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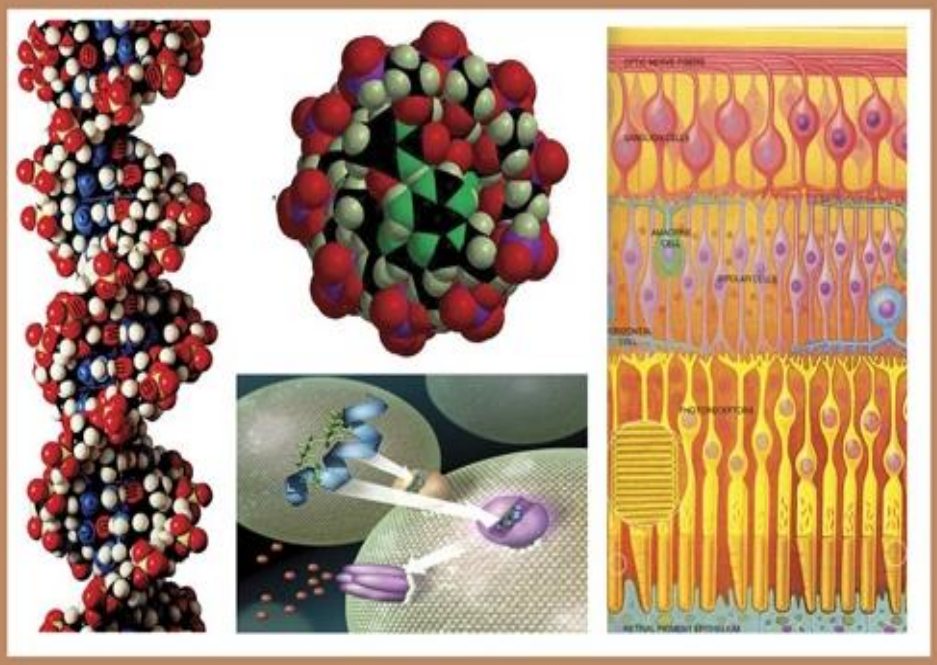

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Anti-Diabetic Effects of Metformin Nanoemulsion and Cell-Based Therapy on the Insulin Signaling Pathway (IRS1/AKT) and Apoptotic Related Genes in Type 2 Diabetic Rat Model

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ABSTRACT

Restoration of the normoglycemic state is the basic tenet of diabetes research. Nowadays, using nanoemulsion drug delivery systems represent an alternative effective tool in Type 2 diabetes mellitus management. We aimed to investigate the anti-diabetic potential of metformin nanoemulsion and mesenchymal stem cells (MSCs) therapy on streptozotocin (STZ)-induced diabetic rat model. Nanoemulsion formulation was prepared with oil phase (5%v/v), 1gm metformin with a hydrophilic surfactant, then characterized for particle size and zeta potential. Adult male albino rats were induced for diabetes with 60 mg/kg STZ and were classified into 4 groups: STZ control group; STZ+MET group (orally administered daily dose of metformin 18 mg/200 g b.wt.); STZ+MSCs group (mono-therapeutic dose of BM-MSCs 1×10^6 cells/rat) and STZ+Nano-MET group (daily intraperitoneal dose of metformin nanoemulsion 18 mg/kg b.wt.). After 8 weeks, relative expression levels of hepatic IRS-1, AKT, Bcl-2, and Bax were assessed by qPCR. Also, histopathological investigations were performed. We reported cytoplasmic vacuolation, tissue necrosis, dilated blood sinusoid and congested hepatic vein in STZ group. Experimental animals either treated with metformin nanoemulsion or the mono-therapeutic dose of MSCs exhibited significant up-regulation in IRS-1/AKT pathway and Bcl-2 gene expression levels, as well as significant repression in Bax mRNA levels, besides a remarkable amelioration in a hepatic histological organization. Conclusion: Nano-MET holds an anti-diabetic potential that clearly surpasses metformin *via* affecting the insulin signaling pathway and apoptotic gene expression.

INTRODUCTION

The incidence of diabetes in North Africa/Middle East was 39 million in 2017, 55 million in 2019 (International Diabetes Federation, 2019) and it will upsurge to 82 million by 2045. T2DM accounts for the vast majority of diabetes cases for around 90% (Abdulmalek & Balbaa, 2019) (Aschner *et al.*, 2020).

