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Inducing Macrophage Polarization Using Metformin-Encapsulated Nanostructured Lipid Carriers to Combat Obesity and T2DM: A Research Protocol

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Abstract

Introduction: Obesity and type 2 diabetes mellitus (T2DM) are driven by chronic inflammation in white adipose tissue, characterized by an imbalance between pro-inflammatory (M1) and anti-inflammatory (M2) macrophages. This disruption contributes to insulin resistance and metabolic dysfunction. Metformin, a widely used antidiabetic drug, has demonstrated potential in promoting M2 polarization and inhibiting M1 polarization. This protocol explores an innovative therapeutic approach using Metformin encapsulated in nanostructured lipid carriers (NLCs) to enhance its efficacy.

Methods: Male C57BL/6J mice will be fed a high-fat diet (HFD) to induce obesity and treated with streptozotocin (STZ) to simulate T2DM conditions. The mice will receive daily or weekly doses of Metformin, either via oral gavage or encapsulated in NLCs, over 12 weeks. Key assessments will include glucose tolerance tests (GTT), intraperitoneal insulin tolerance tests (IPITT), body weight monitoring, and insulin and cytokine profiling through ELISA. Adipose tissue will be analyzed post-euthanasia using histological techniques, flow cytometry, and quantitative PCR to evaluate macrophage polarization.

Results: The NLC-encapsulated Metformin is anticipated to demonstrate improved therapeutic efficacy by reducing fasting blood glucose levels, body weight, and pro-inflammatory cytokines while increasing anti-inflammatory cytokines compared to traditional oral Metformin administration. This is attributed to NLCs' targeted delivery, enhanced bioavailability, and sustained drug release.

Discussion: This study aims to establish the superiority of NLC-encapsulated Metformin in mitigating metabolic dysfunction and inflammation compared to traditional methods. The results could underscore the importance of targeted therapies for treating obesity and T2DM and pave the way for translational clinical applications.

Conclusion: Findings from this study may advance the development of targeted therapies for metabolic diseases, providing a foundation for future clinical applications in managing obesity and T2DM.

Keywords: obesity; type 2 diabetes mellitus; insulin resistance; macrophage polarization; inflammation; metformin; nanostructured lipid carriers; bioavailability; metabolic diseases

Introduction

Obesity affects 1.9 billion people worldwide [1]. It is marked by significant adipose tissue expansion resulting from excessive nutrient intake and inadequate energy expenditure. Obesity has been linked to disrupting immune homeostasis, causing a wide array of metabolic disorders. Most notably, it is a major contributing factor to T2DM due to obesity's link to insulin resistance [2].

Insulin resistance is a condition in which insulin-targeting tissues have a decreased responsiveness to high insulin levels [3]. This continued state leads to elevated levels of plasma glucose. Prolonged conditions of elevated blood glucose and insulin levels lead to pancreatic β-cell failure and T2DM. The disrupted immune homeostasis that obese individuals experience is due to elevated levels of pro-inflammatory cytokines in adipose tissue. This leads to chronic inflammation, a key driver of insulin resistance [3].

White adipose tissue (WAT) plays a critical role in the pathophysiology of obesity and T2DM [4]. Its primary function involves storing energy as triglycerides, but it also serves as an active endocrine organ. Adipocytes within WAT respond to physiological and metabolic changes by releasing endocrine factors that regulate processes such as energy expenditure, appetite regulation, glucose homeostasis, insulin sensitivity, and inflammation [4]. Additionally, WAT comprises preadipocytes, adipocytes, and many types of immune cells (T cells, B cells, macrophages, and dendritic cells). Macrophages make up to 40-50% of WAT cells and polarize into the M1 or M2 phenotype based on body weight [3]. In lean individuals, macrophages polarize into the anti-inflammatory M2

phenotype. M2 macrophages secrete anti-inflammatory mediators such as Interleukin-10 (IL-10) and Interleukin-1 (IL-1), which help maintain insulin sensitivity. In obese individuals, macrophages polarize into the pro-inflammatory M1 phenotype, which secrete pro-inflammatory cytokines including Tumor necrosis factor alpha (TNF-α), Interleukin-12 (IL-12), and Interleukin-1 beta (IL-1β) [5].

