RESEARCH PAPER

Designing an oral insulin delivery system based on milk exosomes for managing diabetes: in vitro and in vivo investigation

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ABSTRACT

Objective(s): Oral drug administration is a noninvasive, painless, and convenient strategy that eliminates the need for routine injection routes. In recent years, milk-derived exosomes (Mexos) have gained tremendous interest as vehicles for oral drug delivery due to their low immunogenicity, high biocompatibility, and enhanced stability. Here, we hypothesized to recruit cow Mexos as an oral delivery vehicle for preparing an exosomal insulin formulation (Mexolin).

Materials and Methods: Mexos were isolated using sequential ultracentrifugation steps, and insulin was loaded into the Mexos using the sonication method. Next, the encapsulation efficiency (EE) and drug loading capacity (LC) were measured using the reverse-phase high-performance liquid chromatography (RP-HPLC) strategy. Finally, following inducing and confirming diabetes in male Sprague-Dawley rats, diabetic rats were orally administered the Mexolin NPs, insulin solution, and intact Mexos. Control gropu was received subcutaneous insulin injection.

Results: Findings showed that the Mexolin NPs could release insulin in a sustained manner within a simulated intestinal medium. Meanwhile, Mexolin NPs penetrated a monolayer of polarized Caco-2 cells, confirming their ability to be absorbed through the intestinal epithelium. Animal studies exhibited that the Mexolin NPs caused a hypoglycemic response in Type 1 diabetes mellitus (T1DM) induced by streptozotocin (STZ) in rats. Orally administered Mexolin NPs (100 IU/kg) indicated higher antidiabetic activity compared to subcutaneously injected insulin (5 IU/kg) and oral insulin solution alone (100 IU/kg). In addition, Mexolin NPs showed remarkable insulin bioavailability in T1DM rats.

Conclusion: Mexos could be a cost-effective alternative with better patient compliance to subcutaneous insulin injection for diabetes therapy.

Keywords: *Oral protein drug delivery, Insulin, Extracellular vesicles, Milk exosomes, Diabetes*

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INTRODUCTION

Diabetes mellitus, a chronic metabolic disorder related to disrupted blood glucose regulation, requires alternative methods of insulin administration due to the limitations and complications of multiple subcutaneous injections as current treatments with poor glucose control and patient compliance [1, 2].

Oral insulin administration as a convenient and cost-effective strategy could greatly improve patient compliance, offering the potential for better glucose homeostasis and managing diabetes [1, 2]. Mimicking the physiological secretion route, orally administered insulin is directly delivered to the liver *via* the portal vein, providing higher concentrations than systemic circulation [3].

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However, subcutaneous injection disrupts this gradient, leading to imbalanced glucose metabolism. However, the biological barriers in the gastrointestinal tract pose challenges such as degradation, limited efficiency in passing through the mucus layer, and poor transcellular transport, resulting in low bioavailability and the absence of FDA-approved oral insulin formulations [1-3].

Nanotechnology has emerged as a solution for improving oral insulin bioavailability by protecting its biodegradation in the gastrointestinal tract, improving its absorption, and enabling its controlled release [1, 4]. For instance, nanoparticles (NPs) formed through polyelectrolyte complexes of charged polymers can shield insulin in the gastrointestinal tract and enhance its delivery efficiency [3]. Despite advancements in nanotechnology, current insulin-loaded NPs systems still face challenges regarding oral bioavailability and pharmacodynamic performance. Further improvements are needed to overcome barriers like mucus layer penetration and transcellular transport [1, 5, 6]. An ideal delivery system should protect insulin in the gastrointestinal tract, increase permeability for absorption, and ensure safety [5].

EVs, particularly exosomes, are essential for facilitating intercellular communication and have been utilized as biomarkers for disease monitoring [7, 8]. Exosomes, small membranous nanovesicles originating from endocytic compartments, are released by mammalian cells and found in various biological fluids like plasma, urine, saliva, malignant effusions, and breast milk [8-10].

Exosomes possess advantageous attributes such as the ability to carry microRNAs, mRNAs, DNAs, and proteins, stability in body fluids, and natural targeting properties through direct interaction with target cell receptors [7, 9, 10]. These nanoscale particles are less immunogenic and more compatible than synthetic NPs [7, 11]. Due to their capacity to traverse biological barriers, non-immunogenic nature, and flexibility in administration routes, exosomes are being explored as promising vehicles for loading and transporting drugs. They can protect cargo from enzymatic degradation, reduce mononuclear phagocyte system-related drug clearance, and selectively load various therapeutic substances [11-13]. Modification with homing molecules further enhances their targeting properties, making exosomal delivery systems encouraging therapeutic approaches for diseases like cancer and metabolic disorders [11, 14].

Milk exosomes demonstrate remarkable biocompatibility, negligible toxicity, and minimal influence on immune and inflammatory responses, making them potential carriers for clinical applications compared to liposomes and artificial NPs [15, 16]. This includes exosomes from various mammalian milk sources, especially bovine milk, a scalable and feasible source [15, 17]. Milk exosomes can carry specific chemical, biological, and macromolecular payloads, heightening their therapeutic cargo's safety, bioavailability, and efficacy [15, 18]. These exosomes can avoid homing to the liver, ensure long circulation time, and cross the blood-brain barrier, making them effective delivery carriers [15, 19]. They can carry antitumor drugs and therapeutic nucleic acids, possibly mitigating the toxic effects of traditional chemotherapy [16, 20].

