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# Combinatory effect of hesperetin and mesenchymal stem cells on the deteriorated lipid profile, heart and kidney functions and antioxidant activity in STZ-induced diabetic rats

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**Key words:** BM-MSCs, Hesperetin, Streptozotocin-induced diabetes

**Abstract:** This study aimed to assess the effect of hesperetin and/or bone marrow-derived mesenchymal stem cells (BM-MSCs) on disturbed lipid profile, heart and kidney functions, oxidative stress and antioxidant defense system in streptozotocin (STZ)-induced diabetic rats. Type 1 diabetes mellitus (T1DM) was induced in male Wistar rats by injecting 40 mg/kg body weight (b.w.) STZ dissolved in citrate buffer (pH 4.5). The diabetic rats were treated with hesperetin orally administered at dose 20 mg/kg b.w., BM-MSCs intravenously injected at a dose of 1 x 106 cells/rat/week and their combination for 6 weeks. The diabetic rats exhibited lipid abnormalities manifested by elevated serum levels of total cholesterol, triglycerides, LDL-cholesterol and VLDL-cholesterol and lowered HDL-cholesterol as well as elevated liver cholesterol and triglycerides content in association with the resultant fasting and postprandial hyperglycemia and insulin deficiency. The heart function biomarkers including CK-MB, AST and LDH activities as well as levels of kidney function parameters, creatinine, and urea, were significantly raised in the serum of diabetic rats. These changes were concomitant with abnormal redox balance represented by elevated lipid peroxidation, decreased glutathione content, and suppressed antioxidant enzyme activities in both heart and kidney of diabetic rats. The previous deleterious alterations were significantly ameliorated after the treatment of diabetic rats with hesperetin and BM-MSCs singly or in combination; the treatment with hesperetin together with BM-MSCs was the most potent. Based on these findings, it can be concluded that the use of hesperetin with BM-MSCs may have more additive therapeutic value than their uses singly in T1DM. In addition, the ameliorative effects of hesperetin and BM-MSCs on lipid profile and heart and kidney functions in diabetic rats may be mediated, at least in part, via their suppressive effects on oxidative stress and ameliorative effects on the antioxidant defense system secondary to improvement in the hyperglycemia and insulin secretory response.

## Introduction

Worldwide, diabetes mellitus (DM) prevalence rate has been increased over time (Ogurtsova *et al.*, 2017; Cho *et al.*, 2018). In this regard, the International Diabetic Federation (IDF) expected that the number of people that will be diabetic reaches 35% by 2045 (Cho *et al.*, 2018; IDF, 2018). It was found that diabetes-related complications including hyperlipidemia, atherosclerosis, cardiovasculopathy, and nephropathy are a major reason for mortality and morbidity and substantially result in a heavy economic burden on the health care system (Deshpande *et al.*, 2008). The main goal of treatment is to prevent the appearance or progression of these diabetic complications and simultaneously minimize the risks of severe hyperglycemia that account for the generation of free radicals in DM (Maritim *et al.*, 2003; Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2013).

The use of traditional medicine increases worldwide over time due to the local availability, simple to use, easily accessible, and postulated to be safe (Teklay *et al.*, 2013; Kassahun *et al.*, 2016). However, recent research works are more interesting in the use of active plant constituent rather than crude extracts in the treatment of diseases to avoid the probable side effects of other plant ingredients (Ahmed *et al.*, 2017; Sangeetha, 2019).

Hesperetin, belonging to a group of flavonoids known as flavanones, is one of the richest flavonoids in citrus fruit peels (Jayaraman *et al.*, 2018). Hesperetin is an aglycone of hesperidin, which is the major flavone glycoside found in

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citrus fruit peels (Nielsen *et al.*, 2006). It was demonstrated to possess several pharmacological properties (Nielsen *et al.*, 2006). Recently, Jayaraman *et al.* (2018) have revealed that hesperetin has hypoglycemic, hypolipidemic, and antioxidant potencies in streptozotocin (STZ)-induced diabetic rats.

Cell therapy is stated as an important therapeutic approach for the replacement of lost β-cells in DM, especially type 1 DM (T1DM) (Márquez-Aguirre *et al.*, 2015). However, the immune system attack against donated cells, unfortunately, reduces their viability with time and lastly results in recurrence of DM (Farge, 2008; Márquez-Aguirre *et al.*, 2015). The elaboration of new techniques for isolation and subsequent transplantation of islets of Langerhans failed to support this approach owing to the issues related to major surgery on one hand and lifelong dependence on the immunosuppressive agents on the other (Farooq *et al.*, 2018). Thus, the researchers are directed to focus on stem cells to produce insulin-secreting β-cells to cure diabetes on a permanent basis and to protect against the development of fatal diabetic complications. The intrinsic regenerative potential, together with the immunomodulatory capabilities of stem cells, highlights the feasibility potential of stem cell-based strategies (Farooq *et al.*, 2018).

The use of natural antioxidants such as plant flavonoids in association with stem cells to treat DM was not yet assessed in previous publications. Although various flavonoids have been shown to have an inhibitory effect on the self-renewal potential and survival of cancer stem cells of different origins (Kandhari *et al.*, 2018), their effects on the efficiency of normal stem cells to treat degenerative diseases have not been evaluated.

Therefore, this study was designed to scrutinize the effects of hesperetin and bone marrow-derived mesenchymal stem cells (BM-MSCs), singly or in combination, on the deleterious changes in lipid profile, heart function and kidney function, oxidative stress and antioxidant defense system in STZ-induced diabetic Wistar rats.

## Materials and Methods

*Chemicals*

Hesperetin (3',5,7-trihydroxy-4'-methoxy flavanone) and STZ were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMEM (Dulbecco's modified Eagle's medium), penicillin-streptomycin solution, trypsin/EDTA, and fetal bovine serum (FBS) were obtained from Lonza, Belgium. Sodium hydrogen carbonate was obtained from LOBA Chemie, India. Culture flasks and culture consumables were obtained from Greiner Bio-One (Germany). All other used reagents and chemicals were of analytical grade and high purity.