Type 2 diabetes mellitus affects the vital roles of the liver in maintaining glucose homeostasis and controlling the levels of glucose release, giving rise to hyperglycemia. Within hepatocytes, the metabolic processes are mainly controlled via insulin signaling. The binding of insulin to its receptor(White, 2002), brings about tyrosine phosphorylation of insulin receptor substrate (IRS), which activates IRS and allows its binding to phosphoinositide 3- kinase (PI3K). Protein kinase B (PKB or AKT) represents the main target of PI3K in hepatic cells and plays a key role in glucose uptake. Consequently, PI3K activates AKT and triggers the translocation of intracellular GLUT4 vesicles to the plasma membrane. Once docked at the plasma membrane, GLUT4 begins glucose uptake, improves glucose utilization as well as, reduces serum glucose levels(Bertrand *et al.*, 2008).

Metformin (MET) is an oral anti-hyperglycemic drug that belongs to the biguanide class drugs and is used widely to treat type 2 diabetes (Abbasian *et al.*, 2019). Metformin is affecting blood glucose level in different ways including; the deactivation of hepatic gluconeogenesis and glycogenolysis, restriction of glucose entrance from the intestine, and boosting the insulin function in the peripheral tissues (Defronzo *et al.*, 1991) as well as, it increases glucose uptake in muscle tissue (Bodmer *et al.* 2008). MET was also found to enhance insulin secretion from beta cells (Ilahi *et al.*, 2012).

Mesenchymal stem cells (MSCs) are adult stem cells undergo multipotent differentiation into many somatic cells such as adipocytes, osteocytes and chondrocytes (Xu *et al.*, 2020) (Ahmed *et al.*, 2020). They are considered as an ideal cell type candidate for diabetes mellitus therapy (Zang *et al.*, 2017). Mesenchymal stem cells can be isolated from various tissues such as bone marrow, adipose tissue, umbilical cord, placenta, muscle and tendons (Ahmed *et al.*, 2020) (Zuk *et al.*, 2002). Given their role in maintaining and replenishing tissues, MSCs represent a potential means of restoring tissue function *via* different mechanisms including, antiapoptotic, proangiogenic, anti-inflammatory properties, in addition to immunosuppression and immunomodulation of the immune system (Shi *et al.*, 2008). Such a response is thought to be an inherent one that can be augmented by enhancing the endogenous MSCs pool with exogenously administered MSCs (Fong *et al.*, 2011).

For improving diabetes mellitus management, a drug delivery system using nanoemulsion has been developed to overcome the drawbacks related to conventional drug delivery systems (Ranganathan & Mahalingam 2019). Nanoemulsion is a heterogeneous system consisting of one immiscible liquid dispersed as droplets within another liquid and stabilized by an amphiphilic surfactant. The droplet size of nanoemulsion is between 20 to 500 nm. The diameter and surface properties of droplets of nanoemulsion play an important role in the biological behavior of the formulation (Pagar & Darekar 2019).

The goal of this study is to investigate the anti-diabetic potential of metformin nanoemulsion and MSCs therapy on streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Metformin®,Streptozotocin (STZ) (Sigma-Aldrich), Oleic acid and Tween 80 (Sigma-USA). Bone marrow-derived mesenchymal stem cells (BM- MSCs) were isolated and characterized according to (Aziz *et al.*, 2014) at the biochemistry department, faculty of medicine, Cairo University, Cairo, Egypt. Mesenchymal stem cells were labeled with PKH26 from Sigma Company (Saint Louis, Missouri USA).

Nanoemulsion Formulation:

Oleic acid is a naturally occurring fatty acid usually obtained from both plant and animal sources. Oleic acid nanoemulsion was formulated using an ultrasonic homogenizer. The components of nanoemulsion include the oil phase, aqueous phase and nonionic surfactants. Where, oil phase (5%v/v) was added to aqueous phase containing 1gm metformin with hydrophilic surfactant (Tween 80 – 1.68%v/v) under vortex for 5 min to produce a coarse emulsion. The coarse emulsion was then subjected to Ultrasonic Homogenizer (BioLogics Ultrasonic Homogenizer Model 150 VT; BioLogics Inc, Manassas, VA, USA) for 10 mins at 50% pulsed power and 30% pulsar rate to yield nanoemulsion (Lin & Chen 2008). The metformin nanoemulsion was passed through a 0.22 µm syringe filter for sterilization and evaluated for physical stability during the shelf life.

