Liposome-encapsulated glycyrrhizin alleviates hyperglycemia and glycation-induced iron-catalyzed oxidative reactions in streptozotocin-induced diabetic rats

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ABSTRACT
Glycyrrhizin, a bioactive constituent of Glycyrrhiza glabra has been reported to ameliorate diabetes. Here, the effects of liposome-encapsulated glycyrrhizin on STZ-induced diabetes and associated oxidative stress were investigated. Wistar rats were grouped as control (NC, received placebo), diabetic (DC, STZ-induced), diabetic treated with free glycyrrhizin (DTG, 3 i.v. doses, 1.6 mg/0.5 ml), empty liposomes (DTl, 3 i.v. doses), and liposome-encapsulated glycyrrhizin (DTbd, 3 i.v. doses, 1.6 mg/0.5 ml). Serum glucose, insulin, intraperitoneal glucose tolerance test and glycohemoglobin were estimated. Free iron and iron-mediated oxidative stress were examined. Histological examinations of the kidney and liver were performed. Liposomal-glycyrrhizin treatment caused significant improvement of hyperglycemia (DC vs. DTbd < 0.05), glucose intolerance (DC vs. DTG < 0.01 and DC vs. DTbd < 0.05), insulin (DC vs. DTG < 0.1, DTbd vs. DC < 0.05 and DTbd vs. DTG < 0.1) and glycohemoglobin (DC vs. DTG < 0.1 and DC vs. DTbd < 0.05) levels in the DTbd group. Alleviation of free iron release (DC vs. DTbd < 0.05), lipid peroxidation (DC + H₂O₂ vs. DTbd + H₂O₂ < 0.05), deoxyribose (DC + H₂O₂ vs. DTbd + H₂O₂ < 0.05), and DNA degradation occurred in the DTbd group. The abnormalities of the kidney and liver were abolished in the DTbd group. The inhibitory effects were more pronounced compared to free glycyrrhizin. Liposome-encapsulated glycyrrhizin treatment caused inhibition of diabetic complications through its antioxidant effects and can be exploited for effective treatment of diabetes.

1. Introduction
Diabetes mellitus, characterized by hyperglycemia and long-term complications, is the most common endocrine disorder. The kidneys (nephropathy), eyes (retinopathy), heart (cardiomyopathy), nerves (neuropathy), and blood vessels are affected in diabetes. It has been hypothesized that an increase in oxidative stress, as a result of chronic hyperglycemia, activates several signaling pathways that alter gene expression (Brownlee 2001). There are evidences that high glucose in diabetes mellitus activates multiple pathways such as protein kinase C activation, non-enzymatic glycation, augmented polyol, and hexosamine that lead to the increased generation of reactive oxygen species (ROS) (Ishii et al. 1998a, 1998b, Goldberg et al. 2002).

The synthetic drugs used in the treatment of diabetes mellitus have been reported to produce certain side effects and drug resistance. Hence, considerable research has been going on to find appropriate antidiabetic agents from herbal sources. Licorice (Glycyrrhiza glabra L.) has been used for ages in herbal therapy for curing inflammatory responses, bacterial, and viral diseases (Shibata 2000, Lin 2003). Glycyrrhizin is the main water-soluble constituent of licorice root. On hydrolysis, it releases two molecules of d-glucuronic acid and the aglycone, glycyrrhetinic acid. Glycyrrhizin and 18β-glycyrrhetinic acid have been shown to possess several beneficial pharmacological actions, including antiviral activity (Lto et al. 1988), antihepatotoxic activity (Nose et al. 1994), and protection against autoimmune disorders (Horigome et al. 2001). We have previously demonstrated the ameliorative effects of glycyrrhizin and G. glabra alcoholic root extract on STZ-induced diabetes through their antidiabetic, antihyperlipidemic, and antioxidant activities (Sen et al. 2011b, Sen and Singh 2021). A liposome is a spherical vesicle with a membrane composed of phospholipid and cholesterol bilayer. Liposomes can entrap both hydrophilic and hydrophobic agents, which could protect the entrapped agents from external destructive conditions, such as light, pH, and enzymes. The superiority of liposomes as drug carriers has been widely recognized (Torchilin 2005, Shravan Kumar and Torchilin Vladimir 2014, Zhai and Zhai 2014). Their functional advantages are mainly demonstrated through the following aspects: (1) liposomes have good biocompatibility and safety; (2) liposomes enhance targeted drug delivery to the various tissues; (3) appropriately sized drug-carrier liposomes have...
enhanced permeability and retention effects at sites of inflammation where capillary blood vessel permeability is increased, demonstrating the ability of passive targeting; (4) liposomes can carry both hydrophobic and water-soluble drugs; and (5) the liposome surface can be modified and linked to functional groups. As a result of these advantageous characteristics, many liposomal-drugs have been approved. In the present study, liposome-encapsulated glycyrrhizin was used to further investigate its ameliorative effects on STZ-induced diabetes. Although glycyrrhizin has anti-inflammatory, anti-ulcerative, antidiabetic, and hepatoprotective properties, its main side effect, pseudaldosteronism, as well as its significant first pass metabolism should be moderated (Wang et al. 1994, Shibata 2000). Therefore, liposomal application maybe supplemented in order to enhance the uptake of the glycyrrhizin.