As for oral drug delivery, which presents a challenge for many therapeutic biologics, milk-derived exosomes are being considered due to their ability to cross intestinal barriers [19, 21, 22]. Milk exosomes contain crucial proteins and microRNAs that regulate growth, immunity, and intercellular communication. They remain resistant to degradation by stomach acids, making them potential candidates for oral drug delivery of human medicine [9, 23]. Nonetheless, additional investigation is required to thoroughly unravel their biochemical and lipid content, enhance their tissue and cell specificity, and advance targeted exosome-derived delivery techniques.

Thus, in the current study, we hypothesize that encapsulating insulin in cow Mexos could serve as an oral drug delivery formulation of insulin (Mexolin). We employed *in vitro* and *in vivo* experimental models to compare and characterize the Mexolin NPs in simulated body fluids regarding stability, *in vitro* release, transepithelial transport capability, and the pharmacological response of both free insulin and Mexolin NPs. A scheme depicting the preparation procedure and subsequent experiments has been represented in Figure 1.

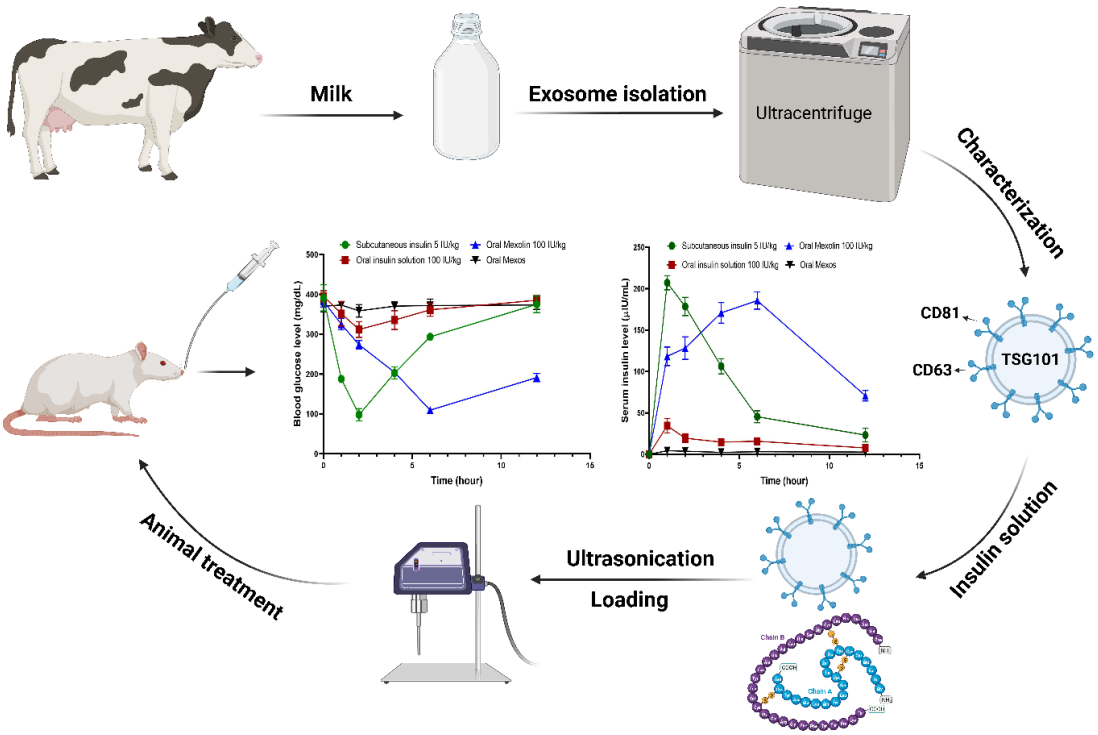

Fig 1. Schematic illustration of isolating milk exosomes, preparing milk exosomal insulin formulation, and designing animal experiments. Created with BioRender.com

MATERIALS AND METHODS

Materials

Recombinant human insulin was purchased from Exir Co., Ltd. (Tehran, Iran). Dulbecco's Modified Eagle's Medium (DMEM) high glucose cell culture medium, fetal bovine serum (FBS), penicillin-streptomycin, and trypsin were from Gibco (Darmstadt, Germany). Bicinchoninic acid (BCA) protein assay kit was obtained from Thermo Fisher Scientific. Human epithelial Caco-2 cells was from the National Cell Bank of Pasteur Institute of Iran (Tehran, Iran). Insulin Rapid AccuBind® ELISA was obtained from Monobind, USA. Acetonitrile, Hank's Balanced Salt Solution (HBSS), and other chemicals and solvents were purchased from Merck (Darmstadt, Germany).

