**Research Paper: A Neurochemical and Electrophysiological Study on the Combined Effects of Caffeine and Nicotine in the Cortex of Rats**

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**Introduction:** Caffeine and nicotine are the most widely consumed psychostimulants worldwide. Although the effects of each drug alone on the central nervous system have been studied extensively, the literature on the neurochemical and electrophysiological effects of their combined treatments is scarce. The present study investigated the cortical electrophysiological and neurochemical alterations induced by acute administration of caffeine and nicotine in rats.

**Methods:** The rats received caffeine and nicotine at a 1-hour interval between the two treatments.

**Results:** Caffeine and nicotine administration resulted in a significant decrease in the concentrations of cortical amino acid neurotransmitters, namely glutamate, aspartate, glycine, and taurine, while γ-aminobutyric acid (GABA) significantly increased. Increased cortical lipid peroxidation and reduced glutathione and nitric oxide levels and acetylcholinesterase and Na⁺/K⁺-ATPase activities were also observed. The Electroencephalogram (EEG) showed an increase in delta frequency power band, whereas theta, beta-1, and beta-2 decreased after caffeine and nicotine treatment.

**Conclusion:** These findings suggest that caffeine and nicotine adversely exacerbate their stimulant effects manifested by the EEG changes mediated by increasing cholinergic transmission and disturbing the balance between the excitatory and inhibitory amino acids leading to oxidative stress.

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**Abstract**

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1. Introduction

Caffeine and nicotine are the two most widely used mood-altering stimulants. Caffeine is readily distributed to all tissues of the body, including the brain where its lipophilic nature enables it to cross the blood-brain barrier (Dager & Friedman, 2000). Brown and Benowitz (1989) observed a tendency toward increased cigarette abuse during caffeine intake, and a tendency toward elevated plasma nicotine levels when administering a low dose of caffeine compared to those not taking any caffeine. Evidence suggests that the most important role of caffeine is blocking the endogenous adenosine-induced inhibition resulting in an increase in the release of dopamine, noradrenaline, and glutamate (Ferre`, Fuxe, Fredholm, Morelli, & Popoli, 1997).

Nicotine, 3-(1-methyl-2-pyrrolidinyl) pyridine, the second most used psychostimulant, is a natural alkaloid component found in tobacco leaves. It is the primary active constituent of tobacco that leads to addiction and is commonly self-administered by smoking or chewing tobacco products. Nicotine is distributed throughout the body and can cross the blood-brain barrier taking only a few seconds to reach the brain (Benowitz, Hukkanen, & Jacob, 2009).

Nicotine acts on the neuronal nicotinic Acetylcholine (nACh) receptors in both the peripheral and central nervous system and can markedly affect cortical activity through complex ways, by the facilitated release of acetylcholine (Bruno et al., 2006), GABA, and glutamate (Fallon, Shearman, Sershen, & Lajtha, 2007). Nicotine elevated heart rate and subjective ratings, which are indicators of aversive effects and also reduced reaction times regardless of the caffeine dose (Blank, Kleykamp, Jennings, & Eisenberg, 2007). The interaction between nicotine and caffeine has shown conflicting results. Caffeine has a promoting (Celik et al., 2006), reverse (Palmatier and Bevins, 2001), or no effect on nicotine responses (Justinova et al., 2009). In addition, the interaction between caffeine and nicotine was most prominent on subjective arousal as the decrease in arousal by nicotine occurred only when caffeine was present (Rose and Behm, 1991). Glutamate is the major excitatory neurotransmitter while GABA is considered the major inhibitory neurotransmitter in the central nervous system. They serve important functions in energy and glucose metabolism, neuronal excitability, and cognitive activity (Hyder et al., 2006).

The brain produces large amounts of Reactive Oxygen Species (ROS). Normally, the generated ROS can be overcome by the brain’s antioxidant mechanism. However, little is known about the effect of the interaction between caffeine and nicotine on the oxidant/antioxidant status of the brain.

Spontaneous Electroencephalogram (EEG) is a non-invasive objective measure of the dynamic activity of
the brain. It has been reported that nicotine elevated EEG power in some of the higher frequency bands, while caffeine reduced EEG power (Gilbert, Dibb, Plath, & Hiyane, 2000). Moreover, EEG signs showed decreased theta power and increased alpha frequency by cigarette smoking and caffeine administration after nicotine withdrawal (Cohen, Pickworth, Bunker, & Henningfield, 1994).