2. Materials and methods

2.1. Chemicals

Glycyrrhizin (monoammonium salt), STZ, phosphatidylcholine, cholesterol, Sephadex G-100, ferrozine (monosodium salt), deoxyribose, thiobarbituric acid (TBA), trichloroacetic acid (TCA), agarose, ethidium bromide, pentobarbital, hematoxylin, and eosis were purchased from Sigma Chemical Company (St. Louis, United States). Glucose estimation kit was purchased from Span Diagnostics Ltd (Mumbai, India). Rat insulin ELISA kit and glycohemoglobin kit were purchased from DRG Diagnostic (Frauenbergstr, Germany) and Eagle Diagnostic (Texas, United States), respectively.

2.2. Preparation of liposome-encapsulated glycyrrhizin

Multilamellar liposomes were prepared with phosphatidylcholine, cholesterol, and glycyrrhizin in a molar ratio of 7:2:1 (Gregoridias and Ryman 1972). In brief, phosphatidylcholine, cholesterol, and glycyrrhizin were dissolved in chloroform and methanol mixture (2:1, v/v) in a round-bottomed flask. The lipid film was made by drying the organic solvents and was desiccated overnight. The thin film was swollen in PBS (pH 7.4) for 1 h and sonicated in a probe-type sonicator for 30 s. The quality control data sheet of the G. glabra extract and TLC data of glycyrrhizin are attached as Supplementary materials. The size of the liposome-encapsulated glycyrrhizin was 80–100 nm.

2.3. Maintenance of animals

For animal experiments, prior approval was obtained from the Institutional Animal Ethics Committee, and the experiments were carried out in accordance with internationally accepted norms, monitored by the committee. Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (eighth edition, National Academies Press). Animals were maintained in accordance with the regulations specified and monitored by the institutional animal ethics committee of the University of Calcutta, Kolkata, India (Registration No. 935/c/06/CPCSEA, 30 June 2009). Male Wistar rats, 100–120 g, were maintained at 24–26 °C, 60–80% relative humidity and on a 12-h light-dark cycle. Rats were fed standard rat chow and allowed free access to water.

2.4. Experimental design

The rats were divided into two groups—normoglycemic and diabetic. Here, type 1 diabetes (T1DM) was induced in rats by a single intraperitoneal injection of 60 mg/kg body wt. of STZ dissolved in citrate buffer (0.01 M, pH 4.5). Control rats were given only the buffer. The normoglycemic rats received an equal volume of the buffer. The glucose concentration in serum taken from the tail vein was determined by using the glucose oxidase/peroxidase (GOD/POD) method. About two weeks after the STZ injection, a stable fasting serum glucose level at 200 mg/dl was considered diabetic. Diabetic rats were divided into four groups. T1DM was also confirmed by measuring the glycohemoglobin, plasma insulin levels, and intraperitoneal glucose tolerance test (IPGTT).

A. Diabetic (DC): Diabetic rats were administered with 50 mM phosphate buffer saline, pH 7.4.
B. Diabetic treated with glycyrrhizin (DTG): The treatment duration was one week. Glycyrrhizin, dissolved in PBS, was administered by intravenous injection (1.6 mg in 0.5 ml) in three consecutive doses on the second, fifth, and seventh day of treatment.
C. Diabetic treated with liposome (DTI): Duration of treatment was one week. Empty liposomes (0.5 ml) were administered in the tail vein on the second, fifth, and seventh day of treatment.
D. Diabetic treated with liposome-encapsulated glycyrrhizin (DTbd): Duration of treatment was one week. The liposome-encapsulated glycyrrhizin (0.5 ml suspension containing 2.88 nmol or 1.6 mg) was injected in the tail vein on the second, fifth, and seventh day of treatment.

Each group was composed of five rats.

2.5. Collection of serum, hemoglobin, and tissue samples

Blood was collected from the lateral tail vein with heparin for estimation of glucose. For estimation of serum insulin level, blood samples were drawn from the retro-orbital plexus using heparinized glass capillary tubes after the rat was properly anaesthetized. Serum was separated from blood samples by centrifugation at 1600 g for 15 min at 4 °C. Hemoglobin was isolated and purified from red blood cells by using Sephadex G-100 column chromatography (Bhattacharya et al. 1998) and its concentration was measured from the Soret absorbance using extinction coefficient, ε415nm as 125 mM−1 cm−1 (monomer basis).

