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Original Article

Neuroprotective effect of RYGB in Zucker fatty diabetic rats

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Abstract: The aim of this study is to explore the therapeutic potential of RYGB, a common used bariatric surgery, on diabetic polyneuropathy (DPN) in streptozotocin (STZ)-induced diabetic rats. In animal model experiments, rats were made diabetic by STZ administration, and after 12 weeks of diabetes, two groups were studied: RYGB and sham surgery control (PF). Change in oral glucose tolerance, insulin sensitivity, and the plasma concentrations of insulin, glucagon, glucagon-like peptide-1 (GLP-1) were measured. Peripheral nerve function was determined by the current perception threshold. Sciatic nerve blood flow (SNBF) and intraepidermal nerve fiber densities (IENFDs) also were evaluated. The results indicated that glucose tolerance and insulin sensitivity were significantly improved in the RYGB group. Fasting total GLP-1 were increased in the RYGB group. The increase seen in current perception threshold vales in RYGB group was reduced. The decreased IENFDs in sole skins of RYGB group were ameliorated by RYGB. In conclusion, the findings indicate that RYGB ameliorates the severity of DPN, which may be associated with increased GLP-1 and improved insulin sensitivity/action.

Keywords: Roux-en-Y gastric bypass, diabetes, glucagon-like peptide-1, insulin sensitivity

Introduction

The prevalence of peripheral neuropathy in diabetic patients approaches 70% and about 50% of these are cases of DPN [1]. The etiology of DPN is unknown and prediction of progression and treatments of the symptoms of DPN are limited [2]. The disease usually progresses to involve cardiac autonomic nerves, and as a result it is a major factor in mortality of diabetic subjects. It is well known that hyperglycemia plays an important role in the beginning and progression of DPN. Indeed, insulin treatment or treatment with insulin-sensitizing drugs to control hyperglycemia reverses some symptoms of DPN and delays its progression in general [3]. However, many large-scale studies suggested that independent factors other than glycemic control are critical to the development of diabetic polyneuropathy [4, 5]. T2DM clusters with other risk factors for coronary heart disease including obesity, hypertension and dyslipidemia; individuals with multiple of these factors are diagnosed with the metabolic syndrome. The common association of T2DM with other aspects of the metabolic syndrome has led to investigations into the effects of the metabolic syndrome and its components on neuropathy. Costa et al. and the Meta screen study team both used cross sectional designs to demonstrate an association between the metabolic syndrome and neuropathy [6, 7]. Smith et al. discovered that patients with idiopathic neuropathy with and without IGT had the same prevalence of metabolic syndrome components [8]. The implication of this study is that metabolic syndrome components other than IGT may play a role in neuropathy. Other groups have demonstrated an independent association between obesity, hypertension, low-density lipoprotein, high-density lipoprotein and/or hypertriglyceridemia with neuropathy [9]. All of

these studies point to factors other than glucose control in the development of neuropathy in patients with T2DM.

The Roux-en-Y gastric bypass (RYGB) is the most common bariatric procedure performed in the United States. The RYGB produces durable weight loss and significant improvements in metabolic conditions including: hypertension, hyperlipidemia, sleep apnea, arthritis, infertility, and type 2 diabetes mellitus (T2DM) [10, 11]. Many clinical studies have different hypothesizes to clarify the mechanisms of glucose homeostasis after RYGB, patients demonstrate significant improvements in T2DM shortly after RYGB surgery, before significant weight loss [12]. However, there was no study to observe the effect of RYGB on the complication of T2DM, such as DPN.

The current study was designed to examine the effects of RYGB on glucose tolerance, insulin sensitivity, and incretin production, and to observe the therapeutic effect of RYGB against peripheral neuropathy using the genetically obese Zuker rat model.

Materials and methods

Animals

Two groups of male Zucker rats, 10 to 12 weeks of age were studied: RYGB, sham surgery pair-fed (PF). Data from 79 rats were included the study: RYGB (n = 44), PF (n = 35). 10 normal rats were used as control group. The number of animals in each experimental group for different experiments is reported in the figure legend. Animals were housed in wire bottom cages to prevent coprophagia. Except for pretest overnight fasting and the immediate postoperative period, animals had free access to water and chow. The experimental protocols were approved by the Institutional Animal Care and Use Committee at Anhui Medical University.

