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Effects of an Insulin-Nanoparticle Complex Administered Orally: Pharmacological Study and Impact on Hemostasis and Tissue Repair in Diabetic Wistar Rats

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Abstract

Diabetes mellitus is a chronic metabolic disorder characterized by persistent hyperglycemia and an imbalance in metabolic homeostasis, often accompanied by various complications. Traditional subcutaneous insulin therapy, while effective in lowering blood glucose levels, often fails to fully mimic natural insulin secretion and address broader metabolic functions. These limitations can contribute to the development of complications associated with diabetes mellitus. To address these limitations, this thesis investigates a novel approach: the oral administration of insulin encapsulated in nCOF particles (Oral nCOF/Insulin).

This study explores the therapeutic potential of Oral nCOF/Insulin in enhancing insulin bioavailability and preserving its efficacy *in vivo*. Our findings indicate that orally administered nCOF/Insulin effectively reduces hyperglycemia without inducing hypoglycemia. It maintains blood glucose levels comparable to non-diabetic states in both fasting and postprandial conditions, demonstrating potential benefits for improving insulin sensitivity and preventing insulin resistance.

Beyond glycemic control, our findings reveal that Oral nCOF/Insulin exerts systemic benefits by mitigating disruptions in hemostasis, as well as by reducing dyslipidemia, oxidative stress, and repairing tissue damage across various organs. This holistic approach to diabetes management underscores the potential of Oral nCOF/Insulin to not only regulate glucose but also restore broader metabolic and physiological homeostasis, offering a comprehensive treatment strategy.

Overall, this thesis presents nCOF/Insulin as a promising candidate for advancing diabetes care. Its ability to address the multifaceted aspects of the disease marks a significant step forward in the management of diabetes.

Keywords: Diabetes; Insulin delivery; Subcutaneous insulin; Oral insulin; Nanoparticles

ملخـــــص

داء السكري هو اضطراب استقلابي مزمن يتميز بفرط سكر الدم المستمر واختلال في التوازن الاستقلابي وغالبًا ما يكون مصحوبًا بمضاعفات مختلفة. على الرغم من أن العلاج التقليدي بالأنسولين تحت الجلد فعال في خفض مستويات الجلوكوز في الدم، إلا أنه غالبًا ما يفشل في محاكاة الإفراز الطبيعي للأنسولين ومعالجة الوظائف الاستقلابية مما يؤدي الي تطور المضاعفات المرتبطة بداء السكري للتغلب على هذه القيود، تستكشف هذه الأطروحة نهجًا جديدًا: تناول الأنسولين عن طريق الفم المحصور في جسيمات nCOF .

تستكشف هذه الدراسة الإمكانيات العلاجية للأنسولين الفموي المحصور داخل nCOF في تعزيز توافر الأنسولين حيويًا والحفاظ على فعاليته في الجسم. تشير نتائجنا إلى أن الأنسولين الفموي المحصور داخل nCOF يقلل من فرط سكر الدم بفعالية دون التسبب في نقص سكر الدم و يحافظ على مستويات الجلوكوز في الدم عند مستويات قابلة للمقارنة مع الحالات غير المصابة بالسكري، سواء في حالات الصيام أو بعد الأكل، مما يظهر فوائد محتملة لتحسين حساسية الأنسولين ومنع مقاومة الأنسولين.

إلى جانب التحكم في نسبة السكر في الدم، تكشف نتائجنا أن الأنسولين الفموي المحصور داخل nCOFيوفر فوائد نظامية من خلال تخفيف الاضطرابات في توازن الدم، وكذلك تقليل خلل شحميات الدم، والإجهاد التأكسدي وإصلاح الأضرار في مختلف الأعضاء. هذا النهج الشامل لإدارة مرض السكري يبرز الإمكانيات التي يوفرها الأنسولين الفموي المحصور داخل nCOF ليس فقط في تقليل فرط سكر ولكن أيضًا في استعادة التوازن الأيضي والفيزيولوجي مما يقدم الأنسولين الفموي المحصور داخلnCOF استراتيجية علاجية كاملة.

بشكل عام، تقدم هذه الأطروحة الأنسولين الفموي المحصور داخل nCOF كمرشح واعد لتعزيز رعاية مرضى السكري وقدرته على معالجة الجوانب المتعددة للمرض و تمثل خطوة مهمة إلى الأمام في إدارة داء السكري.

الكلمات المفتاحية: داء السكري ؛ توصيل الأنسولين ؛ الأنسولين تحت الجلد ؛ الأنسولين الفموي ؛ الجسيمات النانوية

Résumé

Le diabète est un trouble métabolique caractérisé par une hyperglycémie chronique et un déséquilibre de l'homéostasie métabolique, souvent accompagné de diverses complications. Bien que la thérapie traditionnelle à l'insuline sous-cutanée soit efficace pour abaisser les niveaux de glucose dans le sang, elle ne parvient souvent pas à imiter pleinement la sécrétion naturelle de l'insuline ni à réguler correctement les autres fonctions métaboliques, ce qui peut contribuer au développement des complications associées au diabète. Pour surmonter ces limites, cette thèse explore une approche novatrice : l'administration orale d'insuline encapsulée dans des particules nCOF (insuline/nCOF orale).

Cette étude explore le potentiel thérapeutique de l'insuline/nCOF orale pour améliorer la biodisponibilité de l'insuline et préserver son efficacité *in vivo*. Nos résultats indiquent que l'insuline/nCOF orale réduit efficacement l'hyperglycémie sans induire d'hypoglycémie. Elle maintient des niveaux de glucose sanguin comparables à ceux des états non diabétiques, tant en conditions de jeûne qu'après les repas, démontrant des avantages potentiels pour améliorer la sensibilité à l'insuline et prévenir la résistance à l'insuline.

Au-delà du contrôle glycémique, nos résultats révèlent que l'insuline/nCOF administrée oralement exerce des effets systémiques en atténuant les perturbations de l'hémostase, ainsi qu'en réduisant la dyslipidémie, le stress oxydatif et en réparant les dommages tissulaires dans divers organes. Cette approche holistique de la gestion du diabète souligne le potentiel de l'insuline/nCOF orale non seulement pour réguler la glycémie, mais aussi pour restaurer une homéostasie métabolique et physiologique, offrant ainsi une stratégie de traitement complète.

Dans l'ensemble, cette thèse présente l'insuline/nCOF orale comme un traitement prometteur pour améliorer la prise en charge du diabète. Sa capacité à traiter les différentes dimensions de la maladie constitue une avancée significative dans sa gestion.

Mots-clés: Diabète; Délivrance d'insuline; Insuline sous-cutanée; Insuline orale; Nanoparticules

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And lastly, I would like to dedicate a thought to my mother, who is no longer with us but would have been so proud of me today.

Table of content

nCOF particles	Nanoporous Covalent Organic Framework particles
nCOF/Insulin	Insulin loaded into covalent framework nanoparticles
Oral nCOF/Insulin	Oral Insulin loaded into covalent framework nanoparticle
SC Insulin	Subcutaneous Insulin
T1D	Type 1 Diabetes
β-cells	Beta cells
t1/2	Plasma half-life
ROS	Reactive Oxygen Species
TEM	Transmission Electron Microscopy
AUC	Area Under the Curve
HOMA	Homeostatic Model Assessment
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
HOMA-IS	Homeostasis Model Assessment of Insulin Sensitivity
ASAT	Aspartate Aminotransferase
ALAT	Alanine Aminotransferase
PT	Prothrombin Time
APTT	Activated Partial Thromboplastin Time
MDA	Malondialdehyde
***COVID*-19**	*Coronavirus* Disease of 2019
GSH	Glutathione
O_2^-	Anion Superoxide
TC	Total Cholesterol
HDL-C	High-Density Lipoprotein Cholesterol
TG	Triglycerides
LDL-C	Low-Density Lipoprotein Cholesterol

Introduction

The optimal functioning of the human body relies on a consistent and steady supply of energy, primarily sourced from glucose metabolism facilitated by food intake. Essential to this process is the maintenance of glycemic homeostasis, ensuring blood glucose levels remain around 0.8 grams per liter. This regulation, crucial during both fed and fasting states, is primarily overseen by insulin, a pivotal hormone. Disruption of this delicate balance results in a metabolic disorder marked by elevated circulating glucose levels, known as diabetes (Diop, 2018).

Diabetes mellitus is a chronic metabolic disorder characterized by persistent hyperglycemia, resulting from deficiencies in insulin secretion, insulin action, or a combination of both. This global health challenge affects millions of people and is associated with a multitude of complications that have a profound impact on health and mortality (Sun et al., 2024). While several oral anti-diabetic medications exist for treating type 2 diabetes, management of type 1 diabetes (T1D) primarily relies on insulin therapy, often administered via subcutaneous injections.

Although subcutaneous insulin (SC insulin) effectively lowers blood glucose, it is associated with significant challenges, including the risk of hypoglycemia and unpredictable glucose fluctuations. Moreover, its unphysiological distribution in the body may contribute to the development of diabetes-related complications (Antar et al., 2023; Li et al., 2023). SC Insulin therapy often fails to mimic the natural physiological balance between insulin's role in glucose regulation and its broader metabolic functions (Sharma and Singh, 2020; Zhang et al., 2024). Therefore, there is an urgent need for therapeutic strategies that not only regulate blood glucose but also facilitate a more natural distribution of insulin, potentially preventing complications and enhancing the quality of life for patients.

Oral insulin emerges as an ideal method for achieving a more physiological distribution of insulin throughout the body, closely mimicking natural insulin secretion (Limenh 2024; E. Zhang et al. 2024). However, the chemical structure and nature of insulin prevent it from surviving the harsh

digestive environment and crossing the intestinal barrier without being degraded, compromising its bioactivity (Still, 2002). Encapsulation of insulin in nanoparticles offers a potential solution to these challenges (Han et al., 2024; Zhang et al., 2024). The current medical challenge lies in developing a system that can both preserve and deliver insulin effectively while avoiding toxicity to the body (Ramesan and Sharma, 2009; Caturano et al., 2024).

This thesis aims to investigate the oral delivery of insulin using newly synthesized nanoparticle formulations using covalent organic framework (COF) nanoparticles for oral insulin delivery. This innovative approach aims to enhance the delivery and effectiveness of insulin therapy, potentially offering a safer and more efficient method for managing diabetes. These nanoparticles have demonstrated impressive in vitro results, highlighting unique chemical properties that distinguish them from conventional oral insulin formulations and suggest superior biological outcomes. Investigating its efficacy and safety is crucial for establishing a more effective and safer strategy for managing diabetes. This study represents the first exploration of the therapeutic effects of oral nCOF/Insulin in vivo. Initially, we evaluated its efficacy to ascertain whether encapsulation within nCOF maintains insulin's biological activity and effectiveness in both fasting and fed states. Subsequently, we examined the impact of oral nCOF/Insulin on broader metabolic pathways. This thesis also reviews the pivotal role of endogenous insulin in maintaining glucose homeostasis and underscores the limitations associated with current insulin delivery methods. By closely mimicking natural insulin release, this innovative delivery approach holds potential to enhance therapeutic efficacy while mitigating the risks associated with traditional insulin therapies.

Insulin in Diabetes: An Overview

Insulin emerges as the primary hypoglycemic hormone (Qaid and Abdelrahman, 2016), orchestrating a swift restoration of plasma glucose concentrations to basal levels following a meal. Its multifaceted importance extends beyond glucose regulation, encompassing crucial roles in skeletal muscle protein synthesis, glycogenesis, and lipogenesis (Norton et al., 2022).

Vital for human survival, *endogenous insulin* intricately governs glucose metabolism, maintaining a delicate balance essential for sustaining life (Huang et al., 2020). The secretion of endogenous insulin occurs in two distinct phases. During fasting periods, pancreatic β-cells continuously release low concentrations of insulin to prevent excessive catabolism. Postprandially, insulin secretion increases significantly, playing a crucial role in energy storage and inhibiting endogenous glucose production (Mathieu et al., 2021).

This mechanism ensures that blood glucose levels remain tightly regulated. Endogenous insulin possesses unique pharmacological properties that allow for precise control of glucose levels. After a meal, the plasma glucose concentration returns to normal within two hours (Norton et al., 2022). The pulsatile secretion of insulin, characterized by major peaks occurring every 1.5 to 2 hours, is thought to play a significant role in coordinating metabolic responses, particularly in the liver. This rhythmic pattern of insulin release reflects the pharmacodynamic principles of endogenous insulin, as it helps synchronize metabolic processes to maintain glucose homeostasis. Insulin secretion is not solely determined by glucose concentration; it is also influenced by neural, hormonal, and metabolic signals (Henquin et al., 2003; Migrenne et al., 2006; Cantley, 2014), allowing for precise adjustments to meet the body's metabolic needs. Furthermore, insulin secretion does not respond as a linear function of glucose concentration, demonstrating the complex regulation of insulin release. Instead, it follows a sigmoidal curve in response to changes in glucose levels, ensuring that insulin release is finely tuned to prevent extreme fluctuations in

[1] *Endogenous insulin*

blood glucose (Tolic et al., 2000). This non-linear response ensures precise adjustments in insulin secretion to meet the body's metabolic requirements, contributing to glucose homeostasis. Additionally, the short plasma half-life (t1/2) of insulin, approximately four minutes, enables rapid clearance from the bloodstream. This rapid clearance, a key pharmacokinetic characteristic of physiological insulin, allows the body to quickly adjust insulin levels to counter any rise in glucose concentration and maintain balanced glucose levels (Hoffman and Ziv, 1997).

The intricate coordination of both *pharmacodynamics* and *pharmacokinetic* characteristics *of physiological insulin* underscores its pivotal role in maintaining glucose homeostasis. Physiological insulin exhibits a dose-dependent response to glucose elevations, releasing in a pulsatile manner to prevent hypoglycemia. This pattern, characterized by rapid peaks and troughs, is governed by feedback loops and signaling pathways. Additionally, rapid clearance ensures that insulin remains within a tightly controlled range, avoiding excessive fluctuations in glucose levels.

In addition to its dynamic release, the *distribution* of insulin throughout the body is essential for its efficacy. Insulin is primarily secreted into the portal circulation, allowing it to first act on the liver, where it plays a critical role in regulating glucose metabolism before reaching peripheral tissues. Under physiological conditions, after its secretion from the pancreas, insulin enters the portal vein and is partially extracted by the liver—approximately 50%—where hepatocytes are stimulated to store glucose as glycogen. The liver thus serves as the primary site of insulin action. Once insulin passes through the liver, it enters the systemic circulation, where it becomes more diluted. Here, it exerts its effects on skeletal muscle cells and adipocytes, the other major targets of circulating insulin, facilitating glucose uptake and reducing blood glucose levels to baseline. Consequently, in postprandial conditions, insulin concentrations in the portal vein and hepatic sinusoids are higher than those in the systemic circulation, ensuring that the liver efficiently regulates glucose homeostasis before peripheral tissues are engaged. (Lewis et al., 2021; Rahman

[2]*Pharmacodynamics of physiological insulin*
[3]*Pharmacokinetic of physiological insulin*
[4]*Distribution of insulin*

et al., 2021). This distribution pattern is physiologically significant, as it enables precise regulation of hepatic glucose production and uptake, ultimately contributing to overall glucose homeostasis.

Overall, the complex interplay between the pharmacodynamic response of insulin to glucose stimuli and its pharmacokinetic distribution within the body highlights the remarkable precision required for maintaining glucose balance. Understanding these intricacies is essential for elucidating the pathophysiology of insulin-related disorders and developing targeted therapeutic interventions.

Therefore, what happens when there is no insulin? In the absence of insulin, postprandial glucose cannot enter cells effectively, resulting in elevated blood glucose levels. This persistent hyperglycemia can lead to various complications, including damage to blood vessels, nerves, and organs (Giri et al., 2018; Matoori, 2022). Additionally, without insulin's anabolic effects, the body resorts to catabolism, breaking down muscle and fat for energy, which can lead to muscle wasting (Ruegsegger et al., 2018). Insulin deficiency also impairs lipid metabolism, resulting in elevated levels of circulating triglycerides and cholesterol, which increases the risk of cardiovascular disease (Saltiel and Kahn, 2001; Ginsberg et al., 2005; Prince et al., 2011). Furthermore, at the cellular level, the disruption of glucose uptake creates an intracellular glucose deficiency, forcing cells to rely on alternative energy sources, such as fatty acids. This metabolic shift can lead to an overproduction of reactive oxygen species (ROS) (Newsholme et al., 2007; Ruegsegger et al., 2018). When combined with ROS generation induced by hyperglycemia, dyslipidemia, and inflammation, this systemic stress affects the entire body, contributing to metabolic dysregulation (Wronka et al., 2022). Overall, the absence of insulin leads to a cascade of metabolic disturbances, primarily chronic hyperglycemia, which characterizes diabetes (Jiang et al., 2020; Rui-jun et al., 2020; Flenkenthaler et al., 2021).

Diabetes impacts nearly 10% of the population (Wu et al., 2023), with type 1 diabetes mellitus (insulin-dependent) being a significant subset. In type 1 diabetes, individuals do not produce any insulin and depend on exogenously administered insulin for survival (Zhang et al., 2018).

The traditional treatment for type 1 diabetes involves the administration of *exogenous insulin*, primarily through subcutaneous injections. Several *insulin analogs* have been designed to mimic the physiological release of insulin as closely as possible. In a healthy individual, insulin is continuously released at a basal level, with secretion peaking approximately one hour after a meal and then declining over the next two hours. However, in diabetic patients, achieving a 24-hour insulin profile that mimics this natural pattern, both during fasting conditions and in the postprandial state, is challenging.

Diabetic patients require insulin formulations with specific pharmacodynamic and pharmacokinetic properties to effectively manage their blood glucose levels throughout the day and night, both during fasting and postprandial conditions. Insulin analogs must be carefully engineered to possess the right balance of these properties to ensure optimal glucose control (Karmakar et al., 2023). To address the diverse needs of diabetic patients, insulin formulations are designed with specific onset, peak time, and duration of action. Typically, individuals with type 1 diabetes use a combination of long-acting (basal) insulin analogs to provide a steady background insulin level and rapid-acting (bolus) insulin analogs to address postprandial glucose spikes. Additionally, some may also use regular insulin for specific needs (Hirsch et al., 2020).

While subcutaneous insulin delivery remains the most conventional method, its convenience is compromised by numerous adverse effects, including hypoglycemic events, discomfort from multiple daily injections, and peripheral hyperinsulinemia, among others (Karmakar et al., 2023). Moreover, managing insulin therapy for type 1 diabetes patients proves challenging due to the variability in dosage and frequency, influenced by factors such as age, weight, physical activity, and individual insulin sensitivity (Mathieu et al., 2021). This complexity underscores the difficulty in achieving optimal treatment outcomes.

However, the challenges extend beyond mere dosage adjustments. Despite advancements in insulin analogue development, it's crucial to recognize that subcutaneous insulin administration

[5] *Exogenous insulin*
[6] *Insulin analogs*

itself remains inherently non-physiological. This method fails to maintain glycemic balance with the necessary precision due to its mismatch with required pharmacodynamic and pharmacokinetic criteria. In a healthy individual, insulin release is tightly regulated through a complex system of feedback mechanisms. Endogenously secreted insulin not only lowers blood glucose levels but also self-regulates through negative feedback mechanisms to prevent hypoglycemia. Additionally, hormones like glucagon and epinephrine counteract insulin's actions, ensuring glucose levels remain within a narrow range. However, in individuals with type 1 diabetes, these hormones are also impaired. When insulin is administered subcutaneously to individuals with type 1 diabetes, it lacks the dynamic regulation provided by these feedback mechanisms. Without the ability to sense changes in blood glucose levels and adjust insulin secretion accordingly, subcutaneous insulin injection leads to a pharmacodynamic profile that differs from endogenous insulin release. This lack of physiological feedback results in less precise glycemic control and an increased risk of hypoglycemia or hyperglycemia (Guerci and Sauvanet, 2005). In addition to the pharmacodynamic challenges associated with subcutaneous insulin administration in type 1 diabetes, there are also notable pharmacokinetic limitations. While insulin released directly by the pancreas acts solely based on its lifespan, subcutaneously injected insulin undergoes absorption, affecting and modulating peak insulin concentration levels. This alteration heightens the risk of late hypoglycemia, complicating glycemic management for individuals with type 1 diabetes (Hoffman and Ziv, 1997). Furthermore, the central-to-peripheral distribution of insulin is not replicated, leading to significantly higher blood concentrations than those secreted by the pancreas, resulting in non-physiological hyperinsulinemia. Moreover, this *iatrogenic hyperinsulinemia*, commonly observed in type 1 diabetes patients, can lead to a second hidden deviation from the physiological norm: insulin resistance.

The emergence of insulin resistance from insulin injections poses a significant challenge in type 1 diabetes treatment (Paulsen et al., 1979). This insulin resistance often leads to the need for higher insulin doses to achieve adequate glycemic control (Kilpatrick et al., 2007). However, even with

[7] *Iatrogenic hyperinsulinemia*

higher doses, it can be challenging to reach and maintain glucose levels within the target range (Shah et al., 2016). This increased glycemic instability raises the risk of long-term complications such as cardiovascular diseases, kidney diseases, and neuropathies (Cleland et al., 2013; Ndisang et al., 2017). Moreover, alongside diminished insulin sensitivity, this insulin resistance adds another layer of complexity to diabetes management, highlighting the intricate interplay among insulin distribution, sensitivity, and metabolic outcomes. (Donga et al., 2013; Gregory et al., 2020).

What would an ideal treatment entail? An ideal insulin regimen should aim to mimic the natural physiological response to glucose concentrations by releasing insulin accordingly, thereby avoiding the fluctuations between hyperglycemia and hypoglycemia often associated with fixed insulin doses (Wu et al., 2023). It would accurately replicate both basal (background) and prandial/post-prandial (meal-related) insulin secretion profiles to achieve comprehensive glycemic control throughout the day (Guerci and Sauvanet, 2005). Specifically, the treatment should aim to prevent excessive insulin action in peripheral tissues by restoring the normal balance of insulin distribution between hepatic and peripheral tissues. This balance would not only help alleviate insulin resistance caused by hyperinsulinemia but also address hyperglycemia (Gregory et al., 2020). Ultimately, the goal of an ideal insulin regimen is to maintain stable blood glucose levels, closely resembling those of a non-diabetic individual, to promote optimal health and prevent complications associated with diabetes.

Intensive treatments were initially considered as optimal because they involve closely monitoring blood glucose levels and administering multiple daily insulin injections or continuous subcutaneous insulin infusion (insulin pump therapy). This approach aims to mimic the physiological insulin secretion pattern more closely, with both basal (background) and prandial (meal-related) insulin doses adjusted to match individual carbohydrate intake and blood glucose levels. The goal is to achieve optimal glycemic control and minimize the risk of long-term complications associated with diabetes (Gubitosi-Klug et al., 2016; Bratseth et al., 2020). However, intensified insulin therapy was associated with a greater risk of hypoglycemia (Guerci and Sauvanet, 2005). Furthermore, whether administered via pump or subcutaneously, they do not address the issue of distribution, which remains non-physiological due to its lack of central-to-peripheral regulation (Dal et al., 2015).

Among insulin administration methods, the oral route is favored for closely mimicking endogenous insulin. Upon oral intake, insulin is absorbed from the intestinal lumen and travels to the liver through the portal circulation, undergoing first-pass metabolism. This process results in insulin entering the peripheral circulation at lower levels, closely resembling the physiological insulin pathway and enhancing blood glucose control in type 1 diabetes (Elsayed, 2012; Norton et al., 2022; Elsayed et al., 2023).

However, oral delivery faces significant challenges, primarily due to proteolytic degradation in the gastrointestinal tract if unprotected, and insufficient absorption through the intestinal mucosa without chemical or physical enhancement (Karmakar et al., 2023). In addressing these limitations, nanoparticles offer a promising avenue for enhancing the effectiveness of oral insulin delivery (Damgé et al., 2008; Xi et al., 2022; Xu et al., 2022; Vasconcelos et al., 2023).

Indeed, encapsulating insulin in nanoparticles not only shields it from proteolytic degradation and facilitates its passage through the intestinal barrier but also presents a groundbreaking opportunity to precisely control release kinetics, enhance oral bioavailability, and modulate the biological response (Woitiski et al., 2010). Furthermore, it is important to recognize that today's understanding of diabetes extends beyond chronic hyperglycemia. Diabetes is now understood as a complex *dysglycemic disorder*, involving hyperglycemia, glucose variability, and hypoglycemic episodes. Subcutaneous insulin, the traditional treatment, often fails to adequately address these glycemic abnormalities. Not only may subcutaneous insulin not effectively target and address postprandial hyperglycemia (Zhang et al., 2019), but its short duration of action necessitates multiple daily injections (Schofield et al., 2019). Each injection carries the risk of hypoglycemia. Consequently, while insulin is intended to regulate blood glucose levels, it can contribute to glycemic variability in diabetic patients, exacerbating the dysglycemic disorder and becoming part of the disease itself.

[8] *Dysglycemic disorder*

To summarize, oral insulin administration represents a more physiological approach to managing diabetes. However, addressing its associated challenges requires innovative strategies, such as nanoparticle encapsulation, to enhance bioavailability and therapeutic efficacy. Moreover, any effective therapeutic approach must not only manage hyperglycemia but also ensure biocompatibility and mitigate the consequential complications of diabetes.

In vivo oral insulin delivery via covalent organic frameworks

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While oral insulin may appear to be the ideal treatment for type 1 diabetes, it is associated with several persisting issues: toxicity, low bioavailability, absorption challenges, and stability concerns. In their study, Benyettou et al. described the optimal oral insulin delivery method as a biocompatible, high-loading platform that shields insulin from external acidic environments and enzymatic degradation. Furthermore, they emphasized the importance of incorporating targeted drug delivery alongside stimuli-responsive drug release mechanisms, such as hyperglycemia. Their research findings suggest that the nCOF stands as the best vehicle for oral insulin delivery as it fulfills all the stated characteristics.

In their study, they conducted a series of *in vitro* tests to determine the chemical properties of the Oral nCOF/Insulin. They found that it was biocompatible, as it did not induce toxicity or oxidative stress *in vitro* on various cell lines, including liver, colon, cervix, ovary, breast, kidney, and brain. Additionally, it was found to be non-immunotoxic to human blood erythrocytes.

Stability tests revealed that nCOF/Insulin remained stable when exposed to simulated gastric and intestinal fluids, with negligible insulin release observed after 24 hours of incubation. Transmission Electron Microscopy (TEM) imaging confirmed that its size and morphology remained unchanged under stomach-like conditions.

Additionally, the study demonstrated nCOF/Insulin's high loading capacity of over 70%, enabling efficient delivery of large insulin doses in a smaller volume. This capability allows for substantial insulin administration at once, ensuring prolonged coverage.

Finally, Benyettou et al. illustrated that it releases insulin in a dose-response manner to glucose and can adopt a pulsatile release mode, thus meeting all the necessary requirements for an effective oral insulin delivery system.

Drawing upon the extensive groundwork established by our co-authors, we conducted a pharmacological study to investigate the in vivo response of this Oral nCOF/Insulin. To achieve this, we administered Oral nCOF/Insulin and subcutaneous insulin to two groups of diabetic rats and compared the results with those of untreated diabetic rats and non-diabetic rats.

Firstly, we assessed the *bioavailability* of the Oral nCOF/Insulin. This evaluation provided us with insights into the effectiveness of this nCOF for delivering insulin orally, enabling it to reach systemic circulation and exert pharmacological effects. The calculations show that Oral nCOF/Insulin particles exhibit a high bioavailability (24.1%).

Then, we assessed the *pharmacokinetic* to track and compare insulin concentration over time following oral and subcutaneous administration. Plasma insulin levels in diabetic rats treated with Oral nCOF/insulin showed that insulin is released gradually into the bloodstream. Insulin concentrations in the blood increase gradually after administration and stabilize throughout the duration of the experiment. Rats injected with subcutaneous insulin experienced a typical peak in plasma insulin levels within the first hour after injection, after which it rapidly declined. Notably, despite insulin's short half-life, the plasma concentrations of insulin of diabetic rats treated with Oral nCOF/Insulin remained stable throughout the entire experiment, indicating a continuous release of the insulin from the nCOF over time, unlike the single bolus release observed with subcutaneous injection.

Subsequently, we studied the *pharmacodynamics* by measuring fasting blood glucose and postprandial blood glucose levels in diabetic rats after administering both types of insulin, then comparing the results. The objective was to understand how each form of insulin affects blood glucose regulation at different times of the day and after meals. Our investigation revealed that Oral nCOF/Insulin administered under fasting conditions gradually normalized blood glucose levels in diabetic rats, reaching levels comparable to those of non-diabetic rats, and sustained them at low levels throughout the duration of the experiment (10 hours). This indicates that Oral nCOF/Insulin does not induce hypoglycemia even when administered while fasting, further demonstrating its potential to release insulin in a dose-stimulated manner to glycemia. In contrast, subcutaneous insulin administration resulted in an immediate hypoglycemic response followed by a rapid return to hyperglycemic levels characteristic of diabetes.

[9] *Bioavailability*
[10] *Pharmacokinetic of exogenous insulin*
[11] *Pharmacodynamics of exogenous insulin*

To evaluate the postprandial effects and compare Oral nCOF/Insulin with subcutaneous insulin, we conducted an oral glucose tolerance test. Subcutaneous insulin effectively lowered plasma glucose levels initially, however by 150 minutes; levels began to rise, nearing diabetic control values. However, although the hypoglycemic effect of Oral nCOF/Insulin was initially milder, blood glucose levels remained low and stable from 120 minutes onwards until the end of the experiment. These findings indicate that Oral nCOF/Insulin enables diabetic rats to maintain blood glucose levels similar to those of non-diabetic rats, both in fasting and postprandial conditions.

We calculated the areas under the curve (AUC) to comprehensively assess the efficacy of both types of insulin in the long term. The primary benefit of calculating the AUC lies in its ability to synthesize data over an extended period, providing an overview of the overall treatment impact. Specifically, in the context of glycemic regulation, the AUC of glucose levels reflects the treatment's overall effectiveness in stabilizing blood glucose. Our results perfectly demonstrate that Oral nCOF/Insulin maintains glucose levels stable and within a similar range, whether fasting or postprandial. This consistency in glucose maintenance underscores the remarkable ability of Oral nCOF/Insulin to limit glycemic fluctuations, which is crucial for effective diabetes control.

Insulin resistance poses a significant challenge in the treatment of type 1 diabetes, complicating glycemic control and raising the risk of complications. Therefore, it's crucial to develop strategies for managing insulin resistance as part of comprehensive type 1 diabetes treatment, with the ideal treatment meeting this requirement. With this goal in mind, we assessed the sensitivity and resistance of the body to orally administered insulin via nCOF compared to subcutaneous insulin, using the HOMA index.

The homeostatic model assessment (HOMA) is a method used to estimate insulin sensitivity and insulin resistance based on fasting plasma insulin and glucose concentrations. Thus, we aimed to compare insulin resistance, estimated by *HOMA-IR*, and insulin sensitivity, estimated by *HOMA-IS*, calculated from area under the curve and insulin plasma levels.

[12] *HOMA-IR*
[13] *HOMA-IS*

Our results indicated that rats treated with Oral nCOF/insulin exhibited lower HOMA-IR (insulin resistance index) and higher HOMA-IS (insulin sensitivity index) compared to those receiving subcutaneous insulin injections. This suggests that insulin delivered orally via nCOF particles is more effectively absorbed and assimilated by the body compared to subcutaneously injected insulin.