*Preparation of complete culture medium*

To prepare the complete culture medium, FBS, penicillin-streptomycin solution, and sodium hydrogen carbonate were added to 89% DMEM, according to Sun e*t al.* (2007).

*Isolation and culture of BM-MSCs*

The method used for isolation and culture of BM-MSCs, in the present study, is based on the procedure of Chaudhary and Rath (2017) with some modifications. Young male Wistar rats (4-6 weeks old) were used to isolate BM-MSCs. Briefly, animals were killed by decapitation, and the body surface was sterilized by spraying with 70% (vol/vol) ethyl alcohol. The tibiae and femurs were dissected out and cleaned of all associated muscles. In a Biobase vertical laminar flow cabinet (Biobase, Model: BBS V1300; NO-51, South Gongye Road, Jinan, Shandong Province, China) under sterile condition, the epiphysis was cut off just below the growth plate with sterile fine scissors, and then bone marrow cells were harvested by flushing with DMEM, and then collected in a 15 mL Falcon tube. Cells were dislodged and separated by centrifugation at 3000 rpm for 5 min. After decanting the supernatant, the cell pellet was rapidly washed with phosphate buffer saline (PBS) and suspended in complete DMEM (DMEM supplemented with 15% FBS, 0.36% sodium hydrogen carbonate and 1% penicillin-streptomycin solution). By using the hemocytometer at 100X magnification, viable and dead cells were counted after staining with trypan blue solution (0.2%) to assess the cell viability (the number of viable cells relative to the total number of cells). In T-25 $cm^2$ sterile Greiner cell culture flasks with canted neck, 2.5 $\times$ $10^6$ cells were seeded at the cell density of 1 $\times$ $10^6$ cells/$cm^2$ area, and then placed in 5% $CO_2$-humidified incubator (Biobase, Model: BJPX-C50; South Gongye Road, Jinan, Shandong Province, China) at 37°C. Floating and non-adherent cells, as well as dead cells, were removed after 4 days of each incubation. Thereafter, within 7-10 days of culture and incubation, adherent cells were washed twice with sterile PBS (pre-warmed at 37°C), and trypsinized with 1 to 2 mL of trypsin (0.25%)/EDTA (1 mM) (pre-warmed at 37°C) for 2-3 min. The detachment of adherent cells was ensured by checking cells using an inverted biological microscope (Novel, Model: NIB-100; Jiangsu, China). The action of trypsin was stopped by adding 3-5 ml complete DMEM. Then, cells were collected and centrifuged at 3000 rpm for 5 min. The resultant supernatant was decanted, and the cell pellet was washed by adding incomplete DMEM and centrifugation. After 2 times of washing, the cells were re-suspended in an incomplete DMEM and cells were counted and tested for viability by adding an equal volume of 0.2% trypan blue. The BM-MSCs suspended in an incomplete DMEM with viability higher than 95% were rapidly injected in the lateral tail vein of diabetic rats at a dose of 1 $\times$ $10^6$ cells/rat. The isolation of BM-MSC is based on the fact that MSCs adhere to the plastic surface of tissue culture flasks when maintained in standard culture conditions.

*Experimental animals*

Adult male Wistar rats (100-120 g) were obtained from the Egyptian Company for the Production of Sera and Vaccines (Vacsera), Helwan, Egypt. To exclude any interchangeable infection, animals were kept under observation and strict care for one week before the starting of the experiment. Chosen animals were housed in polypropylene cages with well-aerated covers at a temperature of 25 ± 5°C and 12-hour light/dark cycle. They were supplied with free access of standard diet and water. All experimental procedures were performed in accordance with recommendations, guidelines, and instructions stated by the Ethics Committee for Care and Use of Animals, Faculty of Science, Beni-Suef University, Egypt (Ethical Approval Number: BSU/FS/2017/17).

*Induction of T1DM*

After one week of acclimatization, DM was experimentally induced in animals deprived of water and food for 16 h, by intraperitoneal injection of 40 mg/kg body weight (b.w.) STZ dissolved in citrate buffer solution (pH 4.5) (Tariq *et al.*, 2013). To overcome the hypoglycemic shock and to minimize the mortality that occurs during the next 48 h of STZ injection due to massive insulin release from damaged β-cells, 5% glucose solution after 2 h of STZ injection for the next 48 h was added as drinking water. The respective control rats were injected with the same equivalent volume of citrate buffer by the same route of administration. Ten days after STZ injection, animals were deprived of water and food overnight (10-12 h), blood samples were obtained by puncturing the lateral tail vein after 2 h of oral glucose loading (3 g/kg b.w.) and then blood glucose concentrations were detected with a glucometer and GlucoDr.auto strips (All Medicus, Co., Ltd., Anyang-si, Republic of Korea). Rats having a 2-hours blood glucose ≥ 200 mg/dL were considered as diabetic rats and were included in the experiment.

*Animal grouping and experimental design*

After induction of DM, the rats were allocated into 5 groups, each comprised 6 rats. Group I (normal control) was composed of the normal rats that were orally administered the equivalent volume of vehicles [citrate buffer, 1% carboxymethylcellulose (CMC) and DMEM] in which the treatments were dissolved. Group II (diabetic control) consisted of the diabetic rats that were orally administered the same volume of vehicles (1% CMC and DMEM) in which the treatments were dissolved. Group III (Diabetic rats treated with hesperetin) were diabetic rats that were treated with 20 mg/kg b.w. hesperetin dissolved in 1% CMC (Revathy and Sheik Abdullah, 2016) by oral gavage every other day for six weeks. Group IV (diabetic rats treated with BM-MSCs) consisted of diabetic rats which were intravenously injected with BM-MSCs at a dose level of $1\times10^6$ cells/rat weekly (Mohamed *et al.*, 2016) for six weeks. Group V (diabetic rats treated with hesperetin and BM-MSCs) consisted of diabetic rats which were orally treated with hesperetin and were also intravenously injected with BM-MSCs for six weeks, respectively. On the day before sacrifice, fasting blood glucose, and 2-hours blood glucose concentration after oral glucose administration (3 g/kg b.w.) was detected.