Isolation of Mexos

Fresh, unpasteurized cow milk was obtained from a local farm (Mashhad, Iran). Mexos were purified using differential centrifugation and ultracentrifugation steps. Initially, milk underwent centrifugation at 14,000 × g (4 °C/30 min) to remove cell debris and large vesicles. The whey was collected by passing through a cheesecloth. Subsequently, the solution was transferred to polycarbonate centrifuge tubes and ultracentrifuged at 100,000 × g (4 °C/60 min) in an ultracentrifuge (Beckman Coulter). The obtained supernatant was extracted while the pellet was discarded. The supernatant underwent the final centrifugation at 140,000 × g (4 °C/90 min). After discarding the supernatant, the resulting Mexos pellet was washed thrice for 3 min each with PBS (pH 7.4). Next, the Mexos pellets were combined and resuspended in PBS (pH 7.4), and syringe filters (0.22 µm pore size, Sartorius, Goettingen, Germany) were used to filter the suspended Mexos. The quantitative determination of Mexos total protein concentration was conducted by the BCA method. The Mexos concentration was stored at -70 °C until subsequent use.

Physicochemical characterization and identification of Mexos

The Mexos zeta potential, hydrodynamic diameter, and size distribution were determined by dynamic light scattering (DLS) method using a Zetasizer (Nano Zeta-Sizer, Malvern, U.K.). The shape characterization and size investigation of Mexos was performed using a scanning electron microscope (FE-SEM, UK). In summary, a 30 µL sample of Mexos suspension with a concentration of 0.2 mg/ml was placed on an aluminum foil and subjected to drying at 25℃ in an oven. Gold nanoparticles (with sizes ranging from 2-5 nm) were

applied to the dry samples using a vacuum coating process. The identification of Mexos was performed by Western blot analysis to detect membrane-binding proteins, CD81 and CD63, and cytoplasmic protein TSG101 as general exosomal surface markers. The total protein associated with purified Mexos was separated on SDS-PAGE, electrotransferred onto a PVDF membrane and probed with primary mouse monoclonal antibodies for 8h at 4 °C (1:300), and horseradish peroxidase (HRP)-labeled secondary mouse IgG antibodies at RT, 1.5 h (1:1000). Then proteins bands were activated using a chemiluminescence reaction and visualized using ChemiDoc imager (Bio-Rad, USA).

Preparation of insulin-loaded Mexos NPs (Mexolin)

Insulin was encapsulated into Mexos using a sonication strategy. First, insulin was dissolved in 0.1 N HCl (10 mg/mL), adjusting the final pH to 7.4 with 0.1 N NaOH dropwise. Mexos, dispersed in PBS (pH 7.4), was premixed with insulin solution (1 mg/mL stock solution diluted in deionized water) at a Mexos to insulin ratio of 3:2 (w/w), based on Mexos total protein content. Subsequently, the resulting insulin and Mexos mixture was sonicated 8 cycles (500 v, 2 kHz, 20% power, 6 s/3 s on/off) using a probe sonicator, with a 3-minute cooling period on ice. After sonication, Mexolin NPs solution was incubated in a 37 °C incubator for 30 min to facilitate the restoration of the exosomal membrane [24]. A centrifugation step at 7000 rpm for 10 min using ultrafiltration centrifuge tubes (MWCO: 30 kDa) was performed to remove the unloaded insulin. The Mexolin NPs were harvested and kept at 4°C until further experiments. The SEM method was utilized to investigate the size and morphology of the Mexolin NPs by a protocol mentioned earlier.

RP-HPLC: insulin quantification

The encapsulation efficiency (EE) and drug loading capacity (LC) were determined using an indirect method. This involved measuring the concentration of unloaded insulin in the ultrafiltration permeate using a reverse-phase high-performance liquid chromatography (RP-HPLC) technique (Shimadzu, Kyoto, Japan). The analytical column was a MZ-Analysentechnik PerfectSil 300 Column (150 × 4.6 mm, 320 A° pore size) (GmbH, Mainz, Germany), equipped with a photodiode array (PDA) (SPD-M20 A, Shimadzu). The analysis was conducted with UV detection at 214 nm, and the mobile phase was a mixture of phosphate buffer (pH 3.0) and acetonitrile (with a ratio of 70:30 V/V). Injection volume and flow rate were 20 µL and 1 mL/min, respectively. A known amount of pure insulin was injected into the column for the calibration curve. HPLC analyses were carried out in triplicate. The EE and LC were determined as follows:

$$EE\ (\%) = \frac{\text{Insulin (total amount)} - \text{Insulin (unloaded)}}{\text{Insulin (total amount)}} \times 100 \quad (1)$$

$$LC\ (\%) = \frac{\text{Insulin (total amount)} - \text{Insulin (unloaded)}}{\text{NPs (Mexos) mass}} \times 100 \quad (2)$$

Insulin release assay

In vitro insulin release behavior was evaluated by a dialysis procedure. Briefly, freshly prepared Mexolin NPs were placed inside dialysis membranes (MWCO: 14 kDa), sealed, and submerged in either simulated gastric (HCL 0.1N, pH 1.2) or simulated intestinal (PBS, pH 7.4) conditions at 37 °C with continuous shaking at 80 rpm. Samples (1 mL aliquots) were taken at specified time intervals of 15 min-3h from the HCL 0.1N (pH 1.2) release solution and time points of 15 min-30 h from the PBS (pH 7.4) release solution and replaced with that containing fresh solution. The insulin content of the collected samples was determined by the RP-HPLC method. The released insulin content from Mexos was reported as a percentage of cumulative released total insulin, plotted against time. Release experiments were carried out in triplicate.