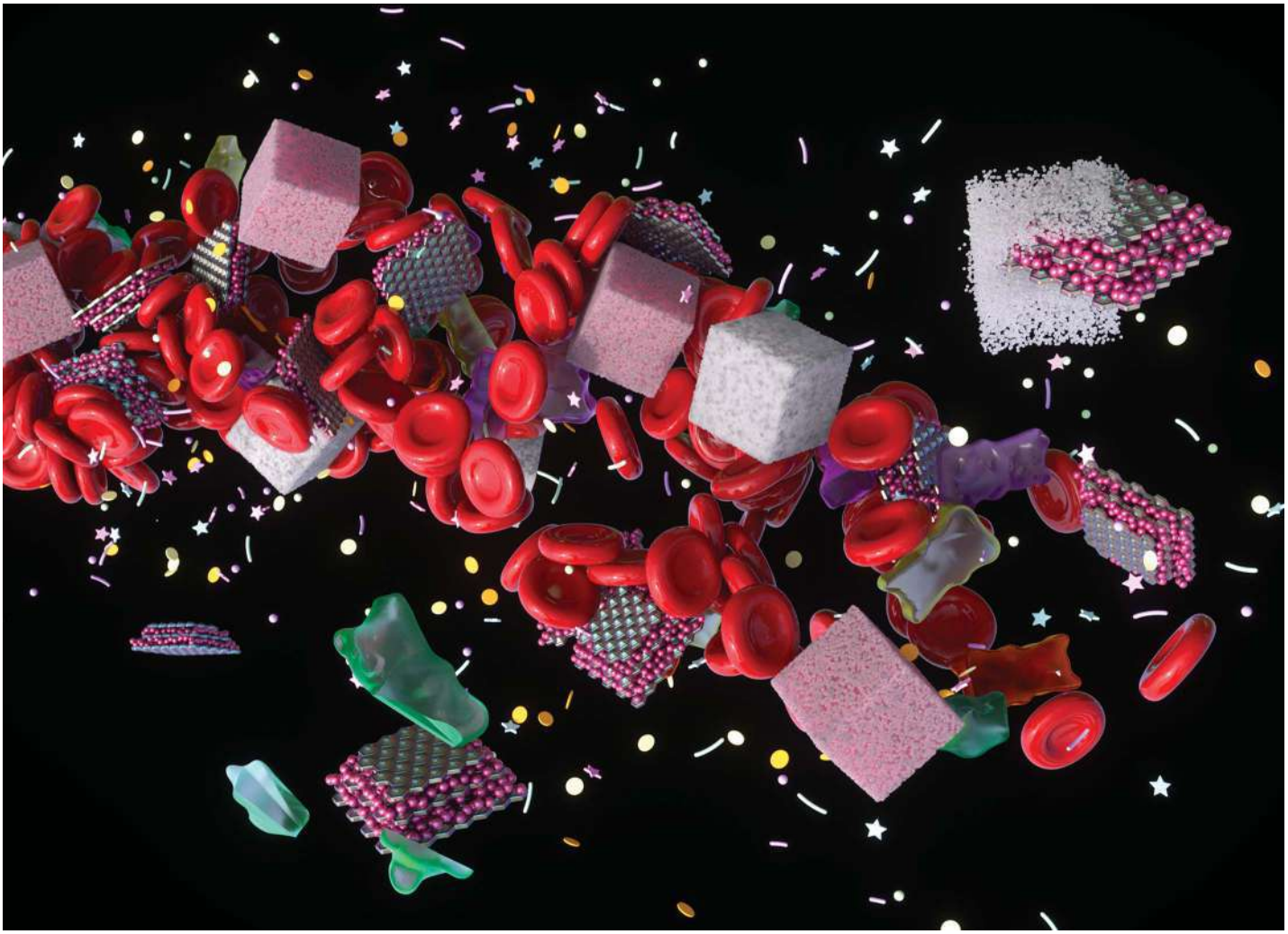
We completed our investigation by conducting preliminary toxicity assessments, focusing specifically on the liver and kidneys. These organs play a crucial role in eliminating foreign substances from the body. The liver metabolizes chemicals to mitigate their toxicity, while the kidneys filter the blood and eliminate waste via urine. Evaluating the safety of organic compounds like nCOF necessitates carefully assessing their impact on these organs. For this purpose, we measured biochemical markers for liver and kidney functions, as they can offer valuable insights into the onset of toxic effects. Specifically, we assessed urea and creatinine levels to detect changes in kidney function, and aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) levels to evaluate liver damage. Additionally, we conducted a histopathological analysis of these two organs to investigate potential histological alterations.

Overall, diabetic rats treated with Oral nCOF/insulin exhibited urea levels comparable to those of diabetic rats treated with subcutaneous insulin, while other parameters such as creatinine, ASAT, and ALAT were more closely aligned with the non-diabetic group. This suggests that, in addition to being non-toxic, nCOF/insulin particles may offer advantages in maintaining kidney and liver functions when compared to traditional subcutaneous insulin administration.

These results are supported by histological findings. Observation of the kidneys of diabetic rats, as well as those treated with subcutaneous insulin, revealed phantom glomeruli and necrotic tubules. Likewise, examination of the livers of diabetic rats and those treated with subcutaneous insulin showed pathological dilation, disrupted hepatocellular architecture, and necrotic or early necrotic hepatocytes. In contrast, observation of the organs of diabetic rats treated with Oral nCOF/Insulin showed not only no particular toxicity but also prevented tissue alterations induced by diabetes. The kidneys of diabetic rats treated with Oral nCOF/Insulin exhibited a structure similar to that of non-diabetic rats, with prominent glomeruli and tubules appearing normal.

Similarly, the liver displayed a healthy architecture, with hepatocytes well-organized around central lobular veins, binucleated with dense nuclei, indicating good metabolic activity and effective assimilation of plasma glucose. Ultimately, our findings demonstrate that Oral nCOF/Insulin not only does not induce toxicity but also prevents functional and tissue alterations induced by diabetes.

In conclusion, this study has provided valuable insights into the pharmacological effects of this newly synthesized Oral nCOF/Insulin, tested for the first time *in vivo*. These highly promising results have enhanced our comprehension of the remarkable biological effects observed in subsequent works of this thesis.



Showcasing research from Professor Ali Trabolsi's laboratory, School of Chemistry, NYU Abu Dhabi, Abu Dhabi, United Arab Emirates.

In vivo oral insulin delivery *via* covalent organic frameworks

We report the successful use of a gastro-resistant covalent organic framework for *in vivo* oral delivery of insulin. The cover represents the journey of the insulin-loaded nanoparticle in the blood of a diabetic patient where the sugar levels are high. The large quantities of sugar trigger the release of the insulin from the nanoparticle allowing the sugar to be metabolized by the body.

As featured in:

See Ali Trabolsi *et al.*, *Chem. Sci.*, 2021, **12**, 6037.

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In vivo oral insulin delivery *via* covalent organic frameworks†

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With diabetes being the 7th leading cause of death worldwide, overcoming issues limiting the oral administration of insulin is of global significance. The development of imine-linked-covalent organic framework (nCOF) nanoparticles for oral insulin delivery to overcome these delivery barriers is herein reported. A gastro-resistant nCOF was prepared from layered nanosheets with insulin loaded between the nanosheet layers. The insulin-loaded nCOF exhibited insulin protection in digestive fluids *in vitro* as well as glucose-responsive release, and this hyperglycemia-induced release was confirmed *in vivo* in diabetic rats without noticeable toxic effects. This is strong evidence that nCOF-based oral insulin delivery systems could replace traditional subcutaneous injections easing insulin therapy.

Introduction

With diabetes being the 7th leading cause of death worldwide affecting nearly 10% of the world's population, with a quadrupling of its prevalence since 1980,[1] and accounting for almost 15% of direct healthcare costs,[2] its treatment is of global significance.[3] Diabetes is a chronic disease occurring when no insulin is produced due to the absence of pancreatic β-cell islets (type 1)[4] or the insulin that is produced is incapable of being effectively utilized by the body (type 2).[5] Coupled with lifestyle changes, insulin therapy remains a key element in controlling and regulating blood glucose levels, with the primary mechanism being insulin injection. However, studies have shown delays in the onset of insulin therapy in a large proportion of people with uncontrolled diabetes, and in those who do eventually undertake treatment, there is a delay of more than 2 years.[6] A fear of needles and self-injection[7] as well as pain and anxiety[8] are some of the many reasons why people are unwilling to start insulin therapy. Insulin pens alleviate some of these worries, as well as overcome dosage issues that exist with vials and syringes;[9] however this method is itself not error free.[10] A shift towards oral delivery of insulin has the potential to improve the uptake of insulin therapy and revolutionize diabetes care since it is a noninvasive therapeutic approach without the side effects caused by frequent subcutaneous (S.C.) injection.[11–14]

Orally delivered insulin is capable of reaching the systemic circulation after passing through the liver similar to physiologically secreted insulin, while subcutaneously injected insulin may result in peripheral hyperinsulinemia and associated complications.[15] However, oral drug delivery faces numerous challenges including dissolution, bioavailability, solubility and its stability in the gastrointestinal (GI) tract.[16] The oral bioavailability of insulin is severely hampered by its inherent instability in the GI tract and its low permeability across biological membranes in the intestine (less than 1%).[17] Despite clinical trials of several oral insulin formulations,[18,19] sufficient commercial development has not yet been achieved.[20]

To be considered an effective oral insulin delivery method, the proposed system must comprise a biocompatible, high-loading platform affording insulin protection against external acidic environments and enzymatic degradation, in addition to targeted drug delivery coupled with stimuli-responsive drug release such as hyperglycemia.[21] Nanocarriers such as polymeric, inorganic and solid-lipid nanoparticles have emerged as effective insulin transporters, circumventing many of the problems associated with oral insulin delivery, and show promise for desirable biopharmaceutical and pharmacokinetic properties.[22–24] However, recent clinical trials have resulted in failure due to toxicology, low levels of oral bioavailability and

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elevated intra-individual differences in insulin absorption, thereby offering strong evidence that challenges still persist.[25–28] Two systems have, so far, been FDA approved for the oral delivery of insulin.[29,30] The first one, developed by Oramed (ORMD-0801), incorporates both a species-specific protease inhibitor that protects active ingredients and a potent absorption enhancer that fosters their absorption across the intestinal epithelium. However, the system is non-specific, and its prolonged use may damage the stomach membrane and may lead to toxicity.[29,30] The second, HDV-I by Diasome, is based on liposomes with hepatic targeting and suffers from instability in the GI tract, high cost and drug release during storage.[29,30]

Nanoparticles offer better storage and physiological stability compared to other nanosized colloidal carriers such as liposomes and emulsions,[27] with nanoscale imine-linked covalent organic frameworks (nCOFs)[31] in particular having shown tremendous potential as emerging nanomedicine candidates for drug delivery.[32–41] nCOFs feature a long-range ordered structure in which the organic building blocks are spatially controlled in two or three dimensions leading to regular pores with diameters facilitating the loading and controlled release of large drugs and proteins/enzymes (Table S1†).[39,42,43] In addition, their high flexibility in molecular architecture and functional design make them versatile and therefore give them unique responsivity to their environment.

Herein, our previously described[44] imine-based nCOF obtained from the co-condensation of 2,6-diformylpyridine (DFP) and 4,4′,4″-(1,3,5-triazine-2,4,6-triyl)trianiline (TTA) (denoted as TTA-DFP-nCOF) was prepared from highly crystalline nanoparticles using a seeded growth method[45,46] and was successfully used as an oral insulin delivery system. The choice of the triazine-based TTA-DFP-nCOF was primarily based on its high stability under harsh conditions including acidic environments.[47,48] The unique features of this delivery method are its high insulin-loading capacity (~65 wt%), biocompatibility, insulin protection under harsh conditions and hyperglycemia-

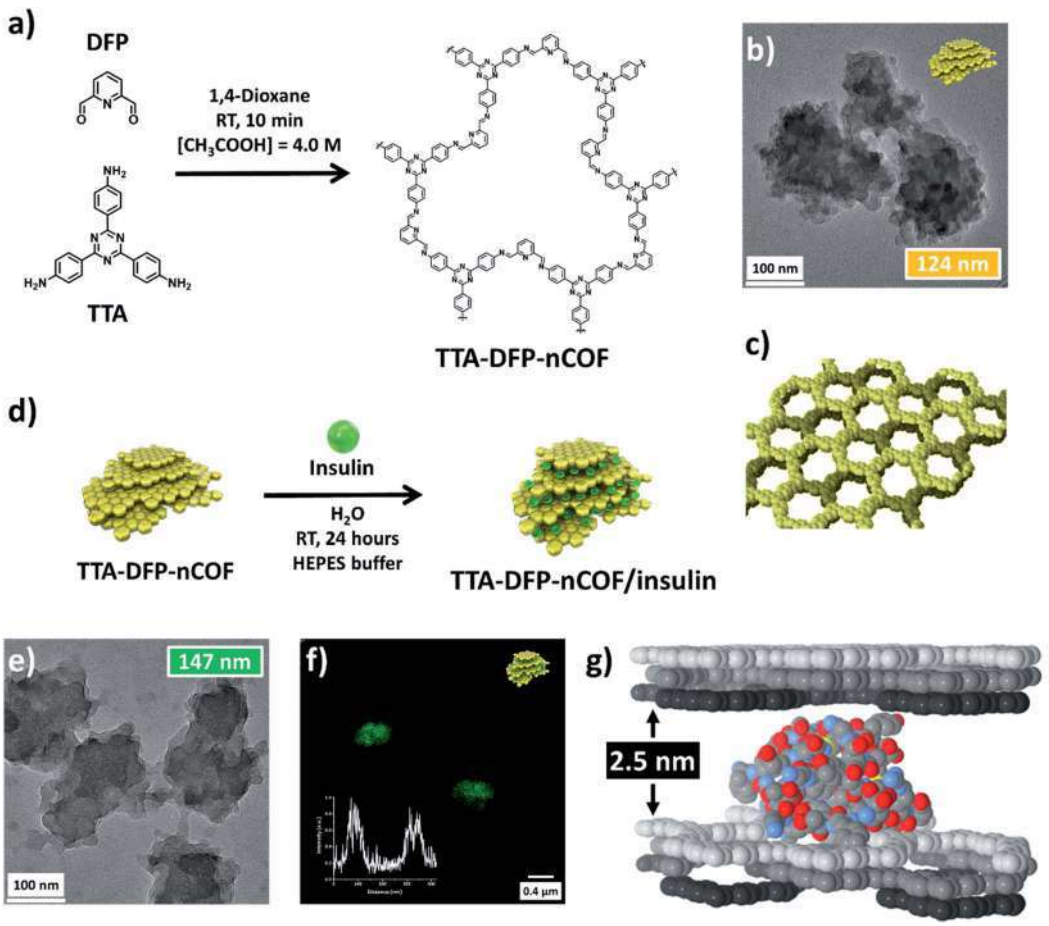

Fig. 1 Insulin is intercalated between TTA-DFP-nCOF layers. (a) Chemical structure and synthesis of the TTA-DFP-nCOF. (b) HR-TEM image of the TTA-DFP-nCOF. Cartoon representation (yellow nanoparticles) of the shape of the TTA-DFP-nCOF. (c) Structural model of the TTA-DFP-nCOF, consisting of hcb layers that are disposed in the abc sequence, generating hexagonal channels along the stacking direction. (d) Schematic representation of the encapsulation of insulin between the layers of the TTA-DFP-nCOF. Cartoon representation (green spheres) represents the insulin. (e) HR-TEM image of TTA-DFP-nCOF/insulin. (f) Confocal microscopy image of TTA-DFP-nCOF/insulin-FITC; inset: fluorescence intensity. (g) van der Waals representation of the optimized location of the insulin monomer molecule intercalated between TTA-DFP-nCOF layers. Atoms belonging to the COF layers are displayed in white, grey, and black color, for each individual layer. For the insulin molecule, the C, N, O, and S atoms are grey, blue, red, and yellow, respectively. H atoms are omitted for clarity.

induced drug release. The insulin-loaded TTA-DFP-nCOF successfully crossed the intestinal barrier and sustainably reduced the blood glucose level *in vivo* in type 1 diabetic (T1D) rat model with the glucose level completely returning to normal as compared to the non-diabetic control group without inducing systemic toxicity. In comparison to the two FDA-approved technologies, our system is biocompatible, highly stable in the stomach, cost effective, specific and glucose-responsive, representing a step forward in the future of oral insulin delivery and a novel pathway toward the treatment of type 1 through nCOF-based oral insulin delivery.

Results and discussion

The TTA-DFP-nCOF was synthesized by co-condensation of DFP (21 mg, 0.15 mmol, 5 equivalents) and TTA (12 mg, 0.03 mmol, 1 equivalent), in anhydrous 1,4-dioxane (3 mL) in the presence of 0.5 mL of 13 M acetic acid ($[\text{acetic acid}]_{\text{final}} = 4.0$ M) at room temperature for 10 min (Fig. 1a). The solution was cleaned using dialysis in H_2O to obtain a stable nanoparticle suspension. After 10 min at room temperature, imine-linked covalent organic nanoparticles with an average diameter of 123.7 nm (Fig. 1b and S2†) emerge from the clear solution without forming amorphous polyimine precipitates. A high concentration of acetic acid ($[\text{acetic acid}]_{\text{final}} = 4.0$ M) induces a rapid imine condensation reaction at room temperature and thus the formation of discrete nCOF crystalline nanosheets (Fig. S2 and S3†). The increased rate of monomer consumption induces both supersaturation in crystalline nanosheets and inhibition of crystallite growth into bigger structures. Subsequently, nanosheets agglomerate by stacking on each other to form polycrystalline nanoparticles of spherical shape with rough surfaces and small protrusions (Fig. S3†); this latter phenomenon is due to the small presence of H_2O co-solvent which favors hydrogen bonding between nanosheets. When the synthesis is performed with pure acetic acid in the absence of H_2O ($[\text{acetic acid}]_{\text{final}} = 5.0$ M), small crystalline nanosheets with limited stacking are obtained without nanoparticle formation (Fig. S4†).

From a drop-cast solution (Fig. S10†), under atomic force microscopy (AFM), the TTA-DFP-nCOF appears as uniform particles with a height of 7 nm, corresponding to the stacking of ~18 nCOF layers. In solution, they present themselves as particles with an average hydrodynamic radius of 82 nm and a polydispersity index (PDI) of 0.1 from dynamic light scattering (DLS) analysis, with no precipitation observed over time. Once isolated as solids using a freeze-drying process, the nanoparticles could be re-dispersed in H_2O and were stable in solution for at least twelve months, during which their size distribution remained unchanged (Fig. S11†). Crystallinity of the as-synthesized material was demonstrated by powder X-ray diffraction (PXRD), which features a strong peak at $2\theta = 4.9°$ assigned to the (110) plane of the regularly ordered lattice, as well as a broad peak centered at $2\theta = 25.6°$, corresponding to the reflection from the (003) plane (Fig. 1c and S12†). Seed-mediated crystallization can explain the increased crystallinity as the amorphous imine polymer formation commonly observed in nCOF powders is not observed.[44,49] N_2 sorption demonstrates the permanent porosity of nCOFs. The shape of the isotherm combines type I and II features, and the Brunauer–Emmett–Teller (BET) surface area is 384.5 $m^2\ g^{-1}$ (Fig. S13†). The isotherm displays an H3-type hysteresis indicative of aggregates of plate-like particles giving rise to slit-shaped pores,[50] similar to those of nanosheet-based materials reported elsewhere.[51,52]

FTIR analysis of the nCOF shows the characteristic imine stretching at 1617 cm^{-1}, a peak at 1582 cm^{-1} attributed to the C–C=N bond of the triazine and pyridine stretching, a broad and strong peak at 1365 cm^{-1} corresponding to the C–N bond of the triazine core and a strong vibration peak at 1507 cm^{-1} corresponding to the C=C aromatic bonds, along with the disappearance of the TTA primary amine N–H stretching at ~3320 cm^{-1} and the DFP aldehyde stretching at 1711 cm^{-1} (Fig. S14†).[53,54] The chemical stability of the TTA-DFP-nCOF under conditions designed to simulate the stomach environment (pH 2.0) as well as the bloodstream (pH 7.4) was assessed. The TTA-DFP-nCOF remained unaffected under both conditions as confirmed through TEM imaging (Fig. S2–S5†), PXRD and the BET analysis (Fig. S17†). These features prompted us to use these nCOFs for oral insulin delivery. Collectively, these bulk characterization experiments indicate that the nanosized TTA-DFP-nCOFs form high quality imine-linked nCOFs.

Insulin-loading of the TTA-DFP-nCOF was first realized by soaking the TTA-DFP-nCOF (5 mg) in insulin solution (5 mg in 2.5 mL HEPES buffer, pH 7.4). Insulin loading was monitored using ^{1}H NMR over a period of 24 hours (Fig. S18†) and showed that the insulin signals progressively decreased following the addition of the nanoparticles due to the encapsulation of insulin inside the TTA-DFP-nCOF. However, no conclusions could be made on the nature of interactions from the NMR experiment.

The use of dye (FITC)-labelled-insulin (insulin-FITC) facilitates the quantification of the amount of loaded insulin. Insulin-FITC uptake was measured using fluorescence spectroscopy of the supernatant to quantify the concentration of unloaded insulin-FITC (Fig. S19 and S20†) with the TTA-DFP-nCOF exhibiting an insulin-FITC loading capacity of 64.6 ± 1.7 wt% (Fig. S20†), comparable to that of previously reported

Table 1 Physicochemical characterization of the TTA-DFP-nCOF before and after insulin loading

	TTA-DFP-nCOF	TTA-DFP-nCOF/insulin
PXRD, 2θ (plane)	4.9° (110), 25.6° (003)	4.9° (110), 25.6° (003)
AFM height (nm)	7	12
TEM width (nm)	124	147
Hydrodynamic radius (nm), (PDI[a])	82, (0.1)	94, (0.2)
ζ-Potential (mV)	~−16	~−17
BET ($m^2\ g^{-1}$), (TPV[b], $m^3\ g^{-1}$)	385, (0.50)	12, (0.02)

[a] PDI: polydispersity index. [b] TPV: total pore volume.

insulin encapsulation in porous materials (Table S2†).[55,56] In order to visually show the presence of insulin-FITC inside the NPs, confocal microscopy analysis was performed. The fluorescent nature of the insulin-FITC loaded TTA-DFP-nCOF (λ_{ex} = 488 nm, Fig. 1f and S21†) confirmed the presence of insulin-FITC in the nanomaterial.

Upon insulin loading, the PXRD pattern of TTA-DFP-nCOF/insulin was nearly flat, as compared to that of the pristine TTA-DFP-nCOF (Fig. S12† and Table 1). It is anticipated that upon loading with insulin, the periodicity in the TTA-DFP-nCOF layers is affected to accommodate insulin molecules, thus losing crystallinity.[57–60]

When the TTA-DFP-nCOF was loaded with a reduced quantity of insulin (30% loading capacity, ESI†), the PXRD pattern showed the presence of $2\theta = 4.9^\circ$ and 25.6° peaks, although their intensities decreased significantly compared to those of the pristine nCOF, confirming our hypothesis. The uniformity of TTA-DFP-nCOF/insulin is evident from AFM analysis and increased to 12 nm upon insulin encapsulation compared to that of the pristine nCOF (Fig. S10† and Table 1). AFM images also show an increase in the nanoparticle width upon insulin loading, corroborated by TEM (Fig. 1e, S6–S8,† and Table 1). This suggests the slipping of nanosheets to accommodate the insulin molecules, a fact that is supported by the loss of crystallinity shown by PXRD. The hydrodynamic radii of the nanoparticles without and with insulin loading show an increase in solvated particle size as well as an increase in polydispersity from narrowly monodisperse to moderately polydisperse, showing a slight distribution in insulin encapsulation (Table 1 and Fig. S22†). ζ-Potential measurements show no statistically significant difference in pristine- and insulin-loaded nCOF yet they are both markedly different from insulin on its own, strongly supporting insulin encapsulation within the nanoparticles, rather than on the surface (Table 1 and Fig. S23†). Following insulin loading, N_2 sorption (Fig. S13†) exhibits a Type IV isotherm, typical of mesoporous materials, with a low-pressure H4-type hysteresis which either indicates swelling of a non-rigid porous structure[50] or, as observed by Bertier *et al.* for shales,[61] that insufficient equilibration is achieved during measurements because of slow N_2 diffusion in ultramicropores or that significant micropores exist but whose access is blocked.

Further evidence for insulin-loading between the nanosheets *versus* inclusion or diffusion through the micropore channels formed by stacking of nCOF layers is obtained from the size of the protein molecules (2.5–3 nm)[62] compared to the diameter of the micropore channels (1.7 nm, Fig. S13†). Insulin molecules are likely intercalated between several layers, favored by the small size of the TTA-DFP-nCOF particles. Simulation of the adsorption of insulin molecules between TTA-DFP-nCOF layers was performed following a simulated annealing process, where one insulin monomer was included between two sets of three ABC-stacked layers (Fig. 1g). A loading amount corresponding to ~70 wt% was calculated which is comparable to the maximum loading amount achieved experimentally (calculation details in the ESI†). To accommodate the insulin molecules, each set of layers was separated by 2.5 nm along the stacking direction during the simulation process. The resulting minimum energy conformation displayed in Fig. 1g shows how the insulin molecules are accommodated and interact with the nCOF layer atoms, which is also favored by the presence of pores, towards which some of the insulin terminal peptides are pointing. TEM dispersive X-ray spectroscopy (TEM-EDX) indicates that insulin is uniformly distributed throughout the TTA-DFP-nCOF (Fig. S9†).

In order to identify the nature of interactions between the TTA-DFP-nCOF and insulin, we conducted Fourier-transform infrared spectroscopy (FTIR) and X-ray photoelectron spectroscopy (XPS) studies. The FTIR spectrum of insulin displayed characteristic protein peaks: (i) at 2800–3000 cm^{-1} corresponding to the CH_2 stretching bond, (ii) at 1645 cm^{-1} for amide I and (iii) at 1535 cm^{-1} corresponding to amide II primarily due to the C=O stretching vibration (Fig. S15 and S16†).[63] After insulin entrapment in the TTA-DFP-nCOF, we observed the evolution of a new set of peaks at 2800–3000 cm^{-1} and at 1657 cm^{-1} corresponding to the CH_2 and C=O (amide I) stretching of insulin, with significant shifts (Fig. S15 and S16†). The peak at 1535 cm^{-1} overlaps with the aromatic C=C stretching of the TTA-DFP-nCOF. In addition, the disappearance of the nCOF imine bond at 1617 cm^{-1} was also observed. These observations could be associated with weak insulin–TTA-DFP-nCOF interactions between the amide I C=O group of insulin and the nCOF imine bond, as reported by Sarmento *et al.*[64] and Boonsongrit *et al.*[65]

The presence of sulfur along with the net increase of oxygen (around 10-fold) seen in the XPS survey spectrum of the TTA-DFP-nCOF/insulin compared with the survey spectrum of the TTA-DFP-nCOF represents clear evidence of insulin's presence within the nanoparticles (Fig. S24a and S26a†), since sulfur is present in insulin due to cysteine amino acids while it is absent in the nCOF (Fig. S25a†). The C 1s high resolution XPS spectrum of TTA-DFP-nCOF/insulin (Fig. S26b†) comprises four peaks at 283.4, 285.1, 286.3 and 288.2 eV attributed to the C=C sp^2, C–C sp^3, C–O and C–N bonds and carbon in carboxylic or amide groups, respectively.[66] A significant increase in the C sp^3 percentage could be observed in the deconvoluted C 1s peak of TTA-DFP-nCOF/insulin compared with C 1s of the nCOF (Fig. S24b†). This increase could be from the contribution of C sp^3 present in the side chains of amino acids that exist in the structure of insulin. Fig. S25c† shows the O 1s XPS survey spectrum in insulin, where the peak is composed of three components centered at 531.4, 532.3 and 533.5 eV attributed to oxygen in carboxylate, amide and alcohol groups respectively.[67] After loading the TTA-DFP-nCOF with insulin, an important decrease (*ca.* 12%) in the percentage of oxygen assigned to carboxylate was observed (Fig. S26c†). The deconvoluted N 1s spectrum of the TTA-DFP-nCOF (Fig. S24d†) indicates the presence of three distinct peaks at (i) 399.0 eV corresponding to the imine nitrogen, (ii) 399.9 eV associated with pyridinic nitrogen, and (iii) 400.7 eV attributed to quaternary nitrogen.[53] On the other hand, the N 1s spectrum for TTA-DFP-nCOF/insulin exhibits the contribution of nitrogen from amino and amide groups present in insulin (Fig. S26d†). Additionally, a decrease in the percentage of the quaternary amine (~8%) in the N 1s peak of the mixture compared with that of the TTA-

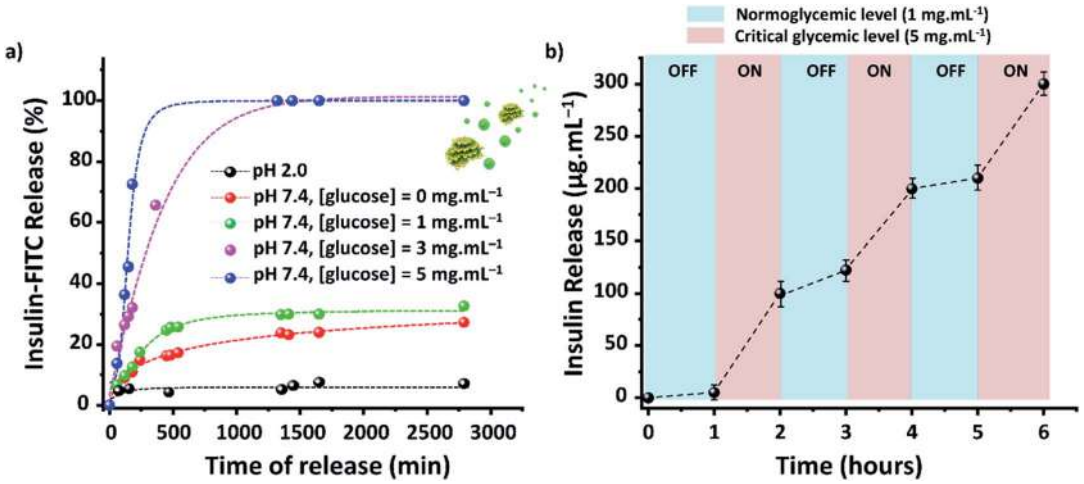

Fig. 2 TTA-DFP-nCOF/insulin presents a glucose controlled release mode with delayed release and pH sensitivity. (a) *In vitro* accumulated insulin-release from the TTA-DFP-nCOF/insulin-FITC at 37 °C in PBS (10 mM) and pH 2.0 (black), or pH 7.4 in several glucose concentrations ([glucose] = 0 (red), 1 (green), 3 (pink) and 5 (blue) mg mL^{-1}). (b) Pulsatile release profile of TTA-DFP-nCOF/insulin-FITC at 37 °C as a function of glucose concentration ([glucose] = 1 *versus* 5 mg mL^{-1}). Error bars indicate ±S.D. of triplicate experiments.

DFP-nCOF was observed. The decrease in the percentage of oxygen assigned to carboxylate existing in insulin and the percentage of quaternary amine existing in the TTA-DFP-nCOF strongly indicate that the interactions occur between the carboxylate groups of insulin and the quaternary amine groups of the TTA-DFP-nCOF.

The efficacy of TTA-DFP-nCOF/insulin-FITC *in vitro* was tested against both simulated gastric and intestinal fluids (SGF, pH 2.0; SIF, pH 7.4). Quantification of insulin-FITC release was carried out using fluorescence spectroscopy (ESI,† Section 4) and the experiments showed insignificant insulin release in both SGF (<5%) and SIF (<15%) following 24 h incubation (Fig. 2a).

The circular dichroism (CD) structure of the recovered insulin from TTA-DFP-nCOF/insulin under acidic conditions for 24 h displays the same pattern as that of native insulin (Fig. S29†). In order to confirm the stability of the TTA-DFP-nCOF/insulin, we performed a DLS study at pHs 7.4 and 2.0 and in the presence of lysozyme (an enzyme abundant in secretions including tears, saliva, and mucus) over a period of 24 hours, followed by TEM imaging. The size and morphology of TTA-DFP-nCOF/insulin do not vary under any of the conditions mimicking that of the stomach. Confinement of insulin between nanosheets of the nCOF nanoparticles protects it from unfolding and degradation, thus providing the safeguard necessary for oral delivery.[55]

Evaluation of the *in vitro* capacity for TTA-DFP-nCOF/insulin-FITC to respond to hyperglycemia-triggered drug release was investigated against various glucose concentrations; control, normal and diabetic, with [glucose] = 0, 1, 3 and 5 mg mL^{-1}, respectively, in PBS (10 mM, pH = 7.4). Under control conditions, only 12% of insulin was released over a 24 h incubation period, rising to 28% for normoglycemia, indicating the slow, natural release of insulin. Importantly, however, under hyperglycemic conditions exhibited by diabetic patients (3 mg mL^{-1}) there was almost 100% insulin release after 7.5 h of incubation (Fig. 2a).

With glucose levels naturally fluctuating between hunger and satiation,[68] the on-off regulation of insulin release was monitored between the normal and hyperglycemic state every 1 h, with the insulin release profile of TTA-DFP-nCOF/insulin-FITC displaying a pulsatile pattern (Fig. 2b).

Compared to the size of insulin (d = 2.5–3.0 nm),[62] glucose is a small molecule (d = 0.8 nm) that can fit inside the nCOF pores (1.7 nm). The TTA-DFP-nCOF can take up to 18 wt% of glucose (24 h, 5 mg mL^{-1}) due to hydrogen bonding between the numerous hydroxyl groups and the nitrogen atoms of the framework (Fig. S32–S35†). To confirm that insulin release is specifically triggered by glucose, TTA-DFP-nCOF/insulin-FITC was incubated at 37 °C for 24 hours in the following: (i) human serum; (ii) a mix of 11 amino acids; and (iii) a saline solution of fructose (3 mg mL^{-1}) or sucrose (3 mg mL^{-1}; Fig. S27 and S28†). There was limited release of insulin observed up to 24 h under all four conditions tested, with a maximum insulin-FITC release of 15% in human serum, 3% in amino acids, 22% in fructose, and 13% in sucrose. However, after incubation with fructose or sucrose, and adjusting the glucose concentration to mimic hyperglycemia (3 mg mL^{-1}), a burst release of insulin was observed, confirming the specific glucose-responsiveness of this nCOF system. The size and the polarity of the sugar molecules play a key role in the release of insulin. Sucrose is a larger molecule (1.06 nm) than fructose (0.8 nm) and glucose (0.8 nm) and therefore its access to the pores seems hindered. The differences in stereochemistry between fructose and glucose determine a relevant change in polarity, which might have a fundamental impact on the behavior of these molecules with the nCOF structure. Fructose being more hygroscopic than glucose has its hydroxyl groups less available to interact with the nitrogen atoms of the framework, which might explain the selective release of insulin with glucose.[69] In addition, the small

sized amino acids are likely to be zwitterionic at neutral pH, and this neutral-yet-charged species clearly prevents them from entering the nCOF structure.[70,71]

Under hyperglycemic conditions, glucose is forcefully diffused through the micropores of the nCOF displacing insulin from between the nanosheets. After 24 hour of hyperglycemic interactions, the TTA-DFP-nCOF was found to be almost insulin free. TTA-DFP-nCOF/glucose displayed a surface charge of −25.8 mV due to the large number of hydroxyl functional groups of glucose on the surface and within the framework (Fig. S33†). TTA-DFP-nCOF/insulin with glucose incubation displayed a charge of −19.6 mV. This strongly indicates the glucose concentration dependence of insulin displacement. The size of glucose results in preferential filling of nCOF pores with glucose, which are too small for insulin. However, once these pores are filled at normoglycemic concentrations, increasing the glucose concentration leads to glucose molecules penetrating the nanosheets, thereby displacing the insulin and forcing it out of the nanoparticle. However, the extent of glucose absorption into the micropores of the nCOF does not hinder the oral delivery of insulin. The TTA-DFP-nCOF can take up a maximum of 18 wt% of glucose under unrealistic hyperglycemic conditions which are considered unlikely to occur in a patient. Therefore, it is doubtful that TTA-DFP-nCOF/insulin would result in a hypoglycemic state by causing a sudden drop in glucose levels *in vivo* or in a patient. TEM, PXRD and BET analysis of the nCOF after insulin release under hyperglycemic conditions showed a decrease in size, restoration of the crystallinity to a certain degree, and increased BET surface area compared to TTA-DFP-nCOF/insulin (Fig. S35 and S36†). The preservation of insulin's secondary structure following release from the nCOF was assessed using circular dichroism and was found to be similar to that of original insulin (Fig. S30†); thus, nCOF-encapsulated insulin maintains both its structure and properties during transport and release, and, with this system exhibiting a glucose-triggered release mechanism, is an ideal candidate for the treatment of diabetes.

