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Original Article

Early maternal separation promotes apoptosis in dentate gyrus and alters neurological behaviors in adolescent rats

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Abstract: Adverse early-life experience such as maternal separation (MS) affects the behavior of adult, and may also aggravate the outcome of neurological insults. In this study, we aimed to investigate the effects of early MS on hippocampus-related behaviors, and to assess the mechanisms. Newborn rats were randomly divided into normal control and MS groups. Our data showed that MS (P3-P21) impaired learning ability as well as memory retrieval, and caused depression-like activity, but decreased anxiety-like activity. Glutamate receptor 1 (GluR1) expression in the dentate gyrus (DG) region was significantly reduced in the adults (P60). Mechanically, MS promoted apoptosis, and reduced protein kinase B (AKT) phosphorylation in the DG region in the early phase (P21). By contrast, MS did not affect ERK phosphorylation. Our data implicate that the inactivation of AKT pathway and apoptosis of DG cells might contribute to MS-induced behavioral changes. This study would provide useful evidence implicating the pathological changes for MS.

Keywords: Maternal separation, hippocampus, memory, anxiety, depression

Introduction

Maternal separation (MS) is one type of early life stress. With the economic growth, approximate 61 million children are living without their parents' accompanying and the numbers are still rising in China. Among them, 40% of the children are younger than 5 years [1]. In fact, early MS is related to mental development and elicits psychological and physiological disorders [2, 3]. 30% of the patients with anxiety are caused by early adverse experiences [4, 5]. In addition, depression, post-traumatic stress disorder (PTSD) and cognition impairment also symptomize the children stressed by early MS [2, 3].

Animal studies also revealed that early MS caused a serial of neurobehavioral disorders, including anxiety, depression and cognitive dysfunction. Early MS elicited chronic pain in adult rats as well [6]. Additionally, MS also aggravated the symptoms of Alzheimer's disease (AD) [7]. Considering the detrimental effects of early MS on neuronal activity, it is of particular interest to disclose the mechanisms involved. Hypothalamic-pituitary-adrenal (HPA) axis is proposed as one mechanism for the detrimental effect of MS [8]. However, the molecular basis for the long-lasting effects of MS on brain function has not been fully elucidated.

Hippocampus is an important region in the brain responsible for memory formation [9, 10]. As well, hippocampus also functions in some other neuronal activity and the dysfunction of neuronal circuit between hippocampus and functional region contributes to mental disorders [11]. Especially, hippocampus-amygdala circuit has been well studied and the imbalance of this circuit contributes to anxiety-like symptoms [12]. Whether hippocampus is a target region involved in the MS-caused behavioral changes is not confirmed.

In this study, we designed experiments to investigate the detrimental effects of MS on neuro-

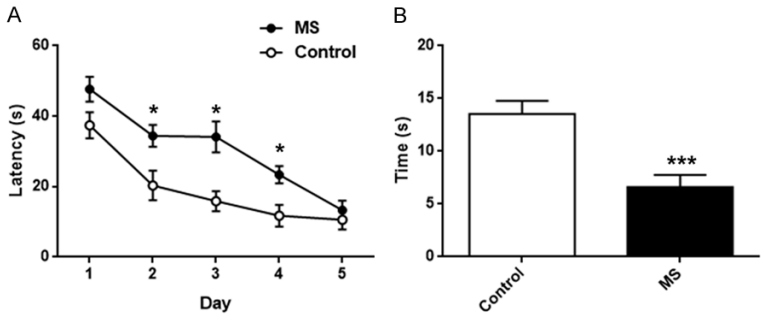

Figure 1. MS reduced learning ability and memory in rats. A. Five-day learning session; B. The time spent in platform quadrant. N=15 in each group. *P<0.05; ***P<0.001 compared with control (Unpaired t test).

development, and to assess the mechanisms involved.

Materials and methods

Animals

Male and female Sprague Dawley (SD) rats were obtained from Animal Center of Anhui Medical University and bred in a pathogen-free facility. Forty infant rats from four litters were randomly assigned to control group (without separation) and MS group. The mice in each litter (half male/female) were equally allocated. MS was performed as previously described [13]. Briefly, the pups were taken away from the maternal cages for 3-h separation each day (9:00 a.m. to 12:00 a.m.) from P3 to P21. In control group, pups were not disturbed. All pups were weaned on P21. Biochemical experiments and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay were performed in P21 rats and behavioral tests were conducted in P60 rats. All experimental procedures were under the approval of Ethnics Committee of Anhui University of Chinese Medicine (China).

