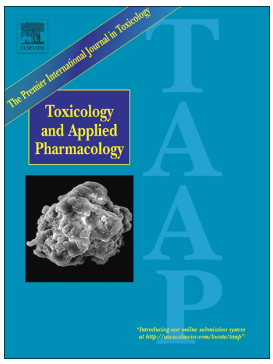

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Novel Oxolane Derivative DMTD Mitigates High Glucose Induced Erythrocytes Apoptosis by Regulating Oxidative Stress

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Abstract:

Chronic hyperglycemia is one of the characteristic conditions associated with Diabetes Mellitus (DM), which often exerts deleterious effects on erythrocyte morphology and hemodynamic properties leading to anemia and diabetes-associated vascular complications. High glucose-induced over production of reactive oxygen species (ROS) can alter the blood cell metabolism and biochemical functions subsequently causing eryptosis (red blood cell death), yet another complication of concern in DM. Therefore, blocking high glucose-induced oxidative damage and subsequent eryptosis is of high importance in the better management of DM and associated vascular complications. In this study, we synthesized an oxolane derivative 1-(2,2-dimethyltetrahydrofuro[2,3][1,3]dioxol-5-yl)ethane-1,2-diol (DMTD), and demonstrated its efficacy to mitigate hyperglycemia-induced ROS generation and subsequent eryptosis. We showed that DMTD effectively inhibits high glucose-induced ROS generation, intracellular calcium levels, PS scrambling, calpain and band 3 activation, LDH leakage, protein glycation and lipid peroxidation, and enhances antioxidant indices, osmotic fragility and Na^+/K^+-ATPase activity in erythrocytes. DMTD dose dependently decreased the glycated hemoglobin level and enhances the glucose utilization by erythrocytes *in vitro*. Further, DMTD alleviated the increase in ROS production, intracellular Ca^{2+} level and PS externalization in the erythrocytes of human diabetic subjects, and enhanced the Na^+/ K^+-ATPase activity. Taken together, the synthesized oxolane derivative DMTD could be a novel synthetic inhibitor of high glucose-induced oxidative stress and eryptosis. Considering the present results DMTD could be a potential therapeutic to treat DM and associated

complications and open new avenues in developing synthetic therapeutic targeting of DM-associated complications.

Key words: Eryptosis; Oxidative stress; Diabetes mellitus; Glycated hemoglobin; DMTD; PS externalization.

Introduction

Diabetes mellitus (DM), which is known for its abnormal glucose levels in the circulation is often associated with significant hemorheological and biochemical malfunctions in red blood cells (RBCs), and platelet hyper-reactivity (Borst et al., 2013; Faggio et al., 2017; Mischitelli et al., 2016a). Chronic hyperglycemia is one of the defining characteristics of DM and is shown to play a crucial role in orchestrating the diabetes-associated vascular and vital organ complications, which results in overproduction and accumulation of ROS (Brownlee, 2001; Green et al., 2004; Nishikawa et al., 2000). Chronic hyperglycemia-induced overproduction or accumulation of ROS is shown to propagate serious pathological conditions such as micro/macro vascular abnormalities (Baynes and Thorpe, 1999; Stratton et al., 2000), endothelial dysfunction (Wong et al., 2010), altered DNA-protein interactions, structural changes in membrane lipids (Hunt et al., 1988) renal polyol formation (Kikkawa et al., 2003), accumulation of advanced glycation end products (AGEs) (Hunt et al., 1988) etc.

RBCs are crucial players in transporting oxygen to all the tissues and elimination of carbon dioxide (Briglia et al., 2015; Pagano and Faggio, 2015; Signoretto et al., 2016). These anuclear cells are highly susceptible to the direct exposure of endogenous and exogenous ROS, which induces oxidative stress (Silva-Herdade et al., 2016; Bonaccorsi et al., 2013). The RBCs are vulnerable to lipid peroxidation and glycation due to the high iron and polyunsaturated fatty acid content (Hunt et

al., 1988). Hyperglycemia also induces RBC membrane protein glycation and decreases Na^+/K^+-ATPase activity defining intracellular Na^+ and K^+ concentration A remarkable decrease in the Na^+/K^+-ATPase activity has been implicated in the pathology of diabetes-induced polyneuropathy and related complications (Vague et al., 2004). The pathological changes due to chronic hyperglycemia and elevated ROS levels are known to induce deleterious effects on the morphology and hemodynamic features of RBCs compromising their lifespan by driving them to suicidal death, a process known as eryptosis. It is characterized by cell shrinkage and scrambling, which are regarded as the hallmark of apoptosis. Activation of Calpain is another crucial event in hyperglycemia-induced eryptosis (Samanta et al., 2012). Calpain, a calcium-dependent thiol protease is involved in fragmentation of RBC cytoskeleton proteins spectrin and band 3 causing severe hemolysis and subsequent anemic condition (Briglia and Faggio, 2017; Faggio et al., 2015; Lang et al., 2013; Starodubtseva et al., 2008).

The key molecular events of oxidative stress-mediated eryptosis include increased formation of endogenous ROS, which activates Ca^{2+} permeable channels resulting in elevated intra-erythrocyte Ca^{2+} concentration and direct activation of calcium sensitive K^+ channels (Bratosin et al., 2001). This results in hyper-polarization of RBCs with K^+ ion leakage and thus raising the electrical driving force for chloride ion exit (Almasry et al., 2017; Bissinger et al., 2014). Finally, the leakage of KCl from the cells with osmotically obliged water decreases the cell volume as well as the loss of membrane integrity, leading to cell membrane scrambling with subsequent externalization of phosphatidylserine (PS) at the cell surface (Borst et al., 2012; Chung et al., 2007; Zwaal et al., 2005). In diabetic patients, the eryptotic erythrocytes bind to endothelial cells via CXCL16/SR-PSO signaling cascade (Borst et al., 2012). The PS-exposing erythrocytes further increase the risk of thrombosis by the activation of blood clotting cascade (Andrews and Low, 1999). Finally,

erythrophagocytosis i.e., the engulfment of these PS-exposing erythrocytes results in the clearance of circulating blood, thus eventually leading to anemia (Lang et al., 2008).

Even though there are many existing plant-derived molecules such as xanthohumal (Qadri et al., 2009), probucol (Shaik et al., 2013), naringin and teriflunomide (Zierle et al., 2016) that can inhibit eryptosis and associated oxidative stress, there is a great demand for synthetic small molecules, which can curb hyperglycemia-induced oxidative stress and eryptosis. Therefore, the present study was aimed to synthesize a potent small molecule that can inhibit hyperglycemia-induced premature death of RBCs. Heterocyclic compounds containing oxolane moiety have been employed to treat variety of inflammatory conditions such as Crohn's disease, HIV, cancer etc. (Luo et al., 2000). There exists a patent on 1, 3 dioxolane derivative as a potent leukotriene B_4 (LTB_4) biosynthesis inhibitor, which is an important proinflammatory metabolite of arachidonic acid pathway. Bioactive molecule Odisolane, an oxolane moiety containing molecule is shown to exhibit anti-angiogenic property by targeting VEGF, AKT and ERK signaling pathways (Dërmaku-Sopjani et al., 2014; Lee et al., 2016; Mischitelli et al., 2016b, 2016c). Therefore, in this study we have synthesized an oxolane derivative 1-(2,2-dimethyltetrahydrofuro[2,3][1,3]dioxol-5-yl)ethane-1,2-diol(DMTD) and evaluated its ability to inhibit hyperglycemia-induced oxidative damage on RBCs and subsequent cell death.

Materials and methods

Chemicals & reagents

All reagents and starting materials were purchased from Sigma Aldrich and SDFCL. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra of isolated compound was recorded on Agilent 400MHz NMR spectrometer using $CDCl_3$ as solvent. Chemical shifts are given as δ value with reference to

tetramethyl silane (TMS) as an internal standard. Infrared spectra were recorded on Shimadzu FT-IR model 8300 spectrophotometer.

All flourogenic, non-flourogenic substrates were from Sigma Chemicals, USA. Polyclonal anti-Band 3 antibody was from Sigma and monoclonal anti-Calpain antibody was from Santa Cruz Biotechnology, Dallas, USA. Calcein-AM, antibodies against GAPDH and β-tubulin were from Cell Signaling and Technology, Danvers, USA. *O*-phthalaldehyde (OPT), phenylmethylsulfonyl fluoride (PMSF), dichlorofluorescein diacetate (DCFDA), reduced glutathione (GSH) and oxidized glutathione (GSSG) were obtained from Sigma Chemicals, St. Louis, USA. Lactate dehydrogenase (LDH), creatine kinase (CK), blood urea nitrogen (BUN), alanine transaminase (ALT), and aspartate transaminase (AST) assay kits were purchased from Agappe Diagnostics Limited, India. Glycated hemoglobin detection kit was purchased from Diatek Healthcare Pvt.Ltd., Kolkata India. Glucose Test kit was purchased from Arkray Healthcare Pvt. Ltd. Surat, India. All other reagents were of analytical grade and procured from Sisco Research Laboratories, India. The synthesis and characterization of the DMTD is provided as the supplementary information. DMTD is a water-soluble compound, hence stock solutions are made in respective assay buffers.

Isolation of human erythrocytes and preparation of erythrocyte ghost samples

Human blood samples were processed as previously described (Lang et al., 2015). Whole blood samples were collected from healthy volunteers within the age group of 20-55, non-smokers and non-alcohol consumers free of any medications, and from routine diabetic patients with plasma glucose level >150 mg/dL. The blood drawn was instantly mixed with acid citrate dextrose (ACD) anti-coagulant (containing 85 mM sodium citrate, 78 mM citric acid and 111 mM D-glucose) in the ratio 6:1 (blood: ACD *v/v*) following centrifugation at 1,000×*g* at 4 °C for 15 min. Then, the plasma and buffy coat layers were discarded. The erythrocyte layers were washed three times with 150 mM

NaCl and centrifuged at 1,000×*g* at 4°C for 10 min. The supernatants were discarded after each centrifugation. The erythrocyte layers were re-suspended in phosphate-buffered saline (PBS) pH 7.4, containing 1 mM NaH_2PO_4, 16 mM Na_2HPO_4 and 140 mM NaCl and washed thrice with PBS. Finally, 0.4% and 10% hematocrit were prepared in Ringer's solution (125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, and 32 mM HEPES/ NaOH, 5 mM glucose, and 1 mM $CaCl_2$ pH 7.4) and used for the assays. The Institutional Human Ethical Committee (IHEC-UOM No.118/Ph.D./2015-16 & IHEC-UOM No.146/Ph.D./2016-17) University of Mysore, Mysuru, has approved the study. All subjects have read and signed a written informed consent before their enrollment into the study.