*Blood and organ sampling*

By the end of the sixth week, blood was collected from the jugular vein of each rat under diethyl ether anesthesia. Then, the rats were decapitated by cervical dislocation and dissected to excise the liver, heart, and kidney that were perfused in sterile isotonic saline (0.9% sodium chloride) and kept in a deep freezer at -20°C pending homogenization. Blood samples collected in gel and clot activator tubes were left to clot and were centrifuged at 3000 rpm for 15 min, and the clear non-hemolyzed supernatant sera were immediately aspirated by Pasteur pipette, fractioned into three portions for each individual animal, and kept frozen at -20°C pending biochemical analyses. Half gram of each frozen liver, heart, and kidney was homogenized in 5 ml 0.9% sterile sodium chloride (10% w/v) using Glas-Col Teflon Homogenizer (Terre Haute, USA). The homogenate supernatants were separated by centrifugation of homogenates at 3000 rpm for 15 min and were used for the estimation of oxidative stress and antioxidant parameters in the heart and kidney as well as for determination of hepatic cholesterol and TG in the liver.

*Estimation of fasting and 2-hours post-prandial blood glucose levels*

Both fasting and 2-hours post-prandial blood glucose concentrations were determined in normal, diabetic control and diabetic treated rats at the day before sacrifice. The blood samples were taken by puncturing lateral tail vein after overnight fasting (10-12 h) and at 2-hours of oral glucose loading (3 g/kg b.w.). Then, blood glucose levels were determined using a glucometer and GlucoDr.auto strips (All Medicus, Co., Ltd., Anyang-si, Republic of Korea).

*Determination of serum insulin level*

Insulin level in serum was detected by ELISA (Enzyme-linked immunosorbent assay) kit (Catalog Number: MBS724709) purchased from MyBioSource (USA) based on the manufacturer's instructions.

*Determination of lipid profile*

Serum cholesterol (TC) and liver cholesterol levels were assayed by using reagent kits obtained from Spectrum Diagnostics (Egypt) based on the method of Ellefson and Caraway (1976). Serum and liver triglycerides (TG) levels were estimated by using a reagent kit obtained from Spectrum Diagnostics (Egypt) based on the method of Bucolo and David (1973). Serum HDL-cholesterol (HDL-C) level was determined using a reagent kit obtained from Spectrum Diagnostics (Egypt) according to Warnick and Wood (1995). LDL-cholesterol (LDL-C) level in serum was estimated based on Friendewald *et al.* (1972) formula. Serum VLDL-cholesterol (VLDL-C) was calculated based on Norbert's (1995) formula. Free fatty acids (FFAs) level in serum was determined based on the method of Duncombe (1963).

*Determination of serum enzymes related to heart function*

Serum creatine kinase-MB (CK-MB) activity was detected using a kinetic reagent kit purchased from BioMed Diagnostics (Badr city, Egypt) according to Wu and Bowers (1982). Aspartate transaminase (AST) activity in serum was assayed using kinetic reagent kit obtained from Biotec Diagnostics Company (Bristol, BS39 5BX, UK) based on the method of Tietz (1976). Lactate dehydrogenase (LDH) activity in serum was estimated using a kinetic reagent kit purchased from Salucea (Haansberg 19, 4874 NJ Etten Leur, Netherlands) according to the method of Zimmerman and Hennery (1979).

*Determination of serum parameters related to kidney function*

Serum creatinine level was estimated by using a reagent kit obtained from Biodiagnostic (Egypt) based on the method of Bartles *et al.* (1972). Urea level was estimated according to Fawcett and Scott's (1960) method by using reagent kits obtained from Biodiagnostic (Egypt).

*Determination of redox system biomarkers*

Lipid peroxidation (LPO) measured by malondialdehyde (MDA) production in the heart and kidney was determined by using a reagent kit obtained from BioDiagnostic (29 Tahreer St., Dokki, Giza, Egypt) based on the method of Ohkawa et al. (1979). GSH (reduced glutathione) content in the heart and kidney was detected by using a reagent kit obtained from BioDiagnostic (Egypt) based on the method of Beutler *et al.* (1963). GPx (glutathione peroxidase), GST (glutathione-S-transferase) and GR (glutathione reductase) activities in heart and kidney was estimated by using reagent kit delivered from BioDiagnostic (29 Tahreer St., Dokki, Giza, Egypt) based on the methods of Paglia and Valentine (1967), Habig and Jakoby (1974) and Goldberg and Spooner (1983), respectively.

*Estimation of various serum cytokine levels*

Serum TNF-α level was detected by using RayBio® Rat TNF-alpha ELISA kit (Catalog Number: ELR-TNFa) imported from RayBiotech, 3607 Parkway Lane, Suite 100 Norcross, GA 30092, based on the manufacturer's instructions. Serum IL-17 level was assayed by using Rat IL-17 ELISA kit (Catalog Number: MBS2503506) purchased from MyBioSource (USA) based on the MyBioSource's instructions. Serum IL-4 level was measured by using Rat IL-4 ELISA kit (Catalog Number: MBS494192) purchased from MyBioSource (USA) based on the method of Brown et al. (1988).

*Data statistical analysis*

Data obtained were represented as mean ± standard error (SEM). Statistical analysis was performed by one-way analysis of variance (ANOVA) using the Statistical Package of Social Sciences (SPSS) program version 22 (SPSS, Cary, NC, USA) followed by Duncan's multiple range test (Duncan, 1957). A significant difference between groups was considered at $p < 0.05$.

## Results

*Effect on fasting and 2-hours postprandial blood glucose levels*

The fasting and 2-hours postprandial glucose levels exhibited a significant elevation ($p < 0.05$) in diabetic rats when compared to normal ones. On the other hand, the treatment of STZ-induced diabetic rats with hesperetin, BM-MSCs, and their combination induced a potential improvement of elevated fasting and 2-hours postprandial glucose levels. The combination of hesperetin and BM-MSCs produced the most potent effect (Fig. 1).