The activation of these pro-inflammatory cytokines interferes with the insulin signaling pathway as they inhibit the downstream signaling molecule Insulin receptor substrate 1 (IRS-1), which causes an impaired ability to take up glucose effectively [6]. The chronic inflammation caused by the secretion of these cytokines can activate stress kinases within the cells such as c-Jun N-terminal kinase (JNK) and Nuclear factor kappa B (NF-κB). These further inhibit insulin signaling proteins, again preventing glucose transport into cells and contributing to insulin resistance [6].

Metformin is a biguanide class of antidiabetic drugs that is commonly prescribed to T2DM patients. It lowers blood glucose levels by reducing glucose production in the liver to enhance insulin sensitivity [7]. Metformin has been found to inhibit macrophage polarization to the M1 phenotype and promote polarization to the M2 phenotype as outlined in Figure 1 [8]. Metformin inhibits complex I of the electron transport chain causing a decrease in ATP production. It also activates liver kinase B1 which goes on to phosphorylate AMPK. The increase in AMP levels and decrease in ATP levels switches the cell into a catabolic state [9,10]. Activated AMPK regulates metabolism as it is linked to energy levels in a cell. AMPK is a negative regulator of inflammation. When active, it inhibits NF-κB, a transcription factor involved in M1 polarization. AMPK also promotes the upregulation of Signal transducer and activator of transcription 6 (STAT6) and Peroxisome proliferator-activated receptor gamma (PPARγ), key transcription factors involved in M2 polarization [11].

Metformin is the most widely prescribed oral drug used to lower glucose. Despite this, the drug has many problems that decrease efficacy, such as low bioavailability, high doses, frequent administration, and poor intestinal absorption [12]. Metformin is currently taken orally in a pill form up to twice daily. The dosage levels range from 500 mg to 2000 mg [13]. It does have side effects, mainly gastrointestinal issues. These include diarrhea, nausea, gas, abdominal pain, and indigestion. More severe side effects include vitamin B12 deficiency and lactic acidosis [14,15].

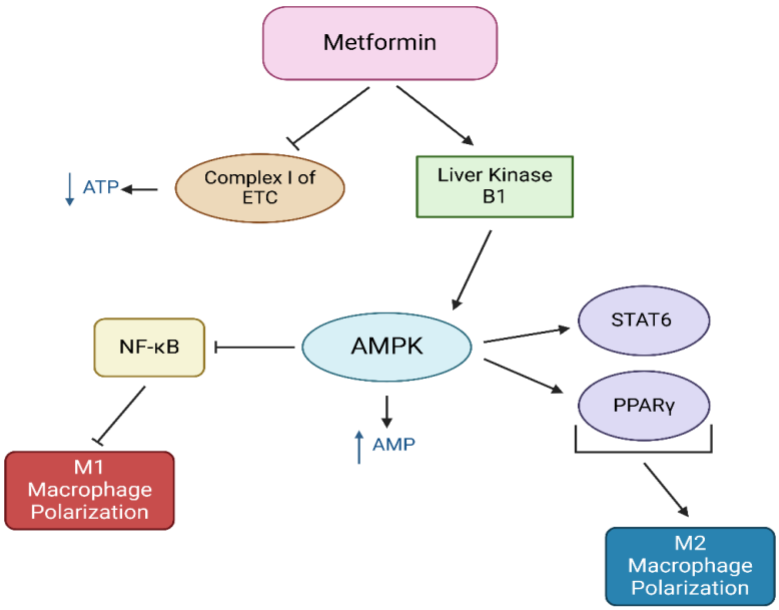

Figure 1. Metformin's Mechanism of Action in Regulating Macrophage Polarization. This pathway illustrates how Metformin regulates macrophage polarization by inhibiting M1 polarization and promoting M2 polarization. Metformin inhibits Complex I of the mitochondrial electron transport chain (ETC), leading to reduced ATP production and increased AMP levels. This shift activates liver kinase B1 (LKB1), which subsequently phosphorylates and activates AMP-activated protein kinase (AMPK) [10]. Activated AMPK induces a catabolic state, negatively regulates inflammation by inhibiting NF-κB (a transcription factor involved in M1 polarization), and upregulates STAT6 and PPARγ (key transcription factors promoting M2 polarization). These processes collectively suppress pro-inflammatory M1 macrophages while enhancing anti-inflammatory M2 macrophages [11]. Created with https://BioRender.com.

