The Profile of MMP-9, MMP-9 mRNA Expression, -1562 C/T Polymorphism and Outcome in High-risk Traumatic Brain Injury: The Effect of Therapeutic Mild Hypothermia

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Abstract

The aim of this study was to investigate the effect of mild hypothermia therapy (34–36°C) and the alterations of matrix metalloproteinase-9 (MMP-9) in 20 patients with high-risk traumatic brain injury (TBI). The neurologic status and outcome were assessed using Full Outline of UnResponsiveness (FOUR) score and Glasgow Coma Scale (GCS). A prospective randomized control study involved patients with high-risk TBI (FOUR score ≤ 7). Patients were randomized into two groups, with and without mild hypothermia therapy which were investigated within 24 and 72 h. The MMP-9 level, MMP-9 mRNA expression and -1562 C/T polymorphism were estimated using enzyme-linked immune sorbent assay (ELISA), reversing transcription polymerase chain reaction (RT-PCR) and PCR-restriction fragment length polymorphism (PCR-RFLP). Different levels of these variables were compared in the two groups. In the hypothermia group, the expression of MMP-9 mRNA and the level of serum MMP-9 were significantly decreased ($P < 0.05$) within 72 h. There was a highly significant correlation between the expression of MMP-9 mRNA and the level of MMP-9 protein ($R^2 = 0.741$, $r = 0.861$, $P < 0.05$). The study did not find in -1562 C/T polymorphism. The patients' outcome was improved significantly after mild hypothermia therapy ($P < 0.05$). The data obtained from this study show that mild hypothermia therapy down regulated the expression of MMP-9 mRNA, the MMP-9 protein level and increased the FOUR score and GCS in high-risk TBI patients within 72 h.

Key words: mild hypothermia therapy, MMP-9, TBI

Introduction

Traumatic brain injury (TBI) is a critical public health and socio-economic problem throughout the world.[1] Following primary insult, severe or high-risk TBI progresses to a secondary brain injury phase associated with biochemical, cellular and molecular changes.

The secondary injury is thought to be responsible for the development of many neurological deficits.[2] The secondary injury involves a complex cascade and biochemical events that contribute to delayed tissue damage and cell death.[3] The time between the two phases of injuries provide a window of opportunity for therapeutic intervention to prevent further damage and improve prognosis.[4,5] Matrix metalloproteinase-9 (MMP-9), a family of extracellular zinc and calcium endopeptidase, is a potential marker as well as an effector of secondary brain injury.[6] Recent experimental studies have

suggested the participation of MMP-9 in TBI.[7] The elevation of MMP-9 levels has been detected in the plasma or serum of TBI patients.[8] Up-regulation of MMP-9 that degrade components of blood brain barrier (BBB) and extracellular matrix (ECM) may be an important pathway associated with secondary brain injury after TBI.[6,8] Although the mechanisms underlying BBB disruption are influenced by many factors, numerous studies have focused on the role of MMP-9 as a major contributor to BBB disruption.[9] MMP-9 can degrade crucial components of cerebrovascular matrix including collagen, laminin and tight junction proteins such as zonula occludens-1(ZO-1), leading to disruption of BBB and exacerbation of edema in acute brain injury.[8,10,11]

In normal physiology conditions, MMP-9 enzyme activities are strictly controlled via gene transcription, pro-enzyme activation and dynamic inhibition by tissue inhibition of metalloproteinases (TIMP).[12,13] After TBI, MMP-9 becomes dis-regulated which causes up-regulation activities of MMP-9 due to the activity of transcription factor, activator protein-1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). Those regulators will get affected in the signal pathway of mitogen activated protein kinase (MAPK) foremost regulation by extracellular signal-regulated kinase (ERK1/2) MAPK in MMP-9 transcriptional level.[14–18]

The MMP-9 was highly expressed at the original sites of human focal ischemic brain tissue[19] and in the patients with cerebral hemorrhage secondary to cerebral infarction.[20] Previous studies have demonstrated that MMP-9 activity is manipulated by several polymorphisms in the promoter, coding, and un-translated regions (UTR),[21–23] with the -1562 C/T polymorphism as the most extensively studied.[24]

An effort was proven to suppress the expression of MMP-9 by choosing mild hypothermia treatment as a potent neuroprotector.[8,17,18] A study of Suehiro et al. (2004) reported that induced hypothermia in severe TBI patients reduced the level of MMP-9 as measured in arterial and jugular venous blood samples.