After completion of treatment, rats were sacrificed by cervical dislocation for collection of liver and kidney. The organs were dissected out and small pieces of tissues (2–3 mm thick) were collected for histological examination.
2.6. Biochemical estimations

HbA1c and insulin levels in serum were measured using commercially available kits following the manufacturer’s direction.

For the intraperitoneal glucose loading, rats fasted overnight were injected (i.p.) with a sterile solution of 20% glucose at a dose of 2 g/kg b.w. Serum glucose and insulin levels in plasma were measured following the intraperitoneal glucose loading as mentioned above.

Free iron in hemoglobin was measured according to the method of Panter (1994) and was calculated from a standard curve using a standard iron solution.

Lipid peroxidation was measured in presence of hemoglobin samples in the presence or absence of H2O2. Aldehydes are produced when lipid hydroperoxides break down in the biological system. Measurement of these compounds is an index of lipid peroxidation caused by free radical reaction (Gutteridge 1986). The reaction mixture (1 ml) contained hemoglobin (80 μM) and arachidonic acid (160 μM). H2O2 (1 mM) was added as indicated. MDA generated from arachidonic acid was quantitated from TBA reaction. MDA generated from arachidonic acid was quantitated from TBA reaction. Data expressed as μmoles/h. The absorbance was measured at 530 nm, and the value was corrected for endogenous TBA reactive substances present in arachidonic acid. The results were calculated from a standard curve obtained with malondialdehyde (MDA) treated similarly.

Deoxyribose degradation was measured in presence of hemoglobin samples in the presence or absence of H2O2 following the method of Gutteridge (1986). Deoxyribose (0.67 mM) in phosphate buffer, 50 mM, pH 7.4 was incubated for 1 h at 37 °C with hemoglobin (4 μM) and H2O2 (0.67 mM). TBA reactivity was developed by adding 0.5 ml each of TBA (1%) and TCA (2.8%) and then heated for 30 min in a boiling water bath. The resulting chromagen was extracted with n-butanol. The product was estimated through fluorescence emission at 553 nm by exciting at 532 nm.

Hemoglobin-mediated DNA (plasmid) breakdown was estimated essentially following a method described previously (Sen et al. 2005). Different forms of DNA were separated by agarose (1%) gel electrophoresis and visualized by ethidium bromide staining.

2.7. Histological studies

After completion of treatment, rats were sacrificed for the collection of kidney and liver. Kidney tissues were fixed in Bouin’s fluid and 10% formalin, respectively. Then, they were dehydrated in a series of ethanol solutions (70, 80, 90, 100%, v/v). The liver tissues were processed by using routine techniques and embedded in paraffin wax. Then, they were sectioned (5 μm) and stained with Haris hematoxylin-eosin. Stained sections were examined and photographed with a light microscope (Olympus).

2.8. Statistical analysis

Statistical significance of the results (mean ± SEM) was determined using unpaired two-tailed Student’s t-test and Kruskal–Wallis one-way ANOVA using NCSS software followed by Tukey post hoc test. Differences were considered to be statistically significant at p < .05.

3. Results

3.1. Liposome-encapsulated glycyrrhizin caused diminution of hyperglycemia and HbA1c level

Treatments with empty liposome, free glycyrrhizin, and liposome-encapsulated glycyrrhizin were started after the rats were rendered diabetic showing serum glucose level 200 mg/dl. The serum glucose levels obtained before and after administration of STZ, empty liposome, only glycyrrhizin, and liposome-encapsulated glycyrrhizin are presented in Figure 1. Serum glucose level diminished significantly after i.v. administration of the second dose of liposome-encapsulated glycyrrhizin in DTbd rats compared to that of DC, DTl, and DTG groups. The serum glucose level in the DTbd group was maintained at the normal level, thereafter (DC vs. DTbd p < .05). The serum glucose level diminished significantly in the DTG group after administration of the first dose of free glycyrrhizin but a gradual enhancement of the same occurred after injection of second and third doses. The serum glucose level in the empty liposome-treated diabetic group (DTl) did not decrease significantly compared to that of diabetic (DC) even after injection of three consecutive doses. The HbA1c level is an important indicator of diabetic condition, and its level was measured two weeks after completion of the treatment regime. HbA1c was found to be significantly higher in the diabetic (DC) rats than in controls (NC) presented in Table 1. HbA1c was significantly