Morris water maze

Spatial memory was tested in the adult animals by Morris water maze. Navigation experiment was carried out for 5 consecutive days (two times per day). Rats were randomly placed in each quadrant. The latency to find the platform was recorded. If the platform was not found in 90 s, the rat was led to the platform and the latency was counted as 90 s. Spatial exploration experiment was carried out to test the memory on day 6. The rats were put in the contralateral quadrant and time spent in the platform quadrant was recorded.

Open-field test

Open-field test was carried out in a plexiglass box (70 × 70 × 31 cm). The animal was placed in the center of the field and time was recorded immediately and continued for a 5-minute interval, and its behavior was recorded by video capture software. The total distance, time in center and border was measured by SuperMaze software. Open field arena was cleaned with 70% ethanol after each test.

Forced swimming test

Forced swimming test was performed according to the method described previously [14]. Briefly, the rats in control and MS groups were individually placed in a glass cylinder filled with water in 25-cm height at room temperature. The animals were forced to swim for 6 min and the video was recorded. The final 4 min was applied to analyze the duration of immobility.

Gait analysis

Rats were trained at the first day to walk through a tunnel and then tested for two trials. Two to four steps from the middle portion of each run were analyzed for hind-stride length and hind-base width manually (distance between the right and left hind-limb strides, sway distance).

TUNEL staining

TUNEL staining was performed in 30-μm hippocampal slices using the ApopTag In Situ Apoptosis Detection Kit (C1089, Beyotime Institute of Biotechnology, Shanghai, China) following the manufacturer's instruction. On P21, hippocampus was isolated for TUNEL staining. Brain was fixed in 4% paraformaldehyde for 1 h. The brain tissues were then cryopreservation in 30% sucrose for 1 h at 4°C, and sectioned on a freezing microtome at 20 μm. After staining, the sections were imaged using FV1000 Olympus Confocal Laser Scanning Microscope (Olympus, Japan). The TUNEL positive cells were normalized to DAPI positive cells.

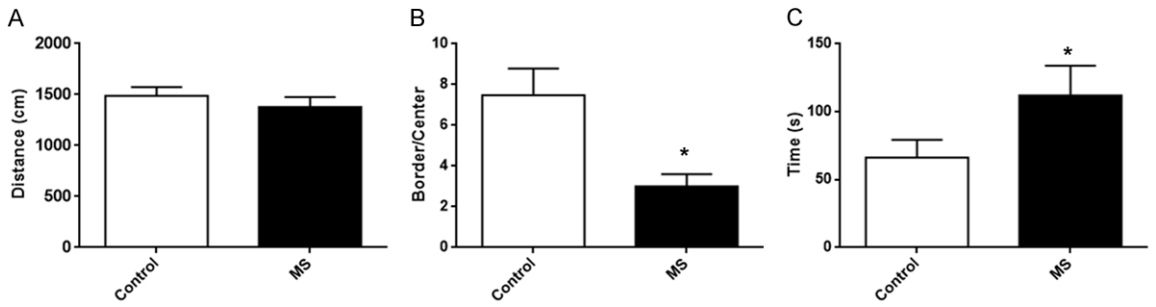

Figure 2. MS reduced anxiety-like activity and promoted depression-like activity. A. Total distance in the open field; B. The time ratio between border and center; C. The immobility time in the forced swim test. N=15 in each group. *P<0.05 compared with control (Unpaired t test).

Protein preparation and western blotting

On P21, some pups from each group were sacrificed. Cortex, cerebellum and hippocampus were isolated for biochemical experiments. The protein was extracted by RIPA cell lysate (containing PMSF). Protein samples were heated at 100°C for 10 min, and the protein concentration was quantified using bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China) as previously described [15]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was processed and proteins were later transferred onto nitrocellulose membrane. The membrane was blocked with 5% defat milk in PBST at room temperature for 2 h. The primary antibodies (anti-AKT, anti-p-AKT, anti-Erk1/2, anti-p-Erk1/2 antibodies, 1:1000, Cell Signaling Technology; Actin antibody 1:500, Zsbio, Beijing, China) were incubated with the membranes overnight at 4°C. After (3 times, 10 min each time) washing, the secondary antibody was incubated with the membrane for 2 h at room temperature. Chemiluminescent substrate detection reagent was applied to assist the staining. The target band was analyzed by Image J software.