The FOUR score was used to assess the patients' outcome. The FOUR score provides greater neurological detail than the Glasgow Coma Scale (GCS), recognizes a locked-in syndrome, and is superior to the GCS due to the availability of brainstem reflexes, breathing patterns, and the ability to recognize the different stages of herniation.[25–27]

We investigated the effect of mild hypothermia therapy toward levels of MMP-9 protein, expression of MMP-9 mRNA, -1562 C/T polymorphism and outcome in high-risk TBI patients.

Materials and Methods

The study covered a period of time, that is, from June 2015 to June 2016. It was designed as a randomized study to assess the effect of therapeutic mild hypothermia toward biomarker in high-risk TBI patients. The protocol and consent procedures were approved by the Human Research Review Committee of Kandou General Hospital. Written informed consents were obtained from patients' family members for inclusion in the study. All patients with high-risk isolated closed TBI (FOUR score ≤ 7) and Marshall computed tomography (CT) score (class I-III) admitted within 2 h of trauma were studied. The predetermined entry criteria, in addition to closed TBI, were age 16 to 45 years and no other chronic illness. Patients who had life threatening injuries to other organs or had systolic blood pressure less than 90 mmHg after resuscitation and planned surgical decompression were excluded. Those patients were then randomly put into mild hypothermia therapeutic group ($n = 10$) and control group ($n = 10$). In this study, we prospectively investigated the expression of MMP-9 mRNA, levels of MMP-9 and -1562 C/T polymorphism in the serum from 20 consecutive patients. The blood samples of the two groups were simultaneously obtained within 24 and 72 h during mild hypothermia therapy.

Standard, intensive care management was followed before applying mild hypothermia therapy and was maintained unchanged throughout the study period. For the patients in the hypothermia group, cooling was administered immediately after the randomization. Mild hypothermia therapy was induced by surface cooling, which was accomplished by placing cooling blanket and ice pack was used to reduce the whole body temperature. The core temperature monitored by a rectal thermometer probe was set at 34–36°C which was achieved within 2 h and the target temperature was maintained for 72 h. After mild hypothermia therapy, the patients were gradually rewarmed at a rate of 1°C every 6 h.

Peripheral blood samples for MMP-9 were drawn at 24 and 72 h from each group. Serum samples were collected under sterile conditions. Serum was immediately separated by centrifugation at 3000 rpm for 10 minutes and stored at −80°C until the analysis was completed. The level of serum MMP-9 protein was measured using a calibrated instrument, Human MMP-9 Quantikine ELISA kit (catalog no. DMP 900), R & D Systems Inc., MN, USA. The inspection procedures followed the procedures in accordance with manufacturer's instructions read using ELISA Reader 270 (Biomerieux, France).

The time course of mRNA for MMP-9 was measured by the real-time quantitative reverse transcription-

polymerase chain reaction (RT-qPCR) at 24 and 72 h from each group. The mRNA expression of MMP-9 gene used Realtime PCR machine (CFX Connect system, Biorad Laboratories, Real Time PCR 96 wells, 0.1 mL, USA) and used the DNA dye SYBR Green (Takara, Shiga, Japan). The results of the test expressed in mRNA levels.

MMP-9 polymorphisms were investigated by PCR-RFLP. Amplicon of locus gene target was digested with restriction enzyme SphI for polymorphisms under conditions recommended by the manufacturer (New England BioLabs, Tokyo, Japan). Restriction-enzyme digestion 435-bp was visualized by electrophoresis in 1.8% agarose gel stained with ethidium bromide.

Data entry and analysis were done using SPSS software V.20.0 (SPSS Inc., Chicago, IL., USA). Data are presented as mean ± SEM. Student's *t*-test for unpaired results. Categorical data were analyzed by Fisher's exact test. Time course differences in the parametric were compared by a nonparametric Wilcoxon rank-sum test. The correlation expression of MMP-9 mRNA and the level of MMP-9 were studied with the Pearson's linear regression method. The Mann-Whitney U one-way analysis of variance by ranks was used to determine whether significance was present. P-values were considered significant when $P < 0.05$.