![Figure 1. Serum glucose levels before (NC) and after injection of STZ (DC), empty liposome DTl, only glycyrrhizin (DTG), and liposome-encapsulated glycyrrhizin in male Wistar rats (DTbd). Diabetes was induced by a single intraperitoneal injection of STZ (60 mg/kg body weight) in 0.01 mM citrate buffer, pH 4.5. Glycyrrhizin, dissolved in PBS, was administered by intravenous injection (1.6 mg in 0.5 ml) in three consecutive doses on the second, fifth, and seventh day of treatment in DTG rats. Empty liposomes (0.5 ml) were administered in the tail vein on the second, fifth, and seventh day of treatment in DTl rats. The liposome-entrapped glycyrrhizin (0.5 ml suspension containing 2.88 nmol or 1.6 mg) was injected in the tail vein on the second, fifth, and seventh day of treatment in DTbd group. Results are mean ± SEM of five experiments in each group (n = 5). For NC vs. DC, p < .01 and DC versus DTl, p < .05. NC: control; DC+: diabetic; DTG#: diabetic treated with glycyrrhizin; DTbd+: diabetic treated with liposomal-glycyrrhizin and DTl: diabetic treated with free liposomes.](image-url)
diminished in the DTbd group compared to that of the DC (Table 1) group \( (p < .1 \text{ for DC vs. DTG and } p < .05 \text{ for DC vs. DTbd}) \). HbA1c in the DTI was not measured as diminution of the glucose level did not occur in this group. Inhibition of HbA1c was found to be lesser pronounced in the diabetic treated with only glycyrrhizin (DTG) compared to that of DTbd \( (p < .1 \text{ for DC vs. DTG and } p < .05 \text{ for DC vs. DTbd}) \). Simultaneously, the corresponding serum glucose level was also estimated in all the groups shown in Table 1.

### 3.2. Liposome-encapsulated glycyrrhizin caused enhancement of plasma insulin and simultaneous non-fasting serum-glucose-lowering following intraperitoneal glucose loading in diabetic rats

Plasma insulin levels were measured after two weeks of liposome-encapsulated glycyrrhizin treatment in the diabetic rats by ELISA method. Insulin level was measured in various groups after 1 h of 20% i.p. glucose injection at a concentration of 2 g/kg b.w. The insulin level significantly increased in liposome-encapsulated glycyrrhizin-treated diabetic (DTbd) rats compared to that of untreated diabetic (DC) presented in Figure 2(a). However, the insulin level did not return to normal in the (DTbd) group. Insulin levels in the DTG rats did not increase significantly compared to that of the diabetic group (DC). A significant difference in serum insulin level between the DTG and DTbd groups was observed (Figure 2(a)) \( (\text{NC vs. DC } p < .05, \text{DC vs. DTG } p < .1, \text{DTG vs. DC } p < .05, \text{and DTbd vs. DTG } p < .1) \). Simultaneously, non-fasting serum glucose level was measured after 1 h of glucose injection presented in Figure 2(b). The non-fasting serum glucose level diminished significantly in liposome-encapsulated glycyrrhizin diabetic (DTbd) rats compared to that of the DC group. In comparison to the DC group, the non-fasting serum glucose level significantly diminished in diabetic rats treated with only glycyrrhizin (DTG) to some extent \( (\text{NC vs. DC } p < .05, \text{DTG vs. DC } p < .05, \text{DC vs. DTG } p < .05, \text{and DTG vs. DTbd } p < .1) \).

### 3.3. Liposome-encapsulated glycyrrhizin diminished the free iron release from hemoglobin in diabetic rats

Table 2 shows that iron release from hemoglobin due to glycation was significantly higher in the DC group in comparison to that of controls (NC). Treatment of diabetic rats with the liposome-encapsulated glycyrrhizin resulted in a significant diminution of the catalytic iron released from hemoglobin samples compared to that of DC, two weeks after administration of its third dose. However, free iron released from the hemoglobin samples of DC and DTG were comparable \( (\text{no significant change}) \) \( (\text{NC vs. DC and DC vs. DTbd } p < .05) \).

### 3.4. Liposome-encapsulated glycyrrhizin caused inhibition of iron-catalyzed free radical-mediated lipid peroxidation and deoxyribose degradation

Table 3 represents the iron-catalyzed free radical-mediated lipid peroxidation in the absence or presence of \( \text{H}_2\text{O}_2 \). Lipid peroxidation was found to be higher in \( \text{DC} + \text{H}_2\text{O}_2 \) samples in comparison to that of \( \text{NC} + \text{H}_2\text{O}_2 \). Treatment of diabetic rats with liposome-encapsulated glycyrrhizin prevented lipid peroxidation and the degree of inhibition was more prominent in DTbd + \( \text{H}_2\text{O}_2 \) samples compared to that of DTG + \( \text{H}_2\text{O}_2 \) \( (\text{NC} + \text{H}_2\text{O}_2 \text{ vs. DC} + \text{H}_2\text{O}_2 \text{ and DC} + \text{H}_2\text{O}_2 \text{ vs. DTbd} + \text{H}_2\text{O}_2 \ p < .05) \). Table 4 represents the iron-catalyzed free radical-mediated deoxyribose degradation in the absence or presence of \( \text{H}_2\text{O}_2 \). TBA reactivity expressed in

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**Table 1.** Blood glucose (BG, mg/dl) and glycohemoglobin (GlycoHb) levels after treatment of diabetic rats with free glycyrrhizin and liposome-encapsulated glycyrrhizin.

