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Antidiabetic, antioxidant, and anti-inflammatory effects of caffeic acid and zinc oxide nanoparticles against high fat diet treated rats.

Loa'i Osama[1], Nashwa W. Yassa[1,2], Doaa A. Ghareeb[1,2, 3,4], Nihal M. Elguindy[1*]

1) Department of Biochemistry, Faculty of Science, Alexandria University, Egypt

2) Bio-Screening and Preclinical Trial Lab, Biochemistry Department, Faculty of Science, Alexandria University, Egypt.

3) Center of Excellence for Drug Preclinical Studies, Pharmaceutical and Fermentation Industry Development Center, City of Scientific Research & Technological Applications, Alexandria, Egypt

4) Research Projects unit, Pharos University, Alexandria, Egypt.

ABSTRACT:

Diabetes and obesity are major global health problems with a well-established link between them. Over the past two decades, research has increasingly recognized obesity as a major risk factor for insulin resistance, which can lead to diabetes. Diabetes, marked by hyperglycemia due to defects in insulin action or secretion, is often complicated by oxidative stress and inflammation. Natural products like caffeic acid (CA), a polyphenolic compound in plants and coffee, have demonstrated antioxidant properties. Similarly, zinc oxide nanoparticles (ZnO-NPs) have gained attention for their ability to penetrate cells and interact with biomolecules, offering potential in treating diseases like diabetes.

This study investigated the therapeutic potential of Zinc Oxide-Caffeic Acid Nanoparticle Conjugates (ZnO-CA NPs) in a rat model of obesity-induced diabetes. Rats were divided into groups based on diet and treated with ZnO-NPs, CA, ZnO-CA NPs, or metformin. The study found that the high-fat diet led to elevated blood glucose, insulin resistance, adipokines such as adiponectin and leptin, and oxidative stress. Treatments with ZnO-NPs, CA, and especially ZnO-CA NPs significantly improved these parameters. ZnO-CA NPs normalized these parameters, suggesting they may offer a promising therapy for obesity-related diabetes. This study suggests ZnO-CA NPs as a promising treatment for obesity and diabetes.

Keywords: Diabetes, Obesity, Zinc oxide -caffeic acid nanoparticles, adipokines, insulin resistance, antioxidant

INTRODUCTION

Obesity remains a significant global health concern, affecting approximately about 14% of adults worldwide and contributing to nearly 5 million deaths annually due to associated health complications (Boutari et al., 2023; Dai et al., 2020). Metabolic disturbances linked to obesity, such as insulin resistance and dysregulated insulin secretion, create a delicate network that predisposes individuals to glucose intolerance and the subsequent development of type 2 diabetes Mellitus (T2DM) (Ruze et al., 2023). The correlation between obesity and its health complications, notably T2D, is a particularly pressing concern. In T2DM, there is a combination of inadequate insulin secretion and peripheral insulin resistance, affecting tissues such as skeletal muscle, adipose tissue (AT), and liver. The progression from insulin resistance to T2DM within the influence of obesity involves the pancreatic β-cells' inability to secrete more insulin to overcome impaired insulin sensitivity. This ultimately leads to elevated blood glucose levels (Kahn et al., 2006) .

In individuals with obesity, adipose tissue releases elevated levels of non-esterified fatty acids (NEFAs), glycerol, hormones, pro-inflammatory cytokines, and other factors that contribute to the development of insulin resistance (Kahn et al., 2006). While NEFAs play a significant role in insulin release, prolonged exposure to NEFAs is associated with substantial impairment in glucose-stimulated insulin secretion pathways and reduced insulin biosynthesis. Furthermore, the presence of insulin resistance in vivo and the failure of compensatory mechanisms in human β-cells contribute to elevated NEFA levels produced by lipids (Saltiel & Kahn, 2001). Additionally, various mechanisms and factors have been proposed to induce insulin resistance, including inflammation, mitochondrial .

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Corresponding author: **Nihal M. Elguindy**

dysfunction, and hyperinsulinemia, all of which are closely associated with obesity.

One of the most chronic complications is the strong link between hyperglycemia, hyperglycemic-induced oxidative stress, inflammation and the development and progression of type 2 diabetes mellitus. Studies reported Oxidative stress as acknowledged pathway in the pathogenesis of diabetic complications (Dludla et al., 2018; Oguntibeju, 2019). Hyperglycemic-induced oxidative stress is believed to increase the levels of pro-inflammatory proteins with infiltrated macrophages secreting inflammatory cytokines which leads to local and systemic inflammation (Wellen & Hotamisligil, 2005). The elevation of reactive oxygen species (ROS) has strong effect for lipid peroxidation, which causes malondialdehyde (MDA) accumulation to increase, and can be exacerbated by a decrease in the efficiency of antioxidant defense mechanisms in DM. Therefore, MDA levels are reported to be significantly increased in T2DM patients, and are among the oxidants known to be associated with T2DM complications (Ladgotra et al., 2016).