For the preparation of erythrocyte ghost samples, after the incubation erythrocyte membranes were isolated from the hematocrit (10% washed RBCs in PBS) in accordance with previously described experimental protocol with slight modifications (Lang et al., 2005). After incubation for 12 h, the control and treated erythrocytes were subjected to centrifugation at 1000×*g* for 10 min and the supernatant was discarded. The cells were then lysed with 30 volumes of ice cold lysis buffer (5 mM phosphate buffer pH 8 containing 1mM EDTA along with protease inhibitor cocktail containing 10 µg/mL pepstatin A, 10 µg/mL leupeptin, 5 µg/mL aprotinin and 0.1 mM PMSF) for 20 min at 4 °C. The ghost membrane and cytosolic fractions were separated by centrifugation at 37,000×*g* for 15 min at 4 °C. Membranes were then pelleted and lysed using a lysis buffer containing 125 mM NaCl, 25 mM HEPES/ NaOH pH 7.3, 10 mM EDTA, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 0.1% SDS, 0.5% deoxycholic acid, 1% Triton X-100, 10 µL β-mercaptoethanol. Protein content in the membrane fractions was determined by using the Lowry's method by using bovine serum albumin (BSA) from sigma as standard. Finally, membrane fractions with equalized protein content, were subjected to SDS-PAGE (10% gel) and immune blotted by western blotting.

Treating erythrocytes with Glucose *In vitro*

Washed erythrocytes were suspended to a final reaction mixture of 0.4% hematocrit in PBS containing varying concentration of glucose (5, 10, 25 and 45 mM) and incubated for 6 h at 37 °C. In a second set of experiments, erythrocytes were treated with high glucose concentration of 45 mM with varying time intervals (2, 6, 12, 24 and 48 h) at 37 °C according to the previously described method (Viskupicova et al., 2015), at the end of the incubation the eryptotic markers such as increase in ROS level, intracellular calcium and PS externalization were measured. Usually, the cytosolic glucose concentration of human erythrocytes is maintained about 4.5-5mM by non-insulin dependent glucose transporter-1 (GLUT-1), which is elevated under hyperglycemia, hence incubation of erythrocytes with high glucose concentration above 45 mM is routinely used as a model to mimic the *in vivo* hyperglycemic condition in diabetes and erythrocytes treated with 5 mM glucose is considered as control samples.

Determination of endogenous ROS, intracellular Calcium and PS scrambling in erythrocytes

In order to determine the oxidative stress-induced erythrocyte death, eryptotic markers such as ROS generation, elevated intracellular calcium level and PS externalization were evaluated in 0.4% hematocrit, as described accordingly (Lupescu et al., 2012). For the induction of eryptosis, erythrocytes were treated with high glucose concentration (45 mM) and for inhibition studies, erythrocytes exposed to high glucose (45 mM) were then treated with DMTD (10 and 25 µM). The final volume was made up to 200 µL with Ringer's solution containing 2 mM $CaCl_2$ and incubated for 6 h at 37 °C with constant shaking. Further, endogenous ROS generation, intracellular calcium and phosphatidylserine (PS) externalization was determined using fluorescent dyes such as DCFDA, Fura2-AM and Annexin V-FITC respectively. After incubation, cell suspensions were centrifuged at 280×*g* for 5 min and supernatants were removed, the settled erythrocytes were then suspended in 200 µL Ringer's solution and stained independently with DCFDA (10 µM), Fura2-AM (5 µM) and

Annexin V-FITC (1 µg/mL) for respective incubation period at 37 °C, finally cells were washed and analyzed by exciting the samples at wavelength of 488 nm and an emission wavelength of 530 nm using FACSVerse^{TM} flow cytometer (BD Biosciences, USA) for ROS generation. The Ca^{2+}-dependent fluorescence intensity was measured with an excitation wavelength of 340 nm and 380 nm and an emission wavelength of 530 nm using a Varioskan multimode plate reader (Thermo Scientific, USA). Similarly, for the determination of PS externalization, erythrocytes were stained using FITC conjugated Annexin V for 20 min, washed and analyzed by FACSVerse^{TM} flow cytometer (BD Biosciences, USA).

Immunoblot

Immunoblot of erythrocytes membrane samples were carried out as described accordingly (Pantaleo et al., 2016). Briefly, 10% hematocrit in PBS were treated with high glucose 45 mM as agonist, for inhibition studies, erythrocytes exposed to high glucose were incubated with DMTD (10 & 25 µM) for 12 h, after incubation erythrocytes ghost samples were isolated and their protein content was determined by Lowry's method. Erythrocyte membrane protein were then separated by SDS-PAGE using 10% polyacrylamide gel and subsequently transferred electrophoretically to PVDF membrane. The membranes were then blocked with 5% skimmed milk in TBST (10 mM Tris-HCl pH 8, containing 150 mM NaCl and 0.05% Tween-20 in double distilled water) for 1 h followed by TBST wash and incubation overnight with primary antibodies calpain (1:3000) and Band 3 (1:5000) at 4 ºC. The membranes were then washed with TBST and incubated with horseradish peroxidase (HRP)-conjugated respective secondary antibodies for 1 h. The blots were then washed thrice with TBST and incubated with a chemiluminescent substrate and visualized using chemiluminescence imaging system (Alliance 2.1, Uvitek, UK).

Imaging of erythrocytes via Atomic force microscopy (AFM)

AFM imaging of erythrocyte samples were carried out as described accordingly (Shi et al., 2013). Briefly, 1% hematocrit in PBS were treated with glucose 5 mM as control and high glucose 45 mM as agonist respectively; for inhibition studies, RBCs pretreated with high glucose were incubated with DMTD (25 µM) for 6 h. Following incubation, erythrocytes were imaged according to the method described previously, using cantilever: ACTA - 40N/m with tip radius of curvature 10-20 nm, along with probe made of silicon pyramidal shape were used to image erythrocytes in non-contact mode with Park systems NX-10 AFM. Suwon, Korea.

Determination of erythrocytes viability *via* calcein fluorescence and LDH release assays

To assess the viability of erythrocytes in 1% hematocrit and PBS, the cells were treated with high glucose (45 mM), and for inhibition studies, high glucose-exposed erythrocytes were incubated with DMTD (0-25 µM) for 6 h at 37°C, followed by staining with calcein AM (10 µM) and incubated for 30 min at 37 °C. Further, cells were washed and measured by exciting the samples at 488 nm, and emission was detected at 585 nm (NaveenKumar et al., 2015). LDH release in erythrocytes was determined by using commercially available LDH kit, according to the manufacturer's protocol. The assay was performed in a time course of decrease in NADH absorbance at 340 nm for 3 min.

Determination of Lipid peroxidation (LPO) and Protein carbonyl content (PCC)

LPO status was assessed in erythrocyte membrane samples by estimating thiobarbituric acid reactive substances (TBARS) as described accordingly (Sharma et al., 2015). Briefly, 10% hematocrit in PBS were treated with high glucose 45 mM as agonist, for inhibition studies, erythrocytes exposed to high glucose were incubated with DMTD (0-25 µM) for 6 h, erythrocytes ghost samples were isolated and their protein content was determined by Lowry's method. Erythrocyte ghost samples (1 mg protein) were added to tubes containing 1.5 mL acetic acid (20%

v/v, pH 3.5), SDS (8% w/v, 0.2 mL) and thiobarbituric acid (0.8% w/v, 1.5 mL). The mixture was heated on a boiling water bath for 45 min. Adducts formed were extracted into 1-butanol (3 mL) and the TBARS formed was read at 532 nm and quantified using TMP as the standard. Values are expressed in terms of malondialdehyde (MDA) equivalents as nmol MDA formed per mg protein.

PCC in erythrocyte membranes was measured as described accordingly [48] using dinitrophenylhydrazine (DNPH). To 0.2 mL of erythrocyte membrane samples (1 mg protein), an equal volume of 10 mM DNPH in 2 N HCl was added and incubated for 1 h, shaking intermittently at room temperature. Corresponding blank was carried out by adding only 2 N HCl to the sample. After incubation, the mixture was precipitated with 20% TCA and centrifuged. The precipitate was washed twice with acetone and dissolved in 1 mL of Tris-buffer (20 mM, pH 7.4 containing 140 mM NaCl, 2% SDS-w/v) and the absorbance was read at 360 nm. The difference in absorbance is determined and expressed as nmol of carbonyl groups per mg protein, using an extinction coefficient of 22 $mM^{-1}cm^{-1}$

Determination Glucose 6 phosphate dehydrogenase (G6PDH) activity and estimation of GSH/GSSG levels

G6PDH activity was estimated by monitoring the increase in absorbance at 340 nm for 3 min due to NADP-dependent glucose 6-phosphate transformation (Sundaram et al., 2014). Briefly, for both G6PDH activity and GSH/GSSG level estimation, washed erythrocytes (5% hematocrit in PBS) were treated with high glucose (45 mM) as agonist, and for inhibition studies, high glucose-exposed erythrocytes were incubated with DMTD (0-25 µM) for 6 h at 37 °C. After incubation, erythrocytes were suspended and lysed in distilled water. The lysates were used to determine G6PDH activity in the reaction mixture (1 mL) containing Tris-HCl buffer (50 µM, pH 7.5 containing 3.8 mM NADP,

3.3 mM glucose-6-phosphate, and 6.3 mM $MgCl_2$). The activity was expressed as nmol NADPH formed/min/mg protein.