*Effect on serum insulin level*

The insulin level in serum exhibited a significant decrease ($p < 0.05$) in diabetic rats as compared to normal rats. On the other hand, the treatment of diabetic rats for 6 weeks with hesperetin, BM-MSCs and their combination significantly increased ($p < 0.05$) the serum insulin level; the treatment with hesperetin and BM-MSCs together or with hesperetin alone were more effective than BM-MSCs in improving insulin level of diabetic rats (Fig. 2).

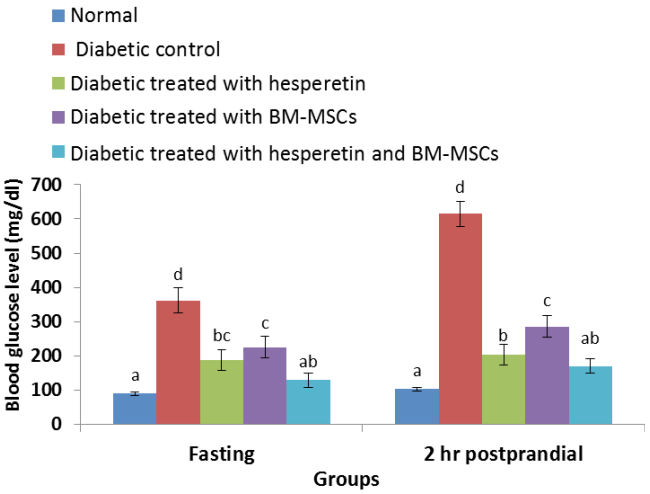


**FIGURE 1.** Effect of hesperetin and BM-MSCs on fasting and post-prandial blood glucose levels in STZ-induced diabetic rats. Mean values with different superscript letters are significantly different at $p < 0.05$. [a,b,c,d] indicated the difference or similarity between groups.

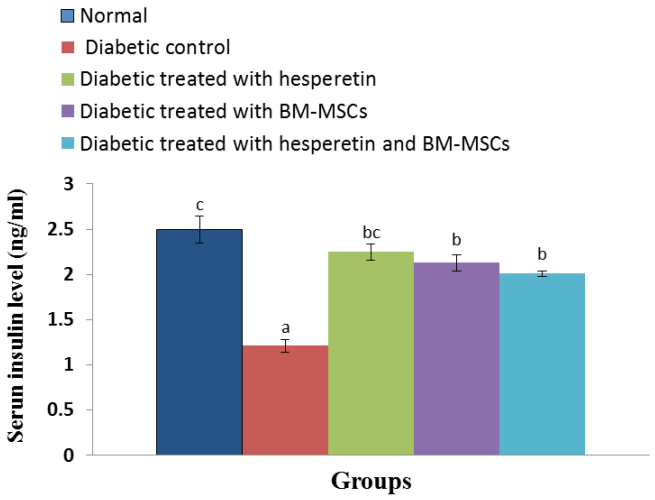


**FIGURE 2.** Effects of hesperetin and BM-MSCs on serum insulin level of STZ-induced diabetic rats. Mean values with different superscript letters are significantly different at $p < 0.05$. [a,b,c] indicated the difference or similarity between groups.

*Effect on lipid profile*

The data of lipid profile depicted a significant ($p < 0.05$) elevation in serum levels of TC, TG, VLDL-C, and LDL-C, as well as the ratio of LDL-C to HDL-C in diabetic rats as compared with the normal rats. The treatment of diabetic rats with hesperetin, BM-MSCs, and their combination produced a significant decrease ($p < 0.05$) of these elevated values; the treatment with hesperetin and BM-MSCs together noticeably was the most potent in alleviating the increased TC, TG, VLDL-C and LDL-C levels. On the other hand, serum HDL-C level was significantly decreased ($p < 0.05$) in diabetic rats and was significantly improved ($p < 0.05$) due to treatment of diabetic rats with hesperetin and/or BM-MSCs (Tab. 1).

**TABLE 1**

**Effects of hesperetin and BM-MSCs on serum TC, TG, vLDL-C, LDL-C, and HDL-C levels, and LDL-C/HDL-C in STZ-induced diabetic rats**

| Group \ Parameter | TC (mg/dL) | TG (mg/dL) | VLDL-C (mg/dL) | LDL-C (mg/dL) | HDL-C (mg/dL) | LDL-C/ HDL-C |
|---|---|---|---|---|---|---|
| Normal | 78.67 ± 0.99[a] | 90.08 ± 3.25[a] | 18.00 ± 0.66[a] | 41.21 ± 0.57[a] | 19.46 ± 0.61[c] | 2.13 ± 0.07[a] |
| Diabetic control | 117.35 ± 3.37[b] | 230.07 ± 4.73[d] | 46.01 ± 0.95[d] | 64.00 ± 3.63[b] | 7.33 ± 0.3[a] | 8.80 ± 0.62[b] |
| Diabetic treated with hesperetin | 87.33 ± 1.60[a] | 143.82 ± 9.92[b] | 28.76 ± 1.98[b] | 41.09 ± 2.58[a] | 17.49 ± 2.03[c] | 2.56 ± 0.38[a] |
| Diabetic treated with BM-MSCs | 89.93 ± 14.94[a] | 178.64 ± 5.68[c] | 35.72 ± 1.14[c] | 35.30 ± 2.44[a] | 16.81 ± 0.33[bc] | 2.10 ± 0.14[a] |
| Diabetic treated with hesperetin and BM-MSCs | 77.36 ± 1.95[a] | 142.62 ± 4.68[b] | 28.52 ± 0.94[b] | 34.57 ± 2.10[a] | 14.26 ± 0.3[b] | 2.44 ± 0.19[a] |

Data are represented as mean ± SEM.
Number of animals in each group is six.
Mean values with different superscript letters are significantly different at $p < 0.05$.
[a,b,c,d] indicated the difference or similarity between groups.