In vitro viability studies were carried out on 10 different cell lines (liver, colon, cervix, ovary, breast, kidney and brain, Fig. S37†) to demonstrate the potential of the TTA-DFP-nCOF as a biocompatible delivery vehicle. Both the TTA-DFP-nCOF and TTA-DFP-nCOF/insulin elicited no cytotoxic effects at TTA-DFP-nCOF concentrations up to 1 mg mL^{-1} following 48 h of incubation, indicating excellent biocompatibility and, therefore, great potential for oral application.

TEM was used to investigate the effects of TTA-DFP-nCOF/insulin on cellular structures and their interactions with organelles on 2 colon cell lines (RKO and HCT-116, 4 h incubation time) and the cells were analyzed 4 h, 24 h and 48 h post-treatment (50 μg mL^{-1}; Fig. S38–S40†) since the material needs to cross the intestinal barrier. All samples treated with TTA-DFP-nCOF/insulin exhibited the regular ultrastructure of the RKO and HCT-116 cells, with a roundish cellular shape, a plasma membrane rich in protrusions (such as microvilli), a well-developed rough endoplasmic reticulum, Golgi apparatus, and mitochondria, which indicate the maintenance of metabolically active cells. Significant amounts of TTA-DFP-nCOF/insulin can be visualized within some of the treated cells and at their surface. Membrane deformation was also observed, confirming the internalization of TTA-DFP-nCOF/insulin by endocytosis. Over time, in both cell lines the TTA-DFP-nCOF/insulin could be found inside cell vacuoles in the perinuclear region but no more on the membrane; cells continued to grow and divide, confirming that TTA-DFP-nCOF/insulin is non-toxic and safe, with no deleterious effect on cell morphology, viability, and mitochondrial health, and does not lead to the production of any reactive oxygen species.

As the purpose of the material is to enter the bloodstream, a hemolysis assay was carried out to determine its biocompatibility and immunotoxicity to human erythrocytes.[72–74] The hemolytic rates (HR) of the samples were found to be <2%, which, according to ASTM F 756-08 (Standard Practice for Assessment of Hemolytic Properties of Materials),[75] is considered to be non-hemolytic as it falls below the HR threshold of <5%. Therefore, the hemolytic test results (Fig. S41†) indicate that the TTA-DFP-nCOF and TTA-DFP-nCOF/insulin are biocompatible and non-immunotoxic to human blood erythrocytes. This could be somehow related to the negatively charged surface of our nanoparticles since nanoparticles with a negative surface charge were proven to be not hemolytic.[76–79]

We next assessed the ability of the TTA-DFP-nCOF/insulin to cross the intestinal barrier in *ex vivo* experiments (Fig. S42†). The use of nanoparticles can improve the transporting ability of proteins through the intestinal wall while protecting them against degradation in gastric fluid.[80,81] TTA-DFP-nCOF/insulin-FITC transportation across the intestinal wall was assessed by measuring the apparent permeability using an *ex vivo* technique in excised rat small intestine using the non-everted mouse small intestine sac model.[82,83] The transportation of TTA-DFP-nCOF/insulin from the mucosal side to the serosal side of the non-everted mouse small intestine sac was measured and quantified by fluorescence measurements. The permeability of TTA-DFP-nCOF/insulin after 3 h was calculated to be 14.76 μg cm^{-2} (corresponding to 60.8% ± 14.2 of the initial dose), while that of pure insulin was reported to be 8.02 μg cm^{-2}.[84] This indicates that incorporation of insulin into the TTA-DFP-nCOF resulted in an approximately two fold increase of the permeability of insulin. Permeation data correlate with accumulation in the gut wall. This can possibly be attributed to enhancement of the surface area leading to a higher rate of insulin-FITC diffusion.[84] The serosal side were collected and TEM imaging was performed, confirming that TTA-DFP-nCOF/insulin-FITC crossed the intestinal barrier. As shown in Fig. S43,† TTA-DFP-nCOF/insulin-FITC was present without modification of morphology or size on the serosal side. This accumulation may result from the potential cellular internalization of nanoparticles. Therefore, upon concluding the experiments, tissues were washed with normal saline, and nanoparticle accumulation in the gut wall was investigated by TEM (Fig. S44†). TEM images of the intestinal sections show their morphology with intact microvilli and the underlying architecture of the ileal mucosa. TTA-DFP-nCOF/insulin was located inside the goblet cells (GCs) of the intestinal tissue and excreted into the gut lumen through the secretion of intestinal GCs.[85] These results

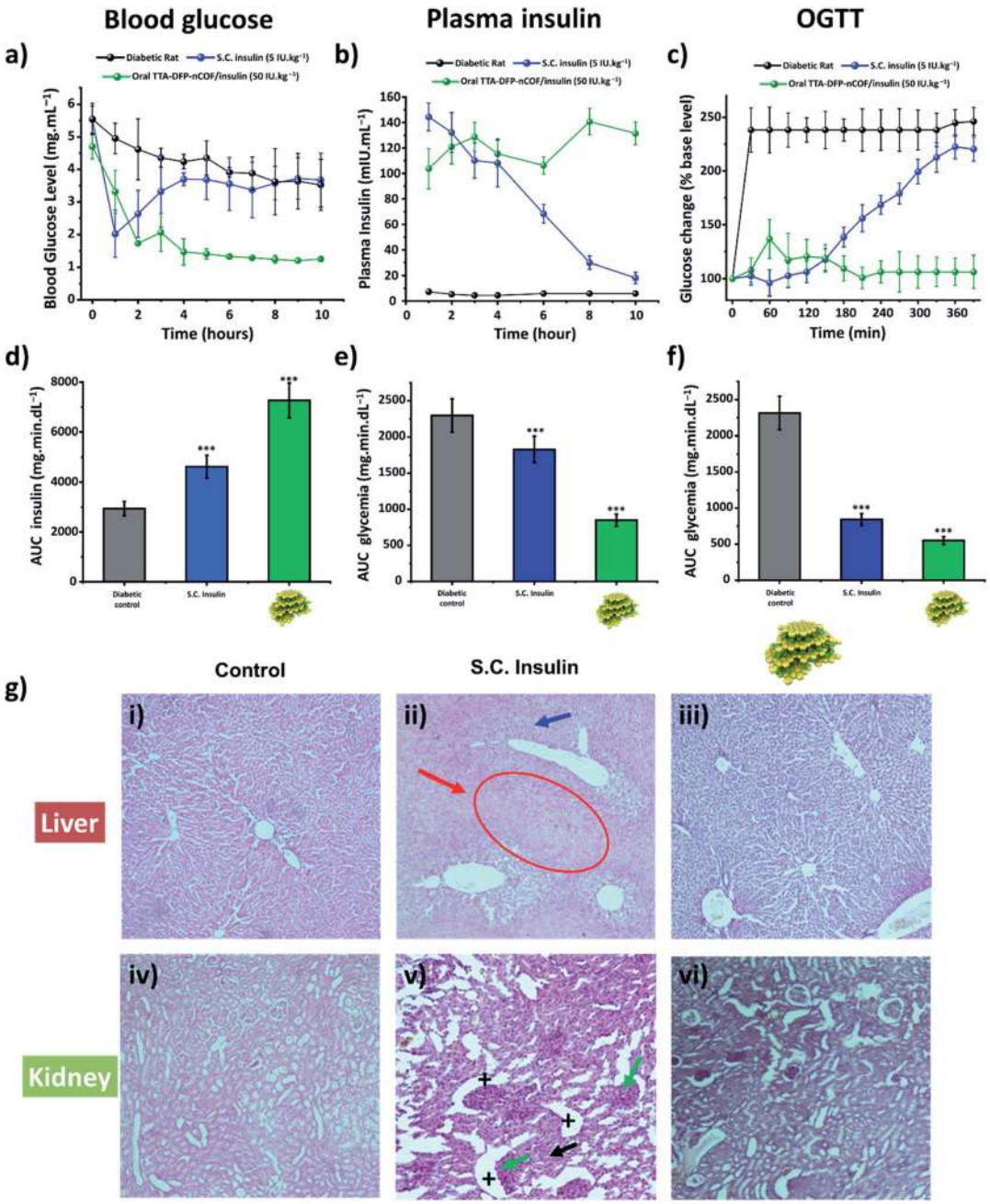

Fig. 3 TTA-DFP-nCOF/insulin regulates glucose uptake *in vivo* without causing toxicity. (a) Blood glucose level evolution, (b) serum insulin level changes and (c) oral glucose tolerance test (OGTT) results of the STZ-induced diabetic rats over time after oral administration of TTA-DFP-nCOF/insulin (green) at an insulin dosage of 50 IU kg^{-1}. The group with subcutaneous injection (S.C., blue) of insulin at 5 IU kg^{-1} was set as the positive control. Glycemic level, plasma insulin level and OGTT results of the diabetic rats (control) are illustrated in black. The corresponding area under the curve (AUC) is depicted in (d)–(f) for glycemic levels, plasma insulin levels and OGTT results, respectively. TTA-DFP-nCOF/insulin showed statistically significant differences in glycemic levels, plasma insulin levels and OGTT results compared with S.C. insulin solution and diabetic control (* p <0.05; ** p <0.01; *** p <0.001). Each value represents mean ± S.D. (n = 3). (g) Histopathological study. Sections of liver (i–iii) and kidney (iv–vi) of diabetic control rats (i and iv), S.C. insulin-injected rats (5 IU kg^{-1}, (ii) and (v) and diabetic rats treated orally with TTA-DFP-nCOF/insulin (50 IU kg^{-1}, (iii) and (vi) In figure (ii), the white arrow points to big hepatocytes, and the red arrow highlights necrosis of hepatocytes and a narrowing of the sinusoids. In figure (v), the + signs indicate Bowman capsules while the green arrow points to glomeruli hypertrophy, and tubule necrosis is indicated by the black arrow.

confirm that TTA-DFP-nCOF/insulin can cross the intestinal barrier carrying the insulin cargo and does not cause obvious pathological changes in intestinal tissues.

The *in vivo* pharmacological effect of glucose-responsive TTA-DFP-nCOF/insulin was evaluated by oral administration to a streptozotocin (STZ)-induced Type 1 diabetic (T1D) rat model (Fig. 3a–d and S45†).[86–89] The changes in blood glucose

Table 2 Measurements by the homeostatic model assessment (HOMA) of insulin resistance (HOMA-IR) and insulin-sensitivity (HOMA-IS) of β-cell function using the changes in insulin and glucose concentrations after subcutaneous insulin and TTA-DFP-nCOF/insulin treatment of diabetic rats. Each value represents mean ± S.D. ($n = 3$)

	S.C. insulin	TTA-DFP-nCOF/insulin
HOMA-IR	0.6 (0.3)	0.5 (0.1)
HOMA-IS	50.4 (11.0)	54.4 (13.2)

levels of the diabetic rats as a function of time following oral administration of various formulations or subcutaneous injection of free-form insulin solution were determined (Fig. 3a–d and S45†). Oral delivery of the insulin-free TTA-DFP-nCOF (2 mg kg^{-1}) or free-form insulin solution (50 IU kg^{-1}) exhibited nearly no oral pharmacological availability as confirmed by the minimal hypoglycemic effect observed. By contrast, subcutaneous injection of free-form insulin solution (5 IU kg^{-1}) resulted in a marked reduction of the blood glucose level within 1 h, though the effect was not sustained, with blood glucose levels rapidly returning to those similar to insulin-free nCOF-injected rats. However, following oral administration of TTA-DFP-nCOF/insulin (50 IU kg^{-1}), a significant reduction in blood glucose levels similar to that in non-diabetic rats was observed within 2 h coupled with a sustained hypoglycemic effect for 10 h, replicating the normal glucose level of the non-diabetic control group (Fig. 3a). These results are in accordance with the plasma insulin levels which showed that TTA-DFP-nCOF/insulin-treated rats presented the highest level of plasmatic insulin. Subcutaneous insulin-injected rats experienced a peak in the plasma insulin level in the first hour after insulin injection, after which it rapidly decreased as described in the literature.[90] The homeostatic model assessment (HOMA) is a method used to yield an estimate of insulin sensitivity, insulin resistance and β-cell function from fasting plasma insulin and glucose concentrations.[91] Rats treated with TTA-DFP-nCOF/insulin presented a lower HOMA-IR (insulin-resistance index) and a higher HOMA-IS (insulin-sensitivity index) than the subcutaneous insulin injected rats, suggesting that the TTA-DFP-nCOF/insulin particles are better assimilated by the body than subcutaneous insulin (Table 2). Oral insulin is directly absorbed by the intestinal epithelium and reaches the liver through the portal vein, allowing maintenance of glucose homeostasis, whereas parenterally administered insulin never mimics naturally secreted insulin as it is first delivered to peripheral tissues.[92] The calculations show that TTA-DFP-nCOF/insulin particles exhibit a high bioavailability (24.1%) compared to insulin-loaded particles described in the literature.[93–95] This is in accordance with the insulin and glucose plasmatic levels observed.

The oral glucose tolerance test (OGTT) was used to assess the ability of the body to take up glucose in post-prandial conditions and to evaluate the sensitivity of the body to endogenous insulin.[96] Test animals first received the TTA-DFP-nCOF/insulin by gavage or subcutaneous insulin injection. Three hours after that, coinciding with the serum insulin peak previously observed, the animals received 2.5 g kg^{-1} of glucose dissolved in 1 mL of water, and then the glycaemic level was evaluated over a period of 280 min (Fig. 3c–f) and compared to subcutaneous insulin-injected group and the diabetic control group. Subcutaneous insulin severely decreases the plasma glucose level within the first two hours after glucose gavage, as described in the literature.[97] Herein, from 150 min onwards, the glycaemic level begins to increase, almost reaching control values. It is reported that recurrent hypoglycaemic episodes caused by subcutaneous insulin therapy compromise the function and integrity of brain cells.[98] The hypoglycaemic activity of TTA-DFP-nCOF/insulin, while initially more moderate than that of subcutaneous insulin, from 120 min onward, results in low and stable glycaemia for the duration of the study. Oral administration of insulin allows high concentrations of insulin to enter the portal vein without sustained peripheral hyperinsulinemia, thereby preventing neuropathy and retinopathy.[68] Thus, oral delivery of TTA-DFP-nCOF/insulin results in low and stable glycaemia from 90 min.

Diabetes mellitus is often associated with alterations of kidney and liver functions in rats; therefore, we studied the impact of TTA-DFP-nCOF/insulin on these vital functions.[99] Fig. 3g shows the histopathological study of the liver and kidney to detect organ pathology in non-diabetic rats and S.C. insulin- and TTA-DFP-nCOF/insulin-treated rats. The S.C.-insulin treated group displays the commonly observed damage to the liver and kidney due to STZ administration to induce diabetes.[100–102] Regarding the TTA-DFP-nCOF/insulin treated rats, it can be seen that there is no damage caused to any of these organs, suggesting that TTA-DFP-nCOF/insulin is non-toxic but also that oral delivery of insulin can in fact inhibit histopathological alterations induced by diabetes in rats.[100,101]

Furthermore, an exploration of biochemical markers for liver and kidney functions in the blood can provide additional useful information to identify the beginnings of toxic effects.[103] Urea and creatinine are biomarkers commonly used to identify alterations in kidney function, while aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) are biomarkers of liver damage; the literature reports elevations of these 4 parameters in diabetic rats (Fig. S46†).[104] Overall, rats treated with TTA-DFP-nCOF/insulin present similar levels of urea, creatinine, ASAT and ALAT to the non-diabetic group (Fig. S46†), demonstrating that TTA-DFP-nCOF/insulin particles are not only non-toxic, but can also enhance kidney and liver functions compared to the diabetic control. Oral insulin delivery is described as being beneficial to kidney and liver functions.[68,105]

Conclusions

In conclusion, we have successfully prepared and tested, *in vitro* and *in vivo*, a nanoscale imine-covalent organic framework (TTA-DFP-nCOF) as an oral insulin delivery system. The TTA-DFP-nCOF's crystalline and porous nature allows the highest loading of insulin to be achieved, with evidence showing that insulin is located between layers of the nCOF nanosheets, rather than in the porous channels. The TTA-DFP-nCOF was

proven to protect encapsulated insulin *in vitro* under harsh conditions mimicking the stomach environment, while the sustainable release of insulin was accomplished under hyperglycemic conditions; importantly, insulin maintained its activity upon release from TTA-DFP-nCOF/insulin. The oral administration of TTA-DFP-nCOF/insulin to STZ-induced diabetic rats led to a continuous decline in the fasting blood glucose level within 2 to 4 h, and the hypoglycemic effect remained unchanged over 10 h *in vivo* showing high insulin bioavailability without systemic toxicity. The potential for this TTA-DFP-nCOF based oral insulin delivery system to replace traditional subcutaneous injections and enhance the uptake of insulin therapy amongst those in need has been demonstrated. We are currently in the process of testing other imine-linked COFs in order to establish a structure–activity relationship and decode the different parameters that make a given COF suitable for oral delivery of insulin.

Author contributions

F. B. and A. T. conceived the idea and led the project; F. B. synthesized and characterized the insulin loaded nCOFs; S. S. and R. J. performed the AFM analysis; T. P. synthesized the DFP linker and performed the PXRD and NMR analysis; G. D. synthesized TTA linker; F. B. and R. P. performed the TEM analysis; H. T. and M. A. performed the XPS analysis and data analysis to understand the interactions between the nanoparticles and the insulin; F. B. performed the *in vitro* biological experiments; M. K. and F. B. performed the gastro-intestinal study; F. B., S. A. T. and R. P. prepared and analyzed the biological samples for TEM study of nanoparticles cell internalization; F. G. performed the simulation of the nCOFs structure as well the insulin and nCOF interactions; N. K., F. B. S. and N. M. S. performed *in vivo* TD1 rat study and participated in data analysis; F. B., J. W. and A. T. prepared the manuscript, and all authors contributed to the discussion of results and the final version.

Conflicts of interest

There are no conflicts to declare.

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Effects of subcutaneous vs. oral nanoparticle-mediated insulin delivery on hemostasis disorders in type 1 diabetes: A rat model study

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During sample collection for our previous study, we made a surprising observation: diabetic rats treated with subcutaneous insulin exhibited apparent hypercoagulability and hemorrhage, while those treated with oral insulin did not manifest these issues. Further investigation into this phenomenon revealed a notable paucity of literature regarding the effects of insulin administration on hemostasis, leaving a significant gap in understanding how insulin therapy may influence coagulation and fibrinolysis in patients with type 1 diabetes. *Given the clinical relevance of hemostatic disturbances in T1D, we were motivated to conduct experiments to elucidate the differential effects of these two insulin delivery methods and to characterize the impact of insulin therapy on the hemostatic profile of diabetic individuals.*

Hemostasis is a vital physiological process aimed at stemming blood loss in response to vascular injury. It consists of three primary stages: primary hemostasis, coagulation, and fibrinolysis. Primary hemostasis initiates the formation of a platelet plug, whereby platelets, in coordination with the endothelium, adhere to the damaged vascular wall and aggregate to form a temporary occlusion to impede bleeding. Subsequently, the coagulation cascade is triggered by a series of reactions involving coagulation factors, culminating in the conversion of fibrinogen to fibrin. This fibrin mesh forms a stable network, solidifying the blood clot. Finally, the fibrinolytic system is activated to facilitate the dissolution of the clot once the injury has been repaired.

To investigate the effects of insulin therapy on the hemostasis of individuals with type 1 diabetes, we divided Wistar rats into four experimental groups: diabetic rats receiving no treatment, diabetic rats treated with subcutaneous insulin, diabetic rats administered oral insulin, and non-diabetic control rats. Our objective was to comprehensively explore each phase of the hemostatic process - primary hemostasis, coagulation, and fibrinolysis. Additionally, to gain a holistic understanding, we considered the endothelium and analyzed the effect of diabetes and insulin therapy on the endothelium's prooxidant/antioxidant balance. We also took into account all factors known to influence homeostasis by assessing the lipid profile, oxidative stress markers, cortisol levels, and by estimating inflammation levels.

The primary hemostasis, which aims to stop bleeding, was assessed by measuring bleeding time. Our results demonstrated that diabetes prolonged bleeding time, and this effect was further

exacerbated by subcutaneous insulin. In contrast, diabetic rats treated with Oral nCOF/Insulin exhibited bleeding times comparable to non-diabetic rats. To investigate the source of this hemorrhagic tendency, platelet counts, Prothrombin Time (PT), and Activated Partial Thromboplastin Time (APTT) were measured to determine whether thrombocytopenia or a loss of coagulation factors could be implicated. The findings revealed no significant differences among the four groups, with the untreated diabetic group showing a slightly higher platelet count than normal. These results rule out the hypothesis that the hemorrhagic tendency is due to a quantitative failure in blood elements (such as a loss of coagulation factors or platelets), pointing instead to a qualitative defect.

Following the assessment of primary hemostasis, our study shifted focus to the coagulation phase. We measured coagulation factor levels and observed that type 1 diabetes led to an increase in factor VIII, a procoagulant factor. This elevation was moderated by Oral nCOF/Insulin treatment. In contrast, subcutaneous insulin not only failed to address the issue but also worsened the procoagulant state by further increasing factor VIII levels and elevating factor IX, another procoagulant factor.

In evaluating fibrinolysis, we measured D-dimer levels, markers of impaired fibrinolysis, finding that orally administered nCOF/Insulin reversed the hypofibrinolysis caused by type 1 diabetes, whereas subcutaneous insulin had no such effect. Additionally, the assessment of fibrinogen concentrations revealed that Oral nCOF/Insulin treatment effectively normalized the diabetes-induced elevation in plasma fibrinogen levels. In contrast, subcutaneous insulin not only failed to reduce this increase but further exacerbated the rise in fibrinogen concentrations. Elevated fibrinogen is a known marker of systemic inflammatory processes.

Furthermore, our evaluation considered several factors impacting hemostatic homeostasis, including the lipid profile, oxidative stress markers, and cortisol levels. Type 1 diabetes disrupts the lipid profile by inducing hypertriglyceridemia, a condition worsened by subcutaneous insulin injections. Similarly, plasma concentrations of malondialdehyde (MDA), a marker of lipid peroxidation, exhibited the same pattern. Additionally, the assessment of plasma cortisol levels, a stress hormone, revealed that diabetes causes an elevation that subcutaneous insulin

treatment does not correct—unlike oral insulin delivered via nCOF, which effectively normalizes these levels. All these results indicate that diabetes causes disturbances in hemostasis, coagulation, and fibrinolysis, which subcutaneous insulin treatment not only fails to correct but also exacerbates. In contrast, insulin administered orally via nCOF appears to rectify these damages.

The comprehensive findings of this study are consistent with our previous research, indicating that, similar to its effects on hepatic and renal complications associated with diabetes, Oral nCOF/Insulin also mitigates diabetes-related hemostatic disturbances. Notably, it effectively restored normal bleeding time in diabetic rats, counteracting hemorrhagic tendencies. Furthermore, Oral nCOF/Insulin treatment ameliorated the hypercoagulable state and hypofibrinolytic milieu characteristic of type 1 diabetes mellitus.

The beneficial effects of Oral nCOF/Insulin on hemostatic parameters can be attributed to its unique pharmacological properties that effectively restore glycemic homeostasis in diabetic rats. Notably, Oral nCOF/Insulin treatment normalizes diabetes-induced dyslipidemia, mitigates oxidative stress, and attenuates inflammatory responses – all of which are critical pathophysiological factors contributing to hemostatic dysregulation in diabetes mellitus. By ameliorating these underlying pathological processes, Oral nCOF/Insulin prevents the development of hemostatic disturbances. In contrast, subcutaneous insulin administration fails to restore glycemic control and metabolic equilibrium, leading to persistent glycemic fluctuations, sustained systemic inflammation, hyperinsulinemia, hypertriglyceridemia, and lipid peroxidation. The persistence of these metabolic derangements exacerbates the spectrum of diabetes-related complications, particularly those pertaining to hemostatic abnormalities.

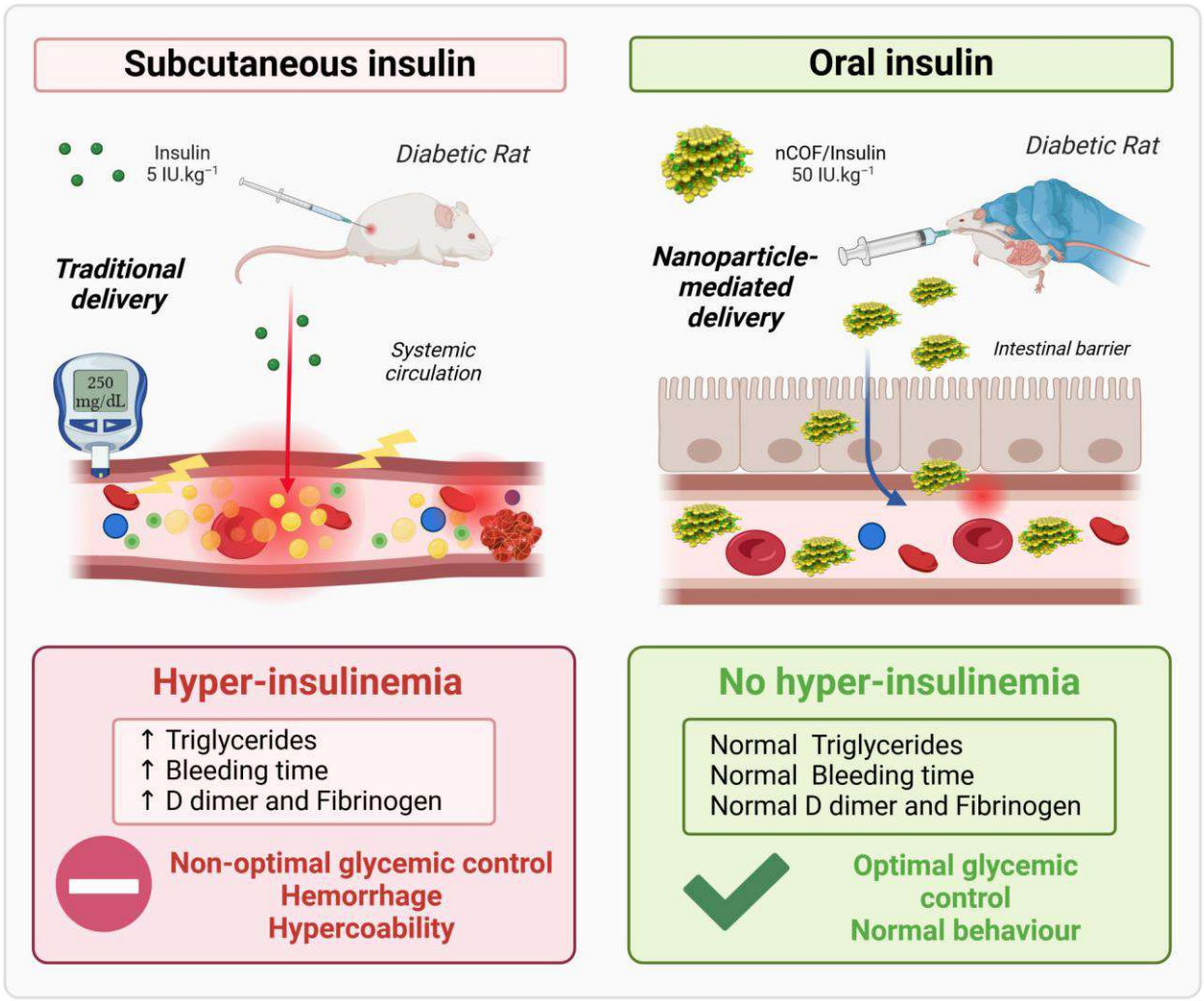
To gain a deeper understanding of the underlying key factor contributing to these hemostatic alterations, we conducted a correlational analysis. The results of this analysis led us to the conclusion that lipid peroxidation represents the primary pathophysiological driver of hemostatic dysregulation in type 1 diabetes mellitus, while also highlighting that hypertriglyceridemia plays a central role in this process. Ultimately, this comprehensive study allowed us to elucidate and draw several pertinent conclusions regarding hemostatic disturbances in type 1 diabetes mellitus,

which had been poorly documented until now, and even identify the pivotal causative factor. This study demonstrated for the first time that insulin therapy in type 1 diabetes affects hemostatic disturbances. Additionally, while the novel oral nCOF/insulin formulation appears to correct these alterations, we showed that traditional subcutaneous insulin administration may exacerbate these issues.

This study offers numerous clinical applications. For instance, while routine tests like APTT and PT may not always provide definitive results for assessing patients' hemostatic status, a more thorough analysis involving plasma triglyceride levels and lipid peroxidation could offer valuable insights. These parameters could serve as cost-effective indicators, helping identify patients who require further hemostatic assessment based on clinical indications.

Moreover, considering that diabetic patients have among the poorest survival rates in major surgeries (such as cardiac surgery) and are particularly vulnerable to conditions like COVID-19, understanding the effects of subcutaneous insulin on coagulation becomes crucial. Traditional protocols often involve administering insulin just before or during surgery, and even COVID-19 patients may receive subcutaneous insulin, regardless of their prior oral anti-diabetic medication. Therefore, comprehending the impact of subcutaneous insulin on coagulation is vital for optimizing patient management and outcomes in various clinical scenarios.

Graphical abstract



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Research article

Effects of subcutaneous vs. oral nanoparticle-mediated insulin delivery on hemostasis disorders in type 1 diabetes: A rat model study

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ABSTRACT

Complications associated with Type 1 diabetes (T1D) have complex origins that revolve around chronic hyperglycemia; these complications involve hemostasis disorders, coagulopathies, and vascular damage. Our study aims to develop innovative approaches to minimize these complications and to compare the outcomes of the new approach with those of traditional methods. To achieve our objective, we designed novel nanoparticles comprising covalent organic frameworks (nCOF) loaded with insulin, termed nCOF/Insulin, and compared it to subcutaneous insulin to elucidate the influence of insulin delivery methods on various parameters, including bleeding time, coagulation factors, platelet counts, cortisol plasma levels, lipid profiles, and oxidative stress parameters. Traditional subcutaneous insulin injections exacerbated hemostasis disorder and vascular injuries in streptozotocin (STZ)-induced diabetic rats through increasing plasma triglycerides and lipid peroxidation. Conversely, oral delivery of nCOF/Insulin ameliorated hemostatic disorders and restored the endothelial oxidant/antioxidant balance by reducing lipid peroxidation and enhancing the lipid profile. Our study pioneers the understanding of how STZ-induced diabetes disrupts bleeding time, induces a hypercoagulable state, and causes vascular damage through lipid peroxidation. Additionally, it provides the first evidence for the involvement of subcutaneous insulin treatment in exacerbating vascular and hemostasis disorders in type 1 diabetes (T1D). Introducing an innovative oral insulin delivery via the nCOF approach represents a potential paradigm shift in diabetes management and patient care and promises to improve treatment strategies for type 1 Diabetes.

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1. Introduction

Complications associated with type 1 diabetes (T1D), encompassing both microvascular and macrovascular issues, remain the foremost contributors to morbidity and mortality among affected individuals. Patients with T1D often exhibit a proinflammatory and

Abbreviations

APTT	Activated Partial Thromboplastin Time
C	Control Rats
DC	Diabetic Rats
DFP	2,6-Diformylpyridine
ELISA	Enzyme-Linked Immunosorbent Assay
GSH	Glutathione
HDL-C:	High-Density Lipoprotein Cholesterol
IACUC	Animal Care and Use Committee
K2-EDTA	Potassium Ethylenediaminetetraacetic Acid
LDL-C:	Low-Density Lipoprotein Cholesterol
MDA	Malondialdehyde
NBT	Nitroblue Tetrazolium
nCOF	Covalent Organic Framework Nanoparticles
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NPs	Nanoparticles
O2•–	Superoxide
OG Ins	Oral Gavage of nCOF/Insulin
Oral nCOF/Insulin	Oral Insulin delivered by Covalent Organic Framework nanoparticles
PBS Buffer	Phosphate Buffered Saline
PPP	Platelet-poor Plasma
PT	Prothrombin Time
RAGE	Receptor for Advanced Glycation End-products
SC Ins	Subcutaneous Insulin
STZ	Streptozotocin
T1D	Type 1 Diabetes
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
TTA	4,4′,4′′-(1,3,5-triazine-2,4,6-triyl)trianiline

pro-coagulant state, characterized by endothelial injury, heightened platelet adhesion, and reduced plasma fibrinolytic potential [1,2].

