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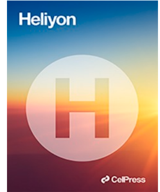

Research article

Oral administration of proniosomal glibenclamide formulation protects testicular tissue from hyperglycemia fluctuations and ROS via *Nrf2/HO-1* pathway

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A R T I C L E I N F O

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Sperms
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A B S T R A C T

Type 2 diabetes causes high blood sugar due to insulin malfunction and is linked to male infertility. Using proniosomes can enhance the effectiveness of Glibenclamide, a medication that stimulates insulin secretion. In our study, male rats with diabetes were treated with GLB with or without proniosomal for 14 days. Proniosomal formulations maintained glucose levels prevented weight loss and showed normal testicular tissue.

GLB-proniosomal reduces ROS caused by T2DM through Nrf2, HO-1 pathway and increases CAT, SOD, and GSH production in response to insulin and glucose uptake. The reference and proniosomal treatments showed CAT and SOD significant enzymatic elevation compared to the positive and negative control. CAT significantly correlated with Gpx4 expression with $P = 0.0169$ and $r = 0.98$; similarly, the enzymatic activity of SOD also showed a positive correlation between the average glucose levels ($r = 0.99$ and $P = 0.0037$). Intestinally, GSH analysis revealed that only proniosomal-GLB samples are significantly elevated from the positive control, with a *P* value of 0.0210. The data showed proniosomal-GLB was more effective than pure GLB, confirmed by higher Nrf2 (2.050 folds), HO-1 (2.148 folds), and GPx4 (1.9 folds) transcript levels relative to the control with less sample diversity compared to the reference samples, indicating proniosomal stabilized GLB in the blood.

Administering GLB and proniosomes formulation has effectively restored testicular function and sperm production in diabetic rats by regulating ROS levels and upregulating anti-ROS in response to glucose uptake. These findings may lead to better treatments for diabetic patients who have infertility issues.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by inadequate insulin production or cellular insulin

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resistance. Hyperglycaemia leads to sugar accumulation in the blood and a relative lack of insulin production, which usually affects middle-aged individuals. The prevalence of T2DM among Saudi Arabian adults has been estimated to be between 15.8 % and 18.2 % from 2016 to 2021, and there are indications that this number is increasing [1].

The risk of developing T2DM depends on various factors, including genetic and lifestyle factors. Lifestyle factors like obesity, inactivity, malnutrition, and stress contribute to an increased risk of T2DM, which can be particularly concerning for individuals of reproductive age. A study focused on Saudi military personnel's lifestyle and obesity and identified a pattern: unhealthy meals, smoking, and low physical activity. The survey also shows that individuals have a family history of T2DM and hypertension significantly correlated with high body mass index (BMI) or obesity [2]. Since then, numerous investigations using demographic features, educational levels, age, and family history with T2DM have shown a high prevalence of obesity among adults and children in Saudi Arabia [3–6].

The World Health Organization (WHO) defines obesity as ''abnormal or excessive fat accumulation that poses a health risk,''. It is also recognized as a chronic relapsing disease that began to rise in the second half of the 20th century. It is estimated that 800 million people worldwide are obese, with rates increasing significantly in the Gulf region, such as Kuwait, the United Emirates, and Saudi Arabia [7,8]. T2DM can negatively impact the male reproductive system, leading to long-term harm and malfunction [9]. Studies have found a strong correlation between T2DM and male infertility, which is caused by abnormalities in the body's oxidant/antioxidant state. This imbalance leads to the formation of oxidative stress, which affects how the testis functions and can result in sexual dysfunction [9–11].

Reactive oxygen species (ROS) are produced in high amounts due to increased glucose auto-oxidation in diabetes. ROS is one of the main causes of diabetes-related issues, such as germ cell death, which negatively affects spermatogenesis and reproductive potential [12]. Despite this, ROS is also a result of important physiological processes in the male reproduction system, including sperm maturation, differentiation, capacitation, acrosome reactions, sperm/oocyte fusion, and regulation of the spermatogenic lineage buffer system. Additionally, ROS reduces mitochondrial sperm energy production, reduces motility, and ultimately induces apoptosis by lowering Adenosine triphosphate (ATP) levels and heat-shock proteins [12].

The antioxidant system protects the testicular tissue from dysfunction, which scavenges excess ROS under normal conditions. The reproductive system requires ROS for reproduction, but uncontrolled generation can pose a risk. The body stimulates antioxidative systems when needed through the transcriptional factor Nrf2. Nuclear factor erythroid 2 related factors 2 (Nrf2) stimulates antioxidant enzymes via Adenylate-uridylate-rich elements (ARE) [13,14]. Nrf2 is a significant protein that helps defend our body against oxidative stress by activating genes to protect against ROS; this regulates cellular resistance to oxidative stress in the testis and affects spermatogenesis. Proper Nrf2 expression is necessary for adequate spermatogenesis and sperm-specific processes, including motility [15–17].

During spermatogenesis, the developing sperm cells are vulnerable to oxidative damage. This damage can be prevented by antioxidant enzymes activated by the Nrf2 transcription factor. This system relies on essential enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and glutathione peroxidase 4 (GPX4) to protect developing sperm cells from oxidative damage and maintain normal sperm function [13].

Oxidation stress due to T2DM has shown several effects on male reproduction that may lead to infertility, so managing diabetes can positively impact sperm health and reduce the risk of excessive ROS. Glibenclamide (GLB) is a medication widely used for treating T2DM and was introduced in 1957. Its mechanism of action involves inhibiting the ATP-sensitive potassium channel (KATP) in the pancreatic beta cells. This helps regulate blood sugar levels by triggering the influx of calcium ions (Ca^{2+}) and promoting insulin release. Consequently, this increases the intracellular calcium influx and insulin secretion, raising blood insulin levels while decreasing glucose levels [18]. Moreover, recent studies have demonstrated other properties of the drug, including anti-oxidative, antinociceptive, anticancer, and platelet aggregation inhibitor properties [19–21]. Despite its beneficial properties, the treatment's effectiveness is reduced by negative aspects such as low water solubility, which leads to destabilization and a short half-life [22].