The physiological effect of either caffeine or nicotine administration has been extensively studied. The physiological effect of their combination did not receive the same attention; however, many people are exposed to both caffeine and nicotine intake by drinking coffee and smoking cigarettes. Therefore, it is worthy to investigate the effect of their combination rather than the effect of each separately to be more consistent with the real human exposure circumstances. For this reason, the present study was performed to reveal the neurochemical and electrophysiological effects of the acute interaction between caffeine and nicotine on the cortex of adult male rats.

2. Methods

Animals

The animals used in this experiment were 21 male Wistar rats whose weights ranged between 230 and 250 g. They were supplied by the Animal House of the National Research Centre, Egypt, and maintained under controlled conditions of temperature and light. The animals were fed with standard rodent chow and were provided with water ad libitum. The approval of the experimental protocol and procedures applied in this study was given by the Cairo University, Faculty of Science Institutional Animal Care and Use Committee (IACUC) (Egypt), (CU/1/F/31/18).

Chemicals

Caffeine was obtained from Global Chemie (Mumbai, India). It was dissolved in saline. Nicotine is nicotine hydrogen tartrate salt and was obtained from BDH Chemicals Ltd (England) and dissolved in saline. Thio-barbituric acid, trichloroacetic acid, perchloric acid, triethylamine, absolute ethyl alcohol, sulfanilamide, and N-1-naphthyl ethylene diamine were supplied by Sigma Aldrich. In addition, glutathione, acetylthiocholine iodide, ethylene diamine tetraacetic acid, 5,50-di-thiobis-(2-nitrobenzoic acid) (DTNB), and phosphate buffers were obtained from Sigma Aldrich. Analytical-grade glacial acetic acid, dansyl chloride, lithium carbonate, and high-performance liquid chromatography (HPLC)-grade acetonitrile were purchased from Fisher (UK). Standard amino acids and HPLC-grade methanol were supplied by BDH (England).

Surgery for electrode implantation

The rats were anesthetized by a single intraperitoneal (i.p.) injection of pentobarbital (40 mg/kg), and placed in the stereotaxic device (David Kopf Instruments, Tujunga, California, USA). Each rat was implanted with two cortical electrodes positioned 2.7 mm lateral to the midline above the hippocampus and 3.5 mm posterior to the bregma in both hemispheres. Stainless steel miniature screws, each 1 mm in diameter, were used as electrodes. A third electrode was placed 2 mm posterior to the lambda on the midline above the cerebellum and was used as a reference to these electrodes. Fixation and isolation of the electrodes in their positions were carried out using dental cement (zinc polycarboxylate, Spofa-Dental-Praha, Czech Republic). The animals were transferred after surgery to separate glass cages and left for a recovery period of 7–10 days and were then used to carry out the experiment.

Experimental design

A group of rats (n=7) was used to evaluate the effect of caffeine and nicotine treatment on the EEG recordings. The recordings of EEG were carried out before and after 30 minutes of treatment (self-control).

The rest of the animals were divided into two groups (n=7): Control and treated animals. Treated animals received an i.p. injection of caffeine (30 mg/kg) followed by subcutaneous injection of nicotine (2 mg/kg) after one hour. Control animals received an i.p. injection of saline followed by subcutaneous injection of saline after one hour. After 30 minutes of the two drug treatments, rats were sacrificed to carry out the neurochemical analyses in the cerebral cortex.

