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Effects of midazolam combined with sufentanil on injury and expression of HMGB1 and NF-κB in rats with pancreatitis

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Abstract. – OBJECTIVE: Midazolam and sufentanil are common analgesic and sedative drugs, but the effects and mechanisms of the combination of these two drugs on pancreatitis injury have not been fully elucidated.

MATERIALS AND METHODS: Rats pancreatitis model were randomly divided into 4 groups, model group, midazolam group, sufentanil group, and combined group, followed by an analysis of the general indicators, the onset time, duration, analgesic time, and adverse reactions, as well as pancreatic serological indicators. In addition, the level of the serum TNF-α and IL-1β was detected by enzyme-linked immunosorbent assay (ELISA), and the reactive oxygen species (ROS) production was assessed by spectrophotometer, together with an analysis of the superoxide dismutase (SOD) activity and the expression of HMGB1 and NF-κB mRNA in pancreatic tissue by Real Time-PCR.

RESULTS: Midazolam alone or in combination with sufentanil improved the general indicators along with long duration of sedative analgesia, reduced serum TNF-α, and IL-1β secretion and few adverse reactions. Meanwhile, the expression of HMGB1 and NF-κB was reduced and the pancreatic serum markers and ROS production were decreased with increased SOD activity. Compared with the model group, the differences were statistically significant ($p<0.05$), with more significant changes in the combined group ($p<0.01$).

CONCLUSIONS: Midazolam combined with sufentanil can inhibit the expression of HMGB1 and NF-κB, inhibit inflammation, thereby improving the sedative and analgesic effects, protecting pancreatic tissue, and reducing acute pancreatitis injury.

Key Words:
Midazolam, Sufentanil, HMGB1, NF-κB, Pancreatitis, Inflammation.

Introduction

Pancreatitis can be divided into acute pancreatitis and chronic pancreatitis. Acute pancreatitis (AP) is a common and frequently occurring disease in the digestive system, especially when it develops into severe acute pancreatitis (SAP). Necrosis of the peripancreatic tissue, damage, dysfunction of multiple important organs, and rapid deterioration of the disease, lead to systemic inflammation and multiple organ failure with a high mortality and poor prognosis[1,2]. The main features of AP are acute upper abdominal pain, nausea, vomiting, and elevated blood urease amylase[3]. In general, mild acute pancreatitis is mainly pancreatic edema, which can heal itself in a short period of time, but if SAP occurs, it can cause serious consequences[4]. Therefore, timely and effective treatment is critically important. In clinic, patients with pancreatitis will have anxiety, agitation, paralysis, and other mental disorders, as well as pain, which often lead to aggravation of the disease[5]. Therefore, patients with pancreatitis receive analgesic sedation treatment to prevent anxiety and fear of patients, etc[6]. The anesthesiologist chooses the appropriate anesthetic, or the method of anesthesia, and the timing of anesthesia, to reduce the patient's pain, after considering the patient's physical function and conditions[7,8].

Midazolam and sufentanil are common analgesic and sedative drugs[9]. Midazolam is a typical pharmacological activity of benzodiazepines, which can produce anti-anxiety, sedation, hypnosis, anticonvulsant, and muscle relaxation. It has both sedative characteristics with anterograde forgetting[10,11]. Midazolam has no drug resistance and withdrawal symptoms or rebound, with a low toxicity and a large

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safety range, and can be used for analgesic sedation treatment of pancreatitis[12,13]. Sufentanil can act on μ-opioid receptors and is used as an analgesic with stronger affinity than fentanyl. Therefore, it not only has greater analgesic intensity, but also has a longer duration of action, faster onset, and faster recovery of anesthesia and ventilation, and it can be applied for pain treatment[14-16]. However, the combined effects of midazolam and sufentanil on pancreatitis injury have not been fully elucidated.

Materials and Methods

Experimental Animals

Forty healthy male Wistar rats, 2 months old, SPF grade, body mass (250 ± 20) g, were purchased by self-calibrated experimental animal centers and fed in our SPF Animal Experiment Center with a temperature of 21 ± 1°C and relative humidity of 50-70%, ensuring a 12/day cycle every 12 hours.