Results

Subject characteristics

Characteristics of patients and the homogeneity of variables of the two groups can be seen from the summary of the analysis in Table 1.

Table 1 shows that male patients were 60–70%, ranging in age between 20 and 44 years old, the onset time of hospitalization from 45 to 120 minutes after the incident GCS and Marshall CT score. There was no significant difference in the characteristics ($P > 0.05$) between the two groups. Both can be considered as homogeneous groups based on the characteristics of sex, age, vitalsigns, onset time of hospitalization, GCS and Marshall CT score.

The effect of mild hypothermia therapy in the expression of MMP-9 mRNA and protein levels of MMP-9 in high-risk TBI patients

The effect of mild hypothermia therapy in the expression of MMP-9 mRNA can be seen in the summary of the analysis of changes in expression of MMP-9 mRNA in Table 2. The differences of the expression of MMP-9 mRNA between two groups at time period 24 and 72 h of mild hypothermia therapy, as well as changes in expression of MMP-9 mRNA within 24–72 h. Similarly, the effects of mild hypothermia therapy in MMP-9 protein level can be seen in Table 3. Linear relationship and correlation of expression of MMP-9 mRNA versus level of MMP-9 protein show in Fig. 1.

As shown in Table 2, that 72 h during mild hypothermia therapy period has proven a significant difference in expression of MMP-9 mRNA ($P < 005$). In the time period of 24–72 h of mild hypothermia therapy, there was significantly suppressed MMP-9 mRNA expression ($P < 0.05$) with mean of 2.63 ng/µL, whereas in the control group in the same time range observation, it was increased significantly with the mean of 0.30 ng/µL ($P < 0.05$). The expression of MMP-9 mRNA tended to decrease in the mild

Table 1 Characteristics of patient subgroups

Characteristics	Group		P
	Controls ($n = 10$)	Hypothermia ($n = 10$)	
Gender: (M/F)	6/4	7/3	0.500*
Age (years) Mean (SD)/Min–Max	29.1 (8.5)/20–44	29.3 (8.4)/20–43	0.958**
Vital signs:			
• Systolic: Mean (SD)/Min–Max	122 (9.19)/110–140	121 (7.38)/110–130	0.511**
• Pulse rate: Mean (SD)/Min–Max	82.6 (4.72)/76–92	82.4 (4.30)/76–90	0.520**
• Respiration rate: Mean (SD)/Min–Max	20 (2.98)/16–24	20 (2.98)/16–24	0.500**
• Body temperature (℃): Mean(SD)/Min–Max	36.9(0.27)/36.6–37.4	36.9(0.29)/36.5–37.4	0.630**
Onset (min): Mean (SD)/Min–Max	75.0 (21.2)/45–120	79.0 (22.8)/45–120	0.690**
GCS: Mean (SD)/Min–Max	5.7 (0.48)/5–6	5.7 (0.48)/5–6	0.566**
CT Marshall: Mean (SD)/Min–Max	2.7 (0.5)/2–3	2.7 (0.5)/2–3	0.680**

*Fisher's exact, **Independent *t* test.

Table 2   The changes expression of MMP-9 mRNA in subgroups

Group	(Mean ± SD) mRNA MMP-9 (ng/µL)			P
	24 h	72 h	Δ	
Control	12.76 ± 0.53	13.06 ± 0.89	(0.30 ± 0.95)	0.074**
Hypothermia	13.24 ± 0.17	10.60 ± 2.06	(2.63 ± 2.09)	0.003*

**Paired *t* test, *Wilcoxon test.
Table 2 shows that at 24 h mild hypothermia therapy, the expression of MMP-9 mRNA mean was slightly elevated in the mild hypothermia group compared to the control. The MMP-9 mRNA expression decreased significantly due to mild hypothermia therapy within 24–72 h. However, in the control group, the expression of MMP-9 mRNA tended to increase.