Surgery

Before surgery, animals were randomized to the RYGB or PF. The RYGB procedure was performed using a modification of the technique described by Xu et al. [13]. The day before surgery rats were made fasted, but provided water. After randomization, rats were weighed, and then anesthetized with isoflurane (3% for induction, 1.5% for maintenance). Ceftriaxone 100 mg/kg intramuscular was given as a prophylactic antibiotic. Under sterile conditions a midline laparotomy was performed. Intestinal manipulation was performed in the 2 sham-surgery groups followed by abdominal closure. In the RYGB group, the stomach was divided using a GIA stapler (ETS-Flex Ethicon Endo surgery 45 mm) to create a 20% gastric pouch, the small bowel was divided to create a 15 cm biliopancreatic limb, a 10 cm alimentary (Roux) limb, and a 33 cm common channel. The gastrojejunal and jejunojejunostomy were performed using interrupted 5-0 silk sutures, followed by abdominal closure using 3-0 silk and 5-0 prolene. Surgical incisions were injected with 0.5 mL of 0.25% bupivacaine to minimize postoperative discomfort. All rats were injected subcutaneously with normal saline [50 mL/kg, before the start of surgery, immediately after surgery, and again on postoperative day (POD) 1]. After surgery, animals were housed individually and body weight and food consumption were monitored daily. To allow the surgical anastomose to heal, animals were not allowed to eat or drink until 24 hours after surgery. Approximately 24 hours after surgery, animals were started on a liquid diet and access to water. Regular chow was started on POD 3, to ensure adequate healing of the stomach and bowel anastomoses. The PF group was given the same amount of food as the RYGB rats consumed.

Oral glucose tolerance tests (OGTT)

OGTTs were performed preoperatively and repeated on POD 21. Blood was collected by tail snip before (to), and 30, 60, 90, and 120 minutes after oral gavage with 1.25 g/kg 25% dextrose in tubes containing 50 mmol/L EDTA, 12 TIU/mL aprotinin, and 100 μmol/mL dipeptidyl peptidase-4 inhibitor. Glucose was measured by glucometer (OneTouch Lifescan, Johnson and Johnson, New Brunswick, NJ). Insulin was measured by enzyme-linked immunosorbent assay (ELISA). Changes in glucose tolerance were compared by analyzing area under the curve (AUC).

Measurement of GLP-1

Plasma levels of intact and total GLP-1 were measured on timed plasma samples before and after surgery. A C-terminal radioimmunoassay for aminated GLP-1 was performed as previously described [14, 15]. Briefly, polyclonal

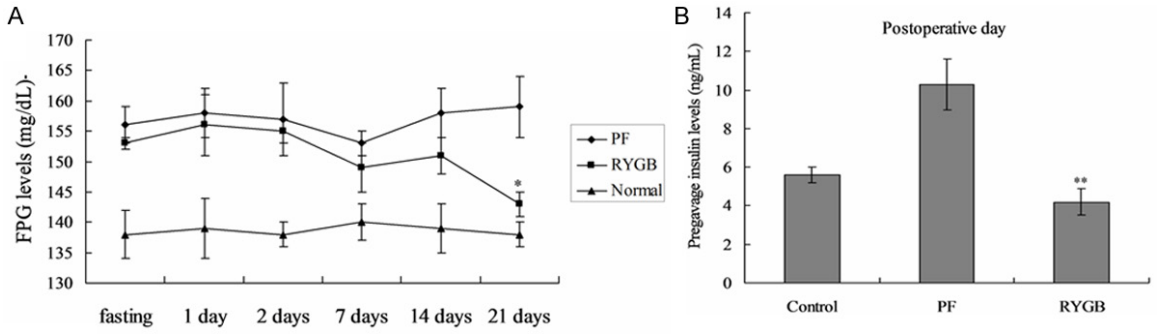

Figure 1. Glucose Tolerance analysis. A. FPG levels in PF, RYGB and Normal groups. B. Pregavage insulin levels in PF, RYGB and Normal groups. $^{*}P < 0.05$, $^{**}P < 0.05$ represents the PF group compared with RYGB group.