Nanostructured lipid carriers (NLCs) are a more recent drug administration method that can be applied to varying treatments [16]. NLCs have several benefits including offering targeted delivery of drugs, protecting sensitive compounds, and increasing the bioavailability of hydrophobic drugs. The structure consists of a solid matrix of lipids with a portion of the internal structure being oil [16]. This improves the NLCs' ability to load drugs and prevents drug oxidation. NLCs offer a prolonged and sustained release of the drug, which can help in maintaining consistent and long-term effects [16]. The characteristics of NLCs make them a better alternative in drug administration. The increased bioavailability in NLCs protects the drugs from degradation and improves absorption to target tissues. The sustained release of the drug reduces the need for frequent doses [17]. These contribute to NLC administrations requiring smaller dose levels and less frequent dosing. This is effective as it allows for reduced side effects without compromising efficacy [18].

This research protocol proposes a potential therapy for obesity and T2DM by targeting macrophages as the therapeutic focus. It is hypothesized that the selective polarization of M1 macrophages to M2 macrophages in the adipose tissue of C57BL/6J mice with STZ-induced T2DM, facilitated by Metformin encapsulated in NLCs, will reduce chronic inflammation in the adipose tissue. This approach is expected to decrease hyperglycemia, hyperinsulinemia, and body weight, offering a novel therapeutic strategy for managing obesity and T2DM. Additionally, it is hypothesized that the NLC-encapsulated Metformin will provide enhanced therapeutic efficacy due to increased bioavailability, targeted delivery, and anti-inflammatory effects compared to traditional oral Metformin administration. Greater reductions in body weight, fasting blood glucose (FBG) levels, and M1 cytokines, as well as increased M2 cytokines, would indicate that NLCs are more effective than standard oral administration.

C57BL/6J mice will be placed on a high-fat diet and will develop T2DM symptoms through STZ injections. NLCs containing Metformin or Metformin via oral gavage will be administered over a 12-week period, with one group receiving the treatment weekly and another group receiving it daily. The inclusion of both daily and weekly drug groups is intended to evaluate the efficacy of less frequent dosing with Metformin encapsulated in NLCs compared to the standard dosing regimen. This approach assesses whether NLCs maintain therapeutic outcomes at reduced dosing frequencies, offering a potential advantage in patient compliance and reducing side effects associated with frequent dosing. The mice's body weight, blood glucose levels, and insulin and adipose cytokine levels will be monitored to observe the effects of Metformin encapsulated in NLCs. Macrophage polarization will be analyzed post-euthanasia through histological analysis, flow cytometry, and real-time quantitative PCR (qPCR).

Methods

Animal Models

Male C57BL/6J mice will be treated with metformin encapsulated in NLCs which will be provided by an industry partner [19]. There will be two cohorts: one cohort receiving daily treatment (n = 60) and the other receiving weekly treatment (n=60). Each cohort will be subdivided into 4 groups (n=15 per group). Mice in each group will be housed together and fed *ad libitum* with the following dietary and treatment conditions: 1) standard diet (STD), 2) HFD (45% calories from fat), 3) HFD + treatment with Metformin, or 4) HFD + treatment with Metformin encapsulated in NLCs (Figure 2A) [20].

Induction of T2DM

Mice in the experimental groups will receive HFD at 6 weeks of age to induce obesity and insulin resistance [21]. This will be followed by treatment with one 40 mg/kg dose of STZ for 5 consecutive days starting at 10 weeks of age to mimic the progression from insulin resistance to T2DM. The mice will be monitored for an additional two weeks to allow hyperglycemia to develop while continuing the HFD. By 12 weeks of age, the mice will have developed hyperglycemia, hyperinsulinemia, and insulin resistance consistent with T2DM [22]. A GTT will be performed to measure the FBG levels, with mice fasted for 6 hours prior. The FBG threshold for mice fed the STD is 79 mg/dL and 250 mg/dL for HFD-fed mice, with anything above this value defined as hyperglycemic [23,24]. An IPITT will assess insulin sensitivity, conducted 72 hours after the GTT to avoid interference from fasting and residual glucose administration. Mice will also be fasted for 6 hours prior to the test. The IPITT FBG threshold is 120 pmol/L for STD-fed mice and 230 pmol/L for HFD-fed mice, with higher values indicating hyperinsulinemia [21]. Mice that do not meet these thresholds will be excluded from the experiment.