Ethical Committee Approval

This study was approved by the Ethics Committee of China-Japan Union Hospital of Jilin University (CJUH20170603EA).

Main Materials and Instruments

Sodium taurocholate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Midazolam and sufentanil were purchased from Jiangsu Enhua Pharmaceutical Co., Ltd (Jiangsu, China). RNA extraction kit, reverse transcription kit, and the superoxide dismutase (SOD) activity detection kit were purchased from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). The reactive oxygen species (ROS) test kit was purchased from Trevigen (Gaithersburg, MD, USA). Enzyme-linked Immune Sorbent Assay (ELISA) kits for TNF-α and IL-1β were purchased from R&D (Minneapolis, MN, USA). Surgical microscopy equipment was purchased from Suzhou Medical Instrument Factory (Suzhou, China). The impark microplate reader was purchased from BD Corporation (San Jose, CA, USA). The Amp PCR System 2400 DNA Amplifier was purchased from PE (Foster City, CA, USA). The MouseOx Small Animal Physiological Monitor was purchased from Shanghai Yuyan Instrument Co., Ltd (Shanghai, China). Altair automatic biochemical detector was purchased from Shanghai Yuyan Instrument Co., Ltd (Shanghai, China). The DR1900 spectrophotometer was purchased from Hach Company (Loveland, CO, USA). Other commonly used reagents were purchased from Shanghai Shenggong Biological Co., Ltd (Shanghai, China).

Grouping and Processing of Experimental Animals

Forty rats were randomly divided into 4 groups, with n=10 rats in each group, model group, midazolam group, sufentanil group, combined group. For the treatment group, based on pancreatitis model, midazolam or sufentanil was administered alone or in combination.

Preparation and Administration of Rat Model of Acute Pancreatitis

According to the literature[7], the model group rats were fasted for 12 hours before modeling. 10% chloral hydrate was intraperitoneally injected into the anesthetized rats, which were then fixed on the operating table, through the mid-abdominal incision layer-by-layer incision, exposing duodenum and pancreaticobiliary duct, double-clamped pancreaticobiliary tube with non-invasive small artery clamp at the proximal hepatic portal. Retrogradely, through the duodenal papilla puncture of the pancreaticobiliary duct, non-invasive small artery clamp fixation, freshly placed 5% sodium taurocholate solution was dropped into the pancreatic duct and pancreas at a constant rate of 0.1 ml/min, so that the concentration in the body reached 0.1 ml/100g. After the non-invasive small artery clamp was clamped for 5 min, the pancreatic lobules were immersed in sodium taurocholate solution, the arterial clip was loosened, the duodenum was also returned, and the abdomen was closed layer by layer. The rats in each group were treated with different anesthesia methods. 100 mg/kg midazolam group or sufentanil alone or combined was administrated through the femoral vein. The rats were maintained analgesic for 1 h. The incision was sutured after surgery followed by intramuscular injection of penicillin 20,000 U/kg for conventional anti-inflammatory.

Specimen Collection

The rat abdominal aorta blood samples were collected into a vacuum biochemical tube using a vacuum collection method and placed at room temperature for 30 min. After the blood was coagulated, blood was centrifuged at 4°C, 3600 rpm for 10 min to obtain serum which was stored in a refrigerator at -20°C. The pancreatic tissue was collected from each group and stored in a refrigerator at -80°C for further use.

Serological Indicators Test

The changes of serum amylase (AMY), creatinine (crea, Cr), and alanine aminotransferase (ALT) in each group were analyzed by automatic biochemical detector.

ELISA Analysis of TNF-α and IL-1β Levels

The serum of each group was collected to detect the expression changes of inflammatory factors TNF-α and IL-1β by ELISA according to the ELISA kit instructions. 50 μl of the sequentially diluted standard was added to 96-well plate to prepare a standard curve. 50 μl of the sample was added and 3 replicates were set for each sample. Then, 50 μl of the enzyme labeling reagent was added to each well, except for blank wells, mixed gently by shaking, and incubated for 30 min at 37°C. After washing the plate 5 times, 50 μl of the developer A was added, followed by addition of 50 μl of the developer B and incubation at 37°C for 10 min in the dark. After that, 50 μl of the stop solution was added to terminate the reaction. The blank value was set to be 0, and the optical density value (OD value) of each well was measured by a microplate reader at a wavelength of 450 nm. The linear regression equation of the standard curve was calculated according to the concentration of the standard product and the corresponding OD value, and the corresponding sample concentration was calculated based on the regression equation according to the OD value of the sample.