Immunochemical staining

After fixation in 4% paraformaldehyde, hippocampus was cryoprotected in 30% sucrose for 1 h at 4°C and sectioned on a freezing microtome. Sections were blocked in 0.1 M PBS containing 10% goat serum and 0.4% Triton X-100, and then incubated with primary antibody (CST; rabbit anti-GluR1, 1:1000, CST) in 0.1 M PBS containing 5% goat serum and 0.4% Triton X-100 overnight at 4°C. Sections were washed (three times, 15 min each time) in PBS and incubated with Alexa Fluor 593 goat anti-mouse IgG (Life Technologies) for 2 h at room temperature. The mean fluorescence intensity (MFI) was analyzed in the DG regions.

Statistical analysis

The data were expressed as mean and S.E.M. and analyzed using GraphPad Prism 6. Statistical significance was assessed using Student's *t* test. *P*<0.05 was considered as statistical significance.

Results

Early MS reduces learning and memory in the adult animals

In order to test the effects of MS on learning and memory in adult rats, we performed Morris water maze test. The latency to find the platform was obviously prolonged in MS group. In the first 4-day training, the latencies to find the platform in MS animals were significantly longer than those in control group (P<0.05) (**Figure 1A**). At the fifth day, the latencies in two groups were comparable (P>0.05). After the removal of the platform, the time spent in platform quadrant was significantly shorter in MS animals (vs. control, *P*<0.001) (**Figure 1B**). These data suggested that the learning ability and memory retrieval were reduced after MS.

Early MS decreases anxiety-like activity and promotes depression-like activity in adult rats

Open-field test was applied to test the mood-related behaviors. As shown in **Figure 2A**, there were no significant differences between control and MS groups regarding the total distance.

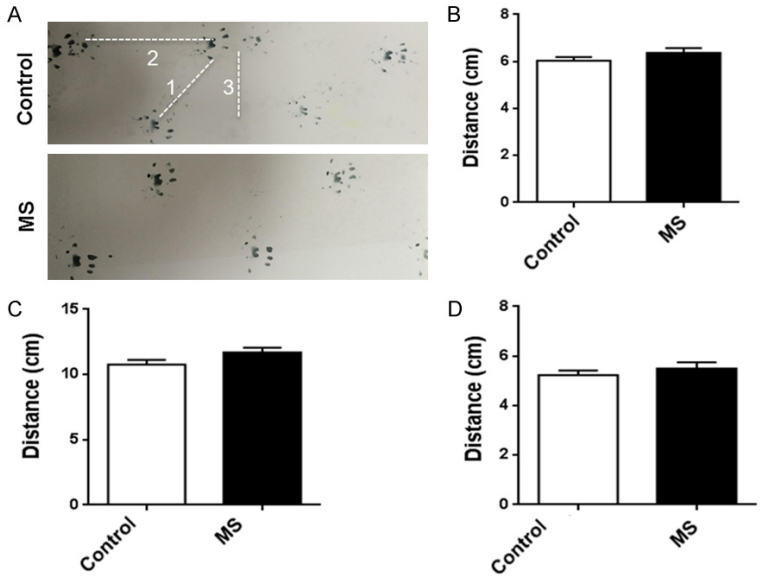

Figure 3. MS did not affect gait characters in rats. A. The representative footprints; B. Stance distance; C. Stride distance; D. Sway distance. 1, 2 and 3 represent stance distance, stride distance and sway distance. N=15 in each group.

