Original Article

Mechanism of Action of Natural Dipeptidyl Peptidase-IV Inhibitors (Berberine and Mangiferin) in Experimentally Induced Diabetes with Metabolic Syndrome

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Abstract

Background: Berberine (BER) and mangiferin are known natural dipeptidyl peptidase (DPP-IV) inhibitors. Hence, the study was designed to elucidate the mechanism of action of natural DPP-IV inhibitors (BER and MNG) in experimentally induced diabetes with metabolic syndrome. **Aim:** The aim of this study was to observe mechanism through which natural DPP-IV inhibitor works in diabetes with metabolic syndrome rat model. **Materials and Methods:** Wistar rats were fed high-fat diet for 10 weeks and challenged with streptozotocin (STZ) (40 mg/kg) at the 3rd week (high-fat diabetic control [HF-DC] group). After the confirmation of metabolic syndrome in the setting of diabetes, monotherapy (metformin [MET], vildagliptin [VIL], BER, and MNG) and combination (MET + VIL, MET + BER, and MET + MNG) therapy was orally fed to these rats from the 4th to 10th weeks. **Results:** Insulin resistance (IR) was seen in the HF-DC group as indicated by raised homeostasis model assessment of IR (HOMA-IR) in HF-DC group as compared with normal control (NC) groups. The treatment groups reduced IR as shown by a decrease in HOMA-IR as compared with HF-DC group rats. The marked reduction ($P < 0.001$) of beta-cell function was observed in the HF-DC group as a reduced level of HOMA for beta-cell function (HOMA-β) was found as compared with the NC group. Increases in HOMA-β as compared to the HFDC group were observed in the therapy groups. The treatment group significantly reduced cholesterol and atherogenic index. The treatment group showed significant preservation of beta-cell mass as per immunohistochemistry and significant anti-apoptotic activity as per Terminal Deoxyribonucleotidyl Transferase-Mediated dUTP Nick End Labeling assay report. The treated rats significantly ($P < 0.05$) reduced high-sensitivity C-reactive protein. Lipid peroxidation (thiobarbituric acid reactive substances) marker ($P < 0.001$) was significantly reduced in the treatment group. **Conclusion:** The natural DPP-IV inhibitors BER and MNG treatment showed beneficial effects on various components of metabolic syndrome.

Keywords: *Berberine, diabetes, dipeptidyl peptidase-IV inhibitor, mangiferin, metabolic syndrome*

Introduction

Dipeptidyl peptidase-IV (DPP-IV) is an ubiquitous multifunctional glycoprotein protease that plays important roles in metabolism, immunology, and nutrition.[1] DPP-IV degrades some enterohormones, the glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide, also known as incretins, in a matter of minutes. Incretins stimulate insulin while inhibiting glucagon secretion. Specific DPP-IV inhibitors can be administered to prevent, in particular, the degradation of GLP-1, allowing a sustained biological activity of this enterohormone to increase glucose-dependent insulin secretion, inhibit glucagon secretion, and slow gastric emptying. Marguet *et al*. confirmed the important role of DPP-IV in regulating glucose metabolism. They demonstrated that CD26 knockout mice show reduced glycemic spikes and increased glucose-dependent insulin levels after an oral glucose load.[2] DPP-IV inhibitors can reduce glycated hemoglobin (HbA1c) by 0.5%–0.9%, inducing a progressive but mild reduction in body weight.[3] Many DPP-IV inhibitors are now available in several countries: sitagliptin, vildagliptin (VIL), saxagliptin, linagliptin, and alogliptin, while others are under testing. DPP-IV inhibitors have been approved for the treatment of type II diabetes mellitus (TIIDM), both in monotherapy and in association with

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metformin (MET), glitazones, sulfonylureas, and insulin. The DPP-IV inhibitors are shown to have a positive effect on cardiovascular system through increasing GLP-1 levels and by inhibiting the degradation of other substrates involved in cardiovascular homeostasis, in addition to its effects on glucose metabolism.

Circulating DPP-IV activity has been reported to be increased in patients with obesity and TIIDM, positively correlating with HbA1c levels, degree of obesity, and measures of insulin resistance (IR) and inflammation.[4] According to Lugari *et al*., diabetes patients have higher levels of circulating DPP-IV activity, which lowers plasma GLP-1 (both during fasting and in response to meals).[5] In addition to circulating DPP-IV activity, the expression of DPP-IV on T-cells and dendritic cells is also increased in patients with TIIDM.[6] However, there are also reports suggesting a decrease of circulating DPP-IV activity in patients with TIIDM.[7] This potential contradiction may relate to the fact that, in these studies, many patients were on concomitant medications. Several widely used antidiabetic medications including thiazolidinedione, pioglitazone, and MET have been reported to reduce circulating DPP-IV.[8-10] This may reflect improvements in glycemic control and other measures of inflammation resulting in the reciprocal decrease in DPP-IV expression. An enhanced expression of hepatic DPP-IV has also been reported in nonalcoholic fatty liver disease and its expression may adversely affect glucose metabolism in this condition.[11]

DPP-IV inhibitors, a novel family of antidiabetic medications with a distinctive mode of action, have been released on the market. In the current situation, it would be ideal to have DPP-IV inhibitors derived from natural sources that have the same positive effects but lack the intolerable negative effects, are more affordable, and cooperate with the body's defense mechanisms. It is well-known that mangiferin (MNG) and berberine (BER) have antidiabetic properties with DPP-IV inhibitory activity.[12,13] BER, an isoquinoline alkaloid isolated from the root of *Berberis aristata*, is a member of the Berberidaceae family. It has demonstrated a broad range of pharmacological activities, including antimicrobial, antitumor, anti-inflammation, and antidiabetic activity.[14] MNG, a significant phytochemical found in *Mangifera indica,* is a member of the Anacardiaceae family. It has potent antioxidant, anti-lipid peroxidation, immunomodulatory, antidiabetic, cardiotonic, hypotensive, wound healing, antihyperlipidemic, anti-atherogenic, and anti-degenerative characteristics since it is a glucosylxanthone.[12] In this scenario, research to explore DPP-IV inhibitors from alternate sources is of paramount importance. Hence, the study was designed to elucidate the mechanism of action of natural DPP-IV inhibitors (BER and MNG) in experimentally induced diabetes with metabolic syndrome.