Liposomal delivery systems, such as transferosomes, niosomes, and ethosomes, are nanocarriers that have gained significant attention due to their ability to enhance oral drug bioavailability and improve transdermal permeation. Proniosomes, which are vesicular drug delivery systems, consist of a bilayer of a non-ionic surfactant, with a hydrophilic end oriented outward and a hydrophobic end that remains in the core. Importantly, proniosomes are physically more stable than niosomes, providing a reassuring level of robustness that can withstand storage and transportation [22]. In our recent publication in 2023, we developed a novel proniosomal delivery system that is chemically and physically stable during storage, and the dissolution rate was 74.456 %, significantly higher than the pure drug [22]. A novel proniosomal delivery system for carrying the nanosize GLB will enhance the pharmacological efficacy of the drug by ensuring a constant level at optimum concentration during the day [22]. We hypothesize that utilizing proniosomal as drug carriers for GLB will protect the testicular tissue from ROS stress generated from blood glucose fluctuation and improve spermatogenesis in individuals with T2MD by tracking the anti-ROS pathway via the Nrf2 pathway.

2. Materials and methods

2.1. Animal model

Male *Sprague Dawley* rats with an average weight of 260 g and ten weeks old were used for this investigation. The rats were housed in a cage with a temperature (of 23 ± 1 °C), provided a standard rat chow diet and water *ad libitum*, and subjected to a natural 12:12 h (hr) light/dark cycle for a week before starting the experiment. All animals were treated according to our instructional animal care guidelines by the Research Ethics Committee of the Animal Care Centre at King Saud University, Riyadh, Saudi Arabia (Approval #

KSU-SE-22-34).

2.2. Induction of type 2 diabetes

Experimental diabetes rats were induced by a single intraperitoneal injection (i.p) of Streptozotocin (STZ, Cas. 18883-66-4, Merck) freshly prepared in citrate buffer (0.1 M, pH 4.5) at a dose of 55 mg/kg body weight. The induction of diabetes was confirmed after 72 h by evaluating the blood glucose level in a drop of blood from the tail vein of the rats using the MEDISAFE MINI Blood Glucose Reader. Eventually, rats with fasting blood glucose concentration >200 mg/dl were considered diabetic and included in this investigation study, as referred to by Ibrahim et al., 2019 [23].

2.3. The GLB-proniosomal formulation

In a study conducted by Alshora et al., in 2023, the pharmacological activity and characterization of GLB proniosomal formulation were described and performed in this study. The slurry method produced a free-flowing powder with a particle size of 190.050 ± 43.204 to 1369.333 ± 150.407 nm, and the zeta potential was above 20 mV (−24 to −58 mV). This formulation greatly increased the drug dissolution rate compared to the pure drug. In *vitro* and in *vivo* investigations using GLB proniosomes also showed a significant reduction in fasting blood glucose levels after treatment with the pure drug. Furthermore, GLB proniosomal showed no sign of toxicity or histopathology changes after daily treatment (gavage) for 14 days. The effective dosage was determined to be 5 mg/kg pure GLB and 137.6 mg/kg GLB proniosomal, equivalent to human dosages at 5 mg [22].

2.4. Experimental design

Twenty healthy adult male *Sprague Dawley* rats were randomly allocated into four groups of 5 rats (Fig. 1); each was orally treated for two weeks as follows:

Group I (Control): Normal rats administrated 0.5 % w/v of carboxymethyl cellulose (CMC).

Group II (Diabetic control): Diabetic rats that received STZ given i.p.

Group III (GLB pure drug): Diabetic rats treated with reference drug, Glibenclamide (GLB), at 5 mg/kg.

Group IV (GLB-proniosomes): Diabetic rats treated with GLB-proniosomal formulations at 137.6 mg/kg.

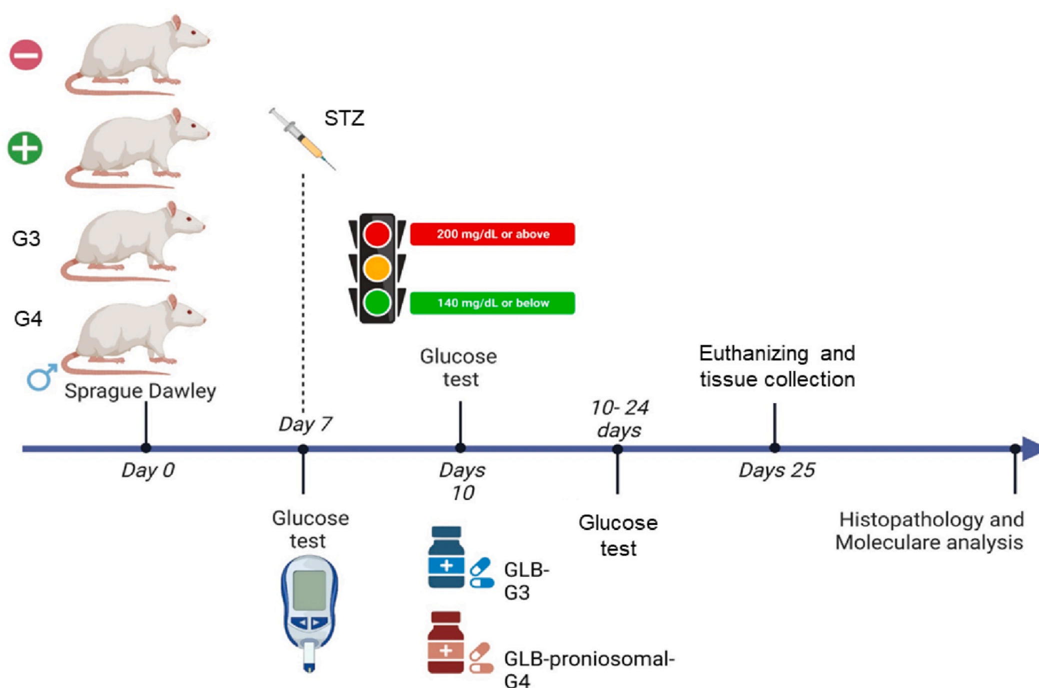

Fig. 1. *Experimental design time of induction, illustrating sugar level and euthanizing.* After seven days' glucose levels were taken after STZ-induction (Initial) and two weeks of treatments (End). The negative control illustrated the normal glucose levels in the blood, which is 140 mg/dl and below, and 200 mg/dl and above are diabetic rats for positive, G3, and G3 groups (n = 5). Rats were anesthetized using a ketamine/xylazine mixture at the end of the experiment (End).

2.5. Euthanizing of experimental animals

At the end of the experimental study, we used a ketamine/xylazine mixture of 9.1/91 mg/kg injected i.p to anesthetize the rats and then collect the blood by direct heart puncture. Immediately, a portion of the testis was carefully removed and placed in 10 % neutral buffered formalin (NBF) for histological examination. Another piece was washed with cold phosphate-buffered saline (PBS) three times and stored at −80 °C until Ribonucleic acid (RNA) extraction was performed or to detect enzymatic activity.