Table 3   The changes level of MMP-9 protein in subgroups

Group	(Mean ± SD) MMP-9 (pg/mL)			P
	24 h	72 h	Δ	
Control	455.27 ± 74.76	553.37 ± 198.87	98.10	0.037**
Hypothermia	460.57 ± 62.00	309.98 ± 226.84	−150.59	0.203**

**Wilcoxon test.
Table 3 shows that in the time period of 24 h of mild hypothermia therapy, the mean level of MMP-9 protein was slightly higher in the hypothermia group compared to the control. In the time period of 24–72 h of mild hypothermia therapy, the mean levels of MMP-9 protein were decreased, whereas that of the control group was actually increased.
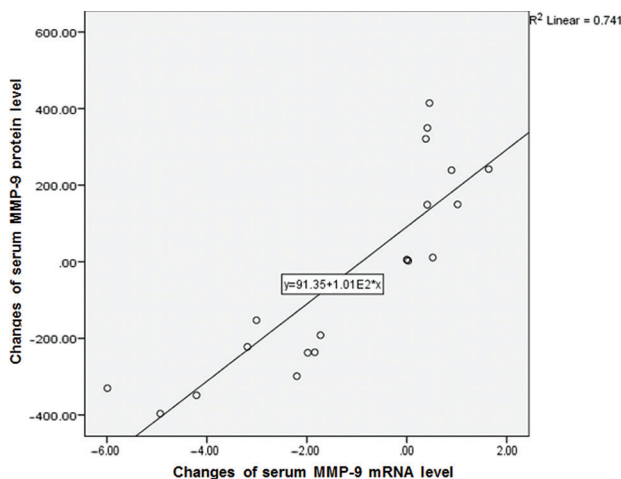

Fig. 1   A linear regression curve of changes of MMP-9 mRNA expression versus changes of MMP-9 protein levels during the investigation period.

hypothermia group, on the contrary, the expression of MMP-9 mRNA were elevated significantly in the control group. Mann-Whitney test results showed significant changes in expression of MMP-9 mRNA ($P < 0.05$) between the two groups. In this study, the mild hypothermia therapy significantly reduced MMP-9 mRNA expression within 72 h.

Table 3 shows that the results of mild hypothermia therapy within 24 h have not shown the effects of the changes in the level of MMP-9 protein; it was not significantly different in the level of MMP-9 protein ($P > 0.05$) between the mild hypothermia and control group. The effect is noticeable after the mild hypothermia therapy takes time at 72 h; there was a significant difference in the level of MMP-9 ($P < 0.05$) between the two groups and the level of MMP-9 protein in the hypothermia group (309.98 ± 226.84) pg/mL was lower than in the control group (553.37 ± 198.87) pg/mL. In the time period of 24–72 h, the level of MMP-9 protein was not significantly decreased ($P > 0.05$) with the mean level of −150.59 pg/mL whereas in the control group in the same time range observation, MMP-9 protein levels were up-regulated significantly ($P < 0.05$) with the mean level of 98.10 pg/mL. Mann-Whitney test results show that changes in the level of MMP-9 protein are significantly different ($P < 0.05$) between the two groups. In this study, the mild hypothermia therapy decreased significantly in MMP-9 protein level within 72 h.

As shown in Fig. 1, the high changes of the level of MMP-9 protein are due to the high changes of MMP-9 mRNA expression, with determinant coefficient (R^2) of 0.741. It means that 74.1% change in the levels of MMP-9 protein is clearly determined by the changes in MMP-9 mRNA expression. To determine if increased expression of MMP-9 mRNA and higher level of MMP-9 protein were related, we performed correlation analysis. The correlation coefficient is 0.861 (0.800 to 1.000), which means that changes in the expression of MMP-9 mRNA

and the level of MMP-9 protein have a very strong linear relationship.

Mild hypothermia therapy influence on genotype polymorphism -1562 C/T gene MMP-9 in high-risk TBI patients

Figure 2 shows that the results of PCR-RFLP (restriction fragment length polymorphisms) were not found in polymorphism -1562 C/T gene MMP-9 on the mild hypothermia in the high-risk TBI patients.

The effect of mild hypothermia therapy on the FOUR score and GCS in high-risk TBI patients

Table 4 confirms that there were no significant effects of the change in the FOUR score ($P > 0.05$) within 24 h in the mild hypothermia therapy and control groups. But, the different effect of the mild hypothermia therapy and control groups to the FOUR score was significantly increased within 72 h.

Figure 3 presents of the FOUR score and GCS between the mild hypothermia therapy and the control group within 24–72 h. In period 72 h, FOUR score and GCS significantly improve.