| Parameters | NC          | DC                  | DTG                  | DTbd                  |
|------------|-------------|---------------------|----------------------|-----------------------|
| BG (mg/dl) | 110 ± 7.50  | 340 ± 25.00*        | 225 ± 22.50*         | 120 ± 15.00*          |
| GlycoHb conc. (%) | 5.75 ± 0.50 | 15 ± 1.00*          | 10 ± 0.50*           | 5.5 ± 0.50*           |

NC: control; DC*: diabetic; DTG#: diabetic treated with free glycyrrhizin; DTbd+: diabetic treated with liposome-encapsulated glycyrrhizin; BG: blood glucose; GlycoHb: glycohemoglobin.

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**Figure 2.** Plasma insulin level (a) after 20% intraperitoneal glucose injection and (b) non-fasting serum glucose of control (NC) diabetic (DC), diabetic rats treated with glycyrrhizin (DTG), and liposome-encapsulated glycyrrhizin (DTbd). Results are mean ± SEM of five experiments \( (n = 5) \). For plasma insulin, NC versus DC \( p < .05, \text{DC versus DTG } p < .1, \text{DTbd versus DC } p < .05, \text{and DTbd versus DTG } p < .1 \). For non-fasting serum glucose, NC versus DC \( p < .05, \text{DTbd versus DC } p < .05, \text{DC versus DTG } p < .05, \text{and DTG versus DTbd } p < .1 \).
terms of fluorescence arbitrary units was found to be significantly higher in DC samples compared to that of controls (NC). Deoxyribose degradation was found to be higher in DC + H₂O₂ samples in comparison to that of NC + H₂O₂.

Treatment of diabetic rats with free glycyrrhizin or liposome-encapsulated glycyrrhizin prevented deoxyribose degradation though the degree of inhibition was more prominent in DTbd + H₂O₂ samples compared to that of DTG + H₂O₂ (NC + H₂O₂ vs. DC + H₂O₂ and DC + H₂O₂ vs. DTbd + H₂O₂ p < .05).

3.5. Effect of liposome-encapsulated glycyrrhizin on DNA (plasmid) degradation

DNA (plasmid, 3.7 Kb) degradation in presence of hemoglobin samples isolated from NC, DC, DTG, DTl, and DTbd groups was performed. DNA was incubated with the hemoglobin samples and subjected to agarose gel electrophoresis followed by ethidium bromide staining. The result of a representative experiment is shown in Figure 3. DNA degradation was evident, with the conversion of form I (covalently closed circular DNA) to form II (open circular with a single nick) and form III (open circular with double strand nick) as compared between control hemoglobin-mediated reaction (lane 3) and diabetic hemoglobin-mediated reaction (lane 4). However, DNA breakdown was also found in the control hemoglobin-mediated reaction (lane 3), although the intensity of form II was lesser than that of lane 2 (DNA + H₂O₂). Treatment with liposome-encapsulated glycyrrhizin prevented the degradation process (lane 7). Free glycyrrhizin administration did not prevent DNA degradation as indicated by the presence of form III (lane 6).

3.6. Liposome-encapsulated glycyrrhizin prevented the histological abnormalities of the kidney and liver

Histopathological examination of the kidney revealed diffused mesangium of glomeruli in DC rats and thicker basal membrane in comparison to controls (Figure 4(a,b)). Diffused mesangium and basal membrane thickening were also found in DTG kidney sections although lesser compared to that of DC (Figure 4(b,c)). The kidney sections of diabetic rats treated with liposome-encapsulated glycyrrhizin (DTbd) showed considerable prevention of these abnormalities compared to those of DC and DTG (Figure 4(d)). Liver sections of the DC rats exhibited nuclear chromatin with coarse granules, a nucleus with the diffused nucleolus, weak staining of
the cytoplasm, and fibrosis (Figure 5(a,b)). However, these abnormalities were prominently milder in diabetic treated with only glycyrrhizin (DTG) and to a greater extent when liposome-entrapped glycyrrhizin (DTbd) was used shown in Figure 5(c,d).

4. Discussion

In the present study, we demonstrated the ameliorative effects of liposome-encapsulated glycyrrhizin on STZ-induced diabetic rats. Elevated serum glucose in diabetes causes

Figure 4. Histopathological analysis of kidney sections showing prevention of diffused mesangium and basement thickening in (d) DTbd compared to those of free glycyrrhizin treated (c) and diabetic (DC). Magnification × 10. Yellow arrows indicate diffused mesangium and the black ones indicate membrane thickening.