Chronic hyperglycemia, while a significant contributor to these complications, represents only part of the multifaceted problem. Other factors, such as alterations in lipid metabolism [3] and the oxidative stress associated with diabetes [4,5], play equally important roles. Specifically, lipid peroxidation can intensify the pro-coagulant state by inhibiting the natural anti-coagulant defences, disrupting anti-thrombotic mechanisms [6], and inducing platelet hyperactivation [7,8]. Additionally, the combination of hyperglycemia and dyslipidemia elevates plasma concentrations of pro-coagulant factors [9] while reducing levels of anticoagulant proteins [10].

This interplay of factors does not act in isolation; rather, they collectively contribute to the complex network of complications associated with T1D. Chronic hyperglycemia further compounds the situation by inducing endothelial damage through two primary mechanisms [11]. First, glucose permeates endothelial cells independently of insulin, triggering multiple signaling pathways, including the generation of reactive oxygen species such as superoxide ($O_2^{\bullet}$). This disrupts the delicate equilibrium between vasodilators and vasoconstrictors within the vasculature. Second, hyperglycemia directly initiates vascular inflammation [12], worsening endothelial dysfunction. Elevated stress hormone levels, such as cortisol, further contribute to the intensification of endothelial dysfunction [11].

From a therapeutic perspective, the impact of traditional subcutaneous insulin injections on hemostasis-related complications in T1D has not been extensively explored. Moreover, while certain studies suggest that intensive insulin therapy may provide cardiovascular benefits in diabetic patients [13] or animals treated with streptozotocin [14], it is essential to consider the existing controversy surrounding this approach due to the associated risk of hypoglycemia [15,16].

Furthermore, the management of diabetic patients extends beyond addressing hyperglycemia; it also involves effectively managing its complications. In this context, the field of nanomedicine offers innovative avenues that can enhance treatment effectiveness [17–19] while concurrently reducing the risk of complications [20,21].

In our previous study, our pharmacokinetic analysis showed that the glycemic response to nCOF/insulin is balanced, so that blood glucose levels can be maintained within a normal range without causing extreme fluctuations. In contrast, with subcutaneous insulin, a hypoglycemic spike was observed within 1 h of injection, followed by a rapid return to a hyperglycemic state characteristic of diabetes.

These results suggest that nCOF/insulin provides more stable glycemic control compared to subcutaneous insulin. In addition, our observations showed different insulinemic profiles for the two insulins. In fasting rats, nCOF/insulin led to a gradual increase in blood insulin concentration, whereas subcutaneous insulin led to a hyperinsulinemic peak after injection. This difference in insulin distribution and the organism's response to administration leads to different biological responses. Thus, our previous study suggests that nCOF/insulin may prevent hepatic and renal complications associated with diabetes compared to conventional subcutaneous insulin injection [21].

Building upon these findings, our current research assesses the impact of administering insulin through oral nanoparticle delivery on key parameters such as lipid profile, lipid peroxidation, bleeding time, vascular damage, and coagulation-fibrinolysis processes, all intertwined with diabetes. To achieve this, we conducted a comparative analysis between the outcomes of subcutaneous insulin injections and oral nCOF/Insulin treatments in rats with experimentally induced T1D.

To assess platelet activity, we measured bleeding time and platelet count. Furthermore, we delved into the potential vascular alterations triggered by diabetes by evaluating the equilibrium between prooxidants and antioxidants. This equilibrium was precisely determined through the measurement of $O_2^{\bullet-}$ and glutathione (GSH) levels in the abdominal aortas of the experimental rats. Our investigation of blood coagulation encompassed a comprehensive approach. We employed global tests such as activated partial thromboplastin time (APTT) and prothrombin time (PT). Additionally, we performed specialized tests designed to gauge the coagulant activities of specific factors, including II, V, VII, VIII, IX, and X. In order to evaluate the hemostatic profile, we analysed fibrinogen levels to assess acute phase response, providing insights into inflammation. Furthermore, we examined d-dimer levels as an indirect indicator of fibrinolysis and cortisol levels to reflect stress levels. In parallel, we conducted a lipid profile analysis, measuring plasma levels of total cholesterol (TC), High-Density Lipoprotein Cholesterol (HDL-C), and triglycerides (TG), as well as determining Low-Density Lipoprotein Cholesterol (LDL-C). Finally, lipid peroxidation was evaluated by measuring Malondialdehyde (MDA) levels.

Our findings reveal that subcutaneous insulin injections intensify hemostasis disorders and vascular damage in diabetic rats. This aggravation primarily manifests through elevated plasma triglycerides and increased lipid peroxidation levels. Conversely, the oral administration of nCOF/Insulin enhances the coagulation profile, fibrinolysis, and bleeding time of diabetic rats while also restoring the equilibrium between oxidants and antioxidants by mitigating lipid peroxidation and enhancing the overall lipid profile.

Our study is among the first to clearly demonstrate how STZ-induced diabetes in rats leads to significant disruption of the lipid profile, increased lipid peroxidation, and prolonged bleeding times, culminating in a hypercoagulable state that increases oxidative stress within the endothelium. The introduction of oral insulin delivery via the nCOF approach effectively addresses vascular injury and corrects hemostatic disorders in diabetic rats. It improves bleeding time, coagulation profile, and fibrinolysis. Additionally, it mitigates dyslipidemia, suppresses lipid peroxidation, and prevents superoxide accumulation in the aorta of diabetic rats.

Overall, our research brings significant advances in our understanding of diabetes-related vascular complications and opens new avenues for safer and more effective insulin therapy methods.

2. Materials and methods

2.1. Synthesis of insulin loaded nCOF nanoparticles (nCOF/Insulin)

nCOFs nanoparticles were synthesized by co-condensation of 2,6-diformylpyridine (DFP, 21 mg, 0.15 mmol, 5 equivalents) and

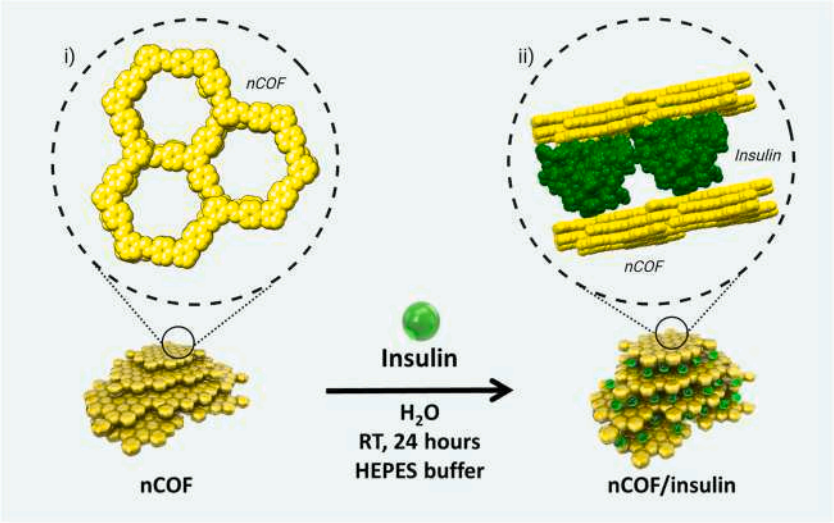

Fig. 1. Schematic representation of the encapsulation of insulin between the layers of the nCOF. Cartoon representation (green spheres) represents the insulin. Inset i) chemical structure of the nCOF. Inset ii) van der Waals representation of the optimized location of insulin monomer molecule intercalated between nCOF layers.

4,4′,4′′-(1,3,5-triazine-2,4,6-triyl)trianiline (TTA, 12 mg, 0.03 mmol, 1 equivalent), in 3 mL of anhydrous 1,4-dioxane in the presence of 0.5 mL of acetic acid (13 M, $[\text{acetic acid}]_{\text{final}} = 4.0$ M) at room temperature for 10 min (Fig. 1). The solution was cleaned using dialysis in H_2O to obtain a stable colloidal suspension. Characterization revealed nanoparticles with an average diameter of 123.7 nm, a crystalline structure with distinct PXRD peaks, and a significant BET surface area indicating permanent porosity.

Insulin was loaded into nCOF by a simple impregnation method. nCOF (5 mg) was suspended in 2 mL HEPES buffer. A HEPES-buffered aqueous insulin solution ([insulin] = 10 mg mL^{-1}, 1 mL) was added (nCOF:Insulin ratio = 1:2). The solution (pH 7.4) was stirred overnight at room temperature, cleaned with water several times by centrifugation, and finally washed with deionized H_2O to remove unloaded insulin molecules. The nCOF/Insulin nanoparticles exhibited a high insulin-loading capacity (64.6 ± 1.7 wt%) and there is evidence that the insulin is located between the layers of the nCOF nanosheets (Fig. 1). Stability assessments showed the nanoparticles are resilient under simulated gastrointestinal and bloodstream conditions. Advanced characterization techniques, including FTIR and XPS analyses, confirmed the encapsulation of insulin and the presence of specific chemical interactions between insulin and the nCOF structure. Additionally, computational simulations supported the experimental loading data, suggesting insulin intercalation between the nCOF layers. This comprehensive preparation and characterization makes the nCOF system a promising candidate for oral insulin delivery.

The efficacy and release mechanism of nCOF/insulin FITC nanoparticles were investigated in vitro. This showed minimal insulin release in simulated gastric and intestinal fluids, so that the structure of the insulin was preserved under acidic, stomach-like conditions. Dynamic light scattering and TEM confirmed the stability and unchanged morphology of the nanoparticles in these environments. Remarkably, the system showed glucose-responsive insulin release, especially under hyperglycemic conditions. Nearly 100 % release was achieved, with a pulsatile release pattern observed between normal and hyperglycemic conditions. This glucose-triggered release mechanism is favored by the smaller size of the glucose molecules, which allows them to penetrate the nCOF structure and displace insulin. Studies in different solutions showed that insulin release is specifically triggered by glucose and not by other sugars or amino acids, underlining the potential of the system for targeted diabetes therapy. The maintained structural integrity of the insulin after release confirms the clinical relevance of this nCOF system for diabetes treatment.

In vitro and *in vivo* studies confirmed the biocompatibility and efficacy of nCOF/insulin as an oral insulin delivery system. In vitro studies on the viability of several cell lines showed no cytotoxic effects, indicating the suitability of the system for oral use. TEM analysis showed that nCOF/insulin did not alter cellular ultrastructure, suggesting safe endocytosis and internal cellular processing. Hemolytic assays also showed that it is not immunotoxic to human erythrocytes, highlighting its safety for entry into the bloodstream. Ex vivo experiments have demonstrated that the nanoparticles can effectively cross the intestinal barrier, which increases the permeability of insulin and represents a promising approach for the treatment of diabetes. Oral administration to diabetic rats (T1D) resulted in a significant reduction in blood glucose levels without any organ damage or changes in kidney and liver functions being observed. This demonstrates the potential of nCOF/insulin to safely maintain glucose homeostasis and replace subcutaneous injections [21].

2.2. Animals

Male and female Wistar rats (12 weeks, 200 g ± 20) from Pasteur Institute were used for this study. Rats were housed individually in wood-chip bedded plastic cages at constant temperature (25 °C), maintained on a 12:12 h light/dark cycle, and fed with a standard pellet diet with water *ad libitum*. The study was conducted following the policies of the University of Tlemcen Institutional Animal Care and Use Committee (IACUC) (accreditation number: D01N01UN130120150006)) and complies with the ARRIVE guidelines.

2.3. T1D induction

T1D was induced via a single intraperitoneal injection of streptozotocin (STZ, dissolved in 10 mM citrate buffer at pH 4.5) at an STZ dose of 45 mg kg^{-1} of body weight. Rats were returned to their cages and given food and water till the onset of diabetes. Blood glucose levels were monitored using a blood glucose monitoring system (AccuChek Performa, Hoffman-La Roche) by taking samples from a rat tail vein. Rats showing fasting blood glucose levels ≥250 mg/dL (13.7 $mmol.L^{-1}$) were considered diabetic and selected for the study ($N = 20$).

Table 1
Rat groups assigned randomly: insulin administration methods and injected concentrations. Wistar rats were randomly divided into 4 groups: normal control (C), diabetic control (DC), diabetic treated with subcutaneous insulin (SC Ins, 5 $IU.kg^{-1}$), and diabetic treated with Oral nCOF/Insulin (OG Ins, 50 $IU.kg^{-1}$).

Group	Insulin Administration Method	Dose	Comments
C (Control)	N/A (Not Applicable)	N/A	Non-diabetic rats serving as a positive control group.
DC (Diabetic Control)	N/A (Not Applicable)	N/A	Untreated diabetic rats serving as a negative control group.
SC Ins (Subcutaneous insulin)	SC (Subcutaneous injection)	5 $IU.kg^{-1}$	Diabetic rats treated with insulin injections under their skin.
OG Ins (Oral nCOF/Insulin)	OG (Oral gavage)	50 $IU.kg^{-1}$	Diabetic rats treated with insulin delivered orally using nCOF nanoparticles.

2.4. Study design

Rats were randomly divided into four groups (n = 5) as shown in Table 1: standard control (C), diabetic control (DC), diabetic treated with subcutaneous insulin (SC Ins, 5 IU.kg^{-1}), and diabetic treated with oral gavage of nCOF/Insulin (OG Ins, 50 IU.kg^{-1}). In the treated groups, the diabetic rats received a single dose of either SC Ins or OG Ins on day 1, as described in the experimental protocol summarized in Fig. 2. Our decision to use different doses for subcutaneous (5 IU/kg) and oral (50 IU/kg) insulin delivery is based on the difference in bioavailability. Subcutaneous insulin, which has nearly 100 % bioavailability due to bypassing the digestive system and first-pass metabolism, requires lower doses to be effective. Oral insulin faces degradation and significant first-pass metabolism, resulting in lower bioavailability. Therefore, a higher dose is used to compensate for this and ensure sufficient systemic levels.

2.5. Bleeding time (BT) measurement

Following the administration of the insulin doses, a bleeding time measurements were performed as described by Ayodele et al. [22]. A cut was made on each rat's tail at 1–2 cm proximal from the end, and the stopwatch was started immediately upon the onset of bleeding. At intervals of 15 s, blood spots were made with the bleeding tail on a blotting paper until the bleeding stopped. Bleeding time was recorded as the time taken for bleeding to stop.

Bleeding time measurements were conducted 1 h after insulin administration on day 1. We specifically chose this time point based on our previous findings [21], which showed that 1 h corresponds with the highest insulin levels in the bloodstream following subcutaneous insulin injection, while oral insulin administration effectively regulates blood glucose levels.

2.6. Sample collection

After an overnight fast, the animals were sacrificed on day 2 in deep isoflurane anaesthesia (approximately 2.5 % v/v). Blood samples were collected via cardiac puncture. From each rat, two tubes of blood were collected and treated as follows.

- **tube 1** which contained 3.2 % sodium citrate in a ratio of 1:9 anticoagulant: blood
- **tube 2** which had K2-EDTA as an anticoagulant.

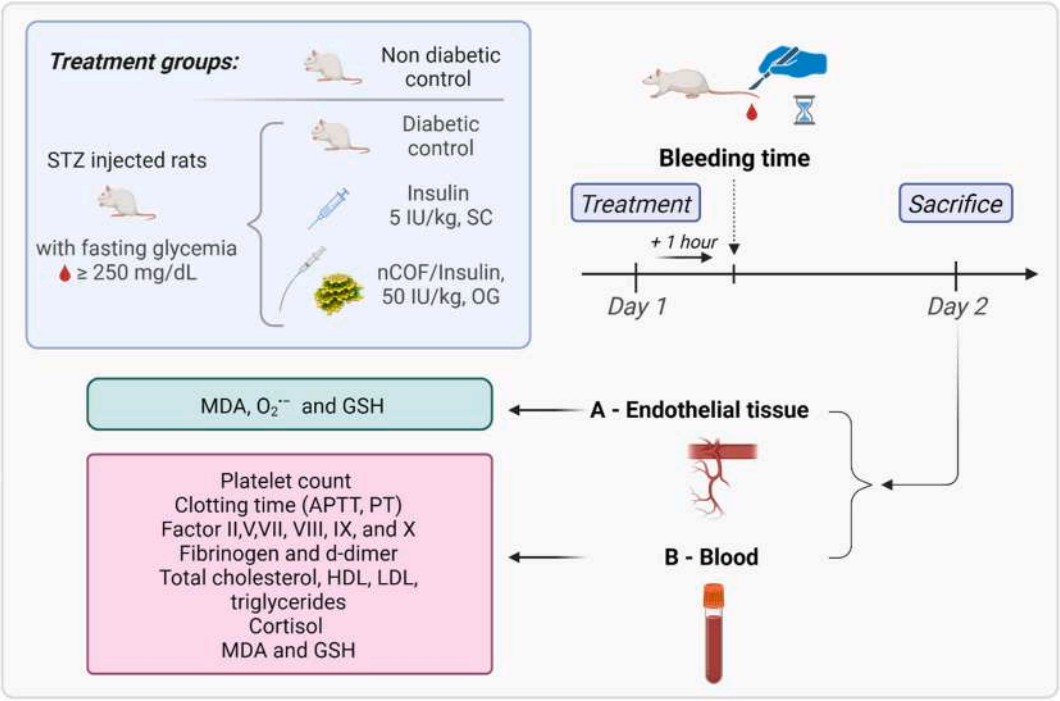

Fig. 2. Schematic representation of T1D induction and subsequent study protocol in rats. This diagram illustrates the sequential methodology employed in our study. Starting with the induction of T1D using streptozotocin (STZ), we monitored and selected diabetic rats based on specific blood glucose levels (≥250 mg/dL). The study design categorizes rats into four distinct groups, each with varied insulin administration methods, as detailed in Table 1. We then measured bleeding times 1-h post-insulin treatment, employing a precise protocol based on prior research findings. Following overnight fasting and subsequent sacrifice of the rats, various samples were collected. Using these samples, an array of tests was conducted including coagulation profiles, endothelial lysate preparations, thrombocytes counting, cortisol levels, lipid profiling, and markers of oxidative stress. Statistical analyses were performed using SPSS.

The abdominal aorta was also removed and cleaned for further analysis (endothelial lysate). All tests were performed immediately following sacrifice.

2.7. Sample preparation

2.7.1. Preparation of platelet-poor plasma samples

Platelet-poor plasma (PPP) was obtained from **tube 1** containing 3.2 % sodium citrate by blood centrifugation (3000 rpm, 20 min, room temperature). PPP was used for coagulation tests by performing the following analysis: quick time; activated partial thromboplastin time (APTT); concentration of fibrinogen; concentration of d-dimers and clotting factors (factor II, factor V, factor VII, factor VIII, factor IX, factor X).

2.7.2. K2-EDTA blood samples

Blood from **tube 2** containing K2-EDTA as an anticoagulant was used for counting platelets from whole blood. Blood samples were centrifuged (1500 rpm, 15 min, room temperature) to obtain plasma. From the obtained plasma was measured: levels of total cholesterol, triglycerides, high-density lipoproteins (HDL), thiobarbituric acid reactive substances (TBARS), and glutathione (GSH).

2.7.3. Endothelial lysate

Abdominal aorta samples were collected from a descending thoracic fragment. After gentle rinsing and removal of surrounding tissue, the samples were homogenized in pH 7.4 PBS buffer using an ultra-Turrax homogenizer (Bioblock Scientific, Illkirch, France) for three cycles of 10 s each, and subsequently sonicated for 10 s using an ultrasonic homogenizer (SONICS, USA) and then centrifuged (3000 rpm, 5 min). The supernatant was used to estimate GSH and reactive oxygen species superoxide ($O_2^{\bullet-}$).

2.8. Coagulation tests

Quantitative determination of fibrinogen levels in plasma was conducted using the clotting method developed by Clauss [23]. An immunoturbidimetric assay determined d-dimer plasma levels. APTT and Prothrombin Time (PT) were assayed using Stago reagents [24]. Coagulant activities of factors II, V, VII, VIII, IX–X were determined in one-stage clotting assays with factor–deficient plasmas (Stago reagents). All measurements were made on an STA coagulometer Stago (^{STA} Compact Max^2).

2.9. Thrombocytes counting

Thrombocytes count from complete blood was performed on an automated hematology analyzer SIEMENS (ADVIA 2120i, France) using well-mixed whole blood with K2-EDTA to prevent blood clotting.

2.10. Cortisol

Cortisol plasma levels were quantified using an enzyme-linked immunosorbent assay (ELISA) kit from Sigma-Aldrich.

2.11. Lipid profile

Plasmatic total cholesterol (TC), High-Density Lipoprotein Cholesterol (HDL-C), and triglyceride (TG) levels were determined using SPINREACT Kit. Low-Density Lipoprotein Cholesterol (LDL-C) plasma level was determined based on Friedewald et al.'s formula [25]:

$$LDL - C = TC - HDL - C - TG/5$$

2.12. Thiobarbituric acid reactive substances (TBARS)

Plasma and endothelial thiobarbituric acid reactive substances (TBARS) were estimated according to literature [26,27] and expressed as malondialdehyde (MDA). TBARS were quantitated by their reactivity with thiobarbituric acid (TBA) in acidic conditions to generate a pink-colored chromophore, monitored at 532 nm.

2.13. Glutathione (GSH)

Estimation of GSH in plasma and endothelial tissue was performed according to Ellman [28]. This method is based on developing a yellow color, monitored at 412 nm when Ellman's reagent (dithionite benzoic acid) is added to compounds containing sulfhydryl groups.

2.14. Reactive oxygen species superoxide ($O_2^{\bullet-}$)

The production of $O_2^{\bullet-}$ endothelial tissue was measured at pH 7 and 25 °C in the presence of nitroblue tetrazolium (NBT). The superoxide anion reduces NBT produced by the tissue into blue formazan [29,30], a chromophore that absorbs at 550 nm.

2.15. Statistics

All data were analysed using SPSS (IBM, SPSS Statistics, version 23, USA) and expressed as means ± standard error of the mean (SEM). Data were analysed using one-way ANOVA with post hoc Tukey's tests for multiple comparisons. Pearson correlation coefficient analysis was performed between triglycerides or MDA in plasma levels and the different biomarkers. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Hemostasis analysis

Hemostasis analysis was performed on the four different groups considered in this study (Table 1): control rats (C), diabetic rats (DC), diabetic rats treated with subcutaneous insulin (SC Ins), and diabetic rats treated with oral insulin loaded in nCOFs (OG Ins). Table 2 shows no statistically significant difference between the four groups for Quick time, APTT, factor V, and factor VII plasma levels. However, statistically significant differences were found between the four groups in factors II ($p < 0.001$), VIII ($p < 0.001$), IX ($p < 0.01$), X ($p < 0.001$), fibrinogen and d-dimers ($p < 0.001$).

Specifically, we found that factor II plasma levels were higher in OG Ins and lower in SC Ins rats compared to C ($p < 0.001$ and $p < 0.05$ respectively). Factor VIII levels were higher in the three diabetic groups compared to the control group (C). Furthermore, diabetic rats treated with subcutaneous insulin (SC Ins) exhibited even higher factor VIII levels compared to untreated diabetic rats (DC), while diabetic rats treated with oral insulin (OG Ins) had lower levels ($p < 0.05$). Factor IX plasma levels were particularly elevated in diabetic SC Ins ($p < 0.05$) and those of factor X were elevated in OG Ins ($p < 0.0001$). Fibrinogen and d-dimer plasma levels were higher in both DC and SC Ins groups when compared to the control group (C), with no statistically significant difference observed in OG Ins group. Additionally, SC Ins exhibited even higher levels of fibrinogen than untreated diabetics rats (DC).

3.2. Lipid profile analysis

There were no statistically significant differences observed among the four groups in terms of total cholesterol, LDL-C, and HDL-C plasma levels (Fig. 3 a-c). However, a statistically significant difference was evident in the plasma triglyceride levels between groups (Fig. 3d). Specifically, plasma triglyceride levels increased in DC and SC Ins compared to C. In contrast, the oral insulin-treated group (OG Ins) exhibited a significant reduction in plasma triglyceride levels when compared to both the DC and SC Ins groups ($p < 0.05$).

3.3. Platelets activity

As shown in Fig. 4a, DC platelet counts exhibited a significant increase when compared to C, SC Ins, and OG Ins. Additionally, the bleeding time in the DC group was longer in comparison to the C and the OG Ins groups. Notably, the bleeding time was significantly longer in SC Ins when compared to all other groups ($p < 0.05$) (Fig. 4b).

Table 2
Hemostasis analysis in control rats (C), diabetic rats (DC), diabetic rats treated with subcutaneous insulin (SC Ins), and diabetic rats treated with oral insulin loaded in nCOFs (OG Ins).

Tests	C	DC	SC Ins	OG Ins
Quick time (s)	15.1 ± 0.5	15.2 ± 0.7	15.3 ± 0.9	14.3 ± 0.6
APTT (s)	25.3 ± 1.0	24.2 ± 1.2	23.9 ± 3.1	23.9 ± 2.1
Factor II (%)	97.7 ± 1.1	85.6 ± 4.0	80.4[a]* ± 12.7	109.9[b]† ± 11.9
Factor V (%)	99.0 ± 0.7	98.3 ± 2.4	99.6 ± 1.1	100.4 ± 1.5
Factor VII (%)	80.0 ± 1.2	79.7 ± 1.8	79.6 ± 1.1	79.8 ± 1.3
Factor VIII (%)	118.7 ± 32.0	294.3[a]† ± 42.6	330.0[a]† ± 0.0	211.5[a,b]* ± 68.8
Factor IX (%)	116.4 ± 1.3	110.4 ± 12.3	203.8[a,b]* ± 18.8	146.7 ± 74.8
Factor X (%)	86.2 ± 2.4	94.2 ± 21.2	104.7 ± 3.7	150.7[a,b]† ± 5.5
Fibrinogen (mg/d)	249.1 ± 28.5	369.4[a]† ± 32.2	555.8[a,b]† ± 47.7	289.0[b]* ± 24.8
d-dimer (μg/ml)	0.33 ± 0.02	0.42[a]* ± 0.01	0.43[a]* ± 0.08	0.32[b]* ± 0.01

Results are expressed as mean ± SEM
(*$p < 0.05$); (†$p < 0.001$).
[a] versus C,.
[b] versus DC..

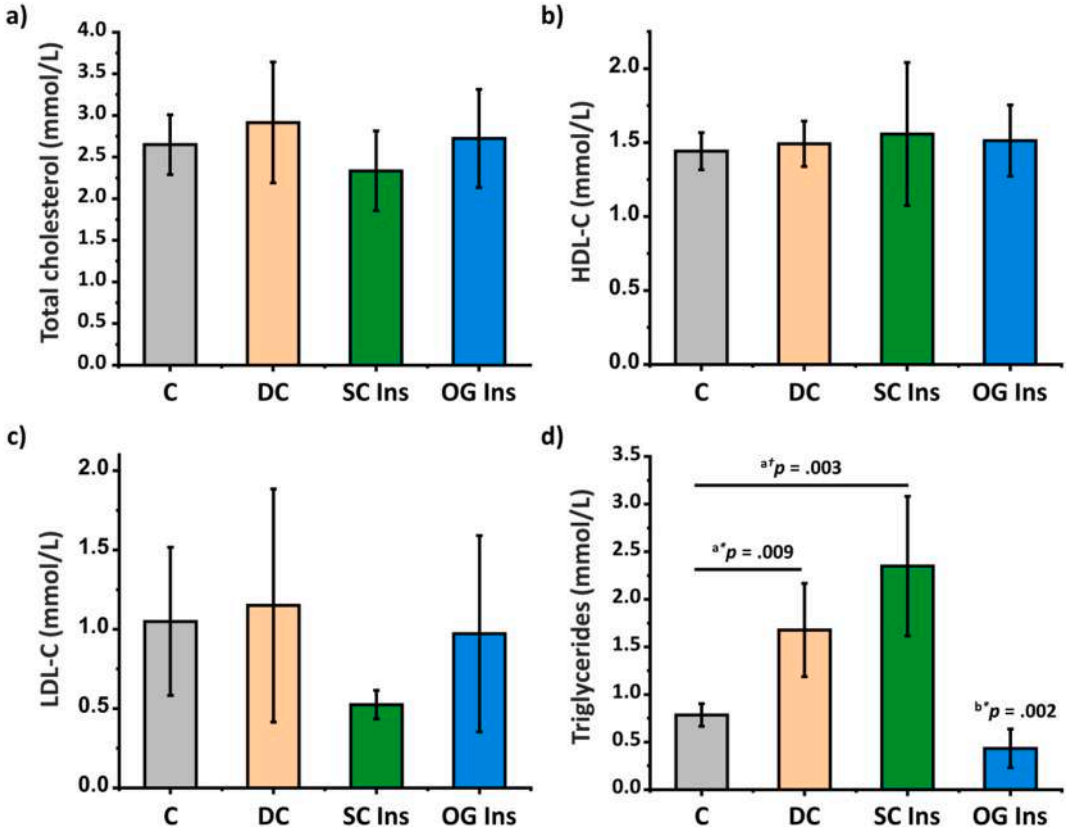

Fig. 3. Effect of diabetes and insulin treatments on plasma biochemical parameters. a) Total cholesterol, b) High-Density Lipoprotein Cholesterol (HDL-C), c) Low-Density Lipoprotein Cholesterol (LDL-C) plasma and d) triglycerides levels of control rats (grey, C), diabetic rats (orange, DC), diabetic rats treated with subcutaneous-insulin (green, SC Ins) and diabetic rats treated with oral insulin loaded in nCOFs (blue, OG Ins). Results are expressed as mean ± SEM, [a]versus C, [b]versus DC (*$p < 0.05$); (†$p < 0.001$).

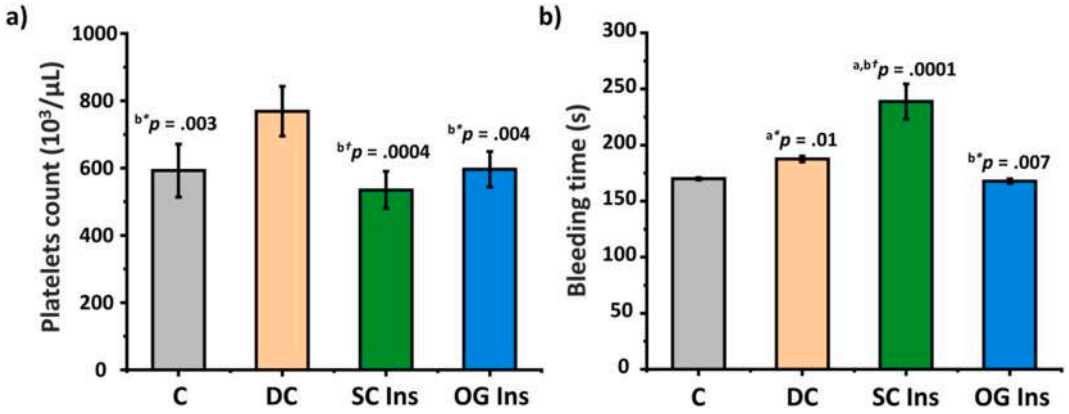

Fig. 4. Effect of diabetes and insulin treatments on platelets activity. a) Platelets count and b) bleeding time of control rats (grey, C), diabetic rats (orange, DC), diabetic rats treated with subcutaneous insulin (green, SC Ins), and diabetic rats treated with oral insulin loaded in nCOFs (blue, OG Ins). Results are expressed as mean ± SEM, [a]versus C, [b]versus DC (*$p < 0.05$); (†$p < 0.001$).

3.4. MDA and cortisol plasma levels

MDA plasma levels exhibited a notable increase in DC and SC Ins ($p < 0.05$) compared to C and OG Ins (Fig. 5a). Additionally, when comparing SC Ins to DC, a further elevation in MDA plasma levels was observed. As shown in Fig. 5b, cortisol plasma levels increased in DC and SC Ins compared to C. However, no statistically significant difference was observed when comparing the OG Ins group to the DC

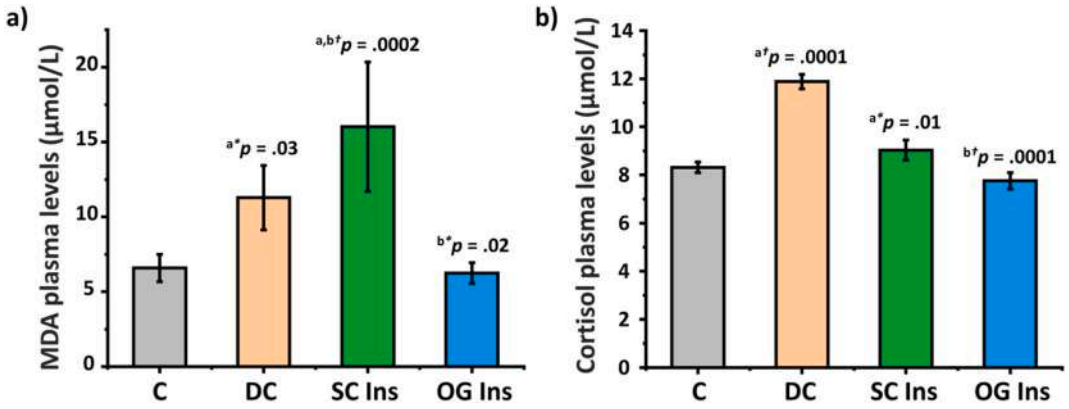

Fig. 5. Effect of diabetes and insulin treatments on plasma lipid peroxidation and plasma cortisol levels. a) MDA and b) cortisol plasma levels of control rats (grey, C), diabetic rats (orange, DC), diabetic rats treated with subcutaneous insulin (green, SC Ins), and diabetic rats treated with oral insulin (blue, OG Ins). Results are expressed as mean ± SEM, [a]versus C, [b]versus DC (*$p < 0.05$); (†$p < 0.001$).

group.