2.6. Histopathological examination

The fixed tissues (testis) underwent dehydration in ascending concentrations of alcohol, cleared by xylene before embedding into paraffin wax for infiltration. Finally, the tissue blocks were cut using microtome to produce six μm sections fixed on the slides and stained using hematoxylin and eosin according to Mayer's modified staining method [24,25]. The slides were observed under a light microscope, and the images were captured using a digital camera.

2.7. RNA extraction and cDNA synthesis

Total RNA was extracted from 30 mg of testicular tissue using TRIzol™ Reagent (Cat no. 15596026, Thermo Fisher) according to the manufacturer's instructions. The purity and concentration of total RNA were assessed using NanoDrop (NanoDrop™ 8000, Thermo Fisher Scientific) at a 260/280 nm wavelength. For the complementary Deoxyribonucleic acid DNA (cDNA) synthesis, the total RNA was reverse-transcribed using a cDNA synthesis kit (Cat no.5001, GoScript™ Reverse Transcriptase) according to the manufacturer's protocol.

2.8. Real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

The synthesized cDNA was subjected to RT-qPCR in a 12 μl reaction mixture containing Eva Green PCR master mix (Cat no. 08-24-00001, Solis Biodyne), ten pmol/μl forward. It reversed primer (Table 1), cDNA (25 ng) double distilled water up to 12 μl. The mean of duplicate target PCR threshold cycle (CT) values was normalized by subtracting the mean value of the DNA-directed RNA polymerase II subunit (*Polr2a*) gene as a housekeeping gene to quantify the expression of the target genes: nuclear factor erythroid 2-related factor 2 (*Nrf2*), Heme oxygenase-1 (*HO-1*), and glutathione peroxidase 4 (*GPx4*). The relative expression fold change of interesting genes was calculated by the $2^{-\Delta\Delta Ct}$ equation [26].

2.9. Enzyme activity detection

Using the frozen portion of the testis and following the kit protocol from Cayman Chemical Company, Inc for detecting the following enzymes; superoxide dismutase 1 (SOD) (Cat no. 706002), Catalase (Cat no. 707002) and Glutathione (Cat no. 703002).

2.10. Statistical analysis

All statistical data were executed using One-way ANOVA (and posttest) or unpaired t-tests with GraphPad Prism software. The values were expressed as the mean ± standard error (SEM). The differences were considered statistically significant if the *P* value was ≤0.05.

Table 1
RT-qPCR targeted genes, primer sequences, and product size in base pairs (bp).

Gene	Primers	Product size (base pair)	References
Nrf2	**Forward** 5′-GCTGCCATTAGTCAGTCGCTCTC-3′ **Reverse** 5′-ACCGTGCCTTCAGTGTGCTTC-3′	104 bp	[27]
HO-1	**Forward** 5′-TTAAGCTGGTGATGGCCTCC-3′ **Reverse** 5′-GTGGGGCATAGACTGGGTTC-3′	90 bp	[27]
GPx4	**Forward** 5′-TCTGTGTAAATGGGGACGATGC-3′ **Reverse** 5′-TCTCTATCACCTGGGGCTCCTC-3′	174 bp	[28]
Polr2a	**Forward** 5′-ATCAACAATCAGCTGCGGCG-3′ **Reverse** 5′-GCCAGACTTCTGCATGGCAC-3′	144 bp	[29]

3. Results

3.1. Body weight and general health analysis

The GLB-proniosomal formulation is stable and maintains glucose levels in the blood.

Fig. 2A and B displays glucose levels between groups at initial and end points and identify any significant variation. The results