EEG recording and analysis

Each rat was placed in an electrically-shielded sound-attenuated cage (25 cm ×25 cm×30 cm) throughout the recording session. The recording electrodes were joined to the amplifier, which in turn was attached to the analog-to-digital conversion (A/D) card (National Instruments, USA). The EEG signals were acquired using Biobench software (National Instruments, USA). Normal EEG was recorded from each of the normal animals and then repeated for the same animals directly after treatment. Each EEG recording session lasted for 30 minutes.
The gain of the EEG amplifier (Coulbourn Instruments, L.L.C., Allentown, PA, USA) was adjusted to amplify the signal by 103 to be suitable for digitalization by the Analog to Digital Card (ADC) (National Instruments Inc., PCI-MIO-16E-4, Austin, TX, USA), which was connected to a personal computer. The EEG signals were acquired at a rate of 200 samples/sec to avoid signal aliasing. The sampled data were filtered through a notch filter at 50 Hz to eliminate the line frequency noise. Offline analysis of the EEG signals was done by transforming the signals from the time domain into the frequency domain via Fast Fourier Transform (FFT) software. The signals were windowed using the hamming window with a frame size of 1024 samples and the output FFT frames were averaged in one power spectrum. The power spectrum represents the signal power (amplitude squared) as a function of its frequency. Through a custom-made software, the power spectrum was then segmented into five frequency bands, namely Delta (0.1-4 Hz), Theta (4.1-8 Hz), Alpha (8.1-13 Hz), Beta-1 (13.1-18 Hz), and Beta-2 (18.1-30 Hz). The power in each frequency band is called absolute band power and is calculated by summing the power at each individual frequency of the band. The relative band power (i.e. the percentage power) was calculated and normalized for each animal as follows:

Relative Band power = Power in the frequency band/Power in all bands × 100

Neurochemical analyses

Handling of tissue samples

Each of the treated animals was sacrificed after 30 minutes of the last injection with a group of the control animals. The brain of each animal was moved to an ice-cold Petri dish to remove the cortex. Each cortex was cut longitudinally into two equal halves. Each half was weighed and stored at –60°C until analysis. Each half of the cortex was homogenized in 5% w/v 20 mM Tris-hydrochloric acid buffer (pH 7.6) and centrifuged at 4000 rpm for 15 minutes. The supernatant was used to measure reduced glutathione, lipid peroxidation, and Nitric Oxide (NO) and acetylcholinesterase (AChE), and Na+/K+-ATPase activities. The right half of the cortex was homogenized in 75% ethyl alcohol and centrifuged at 15777 g at 4°C in a high-speed cooling centrifuge (Type 3k-30, Sigma, Germany) for 30 minutes. The clear supernatant was used for the analysis of amino acid neurotransmitters.

Determination of lipid peroxidation

The method of Ruiz-Larrea, Leal, Liza, Lacort, and de Groot (1994) was used to measure malondialdehyde, an indicator of lipid peroxidation, in the form of thiobarbituric acid-reactive substances. These reactive substances react with thiobarbituric acid in the acidic medium at a temperature of 95 °C for 30 minutes to form a red-colored complex whose absorbance was read at 532 nm in a UV-Visible spectrophotometer (Thermo Spectronic, England).

Determination of reduced glutathione

Reduced glutathione was assayed by the method of Ellman (1959). This method is based on the reduction of 5,5’-dithiobis (2 - nitrobenzoic acid) (DTNB) with glutathione to produce a yellow compound. The reduced chromogen is directly proportional to reduced glutathione concentration and its absorbance can be determined spectrophotometrically at 405 nm.

Determination of nitric oxide level

NO level was measured by Griess reagent according to the technique of Moshage, Kok, Huizenga, and Jansen (1995). NO, as nitrate is transformed to a deep purple azo compound by Griess reagent. The color formed was read spectrophotometrically at 540 nm.

Determination of acetylcholinesterase activity

The technique used for the measurement of Acetylcholinesterase (AChE) activity was based on the method of Gorun, Proinov, Băltescu, Balaban, and Bârzu (1978). In this method, acetylthiocholine iodide is hydrolyzed by the enzyme to thiocholine, which reacts with 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB), reducing it to thionitrobenzoic acid whose yellow color was measured spectrophotometrically at 412 nm.

Determination of Na+/K+-ATPase activity

The spectrophotometric method of Tsakiris, Angelogiani, Schulpis, and Behrakis (2000) was used to measure Na+-K+-ATPase activity. The color obtained was read at 640 nm.

Determination of amino acid concentrations

The quantitative analysis of the neurotransmitter amino acids (glutamate, aspartate, glutamine, GABA, glycine, and taurine) was performed by the high-performance liq-
uid chromatography (HPLC) method of Márquez, Que- sada, Sánchez-Jiménez, and De Castro (1986).