3.5. Pro-oxidant/antioxidant profile analysis in the abdominal aorta

Table 3 reveals significant increases in $O_2^{\bullet-}$ content within the abdominal aorta of the DC, OG Ins, and SC Ins groups when compared to the control group. Notably, the increase in $O_2^{\bullet-}$ content is more pronounced in the SC Ins group compared to both the C and the OG Ins groups. Conversely, GSH content in the abdominal aorta displayed a reduction in all three diabetic groups when compared to C.

3.6. Correlation analysis

Table 4 presents a comprehensive correlation analysis revealing associations between elevated plasma triglyceride levels and various biomarkers. Notably, high plasma triglyceride levels are positively correlated with increased plasma MDA, fibrinogen, d-dimers, factor VIII, and factor IX levels. Furthermore, elevated plasma triglycerides are associated with longer bleeding times, prolonged clotting times, and higher $O_2^{\bullet-}$ content levels in the abdominal aorta. Interestingly, an inverse correlation was observed between plasma factor II concentration and plasma triglyceride levels.

Table 5 continues this analysis, demonstrating significant correlations between MDA plasma levels and multiple biomarkers. These include triglyceride levels, fibrinogen plasma levels, bleeding times, and various other factors, such as VIII, IX, d-dimers, and $O_2^{\bullet-}$ content in the abdominal aorta. Similarly, an inverse relationship between plasma factor II and MDA plasma levels was noted, mirroring the trend observed with triglyceride in Table 4.

4. Discussion

This study delved into the impact of insulin delivery routes, specifically oral and subcutaneous, on various parameters in a T1D rat model, including hemostasis disorders, bleeding time, platelet count, lipid profile, and oxidant/antioxidant balance. Notably, we are the first to demonstrate that subcutaneous insulin injections exacerbate hemostasis disorders and disrupt the oxidant/antioxidant balance in the aortas of diabetic rats. This disturbance primarily results from the deterioration of their lipid profile and the increased levels of lipid peroxidation. In contrast, oral insulin administration improved the coagulation and fibrinolysis as well as the oxidant/antioxidant balance in the abdominal aorta of diabetic rats. This improvement can be attributed to the reduction in lipid peroxidation and the enhancement of their lipid profile. A comprehensive summary of these key findings is presented in Fig. 6.

To explore platelet function and primary hemostasis in diabetic rats, we conducted bleeding time and platelet count tests [31]. Our findings revealed that diabetes is associated with an extended bleeding time, consistent with previous research suggesting that diabetes

Table 3
Pro-oxidant/antioxidant profile analysis in the abdominal aorta of control rats (C), diabetic rats (DC), diabetic rats with subcutaneous insulin (SC Ins), and diabetic rats with oral insulin (OG Ins).

Tests	C	DC	SC Ins	OG Ins
$O_2^{\bullet-}$ (µmol/g)	33.0 ± 7.7	48.1[a]* ± 1.3	62.9[a]† ± 3.2	47.8[a]* ± 10.2
GSH (mmol/g)	2.8 ± 1.0	1.0[a]† ± 0.1	1.4[a]* ± 0.2	1.3[a]* ± 0.2

Results are expressed as mean ± SEM
(*$p < 0.05$); (†$p < 0.001$).
[a] versus C.

Table 4
Correlation (r) between triglycerides plasma levels and various biomarkers: plasma MDA, fibrinogen, d-dimers levels, bleeding time, quick time, hemostasis biomarkers, and $O_2^{\bullet-}$ in the abdominal aorta.

Test	r	*P*
Plasma MDA	0.88	<0.0001
Fibrinogen	0.80	<0.0001
d-dimer	0.79	<0.0001
Bleeding time	0.72	<0.0001
Quick time	0.61	<0.01
APTT	−0.21	N.S.
Factor II	−0.81	<0.0001
Factor V	−0.57	N.S.
Factor VII	−0.15	N.S.
Factor VIII	0.64	<0.01
Factor IX	0.48	<0.05
Factor X	−0.34	N.S.
$O_2^{\bullet-}$ in the abdominal aorta	0.54	<0.05

N.S., not statistically significant.

Table 5
Correlation (r) between MDA plasma levels and various biomarkers: triglycerides, fibrinogen, d-dimers levels, bleeding time, quick time, hemostasis biomarkers, and $O_2^{\bullet-}$ in the abdominal aorta.

Test	r	*P*
Triglycerides	0.88	<0.0001
Fibrinogen	0.86	<0.0001
d-dimer	0.59	<0.01
Bleeding time	0.78	<0.0001
Quick time	0.38	N.S.
APTT	−0.40	N.S.
Factor II	−0.81	<0.0001
Factor V	−0.06	N.S.
Factor VII	−0.19	N.S.
Factor VIII	0.70	<0.001
Factor IX	0.54	<0.05
Factor X	−0.22	N.S.
$O_2^{\bullet-}$ in the abdominal aorta	0.62	<0.01

N.S., not statistically significant.

alters the kinetics of clot formation [11]. Furthermore, we observed an increased platelet count, indicating that the prolonged bleeding cannot be attributed to a deficiency in platelets. Additional assessments included APTT and PT measurements, indicative of factor deficiencies related to bleeding [32]. However, no significant alterations were noted in these parameters. This shows that the prolonged bleeding time associated with diabetes results from functional alterations in platelet activity rather than quantitative changes, aligning with prior research highlighting discernible platelet changes in diabetes [11,33].

The evaluation of $O_2^{\bullet-}$ and GSH levels revealed an imbalance in favor of pro-oxidants within the abdominal aortas of diabetic rats. This imbalance is likely attributed to changes in the lipid profile and increased lipid peroxidation induced by STZ. Streptozotocin-induced diabetes is known to disrupt the lipid profile, elevate lipid peroxidation, and trigger chronic inflammation. These factors, in conjunction with chronic hyperglycemia, collectively contribute to multifactorial vasculopathy [34].

Untreated diabetic rats exhibited a hypercoagulable state, as evidenced by elevated levels of factor VIII and fibrinogen, both considered prothrombotic agents. Additionally, they displayed increased d-dimers levels, a marker associated with reduced fibrinolysis [35]. Furthermore, untreated diabetic rats exhibited elevated cortisol plasma levels, indicating an inflammatory state [11,36,37].

Our results showed that diabetic rats treated with subcutaneous insulin exhibit an extended bleeding time compared to untreated diabetic rats. This observation suggests that insulin injection amplifies platelet alterations induced by diabetes. In a previous study conducted by our team [21], we demonstrated that subcutaneous insulin injections in diabetic rats induce hyper-insulinemia. It is widely accepted that hyper-insulinemia, as with hyperglycemia, can lead to alterations in platelet function [38]. The prolonged bleeding time observed in rats treated with subcutaneous insulin, compared to untreated diabetic rats, is likely due to a combination of factors: the hyperglycemia caused by diabetes and the hyper-insulinemia induced by subcutaneous insulin injection. Furthermore, our research demonstrated that subcutaneous insulin injections exacerbate the levels of plasma factor VIII, d-dimers, and fibrinogen. Additionally, they lead to an increase in factor IX, indicating an aggravation of the overall hemostatic state.

Interestingly, we observed significant correlations between bleeding times, factor IX, factor VIII, fibrinogen, and d-dimer levels with both triglycerides and MDA plasma concentrations. These findings provide strong evidence supporting the notion that subcutaneous insulin injections, which induce peripheral hyperinsulinemia and subsequent hypertriglyceridemia [39], play a critical role in the hemostasis disorder witnessed in T1D patients.

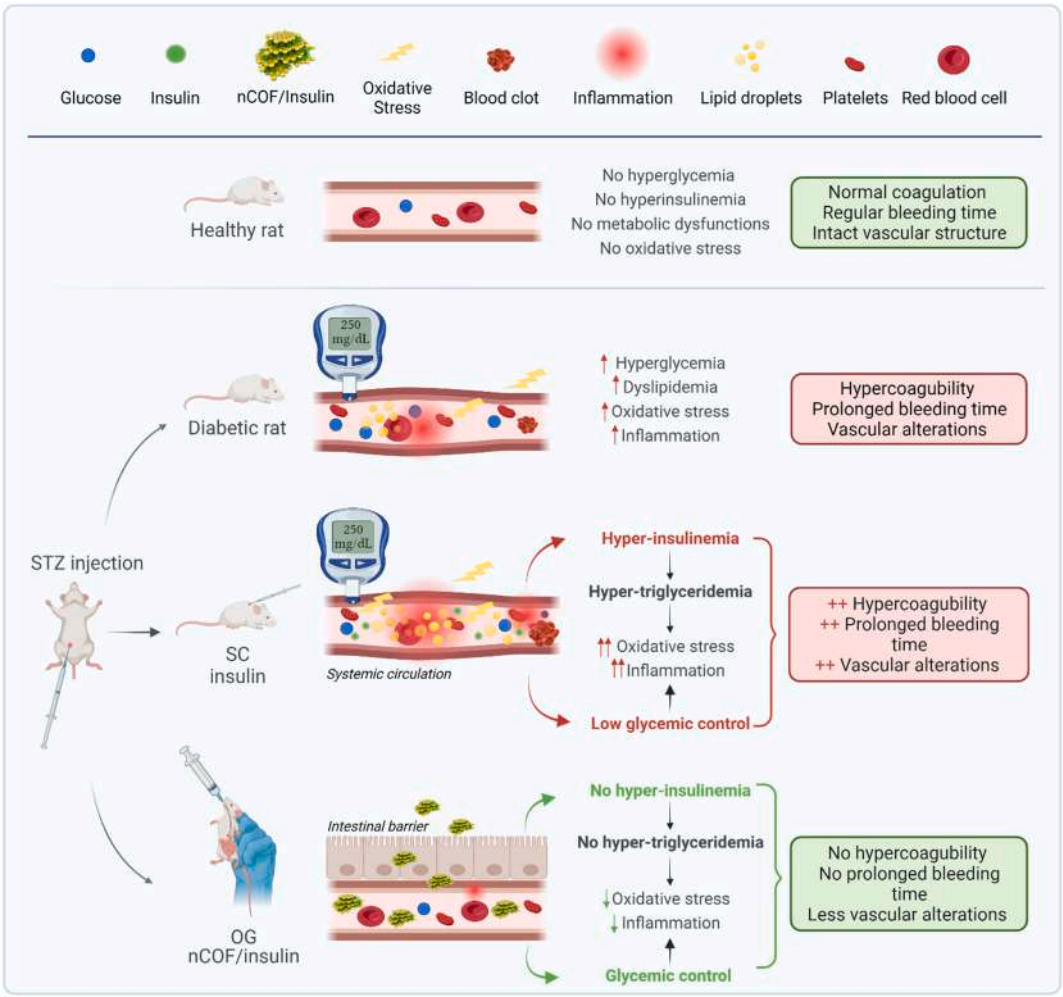

Fig. 6. Overview of vascular changes and treatment effects in normal and STZ-induced diabetic rats. This figure summarizes the key findings of our study: STZ-induced diabetes disrupts the lipid profile, heightening lipid peroxidation and extending bleeding times, leading to a hypercoagulable state and amplified endothelial oxidative stress. Notably, while subcutaneous insulin aggravates these disturbances, oral insulin delivery via nCOF nanoparticles presents a potential solution. This approach mitigates vascular injuries, restores hemostatic balance, averts dyslipidemia, diminishes lipid peroxidation, shields against superoxide buildup in the aorta, and ameliorates the coagulation profile.

In the diabetic groups, the assessment of intracellular $O_2^{\bullet-}$ and GSH concentrations reveals an imbalance in the intracellular antioxidant/pro-oxidant profile, favoring pro-oxidants. Notably, this imbalance is significantly more pronounced in diabetic rats treated with subcutaneous insulin. Previous studies have demonstrated that platelets activated by STZ disrupt vasodilation through the augmentation of oxidative stress, particularly endothelial superoxide production [40]. One plausible explanation for the exacerbated endothelial injuries mediated by oxidative stress in the subcutaneously-treated group is that insulin injections amplify the platelet alterations induced by STZ. This, in turn, intensifies endothelial disturbances by elevating intracellular $O_2^{\bullet-}$ concentrations.

Numerous experimental studies have explored the impact of induced hyperglycemia and/or induced hyperinsulinemia on coagulation in healthy human subjects [38,41,42]. These studies have shown several noteworthy findings. Firstly, they have showed that the pro-coagulant effects induced by hyperglycemia remain unaltered even with insulin administration, irrespective of insulin dosage. Additionally, these investigations have highlighted the influence of hyperglycemia and hyperinsulinemia on hemostatic equilibrium [41] and platelet functionality [38]. Furthermore, it has been observed that the impact of hyperglycemia and hyperinsulinemia on the hemostatic response is aggravated during systemic inflammation [42]. These collective outcomes suggest that hyperglycemia, hyperinsulinemia, and inflammation may synergistically contribute to disruptions in hemostasis. However, it is imperative to interpret with caution considering two crucial considerations. Firstly, the studies were conducted on healthy volunteers, which limits their ability to account for other metabolic disorders frequently associated with diabetes, such as dyslipidemia and oxidative stress [43]. Secondly, they inadequately replicate the characteristic episodic peripheral hyperinsulinemia observed in T1D, often accompanied by episodes of hypoglycemia and subsequent hyperglycemia. The robustness of our study lies in its ability to faithfully replicate the authentic conditions of diabetes.

Our investigation unveiled that subcutaneous insulin administration induces hypertriglyceridemia, subsequently increasing lipid peroxidation. This cascade of events triggers an inflammatory response, as evidenced by elevated fibrinogen levels [44]. Importantly, it aggravates the preexisting hemostasis disorder caused by diabetes in STZ-induced diabetic rats. This exacerbation manifests as an extension in bleeding time, indicative of platelet dysfunction, an amplification in coagulation, characterized by increased factor IX levels, which is further potentiated by diabetes-induced factor VIII elevation and heightened fibrinogen levels. Additionally, this subcutaneous insulin-induced milieu stimulates hypo-fibrinolysis, a phenomenon unveiled by elevated d-dimers plasma levels. Simultaneously, it accentuates endothelial injuries mediated by oxidative stress.

Conversely, our findings took a marked turn when insulin was administered orally, employing nanoparticles (NPs) as carriers. Oral insulin delivery not only ameliorated lipid metabolism but also recalibrated plasma lipid peroxidation levels, bringing bleeding time back to normal values. Furthermore, rats treated with oral insulin exhibited clotting times within the normal range, along with regular d-dimer levels and balanced factors II, V, VII, and IX plasma concentrations. Notably, factor VIII plasma levels were reduced compared to untreated diabetic rats and those treated with subcutaneous insulin, signifying a decrease in inflammation. The cortisol levels, fibrinogen plasma levels, and intracellular superoxide concentrations in rats treated orally with insulin mirrored those of the control group. It is worth noting that although factor X concentrations were elevated, they did not exert a significant impact on the coagulation process.

Insulin itself is not the primary cause of hemostasis disorders; instead, it is the resultant hyperinsulinemia that plays a pivotal role. Notably, oral insulin administration, known for its capacity to establish glycemic homeostasis [21], serves as a prophylactic measure against these complications. Administering the same insulin in two different ways - either by direct subcutaneous injection or orally after encapsulation - results in a different insulinemic profile. This difference significantly affects blood glucose levels, as shown in our previous study [21], and lipid metabolism, as shown in the current study. These differences lead to completely different effects on diabetes-induced hemostasis disorders. This finding aligns with prior research, suggesting the protective potential of intensive insulin therapy. This therapeutic approach, characterized by multiple dose-adjusted injections based on blood glucose levels and stringent glycemic control, has demonstrated the ability to minimize vascular events by reducing oxidative stress and inflammation [13]. Further substantiating this concept, Bratseth et al. also observed that children and adolescents undergoing intensive insulin therapy exhibited pro-coagulant activities similar to those of healthy individuals [45]. Importantly, both studies revealed improvements in lipid profiles as a result of intensive therapy. In rodent experimentations, it has been shown that even a low dose of insulin can enhance triglyceride metabolism, glucose metabolism, and oxidative stress. This effect is accompanied by alterations in the expression of key transcription factors, including NF-κB and RAGE, which are implicated in arterial damage [46]. These collective findings emphasize the multifaceted impact of insulin, with hyperinsulinemia emerging as a critical factor influencing hemostasis and overall vascular health.

5. Conclusion

STZ-induced diabetes in rats disrupts lipid profiles: Our study demonstrates that STZ-induced diabetes in rats disrupts the lipid profile, and oxidative stress within the endothelium. This disruption results in a hypercoagulable state, hypofibrinolysis and prolonged bleeding times.

Detrimental effects of subcutaneous insulin therapy: Our research has shed significant light on the potential detrimental role of subcutaneous insulin therapy in exacerbating these diabetes-related abnormalities. This study highlights, the adverse impact of subcutaneous insulin treatment on amplifying vascular alterations and hemostasis disorder observed in diabetes.

Promising results of oral insulin delivery via nanoparticle carriers: On the other hand, oral delivery of insulin through nanoparticles carriers, in particular the nCOF approach used in our study, represents a promising therapeutic alternative. It effectively counters vascular injuries, corrects hemostatic disorders, alleviates dyslipidemia, suppresses plasma lipid peroxidation, protects against superoxide accumulation in the aorta, and improves the coagulation profile of diabetic rats.

Potential for significant societal and healthcare impact: Our results suggest that the introduction of alternative insulin delivery methods, such as the oral nCOF approach, could lead to a paradigm shift in diabetes care, with far-reaching implications for patient care and research directions.

Foundation for future research and improved patient care: This study not only opens new avenues for insulin-related research but also provides a basis for developing tailored treatment strategies and disease management plans for individuals with T1D, with the goal of improving patient outcomes and quality of life.

Limitations: It is important to note that the sample size of $n = 5$ per group and the short interval between treatment and subsequent analysis (treatment on day 1 and sacrifice on day 2) were determined based on standard practices for preliminary animal studies and the ethical mandate to minimize harm to the animal. While these choices were supported by established methods for initial investigations [47,48], they may limit the generalizability of our results and highlight the need for further research with larger sample sizes and longer durations to fully understand the long-term effects and efficacy of different insulin administration methods.

Ethical statement

All procedures involving animals were conducted in accordance with ethical principles and regulations. The study was approved by the University of Tlemcen Institutional Animal Care and Use Committee (IACUC) (accreditation number: D01N01UN130120150006) and complies with the ARRIVE guidelines.

The data associated with this study are included in the article.

CRediT authorship contribution statement

Nawel Kaddour: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Farah Benyettou:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Kawtar Moulai:** Methodology, Investigation, Formal analysis, Data curation. **Abdelouahab Mebarki:** Validation, Methodology, Formal analysis, Data curation. **Katia Allal-Taouli:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Rose Ghemrawi:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Jamie Whelan:** Writing – review & editing, Investigation, Formal analysis. **Hafida Merzouk:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **Ali Trabolsi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Nassima Amel Mokhtari-Soulimane:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Ali Trabolsi reports financial support was provided by New York University Abu Dhabi. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Efficacy of Oral Nanoparticle-Encapsulated Insulin in Reducing Oxidative Stress and Enhancing Tissue Integrity in a Diabetic Rat Model

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Kaddour N, Benyettou F, Moulai K, Mebarki A, Ghemrawi R, Amir ZC, Merzouk H, Trabolsi A, Mokhtari-Soulimane NA. Efficacy of Oral Nanoparticle-Encapsulated Insulin in Reducing Oxidative Stress and Enhancing Tissue Integrity in a Diabetic Rat Model. *Int J Nanomedicine*. 2024;19:10961-10981

Mortality and morbidity in type 1 diabetes result not only from chronic hyperglycemia but also from the associated complications of the disease. Our findings demonstrated that standard insulin treatment in T1D can have markedly different effects on these complications depending on the route of administration. To further investigate, we examined how the method of insulin delivery (oral versus subcutaneous) influences diabetes-induced organ damage. Diabetes significantly affects multiple organs, leading to toxicity and dysfunction primarily due to insulin deficiency. *In this study, we aimed to evaluate these impacts by examining three key indicators: weight changes, oxidative stress, and histological alterations in vital organs.*

Weight loss is a direct indicator of muscle mass reduction, commonly observed in type 1 diabetes. Additionally, organ weight is a significant indicator of toxicity in preclinical studies. Notable increases in the weight of vital organs such as the liver, kidneys, spleen, or heart may signal toxicity, as the accumulation of toxic substances or cellular damage can cause changes in organ size. Tracking these changes offers valuable insights into the extent of diabetes-induced damage on the body's vital systems.

Oxidative stress, which arises from glucose deprivation in various tissues, provides important insights into the metabolic disturbances caused by diabetes. In type 1 diabetes, insulin deficiency forces cells to rely on alternative energy sources like fatty acids. This metabolic shift leads to the overproduction of ROS, which overwhelms the body’s antioxidant defenses. The resulting oxidative stress plays a key role in organ damage and the progression of diabetes-related complications. By assessing oxidative stress, we can better understand the broader metabolic consequences and tissue damage induced by diabetes

Histological analysis offers a detailed assessment of the structural integrity and overall condition of organs. In the context of diabetes, microscopic examination can reveal critical changes such as cellular hypertrophy, atrophy, necrosis, and inflammation, which often correlate with functional impairments. These observations provide valuable insights into the severity of disease progression. Furthermore, histology helps elucidate the impact of oxidative stress and metabolic dysregulation at the cellular level, highlighting the intricate relationship between oxidative stress and structural damage.

In this study, our objective was to gain a comprehensive understanding of how insulin deficiency impacts organ health and to evaluate the potential of insulin therapy in reversing these diabetes-induced alterations and preventing related complications. To this end, we divided Wistar rats into four experimental groups: diabetic rats receiving no treatment, diabetic rats treated with subcutaneous insulin, diabetic rats administered oral insulin, and non-diabetic control rats. We compared weight changes and oxidative stress profiles in the intestine, spleen, heart, liver, kidneys, brain, and soleus muscle. Furthermore, we conducted histological analyses across these organs and performed immunohistochemical staining of E-cadherin in the intestine to compare results across the four groups. This multifaceted approach provided a comprehensive understanding of the systemic impact of different insulin administration routes on T1D-induced alterations.

Our results show that diabetes decreases body weight and increases the relative weight of organs, indicating metabolic distress. Subcutaneous insulin only partially corrects these changes, especially failing to restore overall body weight and the relative weight of the liver, the main metabolic organ, suggesting it does not fully restore metabolic function. Additionally, our results demonstrated that diabetes disrupts the antioxidant/pro-oxidant balance, particularly by increasing lipid peroxidation in all the studied organs and altering the concentrations of glutathione (GSH) and catalase in certain tissues. Subcutaneous insulin treatment mitigates this imbalance in some organs, notably by reducing MDA levels, but it also increases protein carbonyl concentrations in the intestine and muscle.

Finally, our results showed that T1D induced histological alterations in the intestine, spleen, heart, liver, kidneys, and adrenal glands of untreated diabetic rats. Our histological analysis revealed that subcutaneous insulin treatment corrected the diabetes-induced alterations in the heart and partially restored those in the intestine and spleen, but failed to do so in the liver, kidneys, and adrenal glands.

Our findings suggest that the innovative Oral nCOF/Insulin treatment may help reverse these detrimental effects and facilitate a return to normal physiological functions. Notably, our results indicate that Oral nCOF/Insulin restores the body weight of diabetic rats to levels comparable to

non-diabetic rats after treatment, as well as the relative weight of the studied organs. Additionally, it supports brain health by reducing lipid peroxidation, mitigates muscle wasting by lowering oxidative stress, and restores intestinal integrity. Furthermore, it appears effective in improving splenic immune function, myocardial structure, hepatic architecture, renal integrity, and adrenal gland composition. The ability of oral insulin to positively impact both the adrenal glands—key hormonal organs—and the liver, the largest gland in the body, underscores its extensive effects. These findings highlight insulin's influence across a wide spectrum of interconnected metabolic mechanisms, all working together to regulate metabolic balance.

Finally, our immunohistochemical staining for E-cadherin reveals no depletion of these markers during treatment with insulin-loaded nanoparticles, further highlighting the non-toxicity of our Oral nCOF/Insulin. These findings emphasize both the safety and effectiveness of Oral nCOF/Insulin as a therapeutic option for diabetes management.

These results suggest that oral insulin administration, which restores glycemic balance without inducing hyperinsulinemia or hypertriglyceridemia—due to its central-to-peripheral, dose-responsive delivery—can yield beneficial effects across multiple organs. This controlled, uniform delivery of insulin, as we have previously demonstrated, has the potential to repair diabetes-induced damage in various organs, thereby helping to prevent complications related to diabetes.

Our study underscores the remarkable efficacy of oral nCOF/Insulin in preserving organ health, revealing unprecedented outcomes that have not been previously explored. It presents a comprehensive analysis of the effects of nanoparticle-mediated oral insulin on multiple organs, offering novel insights into diabetes treatment and the prevention of associated complications. To our knowledge, this research represents the most extensive examination of the effects of nanoparticle-mediated oral insulin on multiple organs simultaneously, many of which have not been previously studied in this context. Our work stands as a pioneering effort in the exploration of nanoparticle-based therapies for diabetes management.

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Open Access Full Text Article ORIGINAL RESEARCH

Efficacy of Oral Nanoparticle-Encapsulated Insulin in Reducing Oxidative Stress and Enhancing Tissue Integrity in a Diabetic Rat Model

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Introduction: Diabetes mellitus, a chronic metabolic disorder, leads to systemic organ damage characterized by oxidative stress and structural alterations, contributing to increased morbidity and mortality. Traditional subcutaneous insulin therapy, while managing hyperglycemia, often falls short in addressing the oxidative damage and preventing organ-specific complications. This study evaluates the therapeutic efficacy of a novel oral nanoparticle-mediated insulin (nCOF/Insulin) against these diabetes-induced changes, comparing it with traditional subcutaneous insulin in a streptozotocin (STZ)-induced diabetic rat model.

Methods: We induced diabetes in Wistar rats, dividing them into four groups: standard control, diabetic control, diabetic treated with subcutaneous insulin, and diabetic treated with oral nanoparticle-mediated insulin (nCOF/Insulin). Assessments included organ and body weights, histopathological examinations, and oxidative stress markers (MDA and PCOs) across various organs, including the brain, muscle, intestine, spleen, heart, liver, kidney, and adrenal glands. Additionally, we evaluated antioxidant parameters (GSH and catalase) and conducted immunohistochemical analysis of E-cadherin to assess intestinal integrity.

Results: Our findings reveal that STZ-induced diabetes significantly impacts organ health, with subcutaneous insulin providing limited mitigation and, in some cases, exacerbating oxidative stress. Conversely, oral nCOF/Insulin treatment effectively restored organ and body weights, reduced oxidative stress markers, and mitigated histological damage. This suggests that oral nCOF/Insulin not only offers superior glycemic control but also addresses the underlying oxidative stress.

Conclusion: nCOF/Insulin emerges as a promising treatment for diabetes, with the potential to improve patient quality of life by ameliorating oxidative stress and preventing organ-specific complications. This study underscores the need for further investigation into the long-term effects and mechanisms of action of oral nCOF/Insulin, aiming to revolutionize diabetes management and treatment strategies.

Keywords: oral nanoparticle-mediated insulin, diabetes mellitus, oxidative stress, organ toxicity, diabetic complications

Introduction

Diabetes mellitus is a group of metabolic disorders marked by persistent hyperglycemia.[1] Over half a billion people are living with diabetes worldwide, which represents more than 10.5% of the world's adult population.[2] This widespread prevalence underscores its status as a major global health challenge.[1]

Diabetes mellitus, a complex metabolic disorder, is intricately linked to systemic oxidative stress, a key factor in its pathogenesis. It is now understood that the onset of diabetes originates from oxidative stress within pancreatic cells, which triggers inflammation and prompts the release of pro-inflammatory cytokines, ultimately leading to the death of

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pancreatic beta cells.[3,4] This loss of beta cells results in hypoinsulinemia and hyperglycemia, which in turn creates a vicious cycle of oxidative stress throughout the organism.[5,6] This systemic oxidative stress then triggers functional and structural alterations in various organs, leading to diabetes-related complications and consequently making diabetes one of the principal causes of mortality and morbidity worldwide.[7–9]

The contemporary understanding of diabetes has evolved to recognize it as a complex dysglycemic disorder, not just defined by chronic high blood sugar levels (hyperglycemia) but also involving two additional critical mechanisms: glucose variability, and hypoglycemic incidents. These mechanisms collectively contribute to triggering oxidative stress in diabetic patients.[5] Oxidative stress is implicated in inducing various alterations across organs,[10,11] amplifying the stress response, and perpetuating a cycle of tissue damage that compromises organ function.[12–15] A self-sustaining pattern of oxidative stress pervades the entire body,[6,16] ultimately resulting in diabetic complications.[5,17]

These mechanisms are particularly pertinent to type 1 diabetes (TD1), where therapeutic management primarily relies on subcutaneous insulin (SC Ins). Understanding these mechanisms not only sheds light on the complexities of diabetes pathology but also underscores the challenges inherent in current insulin therapy. While subcutaneous insulin effectively targets hyperglycemia, its limitations become apparent when considering the multifactorial nature of diabetes. The management of diabetes should not only be limited to addressing chronic hyperglycemia but should systematically consider glucose variability, and hypoglycemic incidents. However, the reliance on subcutaneous insulin injections fails to address the underlying dysglycemic mechanisms and oxidative stress induced by diabetes.[3,12] Furthermore, the pharmacokinetic limitations of subcutaneous insulin necessitate frequent administrations to achieve glycemic control, inherently associated with risks of hypoglycemia and inadequate postprandial glucose management.[18,19] Consequently, insulin therapy, as currently administered, does not adequately prevent diabetes-related complications and may even exacerbate them by further exacerbating oxidative stress.[5,20] The challenge today is to develop therapies that address all the pathophysiological mechanisms of diabetes.