Discussion

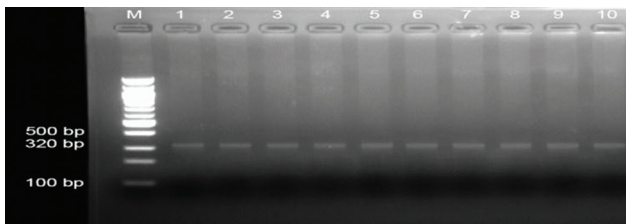MMPs are a family of extracellular zinc and calcium-dependent proteases that degrade ECM and other

Fig. 2   RFLP-PCR test results.

Table 4 The comparison of the FOUR score changes in subgroups

Group	(Mean ± SD) FOUR score			P
	24 h	72 h	Δ	
Control	6.7 ± 0.5	9.1 ± 2.0	2.4	0.005**
Hypothermia	6.7 ± 0.5	12.7 ± 23.1	6.0	0.005**

**Wilcoxon test.
Table 4 presents that the FOUR score was starting to coincide during the period of 24 h in the mild hypothermia therapy and control groups. The mild hypothermia therapy and control groups indicated that the FOUR score tended to increase concurrently throughout 24–72 h. There was a conspicuous difference, the FOUR score in the mild hypothermia therapy group was greater increased than the control group within 72 h.
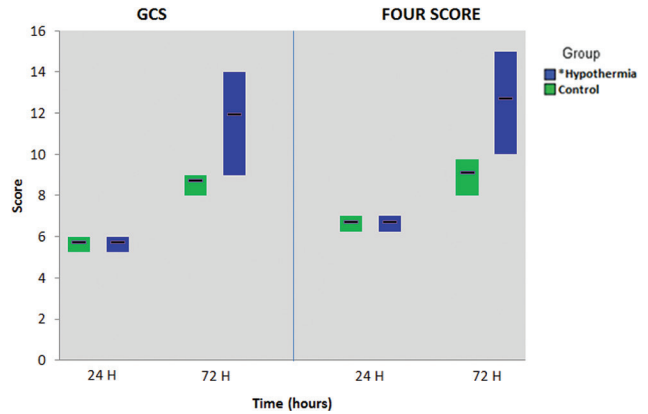
Fig. 3   Comparison of the FOUR score and GCS within 24–72 h.

extracellular proteins.[8,9,28] Under a normal physiologic condition, MMP-9 enzyme are activities that are strictly controlled via gene transcription, pro-enzyme activation and dynamic inhibition TIMP.[11] After TBI, MMP-9 become dis-regulated and elevated. The elevation of MMP-9 can cause the increase in capillary permeability, breakdown of BBB and will also lead to brain edema, a typical symptom of secondary injury after TBI.[8,10,11,29]

Mild hypothermia therapy is a new developed way for treating TBI in a major area of research during the last decade.[8,30–32] Beneficial effects of mild hypothermia therapy in experimental models of TBI have been shown in a large number of laboratories.[33–36] Mild hypothermia therapy has recently reduced MMP-9 after experimental focal ischemia and severe TBI.[8,17,18]

It was shown in our study that mild hypothermia therapy significantly decreased the MMP-9 mRNA expression within 72 h ($P < 0.05$). The effect of the mild hypothermia at 72 h was significantly different in MMP-9 protein levels ($P < 0.05$) between two groups. At a time period of 24–72 h, MMP-9 protein levels in mild hypothermia group were decreased but not significantly ($P > 0.05$). In an animal study, Jia et al. (2010) reported that TBI induced significant increases in MMP-9 mRNA and protein levels in brain tissue with 24–72 h due to secondary damage of TBI. It is known that levels of MMP-9 in the brain begin to increase at 3 h after a traumatic brain insult.[37] Mild hypothermia significantly decreased the microglial response 72 h after ischemia.[38] MMP-9 can also be produced predominantly by microglial cells as well as by infiltrating leucocytes.[18,39,40] Vitro studies have showed that MMP-9 can be produced by cultured microglia upon stimulation with such inflammatory components.[37]

Evolution of secondary damage mechanism has been studied in the experimental setting and can be

divided into three distinct phases: acute, sub-acute and chronic. The evolution supports that an important point of neuroprotective treatments for TBI should be extended up to 72 h.[41] In theory, protective effects could be achieved for a period up to 48–72 h as this is the period of time during which secondary brain injury develops.[42] It was the basic concept, most studies used cooling period in the 24–48-h range, while some studies have used a cooling period longer than 48 h. One reason for using more long-term cooling is that cerebral swelling and edema often are the greatest in 3–5 days after injury.[43] Peterson et al. (2008) reported that reduction in risk mortality was the greatest and most favorable neurologic outcomes, much more common when hypothermia was maintained for more than 48 h.