Materials and Methods

Experimental animal

Adult male Wistar rats, 10–12 weeks old, weighing 150–200 g were used in the study. The rats were housed in the Central Animal Facility, and they were maintained under standard laboratory conditions in the animal house. The study protocol was approved by the Institutional Animal Ethics Committee and conforms to the Committee for the Purpose of Control and Supervision of Experiments on Animals and Indian National Science Academy and Guidelines for the Use and Care of Experimental Animals in Research. The animals were allowed free access to a standard diet or high-fat diet (HFD) as the case may be and water *ad libitum*.

Preparation of high-fat diet

The HFD was prepared indigenously in our laboratory by using normal pellet diet, raw cholesterol, and a mixture of Vanaspati ghee and coconut oil (2:1). Normal rat pellet diet was powdered by grinding and mixed with 2.5% cholesterol and a mixture of Vanaspati ghee and coconut oil (5%). The mixture was made into pellets and put into the freezer to solidify. In addition, 2% raw cholesterol powder was mixed with coconut oil and administered to the rats by oral route (3 mL/kg).

Standardization of streptozotocin dose for induction of diabetes mellitus

The HFD along with 2% liquid cholesterol (3 mL/kg) was orally fed to rats for 3 weeks to induce metabolic syndrome. A pilot study was conducted with different doses of streptozotocin (STZ) (30, 35, and 40 mg/kg) in order to determine the optimal dose for induction of diabetes with STZ. Based on the pilot study results, it was found that 40 mg/kg STZ produced diabetes in experimental rats. Therefore, a single STZ injection (40 mg/kg body weight, i.p., dissolved in 0.01 M citrate buffer, pH 4.5) was standardized to induce diabetes mellitus.

Experimental model of diabetes with metabolic syndrome

After 3 weeks of dietary manipulation, rats were injected intraperitoneally with STZ (40 mg/kg). The body weight and biochemical parameters (blood glucose and total cholesterol [TC]) were estimated 7 days after the vehicle or STZ injection, i.e. on 4 weeks of dietary manipulation in rats. The rats with blood glucose (>200 mg/dL), TC (>110 mg/dL), triglyceride (TG) (>150 mg/dL), change in body weight (8% of initial weight), systolic blood pressure (>130 mmHg), and reduced high-density lipoprotein (HDL) levels (<35 mg/dL) confirmed the presence of metabolic syndrome with diabetes. Thereafter, the rats were either fed normal diet or HFD as per the protocol for 10 weeks. Blood samples were collected from the retro-orbital plexus under light anesthesia at 0, 4, 7, and 10 weeks for estimation of biochemical parameters.

Experimental design

Group 1: Normal control

In the normal control (NC) group, rats were administered distilled water per orally using a feeding cannula for a study period of 10 weeks. To imitate the STZ injection, 0.1 M citrate buffer, pH 4.5, was injected intraperitoneally at the end of 3 weeks. There were 8 animals in this group.

Group 2: High-fat diabetic control

The HFD was fed for 10 weeks to produce metabolic syndrome. At the end of 3 weeks, diabetes was induced by a single STZ injection (40 mg/kg body weight, i.p., dissolved in 0.1 M citrate buffer, pH 4.5). There were 14 animals in this group.

Group 3: Metformin

The HFD was fed for 10 weeks to produce metabolic syndrome. At the end of 3 weeks, diabetes was induced by a single STZ injection (40 mg/kg body weight, i.p., dissolved in 0.1 M citrate buffer, pH 4.5). The standard drug MET (100 mg/kg) was administered orally using a feeding cannula from the 5th week to the 10th week (6 weeks). There were 13 animals in this group.

Group 4: Vildagliptin

The HFD was fed for 10 weeks to produce metabolic syndrome. At the end of 3 weeks, diabetes was induced by a single STZ injection (40 mg/kg body weight, i.p., dissolved in 0.1 M citrate buffer, pH 4.5). The standard drug VIL (10 mg/kg) was administered orally using a feeding cannula from the 5th week to the 10th week (6 weeks). There were 13 animals in this group.

Group 5: Berberine

The HFD was fed for 10 weeks to produce metabolic syndrome. At the end of 3 weeks, diabetes was induced by a single STZ injection (40 mg/kg body weight, i.p., dissolved in 0.1 M citrate buffer, pH 4.5). The BER (100 mg/kg) was administered orally using a feeding cannula from the 5th week to the 10th week (6 weeks). There were 14 animals in this group.

Group 6: Mangiferin

The HFD was fed for 10 weeks to produce metabolic syndrome. At the end of 3 weeks, diabetes was induced by a single STZ injection (40 mg/kg body weight, i.p., dissolved in 0.1 M citrate buffer, pH 4.5). The standard drug MNG (40 mg/kg) was administered orally using a feeding cannula from the 5th week to the 10th week (6 weeks). There were 14 animals in this group.

Group 7: Metformin + Vildagliptin

The HFD was fed for 10 weeks to produce metabolic syndrome. At the end of 3 weeks, diabetes was induced by a single STZ injection (40 mg/kg body weight, i.p., dissolved in 0.1 M citrate buffer, pH 4.5). The standard drugs MET (100 mg/kg) + VIL (10 mg/kg) were administered orally using a feeding cannula from the 5th week to the 10th week (6 weeks). There were 13 animals in this group.

Group 8: Metformin + Berberine

The HFD was fed to rats for 10 weeks to produce metabolic syndrome. At the end of 3 weeks, diabetes was induced by a single streptozotocin injection (40 mg/kg body weight, i.p., dissolved in 0.1 M citrate buffer, pH 4.5). The drugs MET (100 mg/kg) + BER (100 mg/kg) were administered orally using a feeding cannula from the 5th week to the 10th week (6 weeks). There were 13 animals in this group.

Group 9: Metformin + Mangiferin

The HFD was fed for 10 weeks to produce metabolic syndrome. At the end of 3 weeks, diabetes was induced by a single streptozotocin injection (40 mg/kg body weight, i.p., dissolved in 0.1 M citrate buffer, pH 4.5). The drugs MET (100 mg/kg) + MNG (40 mg/kg) were administered orally using a feeding cannula from the 5th week to the 10th week (6 weeks). There were 13 animals in this group.