An ideal diabetes management system requires swift glucose level adjustments without causing insulin overdose or hypoglycemia. It must support lifelong therapy with easy, predictable use, minimal side effects, and stable performance over time. These features are crucial in developing a glucose-responsive insulin delivery system aimed at achieving a synthetic pancreas, addressing challenges like preventing burst release, ensuring ease of administration, and managing degradation and immune responses. This is why alternatives are currently being sought, with nanoparticle-based approache for oral insulin emerging as a promising option due to its ability to closely mimic physiological insulin.[21–23] Nanoparticles can be engineered to encapsulate and deliver insulin in a controlled manner, offering several advantages over conventional insulin therapy (Figure 1). These advantages include improved pharmacokinetics, enhanced bioavailability, and targeted delivery to specific tissues or cells. To address these needs, covalent organic framework nanoparticles (nCOFs), have shown great promise. nCOFs, known for their robustness, tunability, and suitability for biological applications due to their stability in water and acidic environments, offer a novel approach to insulin delivery. In a previous study, we highlighted the use of a specific nCOF, synthesized from 2,6-diformylpyridine and 4,4′4″-(1,3,5-triazine-2,4,6-triyl)trianiline, which demonstrated high insulin loading capacity, biocompatibility, and effective glucose-triggered insulin release (nCOF/Insulin).[19] The system allows insulin to be stored between nanosheet layers, enhancing its protection in the stomach's harsh conditions and subsequent controlled delivery. Furthermore, this method directly absorbs insulin through the intestinal epithelium and delivers it to the liver, more accurately mimicking the natural central-to-peripheral insulin distribution. This approach ensures that the liver receives the highest concentration of insulin, acting as a buffer to prevent peripheral hyperinsulinemia. Moreover, the insulin release from the nCOF/Insulin system closely mimics physiological processes. Under natural conditions, insulin secretion is regulated in a pulsatile, non-linear manner in response to blood glucose levels, a pattern crucial for maintaining glucose homeostasis and aligning insulin levels with metabolic needs. Subcutaneous insulin (SC Ins) often results in a rapid spike in insulin levels, which does not replicate this natural pulsatile secretion. Our oral nCOF/Insulin formulation, however, demonstrates a controlled and gradual release of insulin, thereby reducing the risk of hypoglycemic episodes and offering a more natural and physiologically relevant delivery method. Additionally, the nCOF/Insulin system features a dose-dependent and self-regulated release mechanism. This on-off regulation mimics the pulsatile release of endogenous insulin, ensuring tight control of insulin levels and preventing both hypo- and

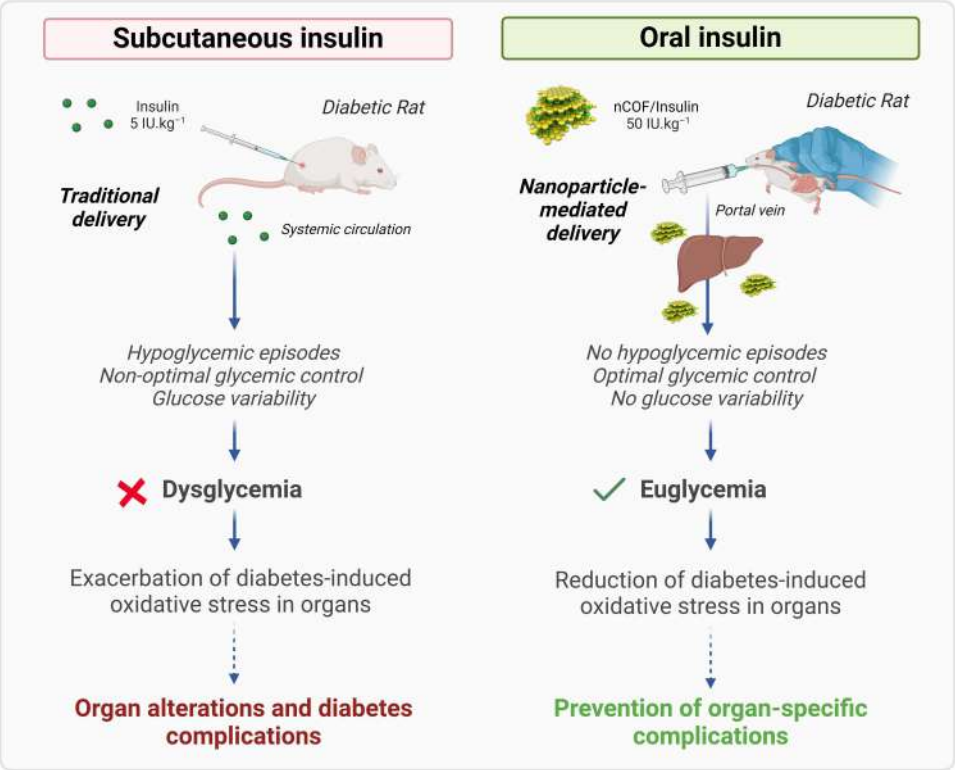

Figure 1 Comparative Effects of Oral and Subcutaneous Insulin Delivery on Diabetic Complications in Rats. The left panel shows that subcutaneous insulin delivery in diabetic rats results in hypoglycemic episodes, non-optimal glycemic control, and glucose variability, leading to dysglycemia and subsequently exacerbating diabetes-induced oxidative stress, which may contribute to organ alterations and long-term diabetes complications. In contrast, the right panel demonstrates that oral administration of nCOF/Insulin has the potential to improve glycemic control with fewer hypoglycemic episodes and reduced glucose variability, thereby promoting euglycemia. This method reduces diabetes-induced oxidative stress in organs which could help mitigate the severity of organ-specific complications.

hyperglycemia. These unique properties of the nCOF/Insulin system—central-to-peripheral distribution, non-linear and pulsatile release, and dose-dependent response—play a crucial role in its physiological efficacy. Collectively, these mechanisms enable the oral nCOF/Insulin formulation to more closely replicate natural insulin secretion, highlighting its advancement over traditional subcutaneous injections.[19] Furthermore, our last study showed that the nCOF can prevent hemostasis disorder and endothelial injuries related to type 1 diabetes, while the traditional subcutaneous treatment exacerbates them. Specifically, we have shown that oral insulin delivered via nCOF addresses various mechanisms in the systemic circulation, such as dyslipidemia, inflammation, oxidative stress, and cortisol levels.[24]

This intriguing observation underscores the intricate interplay between insulin delivery methods and resulting physiological responses. Building upon these findings, the present study aims to delve deeper into this phenomenon by comparing the effects of subcutaneous and oral nCOF/Insulin on oxidative stress profiles and tissue integrity in a diabetic rat model. Through detailed histopathological analysis, we aim to elucidate the specific impacts of oral nCOF/Insulin, particularly its potential to mitigate diabetes-induced tissue alterations. Additionally, this study examines the non-toxicological profile of oral nCOF/Insulin compared to SC insulin, reinforcing its promise as a viable and effective alternative for diabetes management.

By offering a comprehensive evaluation of oral nCOF/Insulin's efficacy and safety, this research provides critical insights into the potential of innovative nanoparticle insulin delivery strategies. While the study demonstrates promising short-term effects, the findings suggest that such strategies may also contribute to addressing the complex challenges of diabetes treatment and potentially mitigating diabetic complications in the long term, thereby advancing the field toward more effective and patient-friendly therapeutic options.

Methods

Insulin Loaded nCOF Nanoparticles (nCOF/Insulin)

Synthesis and Characterization of Insulin-Loaded nCOF Nanoparticles (nCOF/Insulin)

nCOFs were synthesized through a co-condensation of 2.6-diformylpyridine (DFP, 21 mg, 0.15 mmol) and 4,4′,4″-(1,3,5-triazine-2,4,6-triyl)trianiline (TTA, 12 mg, 0.03 mmol) in anhydrous 1,4-dioxane (3 mL) with 0.5 mL of acetic acid (13 M) to achieve a final concentration of 4.0 M. This mixture was reacted at room temperature for 10 minutes and subsequently dialyzed in water to remove unreacted materials, yielding a stable colloidal suspension of nCOFs. The resultant nanoparticles exhibited an average diameter of 123.7 ± 18.1 nm and a spherical morphology confirmed via Transmission Electron Microscopy (TEM). The surface charge was determined by zeta potential measurements, indicating a value of −16 mV, which suggests good colloidal stability.[19]

Drug Loading and Encapsulation Efficiency

For insulin loading, nCOFs (5 mg) were dispersed in 2 mL of HEPES buffer, to which a HEPES-buffered aqueous solution of insulin (10 mg/mL, 1 mL) was added, maintaining an nCOF ratio of 1:2. This mixture was stirred overnight at pH 7.4 and room temperature. The unbound insulin was removed by multiple centrifugation washes with deionized water. The insulin loading capacity reached 64.6 ± 1.7 wt%, with encapsulation efficiency confirmed through various advanced characterization techniques such as fluorescence and Fourier Transform Infrared (FTIR) Spectroscopies as well as X-ray Photoelectron Spectroscopy (XPS), which indicated specific interactions between insulin and the nCOF framework. Additional studies utilizing Powder X-ray Diffraction (PXRD) showed distinct peaks confirming the crystalline structure, while Brunauer-Emmett-Teller (BET) analysis demonstrated a significant surface area, ensuring the structural integrity and porosity essential for insulin encapsulation.[19]

Stability and Release Profile

Stability assessments under simulated gastrointestinal and bloodstream conditions confirmed that the nanoparticles maintained their integrity and protected the insulin payload. Insulin release studies demonstrated a controlled release profile, particularly under hyperglycemic conditions, suggesting a glucose-responsive release mechanism facilitated by the nanoscale dimensions and porous structure of the nCOFs, which enable rapid glucose-mediated insulin displacement.[19]

Biocompatibility and Efficacy Studies

In vitro and in vivo studies confirmed the biocompatibility and efficacy of the nCOF/insulin system for oral insulin delivery. Viability assays on several cell lines (Hep-G2, HCT-116, HCT-8, RKO, HeLa, A2780, MDAMB-231, MCF-7, HEK-293 and U251-MG) showed no cytotoxic effects, affirming the system's suitability for oral use. Further TEM analysis demonstrated that nCOF/insulin did not alter cellular ultrastructure, suggesting safe endocytosis and internal processing. Hemolytic assays confirmed the formulation's non-immunotoxic nature, underlining its safety for bloodstream entry. Ex vivo experiments showed that the nanoparticles effectively crossed the intestinal barrier, enhancing insulin permeability and demonstrating a promising method for diabetes treatment. Oral administration in diabetic rats led to significant reductions in blood glucose levels up to 10 hours without causing organ damage or affecting kidney and liver functions, highlighting the potential of nCOF/insulin to maintain glucose homeostasis and serve as a substitute for subcutaneous injections.[19]

In vivo Experiments

Animals

This study utilized twenty Wistar rats, comprising ten males and ten females, aged 12 weeks and weighing approximately 200 ± 20 g, which were obtained from the Pasteur Institute. The rats were housed in plastic cages with wood-chip bedding, under a constant temperature of 25 °C and a 12:12-hour light/dark cycle. The animals were provided with a standard pellet diet (Teklad Global 18% protein rodent diet, Harlan Laboratories, 58% carbohydrate, 24% protein, 18%

fat) and had unrestricted access to water throughout the study. All procedures were conducted in strict accordance with national standards for the care and use of laboratory animals, and the study protocol was approved by the Animal Research Ethics Committee of the University Abou Bekr Belkaid of Tlemcen in 2020 (accreditation number: D01N01UN130120150006).

T1D Induction

T1D was induced via a single intraperitoneal injection of streptozotocin (STZ, dissolved in 10 mM citrate buffer at pH 4.5) at a dose of 45 mg.kg^{-1} of body weight.[25,26] The rats were then returned to their cages and provided with food and water until the onset of diabetes. Blood glucose levels were monitored using a blood glucose monitoring system (AccuChek Performa, Hoffman-La Roche) by sampling from the rat's tail vein. Rats with fasting glycemia ≥ 250 mg.dL^{-1} (13.7 mmol.L^{-1}) were considered diabetic and selected for the study; the experimental protocol is summarized in Figure 2.

Study Design

Following the induction of diabetes, the rats were categorized into four distinct groups, each consisting of five animals. The groups were organized as follows: standard control (C), diabetic control (DC), diabetic rats treated with subcutaneous insulin (SC Ins, at a dosage of 5 IU kg^{-1}), and diabetic rats treated with oral administration of nanoparticle-formulated insulin (Oral nCOF/Ins, at a dosage of 50 IU kg^{-1}). After their allocation into groups, the rats underwent an overnight fasting period (12 hours) with free access to water. Subsequently, they received a single dose of insulin when applicable in the early morning. Throughout the remainder of the day, the rats had access to food and water. They were

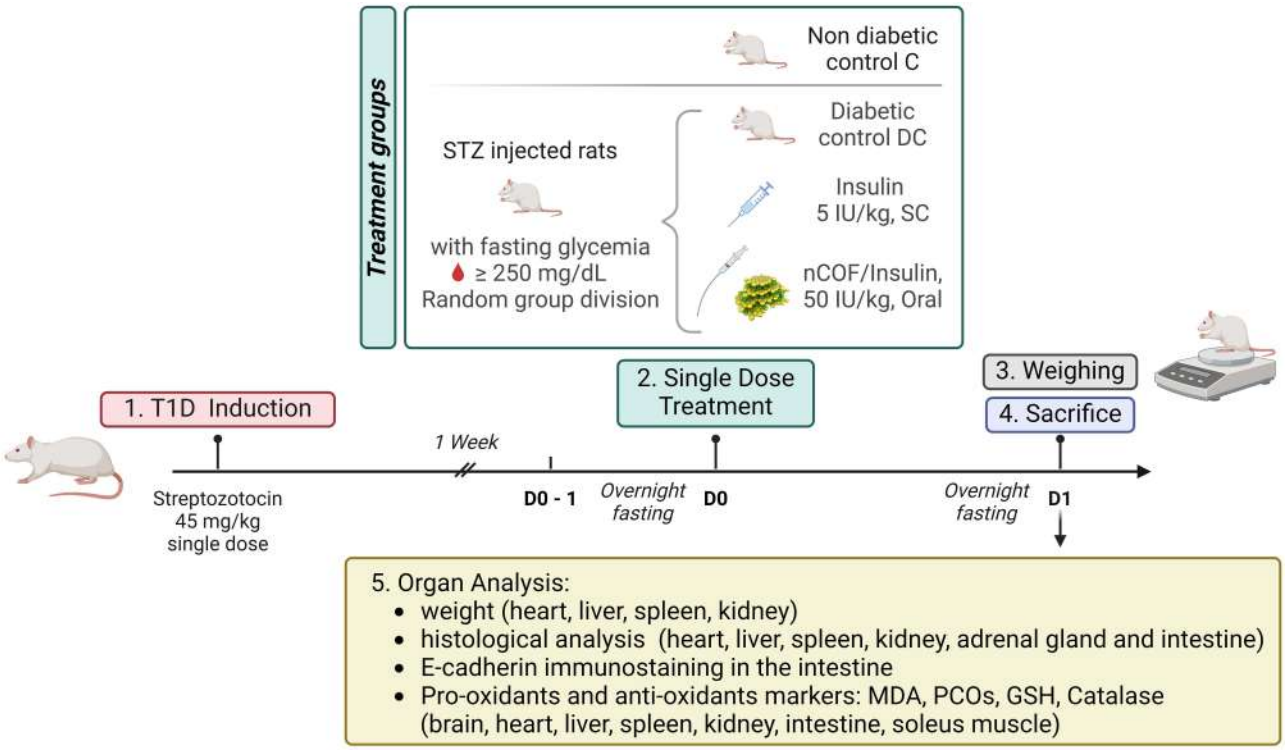

Figure 2 Overview of the Study Protocol. The diagram illustrates the ordered process employed in our investigation. The induction of type 1 diabetes (T1D) in rats was achieved through a single dose of streptozotocin (STZ). Five rats were randomly selected and set as the non-diabetic control group. The remaining 15 rats were administered STZ, and after confirming that their fasting glucose levels had exceeded 250 mg/dL, they were randomly assigned to three different treatment groups for subsequent analysis. These groups were subjected to various insulin treatment protocols. Post-treatment, following an overnight fast, the rats underwent weighing and were subsequently euthanized. A comprehensive collection of organs was undertaken, including the brain, heart, liver, spleen, kidneys with their adrenal glands, intestines, and soleus muscle, which were all washed with ice-cold 0.1 M phosphate-buffered saline. The four treatment groups' heart, liver, spleen, and kidneys were weighed to determine relative organ weights. For histological evaluation, sections of each organ were dissected and preserved in 10% formalin. The intestine was specifically assessed via immunohistochemical analysis for E-cadherin, and the brain, heart, liver, spleen, kidneys, intestines, and soleus muscle were examined for oxidative stress markers, including MDA and PCOs, as well as antioxidant defense enzymes such as GSH and catalase. The statistical analysis of the data was executed using SPSS software.

then fasted overnight once again. The sacrifice was conducted in the early morning following this overnight fasting period, during which the rats had free access to water.

In our study design, the dosages for subcutaneous (5 IU/kg) and oral (50 IU/kg) insulin delivery were selected based on the distinct bioavailability profiles of these administration routes. Subcutaneous delivery provides nearly 100% bioavailability, allowing lower doses to be effective. In contrast, oral administration involves challenges such as significant enzymatic degradation and first-pass metabolism, leading to markedly lower bioavailability. To compensate for this and ensure sufficient systemic insulin levels, a higher dose is used for oral administration. This dosage strategy is critical for comparing the therapeutic efficacy of the two delivery methods under equivalent systemic exposure conditions.

Sample Collection

Following an overnight fast, the animals were weighted and then euthanized under deep anesthesia with isoflurane, at a concentration of approximately 2.5% v/v.[27,28] Then, various organs were harvested for analysis. The organs collected included the intestine, spleen, heart, liver, kidney, brain, and soleus muscle, all of which were immediately rinsed with ice-cold 0.1 M phosphate-buffered saline (PBS; pH 7.4) to remove any residual blood and debris. The weights of the spleen, heart, liver, and kidneys were accurately measured. For biochemical analyses, a portion of each tissue was homogenized in a solution of ice-cold 10 mM phosphate-buffered saline. This process was performed using an ultra-Turrax homogenizer (Bioblock Scientific, Illkirch, France) for three cycles of 10 seconds each, ensuring thorough disruption of the tissue. The homogenates were then centrifuged at 3000 rpm and 4 °C for 5 minutes to separate the supernatant fractions, which were collected for subsequent redox marker determinations.

For histological examination, samples were carefully sectioned using a sharp razor blade and fixed in a 10% formalin solution to preserve tissue architecture. These samples were then subjected to a dehydration process through a series of alcohol baths of increasing concentration, preparing them for embedding in paraffin blocks. This meticulous preparation allows for detailed microscopic examination of tissue structure and pathology.[29,30]

Biomarkers of Oxidative Stress and Antioxidant Defense

Malondialdehyde (MDA), a biomarker of lipid peroxidation, was quantified in tissue samples by measuring thiobarbituric acid reactive substances (TBARS). The procedure involved treating the samples overnight with Sodium Dodecyl Sulfate (SDS), followed by quantification of TBARS through their reaction with thiobarbituric acid (TBA) under acidic conditions. This reaction produces a pink chromophore, the intensity of which was measured spectrophotometrically at a wavelength of 532 nm.[31] The MDA concentration was reported as micromoles of MDA per mg protein and expressed as percentage of the Control Protein Carbonyls (PCOs), indicators of protein oxidation, were assessed by determining the carbonyl group content via reaction with dinitrophenylhydrazine (DNPH), according to a well-established protocol.[32] Protein precipitation was achieved using 20% trichloroacetic acid, followed by redissolution in DNPH. The absorbance of the resulting solution was measured spectrophotometrically at 370 nm, with PCO concentration expressed as nanomoles of PCOs per milligram of protein and expressed as percentage of the Control.

Catalase activity, an important enzymatic antioxidant defense, was assessed by measuring the rate of hydrogen peroxide decomposition using a spectrophotometric kinetic method.[33] The decrease in absorbance due to hydrogen peroxide breakdown was monitored at 240 nm. Catalase activity was expressed as micromoles of hydrogen peroxide degraded per minute per milligram of protein and expressed as percentage of the Control.

Glutathione (GSH) levels, reflecting the tissue's antioxidant capacity, were determined using a method that involves the reduction of 5.5′-dithiobis(2-nitrobenzoic acid) (DTNB) by reduced glutathione, leading to the formation of a yellow compound.[32] The intensity of this color, directly proportional to the GSH concentration, was measured at 405 nm. GSH concentration was quantified as micromoles of GSH per milligram of protein and expressed as a percentage of the Control.

Histopathological Study

For the histopathological analysis, sections with a thickness of 5 μm were meticulously prepared from the paraffin-embedded blocks utilizing a microtome. Following preparation, these sections were stained with hematoxylin and eosin (H&E) to enhance the visualization of cellular and tissue structures.[29,30] The stained sections were then examined and documented using an optical microscope (AX80, Olympus, Tokyo, Japan). During the microscopic examination, observations were documented based on criteria established through a comparison of the histological features of the samples to those of the control group. Each notable observation, such as deviations from normal tissue architecture or the presence of specific pathological features, was recorded with a photograph. To ensure a comprehensive assessment of tissue alterations, we systematically examined sections from each organ of every rat used in the study. This was followed by comparative imaging across other samples to ensure consistency and representativeness of the findings.

Immunohistochemical Staining

Immunohistochemical staining was employed to investigate the impact of nCOF/Insulin on the expression of E-cadherin, a critical cell adhesion protein essential for the cohesion and integrity of epithelial cell layers, in the intestines of diabetic rats. The procedure began with the collection of intestinal tissue samples from both the treatment group, receiving nCOF/Insulin, and the control group. These samples were then fixed in buffered paraformaldehyde, dehydrated through a graded series of ethanol, cleared in xylene, and finally embedded in paraffin. Tissue sections of 4–5 μm thickness were prepared using a microtome, deparaffinized, and rehydrated. Antigen retrieval was performed by heating the sections in a citrate buffer, followed by blocking of non-specific binding sites. The sections were incubated with Dako's E-Cadherin antibody (Clone: NCH-38, Cat. No. M3612), according to the manufacturer's recommendations. E-cadherin expression was visualized as violet staining.

Statistical Analysis

The data were analyzed using SPSS (IBM, SPSS Statistics, version 23, USA) and expressed as means ± standard error of the mean (SEM). Data were analyzed using one-way ANOVA with post hoc Tukey's tests for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

Results

Rat Weight and Organ Weight

Diabetes significantly reduced the body weight of rats (Figure 3a) and increased the relative weights of their organs (Figure 3b), indicating metabolic distress. Subcutaneous insulin fails to restore body weights, and while it does normalize relative organ weights of the rat, heart, and kidney, it does not achieve the same effect in the liver. Notably, oral administration of nCOF/Insulin effectively counteracted these effects, reinstating body and relative organ weight to near-normal levels.

Oxidative Stress

Our findings suggest that diabetes disrupts the balance between oxidants and antioxidants. Diabetes induces oxidative stress, as evidenced by disturbances in MDA levels in all organs of untreated diabetic rats (Figure 3c, D.C.), along with an increase in protein carbonyl concentrations in the spleen, heart, and muscle (Figure 3d). Furthermore, antioxidant concentrations in certain organs are impacted, particularly in the intestine for GSH (glutathione), and in the heart and muscle for GSH and catalase, respectively (Figure 3e and f).

Injections of insulin in diabetic rats, administered subcutaneously (SC Ins), appeared to mitigate the increases in MDA induced by diabetes in some organs (liver, kidney, and brain, Figure 3c), as well as protein carbonyls in the spleen and heart, although not in the muscles (Figure 3d). However, subcutaneous insulin treatment also increased protein carbonyl concentrations in the intestine and muscle.

Administration of Oral nCOF/Insulin resulted in a reduction of oxidative stress levels induced by diabetes. MDA levels (Figure 3c) and protein carbonyl concentrations (Figure 3d) in diabetic rats treated with Oral nCOF/Insulin closely

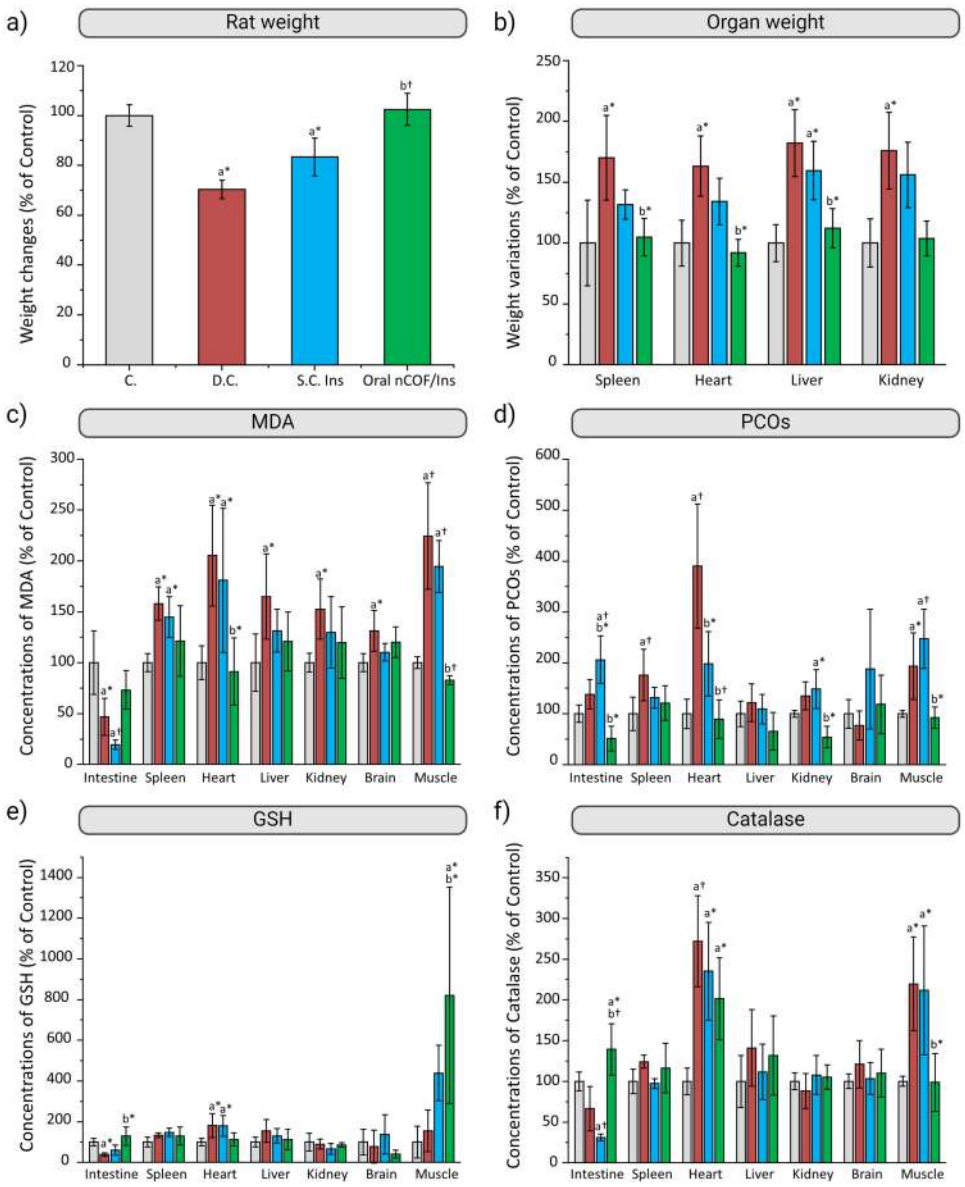

Figure 3 Body and organ weight, and biochemical alterations in rat models. (**a**) Rat body weight changes across Control (C., blue), Diabetic Control (D.C., red), Subcutaneous Insulin-treated Diabetic (SC Ins, blue), and Oral nCOF/Insulin-treated Diabetic (Oral nCOF/Ins) groups, shown as a percentage of the Control. (**b**) Organ weight variations for spleen, heart, liver, and kidney. (**c**) Malondialdehyde (MDA), (**d**) Protein Carbonyls (PCOs), (**e**) Glutathione (GSH), and (**f**) Catalase concentration variations in intestine, spleen, heart, liver, kidney, brain, and muscle. Results are mean ± SEM, expressed as a percentage of the Control group. Comparisons: a vs C., b vs D.C. (*$p < 0.05$); ($^{\dagger}p < 0.001$).

resembled those of non-diabetic rats (C.), indicating a restoration of oxidative balance. Moreover, the increased concentrations of GSH in the muscle (Figure 3e) and catalase in the intestine and heart (Figure 3f) underscore the antioxidant properties of Oral nCOF/Insulin on diabetic organs.

Histological Sections of Rat Intestine

Under microscopic examination, the intestinal lining of control rats displayed a highly organized structure, characterized by well-defined epithelial villi and a pronounced muscular layer (Figure 4, Control). At high magnification of the villi, lymphoid elements were observed in the lamina propria, a connective tissue layer at the core of the villi. Additionally, glands responsible for the secretion of digestive enzymes and mucus were located within the epithelial layer.

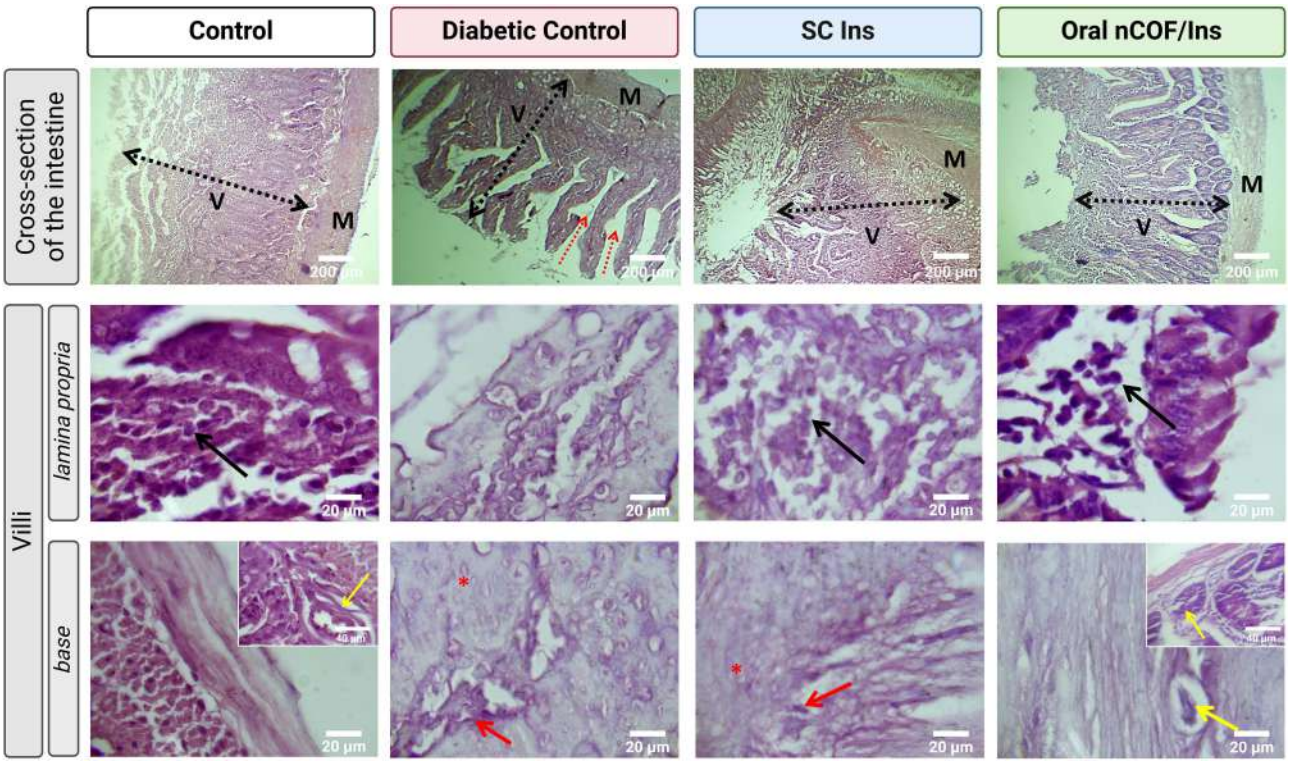

Figure 4 Histological Comparison of Rat Intestinal Sections Stained with Hematoxylin and Eosin across Four Experimental Conditions: Non-diabetic Control, Diabetic Control, Diabetic Treated with Subcutaneous Insulin (SC Ins), and Diabetic Treated with Oral nCOF/Insulin (Oral nCOF/Ins). Annotations: V for villi, M for muscular layer, with the villi borders highlighted by double dashed black arrows. The upper panel (X50) displays the cross-section of the intestine, demonstrating the general structure and condition of the tissue. The non-diabetic control shows healthy, a well-defined villus (V) and an underlying muscular layer (M), representing a normal intestinal architecture. Diabetic control sections reveal villi degeneration, as indicated by dashed red arrows. The middle panel (X500) provides a higher magnification of the villi within the lamina propria, where lymphoid elements (black arrows) are observed in the Control and insulin-treated specimens, suggesting preserved immune cell populations. The lower panel (X500) offers a closer look at the base of the villi. Healthy glandular structures (yellow arrows) are evident in the Control and Oral nCOF/Ins-treated groups. In stark contrast, the diabetic control and SC Ins-treated groups exhibit clear signs of pathology with necrotic glands (red arrows) and extensive necrotic zones (red asterisks), indicating significant histological damage associated with diabetic conditions.

Diabetes markedly induced epithelial degeneration within the intestine, as evidenced by increased crypt depth and villus height in untreated diabetic rats (Figure 4, Diabetic Control), compared to control and insulin-treated groups. Detailed examination at higher magnification revealed a notable decrease in lymphoid elements in the Diabetic Control group, contrasting sharply with the Control and insulin-treated rats. Lymphoid elements are crucial for antibody synthesis, playing a vital role in regulating microbial growth and maintaining intestinal health.

In diabetic rats treated with oral nanoparticle-mediated insulin (Figure 4, Oral nCOF/Ins), the intestinal villi showcased endocrine cells and glands mirroring the structural integrity observed in non-diabetic controls (yellow arrows). This contrasts with the untreated diabetic group and those receiving subcutaneous insulin (Figure 4, Diabetic Control and SC Ins), where significant architectural disruption and cellular necrosis were observed (red asterisks), leading to obscured intestinal glands (red arrows).

Histological Sections of Rat Spleen

Microscopic examination of the spleen in control rats revealed a well-defined architecture, characterized by a clear distinction between the red pulp and the white pulp, the latter marked by white circles for identification (Figure 5, Control). The white pulp is further organized into a germinal zone (GZ) encircled by a marginal zone (MZ), reflecting the typical histological features of a healthy spleen.