Real Time-PCR Detection of High Mobility Group Box 1 Protein (HMGB1) and NF-κB mRNA Expression

Under sterile conditions, pancreatic tissue was washed with PBS and ground with liquid nitrogen. mRNA was extracted using TRIzol reagent and cDNA was synthesized according to the relevant primers (Table I). Real Time-PCR was used to detect the expression of the target gene with reaction conditions: 52°C 1 min, 90°C 30 s, 58°C 50 s, 72°C 35 s, for a total of 35 cycles. Fluorescence quantitative PCR reactor software was used to collect relevant data. According to the internal reference GAPDH, the standard cycle number (CT) of the standard was calculated, and the standard curve was drawn. The quantitative analysis was analyzed by $2^{-\Delta Ct}$ method.

Analysis of SOD Activityin Pancreatic Tissue of Rats in Each Group

The changes in SOD activity in pancreatic tissue of each group were examined according to the kit instructions. The total protein of the tissue was extracted, and boiled at 95°C for 40 minutes, followed by being rinsed with cold water and subsequent centrifuged at 4000 rpm for 10 minutes. The ethanol phase in the tissue homogenate was extracted using an ethanol-chloroform mixture (5:3, v/v volume ratio 5:3) for detection of total SOD activity.

Detection of ROS in Pancreatic Tissue of Rats in Each Group

The treated tissue was subjected to a 95°C water bath. After 40 minutes, it was rinsed with cold water and centrifuged at 4000 rpm for 10 minutes. The tissue homogenate was incubated with 2', 7'-dichlorofluorescein diacetate (DCF-DA) for 15 min at 37°C, centrifuged at 10,000 rpm for 15 min, and the pellet was resuspended in sterile PBS phosphate buffer for 60 min at 37°C. The level of ROS was measured using a spectrophotometer.

General Indicator Observation

The heart rate, respiration, blood oxygen saturation, and body temperature of each group of rats were measured by small animal physiological monitor. The occurrence and number of adverse reactions, such as bradycardia, itching, hypotension, chills, and respiratory depression were observed. The time, duration, and analgesic time of the drug were evaluated separately.

Statistical Analysis

Data were processed by Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) 16.0 software. The measurement data were expressed as mean ± standard deviation (SD), and the comparison of difference among multiple groups was performed by One-way ANOVA with Bonferroni as post-hoc analysis. $p<0.05$ indicated a statistically significant difference.

Table I. Primer sequences.

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	AGTGCCAGCCTCGTCTCATAG	CGTTGAACTTGCCGTGGGTAG
NF-κB	CTCATCTAAGCGGAACAATGG	GCACATTCTCTCCGTAGCG
HMGB1	ATCTACTCATAGTTGCCTCTC	ACATTCGGAACTCGTCTC

Table II. Effect of midazolam combined with sufentanil on general conditions of rats with pancreatitis.

Group	Hear rate (times/min)	Breath rate (times/min)	Oxygen saturation	Body temperature (°C)
Model	471±38	92±12	69.7±2.5	40.2±1.6
Midazolam	361±22*	81±17*	85.6±1.6*	38.1±1.2*
Sufentanil	372±27*	80±15*	82.3±1.9*	38.5±1.5*
Combination	359±31*	76±14*	89.4±3.1*	37.9±1.1*

Compared with the model group, *p<0.05.

Results

Effects of Midazolam Combined with Sufentanil on General Conditions of Rats with Pancreatitis

The effects of midazolam and sufentanil alone or in combination on heart rate, respiration, oxygen saturation, and body temperature in rats with pancreatitis were analyzed. The results showed that midazolam and sufentanil alone or in combination can significantly improve heart rate, respiration, oxygen saturation, body temperature index in rats with pancreatitis. Compared with the model group, the differences were statistically significant (p<0.05). However, there were no statistically significant differences between the mice treated with midazolam and sufentanil alone or in combination (Table II).

