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Research Article

Metformin Effect Against Rotenone-Induced Parkinsonism-Like Symptoms in a Mouse Model

Israa Hussein Al-Mammory[1]*, Ghaith Ali Jasim[2], Wassan Abdulkareem Abbas[3]
[1]*Department of Pharmacology and Toxicology, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq;*
[2]*Department of Pharmacology and Toxicology, Faculty of Pharmacy, Al-Bayan University, Baghdad, Iraq;*
[3]*Department of Clinical Lab Sciences, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq*
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Abstract

Background: Targeting problems in lipid metabolism for the treatment of Parkinson's disease (PD) has advanced significantly in recent years through the use of medications like metformin (MET). In a mouse model of rotenone-induced PD, MET, a commonly prescribed antidiabetic medication, may have a neuroprotective effect. Rotenone is an inhibitor of mitochondrial complex I that can lead to PD and dopaminergic (DA) cell loss. ***Objective***: To evaluate the mechanisms behind the MET neuroprotective effect and possible additive benefits of MET/levodopa-carbidopa (L-DOPA/carbidopa) in rotenone-induced parkinsonism in male mice. ***Methods***: Albino mice were given rotenone (1 mg/kg/48hr, subcutaneous) for 17 days. Following the administration of rotenone, a 30-day oral MET treatment (500 mg/kg/day) was initiated. The neuroprotective effect of MET on rotenone-induced dopaminergic toxicity was assessed by detection of α-synuclein and the neuroinflammatory marker tumor necrosis factor-α (TNF-α), and we also showed that DOPA decarboxylase (DDC) levels in plasma could detect PD using enzyme-linked immunosorbent assay (ELISA) kits. The behavioral tests were performed by wire hanging, catalepsy, and pole tests. ***Results***: Metformin ameliorated the behavioral deficits in the Parkinsonian mouse model, significantly decreased the levels of α-synuclein and tumor necrosis factor-α (TNF-α), and serum DDC levels were significantly reduced. ***Conclusions***: Metformin can alleviate rotenone-induced Parkinson's-like symptoms in a mouse model.

Keywords: α-Synuclein, Dopa decarboxylase, Metformin, Parkinson's disease, Rotenone, TNF-α.

تأثير الميتفورمين ضد الأعراض الشبيهة بالشلل الرعاش الناجم عن الروتينون في نموذج الفأر

الخلاصة

الخلفية: لقد تقدم استهداف مشاكل التمثيل الغذائي للدهون لعلاج مرض باركنسون (PD) بشكل كبير في السنوات الأخيرة من خلال استخدام أدوية مثل الميتفورمين (MET). في نموذج الفأر لمرض باركنسون الناجم عن روتينون, MET, دواء مضاد لمرض السكري يوصف بشكل شائع, قد يكون لها تأثير وقائي للأعصاب. الروتينون هو مثبط لمركب الميتوكوندريا الأول الذي يمكن أن يؤدي إلى فقدان الخلايا والدوبامين (DA). **الهدف**: تقييم الآليات الكامنة وراء تأثير MET العصبي والفوائد المضافة المحتملة ل MET/ ليفودوبا كاربيدوبا في الشلل الرعاش الناجم عن روتينون في ذكور الفئران. **ألطرائق**: أعطيت الفئران البيضاء روتينون (1 مجم/ كجم/ 48 ساعة ، تحت الجلد) لمدة 17 يوما. بعد إعطاء الروتينون، بدأ علاج MET الفموي لمدة 30 يوما (500 ملغ/كغ/يوم). تم تقييم التأثير الوقائي العصبي ل MET على سمية الدوبامين الناجمة عن الروتينون عن طريق الكشف عن α-سينوكلين وعامل نخر الورم الالتهابي العصبي α، وأظهرنا أيضا أن مستويات ديكاربوكسيلاز DOPA (DDC) في البلازما يمكن أن تكتشف مرض باركنسون باستخدام مجموعات مقايسة الممتز المناعي المرتبط بالإنزيم (ELISA). تم إجراء الاختبارات السلوكية عن طريق تعليق الأسلاك، والكتاليبسي، واختبارات القطب. **النتائج**: خفف الميتفورمين من العجز السلوكي في نموذج باركنسون في الفأر، وخفض بشكل كبير مستويات α-سينوكلين وعامل نخر الورم α ، وانخفضت مستويات DDC في الدم بشكل كبير. **الاستنتاجات**: يمكن للميتفورمين أن يخفف من أعراض مرض باركنسون التي يسببها الروتينون في الفئران.