Evaluation parameters

Biochemical parameters

The rat blood samples of all experimental groups were collected from the retro-orbital plexus under light anesthesia at 0, 4, 7, and 10 weeks for estimation of blood glucose. In addition, after the completion of the experimental duration (10 weeks), serum was used for the determination of the following parameters such as serum insulin, C-peptide, high-sensitivity C-reactive protein (hs-CRP), lipid profile by auto-analyzer, or enzyme-linked immunosorbent assay (ELISA) kits in the pathology (NABL accredited) or pharmacology laboratory.

Derived parameters: Homeostasis model assessment of IR (HOMA-IR), HOMA for beta-cell function (HOMA-β), and atherogenic index (AI).

HOMA-IR = (serum glucose, mmol/L × serum insulin, μIU/mL)/22.5.

HOMA-β = (serum insulin, μIU/mL × 20)/(serum glucose, mmol/L)−3.5.

AI = TC − HDL-C/HDL-C.

Immunohistochemical localization of insulin

The pancreas was immediately fixed in a 10% buffered neutral formalin solution after scarification. The tissues were carefully cut 3 μm thick and placed on poly-L-lysine-coated slides before being transferred to three changes of xylene for 30 min and rehydrating with decreasing grades of alcohol. The antigen retrieval was in microwave oven 800 watt for 10 min, 420 watt for 10 min, and 360 watt for 5 min in citrate buffer pH 6. Immunostaining was performed by blocking peroxidase

with 3% hydrogen peroxide in methanol for 5 min and incubated sections for 10 min. The primary antibody was incubated for 30 min at room temperature before being incubated with superenhancer for 10 min. The tissues were incubated with poly-horseradish peroxidase (HRP) for 30 min followed by substrate diaminobenzidine (DAB). The slides were then visualized under light microscope to study the immunohistochemical localization of insulin.

Terminal Deoxyribonucleotidyl Transferase-Mediated dUTP Nick End Labeling assay

Pancreatic cell apoptosis was quantitatively analyzed by detection of DNA fragmentation using a commercially available Terminal Deoxyribonucleotidyl Transferase-Mediated dUTP Nick End Labeling assay (TUNEL) assay kit (Promega Co., USA). The DeadEnd™ colorimetric TUNEL system end-labels the fragmented DNA of apoptotic cells. Biotinylated nucleotide is incorporated at the 3'-OH DNA ends using the terminal deoxynucleotidyl transferase, recombinant, enzyme. HRP-labeled streptavidin (streptavidin HRP) is then bound to these biotinylated nucleotides, which were detected using the peroxidase substrate, hydrogen peroxide, and the stable chromogen, DAB. Using this procedure, apoptotic nuclei were stained dark brown. This method takes advantage of DNA fragmentation, characteristic of apoptosis.[15]

Estimation of serum thiobarbituric acid reactive substances assay

Standard ELISA assay kits (MyBiosource, USA) were utilized to estimate the thiobarbituric acid reactive substances (TBARS). The plate has been precoated with rat TBARS antibody. TBARS present in the sample is added and binds to antibodies coated on the wells. And then biotinylated rat TBARS antibody is added and binds to TBARS in the sample. Then streptavidin-HRP is added and binds to the biotinylated TBARS antibody. After incubation, unbound streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of rat TBARS. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

Results

Antidiabetic variables

Insulin resistance

IR was measured by serum insulin levels and HOMA-IR scores. The high-fat diabetic control (HF-DC) group significantly reduced the serum insulin as compared with the NC group. Monotherapy groups MET (100 mg/kg), VIL (10 mg/kg), BER (100 mg/kg), and MNG (40 mg/kg) treatment significantly increased ($P < 0.05$) the insulin levels as compared to HF-DC at the end of the 10th week, However, increase in serum insulin was highly significant ($P < 0.001$) in combination groups MET + VIL (100 + 10 mg/kg), MET + BER (100 + 100 mg/kg), and MET + MNG (100 + 40 mg/kg) insulin as compared with HF-DC group at the end of the 10th week. The IR was seen in the HF-DC group as indicated by raised HOMA-IR in the HF-DC group as compared with the NC group. However, it was not statistically significant. The treatment groups MET (100 mg/kg), VIL (10 mg/kg), BER (100 mg/kg) and MNG (40 mg/kg), MET + VIL (100 + 10 mg/kg), MET + BER (100 + 100 mg/kg), and MET + MNG (100 + 40 mg/kg) reduced IR as shown by a decrease in HOMA-IR as compared with HF-DC group rats. However, the results were not statistically significant [Figures 1 and 2].

Beta-cell function

The beta-cell function was assessed using HOMA-β scores and C-peptide levels. The marked reduction ($P < 0.001$) of beta-cell function was observed in the HF-DC group as a reduced level of HOMA-β was found as compared with the NC group. At the end of the study, increases in HOMA as compared to HFDC group were found in the monotherapy treatment groups MET (100 mg/kg), VIL (10 mg/kg), BER (100 mg/kg), and MNG (40 mg/kg) (P 0.001), as well as the combination therapy groups MET + VIL (100 + 10 mg/kg), MET + BER (100 + 100 mg/kg), and MET + MNG (100 + 40 mg/kg). The restoration in beta-cell function was more with VIL (10 mg/kg), as compared with BER (100 mg/kg). When compared to MET (100 mg/kg) groups, the MET + VIL (100 + 10 mg/kg) group had a greater impact on beta-cell activity. Rats in the HF-DC group had lower C-peptide levels than those in the NC group, although this difference was not statistically significant. In comparison to HF-DC, an increase in C-peptide level was seen in all treatment experimental groups, though statistically not significantly [Figure 3 and Table 1].