The chronic and low-grade inflammation observed in obesity plays a crucial role in the pathogenesis of T2DM. It inhibits the insulin signaling activity in adipocytes or hepatocytes by suppressing insulin receptor substrate-1 (IRS-1) and insulin receptor in the signaling pathway (Ye & Gimble, 2011). Furthermore, inflammation impairs the function of peroxisome proliferator-activated receptor-gamma (PPAR-γ), a nuclear receptor crucial for lipid synthesis and intracellular fat storage (Hensley & Floyd, 2002). Additionally, inflammation contributes to elevated levels of free fatty acids (FFA) by promoting lipolysis and inhibiting triglyceride synthesis (Ye, 2007).

Inflammation in obesity originated from increased levels of proinflammatory cytokines, endoplasmic reticulum (ER) stress, adipocyte apoptosis, macrophage accumulation, and enhanced lipolysis (Skurk et al., 2007). In individuals affected by obesity, adipose tissue (AT) and the macrophages within it primarily release pro- and anti-inflammatory molecules (adipokines). **Adipokines** are bioactive proteins intimately connected with obesity and insulin resistance. These compounds exert systemic effects on multiple organs and systems, modulating inflammation and regulating endocrine processes (Boutari & Mantzoros, 2018). Adiponectin and leptin are both adipokines, or hormones synthesized and released from adipose tissues. **Adiponectin** acts as an anti-inflammatory hormone with insulin-sensitizing properties (Al-Hamodi et al., 2014; Patané et al., 2013). Also, it affects the pancreas by promoting beta cell function and survival and increases glucose-mediated insulin secretion (Okamoto et al., 2008; Yamauchi et al., 2003). In addition, adiponectin increases fatty acid oxidation while reducing the synthesis of glucose in the liver (Scotece et al., 2014). Like adiponectin, abnormal levels of leptin are associated with obesity. Leptin, serves as a crucial hormone in conveying adiposity signals to the brain, ultimately dampening appetite and reducing food intake (Blüher & Mantzoros, 2009; Jung et al., 2014). However, elevated levels of leptin observed in obese individuals indicate the presence of leptin resistance (Gao et al., 2000; Schmidt et al., 2006). Leptin regulates food intake, body mass, reproductive functioning and plays a vital role in fetal growth, proinflammatory immune responses, angiogenesis, and lipolysis (Farr et al., 2015).

Medicinal plants, in particular, have demonstrated numerous beneficial activities. They can influence carbohydrate metabolism through various mechanisms, aid in the prevention and restoration of cell integrity and function, regulate insulin release, enhance glucose uptake and utilization, and exhibit antioxidant properties. This multifaceted nature makes them promising candidates for drug development and underscores the importance of exploring natural products in pharmaceutical research (Zhang et al., 2015). Caffeic acid is a natural phytochemical polyphenolic constituent in plants found in high concentrations in coffee, wine, and tea as well as in propolis. Several plants also contain caffeic acid, such as apples, plums, lingonberries, black chokeberries, and several herbs from the mint family, including sage, thyme, oregano, marjoram, and spearmint (Belay et al., 2016).CA (CA, 3,4-dihydroxyphenyl-2-propenoic acid or 3,4-dihydroxycinnamic acid, $C_9H_8O_4$; molecular weight (M.wt) 180.16 is a near molecular analog of dihydroxyphenylalanine (DOPA) (El-Seedi et al., 2012).

It exhibits antioxidant and free radical scavenging properties, due to its rich chemical structure of phenolic-hydroxyls (Almeida et al., 2006; Espíndola et al., 2019). Accordingly, it has various biological functions, comprising antibacterial, anti-inflammatory, immunomodulatory, antidiabetic, and anticancer activities (Mosquera et al., 2018; Oršolic et al., 2005; Salau et al., 2022). Nanoparticles are broadly applicable in various biological fields due to their ultra -small size, which enables them to penetrate the cells. This characteristic allows them to interact with biomolecules and potentially trigger specific cellular responses (Smijs & Pavel, 2011). Zinc oxide nanoparticles (ZnONPs), as a novel method for zinc delivery, hold significant potential for treating various diseases, including cancer and diabetes (San Tang, 2019; Uyoyo Ukperoro et al., 2010). The advancement of zinc-based therapies shows promise for managing diabetes and its related complications, with preclinical studies demonstrating beneficial effects of zinc supplementation (Mishra et al., 2017).

Our study aims to investigate the ameliorating effect of ZO-CA NPs treatment on the diabetic profile, lipid profile, oxidant and antioxidant parameters and its effect on liver and kidney function in HFD- induced type 2 diabetic rats

Animals and Methods:

Animals

Sixty-three male Sprague Dawley rats, 3 months old, weighing between 80-120 g were used for this study. The animals were purchased from the Medical Research Institute, Alexandria, Egypt. Animals were housed 4 per cage and maintained under controlled temperature (25± 2^{o} C) and constant photoperiodic conditions (12:12-h daylight/darkness). The rats had free access to water and standard commercial diet 4.1% fat, 22.2% protein, and 12.1% carbohydrates or high fat diet (HFD) containing protein 25%, carbohydrate 17%, and fat 58%, as a percentage of total Kcal.