Insulin transepithelial transport

To study the efficiency of the intestinal epithelial transport of insulin-loaded Mexos, human epithelial Caco-2 cells were grown in Transwell® insert (Costars, Corning Incorporated) with a pore size of 0.4 µm following the previous description [25]. Caco-2 cells were seeded and cultured for 21 days in DMEM high glucose supplemented with 10% FBS, L-glutamine, non-essential amino acids, and penicillin-streptomycin at 37 °C in a 24-well plate at a density of 1.5×10^5 cell per insert. The apical (350 µL) and basolateral (1 mL) media were exchanged every four days. The inserts were visually inspected by daily examination

under optical microscopy to ensure a monolayer formation. The cell monolayers were equilibrated for transport studies with HBSS at 37 °C for 45 min. Then, the apical media was removed and replaced by the 300 µL media, including either 0.20 mg/mL free insulin or Mexolin NPs (containing 0.20 mg/mL insulin). The blank HBSS was used as a negative control. At predefined time intervals (0.5 to 4 h), samples of 50 µL were withdrawn from the basolateral compartments and replaced with the same volume of HBSS. For quantitative determination, after lysis of the transported Mexolin NPs with isopropanol, the insulin content was measured using the RP-HPLC method. The amount of permeated insulin across the Caco-2 monolayer was expressed as a percentage of cumulative total insulin transported versus time. All transport experiments were conducted in triplicate.

In vivo pharmacological studies

Animal procedures and treatments

Healthy male Sprague-Dawley rats (250 ± 20 g) were used for pharmacological response evaluation of prepared exosomal insulin. Animal experimentations followed the ethics committee guidelines of Mashhad University of Medical Sciences for laboratory animal care and use. Rats were given a single dose of 60 mg/kg of STZ (prepared in 100 mM citrate buffer at pH 4.5) via intraperitoneal administration to induce diabetes. STZ-induced diabetic rats were confirmed when their fasting blood glucose levels (BGLs) were > 250 mg/dL. Four groups of diabetic rats (n = 5 per group) were randomly created and fasted overnight before the study. During the experiment, the animals continued fasting for 24 h while having unrestricted access to water. Group 1 received Mexolin NPs orally at 100 IU/kg (rat body weight) of insulin. Group 2, which served as the negative control, received oral insulin solution at a dosage of 100 IU/kg, while group 3, the positive control group, received a subcutaneous insulin injection (5.0 IU/kg). Additionally, group 4 was administered with oral gavage of intact Mexos equivalent to the amount of Mexos in group 1.

Blood glucose and insulin levels

After dosing, blood samples at different intervals (0, 1, 2, 4, 6, and 12 h) were collected from the posterior orbital vein under mild anesthesia. BGLs (mg/dL) were evaluated using an electronic glucose meter. The collected blood was centrifuged at 5000 rpm (10 min/4 °C), and blood serum was stored at -80 °C for subsequent insulin quantification. A commercially available ELISA kit quantified blood insulin levels (µIU/mL). The linear trapezoidal rule was applied to measure the area under the blood concentration-time curve (AUC) for insulin [26]. The relative pharmacological bioavailability of orally administered insulin entrapped in Mexolin NPs in comparison with subcutaneous injection was determined as follows:

Relative pharmacological bioavailability (%) = $AUC_{Oral} \times Dose_{SC} / AUC_{SC} \times Dose_{Oral} \times 100$

where AUC shows the blood insulin area under the concentration-time curve. The pharmacokinetic analysis was based on a non-compartmental model, which is commonly applied in studies involving drug pharmacokinetics [27]. The pharmacokinetic parameters were calculated using GraphPad Prism software.

Statistical analyses

Statistical analyses were carried out using GraphPad Prism statistical software by one-way ANOVA and Tukey tests. All data were obtained from three independent measurements and reported as mean ± standard deviation (SD). Differences associated with $p < 0.05$ were considered statistically significant.

RESULTS

Isolation and characterization of Mexos

In this study, exosomes were isolated from cow milk by ultracentrifugation method. Immunoblotting analysis confirmed the exosomal protein markers CD63, CD81, and TSG101 in the purified exosomal solution (Figure 2). DLS measurement showed that the purified Mexos had a mean hydrodynamic size of 147.9 ± 3.41 nm, with an almost uniform size distribution (polydispersity index, PDI, < 0.4) (Table 1) and a mean surface charge of -3.84 ± 0.17 mV. Consistent with these results, SEM demonstrated 100-200 nm diameter particles with almost spherical morphology (Figure 3a).