GSH and GSSG levels were determined as described previously with slight modifications (Sundaram et al., 2014). In brief, after incubation, erythrocytes samples were treated with *o*-phthalaldehyde (1 mg/mL) at room temperature for 15 min to estimate GSH level and for GSSG level, erythrocytes were treated with *N*-ethylmaleimide (10 µM) prior to *o*-phthalaldehyde addition, and the resulting fluorescence was measured at 430 nm by exciting the samples at 365 nm. The concentration of GSH and GSSG were determined from the standard curve.

Determination of stress marker enzyme activities

Superoxide dismutase (SOD) and Catalase (CAT) activities

SOD and CAT activities in erythrocyte lysates were assessed as described accordingly (Sundaram et al., 2015). In brief, 5 % hematocrit in PBS were treated with glucose 5 mM as control and high glucose 45 mM as agonist respectively, for inhibition studies, erythrocytes exposed to high glucose were incubated with DMTD (5 - 25 µM) for 6 h, after incubation erythrocytes were lysed in distilled water and were used for the determination of enzyme activities such as SOD, CAT, GRd, and GPx. To determine SOD activity, 0.1 mL of erythrocyte lysate (0.05 mg protein) was added to the reaction mixture (1 mL) consisting of phosphate buffer (16 mM, pH 7.8) containing TEMED–EDTA (8 mM/0.08 mM) mixture and quercetin (0.15% w/v). The decrease in absorbance was monitored for 1 min at 406 nm. The amount of protein that inhibits quercetin autoxidation by 50% is defined as one unit. Similarly, The CAT activity in erythrocyte lysate was determined by measuring the hydrolysis rate of H_2O_2 at 240 nm. To the reaction mixture (1 mL) containing sodium phosphate buffer (100 mM, pH 7.4), H_2O_2 (8.8 mM), erythrocyte samples (0.05 mg protein) was added. The

decrease in absorbance was monitored for 3 min at 240 nm and the activity was expressed as mmol H_2O_2 decomposed per min per mg protein (ε=43.6 $mM^{-1}cm^{-1}$).

Glutathione reductase (GRd) and Glutathione peroxidase (GPx) activity activities

The glutathione reductase (GRd) activity was measured in erythrocyte lysates as described accordingly (Sundaram et al., 2015). Briefly, 0.1mL of erythrocyte lysate (0.05 mg protein) was added to the reaction mixture (1 mL) containing phosphate buffer [100 mM, pH 7.0 containing 2 mM EDTA, 20 mM GSSG and 2 mM NADPH (in 0.1% $NaHCO_3$(w/v)]. The decrease in absorbance was monitored at 340 nm for 3 min and the activity was expressed as µmol GSSG reduced per min per mg protein.

GPx activity was measured with a coupled enzyme assay as described accordingly (Tung et al., 2017). The assay mixture (1 mL) contained 770 µL of 50 mM sodium phosphate (pH 7.0), 100 µL of 10 mM GSH, 100 µL of 2 mM NADPH, 10 µL of 1.125 M sodium azide, 10 µL 100 U/mL glutathione reductase and 10 µL erythrocyte lysates. The mixture was allowed to equilibrate for 10 min. The reaction was started by adding 50 µL of 5 mM H_2O_2 to the mixture and NADPH oxidation was measured during 5 min at 340 nm. One unit of glutathione peroxidase is defined as the amount of enzyme able to produce 1.0 µmol $NADP^+$ from NADPH per min. GPx activity was determined using the molar extinction coefficient 6.22 $M^{-1}cm^{-1}$ for NADPH at 340 nm and expressed as µmol NADPH oxidized per min per mg protein.

Measurement of Glycated hemoglobin/HbA_{1C} and glucose utilization in erythrocytes

The levels of glycated hemoglobin or HbA_{1c}, an amadori product of protein glycation in hemoglobin was detected by using Glycohemoglobin detection kit. The assay was carried by ion exchange resin method according to manufacturer's protocol. Briefly, 10% hematocrit in PBS were

treated with glucose (5-45mM), for inhibition studies, erythrocytes exposed to high glucose (45 mM) were incubated with DMTD (10-50 µM) for 6 h. After incubation the erythrocyte samples were lysed using hemolysis reagent and incubated with latex reagent at 37 °C for 5 min. The absorbance was measured at 415 nm. The levels of HbA_{1c} were calculated from a standard curve using HbA_{1c} and expressed as % $HbA_{1c.}$

The glucose utilization levels were measured using a commercially available glucose test kit. Briefly, 10% hematocrit in PBS were treated with glucose (5-45 mM), for inhibition studies, erythrocytes exposed to high glucose (45 mM) were incubated with DMTD (10 & 25 µM) for 24 h. The concentrations of glucose were measured before and after the 24 h incubation by glucose oxidase reagent according to manufacturer's protocol. The absorbance was measured at 505 nm. The levels of glucose utilization were calculated by subtracting glucose levels at 24 h from glucose levels at 0 h using the equation below. The result was expressed as m mol/L.

Glucose utilization (mmol/L) = Glucose levels at 0h - Glucose levels at 24h.

Measurement of erythrocytes osmotic fragility and Na^+/K^+ -ATPase activity

The erythrocyte osmotic fragility was measured as the percentage of hemolysis induced by hypotonic solution. An osmolarity (which produces 50% of hemolysis) was calculated as described accordingly (Jain, 1989). Briefly packed erythrocytes (50 µL) were added to the tubes containing 0.55% NaCl with increasing doses of glucose (5-45 mM), similarly in another set of tubes along with packed erythrocytes, 0.55% NaCl, glucose (5-45 mM) DMTD is added dose dependently ranging from 5-50 µM. All the tubes were then incubated at room temperature for 30 min, the samples were then subjected to centrifugation at 450×*g* for 10 min, supernatants were collected and hemolysis was then estimated by measuring the absorption of hemoglobin released at 540 nm, using a US/Vis Beckman spectrophotometer.

The Na^+/K^+ -ATPase activity was measured accordingly with minor modifications (Sompong et al., 2015). Briefly, 10% hematocrit in PBS were treated with high glucose 45 mM as agonist, for inhibition studies, erythrocytes exposed to high glucose were incubated with DMTD (10 & 25 µM) for 12 h, after incubation erythrocytes ghost samples were isolated and their protein content was determined by Lowry's method. The erythrocyte membranes were incubated with reaction buffer A containing 4 mM $MgCl_2$, 3 mM ATP-Na_2, and 50 mM Tris-HCl, pH 7.4, and buffer B containing 120 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 3 mM ATP-Na_2, and 50 mM Tris-HCl, pH 7.4 at 37 °C for 1 h. After the incubation, the levels of phosphate (Pi) released from ATP-Na_2, were measured according with minor modifications (Sompong et al., 2015). The reaction mixture was incubated with ammonium molybdate (2.5% w/v) at room temperature for 10 min. Then, ascorbic acid (2% w/v) was added and kept at room temperature for 20 min for color development. The absorbance was measured at 725 nm. The levels of Pi release were calculated from a standard curve using KH_2PO_4. Na^+/K^+ -ATPase activity was calculated using the equation below. The results were expressed as nmole Pi/mg protein/h.

Na^+/K^+ -ATPase activity= *Pi* in the reaction buffer B - *Pi* in the reaction buffer A

Recruitment of Diabetes mellitus (DM) subjects

DM subjects (n=20) from Government Ayurvedic Medical College (GAMC), Mysuru were recruited for the study. DM subjects were recruited purely on basis of plasma glucose level (reference range: more than 150-200 mg /dL of blood). Viral and cirrhotic infected patients were excluded from the study. All the experiments were in accordance and approved by the Institutional Human Ethical Committee, (IHEC-UOM No.146/Ph.D./2016-17) University of Mysore, Mysuru. Venous blood was drawn from DM subjects and non-smoking/drug-free healthy subjects (HS, n = 20) by trained professionals from GAMC, Mysuru. The draw blood was immediately mixed with

ACD in the ratio of 6:1 (blood: ACD, v/v). About 100 µL aliquots of anti-coagulated blood was used to determine cell count in an automated hemo-analyser (Sysmex KX-21, Japan). Further, the remaining anti-coagulated blood was then centrifuged to separate plasma. The packed RBCs were further washed thrice with PBS and finally suspended to 0.4 % hematocrit for further fluorescence assays.

Measurement of eryptosis markers and Na^+/K^+-ATPase activity in DM patient's erythrocyte samples

Eryptosis markers like elevation in ROS, intracellular calcium, PS scrambling and Na^+/K^+ -ATPase activity in erythrocytes from DM patients were determined as described previously using Varioskan multimode plate reader (Thermo Scientifics, USA) (Lupescu et al., 2012; Sompong et al., 2015). Briefly, for inhibition studies isolated and washed erythrocytes from DM patients were treated with DMTD (50 µM) after incubation for 6 h, the samples were treated with respective fluorescent probes. Finally, cells were collected by centrifugation and fluorescence was measured.