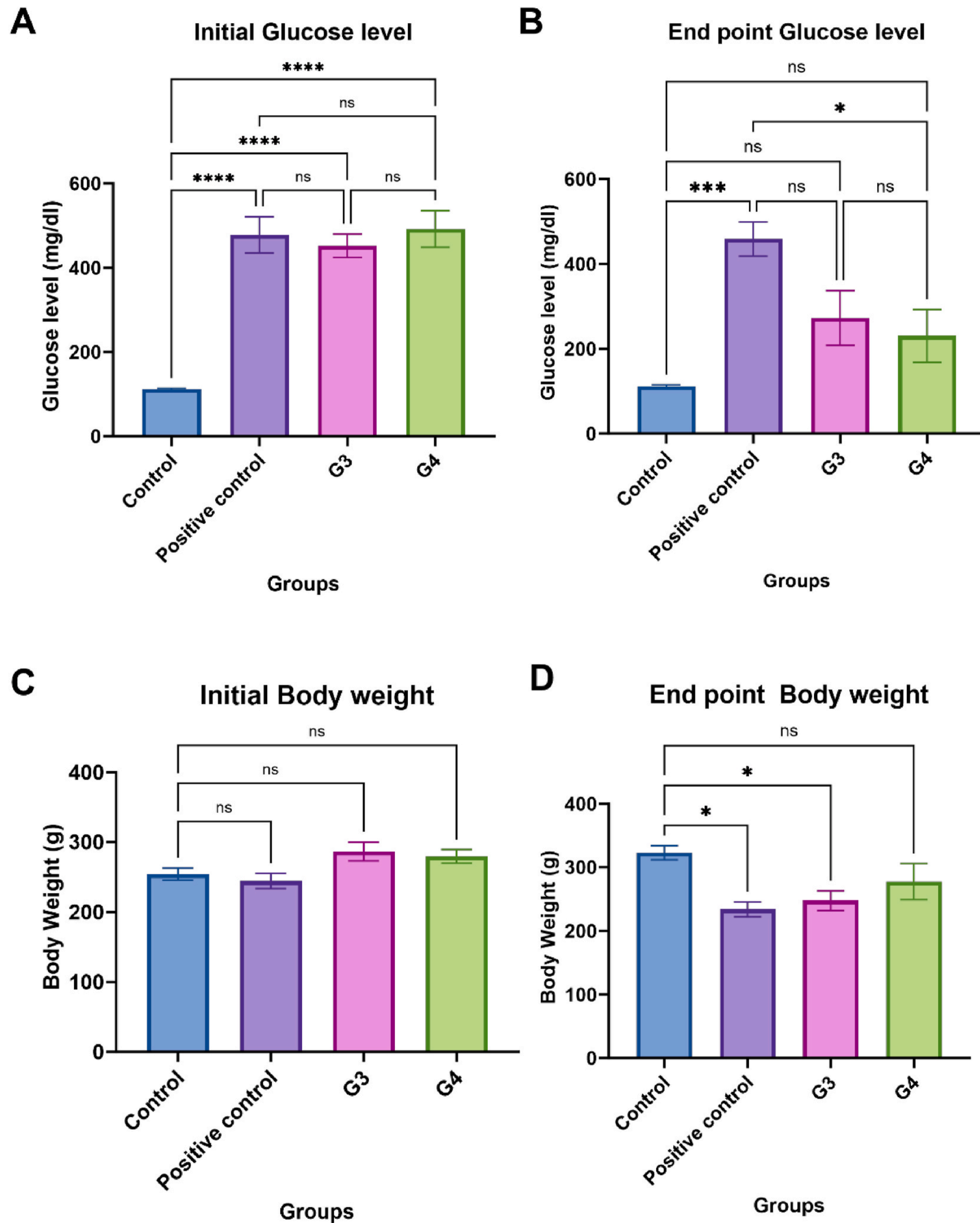

Fig. 2. A and B): *GLB-proniosomal formulation is stable and maintains glucose levels in the blood.* Showing glucose levels after STZ-induction (Initial) and two weeks of treatments (End). The negative control illustrated the normal glucose levels in the blood, which is 140 mg/dl and below. **C and D):** *Normal increase in body weight in the treated group with GLB-proniosomal formulation.* Analogous to Fig. 2A and B, presenting body weight after STZ-induction (Initial) and two weeks of treatments (End). The analysis illustrated a no bodyweight gain between the beginning (Initial) and the end of the experiments compared to the control except group G4. Data represent the mean ± SEM (n = 5). (ns) no significant, *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, and ****$P < 0.0001$ compared carried out in triplicate) was considered significant using Two-way ANOVA and Tukey's multiple.

show STZ-induction significantly reduces insulin secretion, elevating sugar in the blood in positive, G3, and G4 groups compared to the control with a P value smaller than 0.0001, illustrated in Fig. 2A. After two weeks of treatments, only group G4 showed significant differences with the positive control, with mean differences of 228.4 and a *P* value of 0.022 (Fig. 2B).

3.2. A normal increase in body weight with GLB-proniosomal formulation

Body weight was monitored weekly, and there was no sign of dramatic changes within the groups treated with STZ; on the other hand, no significant weight gain was absorbed, as shown in Fig. 2C and D. As expected in the untreated individual, a substantial increase in the rat's weight was observed compared to the initial point ($P = 0.0094$). However, when comparing the control final body weight with the treated groups, it showed no body weight gain with *P* values of 0.0146 and 0.042 and mean differences reaching up to 70 folds with positive and G3, respectively, except G4 ($P = 0.2124$).

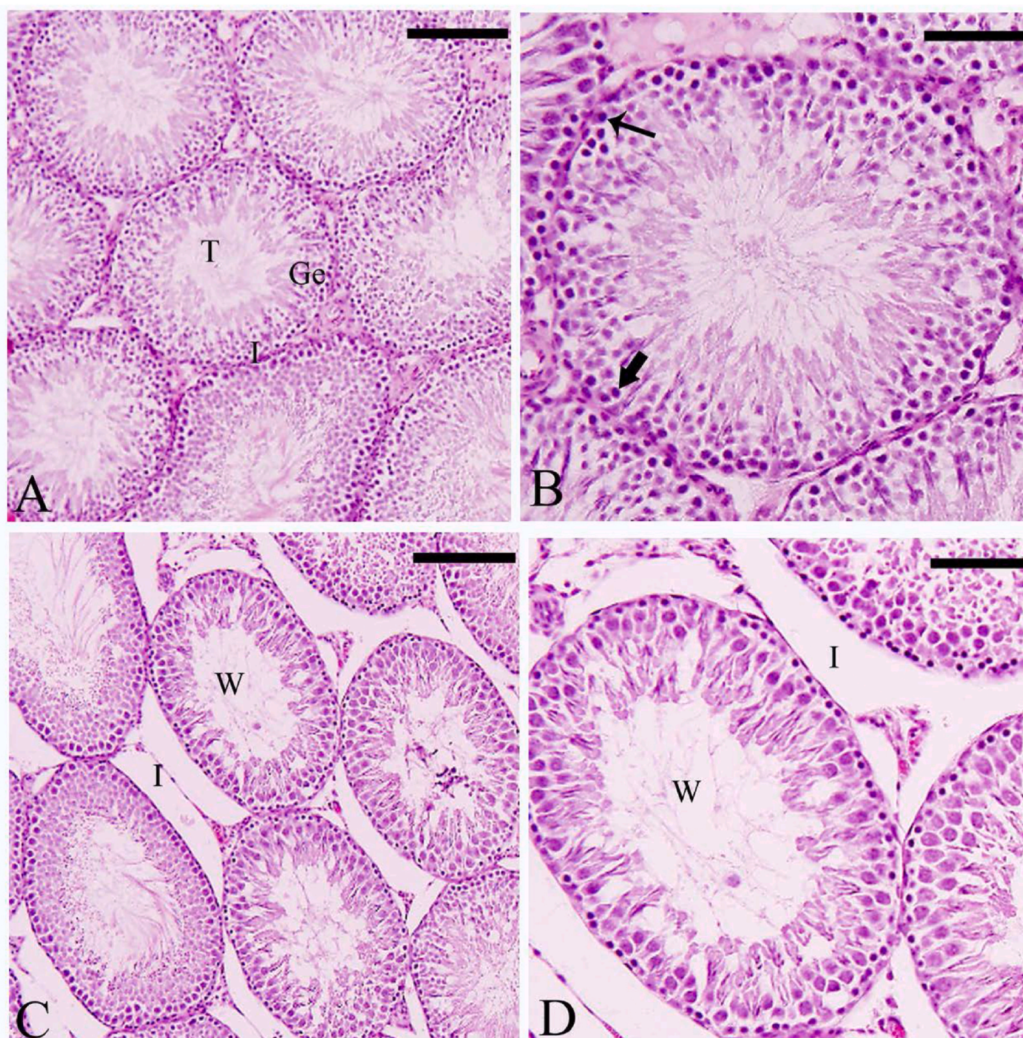

Fig. 3. *GLB-proniosomal formulation significantly protects testicular tissue from glucose fluctuation.* A and B) Control group presenting a normal seminiferous tubule (T) and lined by stratified germinal epithelium (Ge). Spermatogonia rests on a thin basement membrane; sperms are seen in the Lumen of the tubules and interstitial spaces (I). Spermatogonia (Thin arrow) and primary spermatocytes (Thick arrow). C and D) Positive control, showing some tubule changes in shape (oval) and size (elongated) with a wide lumen (W) with a few sperm and wide interstitial spaces (I). The Lumen of some tubules is filled with degenerated germ cells (T). E and F) A cross-section of the teste for G3, illustrating a normal testicular formation. Similar G and H) presenting a healthy arrangement of the seminiferous tubules for group G4. Sections are stained with hematoxylin and eosin. Scale bar = 400 μm and 100X in (A, C, E, G) & Scale bar = 200 μm and 200X in (B, D, F, H).