Pancreatic beta-cell preservation: Immunohistochemistry of pancreas for insulin localization

Immunohistochemistry of the HF-DC group pancreas showed decreased localization of insulin ($P < 0.001$)

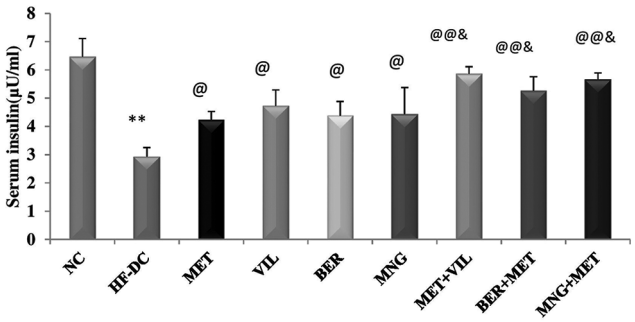

Figure 1: Estimation of serum insulin among various experimental groups. **$P < 0.01$ NC versus HF-DC, @@$P < 0.01$, @$P < 0.05$ MET, VIL, BER, MNG, MET + VIL, MET + BER, MET + MNG versus HF-DC, $P < 0.05$ MET + VIL MET + BER, MET + MNG versus MET. NC: Normal control; HF-DC: High-fat diabetic control; MET: Metformin; VIL: Vildagliptin; BER: Berberine; MNG: Mangiferin

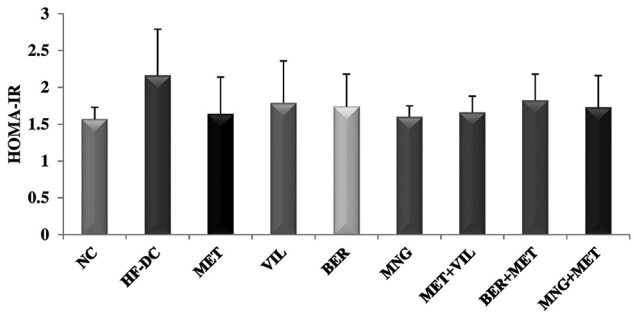

Figure 2: Estimation of HOMA-IR among various experimental groups. NC: Normal control; HF-DC: High-fat diabetic control; MET: Metformin; VIL: Vildagliptin; BER: Berberine; MNG: Mangiferin; HOMA-IR: Homeostasis model assessment of insulin resistance

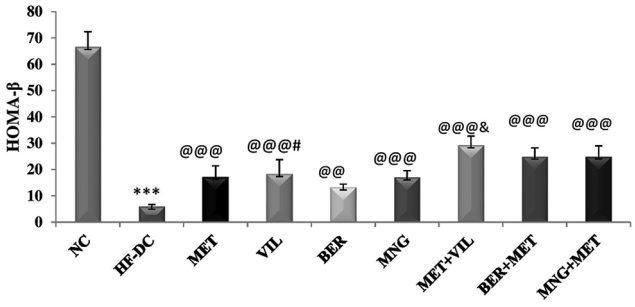

Figure 3: Estimation of HOMA-β among various experimental groups. ***$P < 0.001$ HF-DC versus HF-DC; @@@$P < 0.001$, @@$P < 0.01$ MET + VIL, MET + BER, MET + MNG versus HF-DC. &$P < 0.05$ MET + VIL versus MET, #$P < 0.05$ BER versus VIL. NC: Normal control; HF-DC: High-fat diabetic control; MET: Metformin; VIL: Vildagliptin; BER: Berberine; MNG: Mangiferin; HOMA-β: Homeostasis model assessment of beta-cell function

Table 1: Estimation of C-peptide among various experimental groups

Variable	C-peptide (ng/mL)
NC	0.06±0.01
HF-DC	0.05±0.01
MET	0.07±0.02
VIL	0.06±0.01
BER	0.06±0.01
MNG	0.07±0.01
MET + VIL	0.08±0.02
MET + BER	0.09±0.03
MET + MNG	0.12±0.02

NC: Normal control; HF-DC: High-fat diabetic control; MET: Metformin; VIL: Vildagliptin; BER: Berberine; MNG: Mangiferin

as compared to NC. The HF-DC group showed loss of beta-cell mass resulting in a decrease in insulin secretion. The treatment group increased the proportion of beta-cell, which was functional and secreting insulin as compared to HF-DC. The immunohistochemical findings of insulin localization were quantified and the percentage of insulin-positive cells was evaluated. Accordingly, were the percentages of insulinpositive cells in each group. NC (85 ± 7.7), HF-DC (8 ± 0.7), MET (12 ± 1.01), VIL (20 ± 1.18), BER (18 ± 1.6), MNG (30 ± 2.7), MET + VIL (40 ± 3.6), MET + BER (32 ± 2.9), and MET + MNG (35 ± 3.8), respectively [Figure 4a and b] (H and E ×40).

Apoptosis

Representative photographs of pancreatic tissue section for nick end-labeling (TUNEL) for DNA breaks of different groups are presented in photographs. TUNEL-positive cells were identified as those pancreatic cells which were stained brownish to black. The TUNEL-positive cells were quantified and expressed in percentage. The results demonstrated that the tunnel-positive cells in HF-DC (32.19% ±2.92%) group were significantly raised ($P < 0.001$) as compared to NC (1.83% ±0.16%). The apoptotic cells in treatment groups reduced significantly ($P < 0.001$) as compared with the HF-DC group. At the end of the study, increases in HOMA as compared to HFDC group were found in the monotherapy treatment groups MET (100 mg/kg), VIL (10 mg/kg), BER (100 mg/kg), and MNG (40 mg/kg) (P 0.001), as well as the combination therapy groups MET + VIL (100 + 10 mg/kg), MET + BER (100 + 100 mg/kg), and MET + MNG (100 + 40 mg/kg). The TUNEL positivity was significantly reduced in the treatment groups as compared with HF-DC. In addition, a significant decrease ($P < 0.01$) in the number of apoptotic cells in combination groups: MET + VIL (100 + 10 mg/kg), MET + BER (100 + 100 mg/kg), and MET + MNG (100 + 40 mg/kg) as compared with standard drugs MET (100 mg/kg) and VIL (10 mg/kg) was observed [Figure 5a and b].

Dyslipidemia

Lipid parameter

All treatment groups monotherapy MET (100 mg/kg), VIL (10 mg/kg), BER (100 mg/kg), and MNG (40 mg/kg) ($P < 0.001$), as well as the combination therapy treatment groups MET + VIL (100 + 10 mg/kg), MET + BER (100 + 100 mg/kg), and MET + MNG (100 + 40 mg/kg), showed favorable effects on lipid profile. The TC in the HF-DC group was significantly raised ($P < 0.001$) as compared with the NC group. The treatment group significantly reduced ($P < 0.001$) TC as compared with the HF-DC group. The significant improvement in TC in combination groups was observed as compared to the monotherapy MET group [Table 2].