Determination of *in vivo* toxicity of DMTD

Mice model was used to assess the *in vivo* acute toxicity of DMTD. Swiss albino mice (8 weeks old) obtained from Central Animal Facility (University of Mysore, Mysuru) were used in the present study. The animal experiment was reviewed and authorized by Institutional Animal Ethical Committee (UOM/IAEC/26/2016; Dated 30-07-2016) University of Mysore, Mysuru. Animal handling and experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The obtained animals were acclimatized for one week prior to start of the experiment. The animals were grouped into 3 groups consisting of 6 animals per group (n=6). Group I-Saline control mice; Group II and III-Mice treated with DMTD 50 mg/kg and 100 mg/kg body weight respectively. Aqueous solution of

DMTD (in saline) was administered orally to the respective experimental groups using intragastric tube (gavage) once in a day in the morning for 8 consecutive days. Body weight was checked every day throughout the experimental period. On completion of experiment on day 8, animals were starved overnight and were euthanized, and blood was obtained *via* cardiac puncture. The obtained whole blood was allowed to clot at 4 °C and serum was obtained by centrifuging at 1200× *g* for 10 min and stored at -20 °C until further use. A portion of blood was mixed with anticoagulant and subjected to complete blood count namely total leukocyte count (WBC), erythrocyte count (RBC), platelets (PLT), hemoglobin content (Hb) and hematocrit (HCT) using an automated hematology analyzer (Sysmex KX-21, Japan). Immediately after euthanizing the animals, vital organs such as liver, kidney and spleen were carefully dissected out and blotted free of blood in ice cold saline, weighed and photographed to assess the gross appearance. Furthermore, lungs, stomach, colon and small intestine were also dissected out and washed in ice cold saline. The harvested liver, kidney, spleen, lungs, stomach, colon and small intestine were fixed in 10% buffered formalin for overnight. The tissue samples were subjected to dehydration by processing with different grades of alcohol and chloroform mixture. The processed tissues were embedded in paraffin wax, and sections of 5 µm thickness were prepared, stained with hematoxylin-eosin dye (H &E) and observed under an Axio imager.A2 microscope (Oberkochen, Germany) and photographed. From the serum, various biochemical parameters such as levels of lactate dehydrogenase (LDH), creatine kinase (CK), blood urea nitrogen (BUN), alanine transaminase (ALT), aspartate transaminase (AST) were assessed using commercial kits (Agappe) as per manufacturer's protocol.

Protein estimation

Protein estimation was carried out according to the method of Lowry et al. (Lowry et al., 1951), using BSA as standard.

Statistical analysis

Results were expressed as mean ± SEM of five independent experiments. Statistical significance among groups were determined by one-way analysis of variance (ANOVA) followed by Tukey's test for comparison of means [n = 5, p */$^{\#}$< 0.05, p **/$^{\#\#}$ <0.01, p ***/$^{\#\#\#}$<0.001;* significant compared to untreated erythrocytes. # significant compared to high glucose-treated erythrocytes].

Results

Synthesis of 1-(2, 2-dimethyltetrahydrofuro [2, 3] [1, 3] dioxol-5-yl) ethane-1, 2-diol (DMTD)

DMTD was synthesized according to the previously reported method (Barton and McCombie, 1975) with minor modifications (**Fig. 1A**). Synthesis of the target molecule starts with deoxygenation of the hydroxyl group on the C-3 carbon of the compound **1** *via* Barton, McCombie's reaction (Barton and McCombie, 1975), which produces the compound **2**. In order to get the DMDT compound, selective deprotection of the terminal acetonide of compound **2** was achieved with 70% aqueous solution of acetic acid.

Spectral characterization data of DMTD White solid; m.p. 64-66°C; IR (KBr, Cm^{-1}) 3600, 2900, 1100; ^{1}H NMR (400 MHz, $CDCl_3$) δ : 5.70-5.69 (d, J = 3.6 Hz, 1H), 4.65-4.63 (t, J = 4.0 Hz, 1H), 4.09-4.04 (m, 1H), 3.84 (br s, 2H), 3.75-3.73 (t, J = 3.0 Hz, 1H), 3.59-3.55 (dd, J = 2.8 & 3.2 Hz, 1H), 3.44-3.39 (q, J = 6.8 Hz, 1H), 1.97-1.93 (m, 1H), 1.77-1.70 (m, 1H), 1.39 (s, 3H), 1.21 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 111.1, 105.1, 80.4, 78.3, 72.2, 63.5, 33.6, 26.6, 26.0.

Effect of Glucose on erythrocytes *in vitro*

Incubation of erythrocytes under conditions of high glucose concentrations is usually used as a model to mimic the *in vivo* condition of hyperglycemia in diabetes. Exposure of erythrocytes to high glucose significantly increased ROS and cytosolic calcium level with PS translocation to outer

cell surface in a dose-dependent manner when compared to control (**Fig. 1B**). In the next set of experiments, erythrocytes were incubated with 45 mM glucose for a different time interval starting from 0-48 h, which resulted in significant ROS generation, increased cytosolic calcium and PS externalization after 6 h when compared to control samples (**Fig. 1B**). Therefore, for further experiments a dose of 45 mM glucose exposed for 6 h was considered as high glucose level, which mimics the pathological diabetic complication in RBCs inducing oxidative stress. RBCs exposed to 5 mM glucose was employed as control as normal glucose level in individuals (non-diabetic humans) prior to meal is 4 to 5.8 mM.

DMTD alleviates high glucose-induced ROS generation, intracellular calcium levels & PS scrambling

The effect of DMTD on hyperglycemia-induced ROS production, intracellular calcium concentration and PS scrambling in erythrocytes were measured further. As illustrated (**Fig. 1C**), 45 mM glucose-induced significant increase in DCF fluorescence suggesting the elevated generation of ROS. However, glucose-induced DCF fluorescence was significantly diminished by 2 folds in presence of 25 µM DMTD respectively, demonstrating its ability to alleviate hyperglycemia-induced ROS production. Similarly, fluorometric analysis measuring intracellular Ca^{2+} levels demonstrated that DMTD (25 µM) effectively ameliorated high glucose-induced Ca^{2+} levels in erythrocytes (**Fig. 1D**).

A rise in intra-erythrocyte Ca^{2+} level further triggers cell membrane scrambling with PS translocation signaling event in erythrocytes to undergo erythrophagocytosis, which can be identified by Annexin V-FITC binding to apoptotic erythrocytes. As illustrated in **Fig. 1E**, Annexin V binding of erythrocytes was indeed enhanced by high glucose exposure. However, presence of DMTD

significantly inhibited high glucose-induced PS externalization by 2 folds when compared to high glucose treated cells and abrogated the annexin V binding to erythrocytes.

DMTD down-regulates the levels of membrane proteins, Calpain and Band 3

An increase in intracellular calcium level is further accompanied by activation of calcium sensitive cysteine protease calpain, and Band 3, a membrane glycoprotein, which drives erythrocytes towards apoptosis. High glucose pre-treated erythrocytes showed ten folds increase in the protein levels of both calpain and band 3 when compare to control, however DMTD (25 µM) treatment substantially decreased the expression of both calpain and band 3 by five folds when compared to high glucose treated cells, which is evident from immunoblots (**Fig. 2A** and **2B**).

DMTD restores high glucose-induced erythrocyte shape change

High glucose exposure in erythrocytes is followed by the structural and pathophysiological alterations in morphology of erythrocytes, which could be observed using AFM topography. High glucose (45 mM) induced significant alterations in their structure, leading to the loss of their normal biconcave shape when compared to control, however treatment of affected cells with DMTD (25 µM), remarkably restored back the normal shape of erythrocytes (**Fig. 2C**) (**Table. 1**).

DMTD maintains erythrocyte viability upon high glucose stress

To further evaluate the hyperglycemia-induced erythrocyte toxicity and its protective potential of the DMTD, calcein fluorescence assay and LDH activity were carried out. Erythrocytes treated with 45 mM glucose exhibited decreased calcein fluorescence and this was significantly reversed by the presence of DMTD (**Fig. 3A**). Further, LDH leakage is yet another important marker used to evaluate the erythrocyte death. Treatment of erythrocytes with high glucose, induced the release of LDH into the medium whereas, the presence of DMTD was able to significantly inhibit LDH release owing its cytoprotective efficacy (**Fig. 3B**).

DMTD prevents high glucose-induced oxidative damage and restores endogenous antioxidant enzyme activities

An abrupt increase in the intracellular oxidative stress markers defines the severity of the oxidative damage in a cell. Erythrocytes treated with high glucose exhibited a severe elevation in the levels of oxidative stress markers such as LPO and PCC to an extent of 80% and 73.5% respectively, and decrease in G6PDH activity and the ratio of reduced/oxidized glutathione to an extent of 65% and 51% respectively when compared to control (**Fig. 3C-F**). The erythrocytes treated with DMTD (25 µM) lowered the LPO (**Fig. 3C)** and PCC (**Fig. 3D**) content effectively by 75% and 72% respectively when compared to high glucose alone treated erythrocytes. In addition, treatment of DMTD also restored G6PDH (**Fig. 3E**) activity and GSH/GSSG (**Fig. 3F**) ratio very effectively up to 91% and 93% respectively aiding endogenous GSH replenishment.

Furthermore, endogenous antioxidant enzymes play a crucial role in fighting any form of oxidative stress, hence to evaluate the impact of DMTD on hyperglycemia-induced redox imbalance we assessed the activity of key endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GRd) and glutathione peroxidase (GPx), in high glucose-treated erythrocytes in presence and absence of DMTD (**Fig. 4A and 4B**). Severe changes in the homeostasis of all the antioxidant enzymes were evident in high glucose-treated erythrocytes. A drastic decline in the SOD and CAT activities upto 85% and 64% respectively were observed in hyperglycemic erythrocytes, which was revoked effectively by DMTD (25 µM) to the extent of 80% and 75% respectively. Similarly, activities of glutathione replenishing enzymes like GRd and GPx were reduced by 85% and 70% respectively upon treatment of erythrocytes with high glucose when

compared to control cells. Nevertheless, treatment of cells with DMTD (25 µM) restored the altered GRd and GPx activity up to 90% and 92% respectively when compared to hyperglycemic cells.

DMTD reduces protein glycation (Glycated hemoglobin/HbA_{1C}) and enhances glucose utilization

The dose-dependent effect of DMTD on high glucose treated amdori product formation in erythrocytes were shown in **Fig. 5A**. Erythrocytes treated with high glucose (25 and 45 mM) showed significant increase in HbA_{1C} levels when compared to control cells, however incubation of high glucose exposed erythrocytes with DMTD (10-50µM) resulted in decline of HbA_{1C} level in a dose-dependent fashion. Further, effect of DMTD on glucose utilization in erythrocytes treated with glucose for 24 h was determined. We observed a significant enhancement in glucose utilization by RBCs with 45mM glucose. DMTD (10 and 25 µM) incubated along with 45 mM glucose induced a gradual increase in glucose utilization and a more efficient utilization was found at 25 µM concentration **(Fig. 5B**).