All the animals were acclimatized for 2 weeks before the start of the experiment. All procedures involving laboratory animals were followed in accordance with Institutional Animal Care and Use Committee, Alexandria University, Egypt (IACUC, Approval number: **AU04230328101)**. All efforts were taken to reduce the number of animals used and minimize animal suffering.

Induction of Diabetes mellitus

Rats were fed a high-fat diet (HFD) for 6 months, and blood glucose levels were assessed prior to STZ injection. Following an overnight fast, the rats were administered a single intraperitoneal (i.p.) dose of streptozotocin (STZ) of 30 mg/kg body weight, dissolved in freshly prepared 0.1 M citrate buffer (pH 4.5). To prevent severe hypoglycemia, 5% doses of dextrose solution were administered to the rats for 24 hours post-injection. Three days after STZ administration, diabetic state was confirmed by measuring blood glucose levels from the tail using a glucometer. Only animals with blood glucose levels above 200 mg/dl were included in the study.

Experimental design:

All experiments were performed following the guidelines for the ethical treatment and use of laboratory animals. Sixty-three male Sprague Dawley rats were randomly assigned to two main groups: **Normal and diabetic groups**:

I. Normal group: Rats in this group were fed a standard diet for 6 months and were then further divided into the following subgroups:

- **Control group**: These rats Continued on a standard diet and received daily intraperitoneal injection of 0.9% saline (0.5 ml) for 28 days.
- **ZnO NPs group:** Rats received the standard diet and were treated daily with intraperitoneal injections of zinc oxide nanoparticles (ZnO NPs) at a dose of 6.67 mg/Kg for 28 days (Sayed et al., 2021).
- **CA group:** Rats received the standard diet and treated daily with intraperitoneal injections of a dose of 13.3 mg/Kg Caffeic acid for 28 days (Matboli et al., 2017).
- **ZnO-CA NPs group:** Rats received standard diet and treated daily with intraperitoneal injection of Zinc oxide-Caffeic acid nanoparticles (**ZnO-CA NPs)** at a dose of 20 mg/Kg for 28 days (Sayed et al., 2021).

II. Induced diabetic group: rats received high fat diet (HFD) for 6 months then injected intraperitoneally with streptozotocin (STZ) at a single dose of 30 mg/kg body weight, and were then divided into the following subgroups:

- **HFD group:** Rats continued on the HFD and received no further treatment, serving as the untreated diabetic control for 28 days.
- **HFD-ZnO NPs group:** Rats were maintained on the HFD and received daily intraperitoneal injections of ZnO NPs (6.67 mg/Kg) for 28 days
- **HFD-CA group:** Rats received HFD and were treated daily with intraperitoneal injections of caffeic acid at 13.3 mg/Kg for 28 days.
- **HFD-ZnO-CA NPs group:** Rats received the HFD and daily intraperitoneal injections of ZnO-CA NPs (20 mg/Kg) for 28 days.
- **HFD-metformin group:** Rats were given the HFD and treated with daily intraperitoneal injections of metformin at 50 mg/Kg for 28 days (Araújo et al., 2017).

Throughout the entire experiment, the body weight of all rats was monitored weekly using a digital scale.

At the end of the experiment, animals were anesthetized and scarified by cervical dislocation in deep anesthesia and blood samples were collected in plain test tubes by heart puncture to obtain sera for biochemical analysis. Liver tissues were removed, put on ice, weighed and were used for further analysis.

Preparation of Zinc Oxide- Caffeic acid Nanoparticles

Caffeic acid -Zinc oxide nanoparticles were prepared using the following method, First, a zinc oxide suspension was prepared by mixing 0.0893 g of zinc oxide and 30 ml ethanol, followed by sonication for 30 minutes. In a separate step, 0.180 g of caffeic acid was dissolved in 20 ml ethanol through vigorous stirring until completely dissolved. The nanoparticles were then formed by gradually adding the caffeic acid solution drop by drop with a syringe under magnetic stirring at a temperature below 50°C for 2 hours. Finally, the nanoparticles were separated by centrifugation at 12,000 rpm for 30 min at 4ºC (Sayed et al., 2021).

Preparation of liver homogenate

A 500 mg sample of frozen liver tissue was homogenized in 5 ml 0.1M phosphate buffer saline (pH 7.4) containing 2 mM PMSF using homogenizer at a speed of 9500 rpm. The homogenate was centrifuged at 10,000 rpm and 4 ºC for 20 min. The supernatant from the centrifugation was then aliquoted into 300 µl portions and stored at -20 ºC for further use.

Determination of total liver protein

Liver total protein was measured in homogenate by the specific assay kit according to the manufacturer's instructions (N.S Biotec, Egypt). The absorbance of each sample and standard were read against blank at 540 nm using Helios Alpha UV–Visible Spectrophotometer (Thermo Spectronic, Unicam Limited, UK).

Determination of diabetic parameters

Levels of serum glucose in each sample were assessed using an assay kit following the manufacturer's guidelines (Burtis & Ashwood, 1999). Absorbance measurements for both samples and standards were taken at 500 nm using a spectrophotometer. Fasting serum insulin levels were measured using an ELISA kit according to the manufacturer's protocol (Angel, 1988), with absorbance readings taken at 450 nm on a Bio-Tek ELx 800 ELISA microplate reader (Bio-Tek Instruments, Inc., Vermont, USA). A standard curve was constructed to calculate insulin concentrations in µIU/ml. Insulin resistance was evaluated using the homeostasis model assessment method (HOMA-IR), as described by Matthews, Hosker et al. 1985 (Matthews et al., 1985).