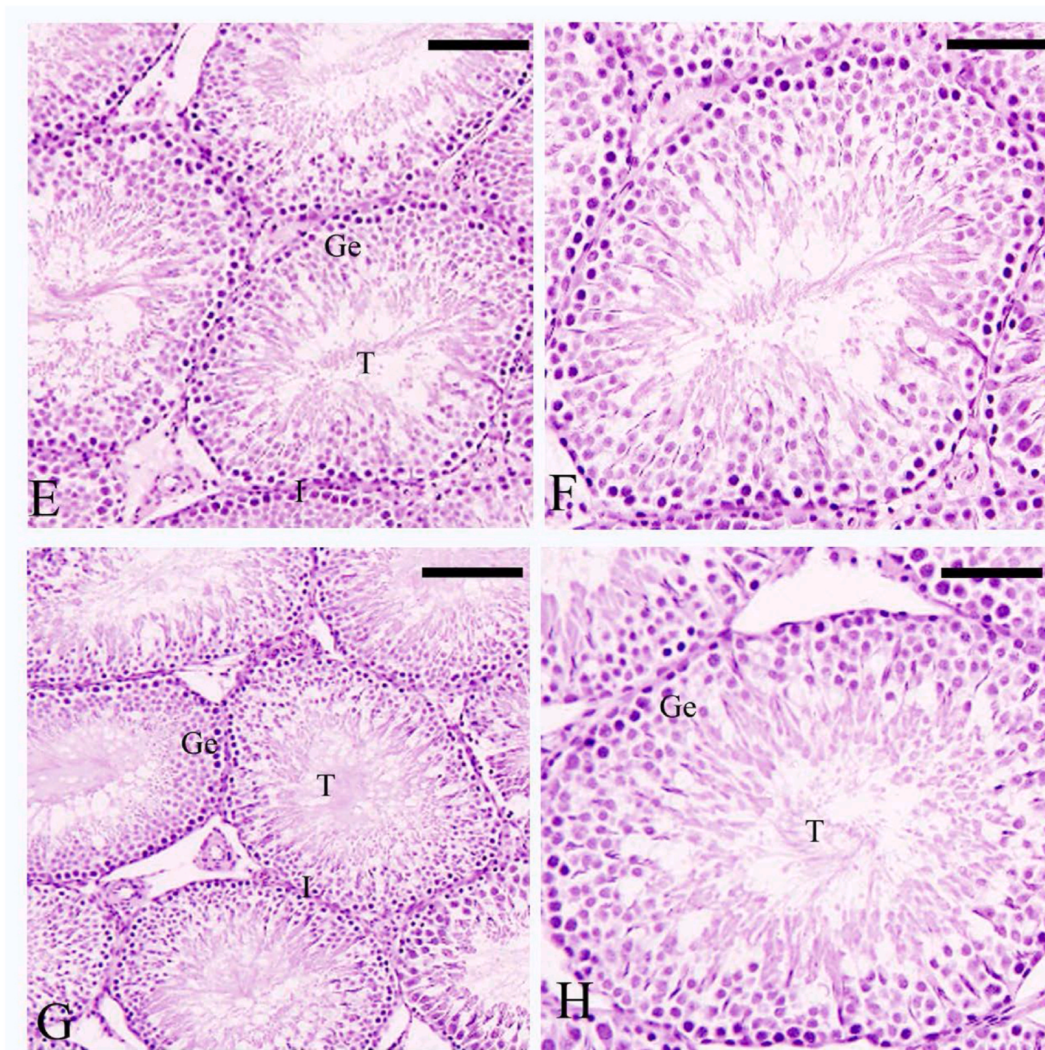

Fig. 3. (*continued*).

3.3. Histopathology analysis

3.3.1. GLB- proniosomal formulation significantly protects testicular tissue from glucose fluctuation

The data obtained from the testicular histopathology analysis from the control and the treated rats showed a range of differences in the tubular organization's shape, size, and uniformity. The control had an undisturbed arrangement and unified shape of the seminiferous tubules, lined with spermatocytes and spermatogonia; a wide lumen filed with spermatozoa flagella; and narrow interstitial space in between (Fig. 3A and B). Conversely, the cross-section of STZ-induced and untreated groups showed a clear change in the tubular formation. The tubules are non-contact arrangements, causing wide interstitial space that changes the seminiferous to an elongated oval shape (Fig. 3C and D). Nevertheless, neither G3 nor G4 demonstrated differences in the seminiferous tubules' structures or content (Fig. 3E–H).

3.4. Enzymatic activity analysis

3.4.1. Anti-oxidative stress markers elevated against ROS

The obtained data from the enzyme activity of SOD present an upregulation in the diabetic rates without treatment as the largest fold difference reaching 1.137 ± 0.2484; furthermore, G3 and G4 showed less fold change of 0.879 compared to the control (Fig. 4A). Interestingly, the resulting average correlated positively (Pearson equals 0.99 and $P > 0.0037$) with glucose Endpoint levels (Fig. 4B). Parallel to SOD results, the high levels of Catalase were detected and correlated positively ($r = 0.98$) and significantly ($P > 0.0169$) with *GPx4* expression (Fig. 4C and D). However, the positive control displays no differences from the normal control, but on the other

hand, G3 and G4 express high enzymatic activity against the negative control (Fig. 4C and D). Finally, the GSH analysis illustrated that only G4 was significant relative to the positive control, with a *P* value of 0.0210, as demonstrated in Fig. 4E.

3.5. Molecular gene expression analysis

3.5.1. Nrf2/HO-1 pathway upregulated against ROS stress

Based on Fig. 5A, the gene expression of *Nrf2* in treated rats with GLB-proniosomes formulation (G4) was significantly higher than that of the positive control and references GLB (G3). The mean differences between G4 and the control were 2.050 ± 0.7085, with a *P* value of 0.0444. In this regard, reference G3 was slightly effective but showed major diversity between samples compared to modification G4. Furthermore, the *HO-1* supported our finding, illustrating the fold change of 2.148 comparing G4 against both treated and untreated groups.

3.6. GPx4 is an antioxidant enzyme upregulated to protect against ROS stress

Administering GLB to diabetic rats restored the antioxidant enzyme activities in the tested compared to diabetic rats. Fig. 5C shows that the gene expression of *GPx4* in treated rats with reference GLB (G4) was elevated compared to the control, with a mean difference of 1.249 ± 0.3327 and a *P* value of 0.0028.