Diabetes significantly alters spleen morphology, most notably reducing the white pulp to red pulp ratio, which is often associated with alterations in immune response (Figure 5, Diabetic Control). The germinal zone (GZ) within the white pulp of untreated diabetic rats appeared diminished and blurred, lacking the distinct marginal zone (MZ) observed in

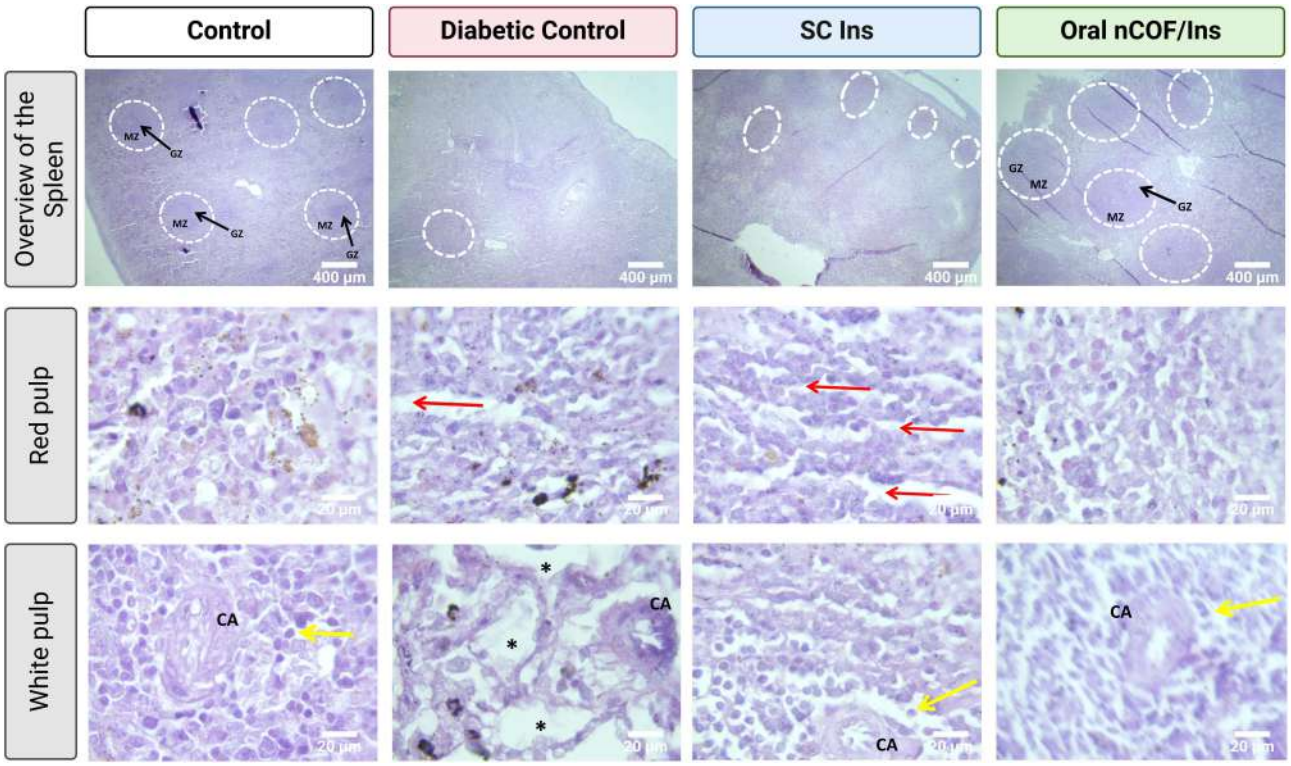

Figure 5 Comparative Histological Analysis of Rat Spleen Sections Stained with Hematoxylin and Eosin across Non-diabetic Control, Diabetic Control, Diabetic Treated with Subcutaneous Insulin (SC Ins), and Diabetic Treated with Oral nCOF/Insulin (Oral nCOF/Ins) Groups. The upper panel provides an overview of the spleen's architecture (X25), while the middle and lower panels offer high-magnification views (X500) of the red and white pulp, respectively. White dashed circles in the upper panel highlight the white pulp, showcasing the Germinal Zones (GZ) and Marginal Zones (MZ) which are particularly discernible in the Control and Oral nCOF/Ins specimens. The middle panel shows the red pulp, where both the Control and Oral nCOF/Ins samples display healthy cellularity. Conversely, the Diabetic Control and SC Ins groups exhibit a contracted tissue with thick Billroth cords, as indicated by red arrows. The lower panel zooms into the white pulp, revealing dilations (black asterisks) around the Central Artery (CA) in the Diabetic Control samples. However, the Control, Oral nCOF/Ins, and SC Ins groups exhibit typical lymphoid tissue (yellow arrows) surrounding the central arteries, while such tissue is notably absent in the Diabetic Control group, suggesting immune depletion.

healthy spleens. This structural degradation was similarly noted in rats receiving subcutaneous insulin, with an unclear demarcation between the two pulp zones (Figure 5, SC Ins).

High-magnification observations revealed densely packed Billroth cords in the red pulp of control specimens, indicative of robust cellular activity (Figure 5, Control). In contrast, diabetic rats exhibited a significant reduction in red pulp cellularity, with noticeable thickening of the Billroth cords which appeared contracted (Figure 5, Diabetic Control, red arrow), suggesting compromised spleen function. This condition was exacerbated in rats treated with subcutaneous insulin (Figure 5, SC Ins, red arrow).

In the white pulp, observation of the central artery (CA) revealed lymphoid tissue surrounding it in control, a typical structure (Figure 5, Control, yellow arrows). However, in untreated diabetic rats, significant dilatation marked by black asterisks and an absence of lymphocytic cells, indicative of immunodepletion, was observed (Figure 5, Diabetic Control). Subcutaneous insulin treatment restored lymphocytes in diabetic rats, indicating a positive effect on lymphocyte presence (Figure 5, SC Ins, yellow arrow).

Remarkably, diabetic rats treated with oral nCOF/Insulin showed a restoration of the spleen's structural integrity, with the white pulp/red pulp ratio and the clarity of the GZ and MZ closely resembling those of control rats and a red pulp with good cellularity and a white pulp rich in lymphocytes (yellow arrow) (Figure 5, Oral nCOF/Ins).

Histological Sections of Rat Heart

Microscopic examination of heart cross-sections from control rats delineated the heart's layered architecture with remarkable clarity (Figure 6, Control), with the innermost layer, the endocardium (Ed), lines the heart chambers, playing a crucial role in maintaining a smooth, frictionless environment for blood flow. The myocardium (My), the thick,

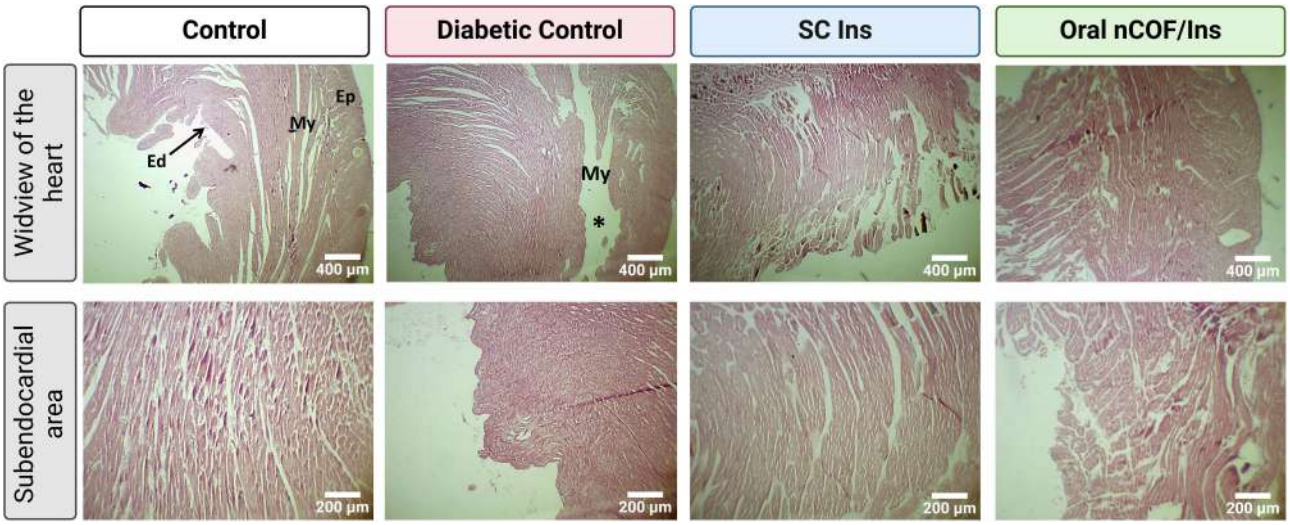

Figure 6 Histological Analysis of Rat Heart Sections Stained with Hematoxylin and Eosin for Different Groups: Non-diabetic Control, Diabetic Control, Diabetic Treated with Subcutaneous Insulin (SC Ins), and Diabetic Treated with Oral nCOF/Insulin (Oral nCOF/Ins). The upper panel presents a wide-field view of the heart's cross-section (X25), illustrating the layers from the endocardium (Ed) inward (indicated by the black arrow) to the epicardium (Ep) outward, including the myocardium (My) in the middle. Myocardial dilation, marked by the black asterisk, is observed prominently in the Diabetic Control specimens. The lower panel (X50) provides a focused view of the subendocardial area, showing the tissue's condition and arrangement in each group.

muscular middle layer, is instrumental in the heart's contraction, facilitating blood circulation throughout the body. The outermost layer, the epicardium (Ep), serves as a protective sheath for the heart.

In contrast, untreated diabetic rats exhibited significant pathological changes, including thickening of the subendocardial area and notable myocardial dilation (Figure 6 Diabetic Control, black asterisk). These changes suggest a compromised cardiac function, likely attributable to the metabolic disturbances associated with diabetes.

Remarkably, both subcutaneous and oral nanoparticle-mediated insulin treatments appeared to mitigate these diabetic-induced cardiac alterations. The treated rats' heart tissues closely resembled the healthy architecture observed in control rats, suggesting that the insulin treatment effectively repairs heart alterations induced by diabetes (Figure 6, SC Ins and Oral nCOF/Ins).

Histological Sections of Rat Liver

Microscopic examination revealed that the liver tissue of control rats and those treated with oral nCOF/Insulin exhibited a normal histological architecture. This was characterized by a well-defined centro-lobular vein (cv) surrounded by hepatocytes, which were evenly spaced by sinuses of regular thickness, indicative of healthy liver function (Figure 7, Control and Oral nCOF/Ins, black arrow).

In contrast, Diabetic Control and diabetic rats treated with subcutaneous insulin (SC Ins) showed severe pathological changes: untreated diabetic rats exhibited atrophied hepatocytes, leading to dilated sinuses (Figure 7, Diabetic Control, red arrow), which are symptoms of cellular degeneration due to insulin deficiency and compromised metabolic activity. Rats treated with subcutaneous insulin also showed signs of cellular distress, including cellular swelling, indicative of impending necrosis, and resulting in a reduction in sinus spacing (Figure 7, SC Ins, red arrow). Additionally, high-magnification of liver sections from untreated diabetic rats and those receiving subcutaneous insulin therapy displayed significant pathological alterations. Notably, these sections were marked by extensive necrotic areas (red asterisks), identifiable by necrotic cells with pale, white nuclei (red arrows), signaling severe tissue damage (Figure 7, Diabetic Control and SC Ins).

Conversely, the liver tissue of diabetic rats treated with oral nCOF/Insulin exhibited significant restoration of its normal structure. Hepatocytes in these rats displayed dark, prominent nuclei. Additionally, there was a notable presence of binucleated cells (double black arrows) which can be linked to enhanced metabolic activity and cellular regeneration (Figure 7, Oral nCOF/Ins).

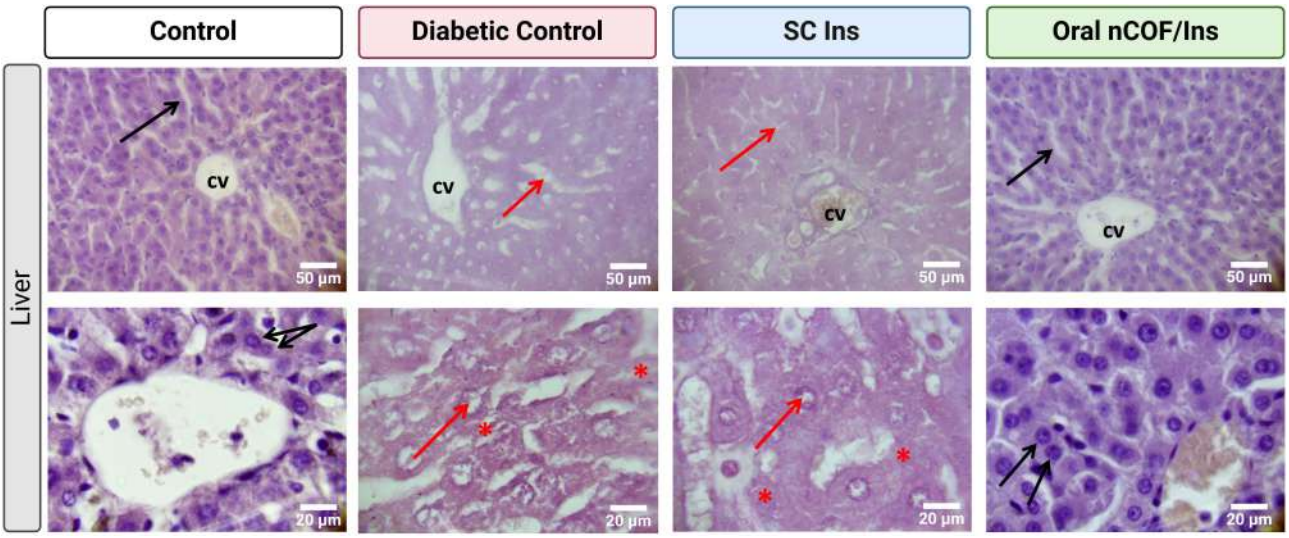

Figure 7 Histological Sections of Rat Liver Stained with Hematoxylin and Eosin across four groups: Non-diabetic Control, Diabetic Control, Diabetic Treated with Subcutaneous Insulin (SC Ins), and Diabetic Treated with Oral nCOF/Insulin (Oral nCOF/Ins). Key identifiers include: cv representing the central vein and black arrows indicating hepatic sinusoids. The upper panel presents an overview of the liver's architecture (X200), and the lower panel provides a higher magnification of the hepatic microstructure (X500). In the non-diabetic control and Oral nCOF/Ins treated rats, the liver architecture around the central vein (cv) appears normal with regular sinusoids (black arrows) between the cells. Pathological changes are evident in the hepatic sinusoids of both the Diabetic Control and SC Ins groups, as indicated by red arrows. In the diabetic group, the sinusoids appear dilated, while the hepatocytes exhibit signs of atrophy. In the SC Ins group, the sinusoids appear retracted, suggesting hepatocyte swelling. Higher magnification in the lower panel reveals necrotic hepatocytes (red asterisks) in both the Diabetic Control and SC Ins groups. Additionally, some cells exhibit pale, white nuclei, indicative of cellular necrosis (highlighted by red arrows). However, in the Control and Oral nCOF/Ins groups, healthy dark nuclei, which are indicative of high metabolism, are observed. Additionally, it is noteworthy that some hepatocytes in these groups appear binucleated, suggesting cellular regeneration or proliferation, as indicated by the double black arrows.

Histological Sections of Rat Kidney

Microscopic analysis of kidney sections from control rats displayed a pristine renal architecture, characterized by healthy glomeruli (G), intact renal tubules, and an intact brush border (black arrow), all indicative of optimal kidney function (Figure 8, Control). The nuclei within these structures were prominently visible underscoring the tissue's health.

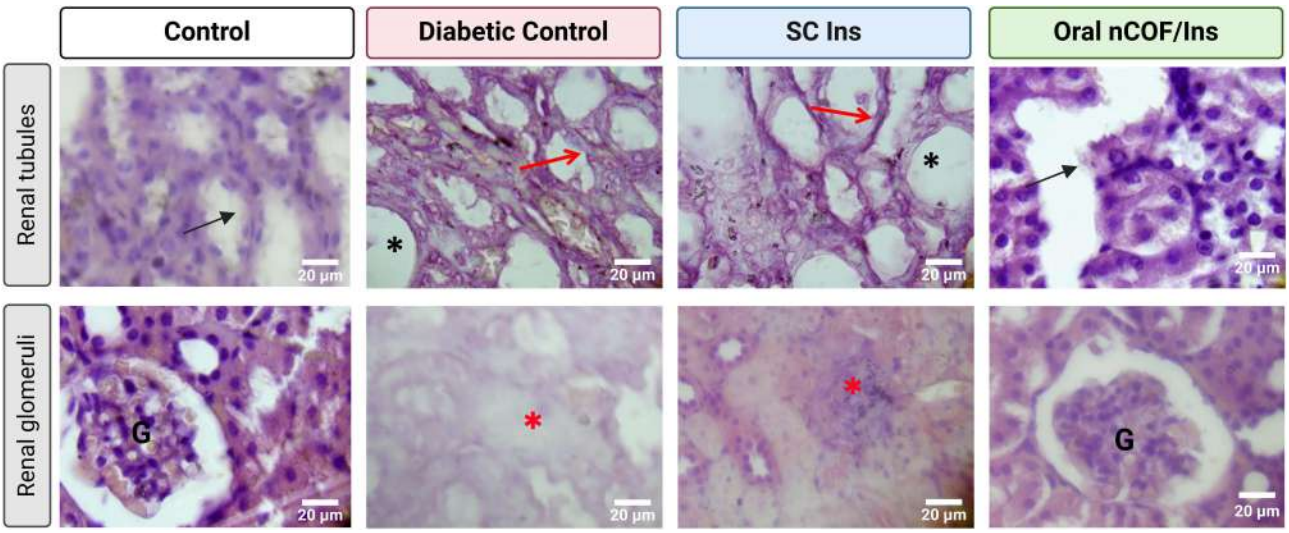

Figure 8 Histological Examination of Rat Kidney Sections Stained with Hematoxylin and Eosin (X500) across four groups: Non-diabetic Control, Diabetic Control, Diabetic Treated with Subcutaneous Insulin (SC Ins), and Diabetic Treated with Oral nCOF/Insulin (Oral nCOF/Ins). The Control and Oral nCOF/Ins sections display healthy glomeruli (G) and tubules with an intact epithelial lining, characterized by dark nuclei indicative of cellular integrity, along with a healthy brush border evident along the tubules (black arrows), indicating normal renal histology. In contrast, the Diabetic Control and SC Ins sections reveal signs of renal damage: glomerular necrosis (red asterisks), tubular dilation (black asterisks), and loss of the brush border (red arrows), suggesting pathological alterations in diabetic conditions.

In contrast, kidney sections from untreated diabetic rats and those treated with subcutaneous insulin revealed significant histological alterations. Notably, these changes included glomerular degeneration (red asterisks), dilation of renal tubules (black asterisk), and a conspicuous loss of the brush border (red arrow) (Figures 8, Diabetic Control and SC Ins). These findings highlight the detrimental impact of diabetes on renal integrity and the insufficiency of subcutaneous insulin in mitigating these effects.

Remarkably, diabetic rats treated with oral nCOF/Insulin exhibited kidney sections that mirrored the healthy architecture observed in control rats, with intact glomeruli (G) and tubules featuring healthy tubular epithelial cells and a healthy brush border (Figure 8, Oral nCOF/Ins, black arrow).

Histological Sections of Rat Adrenal Gland

The adrenal gland of control rats exhibits a well-organized structure, characterized by three distinct cortical zones: the Zona Glomerulosa (ZG), the Zona Fasciculata (ZF), and the Zona Reticularis (ZR), alongside a centrally located medulla (M) (Figure 9, Control). This organization is crucial for the gland's endocrine function, with each zone responsible for producing specific hormones.

In untreated diabetic rats, significant morphological changes were observed in the adrenal glands, suggesting stress-induced alterations in adrenal function. In the first panel, the medulla (M) appeared unusually expanded (Figure 9, Diabetic Control), while alterations in the proportions of the different zones of the cortex were observed (highlighted by double red arrows). Additionally, at high magnification in the third panel, vacuolization within the Zona Fasciculata suggested hyperactivity and hypersecretion of hormones (Figure 9, Diabetic Control, red arrows). Furthermore, diabetic rats treated with subcutaneous insulin exhibited an enlarged adrenal cortex, particularly the ZF, which was approximately twice the size observed in control rats (Figure 9, SC Ins, double red arrows). At lower magnification, the ZF in SC Ins rats appeared indiscernible and resembled a large necrotic zone (red asterisk, first panel); however, higher magnification revealed vacuolated areas similar to those observed in untreated diabetic rats (red arrow, third panel).

Conversely, diabetic rats treated with oral nanoparticle-mediated insulin (nCOF/Insulin) showed adrenal glands with cortical zones (ZG, ZF, and ZR) and a medulla closely resembling the organized structure seen in control rats (Figure 9, Oral nCOF/Ins).

Immunohistochemical Analysis of E-Cadherin Expression

Observation of the intestine section stained with E-cadherin antibody in Figure 10 revealed that the intensity of E-cadherin staining in rats treated with oral nCOF/Insulin matched that of the control group, indicating no depletion of E-cadherin post-treatment. Similarly, untreated diabetic rats and those treated with subcutaneous insulin exhibited E-cadherin expression levels comparable to the control group.

Comprehensive histopathological examinations of muscle and brain tissues across the four groups did not reveal any significant changes.

Discussion

Our comprehensive study on the effects of diabetes and subsequent insulin treatments on various organs in rats provides significant insights into the pathological changes induced by diabetes and the therapeutic potential of oral nanoparticle-mediated insulin (nCOF/Insulin) in comparison to subcutaneous insulin.

Our findings indicate that streptozotocin-induced diabetes causes widespread organ toxicity, as evidenced by notable changes in organ weight, disruption of the oxidative-antioxidative equilibrium, and various histological modifications, primarily including necrosis, vasodilations, and karyolysis (loss of nuclei) in tissues of various organs. The observed reduction in body weight alongside increased organ weights in diabetic rats underscores the metabolic distress caused by diabetes. Additionally, the increase in relative organ weights provides insight into the toxicity within the organs. The restoration of body and organ weights to near-normal levels with oral nCOF/Insulin treatment highlights its efficacy in counteracting the metabolic imbalances and the underlying stress induced by diabetes, suggesting a promising therapeutic avenue for managing diabetes-induced cachexia and organ hypertrophy.

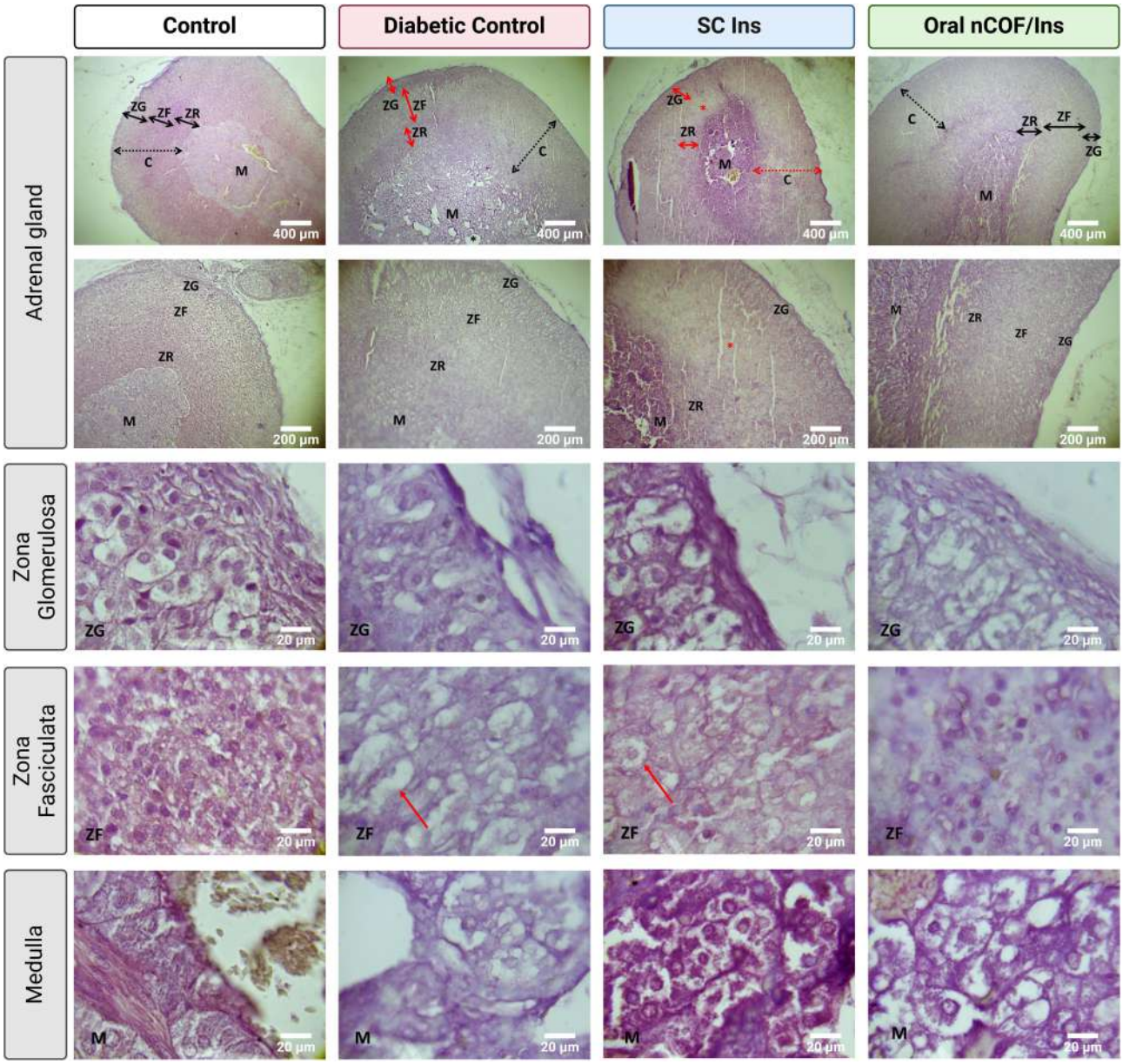

Figure 9 Histological Sections of Rat Adrenal Glands Stained with Hematoxylin and Eosin across four groups: Non-diabetic Control, Untreated Diabetic Control, Diabetic Treated with Subcutaneous Insulin (SC Ins), and Diabetic Treated with Oral nCOF/Insulin (Oral nCOF/Ins). The upper two panels provide an overview of the adrenal glands (X25 and X50), showing the cortex with its three zones: Zona Glomerulosa (ZG), Zona Fasciculata (ZF), and Zona Reticularis (ZR), along with the medulla (M) at the core. In the Control and Oral nCOF/Ins groups, each zone is discernible, and the proportions of the different areas are similar between the two groups. However, in the Diabetic Control and SC Ins groups, the cortex exhibits altered proportions (highlighted by the double red arrows), with increased thickness, particularly in the ZF. The ZF in the SC Ins group shows a poorly defined structure, with regions resembling necrosis (indicated by red asterisks), suggesting severe impairment. Additionally, in the Diabetic Control rats, the medulla appears loosely and dilated compared to the Control and Oral nCOF/Ins groups, indicating potential stress or damage. In the second panel (X500), a high-magnification focus on the ZG reveals its structural integrity. The third panel (X500) focuses on the ZF, where the Diabetic Control and SC Ins samples show altered morphology and the presence of significant lipid droplets (highlighted by red arrows). These observations suggest metabolic disruptions and imply an overproduction of hormones by the ZF. Finally, the fourth panel examines the medulla (X500).

The imbalance between oxidants and antioxidants, leading to heightened oxidative stress, is a hallmark of diabetes pathology. Our findings of elevated malondialdehyde (MDA) levels and protein carbonyl concentrations across various organs in diabetic rats underscore the systemic nature of oxidative damage. Notably, oral nCOF/Insulin administration effectively reduced these oxidative stress markers, aligning them with those observed in non-diabetic rats. This suggests that oral nCOF/Insulin not only mitigates hyperglycemia but also addresses the underlying oxidative stress through

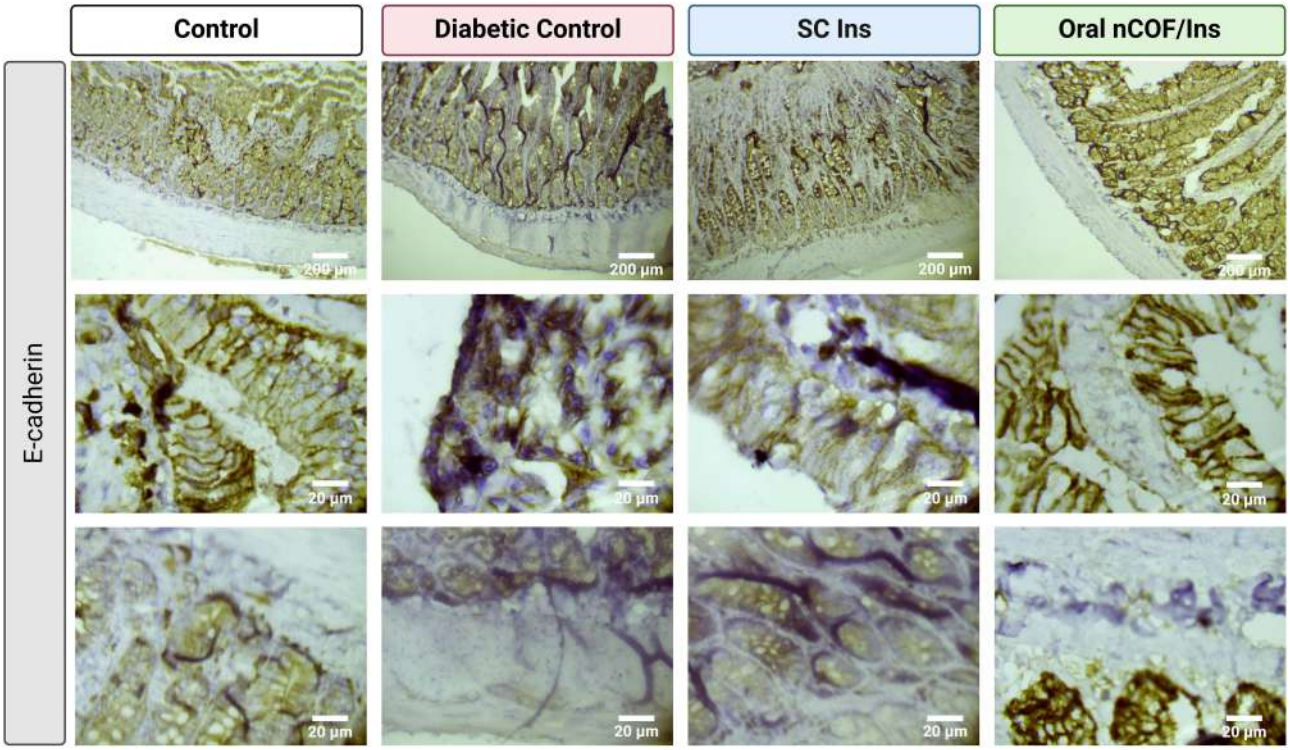

Figure 10 Immunohistochemical Analysis of E-cadherin Expression in Rat Intestinal Tissue across four groups: Non-diabetic Control, Untreated Diabetic Control, Diabetic Treated with Subcutaneous Insulin (SC Ins), and Diabetic Treated with Oral nCOF/Insulin (Oral nCOF/Ins). The staining highlights E-cadherin distribution in the intestinal epithelium across the different treatment conditions. The first row provides an overview at low magnification (X50), while the following rows offer detailed views at higher magnification (X500).

effective insulinization, which implies metabolic improvement. This offers a comprehensive therapeutic strategy for diabetes management.

Brain

The long-term impact of diabetes on brain health is well-documented,[34] with the immediate effects of increased oxidative stress being clearly observable in our findings. During diabetes, the brain undergoes impaired energy metabolism. This impairment can result from a combination of factors, including reduced glucose uptake and increased oxidative stress.[35,36] The brain, with its lipid-rich composition, is especially susceptible to lipid peroxidation due to metabolic imbalances caused by a lack of insulin.[37,38] Our study reveals that insulin therapies, administered both orally and subcutaneously, are effective in mitigating lipid peroxidation. This is evidenced by the reduced malondialdehyde (MDA) levels in the brains of diabetic rats. However, subcutaneous insulin therapy has limitations, particularly in inducing hypoglycemia. In contrast, our findings demonstrate that nCOF administration effectively reduces MDA levels in the brain, akin to subcutaneous insulin, but without the associated risk of hypoglycemia,[36] as evidenced by our previous study.[19] This highlights the potential of nCOF as a safer alternative treatment option for diabetes-related alterations affecting the brain.

Muscle

Streptozotocin is known to trigger oxidative stress in muscle tissue, resulting in functional decline.[39,40] Moreover, the lack of insulin compromises the cellular uptake of glucose and amino acids, leading to increased proteolysis, muscle degradation, and the consequent weight loss observed in diabetes.[41,42] In our study, we observed significant weight loss and increased oxidative stress in the skeletal muscle of diabetic rats and those treated with subcutaneous insulin, with a notably higher level of protein carbonylation in diabetic rats treated with subcutaneous insulin. This aligns with previous research, which has shown that subcutaneous injections of insulin lead to peripheral hyperinsulinemia, linked to

elevated oxidative stress in muscle tissues.[43,44] This phenomenon elucidates why insulin administered subcutaneously does not reestablish the equilibrium between pro-oxidants and antioxidants, but rather exacerbates oxidative stress in the muscles through the hyper-insulinemia it induces. Interestingly, the oral administration of nCOF/Insulin not only restored body weight but also reestablished the balance between pro-oxidant and antioxidant, highlighting the therapeutic potential of this delivery method.

Intestine

Our investigation into the intestinal alterations in diabetic rats reveals a significant deepening of crypts, highlighting the adverse effects of diabetes on intestinal health. These observations align with previous findings that attribute such histological changes to oxidative stress and apoptotic mechanisms induced by diabetes in the intestine.[45,46] Our analysis indicates that these histological alterations are primarily due to an imbalance in the oxidant/antioxidant balance, notably characterized by a reduction in antioxidant levels in individuals with diabetes. Interestingly, insulin treatments, whether administered subcutaneously or orally, appear to mitigate these histological changes. However, with subcutaneous insulin administration, we observed significant necrotic areas within the intestinal tissue, accompanied by elevated levels of carbonylated proteins, a marker associated with chemical alterations in the intestine due to diabetes.[14,47] The effectiveness of oral nCOF/Insulin treatment in restoring intestinal architecture underscores its potential in preventing diabetes-induced gastrointestinal complications.