Nanoemulsion Characterization:

The Metformin nanoemulsions droplet mean particle size, polydispersity index (PdI) and zeta potential were measured by Zetasizer® Nano ZS, Malvern PCS Instruments, UK. The measurement was performed at 25 °C, scattering angle of 160.9°, the material refractive index value of 1.40 and viscosity (cP) value of 0.8872 cPoise. Where, the Dispersant Dielectric Constant and zeta run were 78.5 and 16, respectively. The sample prepared for estimation was diluted from 100 to 200-fold before the measurement, where 10 µL of the sample was added to 1 mL of ultrapure water (Rodrigues *et al.*, 2018)

Experimental Animals:

The study conducted is approved by Helwan University. Adult male albino rats (*Rattus norvegicus*), approximately 100 g $\pm$ 20 g, were purchased from the Medical Ain Shams Research Institute (Cairo, Egypt) and maintained under normal conditions throughout our experimental period. All rats were provided with limited access to food and water to avert the occurrence of diabetic coma during the experiment. For STZ treatment, rats were fasted and injected with a single intraperitoneal dose of freshly prepared STZ solution (60 mg/kg body weight) dissolved in cold citrate buffer (0.9%) (Aziz *et al.* 2014). To overcome the drug-induced hypoglycemia, animals were allowed to drink glucose solution (5%) overnight. Blood glucose levels, 48 h after STZ injection, were measured to confirm diabetes and those with blood glucose levels >200 mg/dl were considered to be diabetic rats that studied further.

Four weeks post STZ injection, our treatments began and the animals were categorized into 4 groups (10 rats/ group) (Fig. 1). (1) *STZ group*: these animals were injected with a single dose of STZ (60 mg/kg); (2) *STZ+MET group*: diabetic animals were orally administered daily dose of metformin (18 mg/200 g b.wt.); (3) *STZ+ MSCs group*: diabetic rats intravenously injected with a single dose of BM-MSCs labeled with PKH26 dye (1×10^6 cells/rat) (Aziz *et al.* 2014) and (4) *STZ+ Nano-MET group*: diabetic rats injected with a daily intraperitoneal dose of metformin nanoemulsion (18 mg/kg b.wt.). Then, the animals were anesthetized and sacrificed after 8 weeks of treatments for molecular and histological assays. All animal protocols in this study were in accordance with Helwan University Institutional Animal Care and Use Committee (HU-IACUC) guidelines and regulations, with an approval number: RS0420-09.

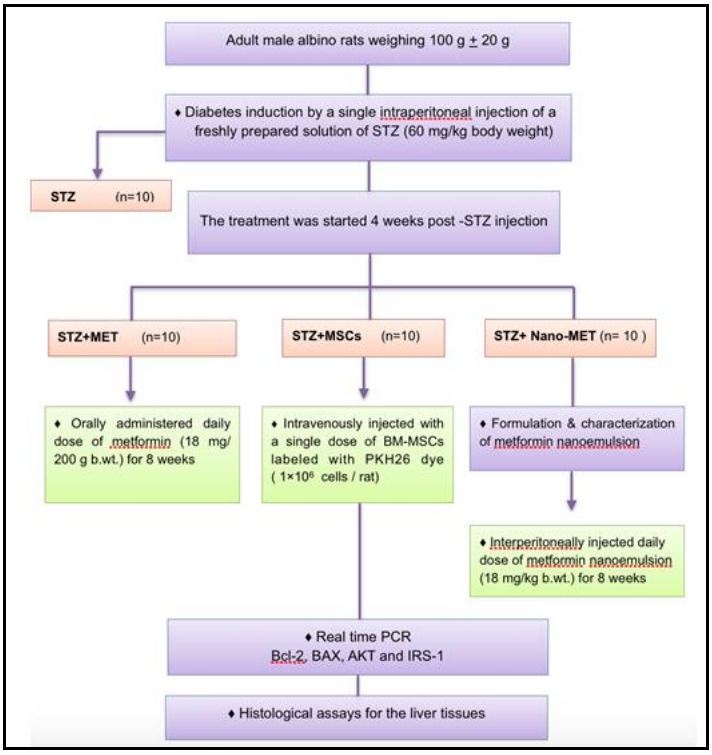

Fig.1. Flow chart of the experimental methodology and study design.

RNA Extraction and qPCR:

Isolation and purification of total RNA from liver tissues were processed using QIAzol Lysis Reagent (Qiagen, Germany). The concentration of RNA was quantified using NanoDrop 2000 Spectrophotometer from Thermo Fisher Scientific™ (United States). Total RNA was reverse-transcribed to cDNA using high-capacity cDNA reverse transcription kit (Qiagen, Germany) as described by the manufacturer's instructions. Relative expression levels for Bcl-2, Bax, AKT and IRS-1 were analyzed using SYBR green PCR kit (Qiagen, Germany). Specific primer pairs for Bcl-2, BAX, AKT and IRS-1 were used (Invitrogen Co., USA) (Table 1). All the quantitative real-time PCR reactions were performed in triplicate on VIIA 7 PCR system (Applied Biosystems, USA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference. The relative expression of mRNA was calculated using the formula $2^{-\Delta\Delta Ct}$ according to Livak method.