Treatment Administration

Metformin will be administered either via oral gavage (Metformin hydrochloride suspended in 0.5% Tween 80) or via intraperitoneal injections of Metformin-NLCs at 12 weeks of age [25]. Table 1 outlines the dosage levels for each cohort and group. Mice receiving free-form Metformin will be administered in a 250 mg/kg dose (split into two 125 mg/kg doses) between the hours of 9-11 am and 7-9 pm [14]. Depending on the cohort, this will be administered this daily or once weekly for 12 weeks. Mice treated with Metformin-NLCs will receive a 15 mg/kg dose administered via intraperitoneal injections between 9-11 am, either daily or weekly for 12 weeks [26].

Table 1. Cohort Structure, Dietary Regimens, and Dosage Information

Cohort	Group	Diet	Dosage
Daily Treatment	Control Group	Standard Diet	No Treatment
	HFD (No Treatment)	High-Fat Diet	No Treatment
	HFD + Oral Metformin	High-Fat Diet	250 mg/kg/day (125 mg/kg twice daily)
	HFD + Metformin-NLCs	High-Fat Diet	15 mg/kg/day
Weekly Treatment	Control Group	Standard Diet	No Treatment
	HFD (No Treatment)	High-Fat Diet	No Treatment
	HFD + Oral Metformin	High-Fat Diet	250 mg/kg/day (125 mg/kg twice daily)
	HFD + Metformin-NLCs	High-Fat Diet	15 mg/kg/day

The table summarizes the experimental design, including two cohorts (daily and weekly treatment), each with four groups: a control group on a standard diet (STD), an untreated high-fat diet (HFD) group, an HFD group treated with Metformin via oral gavage, and an HFD group treated with NLC-encapsulated Metformin. Dosages are provided for Metformin-treated groups based on daily or weekly administration.

In Vivo Monitoring and Measurements

Mice will be monitored once weekly for blood glucose levels and body weights (Figure 2B). In Vivo Imaging System (IVIS) will be used to track the fluorescently labelled NLCs and observe their migration to adipose tissue. A GTT and IPITT will be conducted at Week 0, Week 1, Week 6, and Week 12 for all groups [27]. The IPITT will be performed 72 hours after the GTT to prevent interference from the residual glucose administration [21]. A series of enzyme-linked immunosorbent assays (ELISA) will be performed on blood samples collected from all mice at weeks 0, week 6, and week 12 to measure serum levels of insulin, TNF-α, IL-6, and IL-10 [26]. Mice will be fasted for 6 hours prior to collection of blood for the GTT, IPITT, and ELISA assays.

Histological and Cellular Analysis

At the end of the 12-week treatment period, the mice will be euthanized, and the adipose tissue will be harvested for analysis. To visualize the distribution and phenotype of adipose tissue macrophages, a histological analysis and flow cytometry will be performed. The tissue will be stained for inducible nitric oxide synthase (iNOS) and CD80 using an anti-iNOS antibody for M1 macrophages. An anti-arginase 1 (Arg1) antibody will be used to stain Arg1 and CD206 for M2 macrophages [28]. Flow cytometry will be conducted on the stromal vascular fraction of the harvested adipose tissue to sort and quantify the production of M1 and M2 macrophages. The macrophages will be isolated from the adipose tissue using F4/80 and CD11b (general cell markers for adipose macrophages). iNOS will be used to quantify M1 macrophages and Arg1 and CD206 will be used for M2 macrophages [29]. Additional quantification of cell expression will be conducted through quantitative PCR of extracted RNA from the adipose tissue. TNF-α, IL-1β, IL-6 and CXCL10 will be analyzed for M1 macrophages and IL-10 and Arg1 for M2 macrophages [30].