Analgesic Effect of Midazolam Combined with Sufentanil in Rats with Pancreatitis

The effects of midazolam and sufentanil alone or in combination on analgesic sedation in rats with pancreatitis were analyzed. The results showed that the combination of midazolam and sufentanil had a short onset time and a long duration, which prolonged the pain time. The difference was statistically significant compared with the midazolam and sufentanil alone groups (p<0.05) (Table III).

The Incidence of Adverse Reactions in Rats with Pancreatitis Treated with Midazolam Combined with Sufentanil

The effects of midazolam and sufentanil alone or in combination on adverse reactions in rats with pancreatitis were analyzed and the results showed that the combination of midazolam and sufentanil reduced the adverse reactions during analgesia and sedation in rats with pancreatitis. The differences were statistically significant compared with the midazolam and sufentanil groups (p<0.05) (Table IV).

Analysis of Serum Markers of Pancreatitis in Rats with Midazolam Combined with Sufentanil

The changes of serological indexes after 12 hours of modeling in each group of rats were an-

Table III. Analgesic effect of midazolam combined with sufentanil in rats with pancreatitis.

Group	Onset time (min)	Duration time (min)	Pain time (min)
Midazolam	20±4	58±9	81±11
Sufentanil	21±5	62±11	82±12
Combination	12±6*	78±17*	99±17*

Compared with midazolam or sufentanil alone, *p<0.05.

Table IV. Analgesic effect of midazolam combined with sufentanil in rats with pancreatitis.

Group	Bradycardia	Itching	Hypotension	Chills	Respiratory depression	Incidence (%)
Midazolam	2 (20%)	2 (20%)	3 (30%)	3 (30.0%)	0	8 (80%)
Sufentanil	2 (20%)	1 (10%)	2 (20%)	3 (30.0%)	0	7 (70%)
Combination	1 (10%)	1 (10%)	0 (0)*	1 (10%)	0	2 (20%)*

Compared with midazolam or sufentanil alone, *p<0.05.

Table V. Analysis of serum markers in each group.

Markers	Model	Midazolam	Sufentanil	Combination
AMY(U/L)	7117 ± 316	7617 ± 378*	4659 ±232*#	3159 ±345*#Δ
ALT (U/L)	348 ±35.2	342 ±31.2*	255± 12.1*#	186± 22.4*#Δ
Cr (U/L)	102 ±12.6	97 ±3.6*	71± 6.6*#	51± 4.3*#Δ

Compared with the model group, *p<0.05; compared with midazolam, #p<0.05; compared with midazolam or sufentanil, Δp<0.05.

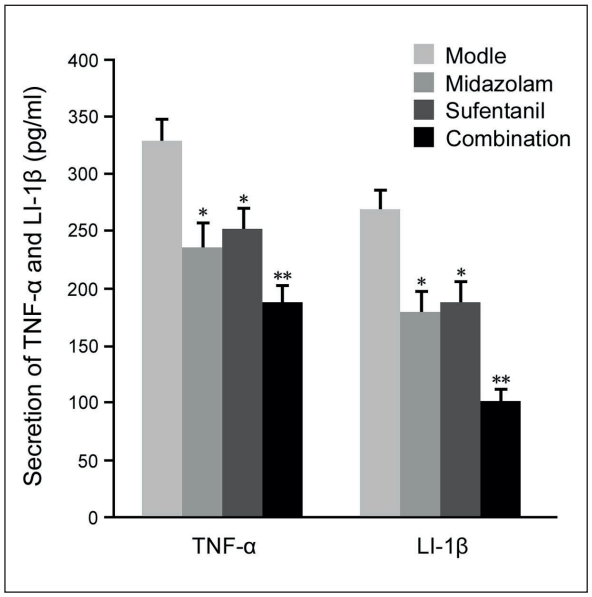

Figure 1. Effect of midazolam combined with sufentanil on serum inflammatory factor secretion in rats with pancreatitis. Compared with the model group, *p<0.05; **p<0.01.

alyzed and showed that midazolam and sufentanil alone or in combination can significantly improve serum AMY, Cr, and ALT, compared with the model group (p<0.05) with more significant changes observed in combined group (p<0.01) (Table V).