Effect of DMTD on osmotic fragility of erythrocytes

Osmotic fragility refers to susceptibility of erythrocytes to hemolysis under oxidative stress conditions by varying the osmolarity of NaCl from 0.25 to 0.9%, as illustrated in (**Fig. 5C**). Osmotic fragility of RBCs increases with high glucose level (25 and 45 mM), however there was a significant recovery in the percentage of RBCs fragility in DMTD-treated erythrocytes when compared to high glucose-treated RBCs in 0.55% NaCl concentration. There was a dose-dependent inhibition of hemolysis in DMTD-treated erythrocytes.

Effect of DMTD Na^+/K^+ -ATPase activity

The effects of DMTD on Na^+/ K^+ -ATPase activity in erythrocytes treated with high glucose are shown in **(Fig. 5D)**. A remarkable reduction in Na^+/K^+-ATPase activity was observed with 45

mM glucose when compared to control. However, incubation of erythrocytes with DMTD reversed the inhibitory effect of high glucose on Na^+/K^+-ATPase activity dose dependently as compared to 45 mM glucose-treated erythrocytes.

Effect of DMTD on erythrocyte of Diabetic patients

About 20 diabetic patient samples with plasma glucose level more than 150 mg/dL were evaluated for their erythrocytes ROS level, intracellular calcium concentration, PS scrambling and Na^+/K^+ -ATPase activity. As illustrated in **Fig. 6**, there is a significant increase in ROS levels (**Fig. 6A**), intracellular calcium concentration (**Fig. 6B**), PS scrambling (**Fig. 6C**) in erythrocytes of diabetic patients. In contrast, Na^+/K^+ -ATPase activity (**Fig. 6D**) was reduced significantly in diabetic patients when compared to normal individuals; however, treatment of erythrocyte samples with 50 µM DMTD reduced the increased levels of ROS, intracellular Ca^{2+}, PS scrambling and recovered back the Na^+/K^+ -ATPase activity.

DMTD is non-toxic *in vivo* as tested in a mice model

In order to assess the acute toxicity of DMTD, mice were orally treated with two dosages of DMTD for 8 days concurrently and monitored for the changes in body weight, toxic symptoms and mortality rate. **Fig. S1** represents the relative body weight in percentage from day 1 to day 9 (**Fig. S1 A**) and percentage change in body weight on day 9 alone of experimental animals (**Fig. S1 B**). Results clearly indicate that there was no significant change in the body weight of mice treated with DMTD throughout the experimental period with respect to saline. Water and feed intake were also monitored in experimental mice, DMTD treated mice exhibited normal water and feed intake as compared to saline control. Furthermore, no mortality and toxic symptoms were observed in DMTD treated mice confirming the non-toxic nature of DMTD. (All the graphs and data related to *in vivo* studies were submitted as supplementary figures)

Anti-coagulated blood isolated from mice through heart puncture was analyzed for total blood cell count to assess the possible effect of DMTD on blood components. Supplementary table S1 represents the total cell count including WBC, RBC, PLT, Hb and HCT of mice treated with different dosages of DMTD (**Table. S1**). Results clearly indicate that there was no statistical difference between the saline- and DMTD- treated groups. LDH, a cytoplasmic biomarker enzyme, is widely used in toxicology and in various pathologies to assess the cell, tissue and organ damage. Likewise, CK, a central controller of cellular energy homeostasis, plays particularly important role in tissues with large and fluctuating energy demands. Both serum LDH (**Fig. S1 C**) and CK (**Fig. S1 D**) activities were estimated in mice treated with DMTD. The results suggested that there was no change in the levels of these enzymes in comparison with saline control.

Liver toxicity was assessed by estimating the serum levels of ALT, AST along with the gross appearance and histological analysis. Liver from DMTD-treated groups showed no significant difference in weight (**Fig. S2 A**) in comparison with saline control group. Serum ALT and AST levels of DMTD-treated mice showed no difference statistically in comparison to saline control (**Fig. S2 B & C**). The histological analysis of DMTD-treated liver tissue sections revealed no signs of congestion, vacuolization, hepatocyte necrosis and infiltration of inflammatory cells. In addition, DMTD-treated liver sections exhibited normal hepatic architecture with respect to saline control group (**Fig. S2 D**). Furthermore, kidney toxicity was assessed by estimating the levels of BUN along with the gross appearance and histological analysis. DMTD treated mice showed no significant change in kidney weight (**Fig. S3 A**) and BUN levels (**Fig. S3 B**). Macroscopic and microscopic analysis also revealed that DMTD-treated mice kidneys showed normal morphology and devoid of Bowmans's capsule damage, urinary and vesicle pole damage, necrosis, constriction of distal tubules and interstitial nephritis, and had normal renal architecture in comparison with saline control group

(**Fig. S3 C**). In addition, splenic damage was assessed by gross appearance, weight and histological analysis. DMTD-treated mice showed no significant difference in spleen weight (**Fig. S4 A**) and the longitudinal section of spleen revealed normal white and red pulp architecture, and were devoid of vacuolization of splenic histocytes, hyperplasia lesions and red pulp pigmentation (**Fig. S4 B**).

Furthermore, the effect of DMTD on lungs, stomach, colon and small intestine were histomorphologically assessed. The obtained result clearly indicates that there was no significant difference in any of these histological parameters between DMTD treated and saline control groups (**Fig. S5 & S6**). The *in vivo* acute toxicity study clearly demonstrates that DMTD is non-toxic in nature.

Discussion

Although RBCs lack nuclei and mitochondria, they are very sensitive and are also chief markers of health. The RBCs with their highly dedicated membrane structure are persistently exposed to various components in the circulatory system. Therefore, they frequently interact or react to certain components, which might trigger the process of eryptosis. Eryptosis studies usually involves the determination of ROS generation, increased intracellular calcium level, externalization of PS from inner leaflet to outer leaflet of plasma membrane, activation of cytoskeleton degrading enzymes, which ultimately lead to cell shrinkage and death of erythrocytes. Some of the pathological conditions associated with elevated rate of eryptosis include type-2 diabetes, malaria, chronic renal failure, hemolytic uremic syndrome, sepsis, sickle cell anemia, thalassemia etc (Pretorius et al., 2016). Our continuing efforts and interests in developing multicomponent reaction for the synthesis of various organic compounds and to check their potency for different biological activities (Girish et al., 2015; Jagadish et al., 2016; Narasimhamurthy et al., 2013), prompted us to adopt the simple synthetic route to produce oxolane derivative (DMTD) and evaluated its protective efficacy on

hyperglycemia-induced premature death of RBCs. In the present study, we have probed the effect of chronic diabetes-associated high glucose concentration on RBCs, particularly the events of eryptosis and its amelioration by DMTD. Apart from xenobiotics, oxidative stress is one of the key triggers of eryptosis (Bartoskova et al., 2013; Faggio et al., 2016; Mischitelli et al., 2017). It was found that hyperglycemic conditions indeed increased the ROS levels, and there was a concurrent increase in intracellular Ca^{2+}levels followed by PS scrambling, which are the major sign of eryptosis. Previous studies show that during hyperglycemic conditions, there is an increased generation of advanced glycation end-products (AGEs), which are known pro-oxidants, and their buildup leads to chronic oxidative stress (Vlassara et al., 1992). When RBCs are persistently exposed these AGEs, the former undergo changes in their morphology and damage in their membranes, resulting in abnormally high rate of eryptosis. Thus, anemia is frequently seen in diabetic patients (Awasthi et al., 2015). Therefore, regulating oxidative stress seems to be the key to combat anemia in diabetics. Till date, studies have demonstrated the anti-eryptotic effects of different plant-derived/synthetic/anti-oxidant molecules including resveratrol, naringin, xanthohumol, probucol, nitric oxide, ferulic acid, zidovudine, blebbistatin, salidroside etc., which were tested under various *in vitro* conditions (Qadri et al., 2009; Shaik et al., 2013; Zierle et al., 2016). To this end, we synthesized a potent small molecule 1-(2,2-dimethyltetrahydrofuro[2,3][1,3]dioxol-5-yl)ethane-1,2-diol(DMTD), an oxolane derivative, and demonstrated for the first time its anti-eryptotic effects on hyperglycemia-induced RBCs as well on RBCs from blood samples of diabetics under *in vitro* conditions.

In the present study, we observed an increased production of ROS and intracellular Ca^{2+}, externalization of PS, altered levels of oxidative stress markers such as LPO, PCC, G6PDH and GSH/GSSG ratio, and decreased activities of key endogenous antioxidant enzymes such as SOD, CAT, GRd and GPx in hyperglycemia-induced RBCs. Upon treatment of these RBCs with DMTD,

there was remarkable reduction in ROS levels, stabilization of oxidative stress markers and maintains the antioxidant enzyme activities. The protective efficacy of DMTD can be attributed to the presence of hydroxyl group in its side chain. These hydroxyl groups might be able to quench the free radicals generated during oxidative stress and by the autoxidation of glucose in presence of transition metal ions. In addition, DMTD may also quench the free radicals generated by autoxidation of glycated proteins, which are formed during hyperglycemia. DMTD further restores the anti-oxidant enzymes by maintaining the osmotic fragility of erythrocytes via revoking the Na^+/ K^+-ATPase activity (Waczulíkova et al., 2000). Thus, we can infer that DMTD has the potential to effectively regulate oxidative stress in RBCs under hyperglycemic conditions and maintains the antioxidant indices. To further determine whether this inhibition of oxidative stress by DMTD could successfully reduce the rate of eryptosis, we evaluated its effect on the eryptotic events such as increase in cytosolic Ca^{2+}, activation of calpain and Band 3 glycoprotein, PS membrane scrambling, and morphological changes in hyperglycemia-induced RBCs. DMTD considerably averted the above-mentioned key events of eryptosis. These findings suggest that DMTD has the potential to treat anemia and associated micro vascular complications such as RBC aggregation and endothelial adhesion in diabetics.