Figure 5. Histopathological examination of the hematoxylin-eosin stained liver sections showing nucleus with diffused nucleolus in diabetic rats (b) compared to controls (NC). Liposome-encapsulated (d) treated resulted in milder abnormalities in comparison to the DC rats (b). Magnifications: × 10 and × 40 (for DTG and DTbd groups). Black arrows indicate a nucleus with the nucleolus.
various complications. Therefore, the primary objective is to maintain the serum glucose concentration within the normal range. Liposomes are accepted as potent drug carriers not only because of their biochemical nature but also those phospholipid vesicles do not elicit a negative biological response that generally occurs when a foreign material is introduced within the physiological system. Moreover, these lipid vesicles are non-toxic, non-immunogenic, non-thrombogenic, and biodegradable. In the present study, the antihyperglycemic effects of liposome-encapsulated glycyrrhizin in STZ-induced diabetes are evident from the reduction of serum glucose in the DTbd group (DC vs. DTbd \( p < .05 \)).

Liposome vesicle size was always \( \leq 100 \) nm: and the incorporation of raw glycyrrhizin led to a decrease in the mean diameter. Glycyrrhizin is a single molecule with a good solubility in water. The stability of the formulation represents an important issue, which becomes essential when developing an industrial product. We (Sen et al. 2011b, Sen and Singh, 2021) and others (Takii et al. 2001; Ao et al. 2008) have previously demonstrated the glucose-lowering effects of free glycyrrhizin and \( G. \ glabra \) root extract in diabetes. The glucose-lowering effect of liposome-encapsulated glycyrrhizin in diabetic rats further supports the antidiabetic potential of glycyrrhizin (DC vs. DTbd \( p < .05 \)). It also indicates the sustained delivery of the aforementioned liposome preparation that gradually causes inhibition of the high glucose level in diabetes. Diminution of HbA1c level in the diabetic treated with liposome-encapsulated glycyrrhizin further supports the anti-hyperglycemic potential of this liposome preparation (\( p < .1 \) for DC vs. DTG and \( p < .05 \) for DC vs. DTbd).

Here, we have studied several parameters associated with hyperglycemia to understand further the actions of the liposome-encapsulated glycyrrhizin. Plasma insulin was increased significantly after treatment with the aforementioned liposome preparation following intraperitoneal glucose injection in comparison to the STZ-induced and diabetic rats treated with only glycyrrhizin groups (NC vs. DC \( p < .05 \), DC vs. DTG \( p < .1 \), DTbd vs. DC \( p < .05 \), and DTbd vs. DTG \( p < .1 \)). Simultaneously, more effective diminution of the non-fasting serum glucose level in the liposome-encapsulated glycyrrhizin-treated diabetic group in comparison to DC and DTG further substantiates its higher antihyperglycemic potential than free glycyrrhizin (NC vs. DC \( p < .05 \), DTbd vs. DC \( p < .05 \), DC vs. DTG \( p < .05 \), and DTG vs. DTbd \( p < .1 \)). We have already reported that free glycyrrhizin (100 mg/kg b.w.) and \( G. \ glabra \) root extract (60 mg/kg b.w) treatment of diabetic rats can cause an increment of plasma insulin and the number of islet cells (including \( \beta \)-cells) of the pancreas (Sen et al. 2011b, Sen and Singh 2021) in STZ-diabetes. It has been reported by another group that glycyrrhizin caused reduction of inflammation in diabetic kidney disease through inhibition of high mobility group box 1 (HMGB1) protein a key facilitator of renal inflammation, Toll-like receptor 4 (TLR4) and NFXb in glucose-induced Normal rat kidney (NRK-52E) cells and Zucker diabetic fatty (ZDF) rat (Thakur et al. 2017).

Recently the same group also reported about the cardioprotective effects of glycyrrhizin on myocardial remodeling in ZFD rat mediated through activation of Nrf2 and inhibition of CXCR4/SDF1 as well as TGF-\( \beta \)/p38MAPK signaling pathway (Thakur et al. 2021). The protective effects of glycyrrhizin on diabetic retina against permeability, neuronal, and vascular damage through anti-inflammatory mechanisms were reported by Liu et al (2019).

The main cause of STZ-induced diabetes is the alkylation of DNA by the nitrosourea moiety of this compound. This causes DNA strand breaks followed by nuclear poly (ADP-ribose) synthetase stimulation, depletion of intracellular NAD\(^+\), and inhibition of proinsulin synthesis (Yamamoto et al. 1981). Diminution of functional \( \beta \)-cells associated with lowered insulin secretion thus leads to hyperglycemia in STZ-diabetes. The possible mode of action of glycyrrhizin may be regeneration or sensitization of pancreatic \( \beta \)-cells that elevates insulin and thereby rectifying the hyperglycemic condition. In the present study, histopathological examination of the pancreas was not done, and instead, we measured the non-fasting serum glucose level.