**TABLE 2**

**Effects of hesperetin and BM-MSCs on serum FFAs level and hepatic cholesterol and TG levels in STZ-induced diabetic rats**

| Group \ Parameter | Serum FFAs (mg/dL) | Hepatic cholesterol (mg/g tissue) | Hepatic TG (mg/g tissue) |
|---|---|---|---|
| Normal | 18.49 ± 1.49[a] | 6.02 ± 0.33[ab] | 26.82 ± 2.11[b] |
| Diabetic control | 26.63 ± 0.96[c] | 16.12 ± 0.48[c] | 40.31 ± 0.89[c] |
| Diabetic treated with hesperetin | 21.52 ± 1.06[b] | 7.57 ± 0.73[b] | 28.10 ± 1.88[b] |
| Diabetic treated with BM-MSCs | 22.71 ± 0.71[b] | 6.51 ± 0.82[ab] | 14.92 ± 1.39[a] |
| Diabetic treated with hesperetin and BM-MSCs | 18.17 ± 0.31[a] | 5.45 ± 0.59[a] | 15.29 ± 0.81[a] |

Data are represented as mean ± SEM.
Number of animals in each group is six.
Mean values with different superscript letters are significantly different at $p < 0.05$.
[a,b,c] indicated the difference or similarity between groups.

**TABLE 3**

**Effects of hesperetin and BM-MSCs on serum CK-MB, AST and LDH activities in STZ-induced diabetic rats**

| Group \ Parameter | CK-MB (U/L) | AST (U/L) | LDH (U/L) |
|---|---|---|---|
| Normal | 31.64 ± 0.63[a] | 87.12 ± 2.06[a] | 328.08 ± 4.23[a] |
| Diabetic control | 162 ± 13.29[d] | 178.43 ± 4.75[d] | 682.28 ± 9.46[d] |
| Diabetic treated with hesperetin | 112.70 ± 7.28[c] | 128.23 ± 4.11[c] | 377.50 ± 10.31[b] |
| Diabetic treated with BM-MSCs | 100.10 ± 5.22[c] | 131.90 ± 7.63[c] | 412.47 ± 16.12[bc] |
| Diabetic treated with hesperetin and BM-MSCs | 68.85 ± 4.87[b] | 107.27 ± 2.32[b] | 455.35 ± 26.52[c] |

Data are represented as mean ± SEM.
Number of animals in each group is six.
Mean values with different superscript letters are significantly different at $p < 0.05$.
[a,b,c,d] indicated the difference or similarity between groups.

**TABLE 4**

**Effects of hesperetin and BM-MSCs on serum creatinine and urea levels in STZ-induced diabetic rats**

| Group \ Parameter | Creatinine (mg/dL) | Urea (mg/dL) |
|---|---|---|
| Normal | 0.43 ± 0.02[a] | 33.34 ± 3.19[a] |
| Diabetic control | 0.83 ± 0.01[c] | 91.79 ± 3.38[d] |
| Diabetic treated with hesperetin | 0.69 ± 0.01[b] | 42.34 ± 7.05[ab] |
| Diabetic treated with BM-MSCs | 0.66 ± 0.01[b] | 54.52 ± 8.42[bc] |
| Diabetic treated with hesperetin and BM-MSCs | 0.67 ± 0.03[b] | 65.78 ± 3.98[c] |

Data are represented as mean ± SEM.
Number of animals in each group is six.
Mean values with different superscript letters are significantly different at $p < 0.05$.
[a,b,c] indicated the difference or similarity between groups.

**TABLE 5**

**Effects of hesperetin and BM-MSCs on LPO, GSH content and GPx, GST and GR activities in heart of STZ-induced diabetic rats**

| Group \ Parameter | LPO (nmol MDA/100 mg tissue) | GSH (nmol/100 mg tissue) | GPx (mU/100 mg) | GST (U/100 mg tissue) | GR (mU/100 mg tissue) |
|---|---|---|---|---|---|
| Normal | 13.92 ± 0.96[a] | 72.1 ± 0.96[d] | 137.15 ± 8.56[b] | 78.82 ± 1.95[c] | 55 ± 0.73[d] |
| Diabetic control | 32.09 ± 0.40[c] | 24.93 ± 0.75[a] | 75.66 ± 5.78[a] | 39.03 ± 1.37[a] | 12.67 ± 1.15[a] |
| Diabetic treated with hesperetin | 17.19 ± 1.34[b] | 48.1 ± 1.86[c] | 65.35 ± 4.45[a] | 66.88 ± 0.49[b] | 25.5 ± 1.26[b] |
| Diabetic treated with BM-MSCs | 12.94 ± 0.34[a] | 35.27 ± 0.88[b] | 77.17 ± 1.49[a] | 67.25 ± 0.67[b] | 26.83 ± 2.36[b] |
| Diabetic treated with hesperetin and BM-MSCs | 17.59 ± 1.42[b] | 37.03 ± 1.51[b] | 77.00 ± 2.88[a] | 66.33 ± 1.86[b] | 39.67 ± 3.57[c] |

Data are represented as mean ± SEM.
Number of animals in each group is six.
Mean values with different superscript letters are significantly different at $p < 0.05$.
[a,b,c,d] indicated the difference or similarity between groups.

**TABLE 6**

**Effects of hesperetin and BM-MSCs on LPO, GSH content and GPx, GST and GR activities in kidney of STZ-induced diabetic rats**

| Parameter / Group | LPO (nmol MDA/100 mg tissue) | GSH (nmol/100 mg tissue) | GPx (mU/100 mg) | GST (U/100 mg tissue) | GR (mU/100 mg tissue) |
|---|---|---|---|---|---|
| Normal | 66.29 ± 5.01[a] | 126.63 ± 4.44[d] | 29.13 ± 0.92[b] | 70.63 ± 1.48[c] | 31 ± 2.28[b] |
| Diabetic control | 146.27 ± 15.93[c] | 59.75 ± 3.32[a] | 21.47 ± 0.74[a] | 23.97 ± 1.86[a] | 12.83 ± 1.28[a] |
| Diabetic treated with hesperetin | 97.31 ± 7.69[b] | 102.53 ± 5.71[c] | 28.45 ± 1.71[b] | 53.77 ± 0.54[b] | 25.5 ± 1.45[b] |
| Diabetic treated with BM-MSCs | 104.30 ± 4.29[b] | 85.85 ± 2.29[b] | 28.45 ± 2.01[b] | 54.00 ± 0.48[b] | 25.33 ± 2.49[b] |
| Diabetic treated with hesperetin and BM-MSCs | 95.87 ± 3.35[b] | 105.75 ± 1.38[c] | 26.96 ± 1.13[b] | 55.00 ± 0.72[b] | 31.17 ± 2.33[b] |

Data are represented as mean ± SEM.