Hyperglycemic condition in diabetics is also shown to cause increased rate of non-enzymatic glycation, the process of glucose binding to amino groups of proteins resulting in the formation and accumulation of AGEs. This in turn leads to free radical generation via auto-oxidation of glucose and glycated proteins. AGEs also interact with cell surface AGE receptors, the ensuing events being oxidative stress, vascular dysfunction and inflammation, which further add to diabetic complications (Niedowicz and Daleke, 2005). The free radicals and oxidative stress cause alterations in erythrocytes morphology and function including shape change, membrane lipid peroxidation,

increased membrane fragility and rigidity, and lipid fluidity, this results in decreased levels of antioxidant enzymes (Waczulíkova et al., 2000).The results of the present study demonstrate that DMTD significantly decreased glycated hemoglobin (HbA_{1C}) level and increased glucose utilization by erythrocytes. Further, DMTD was also able to revoke osmotic fragility and shape change, and conferred cytoprotective effect on hyperglycemia-induced erythrocytes.

Conclusion

Taken together, our findings confirm that high glucose or chronic hyperglycemia associated with type 2 DM can induce oxidative damage on erythrocytes causing eryptosis. We also demonstrate that the synthesized small molecule DMTD effectively revoked high glucose-induced oxidative damage and subsequent apoptosis of erythrocytes. In addition, the study also demonstrates that DMTD significantly decreased glycated hemoglobin (HbA_{1C}) level and increased glucose utilization and was able to revoke high glucose-induced osmotic fragility and shape change, and conferred cytoprotective effect on erythrocytes. The *in vivo* studies to evaluate the toxicity of DMTD confirmed that the molecule is non-toxic to treated mice and its major vital organs. With this, our study suggests that DMTD could be a potential therapeutic candidate to treat chronic hyperglycemia-induced anemia and associated microvascular complications such as RBC aggregation and endothelial adhesion in diabetics.

Competing interests

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Legends

Table 1

Depicting the Morphological and biophysical parameters of control erythrocytes and glucose (45 mM) treated erythrocytes in presence or absence of DMTD detected by AFM. In an average 5- 10 cells were considered with mean height, diameter, membrane roughness and concave depth. Values are presented as mean ± SEM (n = 5), expressed as percentage increase in morphological changes relative to control. $^{**}p<0.001$ significant compared to control. $^{\#}p<0.05$, $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ significant compared to high glucose alone. One-way analysis of variance (ANOVA) followed by Tukey's test was used to determine significance.

Figure 1. Synthesis of 1-(2,2-dimethyltetrahydrofuro[2,3][1,3]dioxol-5-yl)ethane-1,2-diol (DMTD) and its effects on high glucose treated erythrocytes. (A) Reaction scheme for the Synthesis of 1-(2,2-dimethyltetrahydrofuro[2,3][1,3]dioxol-5-yl)ethane-1,2-diol (DMTD). **(B)** Dose and time dependent effect of glucose on erythrocytes. Exposure of erythrocytes to various concentrations of glucose (0-45 mM) induces dose dependent generation of ROS, intracellular calcium and PS externalization as compared to control and exposure of erythrocytes to glucose (45 mM) for varying time intervals (0-48 h) induces time dependent increase in generation of ROS, intracellular calcium and PS externalization as compared to control. **(C)** Effect of DMTD on the high glucose induced endogenous ROS generation and its representative bar diagram showing median changes of DCF fluorescence in folds. **(D)** Effect of DMTD on the high glucose induced intracellular calcium levels. **(E)** Effect of DMTD on the high glucose induced PS externalization in erythrocytes and its representative bar diagram showing median changes of FITC conjugated Annexin-V fluorescence in folds. Briefly, washed erythrocytes were incubated to a final reaction mixture of 0.4 % hematocrit in ringer's solution supplemented with glucose (45 mM) in the presence and absence of DMTD. Values are presented as mean ± SEM (n = 5), expressed as percentage increase in fluorescence of DCF, Fura-2/AM and annexin relative to control. ***p<0.001 significant compared to control. $^{\#}p$<0.05, $^{\#\#}p$< 0.01, $^{\#\#\#}p$< 0.001 significant compared to high glucose alone. One-way analysis of variance (ANOVA) followed by Tukey's test was used to determine significance.

Figure 2. Effect of DMTD on high glucose induced activation of calpain, Band-3 and morphological changes in erythrocytes. (A) & (B) Immunoblot showing the activation of calpain and Band 3 in erythrocytes membrane samples. Briefly, washed erythrocytes were incubated to a final reaction mixture of 10% hematocrit in Ringer's solution supplemented with glucose (45mM) in the presence and absence of DMTD for 12 h at 37 °C. After the incubation, cells were lysed and ghost membranes were isolated, protein content in ghost membrane fractions were determined, 50 µg of proteins were loaded, separated by SDS-PAGE and immunoblotted. Band 3 and calpain proteins were detected using anti-band 3 and anti µ-calpain antibodies respectively, GAPDH and β-actin were used as loading controls. **(C)** Morphological alterations of erythrocytes treated with glucose 45 mM for 6 h at 37 °C in presence or absence of DMTD as determined by AFM imaging (Park systems NX-10 AFM).

Figure 3. Effect of DMTD on high glucose induced cytotoxicity in erythrocytes and assessment of stress markers. (**A)** Determination of cell viability using Calcein fluorescence and (**B)** LDH leakage. Briefly, washed erythrocytes were incubated to a final reaction mixture of 1% hematocrit in PBS supplemented with glucose (45mM) in the presence and absence of dose-dependent DMTD. Effect of DMTD on high glucose induced **(C)** Lipid peroxidation (LPO), **(D)** Protein carbonyl content (PCC), (**E)** Glucose 6 phosphate dehydrogenase activity (G6PDH) and **(F)** GSH/GSSG ratio in erythrocytes. Briefly, washed erythrocytes were incubated to a final reaction mixture of 5% hematocrit in PBS supplemented with glucose (45mM) in the presence and absence of DMTD. LPO

and PCC were determined using erythrocyte membrane samples (1mg/mL). For the determination of G6PDH and GSH/GSSG ratio erythrocytes lysates were used. Values are represented as mean ± SEM (n = 5). $p^{*}< 0.05$, $p^{**}< 0.01$, $p^{***}< 0.001$; significant compared to control. $^{\#}p < 0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ significant compared to high glucose alone. One-way analysis of variance (ANOVA) followed by Tukey's test was used to determine significance among each groups.

Figure 4. Effect of DMTD on Antioxidant enzyme activities in high glucose treated erythrocytes. Briefly, washed erythrocytes were incubated to a final reaction mixture of 5% hematocrit in PBS supplemented with glucose (45 mM) in the presence and absence of DMTD. (**A)** Superoxide dismutase and Catalase activity, **(B)** Glutathione reductase (GRd) and Glutathione peroxidase (GPx) activity. Values are represented as mean ± SEM (n=5). *** $p<0.001$ significant compared to control. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ significant compared to high glucose. One-way analysis of variance (ANOVA) followed by Tukey's test was used to determine significance.

Figure 5. Effect of DMTD on High glucose induced hemoglobin glycation, glucose uptake, osmotic fragility and Na^{+}/k^{+}-ATPase activity in erthrocytes. (A) Protein glycation (glycated hemoglobin or HbA_{1C}), **(B)** Estimation of glucose utilization in erythrocytes treated with 45 mM glucose in presence or absence of DMTD by GOD-POD method **(C)** Assessment of osmotic fragility in erythrocytes treated with dose dependent glucose with 0.55% NaCl in presence or absence of DMTD. **(D)** Estimation of Na^{+}/k^{+} ATPase activity in high glucose treated erythrocytes membrane in presence or absence of DMTD. The results are expressed as mean ± SEM (n = 5). $p^{*}< 0.05$, $p^{**}< 0.01$, $p^{***}< 0.001$; significant compared to control. $^{\#}p < 0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ significant compared to high glucose alone. One-way analysis of variance (ANOVA) followed by Tukey's test was used to determine significance.

Figure 6. Effect of DMTD on early eryptotic markers in RBCs of human hyperglycemic (HG) subjects. Hyperglycemia induces early eryptosis as assessed by fluorometric assays and presented as percentage increase/decrease in fluorescence of (**A)** ROS, (**B)** intracellular Ca^{2+}, **(C)** PS externalization and (**D)** Na^{+}/K^{+} activity. Values are presented as mean ± SEM and expressed as percentage increase/decrease in DCF, fura and annexin. *P**< 0.05, p**< 0.01, *p****< 0.001; significant compared to Control (healthy subjects). $^{\#}p$ <0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 significant compared to HG. One-way analysis of variance (ANOVA) followed by Tukey's test was used to determine significance among each groups.

