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Neutrophil Apoptosis and Inducible Nitric Oxide Synthase in Type II Diabetic Patients with Periodontitis

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ORIGINAL STUDY

Neutrophil Apoptosis and Inducible Nitric Oxide Synthase in Type II Diabetic Patients with Periodontitis

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Abstract

Purpose: The current study was to evaluate the glycemic status, inducible nitric oxide synthase, and spontaneous neutrophil apoptosis in tested groups, which were divided into four groups: group (1) healthy group, group (2) periodontitis, group (3) diabetic group, and group (4) diabetic with periodontitis. **Patients and methods:** There were 40 patients in all, and they were separated into four groups based on their state of health: group (1) healthy, group (2) periodontitis, group (3) type 2 diabetic patients, and group (4) diabetic patients with periodontitis. Periodontal parameters, glycemic condition assessment using HbA1c, evaluation of polymorphous neutrophil (PMN) apoptotic activity using flow cytometry, and measurement of inducible nitric oxide synthase were all carried out. **Results:** Glycated hemoglobin (HbA1C) level and spontaneous apoptosis percentage had a strong negative association ($r = -0.813$). The amount of spontaneous apoptosis and the duration of diabetes had a moderately negative connection ($r = -0.622$). HbA1C level and the duration of diabetes were strongly positively correlated ($r = 0.933$). Age and spontaneous apoptosis showed a moderately negative link. Moreover, there was a moderately negative connection ($r = -0.725$) between iNos and spontaneous apoptosis percentage. **Conclusion:** There is decreased apoptosis in a diabetic with periodontitis group compared with the healthy group. Moreover, there were high concentrations of iNos in the diabetic with periodontitis group compared to healthy group. Also, disturbance of apoptosis related to inflammation. The decline of apoptosis is associated with chronic disorders like diabetes and periodontal diseases. Besides, high concentrations of iNos related to destructive mechanisms included more periodontal tissue destruction.

Keywords: Apoptosis, iNos, Periodontitis, Polymorphous neutrophil, Type 2 diabetes

1. Introduction

Metaflammation is a key feature of type 2 diabetes mellitus (T2DM) and a significant contributor to the development of both diabetes and its comorbidities. In terms of clinical presentation and molecular profile, metaflammation is similar to age-induced inflammation, referred to as inflammaging, indicating a common etiology for co-occurring disorders [1,2].

With or without insulin resistance in peripheral cells, the underlying pathophysiological mechanisms in T2DM reduce the ability of pancreatic cells to produce insulin; this latter factor has a direct impact on high levels of peripheral glucose or free fatty acids. Patients may experience episodes of adephagia during this metabolic change, which would further encourage fat and adiposity [3].

It is interesting to note that the metabolic system substantially influences the direct effects of phagocyte population responses and regulation [4]. The functions of myeloid cells in this environment, particularly macrophages, are well known [5]. Macrophage and immune cells with changed functioning that are precursors to T2DM become regulated in early metabolic syndrome. Besides, areas of inflammation promote a variety of outcomes, such as the release of cytokines and chemokines or the attraction and activation of leukocytes [6,7].

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Leukocyte–endothelial interactions are better and there are more adhesion antigens on polymorph nuclear cell membranes in T2DM patients [7] and murine models [8,9]. As a result of these molecular changes, the magnitudes of polymorphous neutrophil (PMN) functions may change [10].

Type 2 diabetes and chronic periodontitis share comparable pathophysiologies; these are covered elsewhere [11,12]. The underlying mechanisms of host tissue destruction are significantly impacted by the pathogenesis of chronic periodontitis hyperinflammation, notably that generated by hyperactive PMN [13].

Among other disorders, these PMN phenotypes may have an impact on metabolic and central or peripheral artery obstructive diseases. Therefore, PMN may serve as a putative pathogenic connection, enabling chronic periodontitis to affect comorbidities.

Inflammatory cells quickly and persistently express the inducible variation of NOS in response to bacteria or their byproducts, such as lipopolysaccharides [14]. While excess iNOS-induced nitric oxide can cause cell and tissue damage, small levels of NO produced by constitutive NOS are thought to be helpful.