Assessement of lipid profile

The biochemical measurements of triglycerides and total cholesterol are carried out using available commercial kits.

Assay of oxidant and antioxidant parameters

Liver Glutathione (GSH) is assayed using the method of Jollow et al.,1974 (Jollow et al., 1974) which depends on the oxidation of GSH by 5,5`-dithiobis-(2-nitrobenzoic acid) (DTNB) to yield GSSG and 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB that is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction which in turn is directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 412 nm provides an accurate estimation of GSH in the sample. Liver and serum MDA was evaluated by an assay kit (Tappel & Zalkin, 1959), Superoxide dismutase (SOD) determination in liver homogenate was estimated according method of Marklund, S., & Marklund, G. (1974) (Marklund & Marklund, 1974).

Determination of adiponectin and leptin

Serum adiponectin and leptin were determined by the rat ELISA kit from Nova China. Absorbance values were read at 450 nm using Bio-Tek ELx 800 ELISA microplate reader. Standard curves were constructed corresponding to each parameter. An absorbance value for each specimen was used to determine the corresponding concentration of each sample.

Determination of liver and kidney function

Activities of ALT and AST and levels of Urea, creatinin and uric acids are determined using kits from Diamond Diagnostic-ARE.

Statistical analysis:

All data are presented as mean±SD. The Shapiro-Wilk test was used to verify the normal distribution of the studied variables. A one-way analysis of variance (ANOVA) for normally distributed quantitative variables, to compare between more than two groups, and Post Hoc test **(Tukey)** for pairwise comparisons. Differences were considered significant at $P<0.05$.All statistical analyses were performed using IBM SPSS software package version 20.0**. (**Armonk, NY: IBM Corp**)**.

Results:

Amelioration of diabetic profile in HFD-fed Rats by treatment with Zinc Oxide Nanoparticles (ZnO-NPs), Caffeic Acid (CA), and Zinc Oxide-Caffeic Acid Nanoparticle Conjugate (ZnO-CA NPs).

The results of fasting blood glucose, insulin, and HOMA IR are presented in Table 1. No significant differences were observed in glucose, insulin, or HOMA-IR levels between the control group and the control group treated with ZnO-NPs, caffeic acid (CA), or ZnO-CA NPs. However, in the high-fat diet (HFD) group, the induction of diabetes led to a significant ($p \leq 0.05$) increase in fasting glucose, insulin, and HOMA-IR levels compared to the control group. Treatment with caffeic acid (CA), ZnO-CA NPs, and metformin significantly ($p \leq 0.05$) reduced these levels. Notably, the glucose and HOMA-IR levels in diabetic rats treated with ZnO-CA NPs, or metformin were not significantly different from control levels. Among the treatments, ZnO-CA NPs demonstrated the most pronounced and significant effect on reducing glucose and HOMA-IR levels compared to the other diabetic treatment groups

Table 1. Diabetic profile parameters (glucose, insulin and HOMA-IR) in the experimental animal groups.

Groups	Glucose (mg/dL)	Fasting insulin (µIU/ml)	Homa IR
Control	126.3 ± 13.20	4.04 ± 0.17	1.26 ± 0.13
ZnO-NPs	155.3 ± 8.24	4.04 ± 0.19	1.55 ± 0.10
CA	125.2 ± 20.48	4.01 ± 0.13	1.24 ± 0.20
ZnO-Ca NPs	139.8 ± 12.07	3.76 ± 0.17	1.30 ± 0.15
HFD	457.8^{abcd} ± 73.68	7.40^{abcd} ± 0.36	8.34^{abcd} ± 1.20
HFD-ZnO NPs	397.5^{abcd} ± 55.10	6.33^{abcde} ± 0.15	6.21^{abcde} ± 0.85
HFD-CA	278.4^{abcdef} ± 65.30	5.26^{abcdef} ± 0.26	3.60^{abcdef} ± 0.82
HFD-ZnO-CA NPs	141.9^{efg} ± 25.14	5.34^{abcdef} ± 0.29	1.88^{efg} ± 0.38
HFD-met	172.4^{efg} ± 12.05	5.31^{abcdef} ± 0.31	2.26^{cefg} ± 0.25

Data was expressed using Mean ± SD and n of rats in each group =7, Statistically significant at $p \leq 0.05$

a: Significant with control, b: Significant with ZnO-NPs, c: Significant with CA, d: Significant with ZnO-Ca NPs, e: Significant with HFD, f: Significant with HFD-ZnO NPs, g: Significant with HFD-CA, h: Significant with HFD-ZnO-CA NPs and HFD-met

Changes in Cholesterol and Triglyceride in HFD fed rats after treatment with Zinc Oxide Nanoparticles (ZnO-NPs), Caffeic Acid (CA), and Zinc Oxide-Caffeic Acid Nanoparticle Conjugate (ZnO-CA NPs).