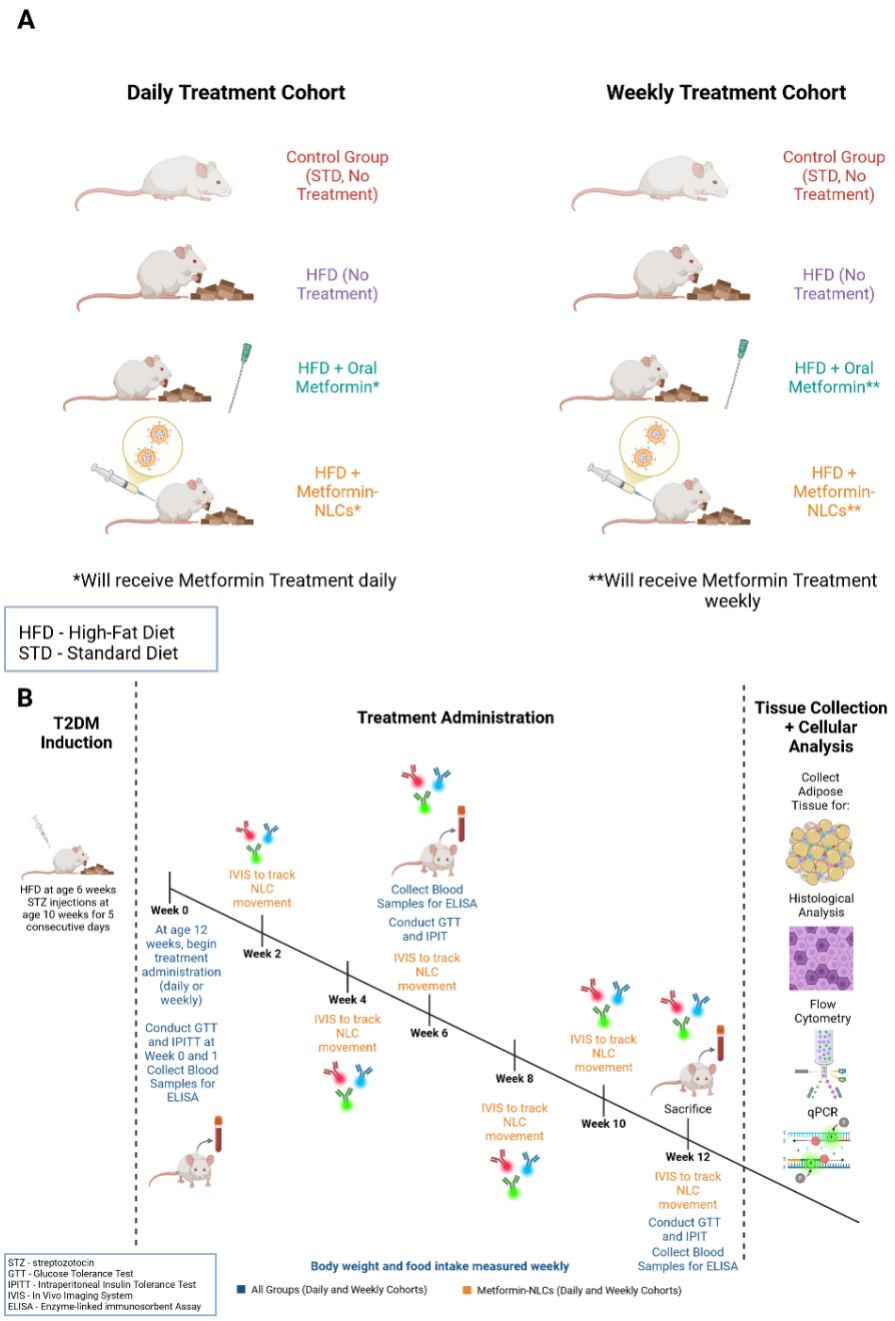

Figure 2. Cohort Structure and Experimental Timeline. **(A)** Cohort and group structure. The study includes two treatment cohorts: daily and weekly. Each cohort consists of four groups: a control group on a standard diet (STD, no treatment), a high-fat diet (HFD, no treatment) group, a high-fat diet group treated with Metformin via oral gavage, and a high-fat diet group treated with NLC-encapsulated Metformin. The dietary and treatment regimens are detailed for each group.
(B) Timeline and methods. The experimental timeline outlines key events, starting with T2DM induction via high-fat diet (HFD) at age 6 weeks and streptozotocin (STZ) injection at age 10 weeks. At age 12 weeks, treatment administration begins at Week 0 and continues for 12 weeks, with Metformin provided either daily or weekly. Regular monitoring includes body weight and food intake (weekly), glucose tolerance tests (GTT) and intraperitoneal insulin tolerance tests (IPITT)at Weeks 0, 6, and 12, and in vivo imaging (IVIS) to track NLC distribution every two weeks. Blood samples for insulin and cytokine analysis (ELISA) are collected at Weeks 0, 6, and 12. Adipose tissue is harvested at Week 12 for histological analysis, flow cytometry, and quantitative PCR (qPCR) to assess macrophage polarization. Created with https://BioRender.com.