The HPLC system used in this study consisted of a Wellchrom Mini-star K-501 pump (Knauer, Germany), equipped with a column thermostat 5–85°C having a 20 μl loop injector (Knauer, Germany). The column used was a Luna 5μ C-18 reversed-phase column (5 μm particle size, 150 × 4.6 mm I.D.) supplied by Phenomenex (USA). The separated amino acids were detected by a Wellchrom spectrophotometer K-2600 with variable wavelength (Knauer, Germany). A chromatography workstation (Eurochrom 2000) was used to quantify the height of the amino acids. The mobile phase consisted of methanol and water (50/50 v/v), to which glacial acetic acid (0.6%) and triethylamine (0.008%) were added. The height ratio of each amino acid was quantified (height of amino acid divided by the height of internal standard) and the concentrations (μmol/g fresh tissue) were then calculated by the internal standard method.

Statistical analysis

The data of each group were expressed as Means±SEM. Statistical comparisons between the control and treated animals and the levels of significance were determined by the independent student’s t-test employing the Statistical Package for the Social Sciences (SPSS). Statistical significance was set at P<0.05.

Percentage difference = (treated conc. - control conc. / control conc. X 100)

3. Results

After the i.p. injection of caffeine, the rats became hyperactive with increased exploratory behavior, grooming, and sniffing. However, immediately after nicotine injection, the rats suffered from tremors, rapid breathing, and disturbances in locomotor activity. This was followed by complete relaxation, which continued throughout the EEG recording session (30 minutes).

Table 1 shows the effect of the i.p. injection of caffeine followed by the s.c. injection of nicotine after 1 hour on cortical amino acid neurotransmitters and glutamine concentrations in adult male rats. The injection of caffeine followed by nicotine after 1 hour resulted in a significant decrease in the concentrations of the excitatory neurotransmitters glutamate (P<0.01) and aspartate (P<0.05) in the cortex of rats. In addition, a significant decrease in glycine (P<0.05) and taurine (P<0.05) levels was obtained, recording – 20.99 % and – 13.99 % below the control levels, respectively. However, GABA levels showed a significant increase (P<0.05).

Figure 1 shows that the i.p. injection of caffeine followed by the s.c. injection of nicotine increased cortical lipid peroxidation levels by 22.73 % (P<0.05) but decreased glutathione and NO levels by 12.93 % (P<0.01) and 34.48 % (P<0.001), respectively, in comparison with control levels. A significant decrease was also obtained in cortical AChE (P<0.01) and Na⁺-K⁺-ATPase (P<0.05) activities (Figure 2). The injection of caffeine followed by nicotine after 1 hour caused a significant increase in the band power of the delta wave (P<0.05) (Figure 3). However, significant decreases (P<0.05) in the band powers of theta, beta-1, and beta-2 were obtained in treated rats recording -37.40 %, -27.18 %, and – 37.63 % below the control, respectively.

4. Discussion

The present study revealed that the interaction between the acute doses of caffeine and nicotine induced significant decreases in the levels of glutamate, aspartate, glycine, and taurine besides a significant increase in GABA concentration.

The increase in glutamate release and glutamatergic neurotransmission following the administration of each of caffeine (Fredholm, Bättig, Holmén, Nehlig, & Zvartau, 1999) and nicotine (Parikh, Man, Decker, & Sarter, 2008) has been reported previously. In addition, it has been reported that nicotine evoked the release of [3H] D-aspartate in a Ca²⁺- and concentration-dependent manner (Rousseau, Jones, Pullar, & Wonnacott, 2005). Therefore, the present treatment of both caffeine and nicotine might result in excessive release of glutamate and aspartate, and this eventually can lead to a decrease in their cortical concentrations.

Caffeine may directly affect the levels of GABA (Fredholm et al., 1999). Fallon et al. (2007) suggested that the excitatory glutamatergic and inhibitory GABAergic receptors both mediate nicotine-induced neurotransmitter responses. In glial cells, glutamate is metabolized via glutamine synthase into glutamine, which in turn is converted to GABA. The glutamate possibly released under the effect of caffeine and nicotine was converted in glial cells to glutamine, which serves to replenish GABA in an attempt to counteract the hyperexcitability state resulting from the excessive release of the excitatory amino acids.