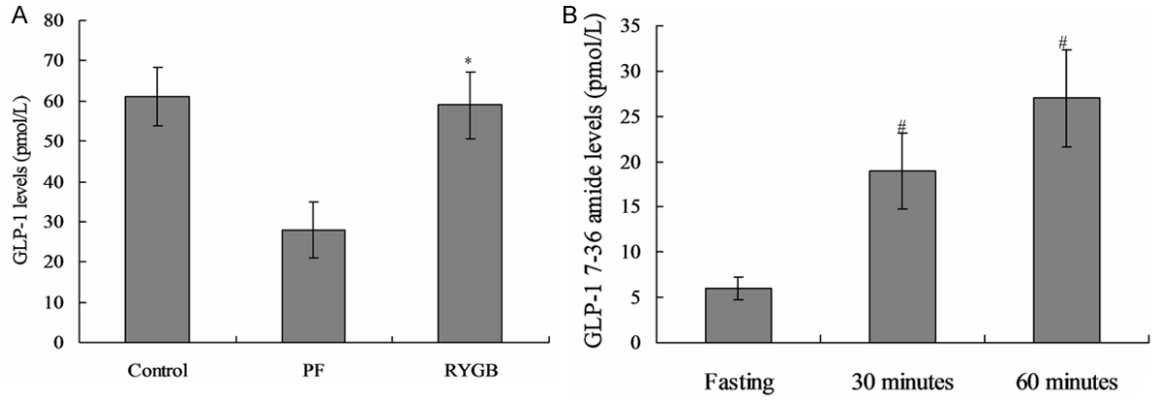

Figure 2. GLP-1 and GLP-1 7-36 amide levels in every groups. A. GLP-1 levels in in PF, RYGB and Normal groups. B. GLP-1 7-36 in PF, RYGB and Normal groups. $^{*}P < 0.05$ represents the PF group compared with RYGB group. $^{\#}P < 0.05$ represents the 30 minutes and 60 minutes compared with fasting group.

antiserum (code 89390) to a synthetic PG 97-107 amide [GLP-1 (26–36) amide] was raised in rabbits, coupled to bovine serum albumin with carbodiimide. Antiserum 89390 has an absolute requirement for amidated C-terminus of GLP-1. Standard and I 125 -labeled tracer are PG 78-107amide [GLP-1 (76-36) amide] and separation of antibody-bound from free peptide was performed using plasma-coated charcoal. The total GLP-1 assay has a detection limit of 1 pmol/L and an ED 50 of 25 pmol/L. Intra- and inter-assay coefficients of variation are < 6% and < 15%, respectively. Intact GLP-1 amide levels were measured on the same samples using a 2-site sandwich assay as previously described. The intact GLP-1 (7-36) amide assay has a detection limit of 0.5 pmol/L with intra- and inter-assay coefficients of variation of 2% and 5%, respectively.

Measurement of current perception threshold

To determine a nociceptive threshold, the current perception threshold (CPT) was measured in 12- and 16-week diabetic and age-matched normal mice using a CPT/laboratory neurometer. The electrodes for stimulation were attached to plantar surfaces. Each mouse was kept in a Ballman cage suitable for light restraint to keep awake. Three transcutaneous-sine-wave stimuli with different frequencies (2,000, 250, and 5 Hz) were applied to the plantar surfaces. The intensity of each stimulation was gradually increased automatically (increments of 0.01 mA for 5 and 250 Hz and increments of 0.02 mA for 2,000 Hz). The minimum intensity at which the mouse withdrew its paw was defined as the CPT. Six consecutive measurements were conducted at each frequency.

Nerve conduction velocity

Mice anesthetized with pentobarbital were placed on a heated pad in a room maintained at 25°C to ensure a constant rectal temperature o f 37°C. Motor nerve conduction velocity (MNCV) was deter-mined between the sciatic notch and ankle with a Neuropak NEM-3102

instrument, as previously described [5, 6]. The sensory nerve conduction velocity (SNCV) was measured between the knee and ankle with retrograde stimulation.

Sciatic nerve blood flow

Sciatic nerve blood flow (SNBF) was measured by laser-Doppler flowmetry. The thigh skin of an anesthetized mouse was cut along the femur and then an incision through the fascia was carefully made to expose the sciatic nerve. Five minutes after this procedure, the blood flow was measured by a laser-Doppler probe placed 1 mm above the nerve. During this measurement, the mouse was placed on a heated pad in a room maintained at 25°C to ensure a constant rectal temperature of 37°C.