Atherogenic index

The marked increase in ($P < 0.001$) AI was observed in HF-DC group rats as compared with NC rats. The treatment groups monotherapy as well as combination therapy significantly reduced the AI as compared with the HF-DC group and MET group. The reduction in AI was most superior in the MET + MNG (100 + 40 mg/kg) group ($P < 0.05$) followed by MET + VIL (100 + 10 mg/kg) [Figure 6].

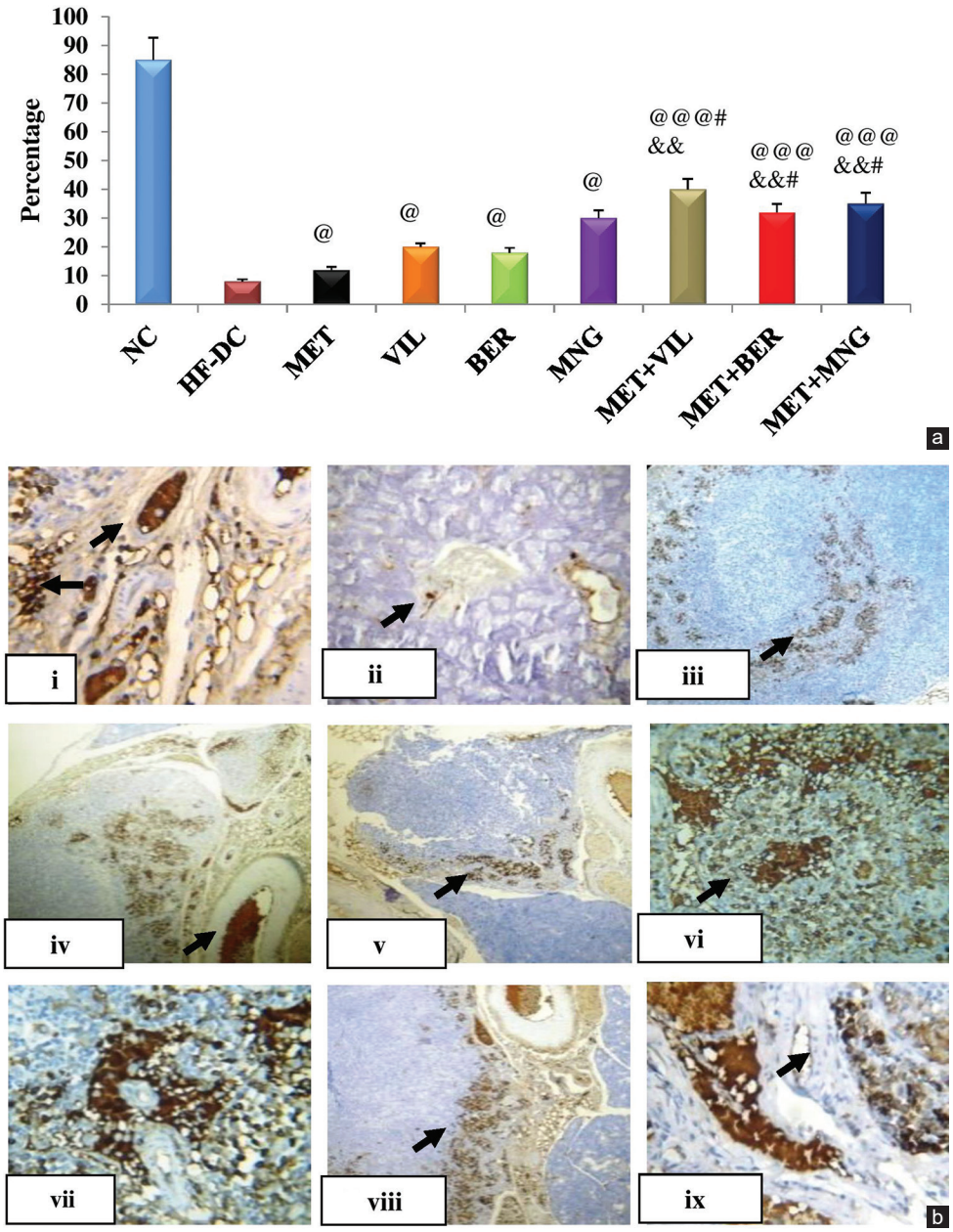

Figure 4: (a) Insulin-positive cells in parenchymal cells among various experimental groups. ******P* **< 0.001 NC versus HF-DC, @@@*P* **< 0.001, @*P* **< 0.05 MET, VIL, BER, MNG, MET + VIL, MET + BER, MET + MNG versus HF-DC, &&*P* **< 0.01 MET + VIL, MET + BER, MET + MNG versus MET. #*P* **< 0.05, #*P* **< 0.05 BER, MNG, MET + VIL, MET + BER, MET + MNG versus VIL. (b) Representative photomicrographs of pancreatic tissue sections stained for Immunohistochemical localization of insulin. Immunohistochemistry of NC group pancreas showed increased localization of Insulin (i). The HF-DC group showed decreased insulin localization and hence loss of beta-cell functions (ii). The MET, VIL, BER, and MNG monotherapy groups showed increased insulin-positive cells (iii-vi). The MET + VIL, MET + BER, and MET + MNG combination groups showed increased localization of insulin in pancreatic tissue (vii, viii, and ix). NC: Normal control; HF-DC: High-fat diabetic control; MET: Metformin; VIL: Vildagliptin; BER: Berberine; MNG: Mangiferin

Inflammatory and oxidative marker

Inflammation (high-sensitivity C-reactive protein)

The inflammatory marker (hs-CRP) ($P < 0.01$) was increased significantly in HF-DC group rats as compared to NC group rats. All treatment groups monotherapy MET (100 mg/kg), VIL (10 mg/kg), and MNG (40 mg/kg) ($P < 0.001$), as well as the combination therapy treatment groups MET + VIL (100 + 10 mg/kg), MET + BER (100 + 100 mg/kg), and MET + MNG (100 + 40 mg/kg) therapy except BER (100 mg/kg)-treated rats significantly ($P < 0.05$) reduced hs-CRP levels as compared to HF-DC rats at the end of study period 10th week [Figure 7].