Free radicals and oxidative stress have been implicated in eliciting diabetic complications. Hyperglycemia, protein glycation, and glucose auto-oxidation have been suggested to induce free radical generation in diabetes (Adachi et al. 1991, Wolff et al. 1991, King and Loken 2004). Previous in vitro and in vivo studies have suggested that glycation of hemoglobin and myoglobin may be a source of catalytic iron and increased free radicals in the diabetic condition (Kar and Chakraborti 1999, Roy et al. 2004, Sen et al. 2005). Here, the free iron level was found to be increased in hemoglobin samples of diabetic rats having a higher glycohemoglobin level. \( H_2O_2 \) generation has been reported to increase in induced diabetes (Takasu et al. 1991). Moreover, \( H_2O_2 \) releases iron from hemoglobin (Gutteridge 1986) and induces more iron release from glycated heme proteins (Kar and Chakraborti 1999, Roy et al. 2004). In liposome-encapsulated glycyrrhizin-treated diabetic rats (DTbd), the free iron level was found to be diminished compared to the diabetic (DC) and diabetic treated glycyrrhizin (DTG) groups (NC vs. DC and DC vs. DTbd \( p < .05 \)). This inhibition of free iron level may be associated with the glucose and HBA1c lowering effects of this liposome preparation.

The iron-catalyzed free radical-mediated lipid peroxidation and deoxyribose degradation in presence of hemoglobin collected from all the groups were performed. Here, lipid peroxidation was found to be higher in DC + \( H_2O_2 \) samples in comparison to that of NC + \( H_2O_2 \). Treatment of diabetic rats with liposome-encapsulated glycyrrhizin prevented lipid peroxidation and the degree of inhibition was more prominent in DTbd + \( H_2O_2 \) samples compared to that of DTG + \( H_2O_2 \). TBA reactivity (in arbitrary fluorescence emission intensity) of the diabetic hemoglobin in presence of \( H_2O_2 \) was found to be higher which indicates that OH\(^-\) radical can specifically attack the pentose sugar 2-deoxy-D-ribose moiety. Iron liberated from the heme of glycated hemoglobin of the diabetic rats by \( H_2O_2 \) acts as a Fenton reagent and form OH\(^-\) radicals, which in turn causes the breakdown of deoxyribose sugar.

The liposome-encapsulated glycyrrhizin by diminishing hyperglycemia and HBA1c levels in diabetic rats causes inhibition of free iron release from heme of glycated hemoglobin.
that may, in turn, prevent the deoxyribose breakdown (NC + H₂O₂ vs. DC + H₂O₂ and DC + H₂O₂ vs. DTbid + H₂O₂ p < .05). DNA (plasmid) degradation is more pronounced by the hemoglobin of diabetic rats than that of controls. Glycated heme proteins auto-oxidize more rapidly than non-glycated proteins (Roy et al. 2004) thus generating more superoxide radicals (O₂•⁻), which may react with water to form H₂O₂. Hemoglobin of diabetic rats containing a higher level of HbA1c may therefore be a source of increased formation of O₂•⁻ and H₂O₂. Free iron may then act as a source of OH radicals through the Fenton reaction: Fe²⁺ + H₂O₂ → Fe³⁺ + OH⁻ + OH⁻ (Halliwell and Gutteridge 1990). Hemoglobin samples from diabetic rats may thus cause enhanced oxidative reactions, as shown by lipid peroxidation, deoxyribose, and DNA degradation assays. We also reported about the increased formation of 8-oxo-2′-deoxyguanosine (8-OHdG), intracellular ROS (fluorescence spectroscopy by dichlorodihydrofluorescein diacetate) and NF-κB p65 in high glucose (25 mM) induced human umbilical vein endothelial cells that was inhibited by the North American ginseng extract treatment (Sen et al. 2011a). High glucose also causes increased production of NO and O₂•⁻, molecules that react to produce peroxynitrite, a potent, long-living oxidant (Beckman and Koppenol 1996). Peroxynitrite can also inhibit electron transport at the mitochondrial level; oxidize sulfhydryl groups in proteins, initiates lipid peroxidation affecting many signal transduction pathways (Ischiropoulos 1998). It is a potent generator of DNA strand breaks (Dandonia et al. 1996). The oxidative stress due to glucose toxicity in turn causes activation of various transcription factors and leading to increased vasoactive factor and ECM protein production. In the present study, liposome-encapsulated glycyrrhizin treatment is much more effective in reversing the aforementioned oxidative reactions and DNA damage in comparison to free glycyrrhizin, which may be associated with lower levels of HbA1c and free iron in the hemoglobin samples of these treated rats. In addition, the liposome-encapsulated glycyrrhizin-treatment may have modified the H-donor ability and ameliorated the oxidized sulfhydryl concentration in the diabetic rats. Overall, the impaired redox balance caused by the oxidant stress in T1DM rats was ameliorated by this liposomal formulation more effectively than the free drug. Liposome-mediated treatment of various polyphenolic compounds derived from plant origin such as a bacopasaponin (Sinha et al. 2002) and quercetin (Mandal and Das 2005) cause prevention of leishmaniasis and CCI4-induced hepatotoxicity in vivo models have been reported. In addition, the galactosylated liposome-encapsulated quercetin was found to be beneficial in diminishing the oxidative stress in the CCI4-induced rat model (Mandal and Das 2005). Glycyrrhizin is also a triterpene saponin and its liposome-encapsulated preparation may similarly exert antioxidant effects as evident from the present findings.