* ***Corresponding author***: Israa H. Al-Mammory, Department of Pharmacology and Toxicology, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq; Email: esraa_hussein@uomustansiriyah.edu.iq
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INTRODUCTION

Parkinson's disease (PD) is a neurological condition that primarily affects the elderly and is slowly progressing and incurable [1]. After Alzheimer's disease, PD is the second most prevalent neurological illness worldwide [2]. While they typically co-occur, signs and symptoms might exist independently at the early stages of the disease (e.g., depression often occurs with anxiety, apathy overlaps with depression and cognitive impairment, and psychosis and depression can exacerbate impulse control difficulties) [3]. Additionally, signs and symptoms can appear at later stages of the disease, when they are often most severe [4]. There are notable differences in PD epidemiology by age, sex, location, ethnicity, and period. Prevalence has risen globally in addition to changes in demographics. The decrease in other competing causes of death is one of many possible explanations for this increase. It is less clear whether the incidence is rising, particularly among women or in many low- and middle-income

nations where high-quality data is limited. Numerous environmental factors, including exposure to neurotoxic chemicals, have been proposed as explanations for the higher prevalence of PD in men and older adults [5]. PD deaths in Iraq totaled 658, or 0.45% of all deaths, according to the most recent WHO data released in 2020. Iraq is ranked #59 in the world with an age-adjusted death rate of 4.74 per 100,000 population [6]. Growth factors, gut microbiota, oxidative stress, inflammatory pathways, and aquaporins are just a few of the molecular and cellular mechanisms responsible for PD pathogenesis [7]. Numerous other neurodegenerative and non-neurodegenerative diseases can also induce parkinsonism, and it can be difficult to distinguish them clinically from PD, particularly in the early stages of the illness [8]. As PD gets worse, more and more α-synuclein (αSyn) builds up in Lewy bodies (LBs), and dopaminergic (DA) neurons die [9]. A pathological change may not be evident for several years. Dopamine levels drop because of dopaminergic neuron death, impairing motor function and possibly adding to the cognitive abnormalities seen in certain patients [10]. The genesis of the disease may also be influenced by several additional factors, such as age, genetics (such as a mutation in the LRRK2 gene), exercise, and environment (including exposure to chemicals and food) [11]. The onset of non-motor symptoms of PD may be related to inflammation. A connection between visuospatial impairment and elevated blood TNF-α levels in PD patients suggests that this pro-inflammatory cytokine plays a role in the neurobiology of cognitive impairment in PD [12]. Scientific studies have demonstrated that carbidopa and levodopa (L-Dopa) are drugs that doctors may prescribe to people with PD [13,14]. At its current state, PD is solely treated to alleviate associated motor symptoms; progression is not inhibited. One significant unmet need in the treatment of PD is a disease-modifying strategy. New therapeutic targets are being evaluated to achieve this target [15]. MET is an insulin-sensitizing drug that can lower insulin resistance (IR) and is used as a first-line treatment for type 2 diabetes mellitus (T2DM) [16]. In addition to type 2 diabetes, polycystic ovarian syndrome, gestational diabetes, type 2 diabetes prevention in prediabetes, non-steroidal anti-inflammatory drugs (NSAID) as adjuvant treatment in patients with knee osteoarthritis, and adjunct therapy for type 1 diabetes are among the current therapeutic applications [17,18] and reduces apoptosis and cell division in all pancreatic cell types [19]. MET is regarded as a cytotoxic and anti-proliferative medication because it can also increase apoptotic factors and decrease proliferation [20]. MET helps break down fatty acids, lowers the production of ROS, and protects apoptosis and energy homeostasis through adenosine monophosphate protein kinase (AMPK) [21]. Repurposing licensed and successful medications, such as MET, may be a useful approach to PD treatment [16]. This study aims to evaluate the mechanisms behind the MET neuroprotective effect and possible additive benefits of MET/levodopa-carbidopa (L-DOPA/carbidopa) in rotenone-induced parkinsonism in male mice**.**

METHODS

Animals

Thirty albino male mice weighing 20-30 gm were obtained from the College of Pharmacy, University of Al-Farahidi. In accordance with the College of Pharmacy's ethics committee at Mustansiriyah University, animals were divided into groups and housed in cages with standard humidity levels, a 12-hour light/dark cycle, and unrestricted access to food (commercial pellets) and water.