The analysis of serum cholesterol and triglyceride levels across the nine experimental groups revealed notable differences, underscoring the influence of various treatments and dietary conditions on lipid metabolism. Levels of serum cholesterol and triglyceride were assessed in all experimental groups, as detailed in Figure1. (A&B). No significant differences in cholesterol and triglyceride levels were found between the control group and the control groups treated with ZnO NPs, caffeic acid (CA), or ZnO-CA NPs. In contrast, a high-fat diet (HFD) led to a significant ($P \leq 0.05$) increase in both cholesterol (Figure 1.A) and triglyceride (Figure 1.B) levels compared to the control group. Treatment of diabetic rats with ZnO-NPs, caffeic acid (CA), ZnO-CA NPs, and metformin led to a significant reduction in cholesterol and

triglyceride levels compared to the HFD group. Interestingly, there were no significant differences in cholesterol and triglyceride levels among the CA, ZnO-CA NPs, and metformin groups when compared to the control group.

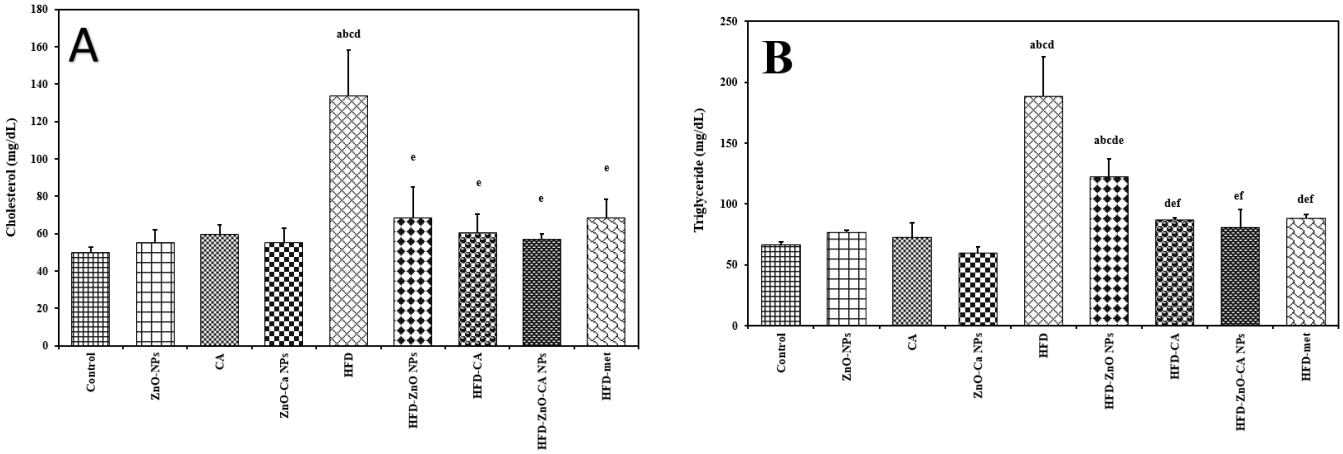

Figure 1. Effect of Zinc Oxide Nanoparticles (ZnO-NPs), Caffeic Acid (CA), and Zinc Oxide-Caffeic Acid Nanoparticle Conjugate (ZnO-CA NPs) treatment on the level of Cholesterol (A) and triglyceride (B) in HFD-induced diabetic rats
Data was expressed using Mean ± SD and n of rats in each group =7, Statistically significant at $p \leq 0.05$
a: Significant with control, b: Significant with ZnO-NPs, c: Significant with CA, d: Significant with ZnO-Ca NPs, e: Significant with HFD, f: Significant with HFD-ZnO NPs, g: Significant with HFD-CA, h: Significant with HFD-ZnO-CA NPs

Impact of Zinc Oxide Nanoparticles (ZnO-NPs), Caffeic Acid (CA), and Zinc Oxide-Caffeic Acid Nanoparticle Conjugates (ZnO-CA NPs) on Oxidant and Antioxidant Parameters in High-Fat Diet-fed rats.

As detailed in Table 2, no significant differences were observed in serum and liver MDA levels between the experimental control groups treated with ZnO-NPs, CA, and ZnO-CA NPs, and the control group. However, high-fat diet (HFD) administration resulted in a significant increase in both liver and serum MDA levels compared to the control group. This increase was significantly reduced by treatment with ZnO NPs, CA, ZnO-CA NPs, or metformin in serum MDA. In terms of liver MDA, significant decreases were observed only when diabetic rats treated with ZnO-NPs and ZnO-CA NPs when compared to the HFD group. HFD administration to rats induced a significant ($p \leq 0.05$) reduction in in the liver GSH accompanied with a significant decline ($p \leq 0.05$) in the activity of liver SOD. In contrast, treating HFD-fed diabetic rats with ZnO NPs, CA, ZnO-CA NPs, or metformin significantly improved liver GSH levels and SOD activity compared to the HFD group.

Table 2. Effect of Zinc Oxide Nanoparticles (ZnO-NPs), Caffeic Acid (CA), and Zinc Oxide-Caffeic Acid Nanoparticle Conjugate (ZnO-CA NPs) on the oxidant and antioxidant parameters in HFD -diabetic rats.