Spontaneous PMN apoptosis is delayed in T2DM and coexpression with localized inflammatory conditions, such as periodontitis, exacerbates delayed spontaneous PMN apoptosis. Functional abnormalities of PMN apoptosis would cause persistence at the site of infection, exacerbating a proinflammatory potential, furthering host tissue destruction, and ultimately causing delayed wound healing [15]. Also, the manipulation of NO concentrations is a particularly promising candidate to alter leukocyte function and rates of apoptosis in inflammatory conditions. This study was carried out to find an association between spontaneous apoptosis of neutrophils and iNOS level in type II diabetic patients with periodontitis.

2. Patients and methods

2.1. Study design

The 40 patients were separated into four groups: the healthy group, the periodontitis group, the diabetic type 2 group, and the diabetic patients with periodontitis group.

2.2. Sample size calculation

ANOVA test or Kruskal–Wallis test was used to compare the groups in the research of PMN apoptosis and iNos in diabetic individuals with periodontitis. The mean PMN apoptosis was 85.33.1 in healthy individuals, 67.33.9 in type 2 diabetic patients, 62.93.5 in periodontitis, and 62.55.4 in T2DM + CP, according to prior work by the researcher [15]. A significant impact size of approximately 0.58 is anticipated.

A total sample size of 40 (10 in each group), using the G power statistical power analysis program (version 3.1.9.7) [16].

2.3. Inclusion criteria of patients with type 2 diabetes [17]

(1) No inflammatory infections in any other organs while they were being treated for T2DM for at least a year.
(2) The current state of diabetes and the recommended dosage were not dramatically altered.
(3) No problems related to the kidney, eye, or peripheral neuropathy were experienced by the individuals.

2.4. Inclusion criteria of type II diabetic patients with periodontal disease [18]

(1) Probing depth (PD) greater than 5 mm.
(2) Loss of periodontal attachment or LPA greater than 4 mm.
(3) Abstaining from hormone and antibacterial medications.
(4) For 6 months, the patient neglected to receive regular periodontal care.
(5) No sexual preferences.

2.5. Exclusion criteria [19]

(1) Smokers with other systemic illnesses
(2) Surgery, trauma, or an acute infection during the previous month.
(3) Use of glucocorticoids, antibiotics, or other immune suppressants within the last month.
(4) Pregnancy or breastfeeding.
(5) Autoimmune illnesses and types of hyperthyroidism.
(6) Periodontitis that is aggressive.

The 40 patients were separated into four groups: the healthy group, the periodontitis group, the diabetic type 2 group, and the diabetic patients with periodontitis group. Patients were chosen for the outpatient clinic run by the Al-Azhar University Faculty of Dental Medicine for Girls', Oral

Medicine, Periodontology, Oral Diagnosis, and Radiology Department.

All subjects received information on the nature, advantages, and/or dangers of participating in the current study before any procedure. Patients provided their written consent. Research Ethics Committee (REC), Faculty of Dental Medicine for Girls, Al-Azhar University approved it under code REC-ME-22-15. All patients met the subsequent criteria.

2.6. Periodontal disease assessment [20]

Periodontal examinations were performed on participants. The periodontal probe is used for clinical measurements.

Probing pocket depth (PPD): The periodontal pocket depth is defined as the distance between the gingival margin and the bottom of the probable pocket to the nearest whole millimeter. Using the Williams periodontal probe, there were six measurements taken for each tooth: three on the facial/buccal side (mid-, mesio- and disto-) and three on the lingual/palatal side (mid-, mesio- and disto-). The deepest point was selected.

Clinical attachment level (CAL): A periodontal pocket is the distance from the base of the pocket to the gingival margin, clinical attachment level is the distance from the base of the pocket to a fixed point on the tooth, usually kept as the cementoenamel junction (CEJ). The CEJ comprises the clinical attachment level. The deepest point was selected.

2.6.1. Plaque score

Four surfaces of each tooth (facial/buccal, mesial, distal, palatal/lingual), excluding all third molars, were checked for the presence of plaque, marking any surface that had plaque presence on the chart. This was divided by the total number of surfaces checked (total number of teeth present × 4) and multiplied by 100. The resulting calculation was the plaque score of the patient, given as a percentage.

2.7. Type II diabetes assessment

The glycated hemoglobin A1C (HbA1c) biochemical parameter was going to be assessed (Biotech-Wuhan).