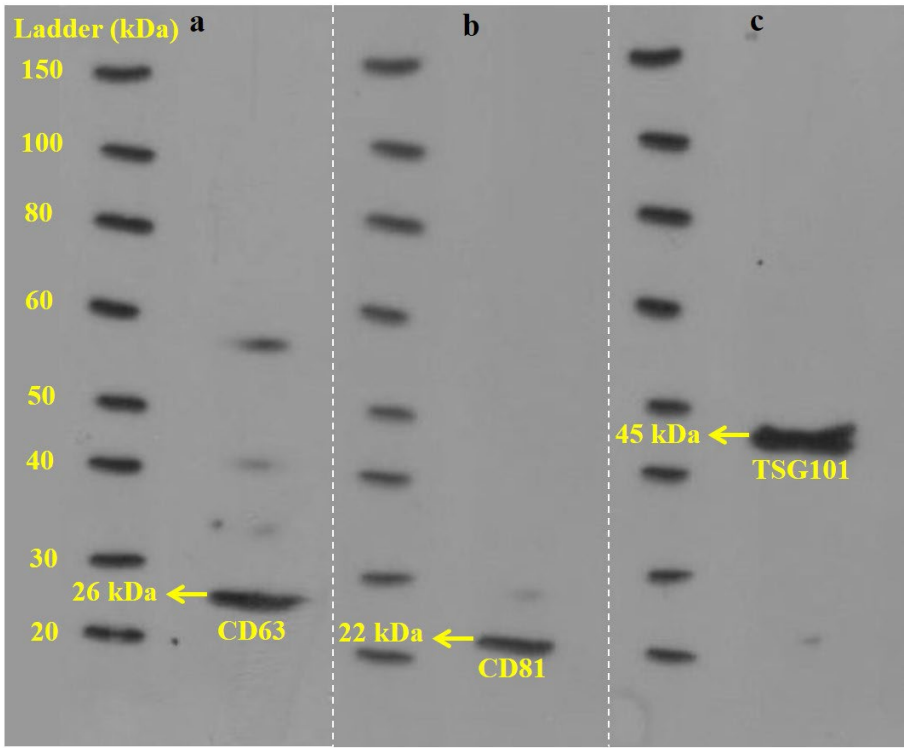

Fig. 2. Mexos characterization using Western blot. Western blot analyzed the total protein associated with purified exosomes isolated from cow milk using common exosome markers. Detection of CD63 (a), CD81 (b), and TSG101 (c) confirmed the exosome preparations. Reproduced from [28] with permission.

Table 1. Size and zeta potential of intact Mexos and insulin-loaded Mexos NPs (Mexolin). The values presented are the mean ± SD, n = 3.

Sample	Size (nm)	Zeta potential (mV)	PDI
Intact Mexos	147.9 ± 3.41	-3.84 ± 0.17	0.36
Mexolin NPs	149.9 ± 4.15	-2.34 ± 0.08	0.47

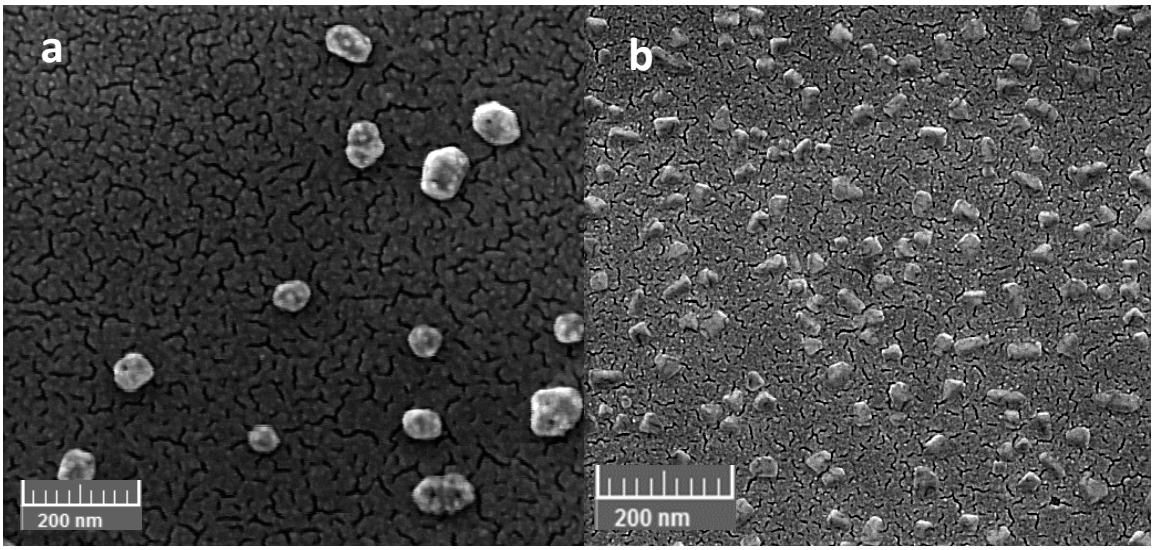

Fig. 3. SEM images of Mexos (a) and Mexolin NPs (b), scale bar 200 nm. SEM investigation shows the spherical morphology of the isolated Mexos before and after insulin loading.

Mexolin NPs preparation and characterization

As a physical approach that mechanical shear force leads to weakened EVs membrane integrity and promotes the loading of cargos, the sonication method [29] was utilized to load insulin into Mexos. The obtained formulation, Mexolin NPs, was assessed in terms of size, morphology, and loading efficiency. Findings showed a slight increase, from 147.9 to 149.9 nm in size, a slight increase in surface charge from -3.84 to -2.34 mV, and a PDI increase from 0.36 to 0.47 following Mexos loading of insulin as measured by DLS (Table 1). SEM analysis (Figure 3b) showed that the Mexos particles maintained their almost spherical shape even after insulin loading. The EE and LC for insulin were 72% ± 3.1 and 46% ± 1.9, respectively.