Number of animals in each group is six.

Mean values with different superscript letters are significantly different at $p < 0.05$.

[a,b,c,d] indicated the difference or similarity between groups.

The serum FFAs level, as well as hepatic cholesterol and TG contents, were significantly elevated in STZ-induced diabetic rats and were significantly ameliorated as a result of the treatment of diabetic rats with hesperetin and/or BM-MSCs. The treatment with hesperetin and BM-MSCs together produced the most potent effect in amending the elevated serum FFAs and hepatic cholesterol content (Tab. 2).

*Effect on the activities of serum enzymes related to heart function*

STZ-induced diabetic rats exhibited a significant elevation ($p < 0.05$) in serum CK-MB, AST, and LDH activities in comparison with the normal control rats. On the other hand, diabetic rats treated with hesperetin, BM-MSCs, and their combination showed a significant decrease in these elevated enzyme activities. The treatment with hesperetin and BM-MSCs in combination was more significantly potent in ameliorating the elevated CK-MB and AST activities than the treatment with either hesperetin or BM-MSCs (Tab. 3).

*Effect on of serum markers of heart function*

The rats administered STZ exhibited a significant increase ($p < 0.05$) in serum creatinine and urea levels in comparison with the normal control rats. On the other hand, STZ-induced diabetic rats treated with hesperetin and BM-MSCs singly or in combination showed a significant improvement ($p < 0.05$) in serum creatinine and urea levels in comparison with diabetic control. While the effect of treatments with hesperetin and BM-MSCs singly or in combination were more or less similar on serum creatinine level, the effect of hesperetin was the most effective in decreasing serum urea level (Tab. 4).

*Effect on heart and kidney oxidative stress and antioxidant defense system*

The STZ-induced diabetic rats exhibited a significant increase ($p < 0.05$) in heart and kidney LPO and a significant decrease in GSH content as well as GPx, GST and GR activities. The treatments of diabetic rats with hesperetin and BM-MSCs singly or in combination induced a significant improvement in the altered values of heart and kidney LPO, GSH, and antioxidant enzymes except for heart GPx (Tabs. 5 and 6). The treatment with hesperetin and BM-MSCs together was more effective in improving kidney LPO, kidney GSH content, kidney GST activity, and heart and kidney GR activities. However, the treatment with BM-MSCs alone and hesperetin alone produced, respectively, the most potent effect on heart LPO and GSH content.

## Discussion

Currently, stem cell therapy is considered as one of the most promising treatments for the near future. This kind of therapy could improve or even reverse some degenerative diseases and have potential applications in replacement and regenerative medicines and DM (Ahmed and Sayed, 2016). Also, the use of plant constituents in the treatment of DM has attracted many researchers because of the side effects of the conventionally used drugs (Ahmed *et al.*, 2017; Sangeetha, 2019; Al-Ishaq *et al.*, 2019).

In the present study, the treatment of STZ-induced diabetic rats with hesperetin produced a significant decrease in the elevated blood glucose level and a significant alleviation in the lowered serum insulin level. These results support Revathy and Sheik Abdullah (2016) who found that oral treatment with hesperetin for 45 consecutive days produced

a significant reduction in plasma glucose levels in diabetic rats. The improvement effect of hesperetin could be due to increased secretion of insulin from the survived pancreatic islet β-cells and/or regenerated β-cells, enhanced insulin sensitivity, activated glucose utilization by peripheral tissues or inhibition of glucose absorption in intestine (Ahmed *et al.*, 2010; *Li et al.*, 2019).

The current study also demonstrated that BM-MSCs therapy of STZ-induced diabetic rats significantly ameliorated the blood glucose level; the combinatory effect of hesperetin and BM-MSCs was more potent. These results are in concurrence with Bhansali *et al.* (2015) who revealed that MSC-treated diabetic Wistar rats showed a significant amendment in the deteriorated serum glucose and insulin levels. In the same way, Hess *et al.* (2003) reported a marked reduction of serum glucose level 4 days after transplantation of bone marrow-derived cells into STZ-treated mice due to the stimulation of endogenous cells to proliferate and produce insulin. Furthermore, Bell *et al.* (2012) attributed the mechanism of the MSCs' therapeutic effect on hyperglycemia to the restoration of islets through direct trans-differentiation into active β-cells. Dong *et al.* (2008) reported that MSCs moved to the pancreas of recipient rats and trans-differentiated into insulin-producing cells. The treatment of diabetic rats with hesperetin together with BM-MSCs may enhance the ability of newly formed β-cells to increase secretion and release of insulin into the blood.

In the present investigation, the therapy of diabetic rats with hesperetin, BM-MSCs, and their combination produced a significant increase in fasting insulin levels in serum. The current data are consistent with Mahmoud *et al.* (2015) who stated that the treatment of diabetic rats with hesperidin (hesperetin-7-O-rutinoside) is followed by significant amelioration in the levels of serum insulin and this may be owing to its potent β-cell protective activity and its stimulatory insulinogenic and insulinotropic effects. In the same regard, Kappel *et al.* (2013) confirmed that flavonoids may produce an enhancement in intracellular accumulation of calcium ions and suppression of channels of ATP-sensitive potassium ions in islets of Langerhans as an initial step in synthesis and release of insulin. Based on such previous findings, it can be elucidated that hesperetin may increase the release of insulin via stimulation of survived pancreatic islet β-cells. The current results are also in accordance with the results of Jurewicz *et al.* (2010) who stated that congenic MSCs therapy reverses hyperglycemia in experimental type 1 DM. In our opinion, the most potent anti-hyperglycemic effect of combination therapy with hesperetin and BM-MSCs may be due to its higher potency to increase insulin secretion and serum insulin levels.