4. Discussion

Type 2 Diabetes Mellitus is a chronic debilitating disorder with a rapid incidence worldwide [3–6]. In the current study, administering a single low-dose STZ has been used in animal models to destroy the pancreatic β-cells partially. This leads to insufficient

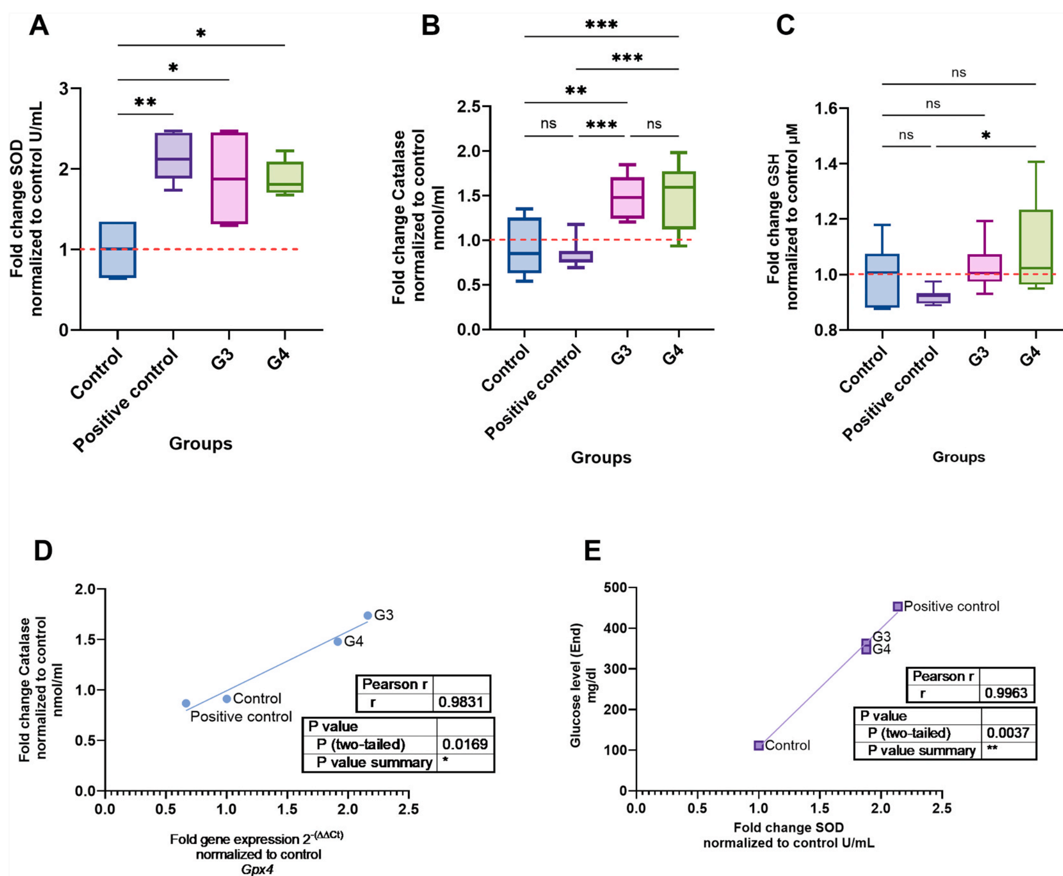

Fig. 4. *Anti-oxidative stress markers upregulated to protect against ROS generated by hyperglycemia.* A) Significant elevation in SOD activity with treated groups compared to the control up to 2 folds. Plus, a positive correlation (r = 0.99) with at *P* of 0.0037 with the glucose level before sacrificing showing in B. C) Catalase activity elevated in both G3 and G4 compared to the positive and negative control with a *P* values of 0.0010 and 0.0009, respectively. D) Using the average fold change of *GPx4* with average Catalase activity, a significant and positive Pearson of 0.98 is used. GSH activity illustrated in E, showing a *P* value of 0.02 for G4 compared to the diabetic untreated rats. Data represent box and whiskers (Max to Min) (n = 5). *$P < 0.05$, **$P < 0.005$, and ***$P < 0.0005$ were considered significant using One-way ANOVA and Tukey's post-test.