Results

The NLCs are anticipated to show efficient distribution by targeting adipose tissue through IVIS. This will be the same in both the weekly and daily cohorts. The weekly and daily HFD + Metformin-NLCs group is anticipated to display a gradual decrease in body weight over the 12-week treatment period. This will also be seen in the groups receiving weekly or daily oral administration of Metformin, however, the weekly group should have a less pronounced decrease in body weight compared to the weekly HFD + Metformin-NLCs group.

The GTT and IPITT will assess insulin sensitivity progression using control group thresholds. STD-fed mice have an FBG of 79 mg/dL (GTT) and 120 pmol/L (IPITT), while HFD-fed mice have an FBG of 250 mg/dL (GTT) and 230 pmol/L (IPITT) [22, 23]. For each Metformin group (NLCs and oral gavage), a gradual decrease in FBG levels is anticipated for both the GTT and IPITT. However, a larger decrease is anticipated in the HFD + Metformin-NLCs groups, exhibiting levels close to the FBG threshold levels of mice on the STD. [31]. Between the weekly and daily NLC groups, the daily NLC group is expected to show a greater reduction in FBG levels compared to the weekly group. By the end of the treatment period, FBG levels in the weekly NLC group are anticipated to be comparable and slightly decreased to those in the daily oral gavage Metformin group, demonstrating a similar effect[32].

The ELISA is anticipated to show a gradual increase in anti-inflammatory cytokines in the weekly and daily Metformin treatment groups over the treatment period. However, a greater increase is expected in the Metformin-NLCs groups compared to the weekly HFD + oral Metformin group. A gradual decrease in serum insulin levels is expected in both weekly and daily Metformin treatment groups, with a larger decrease expected in the Metformin-NLCs group than the oral administration groups.

Post-euthanasia, flow cytometry, qPCR and histological analysis will provide insights into macrophage polarization progression through the quantification and characterization of M1 and M2 cell markers. For the daily cohorts, the HFD + oral Metformin group is anticipated to have greater levels of M2 macrophages than M1 macrophages compared to the HFD control group. The HFD + Metformin-NLCs group is anticipated to show a significantly greater increase in M2 macrophages and a more substantial reduction in M1 macrophages compared to the oral Metformin group. In the weekly cohort, the HFD + Metformin-NLCs group is expected to produce results comparable to the daily NLC group, whereas the weekly oral Metformin group is likely to show a smaller shift toward M2 polarization compared to M1 levels.

Discussion

This research proposal addresses a critical gap in the treatment of obesity and T2DM by targeting macrophages in adipose tissue to combat inflammation and insulin resistance. Our approach of encapsulating Metformin in NLCs represents a novel therapeutic strategy to enhance drug efficacy and improve metabolic outcomes in individuals with T2DM.

The anticipated results demonstrate that Metformin encapsulated in NLCs effectively targets adipose tissue, leading to significant metabolic improvements. Proper distribution ensures that observed changes in body weight, FBG and insulin levels, and cytokine profiles are directly attributable to treatment [33]. By activating AMPK, Metformin promotes a shift from pro-inflammatory M1 to anti-inflammatory M2 macrophages, reducing inflammation and improving insulin sensitivity. These effects are expected to result in reduced FBG and insulin levels, weight loss, and increased anti-inflammatory cytokines (e.g., IL-10). The decrease in insulin levels is attributed to Metformin's ability to reduce inflammation and enhance insulin sensitivity. As a result, the body requires less insulin to facilitate glucose uptake, leading to lower circulating insulin levels [3]. Additionally, the decrease in fasting blood glucose levels indicates an improvement in insulin resistance, as insulin is gradually functioning more effectively to regulate glucose uptake. The superior bioavailability, targeted delivery, and sustained release of NLCs contribute to their enhanced therapeutic efficacy compared to traditional Metformin [17]. This is demonstrated by the comparable FBG levels observed between the weekly NLC group and the daily oral gavage group, indicating that less frequent dosing of NLCs can achieve similar metabolic improvements as daily Metformin administration. Histological analysis, flow cytometry, and qPCR are anticipated to confirm this macrophage polarization shift, reinforcing its role in reducing inflammation and metabolic dysfunction.