Assessment of appoptic activity of polymorphous neutrophil (PMN) [21].

2.8. Isolation of neutrophil from blood

2.8.1. Principle

Blood from the periphery was drawn into vacutainer tubes that contained 10 U/ml heparin using separation media that is a combination of sodium metrizoate and Dextran 500.

Layering blood over the density gradient medium was followed by centrifugation, the separation of the neutrophil layer, and the lysis of any remaining erythrocytes. This is followed by a wash, a count, and resuspension of the cells in the buffer to the appropriate concentration Fig. 1.

2.8.2. Procedure

(1) A centrifuge tube was filled with 5.0 ml of the neutrophil isolation medium, which was a mixture of sodium metrizoate and dextran 500. A measure of 5.0 cc of blood was then gently deposited over the separating medium. To avoid blending the blood with the media, the procedures should be carried out slowly, precisely, and with the pipette tip close to the surface of the media. It was then centrifuged for 35 min between 20 and 25 °C at 500 relative cenrifugal force (RCF). Plasma, monocytes, neutrophils, further isolation medium, and red blood corpuscle pellets are the six distinct bands that the blood should separate into.
(2) The separation procedure may need to be redone if these bands do not appear to be clear.
(3) Pay attention to the top three levels that were removed using a pipette and disposed of.
(4) Carefully remove the neutrophil layer and all of the isolation material underneath the neutrophils using a pipette, and then transfer the 'neutrophil solution' into an appropriately sized centrifuge tube.
(5) Hanks' balanced salt solution without Ca2+/Mg2+ was used to adulterate the neutrophil solution by 10 ml. To suspend the cells, the tube is occasionally turned upside down.
(6) For 10 min, the neutrophil solution was centrifuged at 350 RCF. There should be a crimson pellet with neutrophils and leftover red blood

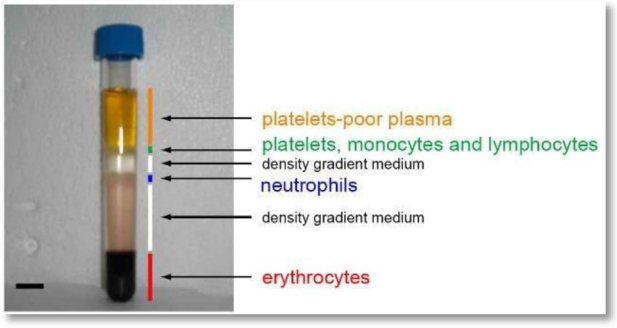

Fig. 1. Isolation of polymorphonuclear cells.

cells at the bottom of the tube. Without disturbing the particle, the supernatant was carefully collected with a pipette.
(7) Red cell lysis buffer (2 ml) was added to the tube to lyse the remaining RBCs. The vial was vortexed to help the pellet stay suspended.
(8) The tube was centrifuged for 5 min at 250 RCF. A pipette was used to extract the supernatant. If necessary, the lysing procedure is repeated.
(9) To each tube, 500 l of HBSS without Ca2+ or Mg2+ were added. Once more, the pellet was vortexed to resuspend it at a setting of 3−4, and it was then diluted to 10 ml with HBSS devoid of Ca2+/Mg2+.
(10) For 5 min, the tubes were centrifuged at 250 RCF. The supernatant was removed and thrown away.
(11) The pellet was resuspended in 250 l of human HSA solution (2 % HAS) in HBSS/albumin solution. The number of cells is then determined, and the concentration is adjusted.

2.9. Assessment of apoptosis using annexin/PI staining by flow cytometry

The Alexa Fluor 488 annexin V/Dead Cell Apoptosis Kit with annexin V and PI for flow cytometry was used to measure apoptosis.

Apoptotic cells showed green fluorescence, dead cells showed red and green fluorescence, and viable cells showed little to no fluorescence after staining a cell population. These populations could be easily identified using flow cytometry and an argon-ion laser's 488 nm line of excitation. Data from flow cytometry were typically analyzed using the Navios programmer.