Groups	Serum MDA (n mol/ ml)	Liver MDA level (n mol/g tissue)	GSH in liver mg/g tissues	Liver SOD (U/mg protein)
Control	5.34 ± 0.52	18.41 ± 4.70	44.65 ± 2.96	272.2 ± 1.75
ZnO-NPs	5.57 ± 0.61	25.49 ± 4.49	37.93 ± 4.06	283.7^{a} ± 4.74
CA	5.69 ± 0.60	21.21 ± 3.36	45.68 ± 2.93	296.1^{ab} ± 1.80
ZnO-Ca NPs	5.43 ± 0.27	19.80 ± 4.84	62.46^{abc} ± 5.98	303.5^{abc} ± 3.18
HFD	8.54^{abcd} ± 0.58	43.12^{abcd} ± 3.31	30.14^{acd} ± 5.76	150.4^{abcd} ± 4.22
HFD-ZnO NPs	7.54^{abcde} ± 0.23	30.57^{e} ± 14.57	43.21^{de} ± 5.05	185.6^{abcde} ± 2.68
HFD-CA	6.39^{abdef} ± 0.22	34.47^{acd} ± 4.26	45.01^{de} ± 2.37	205.1^{abcdef} ± 1.70
HFD-ZnO-CA NPs	5.84^{ef} ± 0.14	25.48^{e} ± 10.41	56.31^{abcefg} ± 10.25	$245.0^{abcdefg}$ ± 3.30
HFD-met	6.17^{adef} ± 0.14	33.84^{acd} ± 4.81	41.20^{deh} ± 3.95	$227.3^{abcdefgh}$ ± 2.65

Data was expressed using Mean ± SD and n of rats in each group =7, Statistically significant at $p \leq 0.05$
a: Significant with control, b: Significant with ZnO-NPs, c: Significant with CA, d: Significant with ZnO-Ca NPs, e: Significant with HFD, f: Significant with HFD-ZnO NPs, g: Significant with HFD-CA, h: Significant with HFD-ZnO-CA NPs

Effect of ZnO-NPs, Caffeic Acid, and ZnO-CA NPs on Adiponectin and Leptin levels in High-Fat Diet-fed Rats.

Serum analysis of rats fed a HFD revealed a significant increase ($p \leq 0.05$) in leptin levels, accompanied by a significant decrease ($p \leq 0.05$) in the anti-inflammatory adiponectin, compared to control rats (Fig. 2, A&B).

Treatment with ZnO-NPs, Caffeic Acid, and ZnO-CA NPs, or metformin demonstrated anti-inflammatory effects by

significantly ($p \leq 0.05$) reducing leptin levels and significantly increasing ($p \leq 0.05$) adiponectin levels comparing with HFD group. Importantly, the levels of leptin and adiponectin in the ZnO-CA NPs treated diabetic group did not differ significantly from those in the control group

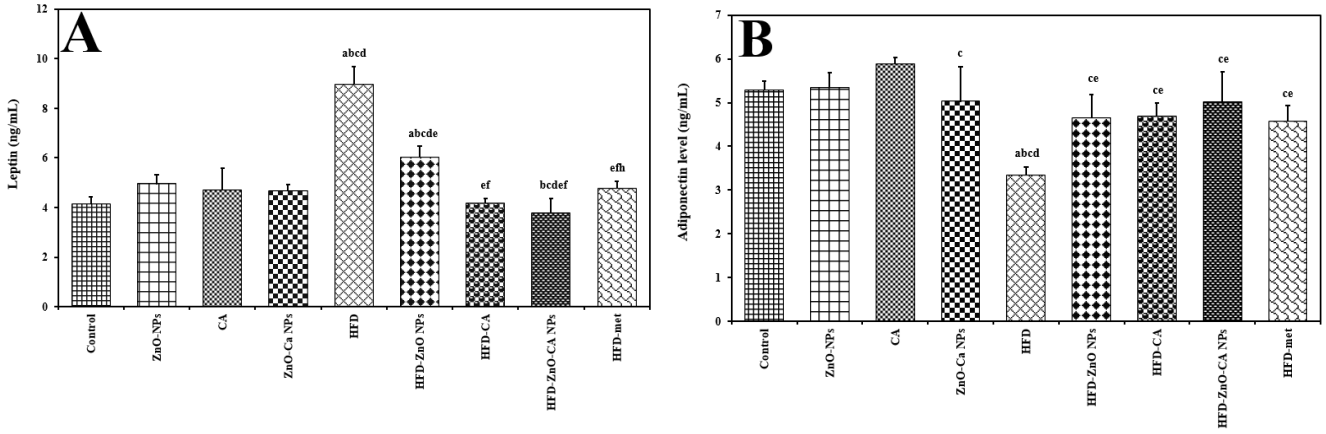

Figure 2. Levels of Leptin (A) and adiponectin (B) in different experimental rat groups.
Data was expressed using Mean ± SD and n of rats in each group =7, Statistically significant at $p \leq 0.05$
a: Significant with control, b: Significant with ZnO-NPs, c: Significant with CA, d: Significant with ZnO-Ca NPs, e: Significant with HFD, f: Significant with HFD-ZnO NPs, g: Significant with HFD-CA, h: Significant with HFD-ZnO-CA NPs