Chemicals and drugs

Rotenone powder pure from Bidepharm, China, was dissolved in commercial sunflower oil. MET pure powder was obtained from Baoji Guokang Bio-Technology Co., Ltd., China, and dissolved in distilled water. L-DOPA was purchased from Xian Sonwu Biotech Co., Ltd., China, and carbidopa from Sandoo Pharmaceuticals and Chemicals, China. The mean body weight for each group was considered to determine the dose of rotenone. 10 mg of rotenone powder was dissolved in 5 ml of absolute ethanol in a glass tube and mixed thoroughly by magnetic stirrer until completely dissolved; then 0.5 ml from the stock solution was diluted by 4.5 ml of sunflower oil to prepare a working solution of concentration (1 mg/5 ml). A dose of 1 mg/kg (about 0.15 ml of the solution according to weight) was injected subcutaneously for each animal.

Study design

Mice were randomly allocated into equal groups of five mice each as follows: Group (1) control group: Mice received nine subcutaneous (s.c.) injections of the vehicle (sunflower oil, 4 ml/kg) every 48 h; group (2) induction group: Include 5 mice who received nine doses of rotenone (1mg/kg, every other day, subcutaneously) dissolved in 99% absolute ethanol; group (3) pretreatment group: include 5 mice who pretreated with MET orally daily for 3 days before rotenone administration, and continued to be treated with MET for 17 days concomitant with rotenone injections; group (4) treatment group: include 5 mice who received nine doses of rotenone (1mg/kg, every other day, subcutaneously) at day 0 then treated daily with 500mg/kg MET which dissolved with D.W per day orally for 30 days after 17 days from rotenone doses. Group (5) standard treatment group: includes 5 mice who received nine doses of rotenone (1 mg/kg, every other day, subcutaneously) and were then treated daily with L-Dopa (100 mg/kg), containing Carbidopa (25 mg/kg) dissolved in distilled water orally for 30 days; and group (6) combination group: includes 5 mice who received nine doses of rotenone (1 mg/kg, every other day, subcutaneously) at day 0 and were treated daily with MET 500 mg/kg plus L-Dopa (100

mg/kg), containing Carbidopa (25 mg/kg) dissolved in distilled water orally for 30 days. The study design is illustrated in Figure 1.

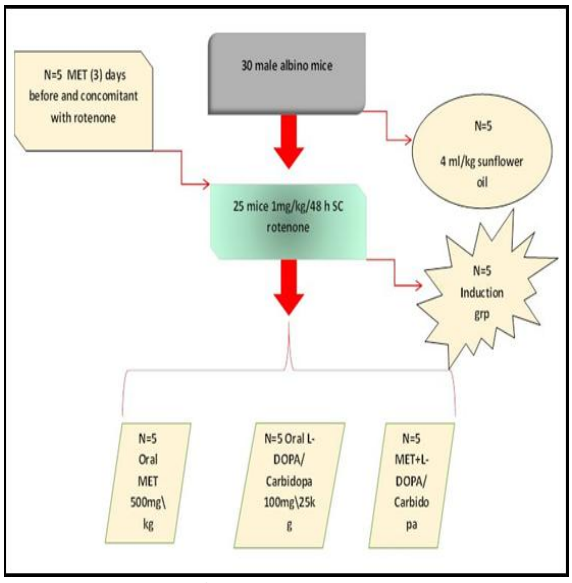

Figure 1: Flow chart of the study.