Table 1. Primers sequences of the studied genes.

Genes	Primers	Ref.
Bcl-2	F: 5'-CACCCCTGGCATCTTCTCCT-3' R: 5'-GTTGACGCTCCCCACACACA-3'	Albeltagy *et al.* 2020
Bax	F: 5′-TGCAGAGGATGATTGCTGAC-3′ R: 5′-GGTGAGCGAGGCGGTGAGGAC-3′	Strauss *et al.*, 2004
AkT	F: 5'-GTGGCAAGATGTGTATGAG-3' R: 5'-CTGGCTGAGTAGGAGAAC-3'	Fan et al. 2015
IRS1	F: 5'-ACCATGGGGACAAGCCCGGCC-3' R: 5'-GGGGCTGCTGGTGTTGGAATC-3'	Paris *et al.* 2004
GAPDH	F: 5′-GCCATCAACGACCCCTTCATT-3′ R: 5′-CGCCTGCTTCACCACCTTCTT-3′	Qi *et al.* 2016

Histological Investigations:

Rats were sacrificed and their liver tissues were excised for histological assay. Then the excised organs were fixed in Bouan's fixative for about 24 hours, washed in 70% alcohol, dehydrated, cleared in xylene and impregnated in parablast for blocking. Serial sections of 5 µm thick were prepared and stained with Hematoxylin and Eosin.

Statistical Analysis:

Data are presented as mean ± standard error (SE). All analyses were conducted via IBM SPSS Statistics program, Version 23. Data were analyzed using a one-way analysis of variance (ANOVA). Statistically significant values are considered at P value <0.05.

RESULTS

Nanoemulsion:

Figure (2A) showed that the mean size of Metformin nanoemulsion was 291 nm. A lower PDI value (near zero) indicates a monodisperse droplet population, whereas a PDI value closer to 1 (one) indicates a wide range of droplet sizes. Despite the fact the droplet size slightly increased after this test, the PDI value remained around 0.220, indicating the homogeneity of the droplet population in both formulations. Zeta potential avalues were -24.9 for Metformin nanoemulsion **(**Fig.2B**)**. The estimated zeta potential is already enough to create a repulsion barrier between Metformin Nano-droplets which have good stable dispersed colloidal systems**.**

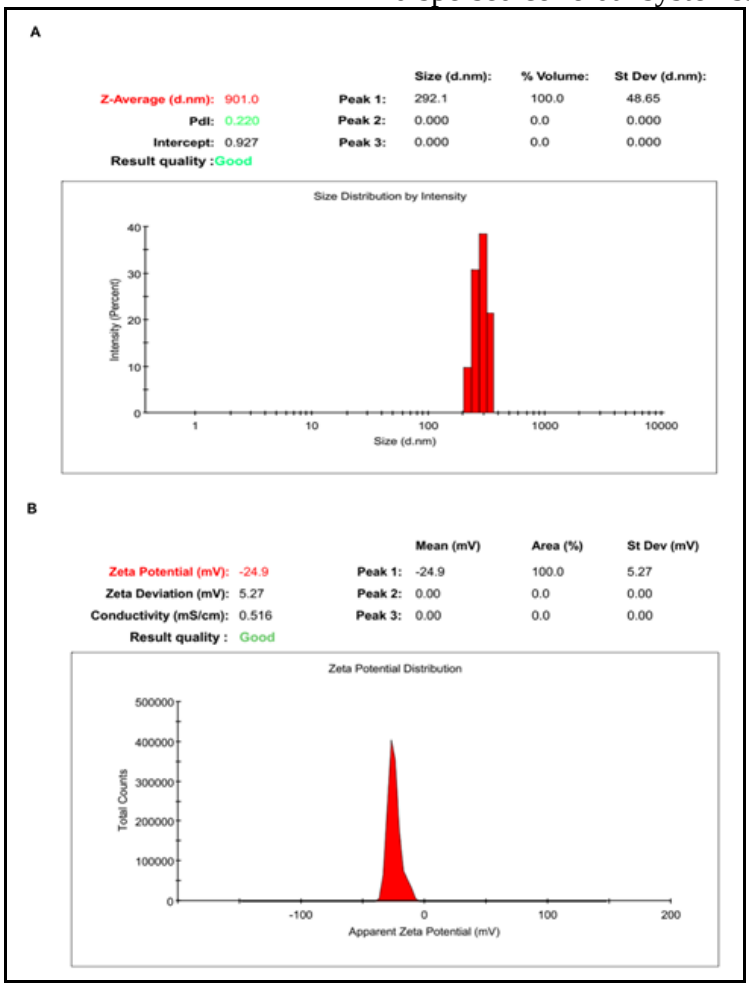

Fig. 2. (**A):** Dynamic Light Scattering (DLS) size distribution of stable Metformin nanoemulsion samples after 45 days. (**B):** Zeta potential graphic of Metformin nanoemulsion.