Effect of Midazolam Combined with Sufentanil on Serum Inflammatory Factor Secretion in Rats with Pancreatitis

The effects of midazolam and sufentanil alone or in combination on the secretion of TNF-α and IL-1β in the serum of rats with pancreatitis were analyzed and showed that midazolam and sufentanil alone or in combination can significantly reduce the secretion of inflammatory factors TNF-α and IL-1β in rats with pancreatitis, compared with the model group (p<0.05). The effect was more pronounced in the combined group (p<0.01) (Figure 1).

Effect of Midazolam Combined with Sufentanil on Redox Index in Rats with Pancreatitis

The effects of midazolam and sufentanil alone or in combination on ROS production and SOD activity in rats with pancreatitis were analyzed and found that midazolam and sufentanil alone or in combination can significantly reduce ROS production and increase SOD activity in rats with pancreatitis. Compared with the model group, the differences were statistically significant (p<0.05), and more significant effect was found in combined group (p<0.01) (Figure 2).

Effect of Midazolam Combined with Sufentanil on the Expression of HMGB1 and NF-κB in Rats with Pancreatitis

Real Time-PCR analysis of the effects of midazolam and sufentanil alone or in combination on the expression of HMGB1 and NF-κB in pancreatic tissue of rats with pancreatitis showed that midazolam and sufentanil alone or in combination significantly reduced the expression of HMGB1 and NF-κB in pancreatic tissue of rats with pancreatitis, compared with the model group (p<0.05), and the combined group showed more significant changes (p<0.01) (Figure 3).

Discussion

Pancreatitis is a disease caused by the self-digestion of trypsin in pancreatic tissue. The pancreas has edema, congestion, or pathological changes, such as hemorrhage and necrosis, which leads to clinical symptoms, such as abdominal pain, infection, shock, and amylase in blood and urine[17]. Patients with pancreatitis often need to enter the ICU. Due to their own symptoms and medical environment, patients are prone to panic, irritability, and difficulty in cooperating with treatment. Therefore, it is necessary to choose analgesic and sedative drugs to make patients enter into a sedative state, thereby reducing patient suffering and adverse reactions[18,19].

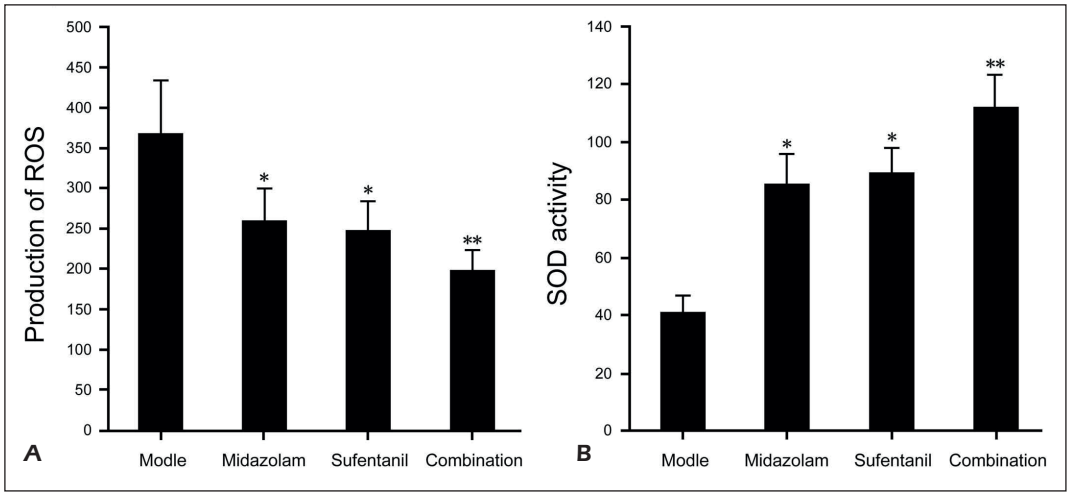

Figure 2. Effect of midazolam combined with sufentanil on redox index in rats with pancreatitis. **A**, The effect of ROS production. **B**, The effect of SOD activity, compared with the model group, *p<0.05; **p<0.01.