The immunohistochemical analysis of E-cadherin was specifically used to assess intestinal barrier integrity, which is crucial for our study given that our newly formulated nanoparticles are absorbed in the intestine. E-cadherins are essential transmembrane adhesion molecules crucial for the cohesion of the intestinal epithelium.[48] Our E-cadherin labeling in the intestine shows that despite the effective absorption of the nanoparticles, the integrity of the intestinal barrier is preserved, as indicated by the comparable levels of E-cadherin across all groups, including the control.

These findings are corroborated by those from our previous in vitro analysis where transmission electron microscopy (TEM) images revealed that the nCOF/insulin nanoparticles were located inside goblet cells of the intestinal tissue and were excreted into the gut lumen through cell secretion.[19] This confirms that our nanoparticle formulation can traverse the intestinal barrier while maintaining its integrity and carrying the insulin cargo without causing significant pathological changes to intestinal tissues.

Spleen

Diabetes is associated with degenerative changes in splenic tissues induced by oxidative stress.[11,49] In our study, streptozotocin-induced diabetes resulted in a marked reduction of the white pulp and the marginal zone within the spleen—key anatomical regions integral to the organ's immune function. This reduction in white pulp has been characterized as stemming from an increase in apoptosis triggered by oxidative stress accumulation due to prolonged hyperglycemia.[50] Such alterations in splenic structure contribute significantly to the immunodeficiency observed in diabetic conditions, underscoring the spleen's critical role in the array of diabetic complications. Furthermore, our findings indicate that while subcutaneous insulin treatment may mitigate some aspects of this degeneration, it appears to offer only limited protection against the comprehensive degradation of splenic tissues. This observation suggests that subcutaneous insulin administration alone may not adequately prevent the onset of immunodeficiency disorders associated with diabetes, highlighting the need for more effective therapeutic strategies to preserve immune function in diabetic patients. The restoration of splenic architecture with oral nCOF/Insulin treatment suggests its role in preserving immune competence in diabetic conditions, potentially mitigating the risk of immunodeficiency and related complications.

Heart

Chronic hyperglycemia associated with diabetes is known to exert pathological effects on the structure and function of the myocardium,[5,51] and for inducing subendocardial stress-related alterations.[52] Microscopic examination of heart sections from diabetic rats treated with either subcutaneous insulin or oral nCOF/Insulin showed normal histological architecture, indicating that both treatments effectively restored cardiac integrity. This observation is consistent with

existing research, which suggests that the pathological alterations in the heart induced by diabetes can potentially be reversed through insulin therapy.[53,54]

Liver

The pathophysiology of diabetes involves an impairment of antioxidants and an increase in oxidative stress at the hepatic cell level, leading to intracellular damage.[55,56] These biochemical imbalances result in observable alterations under the microscope: hepatocyte ballooning, periportal necrosis, and dilatation.[57] Our findings are in line with existing literature, and our histological sections reveal alterations in both untreated diabetic rats and those administered subcutaneous insulin. Although subcutaneous insulin administration was observed to decrease malondialdehyde (MDA) and protein carbonyls (PCOs) levels in the livers of diabetic rats, it failed to ameliorate tissue alterations. This aligns with previous findings suggesting that subcutaneous insulin is ineffective in mitigating liver oxidative stress damage.[12] This collective evidence underscores the limitations of subcutaneous insulin in addressing diverse aspects of hepatic pathology associated with diabetes. This ineffectiveness can be attributed to insufficient insulinization, leading to inadequate restoration of glucose uptake and impairment in liver glucose metabolism.[58] This underscores the critical role of the administration route in insulin assimilation by organs and its metabolism.

Kidney

The impact of diabetes on renal function and structure is extensively documented, aligning with our findings that corroborate previous research indicating significant histological changes within renal tubules, nephrons, and vasculature.[42,59] These alterations are mediated by the impairment of oxidative species in the kidney.[42,60] Notably, Ghavimishamekh et al explored the efficacy of both subcutaneous and oral insulin in mitigating these renal alterations in diabetic rats, reporting no observable improvements.[42] Consistent with these findings, our study also revealed that subcutaneous insulin administration failed to ameliorate the diabetes-induced histological changes in the kidneys. However, our investigation into the effects of oral nCOF/Insulin presents a contrasting outcome, indicating a potential therapeutic advantage of this novel insulin delivery method in preserving renal integrity in the context of diabetes.

Adrenals

The adrenal glands, pivotal in the endocrine system,[61] experience significant structural and compositional changes in the context of diabetes.[62,63] Research indicates that diabetes, particularly when induced by streptozotocin (STZ), leads to the hypersecretion of adrenocorticotropic hormone (ACTH) from the pituitary gland. This process stimulates the growth of the zona fasciculata within the adrenal cortex while concurrently causing atrophy in the zona glomerulosa.[64] Furthermore, it has been observed that subcutaneous insulin treatment impacts the adrenal glands by promoting the secretion of androgens, thereby contributing to structural alterations and notably increasing cortex thickness.[39,65] These results demonstrate that insulin can influence various metabolic processes, including the secretion of other hormones. Interestingly, diabetic rats treated with oral nCOF/Insulin exhibit adrenal glands that resemble those of non-diabetic controls. This suggests that the different assimilation and metabolism of oral insulin, compared to subcutaneous insulin, may modulate hormonal responses in diabetic rats due to its gradual absorption.

Our study primarily focuses on the implications of insulin delivery routes, highlighting how oral nanoparticle-mediated insulin (nCOF/Insulin) can offer distinct advantages over subcutaneous administration. While the rapid repair observed in our study is notable, it should be interpreted in the context of the relatively recent onset of hyperglycemia in our model. It is worth noting that the tissue alterations observed in this study were assessed one week after streptozotocin administration, a period when the hyperglycemic effects of diabetes are becoming fully established. Our findings suggest that the observed damage may still be somewhat reversible. This potential for repair within a short timeframe may reflect the early stages of metabolic imbalance rather than long-term complications.

A key advantage of the nCOF/Insulin formulation is its controlled release of insulin over 10 hours, compared to the approximately one hour of coverage provided by subcutaneous insulin.[19] This sustained release aligns with blood glucose concentrations throughout the 24-hour period when the animals had access to food. By ensuring continuous insulin

delivery, the nCOF/Insulin formulation facilitates glucose uptake and potentially reduces cellular stress, contributing to the restoration of tissue architecture.

Moreover, restoring glycemic homeostasis is crucial for mitigating diabetes-induced damage. Our previous studies have demonstrated that the nCOF/Insulin formulation not only alleviates hyperglycemia but also addresses associated conditions such as hyperinsulinemia, hypertriglyceridemia, and inflammation.[19,24] Additionally, the formulation may influence adrenal hormone levels. All these factors are known to impact tissue damage. By managing these conditions, the nCOF/Insulin formulation supports a more favorable tissue environment, likely contributing to the impressive results observed in our study.

This study serves as an exploratory investigation into the therapeutic potential of oral nCOF/Insulin, providing a comprehensive analysis of its short-term effects on multiple organs and offering novel insights into diabetes treatment. As a pioneering effort in nanoparticle-based therapies, it highlights the effectiveness of oral nCOF/Insulin in ameliorating metabolic disturbances and oxidative stress, demonstrating its potential to significantly impact diabetes management. However, the study's short duration is a limitation that underscores the need for further research to fully understand the long-term efficacy and underlying mechanisms of this delivery method. While the rapid improvements observed are promising, future studies are essential to establish a more comprehensive view of its therapeutic benefits and validate its potential in clinical settings.

Conclusion

In conclusion, our study provides compelling evidence of the effectiveness of oral nanoparticle-mediated insulin delivery (nCOF/Insulin) in mitigating organ damage induced by diabetes in rat models. We have elucidated the pathophysiological changes that diabetes imposes on various organs, including alterations in organ and body weights, disruption of the oxidative-antioxidative balance, and histopathological changes. Our findings suggest that the innovative nCOF/Insulin treatment may help to reverse these detrimental effects and facilitate a return to normative physiological functions. Notably, our results indicate that oral nCOF/Insulin may support brain health by reducing lipid peroxidation, mitigate muscle wasting by reducing oxidative stress, and restore intestinal integrity. Furthermore, it appears effective in improving splenic immune function, myocardial structure, hepatic architecture, renal integrity, and adrenal gland composition.

While our research highlights the limitations of existing diabetes treatments and positions oral nanoparticle-mediated insulin delivery as a promising therapeutic strategy, we recognize that these findings are preliminary. The short treatment period used in this study necessitates further investigation to fully understand the long-term efficacy and safety of this approach. Additional research is essential to unravel the intricate mechanisms of action of nCOF/Insulin and to validate its therapeutic potential in humans. Future studies should aim to expand on our findings, exploring the long-term efficacy and safety of oral nCOF/Insulin, as well as its integration into existing diabetes treatment regimens. The journey towards a more effective diabetes treatment is far from over, but our study marks a significant step forward.

Abbreviations

nCOF/Insulin, Oral Insulin Loaded with Nanoparticles comprising covalent organic frameworks; TD1, Type 1 diabetes; STZ, Streptozotocin; C., Standard Control; D.C., Diabetic Control; SC Ins, Diabetic rats treated with subcutaneous insulin; Oral nCOF/Ins, Diabetic rats treated with oral administration of nanoparticle-formulated insulin; PBS, Phosphate Buffer Saline; MDA, Malondialdehyde; TBARS, Thiobarbituric Acid Reactive Substances; TBA, Thiobarbituric Acid; PCOs, Protein Carbonyls; DNPH, Dinitrophenylhydrazine; GSH, Glutathione; DTNB, 5,5′-Dithiobis(2-Nitrobenzoic Acid); HE, Hematoxylin and Eosin; GZ, Germinal Zone; MZ, Marginal Zone; CA, Central artery; Ed, Endocardium; My, Myocardium; Ep, Epicardium; CV, Centro-Lobular Vein; G, Glomerular Structures; ZG, Zona Glomerulosa; ZF, Zona Fasciculata; ZR, Zona Reticularis; M, Medulla.

Data Sharing Statement

All data are available in the main text.

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Disclosure

The authors declare that they have no competing interests in this work.

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Discussion

In this thesis, we demonstrated that encapsulating insulin in nCOF nanoparticles enabled its oral administration by significantly enhancing its bioavailability while preserving its therapeutic efficacy. Our pharmacological analysis revealed that orally administered nCOF/insulin effectively reduced hyperglycemia progressively without causing hypoglycemia, maintaining blood glucose levels comparable to non-diabetic conditions, both in fasting and postprandial states. This effect was sustained for up to 10 hours post-administration and even showed potential for improving insulin sensitivity, helping to prevent insulin resistance in type 1 diabetic subjects. This suggests that nCOF/insulin is better absorbed by the body, contributing to the prevention of diabetes-related alterations, including disruptions in hemostasis, coagulation, and fibrinolysis, while also reducing oxidative stress and repairing histological damage across various organs. Moreover, this oral insulin formulation plays a key role in restoring metabolic balance by influencing parameters critical to glycemic regulation, such as the lipid and oxidative profiles. It may also exert an impact on hormonal balance, as evidenced by our findings on cortisol levels and adrenal gland analysis.

Bioavailability poses the primary challenge to oral insulin delivery (Masloh et al., 2023; Shetty et al., 2023). Given insulin's delicate nature as a peptide hormone, it struggles to withstand the hostile environment of the digestive system, including the acidic pH of the stomach and enzymatic activity. Previous attempts to address this issue had failed to achieve adequate bioavailability. However, our molecule demonstrated a bioavailability superior to that proposed by the literature (Damgé et al., 2007; Sarmento et al., 2007; Li et al., 2013; and Wu et al., 2023).

Although Oral nCOF/Insulin exhibits high bioavailability, the pharmacokinetic study revealed that blood insulin concentrations increase gradually without inducing hyperinsulinemic peaks, unlike subcutaneous insulin, which triggers a typical peak within an hour of injection but is also rapidly cleared from the bloodstream (Sonaje et al., 2010; Steyn et al., 2023).

The occurrence of a hyperinsulinemic peak followed by rapid clearance, as observed with subcutaneous insulin administration, carries significant metabolic implications. The sudden surge

in insulin levels triggers hyperinsulinemia, which is associated with increased cellular uptake of glucose, potentially leading to hypoglycemia if not adequately managed (Mccall, 2012; Jiao et al., 2022). Prolonged or frequent episodes of hypoglycemia can damage blood vessels and nerves, increasing the risk of cardiovascular disease and neuropathy (Inouye et al., 2005; Mccall, 2012; Kosiborod et al., 2015). Additionally, sustained hyperinsulinemia is linked to metabolic dysregulation, including dyslipidemia (Maahs and Eckel, 2015; Vergès, 2020), increased hepatic glucose production (Gregory et al., 2019; Lewis et al., 2021; Norton et al., 2022), and altered adipose tissue function, contributing to the development of obesity and metabolic syndrome (Barazzoni et al., 2018; Corbin et al., 2018). Furthermore, chronic hyperinsulinemia may promote cellular proliferation and inflammation (Püschel et al., 2022; Szukiewicz, 2023), exacerbating the risk of cardiovascular disease and other metabolic complications (Kosmas et al., 2023; Sun et al., 2024). Moreover, the rapid change in insulin concentration may result in important glycemic fluctuations, with long-term repercussions on metabolism and organ function (Zaccardi et al., 2009; Klimontov et al., 2021).

Our pharmacodynamics analysis reveals that the Oral nCOF/Insulin system functions in a dose-dependent manner, characterized by a gradual and moderate release of insulin, intricately calibrated to blood glucose concentrations. These findings are consistent with the investigations of Benyettou et al., which elucidated the intricate chemical properties of nCOF. Their research elucidated that while the nanosheets pores of nCOF are selectively permeable to glucose over insulin. At normoglycemic conditions, they are filled with insulin. However, as insulin concentrations rise, glucose molecules penetrate the nanosheet, competing with insulin for binding sites and thereby prompting insulin release from nCOF. Consequently, the Oral nCOF/Insulin system intricately regulates insulin release in response to fluctuating glucose levels. Additionally, glucose occupying a portion of the pores restricts insulin release, preventing excessive liberation. Moreover, nCOF can only absorb a maximum of 18% by weight of glucose under surreal hyperglycemic conditions. This implies that even under extreme and unrealistic glycemic scenarios, the amount of glucose absorbed by nCOF is limited, thereby regulating insulin release. These unique chemical properties enable Oral nCOF/Insulin to release insulin in a dose-dependent manner relative to blood glucose concentrations, while maintaining controlled release

without inducing hypoglycemia. Indeed, Benyettou et al. demonstrated that by exposing the nCOF/Insulin to alternating concentrations of glucose (1 *versus* 5 $mg.ml^{-1}$), an on-off regulation of insulin release by nCOF/Insulin is created, thereby mimicking the pulsatile liberation of endogenous insulin.

These unique chemical properties confer to the nCOF/Insulin the remarkable ability to modulate insulin release in direct response to blood glucose dynamics, while maintaining a controlled and predictable release profile, thereby minimizing the risk of hypoglycemia. Additionally, Benyettou et al. identified specific properties of nCOF nanosheets: size reduction post-insulin release, crystallinity restoration, and increased porosity, along with the maintenance of insulin's three-dimensional structure.

The size reduction post-insulin release indicates structural adaptability, enabling insulin encapsulation and release over multiple cycles without damage. Crystallinity restoration ensures durability in functionality of nCOF nanosheets. Increased porosity enhances the surface area available for insulin loading and release, facilitating sustained insulin delivery over extended periods. Finally, preservation of insulin's native conformation by nCOF maintains its bioactivity and stability over time, as it ensures that the released insulin retains its ability to bind to insulin receptors and regulate glucose levels (Benyettou et al. 2021).

Additionally, alongside its high bioavailability and highly interesting pharmacological characteristics, we conducted calculations of the HOMA-IS and HOMA-IR indices for Oral nCOF/Insulin, comparing the values to those obtained with traditional subcutaneous insulin. Our findings revealed that diabetic rats treated with Oral nCOF/Insulin exhibited a higher HOMA-IS index and a lower HOMA-IR index compared to diabetic rats treated with subcutaneous insulin injections. This observation suggests that the oral administration of insulin via nCOF results in superior assimilation by the body. These outcomes align with existing literature indicating that central-to-peripheral insulin distribution enhances insulin sensitivity in type 1 diabetes, while peripheral-to-central distribution fosters insulin resistance development (Liu et al., 2009; Gregory et al., 2020). These results provide additional support for the idea that Oral nCOF/Insulin effectively mimics endogenous insulin at a physiological level.

Therefore, these results demonstrate the insulin resistance induced by subcutaneous insulin injections, primarily due to the peripheral hyperinsulinemia they generate (Shanik et al., 2008; Gregory et al., 2020). This peripheral hyperinsulinemia is a central aspect of the pathophysiological effects associated with subcutaneous insulin therapy. Importantly, the oral administration of nCOF/Insulin bypasses this phenomenon, serving as the primary mechanism by which it mitigates the adverse effects attributed to subcutaneous insulin injections.

To conclude the work of this first article, we conducted a preliminary toxicity study by analyzing biochemical markers of liver and kidney function and performing a histopathological study on the liver and kidney. Our results were surprising; not only did we conclude that Oral nCOF/Insulin did not induce toxicity, but it also demonstrated the ability to prevent alterations in the liver and kidney often associated with diabetes (Ozcelik et al., 2011; Rai et al., 2016). Therefore, Oral nCOF/Insulin could, in addition to perfectly mimicking physiological insulin and maintaining glycemic homeostasis, prevent diabetes-related complications while remaining fully biocompatible.

It is on this conclusion that we based the continuation of our work to better understand the effects of this Oral nCOF/Insulin on alterations related to type 1 diabetes. In this context, we specifically investigated its impact on hemostatic and vascular alterations, an area that has been relatively unexplored in type 1 diabetes research.

Cardiovascular complications are frequently associated with hemostatic and vascular dysfunctions and are leading causes of mortality and morbidity in diabetic patients (Raghavan et al., 2019; Amiel et al., 2019). Disturbances in hemostasis are known to be early and significant indicators of the progression of diabetes-related cardiovascular complications (Page and Ari, 2021). Despite the well-established link between type 1 diabetes and hemostatic dysfunction, the underlying mechanisms remain poorly understood. Our study aims to address this gap by providing new insights into these mechanisms and evaluating the potential benefits of Oral nCOF/Insulin in mitigating these complications.

To fully understand the impact of insulin treatment on hemostatic disorders and vasculopathy associated with type 1 diabetes, we first identified the key factors contributing to these disturbances:

Hyperglycemia has a significant impact on hemostasis by promoting a hypercoagulable state (Li et al., 2021; Pepe et al., 2024). This effect is mediated through an increase in the production of coagulation factors (Boden and Rao, 2007; Lemkes et al., 2010) and a reduction in the activity of natural anticoagulants (Piemontino et al., 1994; Hernández-espinosa et al., 2009), resulting in an imbalance in the coagulation cascade. Consequently, this shift towards a procoagulant environment predisposes diabetic patients to thrombotic events. Hyperglycemia also promotes vascular inflammation (Zhao et al., 2021; Nedosugova et al., 2022). Additionally, elevated plasma glucose can directly infiltrate endothelial cells, triggering the release of superoxide anions (O_2^-), which further exacerbates endothelial dysfunction (Dauth et al., 2023; Ceinos et al., 2024).

Diabetes is often accompanied by **dyslipidemia** (Vergès, 2009; Vergès, 2020), which further exacerbates hemostatic imbalances (Menti et al., 2016; Firani et al., 2022). Elevated triglyceride and cholesterol levels contribute to endothelial dysfunction (Steinberg et al., 1997; Kajikawa et al., 2016; Higashi, 2023), increasing the risk of thrombosis. Dyslipidemia also disrupts the production and degradation of clotting factors, affecting blood clot formation (Puccetti et al., 2002; Siniarski et al., 2024; Zhang et al., 2024), aggravating the hypercoagulable state and impairing fibrinolysis.

Lipid peroxidation leads to platelet hyperactivation (Berger et al., 2018; Kelem et al., 2023; Li, 2024), which impacts clot formation and exacerbates endothelial dysfunction (Ishida et al., 2014). Among the various contributing factors, **inflammation** also plays a crucial role in diabetes-related hemostatic disturbances (Levi, 2017; Gidaro et al., 2023).

In our study, untreated diabetic rats exhibited hypertriglyceridemia, along with increased lipid peroxidation marked by elevated plasma MDA concentrations. Additionally, they showed prolonged bleeding time, coupled with increased plasma levels of fibrinogen, D-dimer, prothrombotic factor VIII, and an imbalance between antioxidants and pro-oxidants in both the

plasma and endothelium. Our results, combined with findings from the literature, highlight that diabetes has a multifactorial impact, involving chronic hyperglycemia, dyslipidemia, lipid peroxidation, and inflammation. These factors disrupt primary hemostasis (leading to bleeding tendencies), cause coagulation-fibrinolysis disturbances (creating a procoagulant state), and disturb the pro-oxidant/antioxidant equilibrium of the endothelium, as observed in untreated diabetic rats.

To comprehensively assess these disturbances, we conducted a detailed analysis using various assays and markers. We evaluated primary hemostasis and platelet activity by measuring bleeding time and platelet count. For vascular alterations, we assessed the balance between pro-oxidants and antioxidants by measuring superoxide (O2-), Malondialdehyde (MDA), and glutathione (GSH) levels in the abdominal aortas of experimental rats.

Our blood coagulation investigation was extensive, employing global tests such as activated partial thromboplastin time (APTT) and prothrombin time (PT). We also performed specialized tests to assess the coagulant activities of specific factors, including factors II, V, VII, VIII, IX, and X. To further evaluate the hemostatic profile, we analyzed fibrinogen levels to gauge the acute phase response, examined d-dimer levels as an indirect marker of fibrinolysis, and measured cortisol levels to reflect stress.

Additionally, we conducted a lipid profile analysis, measuring plasma levels of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG), and determined low-density lipoprotein cholesterol (LDL-C). Finally, lipid peroxidation was evaluated by measuring malondialdehyde (MDA) levels, and plasma cortisol concentrations were measured as an indicator of stress.

We demonstrated that traditional subcutaneous insulin did not correct these disturbances but rather exacerbated them. This aligns with previous findings, which indicate that the effects of hyperglycemia on coagulation are not reversible by insulin injections (Stegenga et al., 2006). Moreover, the **hyperinsulinemia** induced by subcutaneous insulin could be a significant procoagulant factor in itself, potentially further aggravating the hemostatic imbalance (Stegenga

et al., 2008; Schneider, 2023), as could the resulting **insulin resistance** (Kelem et al., 2023; Safdar et al., 2023). Our observations on the effects of subcutaneous insulin on hemostasis and endothelial function are therefore consistent with the previously discussed results, which demonstrated elevated HOMA-IR values and peripheral hyperinsulinemia in rats treated with subcutaneous insulin.

In contrast, diabetic rats treated with Oral nCOF/Insulin showed significant improvements. The restoration of glucose homeostasis, normalization of lipid profiles, correction of lipid peroxidation, and maintenance of metabolic equilibrium without inducing hyperinsulinemia helped prevent diabetes-related hemostatic disturbances. Interestingly, while Oral nCOF/Insulin did not fully correct the pro-oxidant/antioxidant imbalance in the endothelium, it still normalized bleeding time and restored primary hemostasis, as well as coagulation and fibrinolysis.

These results demonstrated for the first time that insulin treatment in type 1 diabetes has a significant impact on hemostasis and endothelial alterations induced by diabetes. This comprehensive study allowed us to observe that the biological implications of insulin therapy are critically dependent on the route of administration and its resulting pharmacological profile. Additionally, our correlational study highlighted that lipid peroxidation is a primary factor driving these disturbances. We also emphasized that routine tests such as PT and APTT may appear normal but are not fully representative of hemostatic status, suggesting the need to consider other markers, particularly triglyceride levels. Finally, our investigation provided valuable insights into the pathophysiological mechanisms underlying these hemostatic complications, showing that Oral nCOF/insulin, despite being an exogenous treatment, can have a markedly different effect on these parameters. This demonstrates that it is hyperinsulinemia, rather than insulin itself, that is primarily responsible for the observed damage. Motivated by these findings, we further explored the potential of Oral nCOF/Insulin to reverse diabetes-induced organ damage.

Oxidative stress is known to impair organ health (Hepel and Andreescu, 2015; Liguori et al., 2018), affecting both structure and function (Ogura and Shimosawa, 2014; Boarescu et al., 2022), which can lead to various complications (Giacco and Brownlee, 2011). In our preliminary toxicity assessments, we observed that oral insulin effectively corrected the diabetes-induced alterations

in liver and kidney tissues, contrasting sharply with the effects associated with subcutaneous insulin administration. To expand our investigation, we assessed oxidative status and histological changes across various organs, while also considering body weight and relative organ weights as indicators of toxicity (Bindhu et al., 2007).

Our findings indicate that streptozotocin-induced diabetes resulted in significant organ toxicity, characterized by alterations in organ weights, an imbalance in oxidative stress, and various histological modifications, including necrosis, vasodilation, and karyolysis in multiple tissues. The reduction in body weight, coupled with an increase in organ weights among diabetic rats, reflects the metabolic stress imposed by diabetes. Notably, treatment with Oral nCOF/Insulin restored both body and organ weights to near-normal levels, emphasizing its potential to mitigate metabolic distress and organ hypertrophy associated with diabetes. Moreover, the imbalance between oxidants and antioxidants, a hallmark of diabetes pathology (Bonnefont-Rousselot et al., 2000; Black, 2022), was evident in our measurements, which revealed elevated malondialdehyde (MDA) and protein carbonyl concentrations across several organs, alongside changes in glutathione (GSH) and catalase levels. Treatment with Oral nCOF/Insulin effectively diminished these oxidative stress markers, aligning their levels with those observed in non-diabetic controls.

Furthermore, our results demonstrate that insulin can influence the structure and function of the adrenal glands, potentially modulating the secretion of other hormones (Mahar et al., 2012; Soliman and Noya, 2020). Indeed, the remodeling of adrenal gland structure provides important insights into its secretory functions. The impact of Oral nCOF/Insulin was evident in the normalization of adrenal gland morphology, which had been altered by diabetes and further exacerbated by subcutaneous insulin treatment. Notably, subcutaneous insulin appeared to promote androgen secretion, leading to an increase in the thickness of the adrenal cortex and contributing to significant structural changes (Unluhizarci et al., 2021). In contrast, diabetic rats treated with Oral nCOF/Insulin exhibited adrenal glands resembling those of non-diabetic controls.

This normalization indicates that the gradual absorption of oral insulin may affect hormonal regulation differently compared to the rapid absorption of subcutaneous administration. The

modulation of adrenal gland structure and function plays crucial roles in metabolism and can impact various organs in the body (Ioakim et al., 2019; Unluhizarci et al., 2021). Thus, the effects of Oral nCOF/Insulin on adrenal morphology could significantly influence overall metabolic health. This underscores its potential not only to enhance glucose regulation but also to improve the essential endocrine balance necessary for maintaining homeostasis in individuals with diabetes.

Our research contributes novel insights into the field of diabetes management by demonstrating that Oral nCOF/Insulin can restore physiological functions across a range of organs. Specifically, we demonstrated that the Oral nCOF/Insulin treatment supports brain health by reducing lipid peroxidation, mitigates muscle wasting by addressing oxidative stress, restores intestinal integrity, and improves the structure and function of the spleen, myocardium, liver, kidneys, and adrenal glands in diabetic rats. These findings highlight the therapeutic potential of Oral nCOF/Insulin beyond glucose regulation, presenting a more comprehensive approach to managing the complications associated with diabetes.

The findings of this research demonstrate that nCOF exhibits effects that extend beyond hyperglycemia, addressing an urgent need in diabetes management. This need encompasses not only the management of hyperglycemia but also the restoration of metabolic homeostasis and the prevention of complications related to diabetes, which are currently major challenges in the field.

Oral nCOF/Insulin treatment fulfills multiple criteria as an optimal insulin treatment by effectively managing dysglycemia and restoring glycemic homeostasis. It prevents fluctuations in blood glucose levels and provides prolonged coverage in both fasting and postprandial states. Additionally, this treatment aids in restoring metabolic balance, impacting several physiological aspects, including hemostasis, coagulation, fibrinolysis, muscle mass, lipid profile, and cellular metabolism across various organs. These effects can significantly influence both the structure and function of these organs.

Moreover, oral nCOF/Insulin positively affects endocrine metabolism and notably reduces oxidative stress—both plasmatic and intracellular—which in turn helps mitigate the associated

consequences of diabetes. By addressing these multifaceted aspects of metabolic health, nCOF/Insulin emerges as a promising candidate for future therapeutic strategies aimed at enhancing diabetes management and preventing related complications.

Ultimately, this study underscores the importance of pharmacological parameters, illustrating how the same insulin molecule can produce markedly different effects depending on the route of administration. This finding suggests that traditional subcutaneous insulin therapy may contribute to the development of diabetic complications, highlighting the need for comprehensive diabetes management that extends beyond mere hyperglycemic control.

Conclusion

This thesis presents significant advancements in diabetes management by demonstrating the efficacy and therapeutic potential of oral insulin encapsulated in nCOF particles (nCOF/Insulin). Our research revealed that this innovative formulation not only enables oral insulin administration but also significantly enhances its bioavailability while maintaining its therapeutic efficacy. Notably, nCOF/Insulin progressively lowered blood glucose levels without causing hypoglycemia, maintaining glycemic control comparable to non-diabetic conditions. The sustained effect, covering both fasting and postprandial states, underscores the superiority of this approach over traditional subcutaneous insulin.

Beyond glycemic control, our findings suggest that oral nCOF/Insulin has the potential to prevent insulin resistance and improve insulin sensitivity, marking an important advancement in type 1 diabetes treatment. Additionally, this formulation addresses broader diabetes-related complications, such as disruptions in hemostasis, coagulation, and oxidative stress. Our study demonstrated the ability of nCOF/Insulin to repair histological damage and restore metabolic balance across various organs. These systemic effects offer a more comprehensive approach to diabetes management, addressing not only glucose regulation but also the underlying metabolic and physiological dysfunctions that contribute to long-term complications.

Furthermore, this research highlights the critical role of pharmacological parameters in determining the outcomes of insulin therapy. The marked differences in efficacy between oral nCOF/Insulin and traditional subcutaneous insulin suggest a need to reconsider current diabetes treatment strategies. While subcutaneous insulin effectively lowers blood glucose, it may inadvertently contribute to the development of complications by failing to replicate insulin's natural physiological roles. Our results offer deeper insights into how conventional treatments may exacerbate metabolic alterations and diabetes-related complications. In contrast, oral nCOF/Insulin presents a more physiological approach that helps restore metabolic homeostasis and prevent the progression of complications.

The therapeutic potential of oral nCOF/Insulin is evident in its ability to prevent diabetes-related complications and improve overall metabolic health. In summary, this thesis provides novel insights into diabetes management, positioning oral nCOF/Insulin as a promising candidate for future therapeutic applications. This treatment has the potential to transform current approaches to diabetes care. Future research should explore its long-term effects, with the ultimate goal of improving patient outcomes and enhancing the quality of life for individuals with diabetes.

Glossary

1. *Endogenous insulin*: physiological insulin produced naturally by the pancreas.
2. *Pharmacodynamics of physiological insulin:* Describes the dynamic interaction between endogenous insulin and the body, including its effects and regulatory mechanisms.
3. *Pharmacokinetic of physiological insulin*: Describes how the body processes endogenous insulin.
4. *Distribution of insulin*: The distribution of insulin refers to the process by which insulin spreads throughout the body after being released into the bloodstream or administered. The distribution determines which tissues are targeted first and influences the concentration of insulin that reaches each tissue.
5. *Exogenous insulin*: refers to insulin that is externally administered to the body. This insulin is synthetic and not produced naturally by the body.
6. *Insulin analogs*: Insulin analogs are synthetic forms of insulin that mimic natural insulin action. They are structurally modified to achieve specific properties, such as rapid onset, prolonged duration, or intermediate action. These modifications enhance their performance in controlling blood glucose levels in individuals with diabetes.
7. *Iatrogenic hyperinsulinemia:* refers to elevated levels of insulin in the blood resulting from medical administration of insulin.
8. *Dysglycemic disorder*: refers to a condition characterized by abnormalities in blood glucose levels, including hyperglycemia (high blood sugar), hypoglycemia (low blood sugar), and glucose variability (fluctuations in blood sugar levels).
9. *Bioavailability*: The bioavailability of Oral nCOF/Insulin refers to the fraction of the administered dose that reaches the bloodstream and is available to produce pharmacological effects, as compared to subcutaneous insulin, which is considered 100% bioavailable due to its direct administration into the systemic circulation. Bioavailability is calculated relative to subcutaneous insulin.
10. *Pharmacokinetic of exogenous insulin*: refers to the study of how externally administered insulin behaves in the body, including its onset, peak, and duration of action, typically analyzed by tracking insulin concentration in the bloodstream over time.
11. *Pharmacodynamics of exogenous insulin:* refers to how externally administered insulin affects blood glucose levels.
12. *HOMA-IR*: is an index used to assess insulin resistance by measuring fasting glucose and insulin levels. A high HOMA-IR value indicates increased insulin resistance, meaning the body requires higher levels of insulin to regulate glucose plasma levels effectively. This is often associated with elevated concentrations of both insulin and glucose in the blood.
13. *HOMA-IS*: is an index used to evaluate insulin sensitivity by measuring fasting glucose and insulin levels. A high HOMA-IS value indicates better insulin sensitivity, meaning the body requires lower levels of insulin to regulate blood glucose effectively. It is typically associated with lower concentrations of both insulin and glucose in the blood.

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