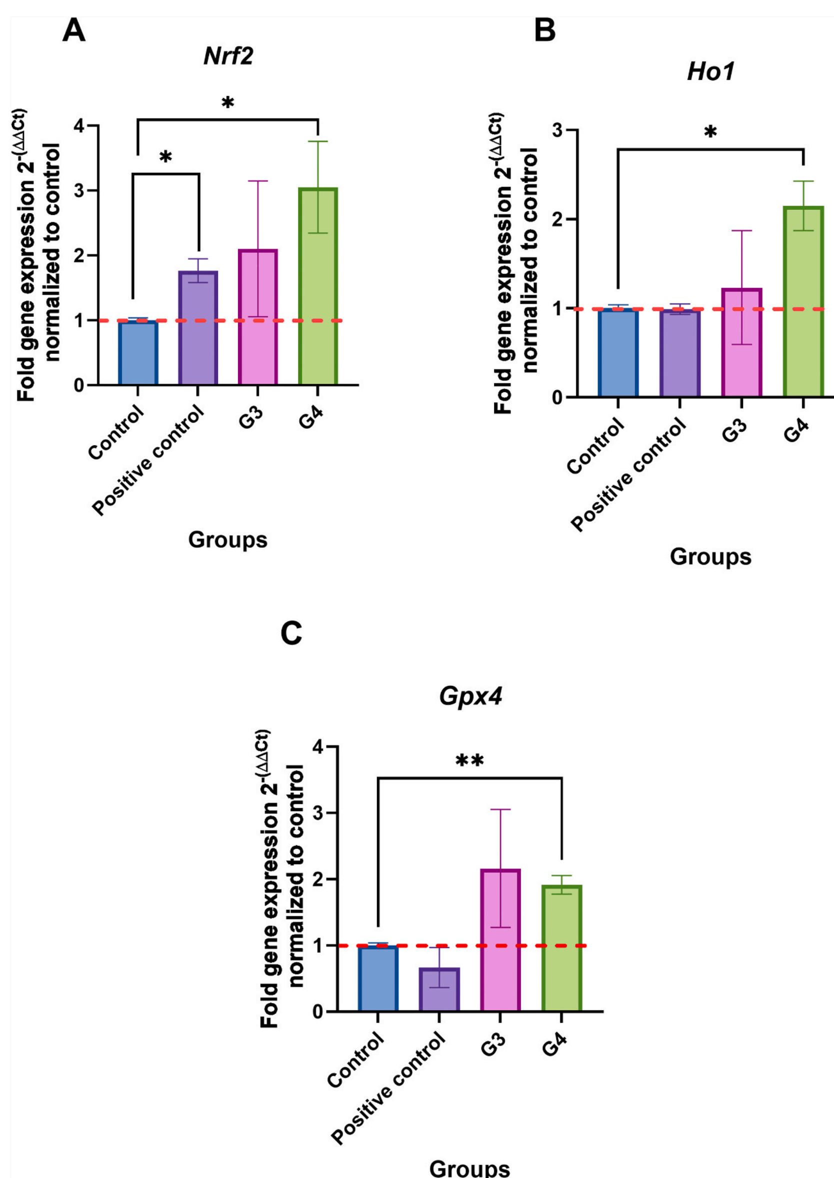

Fig. 5. *Nrf2/HO-1 pathway upregulated to protect against ROS generated by diabetes*. A) Gene expression represented in fold change for *Nrf2* normalized to control shows great significance between the control and G4 by three folds. Plus, there is a positive control by 1.7 fold and large variances between samples in G3. B) *OH-1* elevated by 2.148 compared to the positive and negative control. *The GPx4 pathway is upregulated to protect against ROS generated by diabetes*. C) Gene expression illustrated in fold change for *Gpx4* normalized to control shows significance between the control and G4 by 1.9 folds. Plus, a mean difference of 1.249 ± 0.3327 with the positive control and large variances between samples in G3. Data represent the mean ± SEM (n = 3). $^*P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.0005$ (carried out in triplicate) were considered significant using an unpaired *t*-test. *Polr2a* was used as a housekeeping gene.

insulin secretion, mimicking the symptoms observed in T2DM patients [30].

Glibenclamide (GLB) is one of the oral antidiabetic medications that belongs to the second-generation sulfonylurea and is used to treat diabetic mellites type 2. Despite the convenience and effectiveness of oral administration in improved T2DM patients, GLB is classified as a class II drug according to the Biopharmaceutical Classification System (BCS) owing to its low aqueous solubility, high permeability, and frequent dosing [31]. Collectively, these consequences lead to a reduction in the bioavailability of GLB drugs. However, various vesicular drug delivery systems, including liposomes and cubosomes, have been proposed to enhance the therapeutic efficacies of medications [32,33]. In this regard, a novel proniosomal-GLB formulation was made to improve the bioavailability of GLB. The formulation used sucrose as a carrier and coated the lipid with a zeta potential value of −51.50 ± 7.35 mV and 277.100 ± 36.06 nm partial size. The novel proniosomal-GLB formulation increased the drug dissolution rate in terms of %DE by 74.456 % compared to the pure GLB with no Hepatotoxicity [22]. Therefore, the present work was undertaken to evaluate the possible therapeutic effects of

the proniosomal formulation of Glibenclamide to be stable in the blood and decline glucose levels and oxidative stress in the testicular tissue of STZ-induced rats.

Our results revealed that the enhanced GLB has significantly lowered the blood glucose of diabetic rats compared to untreated diabetic rats. This finding correlates with other studies that confirmed the probable effect of enhanced carriers, such as the metformin-loaded alginate nanoparticles (MLANs) and metformin beeswax PEGylated solid lipid nanoparticles (PEG-SLN) in reducing blood glucose levels compared to pure metformin since the sustained release of drug was observed from the MLANs by only 21 % an hour while 100 % was released within 1 h compared to commercially available pure drug [34,35]. Our study has shown a significant decrease in blood glucose compared to our previous study [22]; these results specify the effect of the enhanced GLB, which was the initial and final blood glucose level (BGL) reduction of 17.6 % compared to the pure GLB. Moreover, with the novel carrier proniosomes formulation, pharmacodynamics analysis *in vivo* reduces the blood glucose level by 73.7 % compared with only 17.6 % after treatment with a pure drug ($P = 0.002$) [22].

On the other hand, the non-significant changes in body weight between GLB-proniosomal formulation-treated rats and untreated normal rats suggest that the main cause of pure GLB did not prevent the significant body weight loss as shown in untreated diabetic rats was drug instability. Indeed, the investigation by [36] reported that weight loss is associated with increased gluconeogenesis and protein degradation as alternative energy sources in response to glucose homeostasis disruption, as shown in our data. Moreover, T2DM is known to induce oxidative stress levels, hindering the efficiency of the antioxidant defense system [37]. Furthermore, the imbalance between oxidants and antioxidants caused by hyperglycemia can shift the status of the stress in favor of oxidants, causing testicular damage and subsequently leading to reproductive disorders [38]. Herein, ROS markers, including SOD, CAT, and GSH, were measured and compared in our groups.

The histopathological analysis of diabetic rats' testis compared to the non-diabetic rats showed the disorganization of seminiferous tubules with a marked reduction in diameter, and the normal stratification of germinal epithelium was destroyed, confirming that the spermatogenesis was arrested. Meanwhile, the testicular section of the GLB-treated groups illustrated a remarkable improvement of seminiferous tubules against glucose fluctuation and a considerable increase in spermatogenic cell numbers. Consistent with this finding, combining Cardamonin (CARD) with GLB provided optimal attenuation against the spermatogenic layers atrophy and the disappearance of spermatids in diabetic rats compared to untreated diabetic rats and rats treated with CARD only [39].

It is acknowledged that enzymatic antioxidants such as CAT, SOD, and GSH have been considered crucial components in the direct elimination of ROS. Our findings revealed that SOD activity was increased in the testis of untreated diabetic rats compared to the normal control groups. In contrast, compared to the control group, pure GLB-treated rats and the enhanced GLB-treated rats showed a significant increase in SOD activity that was in line with the [39] study, suggesting that SOD explored a defense strategy to counteract ROS-induced stress associated with diabetes that was still sustained even after the proniosomal formulation and shown a positive correlation with the average blood glucose levels with SOD enzymatic activity. Research indicates that SOD plays a vital role in the development of testicles and the production of sperm in rats. This enzyme positively correlates with sperm concentration and overall motility [40]. Moreover, SOD mRNA transcripts have been found in the testis of rats, with the highest concentration present in tubules just before spermiation [41,42].

Besides the SOD activity, elevated CAT activity was observed within pure-GLB and proniosomal-GLB-treated rats compared to the control and diabetic-untreated groups. In diabetic conditions, CAT helps mitigate oxidative stress as a cellular defense mechanism against oxidative damage. Our results aligned with the investigation of the herbal drug Momordica charantia (MCFE) and GLB interactions against oxidative stress induction in the pancreatic tissues of diabetic rats [43].