Disturbances in lipid profiles are one of the most common abnormalities of DM found in 40% of diabetic patients, as revealed by Kulkarni *et al.* (2012). Hyperlipidemia is evidenced in both clinical and experimental DM (Solano and Goldberg, 2006). The key components of diabetic dyslipidemia are elevated plasma LDL-C, VLDL-C, TG, circulating FFAs, and lowered HDL-C (Ansar *et al.*, 2011).

The treatment of the STZ-induced diabetic rats with the flavonoid, hesperetin, successfully produced a significant decrease in the elevated serum levels of TG, TC, VLDL-C, LDL-C, and LDL-C:HDL-C ratio, and a significant increase in the lowered level of HDL-C. Such observations are in accordance with Miyake *et al.* (1998) and Fernandes *et al.* (2009), who stated that flavonoids decreased the TG and TC in the blood of rats. In addition, Hossain *et al.* (2016) stated that hesperetin and hesperidin exhibited lipid-lowering activity both in vivo and in vitro. Hesperetin was reported to lower the activity of the key cholesterol-regulating enzyme, acetyl-coenzyme A acetyltransferase, which is implicated in the absorption and esterification of cholesterol (Bilheimer *et al.*, 1983). Moreover, hesperetin suppressed cholesterol biosynthesis and increased the clearance of circulating LDL particles (Bilheimer *et al.*, 1983; Hossain *et al.*, 2016). The present results are also in concordance with Zhu *et al.* (1999), who stated that polyphenols inhibit oxidation of LDL in vitro; LDL oxidation is considered to be a key mechanism in atherosclerosis. The hesperetin antioxidant effects may result in a decreased oxidation of LDL lipids (Scalbert et al., 2005). The decrease in LDL levels may be rationalized to the decrease in VLDL and the increase in hepatic depuration of LDL precursors (Knekt *et al.*, 2002; Fernandes *et al.*, 2009).

The current study also showed the therapeutic effect of stem cells on dyslipidemic changes in diabetic rats and indicated a significant reduction in the elevated levels of serum levels of TC, TG, LDL-C, VLDL-C, LDL-C:HDL-C ratio and a significant increase in lowered serum level of HDL-C in diabetic rats treated with BM-MSCs in comparison with those of the diabetic control. These results support El-Tantawy and Haleem (2014), who found that the treatment of type 1 diabetic rats with MSCs normalized lipid profile. The present results are also in concordance with Pan *et al.* (2015) who reported that adipose tissue-derived stem cell transplantation reduced serum levels of TC and TG secondary to the ameliorative effects of MSCs on hyperglycemia and serum level of insulin which is concerned with activation of LPL (lipoprotein lipase), the enzyme responsible for removal of accumulated TG.

Insulin has a potent inhibitory effect on lipolysis in adipocytes. Insulin deficiency results in excess lipolysis and increased influx of FFAs to the liver (Coppack *et al.*, 1994; Ohno *et al.*, 2000). In the present study, a higher serum level of FFAs in diabetic rats due to increased mobilization of FFAs from peripheral deposits was evidenced since insulin inhibits the hormone-sensitive lipase. These results are in agreement with Revathy and Sheik Abdullah (2017).

The treatment of diabetic rats with hesperetin, BM-MSCs, and their combination, in the present investigation, induced a significant decrease in serum FFAs level. These results are in agreement with Jayaraman *et al.* (2018), who reported that hesperetin effectively alleviated hyperlipidemia by lowering FFAs levels in diabetic rats and with Tantawy and Halem (2014) and Frodermann et al. (2015), who found that treatment of type 1 diabetic rats with MSCs normalized lipid profile pattern. In our opinion, the antihyperlipidemic effect of hesperetin and BM-MSCs may be due to the enhancement of insulin secretion that eventually, in turn, leads to a reduction in serum FFAs, TG, TC, VLDL-C, and HDL-C levels.

The treatment of diabetic rats with hesperetin and/or BM-MSCs decreased the hepatic cholesterol and TG levels as compared to the diabetic control rats. These results are in

agreement with Kim *et al.* (2010) and Jayaraman *et al.* (2018) who found that hesperetin effectively decreased the elevated hepatic cholesterol, TG, FFAs and phospholipids levels in STZ-induced diabetic rats. The antihyperlipidemic effect of hesperetin and BM-MSCs may be due to the enhancement of insulin secretion that eventually leads to a reduction in the synthesis of cholesterol and fatty acids (FAs) (Jayaraman *et al.*, 2018). Our results support this attribution since the treatment of diabetic rats with hesperetin and/or BM-MSCs produced a significant increase in serum insulin levels. In another way, the cholesterol-lowering effect of hesperetin was possibly attributed to the ability of hesperetin to bind to bile acids in the small intestine, thereby increasing bile acid excretion and decreasing the cholesterol level as well as FAs absorption (Jayaraman *et al.*, 2018).

Diagnosis of cardiac enzymes in serum is a prerequisite in the case of diabetic cardiomyopathy. CK-MB, AST, and LDH are commonly used as indicators for impaired myocardial function. These biomarker enzymes are tightly bound to the contractile apparatus of the cardiomyocytes, and any serious insult to the heart muscle will stimulate the leakage of these enzymes into the serum (Edet *et al.*, 2009).