Table. 1

Contents	Control	Glucose (45mM)	Glucose (45mM) + DMTD (25µM)
Diameter	7.69 µm± 0.11 µm	6.51 µm± 0.04 µm**	7.55 µm± 0.12 µm$^{\#\#}$
Height	2.87 µm± 0.09 µm	2.01 µm± 0.08 µm*	2.52 µm± 0.10 µm$^{\#}$
Membrane roughness	2.84 nm± 0.11 nm	2.09 nm± 0.11 nm*	2.71 nm± 0.19 nm$^{\#\#}$
Concave Depth	321.03 nm± 84.6 nm	213.25 nm±32.32 nm**	302.32 nm± 102.6 nm$^{\#\#}$

Figure .1

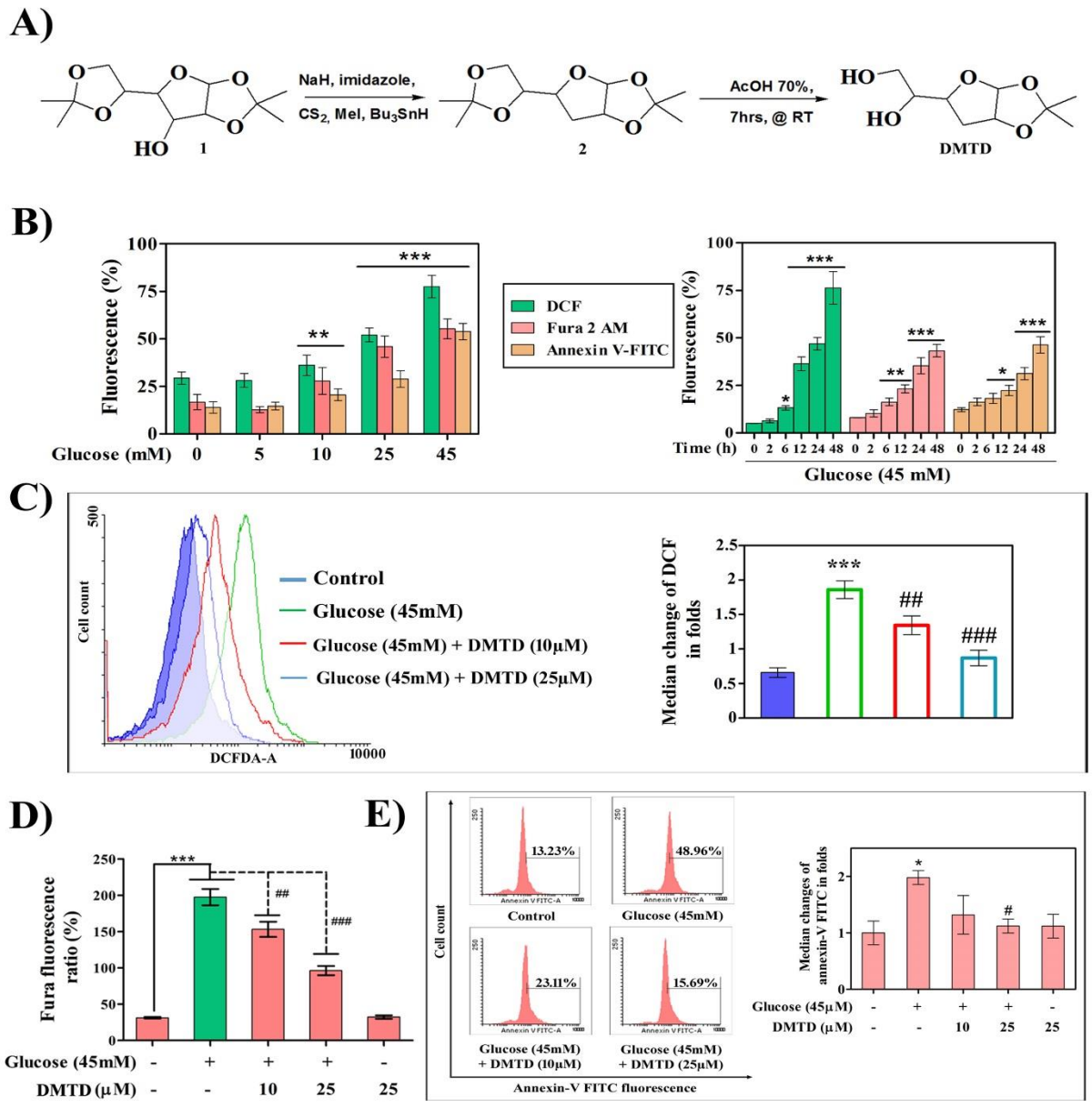


Figure. 2

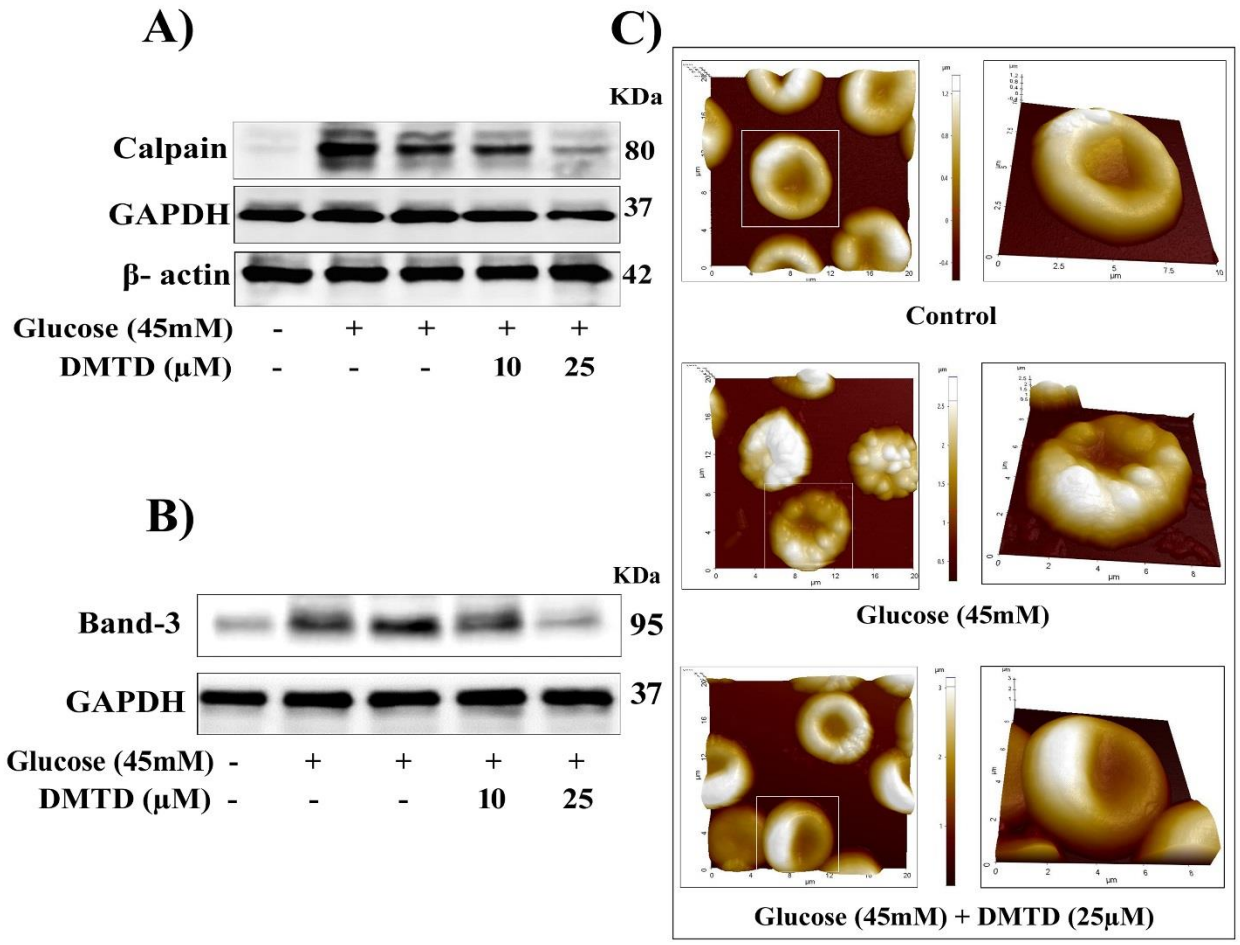


Figure. 3

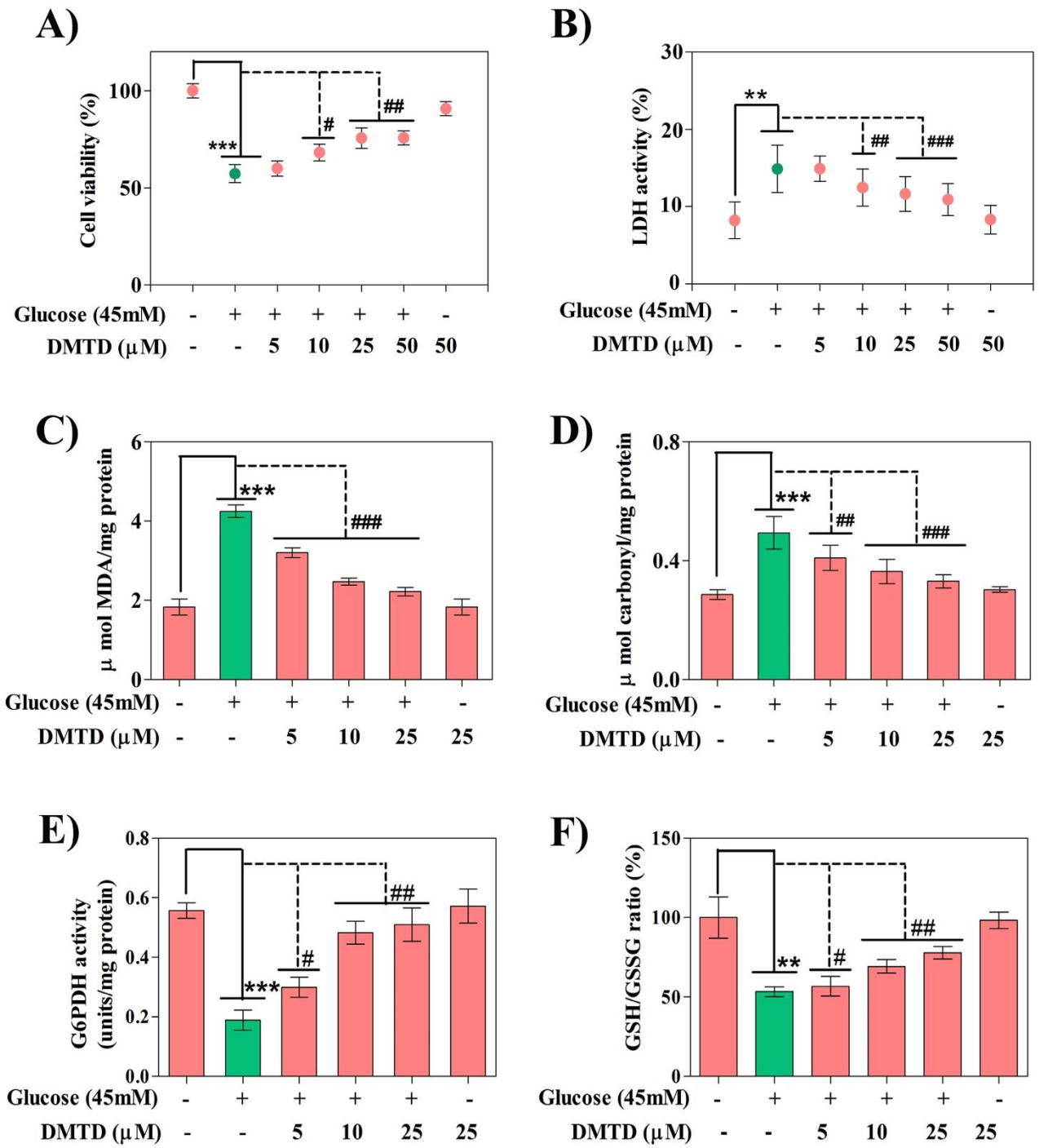
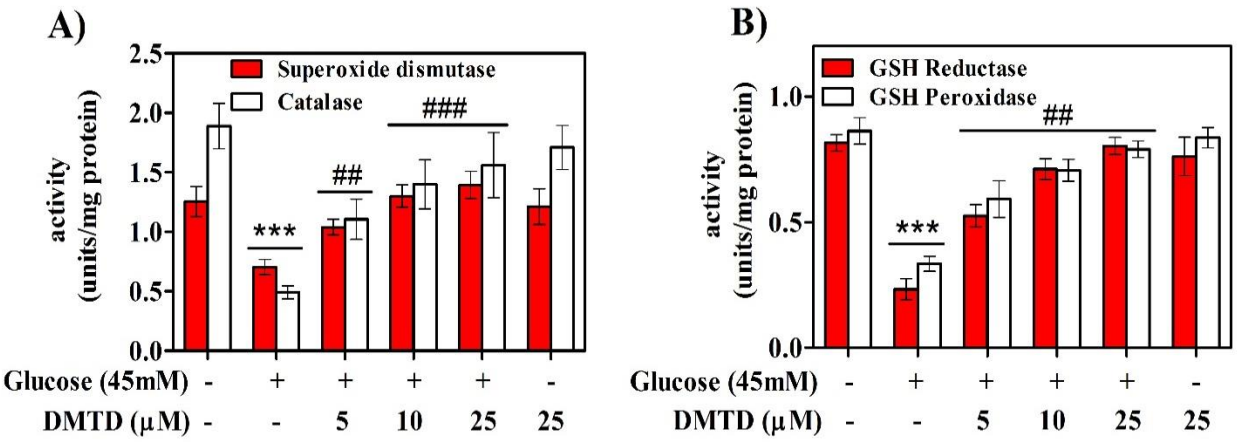