Moreover, the oral administration of MCFE and GLB combination to STZ-diabetic rats increased the antioxidant enzymes compared to rats that received GLB alone. In addition [39], have also shown that the restoration of the elevated level of the oxidative stress-related marker (TBARS) to near-decreased levels and the significant increase in the activity of CAT, SOD, and GSH were evidenced by the protective effect of Cardamonin (CARD) in diabetic-treated rats against diabetes-induced testicular dysfunction. However, our study focused on modifying the GLB stability in the blood, which would eliminate the need to add any herbal or other medication to close the low aqueous solubility gap of Glibenclamide.

Additionally, in diabetic rats, elevated blood glucose levels lead to increased oxidative stress, affecting antioxidant enzyme activities [36]. This study found significant evidence between CAT and *GPx4* that are involved in antioxidant defense mechanisms but operate in different mechanisms in which CAT breaks down hydrogen peroxide. Additionally, the seminal fluid contains CAT enzyme, which is responsible for converting H_2O_2 into oxygen and water, which helps maintain appropriate ROS levels, protecting spermatozoa from harmful ROS in the seminal fluid; also, low sperm quality is correlated with CAT activity [44]. Moreover, male infertility was also brought on by the loss of mitochondrial GPX4 (mGPX4), which, therefore, resulted in decreased sperm motility, severe structural abnormalities, and mitochondrial membrane potential in the testis [45]. At the same time, GPx4 protects the cells from lipid peroxidation. Regarding this study, the positive correlation between CAT and *GPx4* was significantly observed in all subjected rats, where the diabetic rats had the lowest levels of both targets, the highest was proniosomal-GLB, and the normal group was in between groups.

In addition, GSH is a cofactor for glutathione peroxidase (GPx), which protects mammalian cells against oxidative stress by reducing H_2O_2 to H_2O. It is also crucial for sperm's antioxidant defenses and for developing an enzyme in spermatids that becomes a structural protein in mature spermatozoa. A study has shown diabetic individuals experience a reduction in GSH levels within the testis, disrupting spermatozoa membrane integrity [46]. Additionally, the study demonstrated a correlation between oxidative stress and reduced GPx levels in the testicular tissue of rats subjected to a high-fat diet. Notably, the protective impact of bee bread was observed to significantly enhance GPx expression, directly mitigating lipid peroxidation by-products and thereby protecting spermatozoa against ROS production [46]. In our study, diabetic rats treated with proniosomes-GLB had significantly elevated levels of GSH content compared to untreated diabetic rats, which was not seen with the pure-GLB group. Another study used an antidiabetic

treatment known as Astragalin (AG), which was also shown to increase GSH. AG is one of the active flavonoids in *Cuscuta chinensis* medical herbs that demonstrated its antidiabetic activities in the testicular tissue of diabetic mice [47]. However, Han et al. investigation needed the administration of oral supplementation of AG (30 mg/kg) to significantly restored the normal level of GSH activities in the testis of diabetic mice compared to our dose of proniosomal formulations GLB which was only 5 mg/kg and reached the normal levels and exceeded the diabetic untreated group.

Furthermore, we study the gene expression of *Nrf2/HO-1* nuclear levels and downstream signals. Nrf2 transcription factors control the endogenous antioxidant system [48,49]. The antioxidant system protects the testicular tissue from dysfunction, which scavenges excess ROS under normal physiological conditions. While the reproductive system requires ROS for reproduction, the risk posed by an uncontrolled generation by stimulating the physiologically antioxidative systems when needed [50]. The antioxidation system gets stimulated by Nrf2, which plays an important role in stimulating antioxidant enzymes through the ARE [51]. However, if Nrf2 activity is impaired, oxidative stress and inflammation accumulate. Additionally, Nrf2 regulates cellular resistance to oxidative stress in the testis, which affects the physiology and pathology of testicular issues, particularly the spermatogenic process [15–17,52]. It has been proposed that Nrf2 expression is necessary for proper spermatogenesis and sperm-specific processes, including motility.

The current data revealed that proniosomal-GLB-treated rats restrained testicular oxidative stress via *Nrf2/HO-1* pathway expression compared to the pure GLB and untreated groups. Initially, the expression of *Nrf2* level was significantly increased in treated rats with proniosomal-GLB compared to control and untreated rats. This upregulation in Nrf2 expression indicates an adaptive response to oxidative stress associated with diabetes [53]. In contrast, the expression of the *Nrf2* gene in pure GLB-treated rats showed huge diversity among samples that lost the data significantly compared to control and untreated groups. Moreover, the *HO-1* expression that confirmed the protein activation Nrf2 as a transcription factor showed great significance within proniosomal-GLB treated rats compared to control and diabetic-untreated rats, and the diversity between individuals within pure GLB again lost its significance. The findings from the current investigation showed that the administration of proniosomal GLB formulation is markedly superior to pure GLB in eliminating the effect of ROS caused by hyperglycemia. These findings concur with the reported antioxidant potential of sit gliptin against cadmium-triggered testicular dysfunction in rats [54].

Additionally, spermatogenesis is a highly metabolically active process, and sperm cells are vulnerable to oxidative stress. GPx4 protects cell membranes from damage in oxidative stress by reducing lipid hydroperoxide. Its role in protecting developing sperm cells from ROS damage is essential for understanding the mechanism contributing to male fertility and unraveling the potential factors that could affect sperm validity. In the present investigation, the gene expression of *GPx4* was highly significant in pure GLB-treated rats compared to the proniosomal modified GLB groups. Likewise, compared to control and diabetic untreated rats, the expression of *GPx4* in proniosomal GLB rats was significantly increased. Our results agreed with the study of Shoorei et al., which showed that diabetic carvacrol-treated rats significantly reduced lipid hydroperoxides and increased the activity of the GPx antioxidant enzyme in diabetic conditions [55].

5. Conclusion

Our research indicates that using a proniosomal formulation of Glibenclamide can help reduce oxidative stress in testicular tissues, thereby mitigating restraint stress-induced damage to spermatogenesis. This is achieved by increasing insulin production and upregulating anti-ROS enzymes (SOD, CAT, and GSH) via *Nrf2/HO-1* and *GPx4* signaling pathways. Our findings suggest that this treatment could be a promising strategy for preserving male fertility in individuals with prediabetes or obesity.

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CRediT authorship contribution statement

Nouf M. Alyami: Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Funding acquisition, Conceptualization. **Zainab A. Alnakhli:** Writing – review & editing, Writing – original draft, Project administration, Investigation, Formal analysis. **Noura M. Alshiban:** Visualization, Investigation, Formal analysis. **Saleh Maodaa:** Visualization, Investigation, Formal analysis. **Ghufran A. Almuhaini:** Writing – review & editing. **Rafa Almeer:** Writing – review & editing. **Doaa Alshora:** Resources, Conceptualization. **Mohamed Ibrahim:** Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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