Sufentanil, one of the commonly used analgesic drugs, acts on opioid receptors, has strong analgesic effect, reduces myocardial oxygen consumption, and is safe, but sufentanil is easy to cause hypotension, chills, respiratory depression, etc[20]. The benzodiazepine receptor midazolam is a specific agonist with fast onset and short half-life and can exert sedative, hypnotic, anticonvulsant, anti-anxiety, and anterograde amnesia effects with few side effects on the respiratory system and circulatory system. It is usually combined with opioid analgesic drugs to exert a synergistic effect, thereby significantly reducing the amount of opioids and side effects[21]. Therefore, this paper analyzes the effect of midazolam combined with sufentanil on pancreatitis rats. The results indicated that pancreatitis caused an increase in the secretion of inflammatory factors in rats, impaired liver function, and elevated the levels of serum enzymes. However, midazolam and sufentanil alone or in combination can reduce the expression of TNF-α and IL-1β in serum of rats with pancreatitis, reduce the levels of amylase, Cr and ALT, reduce ROS production, and increase SOD activity and thus ameliorating the damages to pancreatitis. The combination of midazolam and sufentanil can significantly improve the general signs of heart rate, respiration, blood oxygen saturation, and body temperature in rats with pancreatitis. Moreover, the combination of midazolam and sufentanil has a short onset time and long duration, which can relieve pain, suggesting that the combined use of midazolam and sufentanil can increase analgesia and sedation, reduce pain time, and promote the recovery of rats with pancreatitis.

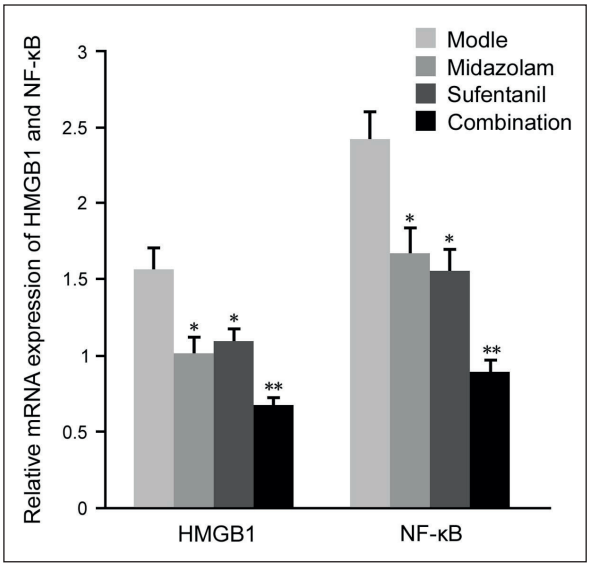

Figure 3. Effect of midazolam combined with sufentanil on the expression of HMGB1 and NF-κB in rats with pancreatitis. Compared with the model group, *p<0.05; **p<0.01.

High mobility group protein B1 (HMGB1) is a highly conserved nuclear protein. Pancreatitis leads to increased expression of HMGB1, which in turn activates downstream signaling pathways, activates NF-κB, and regulates gene transcription, as well as triggers the release of inflammatory factors, such as TNF-N and IL-1β, leading to an inflammatory response[22]. Redox balance plays a role in various diseases of the body, including ischemia-reperfusion injury, inflammation, and tumors. Under the action of

endogenous antioxidant system, ROS are continuously removed to alleviate the tissue damage. SOD is one of the most important antioxidant enzymes for scavenging oxygen free radicals in the body and plays a vital role in the regulation of oxidation and antioxidant balance of the body and its vitality reflects the body's ability to scavenge oxygen free radicals and reduce ROS production[23,24]. This study detected that midazolam and sufentanil in combination with pancreatitis can inhibit the expression of HMGB1/NF-κB, decrease ROS production, promote the increase of SOD activity, and thus inhibiting the release of the inflammatory factors, further alleviating the development of pancreatitis. In a further study, it was proposed to analyze the analgesic sedative effect of midazolam and sufentanil on patients with acute pancreatitis, and related mechanisms in clinical treatment.

Conclusions

Briefly, midazolam combined with sufentanil can inhibit the expression of HMGB1 and NF-κB, decrease inflammation, improve the anesthetic and analgesic effects during and after surgery, protect pancreatic tissue, and reduce acute pancreatitis damage.

Conflict of Interests

The authors declare that they have no conflict of interests.

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