Statistical analysis

Both the group values were expressed as means ± SD. Statistical analyses were made by *Students' t* test. All analyses were performed by personnel who were unaware of the animal identities.

Results

Glucose tolerance

Before surgical intervention, there were no significant differences in glucose tolerance curves between the groups. To assess the effects of RYGB on glucose homeostasis, OGTTs were performed on POD 21 (**Figure 1A**). Mean fasting plasma glucose levels (mg/dL) were 159 ± 9 in the PF group, and 143 ± 6 in the RYGB group, ($P < 0.05$). Glucose tolerance in the RYGB animals was significantly improved as indicated by a 29% reduction in the AUC for blood glucose. On POD 21, pregavage insulin levels were significantly decreased in the RYGB group (4.2 ± 0.7 ng/mL) compared with the PF group (10.3 ± 1.32 ng/mL) (**Figure 1B**, $P < 0.01$). Postgavage insulin levels in the PF remained elevated and stable over time, whereas the insulin levels in the RYGB group more than doubled at 30 minutes (10.7 ± 1.9 ng/mL), then decreased to basal levels from 60 to 120 minutes postgavage. By POD 21, the RYGB animals demonstrate reductions in both basal insulin and glucose levels relative to PF suggesting an improvement in insulin sensitivity.

The level of GLP-1

The dramatic postgavage increase in plasma insulin levels observed in the RYGB animals prompted investigation into the effect of RYGB on plasma incretin levels. Before surgery, total GLP-1 levels were similar in the PF, and RYGB groups. However, after the surgery, the changes in plasma GLP-1 over time differed between the groups. Plasma GLP-1 levels remained between 35 and 22 pmol/L (mean 28 pmol/L) in the PF. In contrast, the 30 minutes postgavage total GLP-1 level increased 2-fold in the RYGB group compared with the PF group (**Figure 2A**) ($P < 0.05$) and gradually decreased over time. Levels of intact, biologically active GLP-17-36 amide were measured on the same samples. Fasting and 30 to 60 minutes postgavage GLP-17-36 amide levels were elevated in the RYGB group (**Figure 2B**) (both $P < 0.05$).

Reduced sensory perception in diabetic mice was ameliorated by RYGB

CPTs of RYGB and PF group at 5, 250, and 2000 Hz were significantly increased compared with those in normal mice (**Figure 3A**) (5 Hz: $P = 0.015$, 250 Hz: $P = 0.019$, and 2,000 Hz: $P = 0.028$), representing hypoalgesia in diabetic mice. After 8 weeks of surgery, these deficits in sensation were significantly improved in RYGB (**Figure 3B**) (5 Hz: $P = 0.0161$, 250 Hz: $P = 0.0012$, and 2,000 Hz: $P = 0.0011$).

RYGB improved delayed NCVs

MNCVs and SNCVs were significantly delayed compared with those of normal mice (**Figure 4**) (MNCV: $P = 0.0341$, SNCV: $P = 0.0489$). The delay in MNCVs and SNCVs was significantly restored by RYGB (**Figure 4**) (MNCV: $P = 0.0289$, SNCV: $P = 0.0201$). However, NCVs did not change in PF group. Nerve fibers in epidermis were preserved by RYGB. IENFDs were evident in both the epidermis and the dermis of the foot skin by the fluorescent imaging. Although IENFDs were decreased in diabetic mice ($P = 0.0011$), this decrement was significantly ameliorated by RYGB ($P = 0.000\,7$) (data not shown).

Discussion

In general agreement with both clinical data on the earliest signs and symptoms of human