In vitro insulin release study

In vitro insulin release kinetics of Mexolin NPs was conducted at different time points in either HCL 0.1N (pH 1.2) or PBS (pH 7.4) corresponding to the gastric and intestinal media, respectively. The findings demonstrated that insulin release occurred consistently and was time-dependent, regardless of the pH of the release medium (Figure 4). After 15 minutes, Mexolin NPs showed a cumulative insulin release of 18% in pH 1.2 and 25% in pH 7.4, resembling gastric and intestinal fluids, respectively. After 2 h, the cumulative release percentage of the insulin was about 27% in the gastric fluid (pH 1.2), which subsequently increased to around 32% after 3 h (Figure 4a). When the cumulative release profile was determined in the intestinal fluid (pH 7.4), Mexolin NPs released almost 45% of the insulin in 6 h (Figure 4b).

Insulin transport across Caco-2 cell monolayer

The efficiency of insulin transport was evaluated using a transwell permeability assay with monolayered Caco2 cells. Caco-2 cells have been widely used as a cell model to investigate oral absorption studies [30-32]. Quantitative determination by RP-HPLC revealed time-dependent insulin permeation across the Caco-2 cell monolayer model (Figure 5, a and b). Results indicated that the maximum percentage of insulin transport through the Caco-2 cell monolayer when cells were incubated with free insulin was almost 5% after 4 h of incubation (Figure 5b). On the other hand, the maximum cumulative percentage of transported insulin through cell monolayer when insulin was encapsulated in Mexolin NPs significantly increased ($P < 0.001$) and reached 12.5% after 4 h of incubation (Figure 5b).

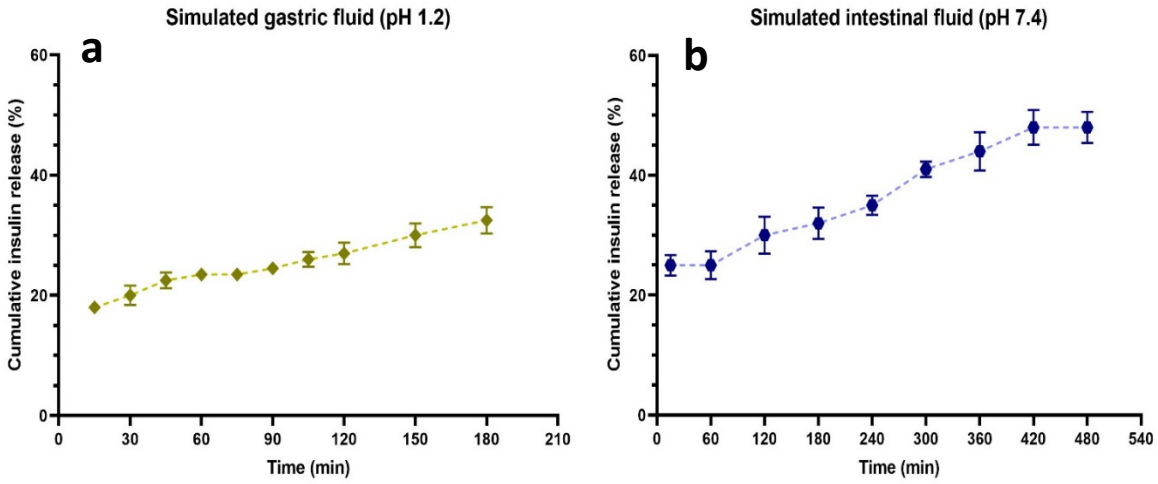

Fig. 4. Cumulative insulin release *versus* time from Mexolin NPs in simulated gastric medium (pH 1.2) (a) and simulated intestinal medium (pH 7.4) (b). The values presented are the mean ± SD, n = 3.

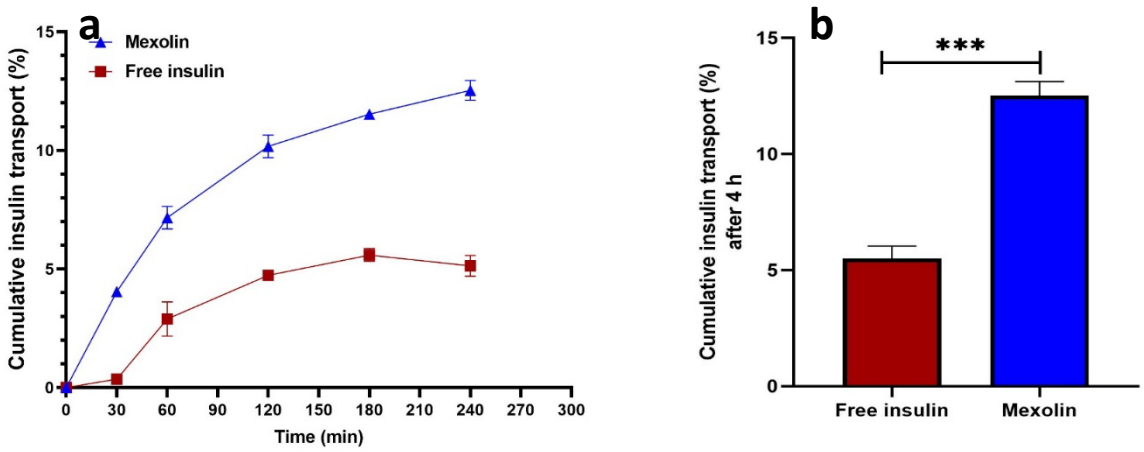

Fig. 5. Cumulative insulin transport (%) across Caco-2 epithelial cell monolayer after incubation with either insulin or Mexolin NPs at 37°C (a) and cumulative insulin transport (%) after 4 h (b). The values presented are the mean ± SD, n = 3. ***P < 0.001