Impact of Zinc Oxide Nanoparticles (ZnO-NPs), Caffeic Acid (CA), and Zinc Oxide-Caffeic Acid Nanoparticle Conjugate (ZnO-CA NPs) on Liver and Kidney Function Biomarkers in High-Fat Diet-Induced Diabetic Rats

The activities of serum ALT and AST, as well as the levels of urea, creatinine, and uric acid in animals, significantly increased ($p \leq 0.05$) by high-fat diet (HFD) compared to the control group. However, treatment with ZnO-NPs, CA, ZnO-CA NPs, or metformin significantly ($p \leq 0.05$) reduced these levels. No significant differences were observed in AST, creatinine, and uric acid between the control group and control groups treated with ZnO-NPs and CA. Notably, the HFD group treated with ZnO-CA NPs showed no significant difference in the levels of ALT, AST, creatinine, and uric acid compared to the control group (Table 3)

Table3. Effect of Zinc oxide nanoparticles (ZnO-NPs), Caffeic acid (CA), and Zinc oxide- caffeic acid nanoparticles conjugate (ZnO-CA NPs) on the liver and kidney function Biomarkers in a High Fat Diet-Induced Diabetic rat Models.

Group	ALT (U/L)	AST (U/L)	Urea (mg/dL)	Creatinine (mg/dL)	Uric acid (mg/dL)
Control	34.96 ± 5.16	44.83 ± 3.07	21.27 ± 0.71	1.09 ± 0.04	1.08 ± 0.22
ZnO-NPs	45.26 ± 3.01	44.35 ± 3.26	32.57^{a} ± 9.05	1.17 ± 0.06	1.37 ± 0.28
CA	45.73 ± 5.57	43.75 ± 3.59	31.94^{a} ± 1.95	1.13 ± 0.09	1.69 ± 0.33
ZnO-Ca NPs	49.02^{a} ± 3.98	44.41 ± 3.58	31.26^{a} ± 1.08	1.06 ± 0.05	1.64 ± 0.07
HFD	94.76^{abcd} ± 3.63	84.41^{abcd} ± 6.71	73.83^{abcd} ± 6.63	1.46^{abcd} ± 0.12	3.56^{abcd} ± 0.60
HFD-ZnO NPs	59.85^{abce} ± 19.04	71.30^{abcde} ± 5.57	65.39^{abcde} ± 6.90	1.30^{acde} ± 0.11	1.93^{ae} ± 0.50
HFD-CA	64.73^{abcde} ± 4.60	63.50^{abcde} ± 5.67	60.41^{abcde} ± 2.51	1.29^{ade} ± 0.17	1.77^{e} ± 0.42
HFD-ZnO-CA NPs	41.18^{efg} ± 6.64	51.90^{cefg} ± 2.56	$41.53^{abcdefg}$ ± 2.02	1.16^{e} ± 0.04	1.58^{e} ± 0.54
HFD-met	65.86^{abcdeh} ± 1.86	57.36^{abcdef} ± 5.36	70.68^{abcdgh} ± 3.68	1.32^{acd} ± 0.09	2.44^{abcdeh} ± 0.38

Data was expressed using Mean ± SD and n of rats in each group =7, Statistically significant at $p \leq 0.05$
a: Significant with control, b: Significant with ZnO-NPs, c: Significant with CA, d: Significant with ZnO-Ca NPs, e: Significant with HFD, f: Significant with HFD-ZnO NPs, g: Significant with HFD-CA, h: Significant with HFD-ZnO-CA NPs

Discussion

The findings of this study demonstrate the potential of Zinc Oxide Nanoparticles (ZnO-NPs), Caffeic Acid (CA), and their conjugates (ZnO-CA NPs) as effective interventions for ameliorating the diabetic profile in high-fat diet (HFD)-induced diabetic rats. The significant elevation in fasting glucose, insulin, and HOMA-IR levels in the HFD group, as compared to controls, highlights the diabetogenic impact of a high-fat diet, consistent with previous studies linking dietary fats to metabolic dysfunctions and increased serum glucose concentrations (Akiyama et al., 1996; Lozano et al., 2016). Notably, the treatment with ZnO-CA NPs and metformin significantly reduced these levels, with ZnO-CA NPs showing the most pronounced effect, bringing glucose and HOMA-IR levels close to those of the control group. This suggests that ZnO-CA NPs might provide a synergistic effect, combining the antioxidant properties of caffeic acid (Hsu et al., 2000; Vera et al., 2023) with the cellular targeting

abilities of ZnO-NPs, potentially enhancing glucose metabolism and insulin sensitivity (Siddiqui et al., 2020). Additionally, CA has been found to enhance insulin sensitivity by reducing proinflammatory cytokines and increasing adiponectin levels, as observed in our work, in a hyperglycemic state (Vanella et al., 2016).