Oxidative stress (lipid peroxidation marker: Thiobarbituric acid reactive substances)

Lipid peroxidation (TBARS) marker ($P < 0.001$) was significantly reduced in the treatment group as compared to HF-DC group rats at the end of study periods. The treatment group BER (100 mg/kg) ($P < 0.01$) and MNG (40 mg/kg) ($P < 0.001$) significantly

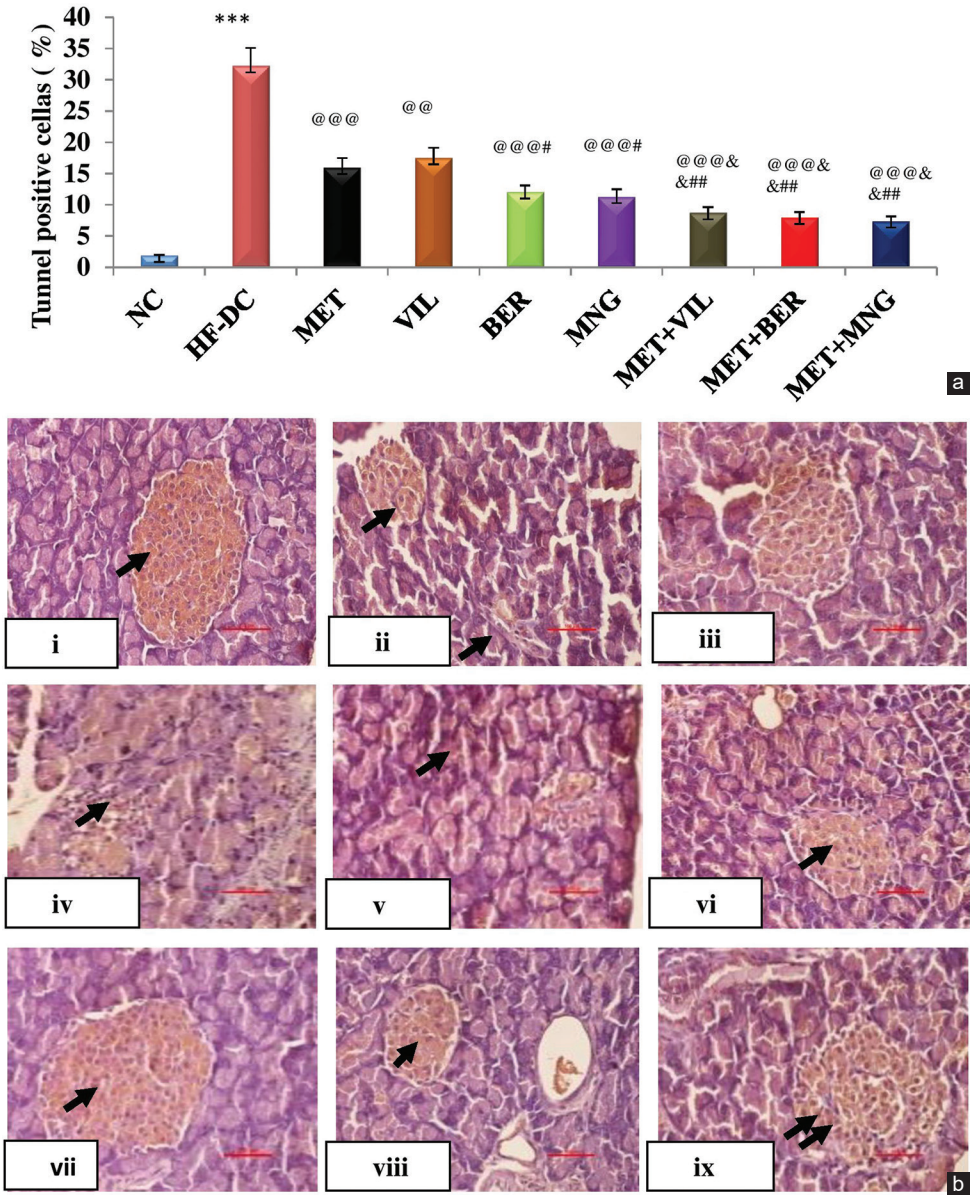

Figure 5: (a) TUNEL-positive cells among various experimental groups. ***$P < 0.001$ HF-DC versus NC; @@@$P < 0.001$, @@$P < 0.01$MET, VIL, BER, MNG, MET + VIL, MET + BER, MET + MNG versus HF-DC, &&$P < 0.01$ MET + VIL, MET + BER, MET + MNG versus MET., #$P < 0.05$, ##$P < 0.05$ BER, MNG, MET + VIL, MET + BER, MET + MNG versus VIL. (b) Representative photomicrographs demonstrating pancreatic tissue section stained using TUNEL assay for apoptosis. Normal control (i). The increased tunnel-positive cells in the HF-DC group (ii). The TUNEL positivity was significantly reduced in the treatment groups (iii-ix). Arrow indicates TUNEL-positive cells the brown-black staining with scale bar = 100 μm. Quantitative analysis of the percentage of apoptosis cells in pancreatic islets. NC: Normal control; HF-DC: High-fat diabetic control; MET: Metformin; VIL: Vildagliptin; BER: Berberine; MNG: Mangiferin; TUNEL: Terminal Deoxyribonucleotidyl Transferase-Mediated dUTP Nick End Labeling

reduced TBARS levels as compared to synthetic DPP-IV inhibitor. Combination therapy MET + BER (100 + 100 mg/kg) and MET + MNG (100 + 40 mg/kg) showed significant antioxidant property as compared to standard drug MET (100 mg/kg) [Figure 8].

Discussion

Mechanism of action of natural dipeptidyl peptidase-IV inhibitors: Berberine and mangiferin

A study of natural DPPIV inhibitors in the context of diabetes with metabolic syndrome was conducted to identify possible mechanisms behind their beneficial effects: antidiabetic (IR, beta-cell function, beta-cell preservation), dyslipidemia (lipid profile, AI, histopathology of thoracic aorta), apoptosis, inflammation, and oxidative stress.