Efficacy of Mexolin NPs in STZ-induced diabetic rats

We assessed the hypoglycemic response and pharmacokinetics parameters following oral administration of Mexolin NPs (100 IU/kg) compared to subcutaneous insulin injection (5 IU/kg), oral free insulin solution (100 IU/kg), and oral intact Mexos in T1DM rats. The blood glucose and serum insulin concentrations were measured using a glucometer and insulin ELISA assay, respectively. As demonstrated in Figure 6a, orally administered free insulin failed to diminish the BGLs. In contrast, subcutaneous insulin injection and oral Mexolin NPs administration elicited a strong hypoglycemic response. The subcutaneous insulin injection caused a sharp decrease in BGLs to about 85 mg/dL after 2 h. The hypoglycemia gradually disappeared, and the BGLs returned to its baseline after 12 h. Compared with the subcutaneous insulin injection, the Mexolin NPs gradually reduced blood glucose to around 106 mg/dL. They led to a prolonged hypoglycemic effect during 6 h, sustaining up to 12 h. In addition, oral Mexos did not alter BGLs in the diabetic rats (Figure 6a).

Figure 6b illustrates the serum insulin profiles over time in diabetic rats treated with various formulations. The maximum serum insulin level of the subcutaneously injected group appeared within 1 h and returned to its minimum value in 6 h. Compared with the subcutaneously injected group, the Mexolin NPs group could maintain a relatively high and sustained serum insulin level for up to 12 h. The pharmacokinetics parameters derived from peripheral serum insulin content are shown in Table 2.

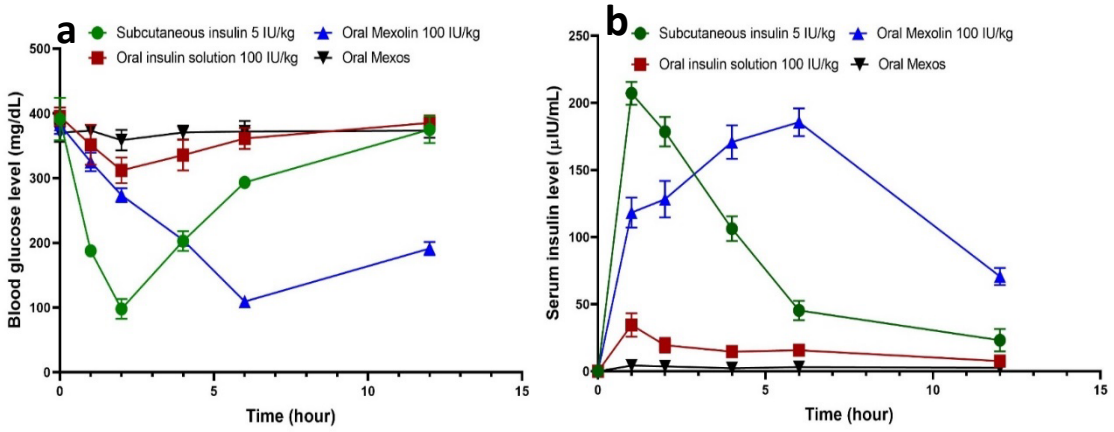

Fig. 6. Time profiles of blood glucose (a) and serum insulin (b) levels in diabetic rats after treating with subcutaneous insulin (5 IU/kg), oral insulin (100 IU/kg), oral Mexolin NPs (100 IU/kg), and oral Mexos at an equivalent dose. The values presented are the mean ± SD, n = 3.

Table 2. Pharmacokinetics parameters for serum insulin level after treatment with different formulations.

Treatments	T_{max} (h)	C_{max} (µIU/mL)	$AUC_{0\text{-}12h}$ (µIU/mL·h)	Bioavailability (%)
Orally administered Mexolin NPs (100 IU/kg)	6	185.61+10.47	1607	8.5
Oral insulin solution (100 IU/kg)	1	34.48+8.69	178.9	0.95
Subcutaneous insulin injection (5 IU/kg)	1	207.12+8.56	938.5	-

DISCUSSION

This study focused on developing a biocompatible milk exosomal oral insulin delivery formulation. Further, we explored whether the oral dosing of insulin encapsulated in this exosomal formulation (Mexolin NPs) could regulate the BGLs of diabetic rats. Mexos were successfully isolated using the differential ultracentrifugation method as the gold standard for EVs isolation and purification [33]. The nanoscale diameter and PDI of Mexos further suggested their utilization as a drug delivery system. Subsequently, insulin was loaded into Mexos by applying mechanical shear force using mild probe ultrasonication to induce transient deformation of the Mexos membrane, thus allowing insulin to enter the Mexos lumen passively. The size and the surface charge of the resulting Mexolin NPS did not alter significantly, indicating that the loading protocol did not change the characteristics of Mexos.

Our initial investigation demonstrated a slow insulin release from Mexolin NPs for 8 h in the medium simulating intestinal conditions compared to the medium simulating stomach conditions. This result suggests that the intestinal environment might promote insulin release to reach the site of action for enhanced therapeutic efficacy. The slight insulin release in the simulated gastric medium might be due to the modest degradation of Mexos under gastric acidity.