Behavioral test

The pole test, catalepsy, and wire hanging test were used to screen for motor functionality in mice one day after the induction phase ended (day 18). Bradykinesia is observed using the pole test. The mice were positioned atop the wooden pole, and the duration in seconds it took them to descend to the ground was noted. The pole measured 50 cm in height and 1 cm in diameter. The test was carried out 3 times, and the average time was calculated. Catalepsy: The test was the bar test. Mice were placed with both front paws on a bar that was 9 cm above and parallel to the base. The mice were placed with both front paws on the bar in a half-rearing position. Here they were timed with the stopwatch. When the animals removed one paw from the bar, the stopwatch was stopped and the time noted [22]. The maximum cutoff for the bar test was fixed at 120 s. The hanging wire test was used to measure muscle strength. The lid of a typical wire cage was used. An animal was put on top of the lid for the test. The meantime it took for the animal to drop off the wire lid was measured after it clutched the lid with all its paws and then flipped over. 5 minutes was set as the maximum latency time.

Sample collection

After seventeen days from the rotenone doses, collect the blood samples from the control, induction, and pre-treatment mice groups and store them at −20°C until the time of analysis. The brain tissue was harvested immediately and washed with distilled water. After thirty days of the rotenone doses, five mice from the treatment, standard treatment, and combination groups were euthanized, and blood samples were collected and then compared.

Statistical analysis

The post-hoc Tukey test was performed after all data analysis (given as mean ± standard deviation) was completed using the one-way analysis of variance (ANOVA) test in SPSS (V25). When the *p*-value was less than 0.05, the statistical significance difference was considered.

RESULTS

In comparison to the control group, all rotenone-injected groups showed a substantial increase in ladder-ascending score and time to turn and descend (seconds) during the pole test. The cataleptic behavior of mice treated with rotenone considerably increased latency. whereas all rotenone-injected groups showed a substantial reduction in climbing time and latency time (seconds) at the wire hanging test as compared to the control group. In contrast to the induced-PD group, the time taken to move from the top to the bottom of the pole significantly decreased in all treated groups. The LADDER score of the group treated with both MET and L-DOPA/carbidopa was significantly lower than that of the other treatment groups. In all treated mouse groups, a notable reduction in catalepsy was noted. Compared to the rotenone-treated group, the co-treatment group had a lower cataleptic score ($p< 0.05$). There were no significant differences between the control and MET-alone-treated animals ($p< 0.05$). Moreover, mice revealed a significant increase in wire hanging test delay time as compared to the induced PD (untreated) group. The delay time was noticeably longer in the combination groups. MET is superior to the L-DOPA group. For each group, values are presented as mean ± SD for four animals, as shown in Table 1.

Table 1: Effect of different treatment approaches on behavior changes

Groups	Pole test	Catalepsy test	Wire hanging test
Control	2.25 ± 0.5^{a}	21.25 ± 0.96^{e}	5.0 ± 0.0^{a}
Induction (Rot)	3.25 ± 0.5^{b}	120.0 ± 0.0^{a}	1.5 ± 0.58^{d}
Pre-treatment (MET)	2.50 ± 0.58^{a}	30.75 ± 1.5^{c}	$3.75\pm0.5^{b,c}$
MET (500 mg/kg)	2.75 ± 0.5^{a}	34.75 ± 1.26^{b}	$3.25\pm0.5^{b,c}$
L-DOPA/Carbidopa (100/25 mg/kg)	2.75 ± 0.5^{a}	36.00 ± 1.4^{b}	3.0 ± 0.0^{c}
MET+L-DOPA/Carbidopa	2.25 ± 0.5^{a}	25.50 ± 0.58^{d}	4.0 ± 0.0^{b}

Values are expressed as mean±SD. Values with different superscripts (a,b,c,d,e) are significantly different among groups ($p<0.05$).

As shown in Table 2, the mean serum SNCα concentration was significantly ($p< 0.05$) higher in the induction group compared to the control group. Before the treatment with MET (500 mg/kg), the average serum SNCα concentration dropped significantly ($p< 0.05$) compared to the induction group. This happened in a dose-dependent way. Furthermore, there was no significant difference ($p> 0.05$) between the group treated with MET and the control group.

Table 2: The effect of MET on serum SNCα levels

Groups	Serum SNCα level (ng/mL)
Control	167.6±4.11[b]
Induction (Rotenone)	298.06±14.71[a]
Pre-treatment (MET)	156.90±15.64[b]
MET (500 mg/kg)	170.3±7.89[b]
L-DOPA/Carbidopa (100/25 mg/kg)	164.73±6.24[b]
MET+L-DOPA/Carbidopa	105.28±6.057[c]

Values are expressed as mean±SD. Values with different superscripts (a,b,c) are significantly different among groups (p<0.05).