Insulin resistance and beta-cell function

TIIDM is a heterogeneous disorder characterized by a progressive decline in insulin action (IR), followed by the inability of pancreatic β-cells to compensate for IR (β-cell dysfunction). The HOMA-IR and HOMA-β scores are validated surrogate measures of IR and beta-cell function,

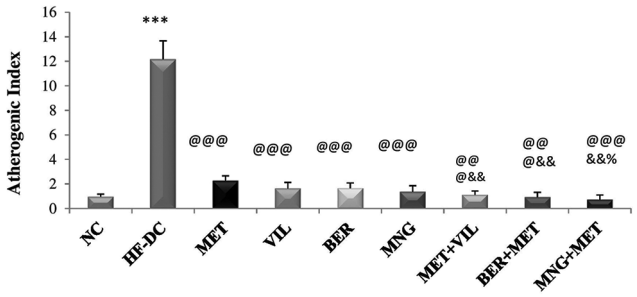

Figure 6: Atherogenic index among various experimental groups. ***P < 0.001 HF-DC versus NC; @@@ P < 0.001 MMET, VIL, BER, MNG, MET + VIL, MET + BER, MET + MNG versus HF-DC. &&P < 0.01 MET + VIL, MET + BER, MET + MNG versus MET. %P < 0.05 MET + VIL versus MET + MNG. NC: Normal control; HF-DC: High-fat diabetic control; MET: Metformin; VIL: Vildagliptin; BER: Berberine; MNG: Mangiferin

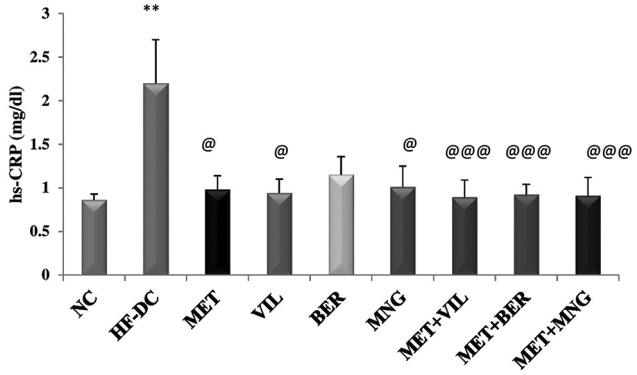

Figure 7: Effect of hs-CRP among various experimental groups. **P < 0.01HF-DC versus NC; @@@ P < 0.001, @P < 0.05 MET, VIL, MNG, MET + VIL, MET + BER, MET + MNG versus HF-DC. NC: Normal control; HF-DC: High-fat diabetic control; MET: Metformin; VIL: Vildagliptin; BER: Berberine; MNG: Mangiferin; hs-CRP: High-sensitivity C-reactive protein

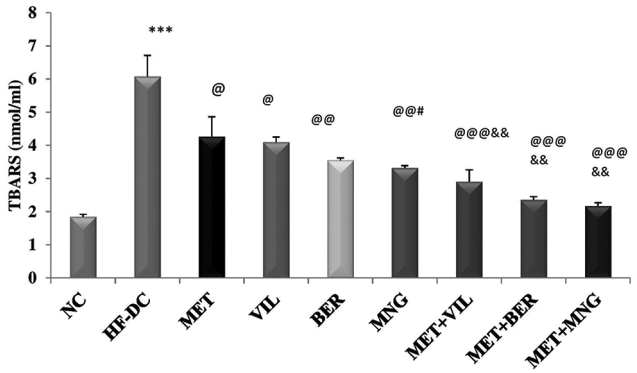

Figure 8: Effect of TBARS levels among various experimental. ***P < 0.001 HF-DC versus NC; @@@P < 0.001, @@P < 0.01, @P < 0.05 MET, VIL, BER, MNG, MET + VIL, MET + BER, MET + MNG versus HF-DC,#P < 0.05 BER, MNG versus VIL; &&P < 0.01 MET + BER, MET + MNG versus MET. NC: Normal control; HF-DC: High-fat diabetic control; MET: Metformin; VIL: Vildagliptin; BER: Berberine; MNG: Mangiferin; TBARS: Thiobarbituric acid reactive substances

respectively. The HF-DC group rats showed a modest increase in HOMA-IR scores (although statistically not significant) and a dramatic decrease in HOMA-β scores in HF-DC as compared to the NC group. In the present study, treatment

Table 2: Lipid profile among various experimental groups

Variable	TC (mg/dL)
NC	64.75±12.02
HF-DC	316.57±34.5***
MET	105.14±13.01$^{@@@}$
VIL	98±8.64$^{@@@}$
BER	94±14.13$^{@@@}$
MNG	85.57±17.34$^{@@@}$
MET + VIL	78.75±9.57$^{@@@,\&\&\&}$
MET + BER	71.57±7.67$^{@@@,\&\&\&}$
MET + MNG	68.28±8.2$^{@@@,\&\&\&,\#}$

**P<0.01 NC versus HF-DC; ***P<0.001; $^{@@}P$<0.01 MET; VIL; BER; MNG; MET + VIL; MET + BER; MET + MNG versus HF-DC; $^{@@@}P$<0.001; $^{\&}$0.05 MET + VIL; MET + BER; MET + MNG versus MET; $^{\&\&\&}P$<0.001; $^{\#}P$<0.05 MET + VIL versus MET + MNG. NC: Normal control; HF-DC: High-fat diabetic control; MET: Metformin; VIL: Vildagliptin; BER: Berberine; MNG: Mangiferin; TC: Total cholesterol

with BER and MNG restored the disturbed glucose homeostasis and improved IR. Wang Y *et al*.[16] (2011) supported the insulinotropic capabilities of BER. Similar actions of MNG were previously reported.[17] C-peptide is considered an important component in the biosynthesis of insulin and is an excellent parameter for evaluating pancreatic β-cells function. Our results demonstrated a significantly lower serum C-peptide concentration paralleling the HOMA-β scores, which further accounts for significant reductions in circulating serum insulin and insulin production in the HF-DC group as compared to the NC group. The study by Yin *et al*.[18] reported that BER has a beneficial effect on beta-cell function. C-peptide was restored by treatment with MET, VIL, and MNG. It is speculated that MNG might possess dual beneficial effects on the pancreatic mechanism, i.e. stimulating insulin release from the pancreatic cells, restoring the pancreatic beta-cell mass, and reducing IR by extrapancreatic mechanisms. Such multiple actions may explain the beneficial effects of MNG. Saleh *et al*. showed that MNG enhanced beta-cell function in dyslipidemia in insulin-resistant rats.[19]

Preservation of pancreatic beta-cell mass and function

While diabetes is diagnosed clinically by elevated plasma glucose levels, loss of β-cell function is progressive over time and β-cell dysfunction is far advanced by the time diabetes is diagnosed. Methods for preserving or restoring β-cell function are important for the prevention and treatment of TIIDM. Insulin immune reactivity was found in the majority of the islets in NC rats, while it was rare in the islets of HF-DC rats. The well-organized islet structure and high insulin immune reactive positive cells in the islets of the treated animals and the scant number of insulin immune reactive positive cells in the islets of HF-DC rats in this study indicate that treatment groups preserved not only the beta-cell mass but also its function of secreting insulin.