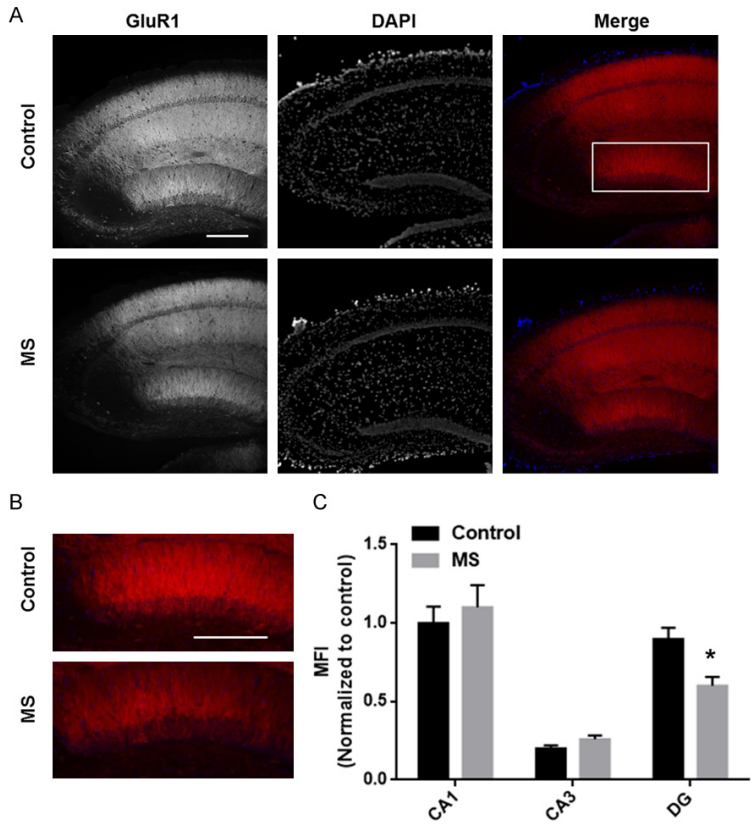

Figure 4. MS decreased GluR1 expression in DG region. A. The representative images of GluR1 expression; B. The DG region showed in A. C. Quantification data of mean fluorescence intensity (MFI). N=15 in each group. *P<0.05 compared with control (Unpaired t test). Scale bar: 100 μm.

However, the time ratio of border/center was significantly lower in MS group than that in control group (P<0.001) (**Figure 2B**). These data implicate that anxiety-like activity was reduced in MS rats.

Forced swimming test was applied to test the depression-like activity in the MS animals. As shown in **Figure 2C**, the immobility time in MS group was significantly decreased compared with control. These data might implicate that depression-like activity was facilitated in MS rats.

Early MS does not affect gait characters in adult rats

Gait characters were also detected in the adult animals after MS. As shown in **Figure 3**, stance length, stride length and sway distance were not affected in the MS animals (P>0.05). These data suggested that the MS might not affect cerebellar function.

Early MS down-regulates GluR1 expression of pyramidal neurons in the DG region

We also detected GluR1 expression in hippocampus. As shown in **Figure 4**, GluR1 expression in DG region was reduced in MS group (vs. control P<0.05), while in CA1 and CA3 was not affected.

Early MS elicits apoptosis of pyramidal neurons in the DG region

We further detected apoptosis in that area. As shown in **Figure 5**, apoptotic cells were rarely observed in control group, while remarkable apoptosis was found in MS group. These data suggested that