Real Time PCR:

The results indicated significant up-regulation of the hepatic Bcl-2, AKT and IRS-1 levels in MET, MSCs as well as Nano-MET groups versus STZ group. While the gene expression of Bax was down-regulated in all treated groups as compared to STZ group. Surprisingly, MSCs treatment exhibited the best amelioration in the tested genes followed by Nano-metformin (Fig. 3).

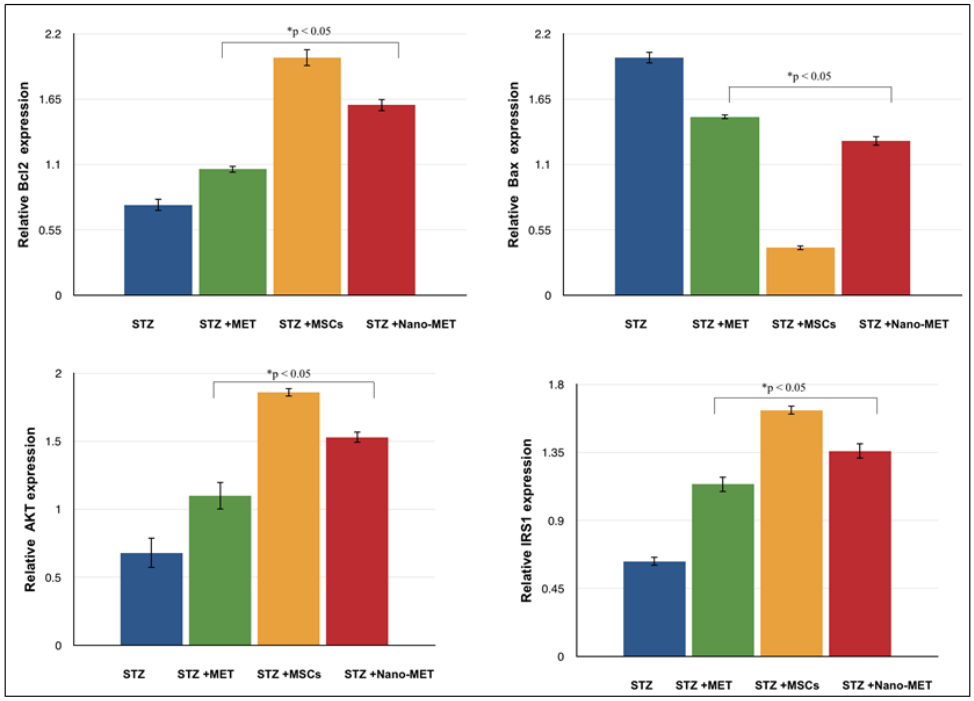

Fig. 3. Effects of MET nanoemulsion and MSCs therapy on hepatic Bcl-2, BAX, AKT and IRS-1 relative mRNA expressions. Data were represented as the mean ± SE, *p < 0.05, statistically significant values compared with STZ group.

Histological Results:

Mesenchymal stem cells labeled with PKH26 fluorescent dye were detected in the hepatic tissue, confirming their homing (Fig. 4).

Diabetic liver demonstrated disturbance of the hepatic lobules. Cytoplasmic vacuolation, tissue necrosis, nuclear pyknosis, karyolysis and dilated blood sinusoids, congested hepatic vein were present (Fig. 5A). MSC treated diabetic group showed organized hepatic cords, normal polyhedral hepatocytes with intact nucleus with peripherally dispersed chromatin and prominent nucleoli and eosinophilic cytoplasm, hepatic sinusoids with clearly visible kupffers cells were also observed (Fig. 5B).

The metformin-treated diabetic group showed some organized hepatic cords and blood sinusoids in-between with phagocytic irregular cells with multiple processes known as Von Kupffer. Normal hepatocytes and intact nucleus but some histopathological changes remained just like disorganized hepatic cords, vacuolated cytoplasm and pyknotic nucleus (Fig. 5C). Nano metformin group showed well organized hepatic cords, normal polyhedral hepatocytes with an intact nucleus and granular, strongly eosinophilic cytoplasm, hepatic sinusoids with clearly visible kupffers cells were also observed (Fig. 5D).