It has been reported that the acute administration of nicotine could evoke endogenous glycine release in...
the hippocampus of rats (Zappettini et al., 2011). This may underlie the decrease in its concentration in the present study after caffeine and nicotine treatments. As glycine acts as a coagonist at the NMDA receptor enhancing NMDA receptor-mediated glutamatergic transmission, its decrease may serve to attenuate the increase in excitatory transmission induced by both caffeine and nicotine.

On the other hand, the injection of caffeine and nicotine reduced the concentration of cortical taurine, which is neuroprotective against glutamate hyperexcitability (El-Idrissi, 2008). The loss of the neuroprotective effect of taurine may further enhance the state of cortical excitability resulting from the excessive release of the excitatory amino acid neurotransmitters.

K⁺/Na⁺-ATPase plays a major role in the maintenance of membrane excitability by restoring ionic gradients on both sides of the membrane. The present results revealed that K⁺/Na⁺-ATPase activity significantly decreased. This could be due to its exhaustion against the state of hyperexcitability induced by caffeine and nicotine administration. Our results are in line with the findings of Wang, Mccomb, Weiss, Mcdonough, and Zlokovic (1994) that nicotine decreased cerebromicrovascular and brain K⁺/Na⁺-ATPase enzymatic activities. In the present investigation, the injection of caffeine followed by nicotine induced a state of oxidative stress as evident from the increased lipid peroxidation and decreased glutathione and NO levels.

Boison (2011) suggested that acute caffeine administration acts mainly on adenosine A1 receptors, enhancing excitotoxicity contrary to caffeine effects on adenosine A2A receptors, which is neuroprotective. Similarly, reports regarding the effects of nicotine on oxidative stress have yielded conflicting results. It has been reported that nicotine, at low concentrations, may behave as an antioxidant and play a neuroprotective role, while high doses of nicotine may result in oxidative stress and neurotoxicity (Guan, Yu, & Nordberg, 2003).

It is obvious from the present data that the decrease in glutathione level may reflect the failure of the antioxidant system of the brain to overcome the excessive production of free radicals. Glutathione is the most abundant antioxidant that maintains cellular redox status.

It has been suggested that enhanced glutamatergic transmission under the effect of nicotine administration could promote NO production, which in turn may increase glutamatergic activity as NO increases the release of glutamate (Meffert, Premack, & Schulman, 1994). NO reacts rapidly with superoxide anion to produce peroxynitrite, which is a strong oxidant (Beckman and Koppenol, 1996). Therefore, a decrease in NO could be explained by its interaction with the superoxide anion generating the potent oxidant derivative; peroxynitrite.

The current state of oxidative stress may also have a role in the reduction in K⁺/Na⁺-ATPase activity since lipid peroxidation has been reported to disrupt numerous membrane-bound transport proteins, including K⁺/Na⁺-ATPase (Marnet, 2002).

### Table 1.

| Variables     | Mean±SD | %D  | Significance |
|---------------|---------|-----|--------------|
|               | Control | Treated |     |
| Glutamine     | 6.45±0.26 (6) | 6.71±0.18 (6) | 4.07 | n.s.  |
| Glutamic Acid | 10.47±0.25 (6) | 9.36±0.21 (6) | -10.560 | * |
| Aspartic Acid | 3.81±0.13 (6) | 3.32±0.11 (6) | -12.920 | * |
| Glycine       | 4.15±0.28 (7) | 3.27±0.12 (7) | -20.988 | * |
| GABA          | 4.22±0.17 (6) | 4.73±0.15 (6) | 12.353 | * |
| Taurine       | 13.69±0.71 (6) | 11.77±0.29 (6) | -13.991 | * |

Values are presented as Mean±SEM with the number of animals in parentheses. *Significant at P<0.05; **: Not Significant.