Due to their similar structure to liposomes, various hydrophilic and lipophilic cargos could be loaded into exosomes [34]. Previous studies have documented the Mexos versatility for encapsulating and carrying small and large molecules [17, 20]. In an *in vitro* investigation, Mexos loaded with hydrophobic small molecule curcumin preserved the integrity of the compound during gastrointestinal digestion. Additionally, Mexos facilitated the enhanced transportation of curcumin across the polarized Caco-2 monolayer compared to free curcumin [35]. The conventional route of administration for paclitaxel is through intravenous injection. Due to its limited water solubility, the therapeutic dose of paclitaxel cannot be achieved with oral administration. The encapsulation of paclitaxel into Mexos presented a potential solution for addressing these challenges through oral delivery [36]. In another investigation, researchers employed colostrum-derived exosomes to deliver paclitaxel orally. The findings demonstrated that tumor-targeted oral formulation of paclitaxel showed a substantial enhancement in both effectiveness and safety, offering a convenient and cost-efficient alternative to the standard intravenous administration of paclitaxel [37]. The delivery of a single dosage of liraglutide-loaded milk EVs showed significant efficiency in decreasing BGLs in diabetic mice when administered sublingually. Conversely, administering the formulation by oral gavage did not yield the desired therapeutic effect [38].

As insulin is a hydrophilic biomolecule, enhancing its lipophilicity can improve its ability to cross the mucosa barrier [39]. We hypothesized that insulin encapsulation into Mexos could be a potential strategy to facilitate the intestinal epithelial mucus penetration of insulin. We then conducted a transport assay in a differentiated intestinal Caco-2 monolayer model. We identified a notable increase in cumulative transported insulin across the established Caco-2 monolayer while loaded in Mexos compared to negligible transport of free insulin. The *in vitro* Caco-2 epithelial cell monolayer permeability assay findings revealed that Mexolin NPs could efficiently overcome the intestinal epithelial barrier to oral delivery, which typically restricts NPs from entering the blood circulation.

Although the precise mechanisms behind the uptake of Mexos after oral administration are currently not fully understood, a recent study has revealed the potential role of the neonatal Fc receptor (FcRn) in the intestinal absorption of cow Mexos. They proposed that the FcRn receptor, facilitating immunoglobulin G (IgG) transport across various epithelia, including the intestinal epithelium, and contributing to the prolonged serum half-life of IgG in adults [40], may be involved in mediating the uptake of Mexos. More precisely, they suggested that Mexos can be internalized as intact particles through a FcRn mechanism [41]. It is worth noting that the expression of FcRn persists throughout adult life in humans, emphasizing its potential significance [42]. If FcRn facilitates the absorption of Mexos

in the intestines, this finding could explain the notably increased transport of Mexolin NPs in differentiated Caco-2 cell monolayer compared to free insulin [43, 44]. Moreover, it is suggested that endocytosis is a potential intestinal transport of Mexos across human Caco-2 cell monolayer and rat Small Intestinal IEC-6 Cells [45].

While the Caco-2 cell line is the principal cell type for assessing the efficacy of drug transport across the intestinal epithelium, it cannot fully replicate the complex *in vivo* interactions [46]. Presently, no *in vitro* experimental design can precisely mimic the intricate epithelial structure and function, particularly the acidic circumstance found at the surface of epithelial cells [47]. In this study, we decided to test the efficiency of Mexolin NPs for oral insulin delivery in rats *via* oral gavage. Findings of *in vivo* therapeutic assessments revealed that Mexolin NPs can efficiently lower BGLs and elicit a hypoglycemic response in T1DM rats after oral delivery. Results from rats receiving free insulin revealed a lower serum insulin concentration, indicating less effective intestinal uptake. However, rats treated with Mexolin NPs showed a serum insulin level lasting about 10 h, demonstrating both efficient uptake and a beneficial sustained release profile. The observed hypoglycemic response indicates that although a part of the orally administered Mexolin NPs may have been degraded in the stomach's acidic conditions, a considerable amount of them reach the intestine and successfully translocate the insulin into the blood circulation, enhancing the bioavailability of oral insulin. Moreover, animal studies confirmed that the antidiabetic function of insulin did not alter after being loaded into Mexos NPs.

It is acknowledged that our research faced several limitations. The use of PEGylation of Mexolin NPs has the potential to serve as a viable approach for augmenting its durability in gastrointestinal conditions, enhancing its ability to traverse mucus barriers, and prolonging the overall circulation time of the formulation. In addition, creating an *ex vivo* mucus penetration experiment would elucidate the activity of Mexolin NPs and provide further insights for implementing *in vivo* experiments. The intestinal transport of Mexolin NPs could be enhanced through the functionalization of these NPs with targeting ligands.

CONCLUSION

In the present study, we successfully isolated and characterized milk-derived exosomes (Mexos) as promising nanocarriers for oral insulin administration. The outstanding oral biocompatibility, stable availability, and feasibility of large-scale production make cow Mexos highly appealing for oral drug delivery purposes, garnering significant attention in this field. Through a series of experiments, this study has offered new perspectives on the potential of Mexos in enhancing insulin transport through mucus membranes and achieving sustained hypoglycemic effects in diabetic rats. This strategy could also be suitable for administering other protein-based antidiabetic medications and insulin analogs frequently used in clinical practice.

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CONFLICTS OF INTEREST

The authors report no declarations of interest.

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