The differences between the daily and weekly cohorts provide insight into the advantages of NLCs compared to traditional oral administration of Metformin. Both cohorts of mice receiving Metformin-NLCs are anticipated to exhibit improved insulin sensitivity due to higher M2 macrophage levels compared to those receiving Metformin via oral gavage. The daily and weekly treatment regimens assess whether less frequent Metformin-NLC dosing achieves comparable or superior outcomes to daily dosing. This evaluation is critical as NLCs, with their ability to target tissues more effectively and sustain drug release, may reduce the need for frequent administration. Frequent dosing of Metformin, currently taken up to twice a day, is associated with increased adverse effects. By reducing the frequency of administration without compromising efficacy, weekly NLC administration could provide a more convenient and less invasive alternative while minimizing the side effects commonly associated with traditional treatment methods [18].

A possible limitation of this experiment includes feeding the mice *ad libitum*. As the mice will eat at their own will, the amount of food from either the STD or HFD they will eat will differ, possibly affecting their body weight, GTT, and

IPITT results. To improve this, fixed eating times for the mice could be implemented instead. Another limitation is the group housing of each experimental group, possibly leading to a hierarchy developing where dominant mice eat more than others. This could be altered by individually housing each mouse. Using injections instead of oral administration may limit the practicality of this treatment for long-term management, as injections are less convenient for patients and may affect adherence. This delivery method is less ideal for routine management of diabetes and obesity, where oral administration is typically preferred for ease of use.

Conclusions

This protocol presents a novel therapeutic approach for managing obesity and T2DM by targeting macrophage polarization through Metformin encapsulated in NLCs. By enhancing Metformin's bioavailability and precision, this protocol demonstrates a promising strategy for reducing chronic inflammation in adipose tissue and improving metabolic health outcomes.

The findings contribute significantly to our understanding of macrophage-driven inflammation in metabolic diseases, with potential clinical implications. Targeted NLC-Metformin delivery could represent a more efficient and patient-friendly alternative to traditional high-dose Metformin therapy, potentially reducing the dosage required and minimizing gastrointestinal side effects. For patients struggling with conventional Metformin regimens, NLC-based delivery offers an innovative pathway to improving insulin sensitivity and metabolic control, thereby enhancing patient adherence and overall quality of life.

Additionally, this study raises important research questions about the long-term effects of NLC-Metformin on metabolic and inflammatory profiles, suggesting the need for further clinical exploration. The protocol lays a foundation for future studies to optimize drug delivery and broaden applications to other metabolic conditions involving inflammation, such as non-alcoholic fatty liver disease (NAFLD) [34]. Overall, this research represents an advancement in the development of targeted therapies for obesity and T2DM, with significant potential to inform future clinical strategies and improve patient outcomes in metabolic disease management.

List of Abbreviations

AMPK: amp-activated protein kinase
Arg1: arginase 1
ELISA: enzyme-linked immunosorbent assay
FBG: fasting blood glucose
GTT: glucose tolerance test
HFD: high-fat diet
IL-1: interleukin-1
IL-10: interleukin-10
IL-12: interleukin-12
IL-1β: interleukin-1 beta
iNOS: inducible nitric oxide synthase
IPITT: intraperitoneal insulin tolerance test
IRS-1: insulin receptor substrate 1
IVIS: in vivo imaging system
JNK: c-Jun N-terminal kinase
NAFLD: non-alcoholic fatty liver disease
NF-κB: nuclear factor kappa b
NLCs: nanostructured lipid carriers
PPARγ: peroxisome proliferator-activated receptor gamma
qPCR: quantitative polymerase chain reaction
STAT6: signal transducer and activator of transcription 6
STD: standard diet
STZ: streptozotocin
T2DM: type 2 diabetes mellitus
TNF-α: tumor necrosis factor alpha
WAT: white adipose tissue

Conflicts of Interest

The author(s) declare that they have no conflict of interests.

Ethics Approval and/or Participant Consent

This research protocol did not require ethics approval and/or participant consent.

Authors' Contributions

SN: made substantial contributions to the design and concept of the proposed study, revised the manuscript critically, and gave final approval of the version to be published.

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