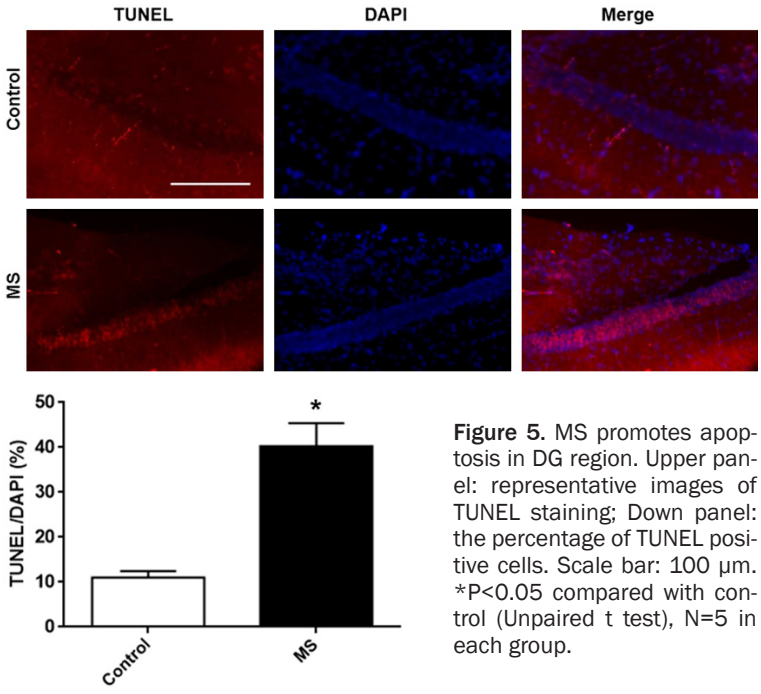

Figure 5. MS promotes apoptosis in DG region. Upper panel: representative images of TUNEL staining; Down panel: the percentage of TUNEL positive cells. Scale bar: 100 μm. *P<0.05 compared with control (Unpaired t test), N=5 in each group.

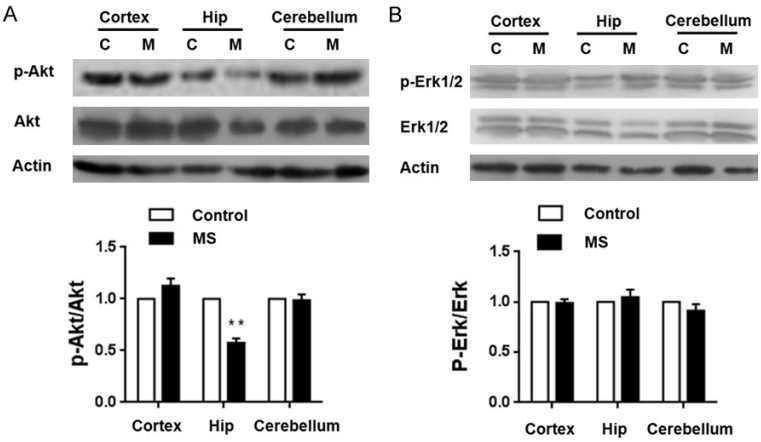

Figure 6. MS decreased p-Akt signaling pathway. A. The upper are representative blots of p-Akt, Akt and Actin; The down is the quantification data of p-Akt/Akt; B. The upper are representative blots of p-Erk1/2, Erk1/2 and Actin; The down is the quantification data of p-Erk1/2/Erk1/2. *P<0.05 compared with control (Unpaired t test). N=5 in each group.

the apoptosis of pyramidal neurons was likely responsible for the cell loss of pyramidal neurons.

Early MS decreases the level of p-AKT in the DG region

We also determined the mechanisms involved in the cell loss caused by MS. The kinase activities of AKT and ERK were tested. As shown in **Figure 6**, total AKT level was not affected in cerebral cortex, hippocampus and cerebellum. By contrast, p-AKT level in hippocampus was significantly reduced by MS (vs. control $P<0.05$). By contrast, p-AKT levels in cerebellum and cortex were not affected. Both of total ERK and p-ERK levels were not affected by MS in cerebellum, cortex and hippocampus.

Discussion

Early MS is one type of early life stress. Based upon the conditions of separation, MS could function positively, as well as negatively [16]. Short-term MS would promote the neurodevelopment and have positive effects on adult life. On the contrary, chronic separation will be detrimental and can cause neurological disorders. In this study, we stressed the rats by delivering three-week separation from the maternal line and disclosed the potential mechanisms. Our data demonstrated that early MS led to decrease of learning ability and anxiety-like activity, and promoted depression, while did not affect cerebellar function. The inactivation of AKT signaling pathway and neuronal apoptosis in the DG region might explain the behavioral changes.

As demonstrated, ten-day separation (P1-P10, 3 h each day) could obviously impair hippocampal synaptic activity and memory [17]. However, opposite results were also reported. For example, short-term separation (P5-P7, 6 h each day) increased the fear memory [18]. In addition, early MS is likely to promote aging and impair the memory [19]. A general acknowledge about postnatal development is the second week after birth. In that period, the neurons are sensitive to environmental factors or endogenous cytokines [20, 21]. Hoffman verified that the

exact period is critical for growth of Purkinje cells and persistent inflammation in that period will cause abnormality of cerebellum development [22]. Morris water maze is widely applied to measure the spatial memory. As reported, MS (P1-P20, 4 h each day) increased the swimming distance and latency [23]. However, Sun *et al* reported that MS (P1-P21, 6 h each day) did not affect the learning ability and memory [24]. We applied a 3-week chronic separation and found that learning ability, as well as memory retrieval was impaired in the adult animals. These inconsistences might be caused by the separation condition, as well as animal strains in different laboratories.