2.10. Measurement of iNos by ELISA [22]

The GCF was collected using paper point number 25. In the gingival sulcus, paper points were placed until resistance was felt and then left there for 30 s. The actual teeth were washed and cleaned using cotton rollers and syringes to remove bacterial plaque and saliva before inserting the paper tip within the gingival sulcus. After that, cotton rolls were used to isolate them. Bloody and salivary paper points were not included. The paper points were then transferred to the lab and placed in microtubes with caps.

They were kept there until the day of the measurement at −80 °C. On the day of each mediator's trial, appropriate microtubes were kept at a temperature below −80 °C, and the prepared GCF samples were tested for iNOS using an enzyme-linked immunosorbent assay technique.

2.11. Statistical analysis

Statistical analysis was performed using one-way Kruskal−Wallis followed by Mann−Whitney test.

3. Results

The 40 patients were split into four groups: the healthy group, the periodontitis group, the diabetic type II group, and the diabetic patients with periodontitis group. The groups included six males and 34 females. Their age ranged from 24 to 67 years Table 1.

3.1. Percentage of spontaneous neutrophil apoptosis

The Kruskal−Wallis test revealed that the overall P value for intergroup comparison was highly significant at the 0.01 level (P > 0.01 and confidence interval, 99 %), indicating that there was a statistically significant difference in the mean neutrophil apoptosis between groups, primarily between healthy and diabetes with periodontitis Table 2.

3.2. Glycated hemoglobin A1C percentage measurement

There was a statistically significant difference in the mean HbA1C percentage between groups,

Table 1. Basic descriptive data, clinical parameters, and laboratory investigations data for all four outcomes.

Classification	Healthy	Periodontitis	Diabetes	Diabetes with periodontitis
Age (year)	23.7 ± 0.48	35.9 ± 5.57	37.8 ± 2.2	66.3 ± 2.00
Sex	100 % F	20 % M and 80 % F	20 % M and 80 % F	20 % M and 80 % F
Duration of diabetes (year)	0	0	4.8 ± 0.79	14.4 ± 2.12
HbA1C	5.18 ± 0.18	5.6 ± 0	6.81 ± 0.4	7.81 ± 0.1
SA	38.97 ± 2.45	25.11 ± 2.17	13.84 ± 1.19	8.41 ± 1.86
CAL	0	5.5 ± 0.53	0	5.7 ± 0.63
Max PD	2.4 ± 0.52	6.5 ± 0.53	2.6 ± 0.52	7.4 ± 0.7
PI	0	38 %	0	41.99 %

CAL, clinical attachment loss; F, female; GI, gingival index; HbA1C, glycated hemoglobin A1C; M, male; Max PD, maximum probing depth; PI, plaque index; SA, percentage of spontaneous neutrophil apoptosis.

Table 2. Mean ± SD of the percentage of spontaneous neutrophil apoptosis for all groups.

Healthy	Periodontitis	Diabetes	Diabetes with periodontitis	P-value**
38.97 ± 2.45^{A}	25.11 ± 2.17^{AB}	13.84 ± 1.19^{BC}	8.41 ± 1.86^{C}	0.000^{HS}

**Highly significant difference (P-value <0.05).

mainly between the diabetes with periodontitis group and the healthy and diabetes group Table 3.

3.3. Concentration of inducible nitric oxide synthase enzyme

The maximum value of iNOS concentration (56.951.93 u/l) was attained in the diabetes with periodontitis group. The mean optical density of iNos was 33.752.91 u/l in the healthy group, 49.371.94 u/l in the periodontitis group, 41.040.55 u/l in the diabetic group, and there was a statistically significant difference in the mean optical density of iNOS between groups, primarily between healthy and diabetes with periodontitis Table 4.

3.4. Effect of HbA1C percentage, duration of diabetic disease, and concentration of iNos on spontaneous apoptosis

We can draw the following conclusions from the findings of the Pearson correlation test between the HbA1C level, duration of diabetic disease, concentration of iNos, and spontaneous apoptosis%. A strong negative connection ($r = -0.813$) that was highly statistically significant ($P > 0.001$) was found between HbA1C level and spontaneous apoptosis percentage. The relationship between the amount of spontaneous apoptosis and the length of the diabetes condition had a moderately negative correlation ($r = -0.622$). The HbA1C level and the length of the diabetic disease had a strong positive association ($r = 0.933$). iNOS and the percentage of spontaneous apoptosis exhibited a moderately negative connection ($r = -0.725$), which was highly statistically significant (P 0.0001) as shown in Table 5.