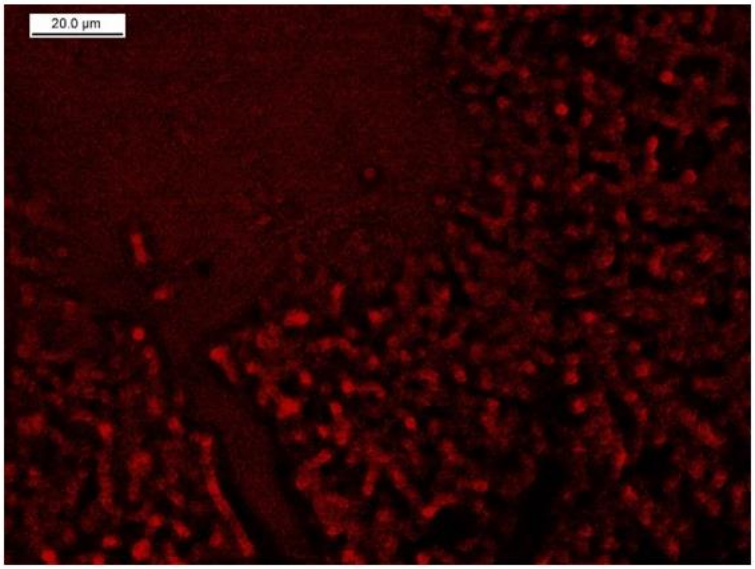

Fig.4. Mesenchymal stem cells homing in liver tissue, labeled with PKH26 fluorescent dye.