The quantification of insulin localization demonstrated an increased percentage of insulin-positive cells in the treatment groups (MET, VIL, BER, MNG, MET + VIL, MET + BER, and MET + MNG) as compared with the HF-DC group. MET + MNG showed superior effects on restoration of pancreatic beta-cell mass and function among the treatment groups. Sufium A *et al*.[20] (2015) demonstrated an increased number of insulin-positive cells in BER-fed rats as compared with the HF-DC control rats. The increase in number of insulin-positive cells is directly linked to the restoration of beta-cell function which supports the biochemical findings.

Apoptosis of pancreatic cell

There is a progressive deterioration in beta-cell function and mass in type II diabetics. It was found that islet function was about 50% of normal at the time of diagnosis, and a reduction in beta-cell mass of about 60% was shown at necropsy. The reduction of beta-cell mass is attributable to accelerated apoptosis. In the present study, TUNEL positivity was studied to delineate the involvement of apoptosis in the setting of diabetes coexisting with metabolic syndrome. The TUNEL assay is a method to quantify the degree of apoptosis in pancreatic cells. In the current data, enhanced, apoptosis as indicated by increased TUNEL-positive nuclei was observed in the HF-DC group as compared to the NC group. The significant reduction of TUNEL-positive cells was reported in the standard drug (MET, VIL, and MET + VIL) and test drug (BER, MNG, MET + BER, and MNG + MET) groups. BER and MNG demonstrated anti-apoptotic activities. Inhibition of beta-cell apoptosis may thus promote the expansion of beta-cell mass. The combination group showed a superior anti-apoptotic effect than monotherapy groups.

Atherogenic dyslipidemia

TIIDM is often linked with abnormal lipid metabolism. Both metabolic syndrome and type II diabetes are commonly associated with an abnormal lipoprotein phenotype which is characterized by increased TG, decreased HDL cholesterol (HDL-C), and an accumulation of small dense LDL particles (the so-called atherogenic dyslipidemia phenotype). The significantly higher serum TC and TG levels with a concomitant decrease in HDL-C levels in the HF-DC group, compared to the NC group, confirm the presence of atherogenic dyslipidemia. In the present study, standard drug and test drug treatments (MET, VIL, and MET + VIL) and test drugs (BER, MET + BER, MNG, and MET + MNG) showed favorable effects on the lipid profile by reducing TC and increasing HDL-C levels. The significant lipid-lowering effect of BER and MNG observed in the present study is consistent with the earlier reports by Hu *et al*.,[21] Weng *et al*.,[16] and Tang *et al*.[22]

The significant increase in AI in HF-DC indicates an increased risk of atherosclerosis. This is in agreement with the fact that IR induces several metabolic changes such as hyperglycemia, dyslipidemia, and perhaps to a lesser extent, hypertension, which all contribute to the development of atherosclerosis. AI was significantly reduced in MET, VIL, BER, MNG and MET + VIL, MET + BER, and MET + MNG group rats as compared to the HF-DC group. Interestingly, the AI was most favorably restored in MET + BER and MET + MNG as compared to MET. The antilipidemic activity of standard drugs (MET, VIL, and MET + VIL) and test drugs (BER, MNG, MET + BER, and MET + MNG) showed intact aortic layer, no hemorrhage, no atherosclerosis, and a reduced degree of inflammation in the aortic layer confirming the antilipidemic mechanism of test drugs. The study is also supported by Li *et al*.[23] and reported that BER improved endothelium arrangements and inflammation in the aorta.

Inflammation

Increasing evidence suggests that chronic, subclinical inflammation is part of the metabolic syndrome. Various components of metabolic syndrome are related to inflammatory markers (CRP, fibrinogen, and white cell count) and dyslipidemia, abdominal obesity, low insulin sensitivity, and hypertension parallel increasing levels of CRP.[24] In the present study, a significant elevation in plasma levels of the systemic inflammatory biomarker hs-CRP was seen in the HF-DC group as compared to the NC. The standard drugs (MET, VIL, and MET + VIL) and test drugs (BER, MNG, MET + BER, and MET + MNG) restored the levels of the inflammatory marker: hs-CRP, BER, and MNG treatment demonstrated anti-inflammatory activity, which may contribute to their favorable effects on dyslipidemia, insulin sensitivity, and hypertension, as the reports by Chen *et al*.[25]

Oxidative stress

Hyperglycemia is a well-known cause of free radical generation, which in turn can stimulate lipid peroxidation (TBARS). The most popularly used method to assay lipid peroxidation and free radical activity in biological samples is TBARS. A significant rise in lipid peroxidation was observed in the serum of HF-DC group rats as compared with the NC group, which was significantly decreased after treatment with standard antidiabetic drugs (MET, VIL, and MET + VIL) and test drugs (BER, MNG, MET + BER, and MET + MNG). These findings demonstrate that BER possesses anti-peroxidative effects. Similar results were reported in a previous study by Wang *et al*.[16] MNG has well-established antioxidant properties because it bears a catechol moiety that enables it to form stable MGF-Fe^{+2} complexes, preventing lipid peroxidation.[26] MNG treatment demonstrated antioxidant activity. Previous studies by Sellamuthu *et al*.,[17] Apontes *et al*.,[26] and Saleh *et al*.[19] supported this finding.

Conclusion

The natural DPP-IV inhibitors BER and MNG were efficacious in attenuating the deleterious changes induced by diabetes and metabolic syndrome. The mechanism of

action of natural DPP-IV inhibitors (berberine and MNG) for contribution to the beneficial effects in the context of metabolic syndrome and diabetes by decreasing IR, improving cell function, preserving pancreatic cells, anti-apoptotic activity, hypolipidemic activity, anti-inflammatory property, and antioxidant property.

Acknowledgment

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Ethical statement

The study was approved by the MGM medical college Institutional animal ethics committee, Navi Mumbai.

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Conflicts of interest

There are no conflicts of interest.

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