In the present study, the treatment of diabetic rats with hesperetin and BM-MSCs resulted in a significant amelioration of the elevated serum CK-MB, AST and LDH activities; the combinatory effect of hesperetin and BM-MSCs was the most potent on CK-MB and AST activities. In agreement with these results, Imaeda *et al.* (2002) reported that the hesperetin treatment prevented both the decrease in AST and LDH activities in the liver and heart and the increase in these enzymatic activities in serum that was caused by STZ injection. They also stated that antioxidants inhibited the increase in serum levels of AST in STZ treated mice (Imaeda *et al.*, 2002). On the other hand, Abdel Aziz et al. (2008) proved that MSCs transplantation improved cardiac function in STZ-induced diabetic rats. Based on the obtained effects, it can be postulated that the treatment with hesperetin and BM-MSCs together in combination has the most potent in improving the deteriorated heart function and integrity in diabetic rats.

Markers commonly and routinely used to test kidney function include the serum levels of creatinine and urea nitrogen (Bagshaw and Gibney, 2008). Changes in these markers have been used to assess renal function for decades, but each marker has limitations (Ronco and Bagshaw, 2009). Diabetic nephropathy (DN) is one of the most common and serious complications of DM and it is a major cause of end-stage renal disease that is reflected by proteinuria, decreased creatinine clearance, elevated creatinine, urea and uric acid levels (Yan *et al.*, 2007; Davey *et al.*, 2014; Khanra *et al.*, 2017; El-Said *et al.*, 2018; Ahmed *et al.*, 2019).

The therapy of diabetic rats with hesperetin and/or BM-MSCs, in the current study, ameliorated these renal markers. These data agree with Jayaraman *et al.* (2018), who demonstrated that the treatment of STZ-injected rats with hesperetin restore serum creatinine and urea near normalcy. The present data are also in parallel with the results of Mousa *et al.* (2016), who found that administration of BM-MSCs in diabetic rats showed a significant improvement of kidney function tests (creatinine, urea, and uric acid) as compared with the diabetic control group and this may be due to homing of MSCs to injured tissue causing its regeneration by its direct differentiation ability or by the paracrine factors released by MSCs that exhibit protective organ-actions (Gnecchi *et al.*, 2009; Tögel and Westenfelder, 2010; Xagorari *et al.*, 2013). Furthermore, the results of Abdel Aziz *et al.* (2014) showed a significant improvement in kidney function (serum creatinine and urea) in the DN group treated intravenously with a single dose of $1 \times 10^6$ MSCs per rat as compared with DN control group. The later authors referred this improvement in kidney function in MSCs treated group to their paracrine action via different growth factors such as vascular endothelial growth factors (VEGF), transforming growth factor-β (TGF-β) and tumor necrosis factor-α (TNF-α) as well as antiapoptotic effects via Bax and Bcl2 genes.

In the current study, there was a significant decrease in heart and kidney LPO in the diabetic rats treated with hesperetin, BM-MSCs, and their combination as compared with the diabetic control group. While BM-MSCs therapy was the most effective on heart LPO, the combination of hesperetin and BM-MSCs was the most potent in improving the kidney LPO. The present data are in concordance with Trivedi *et al.* (2011) who revealed that administration of hesperetin for five weeks reduced cardiac LPO and prevented oxidative DNA damage and apoptosis in doxorubicin-induced rats and with Kumar *et al.* (2017), who found that hesperetin treatment significantly reduced MDA level in renal tissue. In the same regard, hesperetin was reported to attenuate cisplatin-induced renal damage in rats through the inhibition of LPO and inflammatory cytokines (Kumar *et al.*, 2017). Furthermore, the present study is also in agreement with Mansour *et al.* (2017) and Hamza *et al.* (2018), who stated that the treatment with MSCs reduced the oxidative stress in diabetic rats by lowering LPO in comparison with diabetic control rats.

The treatment of diabetic rats with hesperetin, BM-MSCs or both, in the present study, significantly alleviated the lowered GSH content in the heart and kidney. While the treatment with hesperetin produced the most potent effect on heart GSH content, the treatment with hesperetin and BM-MSCs together was the most effective on kidney GSH content. The increase in GSH content has a pivotal role in the protective effect against heart and kidney injuries (Rajadurai *et al.*, 2009; Ognjanovic *et al.*, 2012; Saddala *et al.*, 2013).

Similarly, the activities of antioxidant endogenous enzymes including GST and GR, in the present study, were significantly improved in the heart and kidney as a result of the treatment of diabetic rats with hesperetin and BM-MSCs singly or in combination. GPx activity, on the other hand, was significantly increased in kidney of diabetic rats due to treatment with hesperetin and/or BM-MSCs while it was not significantly affected in the heart. The treatment of diabetic rats with hesperetin and BM-MSCs together was the most potent in improving heart GR activity and kidney GST and GR activities. These results are in agreement with Revathy and Sheik Abdullah (2016), who found a significant elevation of GPx activity in the hesperetin treated diabetic rats which might be due to its scavenging property and with Miyake et al. (1998), who noticed a marked increase in GPx and GST activities in kidney of the diabetic rats treated with lemon

flavonoids as compared with the non-diabetic rats and the diabetic rats. The present results also go parallel with the data of Haidara *et al.* (2015), who stated enhancement of cardiac antioxidant defense system in differentiated BM-MSC-treated diabetic rats and with Yan and Singla (2013), who reported that oxidative stress was significantly counteracted in STZ groups treated with induced pluripotent stem cells as evidenced by diminished pro-oxidant expression and enhanced antioxidants. In our opinion, the enhancement of the antioxidant defense potency as a result of the treatment of diabetic rats with hesperetin and BM-MSCs may have an important role in the improvement effects of heart and kidney functions. This speculation was supported by Ighodaro and Akinloye (2018).

## Conclusion

The STZ diabetes-induced impairments in lipid profile and heart and kidney functions were significantly improved by treatments with hesperetin and BM-MSCs singly or in combination; the treatment with hesperetin and BM-MSCs together was the most significant. The ameliorative effects as a result of the treatment of diabetic rats with hesperetin and BM-MSCs may be mediated, at least in part, to their suppressive effects on the oxidative stress and their enhancement effects on the antioxidant defense system.

## Conflicts of Interest

The authors declare that they have no conflicts of interest to report regarding the present study.

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