Figure. 4



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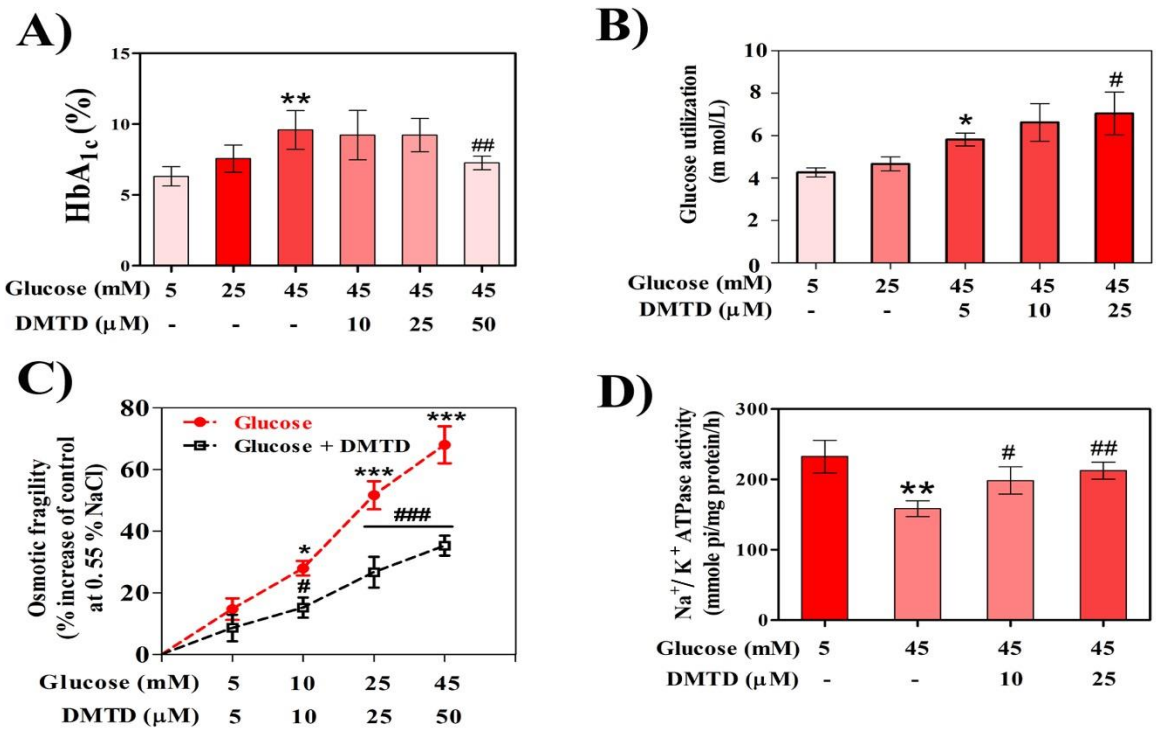

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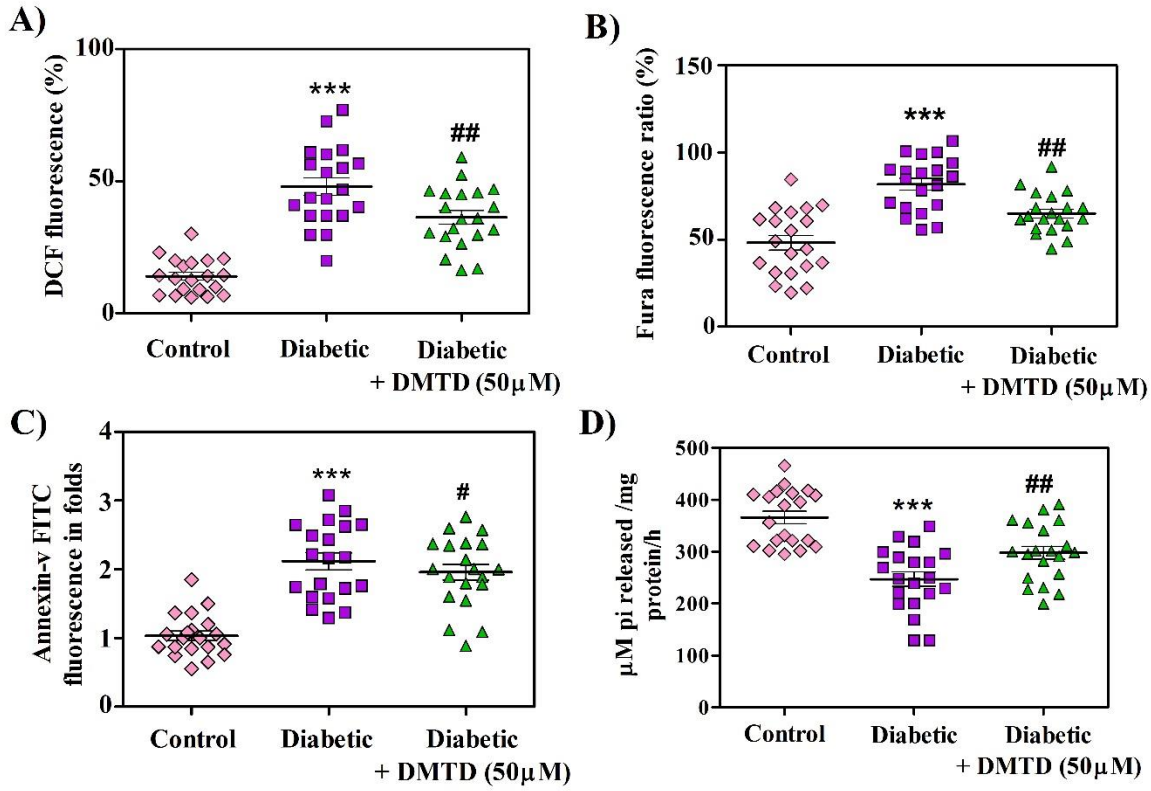
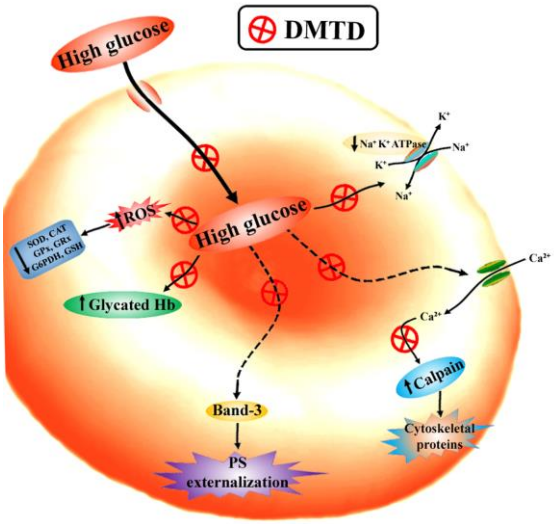


Graphical abstract



Highlights

- Chronic hyperglycemia exerts deleterious effects on erythrocytes leading to anemia
- High glucose induced ROS instigates eryptosis and associated vascular complications
- Novel oxolane derived DMTD ameliorates hyperglycemia induced ROS ensuing eryptosis
- DMTD inhibits hyperglycemia induced Ca^{2+} levels, PS scrambling & calpain activation
- DMTD a promising therapeutic candidate to treat hyperglycemia induced eryptosis