3.5. Effect of age on spontaneous apoptosis %

There was moderate negative correlation between age and spontaneous apoptosis ($r = -0.704$) as shown in Table 6.

4. Discussion

It is well known that the severity of periodontitis and the degree of hyperglycemia have a clear link. Although the mechanisms connecting the two are not fully understood, it is known that components of immunological functioning, neutrophil activity, and cytokine biology are involved. A bidirectional association between diabetes and periodontitis has come to light. Diabetes is known to increase the risk for

Table 6. Correlation between age and spontaneous apoptosis.

	r**	P-value	Correlation type
Age vs. spontaneous apoptosis	−0.704	0.000^{HS}	Moderate Negative

**Highly significant difference (P-value <0.000).

Table 3. Mean ± standard deviation of glycated hemoglobin A1C percentage for all groups.

Healthy	Periodontitis	Diabetes	Diabetes with periodontitis	P-value**
5.18 ± 0.18^{C}	5.6 ± 0.00^{CB}	6.81 ± 0.4^{AB}	7.81 ± 0.1^{A}	0.000^{HS}

**Highly significant difference (P-value <0.000).

Table 4. Mean ± SD of inducible nitric oxide synthase enzyme.

Healthy	Periodontitis	Diabetes	Diabetes with periodontitis	P-value**
33.75 ± 2.91^{A}	49.37 ± 1.94^{BC}	41.04 ± 0.55^{AB}	56.95 ± 1.93^{C}	0.000^{HS}

**Highly significant difference (P-value <0.000).

Table 5. Effect of HbA1C, duration of diabetic disease, and concentration of iNos on spontaneous apoptosis.

	r**	P-value	Correlation type
HbA1C vs. spontaneous apoptosis	−0.813	0.000^{HS}	Strong Negative
Duration of diabetic disease vs spontaneous apoptosis	−0.622	0.000^{HS}	Moderate Negative
HbA1C vs duration of diabetic disease	0.933	0.000^{HS}	Strong positive
iNos vs. spontaneous apoptosis	−0.725	0.000^{HS}	Moderate Negative

**Highly significant difference (P-value <0.000).

periodontitis, and periodontal inflammation negatively affects glycemic control [23]. By causing oxidative stress, inflammation, and subcellular organelle malfunction, hyperglycemia has a direct negative impact on the track organs of DM complications. Moreover, it causes proteins to undergo nonenzymatic glycation, which modifies their structure, function, and turnover. By adhering to their receptor cells, advanced glycation end products that build up in numerous organs can promote both inflammation and oxidative stress [24].

The most often used test to evaluate glucose management in people with diabetes is glycated hemoglobin [25]. The American Academy of Periodontology and the European Federation of Periodontology added diabetes mellitus to their list of risk factors for the development of illness [26], and they advise using HbA1c for periodontitis grading [26]. However, few diabetologists, dentists, and patients are aware of the possibility of periodontitis in DM [27].

The opposite is also true as periodontitis is linked to higher glucose levels in nondiabetic people and an increased risk of both prediabetes and type 2 DM [28]. A vital enzyme involved in the development of neutrophil extracellular traps is also overexpressed in neutrophils from individuals with diabetes mellitus [29]. Periodontitis' etiology has been linked to NETosis [30].

Importantly, several systemic inflammatory disorders, including disorder, autoimmune disease, and type 2 diabetes, are associated with periodontitis. A hyperreactive neutrophil phenotype is thought to play a crucial role in periodontitis [31]. Therefore, 'hyper-inflammation' may be one unintentional way through which periodontitis causes comorbidity [32].

A group of enzymes known as gas synthases (NOS) converts the chemical substance L-arginine into NO. There are three different isoforms of NOS: endothelium (eNOS), neuronal (nNOS), and inducible (iNOS) [33]. Partially understood mechanisms for enhanced oxidative stress in diabetes complications include protein kinase C activation, transcription factor activation, and AGEs. Through protein kinase C, hyperglycemia, either directly or indirectly, modifies the activity of NOS [34]. As iNOS affects osteoclasts in bone modeling, NO is also an important modulator of bone resorption [35].