HFD administration led to dyslipidemic alterations, characterized by elevated serum levels of triglycerides and total cholesterol compared to the control group. These lipid changes contribute to oxidative stress in obesity (Akiyama et al., 1996). the marked reduction in cholesterol and triglyceride levels following treatment with ZnO-CA NPs further supports their therapeutic potential. The lipid-lowering effects observed align with the established role of polyphenolic compounds like caffeic acid in modulating lipid profiles by inhibiting lipid peroxidation, decreasing lipid accumulation, and promoting the white-to-brown transition of adipocytes (Dantas et al., 2018). The absence of significant differences in cholesterol and triglyceride levels between the ZnO-CA NP-treated diabetic group and controls underscores the effectiveness of this treatment in restoring normal lipid homeostasis.

The study also highlights the beneficial impact of ZnO-CA NPs on oxidative stress markers. Oxidative stress plays a crucial role in the pathogenesis of several metabolic disorders, including obesity and type 2 diabetes. In this study, the HFD/STZ-induced group exhibited clear signs of oxidative stress, as evidenced by decreased levels of GSH and SOD, and increased lipid peroxidation, indicated by elevated MDA levels in serum and liver tissues (Table 2). Oxidative stress resulting from the consumption of a high-fat diet is commonly observed in both experimental models and patients with various clinical conditions (Lasker et al., 2019; Maher et al., 2020; Yadav et al., 2008). In the present study, rats fed a high-fat diet exhibited elevated levels of serum and liver malondialdehyde (MDA), indicating increased lipid peroxidation and oxidative stress. This rise in MDA may be linked to the observed decrease in SOD and GSH. These findings align with previous research suggesting that tissue antioxidant defenses may be compromised in animals subjected to a high-fat diet (Lee et al., 2009; Ulla et al., 2017).

ZnO-CA NPs demonstrated significant antioxidant effects by enhancing SOD and GSH levels and reducing lipid peroxidation. These findings are consistent with previous studies, which suggest that the antioxidant capacity of ZnO-CA NPs is due to their ability to scavenge free radicals and chelate metal ions, making them a promising therapeutic alternative for reducing oxidative stress (Askar et al., 2022; Fifere et al., 2021). The reduction in MDA levels and the enhancement of antioxidant enzyme activities in the ZnO-CA NP-treated group further confirm the potent antioxidant properties of these nanoparticles. In addition, Caffeic acid has a structure highly effective at trapping free radicals. Its aromatic core, combined with a conjugated side chain , facilitates the delocalization of unpaired electrons. By donating hydrogen atoms to neutralize radicals, caffeic acid functions as a potent primary antioxidant (Damasceno et al., 2017)**.** Caffeic acid also inhibits the formation of reactive oxygen species (ROS) by blocking the activity of 5-lipoxygenase, an enzyme responsible for converting arachidonic acid into leukotrienes and contributing to ROS production (Cai et al., 2016).

Leptin and adiponectin, primarily produced by adipose tissue, play crucial roles in regulating energy balance and metabolic processes. In obesity, leptin levels are often elevated, contributing to leptin resistance, while adiponectin levels decrease, which can exacerbate metabolic disturbances such as insulin resistance (Obradovic et al., 2021; Peng, Yin, & Wang, 2021). In this study, rats fed an HFD showed a significant increase in leptin levels and a decrease in adiponectin levels compared to control rats. However, treatment with ZnO-CA NPs significantly reduced leptin and increased adiponectin levels, suggesting the anti-inflammatory and metabolic regulatory potential of this treatment. The anti-inflammatory effect of the nanoparticles may be referred to the effect of caffeic acid as it decreased tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), cyclooxygenase-2 (COX-2), and NF-κB levels in mice in a study of Balupillai A et al., 2015 (Balupillai et al., 2015), possibly by inhibiting the expression of peroxisome proliferator-activated receptor gamma (PPARγ). ZnO-CA NPs, effectively normalized these hormonal imbalances, highlighting their therapeutic potential in mitigating obesity-related complications.

Oxidative damage in tissues also leads to hepatocyte damage in high-fat diet-fed rats, as evidenced by the increased plasma activities of the AST, ALT enzymes. These enzymes are considered markers of hepatic dysfunction. Generally, hepatocyte damage causes these enzymes to be transported to the plasma. Also, HFD affect kidney function by elevated urea, creatinine, and uric acid levels. The positive effects of ZnO-CA NPs on liver and kidney function biomarkers further underscore their therapeutic potential. The significant reduction in ALT, AST, urea, creatinine, and uric acid levels in the treated groups compared to the HFD group suggests a protective effect against liver and kidney damage commonly associated with diabetes. This may be attributed to the antioxidant and anti-inflammatory properties of ZnO-CA NPs which alleviate the oxidative stress and inflammation underlying diabetic complications.

Conclusion

In conclusion, the combination of natural antioxidants like caffeic acid with zinc oxide nanoparticles represents a promising approach in nanotherapy for managing diabetes and its complications. The study highlights the potential of ZnO-CA NPs in improving diabetic profiles, lipid metabolism, oxidative stress, and hormonal balance while offering protective effects on liver and kidney functions. Further research is needed to fully understand the mechanisms and optimize the therapeutic use of these nanoparticles in clinical settings

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