Hyperglycemia induces the accumulation of proteins in the mesangial basal membrane of the kidney and by free radicals derived from auto-oxidation of glucose or advanced glycation end products (Trachtman et al. 1995). The liposome-encapsulated glycyrrhizin reduces diabetes-induced abnormalities in a more pronounced manner than free glycyrrhizin. The alleviation of membrane thickening in the kidneys of diabetic rats by the aforementioned liposome preparation suggests its preventive effects in diabetic nephropathy. It has been also indicated that anti-alpha8 integrin-modified immunoliposomes for codelivery of emodin and diammonium glycyrrhizinate have great potential for targeting the kidneys for the treatment of renal fibrosis (Sun et al. 2020). Ameliorative effects of glutathione-liposomes on the oxidative stress, aldose reductase pathway, and sorbitol accumulation in diabetic nephropathy were reported by Shen and Wang (2021). However, semiquantitative analysis of the effect of liposome-encapsulated glycyrrhizin on diabetic nephropathy is required.

Hepatocytes of the diabetic rats exhibited nuclear chromatin with coarse granules, a nucleus with diffused nucleolus, and weak staining of the cytoplasm. These abnormalities were also observed in the liver sections of the diabetic rats treated with free glycyrrhizin although to a lesser extent. Furthermore, the abnormalities were prominently milder in diabetic rats treated with liposome-encapsulated glycyrrhizin. Previous studies also indicate the protective role of glycyrrhizin against CCI4-induced hepatotoxicity (Lee et al. 2007) and galactosamine-induced cytotoxicity in altered hepatocytes (Abe et al. 2008). These hepatoprotective and antioxidant functions of glycyrrhizin are found to be more pronounced by using the liposome-encapsulated glycyrrhizin conjugate. The affinity of glycyrrhizin for the receptors in the liver was demonstrated by Tsuji et al. (1991). Stecanella et al. (2021) reported about the glycyrrhizin and its hydrolyzed metabolite 18β-glycyrrhetinic acid as specific ligands for targeting nanosystems in the treatment of liver cancer. Glycyrrhizin/metformin loaded nanoparticles using the biocompatible polymers chitosan and gum arabic have been proved to possess strong antidiabetic activity in in nicotinamide-streptozotocin-induced diabetic rats (model of T2DM) (Rani et al. 2017). In another study, the same group reported about the enhanced antidiabetic activity of the combinational polymeric nanoformula containing glycyrrhizic acid and thymoquinone in STZ-induced type 1 diabetic rats evidenced by lowering of serum glucose, HbA1c and significant improvements of the body weight and lipid profile (Rani et al. 2019). It was demonstrated that licorice extract loaded in liposome vesicles scavenged DPPH free radical and protected 3T3 fibroblasts against H₂O₂-induced oxidative stress. In addition, the licorice-liposomal formulation promoted the proliferation and migration of 3T3 fibroblasts, favoring the closure of the scratched area. In vivo anti-inflammatory tests on mice confirmed the ability of the proposed liposomal formulation in improving the local efficacy of the extract, favoring the re-epitelization process (Castangia et al. 2015). In another study, the hepatoprotective effect of liposomal-glycyrrhizin formulation in alcoholic liver injury was reported through its antioxidant and transmethylation activities (Kleiner et al. 2016). The main side effect of glycyrrhizin, the pseudoadosteronism was also ameliorated. Wu et al. (2014) reported about the
induced significant effects with a daily dose of 200 mg/kg ad libitum, and the bioavailability of glycyrrhizin was 4%.

5. Conclusion

In the present study, hyperglycemia leads to glycation of hemoglobin that in turn causes enhanced iron release from hemoglobin and iron-catalyzed oxidative reactions, namely, lipid peroxidation, deoxyribose degradation and DNA breakdown associated with histopathological abnormalities of the kidney and liver in diabetic rats. The liposome-encapsulated glycyrrhizin caused significant alleviation of hyperglycemia reflected by lowering of the HBA1c levels, enhancement of serum insulin and normalization of IPGTT in the diabetic group. The free iron level, hemoglobin glycation-induced iron-catalyzed oxidative reactions and histopathological abnormalities of the kidney and liver are abolished by the liposome-encapsulated glycyrrhizin treatment in the diabetic rats. The ameliorative effects of the liposome-glycyrrhizin formulation are more pronounced than free glycyrrhizin; and additionally, the side effects of free glycyrrhizin, namely, pseudoaldosteroneism, are abrogated.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

All data are incorporated into the article and its online supplementary material.

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