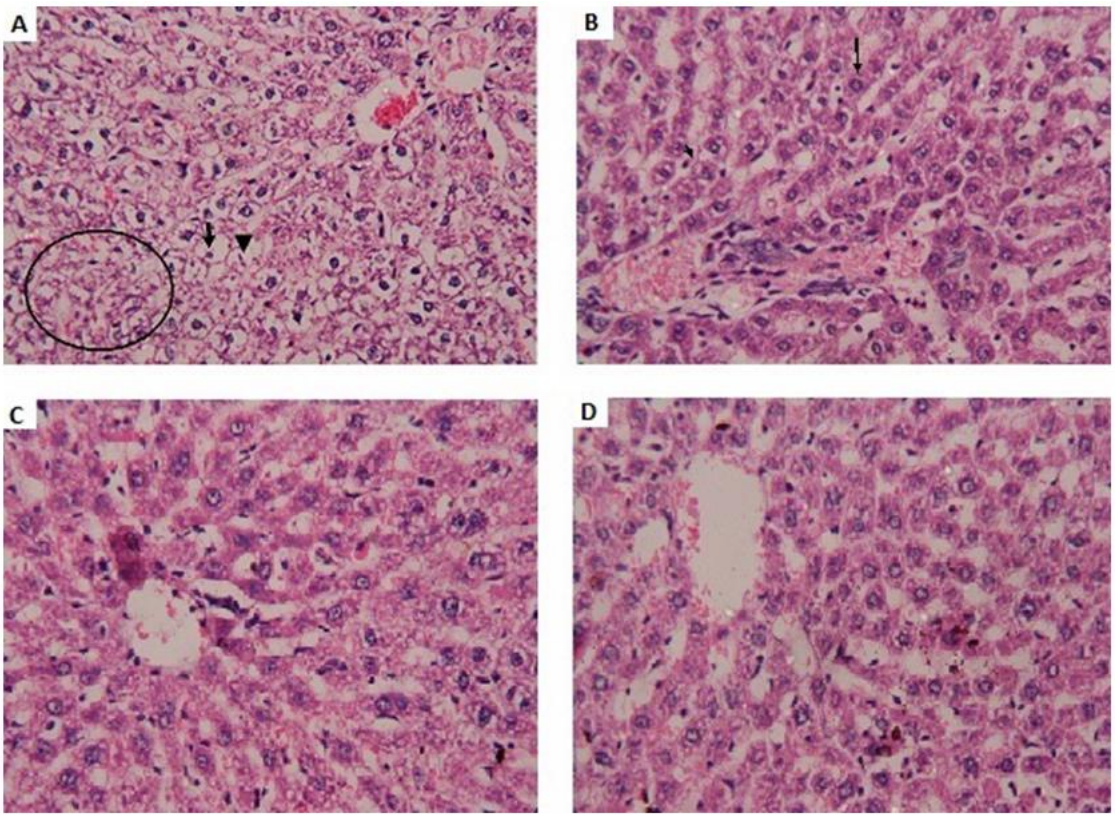

Fig. 5. A: STZ-group liver shows disoriented hepatic cords, congested hepatic vein vacuolated cytoplasm pyknotic (arrow), focal necrosis of hepatocytes (circle) and karyolitic nuclei (arrowhead) obliterated sinusoids. B: MSCs treated diabetic group shows well organized hepatic cords, hepatic sinusoids with clearly visible kupffers cells (arrowhead), normal hepatocytes with an intact nucleus and eosinophilic cytoplasm (arrow). C: metformin-treated diabetic group shows some organized hepatic cords with normal hepatocytes and intact nucleus but some disorganized hepatic cords, vacuolated cytoplasm also presents. D: Nano metformin group shows relatively normal hepatic cords, hepatocytes with centrally located nuclei and eosinophilic cytoplasm open sinusoids with clearly visible kupffers cells.

DISCUSSION

For the being times, a number of nanoparticle-based therapy have been proposed as alternative diabetic therapy with multifunctional biological activities that could alleviate and /or treat diabetic complications. Herein we evaluate the effect of MET nanoemulsion and MSCs therapy versus the oral administration of MET on the streptozotocin-induced diabetic rat model. Our results revealed a disturbance of the hepatic tissue including; cytoplasmic vacuolation, tissue necrosis, nuclear pyknosis, kariolysis, dilated blood sinusoids and congested hepatic vein in STZ group. On the other side, experimental animals either treated with daily intraperitoneal dose of metformin nanoemulsion (18 mg/kg b.wt.) or with a mono-therapeutic dose of BM-MSCs (1×10^6 cells/rat), for 8 weeks, showed significant improvement in IRS-1, AKT, Bcl-2 and Bax gene expressions levels. In addition, a remarkable advance in the hepatic histological organization was observed.

It has been reported that apoptosis plays an important role in diabetes (Rashid & Sil 2015). STZ diabetic rats exhibited an increased expression of the proapoptotic Bax along with a decreased level of antiapoptotic Bcl-2. During hyperglycemic conditions, advanced glycations end products (AGEs) are excessively synthesized and triggered cell-damaging mechanisms. These increased levels of AGEs mediate the modification of the intracellular proteins, extracellular matrix components, as well as, activate nuclear factor-κB and subsequently leading to proinflammatory gene expression (Brownlee, 2001). Additionally, AGEs activate different kinase and NADPH oxidase leading to excessive reactive oxygen species (ROS) synthesis and further promote oxidative stress (Peng *et al.*, 2018). Previous studies indicated that hyperglycaemia induced oxidative stress can stimulate mitochondrial dysfunction, causing the decrease of Bcl-2, as well as the release of Bax, triggers activation of the caspase family and leading to apoptosis (De Ford *et al.,* 2016; Rashid *et al., 2017*).

As a standard anti-diabetic drug, metformin mediates its antioxidant activities by suppressing mitochondrial respiration that increases the antioxidant enzyme activities and diminished the ROS in diabetic rats, as well as suppressing AGEs production *via* an insulin-dependent mechanism directly and its hypoglycemic effect indirectly (Ahmed *et al.,* 2017; Abdulmalek and Balbaa, 2019). In the present study, MET significantly inhibited STZ-induced apoptosis of the liver by decreasing expression of the Bax together with increasing the level of Bcl-2. Similar to our results, several studies have reported that the mitochondrial pathway might be involved in the anti-apoptotic effect of metformin (Chen *et al.*, 2016; feng *et al.,* 2019). Metformin has been reported to participate in the attenuation of ROS and decrease inflammatory markers in diabetic patients (Abdulmalek and Balbaa, 2019; Festa *et al.,* 2002). However, MET is characterized by relatively low bioavailability and some side effects are known to be associated with this drug (Abassian *et al.,* 2019). In addition, He *et al.*, 2019 have reported that metformin-induced MSC apoptosis and is involved in the decreased quantity of endogenous MSCs during intensive glucose control.