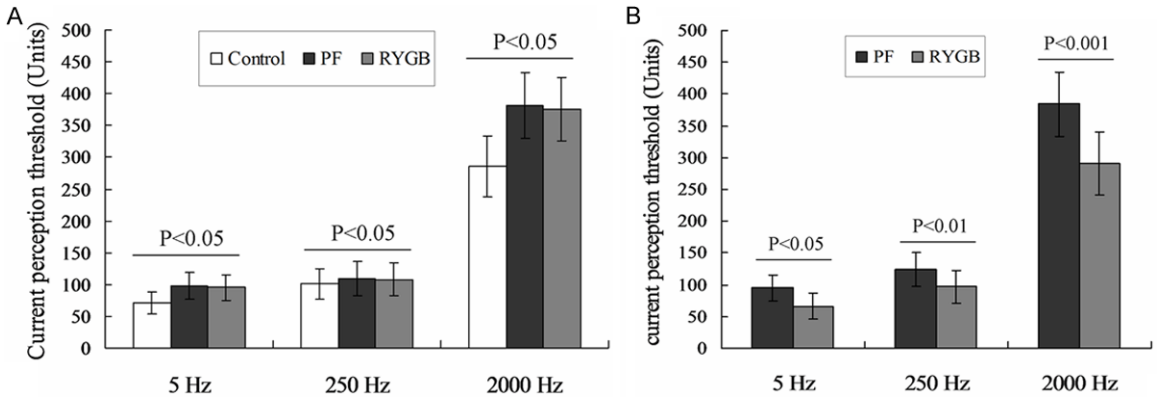

Figure 3. Current perception threshold (CPT) in every group. A. CPTs of RYGB, PF and normal group at 5, 250, and 2000 Hz in 8 weeks. B. CPTs of RYGB and PF group at 5, 250, and 2000 Hz after 8 weeks of surgery.

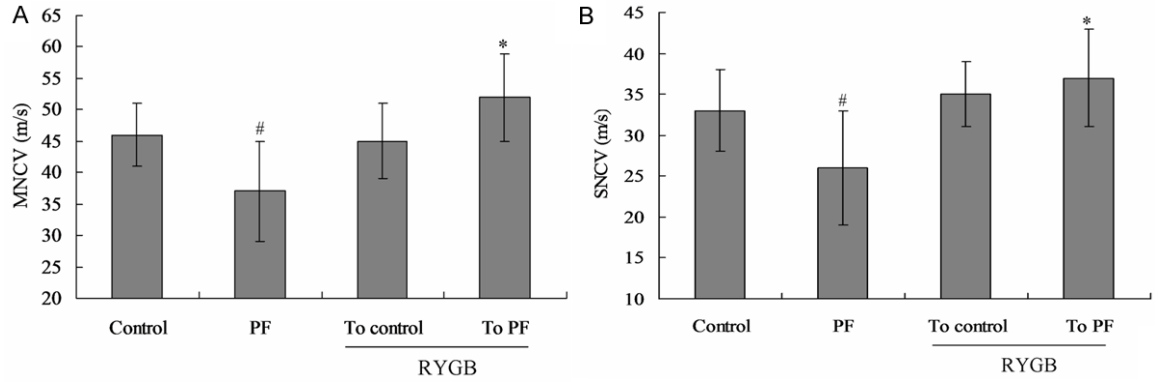

Figure 4. MNCA and SNCV changes in every group. MNCV (A) and SNCV (B) were measured before and after the treatment with RYGB or without. #P represents MNCV or SNCV in PF group compared with in Control group. *P represents MNCV or SNCV in RYGB group compared with in PF group.

DPN, slowing of sensory and motor NCV and manifestations of evoked pain were shown to develop within the first month of onset of hyperglycemia in diabetic rats [16, 17]. With a longer time allowed (six to twelve months of diabetes) signs of axonopathy, demyelination and nerve degeneration can also be detected in diabetic animals [18]. Rat models of type 1 diabetes (STZ-induced or spontaneous in BB-rats) and spontaneous type 2 diabetes in Zucker fatty rats that appear to be the best studied animal models with regard to neuropathy [19]. In our study, Zucker fatty rats were used because the potential mechanism of diabetes was more similar to T2DM in human.

We evaluated sensory nerve functions using a CPT/laboratory neurometer. The neurometer is now widely and clinically used to evaluate the effects of analgesic drugs and peripheral nerve functions in various painful neuropathies, including DPN. In this study, after 12 weeks of diabetes, hypoalgesia at 2000, 250 and 5Hz was observed in the diabetic mice, and RYGB improved these abnormalities. In addition, RYGB ameliorated the decreased IENFDs in diabetic mice. The restoration of sensory functions by RYGB was confirmed by the improvement of IENFDs. In addition, we measured MNCVs and SNCVs that represent relatively large axonal functions. Both the delayed MNCVs and SNCVs in diabetic mice were improved by RYGB, indicating that RYGB had therapeutic effects on impaired motor and sensory nerve functions.