In the current investigation, the tested group's glycemic status, spontaneous neutrophil apoptosis, and iNOS were all measured. A link between the HbA1C level, length of the diabetes condition, aging, iNos, and the percentage of spontaneous apoptosis was also distributed. From the test group, samples of peripheral blood, gingival crevicular fluid, and clinical parameters were obtained.

The high rate of spontaneous neutrophil apoptosis seen in the healthy group is consistent with our current study, and this is consistent with a study conducted by a researcher [15]. This may be explained by the neutrophils typically circulate in the blood for 6–12 h, making it the home to the bone marrow, spleen, or liver where they undergo apoptosis. Subsequently, they are phagocytosed by Kupffer cells within the liver or by red pulp macrophages within the spleen [36].

According to the results of the current study, there is a lower rate of spontaneous neutrophil apoptosis in the periodontitis group compared with healthy individuals, which is consistent with a microarray study that identified the apoptosis cluster including the lone major alteration [37].

Increased neutrophil migratory potential was the outcome of the unregulated inflammatory response to *P. gingivalis* LPS, whereas apoptosis was suppressed as seen by the decreased caspase-3 activity [38]. Given that *P. gingivalis* endotoxin might activate TLR2 rather than TLR4, this could explain why it inhibits apoptosis [39].

In our current investigation, the fraction of neutrophils undergoing apoptosis in the diabetic group with periodontitis was lower than that of those without diabetes, and this might be explained as neutrophils are the most cellular source of the increased collagenase activity within the gingival crevicular fluid of patients with DM [40].

In the current investigation, there was a strong negative association between the HbA1C level and the spontaneous apoptosis percentage. This is consistent with research done by Elsayed and Araby [41], who discovered a strong negative association between the rate of neutrophil apoptosis and HbA1c levels ($r = -0.352$, P 0.01). Also, they found that chronic hyperglycemia is thought to promote tissue injury and increase the risk of microangiopathy by inhibiting neutrophil apoptosis.

According to our research, aging and spontaneous neutrophil apoptosis have a substantial indirect association. This can be explained by the background work done by Sendama [42]. He concluded that aging impairs the processes controlling inflammation [43]. This dysregulation has been inferred in part from the finding that older adults have higher levels of circulating proinflammatory cytokines and acute-phase proteins even in the absence of infection, which suggests a low-level chronic inflammation of aging known as inflammaging [44].

In addition to the aforementioned, there was a strong association between the HbA1C level and the

duration of diabetes (r = 0.933), and this correlation was highly statistically significant (P > 0.001). This is frequently in complete agreement with a study, which observed that the insulin level exhibited a significant link with HbA1c levels after adjusting for age, sex, and diabetes duration [45].

In the group of diabetics with periodontitis in our current investigation, we discovered a high concentration of iNOS. This was in line with the research that the investigator had conducted [46]. This could be explained by the possibility that iNOS produces NO, and that NO's functions are focused on preserving the inflammation. In human neutrophils, NOS activity and NOS mRNA have been identified [47].

NO has been linked to altering the expression of matrix metalloproteinases (MMPs), and it is also believed to suppress the production of tissue inhibitors of MMPs [48].

In conditions where neutrophils play a significant role, such as arthritis, glomerulonephritis, diabetes, stroke, septic shock, respiratory problems, sepsis, encephalitis, and colitis, unwarranted NO production has detrimental effects [49]. Because of an increase in P-selectin and ICAM expression, neutrophil overexpression of iNOS may be to blame for the impaired leukocyte–endothelial interaction in diabetes mellitus [50].

It has been demonstrated beyond a doubt that NO's ability to suppress apoptosis was due to its ability to inhibit caspase-3 activation through both cGMP-dependent and cGMP-independent methods [51].

4.1. Conclusion

Diminishing of apoptosis in a diabetic with periodontitis group compared with the healthy group. High concentrations of iNos in the diabetic with periodontitis group compared with the healthy group. Also, the disturbance of apoptosis is related to inflammaging.

5. Recommendations

Many studies are required to confirm the relationship between disturbances of neutrophil apoptosis, iNOS, and chronic diseases such as diabetes and periodontitis.

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Conflicts of interest

The authors declared that there is no conflict of interest.

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