In our study, we also applied open field test to detect mood-related function. Our results revealed that MS did not affect the total distance in the field, but significantly decreased the border/center time ratio. These results implicated that our MS protocol had anti-anxiety activity. These results seem to contradict to previous publications which showed that MS caused anxiety-like activity. For example, Shin et al found that MS mice (P2-P20, 4 h each day) displayed anxiety-like behavior in elevated plus maze, which was further confirmed by three-day open-field test (P1-P14, 3 h each day) [25]. However, there were also reports showing that MS (P1-P21, 3 h each day) can reduce anxiety, which is consistent with the results from our study [26]. The reasons for these discrepancies require further clarification, although animal strains, MS protocol and experimental environment might explain.

Previous studies revealed that MS elicited depression-like activity [8, 27, 28]. MS (P2-P14, 3 h each day) can increase the immobility time in the swimming test [29]. Lee et al found that immobility duration of MS rats during the first swim test was markedly increased, showing depression-like behavior [25]. In our study, the immobility time of MS rats were significantly increased in forced swimming test. The results showed that MS caused depression-like behavior in adult rats, which were consistent with previous publication [25]. In our study, we also detected the gait character, which is supposed to define cerebellar function [30]. Our study firstly reported that MS did not affect cerebellum-related behaviors.

Hippocampus is a region associated with several brain functions. The abnormal function of hippocampus affects memory formation and consolidation [31]. In addition, hippocampus was also involved in hippocampus-amygdala circuit to mediate anxiety and depression [12, 32]. In our study, we detected the cell numbers in hippocampus after MS. Our data showed that the cell numbers in DG, but not in CA1 (data not shown) were decreased in MS animals, which was consistent with previous publication [33, 34]. DG was important for memory formation and was also the important region taking part in the hippocampus-amygdala circuit involved in anxiety activity. We detected GluR1 expression by immunofluorescence and found that GluR1 was predominantly expressed in CA1 and DG regions. Interestingly, the fluorescence intensity in DG region was remarkably decreased after MS. These data were consistent with the changes of cell numbers.

To identify the underlying mechanisms of MS-induced neuronal loss, we tested programmed cell death. As indicated by previous publication, apoptosis happened in the early phase of development could lead to functional impairments [30]. In our study, apoptosis was obviously observed in the DG region of MS rats, while rarely found in control rats. Therefore, apoptosis might be involved in the MS-caused behavioral changes. ERK1/2 and AKT are two important kinases associated with cell survival, proliferation and plasticity [35, 36]. There were evidences suggesting that ERK activation in amygdala affected the synaptic plasticity [37, 38]. In addition, neonatal MS was also reported to up-regulate ERK phosphorylation to promote cell survival in rat hypothalamus [39]. In our study, we detected the ERK phosphorylation in cerebellum, cortex and hippocampus. However, p-ERK level in those three different regions was not affected after MS. p-AKT is also important for cell survival and synaptic plasticity [35, 36]. In our study, we only found that p-AKT in hippocampus was down-regulated in MS animals. In our previous research, we have confirmed a connection between p-AKT pathway and neuronal apoptosis [36]. Although we did not present direct evidence showing that the depression of AKT pathway was responsible for the apoptosis and behavioral changes, the accumulating evidences would support that the suppression of

p-AKT induced by MS contributes to the apoptosis and behavioral changes [30, 36, 40].

Conclusion

We concluded that early MS could affect neurobehavioral activities, including decreasing learning ability and memory, causing depression-like activity and alleviating anxiety-like activity. The potential mechanism might be related to the down-regulation of AKT signaling pathway and apoptosis of pyramidal neurons in the DG region. Although our data show that MS animals have less anxiety-like activity, whether those animals are more immune to environmental factors-caused anxiety still requires future clarification.

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Disclosure of conflict of interest

None.

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