Also, the group that was given L-DOPA/carbidopa had significantly lower levels of SNCα in their blood than the group that was not treated. Furthermore, it was seen that when MET was combined with L-DOPA and Carbidopa, the concentration of SNCα dropped significantly (p< 0.05) compared to the induction group. As illustrated in Table 3, rotenone injection in the induction group resulted in a significant (p< 0.05) increase in the mean serum DDC concentration compared to the control group.

Table 3: The effects of MET on serum DDC level in treated groups

Groups	Serum DDC level (ng/mL)
Control	120.74±21.89[d]
Induction (Rot)	1305.37±63.07[a]
Pre-treatment (MET)	354.17±55.60[b]
MET (500 mg/kg)	250.95±31.48[c]
L-DOPA/Carbidopa (100/25 mg/kg)	273.19±49.73[b,c]
MET+L-DOPA/carbidopa	68.74±1.77[d]

Values are expressed as mean±SD. Values with different superscripts (a,b,c,d) are significantly different among groups (p<0.05).

An induction group was given 500 mg/kg of MET before treatment (group 3). This caused a significant (p< 0.05) drop in the mean serum concentration of DDC compared to the induction group. Additionally, the group that was given L-DOPA/carbidopa had a lower mean serum DDC concentration than the group that was given induction. This effect of L-DOPA/carbidopa was also not significantly different from the effect seen in the group that was given MET 500 mg/kg. On the other hand, the combination-treated group showed a significant decrease in DDC (p< 0.05) compared with the MET and L-DOPA/Carbidopa alone groups. Table 4 shows that seventeen days after rotenone doses, there was a highly significant difference (p< 0.05) in the serum level of TNF-α between the induction group and the control group. Our findings demonstrated that prophylactic MET treatment counteracted the rise in TNF-α levels and markedly enhanced motor functioning.

Table 4: The effect of MET on serum TNF-α level in different treated groups

Group	Serum TNF-α level (µg/mL)
Control	88.29±4.72[c]
Induction (Rotenone)	611.39±55.87[a]
Pretreatment (MET 500 mg/kg)	140.68±17.64[b]
MET (500mg/kg)	101.58±8.76[b,c]
L-DOPA/Carbidopa (100/25 mg/kg)	107.16±4.32[b,c]
MET+L-DOPA/carbidopa	73.50±5.65[c]

Values are expressed as mean±SD. Values with different superscripts (a,b,c) are significantly different among groups (p<0.05).

The current study also showed that TNF-α levels in the serum of mice in the MET-treated group (500 mg/kg) and the L-DOPA/carbidopa group (100/25 mg/kg) were significantly lower (p< 0.05) than TNF-α levels in the rotenone model group. When the dose of MET was combined with levodopa, there was a significant decrease (p< 0.05) in the level of TNF-α in the blood compared to when the treatment was given alone.

DISCUSSION

A dopamine and catecholamine imbalance in the nigrostriatal pathway causes PD, which is the gradual progression to neuronal death [23]. Aggregated α-synuclein deposition is linked to PD, a progressive neurodegenerative illness [24]. Presently available treatments are only able to alleviate symptoms; they are unable to stop, slow, or prevent the neurodegenerative process of PD [25]. Rotenone liquified in sunflower oil was injected subcutaneously into male albino mice in this investigation to create an experimental PD model [26]. By demonstrating decreased performance duration in the tests used in this investigation, our findings showed that rotenone caused behavioral abnormalities in mice. Additionally, rotenone reduced the number of SN dopamine neurons in mice. These results backed up earlier research by showing that rotenone really did cause harmful effects and strange behaviors in PD animals [27]. Although rotenone has many positive impacts in agriculture, fishery, and animal husbandry, long-term exposure has a negative impact on the health of those who work in these fields. When rotenone is extracted, formulated, or used, it may penetrate the blood-brain barrier, resulting in neurodegeneration and the emergence of symptoms resembling PD [28]. It inhibits complex I in the electron transport chain, potentially causing progressive neurodegenerative disorders and oxidative damage [29]. Research has shown that neuronal malfunction and death can result from oxidative stress, mitochondrial dysfunction, and inflammatory changes [30]. A growing body of research has demonstrated that MET has some interesting qualities in addition to its anti-diabetic effect. These include anti-oxidative, anti-cancer, genomic stability, anti-inflammation, and anti-fibrosis properties. These properties are powerful enough to treat conditions other than diabetes mellitus [31]. The most effective drug to date is L-Dopa, but prolonged use of the drug causes PD to develop uncontrollable movements, or L-DOPA-induced dyskinesias (LID) [32]. In this study, we evaluated the neuroprotective effects of MET against rotenone-induced PD in albino male mice. Moreover, we investigated the potential of both drugs (L-DOPA/carbidopa and MET) in managing PD. The pole test, hanging test, and latency in the cataleptic test, which are reliable techniques for assessing the impact of possible medicinal substances on muscular strength, were employed in this investigation to assess the animals' motor abilities. Dopaminergic depletion affects both motor and non-motor function,