Regarding the effect of metformin nanoformulation on the apoptotic-related genes, our results indicated significant up-regulation of Bcl-2 and down-regulation of Bax mRNA levels with respect to STZ group. Nanoemulsions are known to enhance the delivery of lipophilic compounds with anti-diabetic properties (Souto *et al.,* 2019). Nano-emulsion droplets are characterized by their small particle size in the range of 50–500 nm and show narrow size distributions

(Izquierdo *et al.*, 2002). Rapid adsorption of nonionic surfactant and emulsifier Tween80 results in minimizing the diameter of the emulsion into nano-size (Qian & McClements 2011). In particular, nanoemulsion as a promising carrier for MET, Kumar *et al.*, 2017 study indicated that metformin-loaded alginate nanoparticles (MLANs) with the dose of 46.8 mg/kg resulted in significant hypoglycaemic effects that exceed the triple dose of pure metformin (dose of 150 mg/kg), besides they suggested that the metformin nanoformulation may possess an increased bioavailability in the GI tract, especially at lower doses. Also, Akhtar *et al.*, 2014 study investigated the antidiabetic activity using blood glucose estimation in diabetic rats and they reported that the metformin incorporated into the oil phase of nanoemulsion exhibited a significant hypoglycemic effect. Moreover, Abbasian *et al.*, 2019 illustrated that the physical loading of metformin into a chitosan-based nanosystem showed a higher controlled drug release profile comparable to that observed with conventional formulations of the drug. In addition, the fabricated chitosan-based nanosystem exhibited high potential as *de novo* drug delivery system for diabetes mellitus treatment.

The insulin receptor substrate-1/phosphoinositide 3- kinase/protein kinase B insulin-signaling cascade is closely related to insulin resistance-associated diseases, including diabetes and obesity (Hu *et al.*, 2014). The dysregulation of IRS/AKT insulin signaling pathway is a crucial determinant of the glycemic response in diabetes (Lin *et al.*, 2013; Abdulmalek & Balbaa 2019). At molecular levels, our STZ model treated with MET and nanoMET exhibited significant up-regulation of IRS1 associated with an increased level of AKT1 compared to STZ group. Inconsistent, Lin *et al.*, 2018, stated that the up-regulation of both pIRS1 and pAKT might improve the uptake of glucose and reduce blood glucose levels. As activators of AMP-activated protein kinase (AMPK), metformin promotes the action of insulin on AKT activation (Horike *et al.*, 2008) and inhibits cellular inflammation (Kim *et al.*, 2014). Moreover, It was also indicated that downregulation of AKT and IRS resulted in insulin resistance that is secondary to loss of the effects of insulin on the liver (Lu *et al.*, 2012). PI3-kinase and AKT are well-established activators of survival in numerous cell types, and overexpression of AKT, specifically in beta cells, results in a marked expansion of cell number and size (Tuttlen *et al.*, 2001).

Cell-based therapies, including the multipotent MSCs are being considered as a therapeutic approach for diabetes mellitus and its complications (Peng *et al.*, 2018; Albeltagy *et al.*, 2020). Injury is believed to trigger the mobilization of resident MSCs and recruit abundant MSCs from peripheral circulation to the injury site and contribute to damaged tissue healing (Albeltagy *et al.*, 2020). MSCs have therapeutic effects on glycemic control both *in vivo* and *in vitro*. Impaired quality of MSCs plays a pathogenic role in diabetes (Kim *et al.*, 2016), likewise the circulating MSCs was significantly decreased in patients with T2DM and was correlated with the progression of diabetic complications (He *et al.*, 2019). In the current study, MSCs infusion significantly inhibited STZ-induced apoptosis of the liver by decreasing expression of the Bax together with increasing level of Bcl-2, in addition to significant up-regulation in IRS-1/AKT pathway. Similarly, it has been reported that MSCs have attracted substantial attention for the treatment of DM through inhibiting cell apoptosis. Also, MSCs may improve insulin sensitivity *via* their ability to restore the feeding-induced phosphorylation of IRS-1 and AKT. In addition to increasing the expression level of GLUT4 on the insulin target cell membrane of T2DM rats (Hao, H. *et al.*, 2013; *Gao et al.*, 2014; Si *et al.*, 2012).

Overall, in the current *in vivo* study, the nanoemulsion formulation prominently affects the anti-diabetic behavior of metformin on the insulin signaling pathway (IRS-1/AKT) and apoptotic-related gene expression. Also, we bring attention that, BM-MSCs infusion on demonstrated features of T2DM still represents the best results in our experimental setting. Additional studies with different experimental designs and dosage are needed to provide more insights on Nano-MET therapeutic potential.

Author contributions

All the authors were involved in the study planning, design and execution. B.H, R.A, A.A and M.H were responsible for conducting the animal work. A.F performed the preparation and characterization of the nanoemulsion. R.A, M.H. and S.A contributed to functional evaluation of the molecular parameters and prepared the manuscript. A.A and B.H performed the histological assay. S.A and R.A were responsible for conducting the interpretation and data analysis. All authors contributed to revising the manuscript and reviewing the final draft.

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Ethical approval: The study has followed the guidelines of HU-IACUC, Approval number: *RS0420-09*.

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