Mourad, I. M., et al. (2021). Cortical Acute Effects of Caffeine and Nicotine. BCN, 12(5), 681-692.
Consistent with the previous data, the administration of caffeine and nicotine resulted in a decrease in cortical AChE activity. It has been suggested that the production of free radicals could be associated at least in part with decreased activity of brain AChE (Tsakiris, Angelogianni, Schulpis, & Starridis, 2000). Karadsheh, Kussie, and Linthicum (1991) reported that caffeine inhibited AChE through binding of the N-methyl group of the pyrrolidine ring to AChE. Moreover, it was found that nicotine alone decreased AChE mRNA expression (Jamal et al., 2010). Thus, the oxidative stress, binding of the enzyme, and reduced synthesis may all contribute to the decreased AChE activity induced by caffeine and nicotine, in the present study, thereby augmenting cholinergic activity.

In the neocortex, Acetylcholine (ACh) is known to stimulate arousal and play a major role in working memory and attention (Jones, 2008). Neuropsychological studies on caffeine reported increased arousal, alertness, and concentration (Brunyé et al., 2010). However, caffeine intake has also been associated with anxiety, nervousness, irritability, insomnia, and even panic attacks (Sicard et al., 1996). Similarly, nicotine improves cognitive performance by improving learning, memory, and attention (Swan and Lessov-Schlaggar, 2007). High doses of nicotine were found to be anxiogenic in a social

Figure 1. Effect of the acute treatment with caffeine (30 mg/kg) followed by nicotine (2 mg/kg) on the concentration (Conc.)
A: Lipid peroxidation (nmol/g tissue), B: Reduced glutathione (mmol/g tissue), and C: nitric oxide (µmol/g tissue) in the cortex of rats. Values are expressed as Mean±SEM; n=7; *Indicates significant difference between control and treated rats at P<0.05.

Figure 2. Effect of the acute treatment with caffeine (30 mg/kg) followed by nicotine (2 mg/kg) on the activity
A: Acetylcholinesterase; and B: Na⁺/K⁺-ATPase in the cortex of rats. Values are expressed as Mean±SEM; n=7; *Indicates significant difference between control and treated rats at P<0.05.
interaction test used to induce anxiety (File, Kenny, & Ouagazzal, 1998).

Thus, the present increase in the cortical cholinergic activity, due to decreased AChE activity, together with the present alterations in amino acid neurotransmitters may increase the cortical excitability. This in turn may exaggerate the arousal effect of both agents; however, other aversive effects, such as the present oxidative stress may develop. EEG and behavioral arousal are regulated by adenosinergic and cholinergic neurotransmission (Steriade and McCarley, 2005). The present EEG power spectrum revealed that the delta wave significantly increased while theta, beta-1, and beta-2 waves significantly decreased after caffeine and nicotine.

Existing evidence shows that delta activity increases in pathological conditions correlated with neuronal damage and even in anxiety (Gauthier, Chevrette, Bourier, & Godbout, 2009). On the other hand, cognitive neuroscientists describe delta as a cognitive rhythm (Knyazev, 2007) that is linked to attention (Knyazev, 2012).

It can be suggested that the increase in delta wave power may underlie the increased attention and alertness induced by caffeine and nicotine on one hand, and the development of anxiety on the other hand. It was reported that GABA increases the generation of alpha waves associated with the relaxed and mentally focused state, but decreases beta waves, related to fleeting thoughts, hyperactivity, and nervousness, leading to a highly significant increase in the alpha-to-beta wave ratio (Abdou et al., 2006). Thus, the present increase in GABA concentration may underlie the decrease in beta waves and increase in alpha to beta ratio. Theta oscillations have been correlated with working and long-term memory encoding (Klimesch, Freunberger, Sauseng, & Gruber, 2008). Therefore, the present decrease in theta wave power may be related to the increased excitability and oxidative stress rendering these animals susceptible to impaired memory encoding in spite of the increased alertness.

The present findings demonstrated that the acute treatment of caffeine and nicotine may adversely affect the cortical status of the brain by enhancing excitability,
oxidative stress, and cholinergic transmission and altering the EEG power spectrum. These effects mediate the exacerbated stimulant effect of caffeine and nicotine. Therefore, although the frequent or habitual co-administration of caffeine and nicotine may have a stimulant effect, increased concentration, and good mood, aggravated deleterious effects may be evolved by these stimulants. Future studies are recommended to confirm the deleterious effects of the chronic administration of both stimulants.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Institutional Animal Care and Use Committee (IACUC) (Egypt), Faculty of Science, Cairo University, (Code:CU/I/F/31/18).

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Authors’ contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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