidney tissues of the experimental rats.

A. Control: Normal Intact glomeruli and tubules
B. Control + HSPCNPs (40mg/kg b.w): Well-preserved glomeruli and tubules
C. Diabetic (HFD + STZ): Tubular degeneration, glomerular hypertrophy, interstitial inflammation, and evidence of necrosis.
D. Diabetic + HSPCNPs (40 mg/kg b.w): Reduced tubular degeneration and glomerular changes.
E. Diabetic + Metformin (100 mg/kg b.w): Reduced tubular and glomerular pathology, minimal inflammation.

Conclusion

In conclusion, HSPCNP had a significant protective effect against oxidative stress in STZ-induced diabetic rats fed a high-fat diet. The treatment notably enhanced both enzymatic and non-enzymatic antioxidant defenses, as evidenced by increased activities of SOD, CAT, GPx, GR, and GST, as well as elevated levels of vitamins E and C and GSH. The improvement in antioxidant status was associated with a decrease in markers of lipid peroxidation, indicating a decrease in oxidative damage to cellular structures.

Histopathological analysis further supported the biochemical findings, showing reduced liver and kidney tissue damage in HSPCNP-treated diabetic rats. These results show that HSPCNP can effectively mitigate oxidative stress and related

tissue damage in diabetic conditions, highlighting its potential as a therapeutic agent for managing diabetes and its complications. This study underscores the importance of enhancing antioxidant defenses to counteract the harmful effects of oxidative stress in diabetes mellitus.

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Ethical Approval:
This work has considered all relevant national, international, and institutional criteria for the use and care of animals.

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