which changes behavior [33]. However, all behavioral indicators improved after receiving MET therapy, which reduced locomotor activity. A protein called α-synuclein (αsyn) is found in many brain neurons. In neurodegenerative diseases like Parkinson's, it can fold and clump together in harmful ways, making harmful filaments that stick together in unhealthy ways [34]. suppression of α-Syn aggregation, which would restrict the course of Parkinson's disease by reducing the death of dopaminergic neuronal cells [35]. It was found that serum α-synuclein levels were significantly decreased in the MET-treated group. Serum α-synuclein levels were observed to be considerably lower in the group treated with metformin. Metformin's improved phosphatase activity reduces alpha-synuclein phosphorylation [36]. Many factors can explain why MET inhibits SNCα. First, as demonstrated recently in a C. elegans PD model exposed to 6-hydroxydopamine (6-OHDA), MET decreased dopaminergic neuron loss and α-syn aggregation, suggesting that increased expression of the autophagic pathway by MET may counteract α-syn pathology by quickly eliminating the α-syn aggregates [37]. Second, metformin has been shown to lower phosphorylated α-syn at serine 129 (α-syn pSer129) levels [38]. The findings of the research revealed a significant decline in the levels of DDC in serum samples from the MET-treated group compared to the induction group. DDC, a distinct and extremely promising biomarker for Parkinson's disease, is necessary for the production of dopamine from exogenous L-DOPA. Carbidopa is often given along with L-DOPA therapy. It blocks DDC but doesn't get into the brain or spinal cord, so it lowers the production of dopamine outside of the brain [39]. In particular, long-term use of oral combinations of levodopa and decarboxylase inhibitors helps with the start of movement changes. Additionally, it changes levodopa's turnover to O-methylation, which raises oxidative stress and reduces human methylation ability [40]. Here, we show that oral MET administration normalized the serum DDC to a level that was not statistically different from that of the standard therapy group. According to other research [41] and validated by our findings, we can show the capacity of MET to inhibit neuroinflammation has long been implicated in PD pathogenesis, in part supported by the presence of activated microglia that reside in abundance within the substantia nigra of PD patients [42]. It is also well established that rotenone can cause an inflammatory response in vivo. Moreover, rotenone can activate microglia via proinflammatory signaling pathways [43]. Activated microglia and astrocytes secrete pro-inflammatory cytokines, such as TNF-, in PD [44]. MET therapy has a strong anti-inflammatory effect because it effectively reverses changes in pro-inflammatory cytokine activity caused by rotenone. By lowering inflammation, neurons are protected from the inflammatory damage commonly observed in PD. These findings match with those of research published by Jambi *et al*. and Altharawi *et al*. [45,46].

Conclusion

According to the neurological assessment, the cognitive functions improved as a result of using MET as a treatment. MET was able to lower oxidative stress, stop the loss of dopamine in the striatum caused by rotenone, change neurobehavioral patterns, and raise TLR4 mRNA expression in mice. Its downregulation of TLR4 signaling and antioxidant action at least partially caused this impact. Its effectiveness in the current animal model makes it possible to examine MET's effects in PD models. It may also be a promising treatment for other neurodegenerative illnesses.

Conflict of interests

The authors declared no conflict of interest.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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