More importantly, in our study, there was a significant correlation and a very strong linear relationship between the MMP-9 mRNA expression and the level of MMP-9 protein in the high-risk TBI patients. This supports our current study that mild hypothermia appeared to regulate the MMP-9 expression particularly at the transcriptional level and suppressed its protein activity.

Mori et al. (2002) reported that the ERK and MAPK signaling pathway was rapidly up-regulated after TBI. Schmitt et al. (2007) investigated that ERK-MAPK signaling pathway was significantly influenced by hypothermia and rewarming. Furthermore, inhibition of ERK-MAPK signaling pathway reduced trauma-induced levels of MMP, suggesting that pathway triggered the up-regulation of MMP-9 after TBI. The ERK1/2 specific phosphatase enzyme and the balance activity of kinase to phosphatase of ERK-MAPK signaling pathway during hypothermia therapy were temperature sensitive.[44] Hypothermia and rewarming boost this induction of inflammatory enzyme activation. The inhibition and suppression of phosphorylation by inhibiting synapsin I phosphorylation after pre-cooling will cause diminished ERK-MAPK signaling pathway.[18]

The finding indicates that mild hypothermia therapy attenuated the expression of MMP-9 that its inhibit of the inflammatory response by preventing activation of the transcription factor, NFκB. Webster et al. (2009) reported that hypothermia inhibited NFκBs activating kinases complex, inhibitor kinase (Iκ) β and γ. NFκB is known as a regulator of MMP-9 promoter.[12,45] The activation of NFκB and cerebral edema significantly increased after TBI and the inhibition of NFκB could significantly reduce the cerebral edema after TBI. The dynamic interaction between transcription factors and signal transduction pathways are a key role of mild hypothermia therapy.[46,47,48]

No previous studies have investigated the association between MMP-9 gene promoter polymorphism to the responsiveness of high-risk TBI patients toward mild hypothermia therapy. The MMP-9 gene is located on chromosome 20q11.2—q13 and several polymorphisms in the promoter, coding and un-translated region (UTR) have been reported, with the -1562 C/T polymorphism as the most extensively studied. MMP-9 gene polymorphism is characterized by a single nucleotide change from cytosine to thymidine 1562-bp upstream from the start of transcription (C/T). This polymorphism in the promoter region of the MMP-9 gene is probably functional in transcriptional regulation. A recent study has revealed that CC genotype is responsible for lower activity of MMP-9 and genotypes with the T allele (CT, TT) are responsible for high activity.[21–24] The study of MMP-9 single nucleotide polymorphism -1562 C/T is a new point of view in TBI study; however, the result was negative. The result explained that the pattern of DNA sequences was the same. Secondly, it might be argued that our sample size is too small.

Sadaka et al. (2012) presented that the FOUR score has a high degree of internal consistency and is an accurate predictor of mortality and neurologic outcome in TBI patients. The FOUR score provides greater neurological detail than GCS which could assess brainstem reflexes, breathing pattern and herniation.[26,27]

Our research results indicate that the FOUR score is increased significantly due to the mild hypothermia therapy ($P < 0.05$) within 72 h. Consequently, the mild hypothermia therapy is beneficial to improve the outcome of high-risk TBI.

In summary, we demonstrated that mild hypothermia therapy attenuated the MMP-9 mRNA expression and the MMP-9 protein level, and improved the outcome FOUR score and GCS in high-risk TBI patients within 72 h. Our data support that mild hypothermia therapy down-regulated significantly the expression of MMP-9 mRNA and protein levels. It is a possibility that the enzyme could be used as an appropriate predictive marker of the benefit of mild hypothermia therapy in high-risk TBI patients.

Conflicts of Interest Disclosure

The authors report no conflicts interest. The authors have no personal, financial, or institutional interest in any of the drugs, materials or devices used in the article.

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