From experiments in animals, and by analogy with other neuropathies, it can be suggested that the pathogenesis of negative symptoms and signs of DPN is likely to be associated with demyelination and axonal atrophy and degeneration [20]. Failure of re-innervation will make

these symptoms essentially irreversible [21]. DPN follows both type 1 and type 2 diabetes, and systemic hyperglycemia is the most obvious symptom that these types of the disease have in common [22], suggesting hyperglycemia as a universal trigger for DPN. Most of the data available indicate that all the various pathways activated by hyperglycemia converge in generation of excess reactive oxygen species (ROS). This process eventually overwhelms the intrinsic anti-oxidant mechanisms of the cell and ends in oxidative/nitrosative stress and pro-inflammatory conditions in the tissues [23, 24]. Then the good glucose control by RYGB observed in our study obviously participated in the improvements of DPN in Zucker fatty rats.

Over the long-term, insulin production is impaired in type 2 diabetes further increasing the incidence of DPN in this population. In a ten-year study of the natural history of type 2 diabetic patients, it was found that decreased serum insulin and increased blood glucose concentrations are independent predictors of DPN [25]. However, in early type 2 diabetes there is a compensatory hyperinsulinemia, it suggests that increased production of insulin may fail to compensate for decreased sensitivity of PNS to regulation by insulin. In studies in normal human volunteers, warmth detection threshold correlated with insulin but not fasting or 2-h GTT glucose, leading the authors to suggest that insulin resistance may determine some sensory functions of PNS [26]. This is also supported by observations of decreased NCV [27] and pressure pain threshold [28] and the authors' unpublished observations in the Zucker fatty rat model of T2DM. In the obese Zucker rat, peripheral insulin resistance is because of defective insulin signaling, reductions in the insulin-sensitive GLUT4 expression, and impaired insulin-stimulated GLUT4 membrane translocation [29]. The physiologic response to insulin resistance initially involves a compensatory increase in pancreatic β-cell mass and insulin secretion in the obese Zucker rat [30]. The reduction in fasting insulin observed in the RYGB animals relative to PF control group suggests an improvement in insulin sensitivity as a mechanism for post-RYGB glucose homeostasis. Our data clearly indicate that RYGB improves the ability of insulin to increase peripheral glucose uptake. Then the correction of insulin resistance might also be a factor promoting the amelioration in DPN.

GLP-1, a product of the proglucagon gene is secreted by L cells of the distal ileum and colon in response to intraluminal fats and carbohydrates. GLP-1 induce β-cell proliferation, inhibit apoptosis, and stimulate glucose-dependent β-cell insulin secretion via specific receptor-mediated pathways [31]. GLP-1 previously has been shown to promote neurite outgrowth of rat pheochromocytoma cells [32] and to protect rat primary hippocampal neurons from cell death. It has been reported that exogenous GLP-1 R activation significantly reduces glucose-dependent reactive oxygen species generation in hypothalamus [33]. These antioxidative effects of GLP might yield benefits to central and peripheral nervous systems. We observed the increase in plasma GLP-1 in the RYGB group. GLP-1 is one of the substrates of DPP-IV, several bioactive peptides, such as neuropeptide Y, substance P, glucagon-like peptide-2, and stromal cell-derived factor-1 a also have been reported as substrates of DPP-IV [34]. Among these peptides, neuropeptide Y and substance P are known as neurotransmitters or modulators of peripheral nervous systems regulating leukocyte chemotaxis and modulating neuropathic pain behavior [35, 36]. Therefore, the preventive effects of RYGB on DPN may be attributed to its protective effects on these neurotrophic peptides and mediated through increased levels of GLP-1.

In conclusion, RYGB appeared to ameliorate diabetic peripheral neuropathy in sciatic nerve functional abnormalities, probably through controlling the hyperglycemia, improving the insulin resistance and releasing more GLP-1. The GLP-1 receptor may act through signal pathways involved in apoptosis and cAMP to enhance neuroprotection. These findings have important implications for RYGB in the treatment of diabetic peripheral neuropathy.

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Disclosure of conflict of interest

None.

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