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ПРОТЕКТИВНЫЕ ЭФФЕКТЫ ПРЕПАРАТА НУКЛЕОТИДНОЙ ПРИРОДЫ «ДЕРИНАТ» НА ТЕЧЕНИЕ И КЛЕТОЧНЫЕ МЕХАНИЗМЫ ЧЕРЕПНО-МОЗГОВОЙ ТРАВМЫ В ЭКСПЕРИМЕНТЕ

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Резюме. Черепно-мозговая травма является наиболее частой причиной смерти и инвалидности среди молодых людей, включая спортсменов и солдат, людей в возрасте до 45 лет в промышленно развитых странах, и представляет растущую проблему со здоровьем как в развивающихся странах, так и среди стареющих людей, лечение которых является серьезной проблемой современной медицины. Этот вид травм приводит ко многим видам расстройств и очень часто к инвалидности, что обуславливает необходимость разработки новых методов лечения травм головного мозга. В экспериментах на мышах изучали новый метод лечения травм головного мозга, в частности использовали натриевую соль дезоксирибонуклеиновой кислоты. Этот препарат известен как смесь пептидов с иммуномодулирующим действием, который широко используется для лечения воспалительных, аллергических и аутоаллергических процессов. Натриевая соль дезоксирибонуклеиновой кислоты (DNA) (Деринат), выделенная из икры русского осетра, является препаратом, эффективность применения которого показана при лечении различных заболеваний. В настоящей работе показаны нейропротекторные, антиоксидантные и противовоспалительные эффекты «Дерината» на модели черепно-мозговой травмы (ЧМТ) у крыс. Внутрибрюшинная инъекция «Дерината» в течение 3 дней после ЧМТ снижает объем повреждения ткани мозга. Иммуногистохимический анализ позволил констатировать морфологические изменения клеток микроглии в коре головного мозга и гиппокампе через 7 дней после ЧМТ, которые значительно снижались при введении препарата, как и индуцированное ЧМТ накопление 8-оксогуанина (8-oxoG) – маркера окислительного повреждения. Для изучения клеточного механизма противовоспалительных эффектов использовали первичную культивированную мышиную микроглию с АТФ (50 мкм и 1 мм) в качестве вещества, высвобождающегося в месте повреждения, для имитации воспалительной реакции *in vitro*. Введение «Дерината» обуславливало повышение количества мРНК нейротрофического фактора глиальных клеток (GDNF) и фактора роста нервов (NGF) в присутствии АТФ, а уровень мРНК активатора тканевого плазминогена (tPA) снижался при

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действии АТФ в сочетании с «Деринатом» или без него. Хотя экспрессия мРНК интерлейкина-6 (IL-6) не изменялась при действии АТФ, она возрастала при аппликации «Дерината». Те же показатели фактора-α некроза опухоли (TNFα) были значительно ингибированы. Комплекс полученных данных раскрывает механизмы иммуномодулирующего действия дезоксирибонуклеиновых кислот при ЧМТ.

Ключевые слова: ЧМТ, микроглия, 8-oxoG, ATP, GDNF, NGF, TNFα

PROTECTIVE EFFECTS OF DERINAT, A NUCLEOTIDE-BASED DRUG, ON EXPERIMENTAL TRAUMATIC BRAIN INJURY, AND ITS CELLULAR MECHANISMS

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Abstract. Traumatic brain injury is the most common cause of death and disability in young people including sport athletes and soldiers, people under 45 years of age in the industrialized countries, representing a growing health problem in developing countries, as well as in aging communities. Treatment of the latter is a serious challenge for modern medicine. This type of injury leads to many kinds of disorders and, quite often, to disability. These issue require development of new methods for brain trauma treatment. The new approach to brain trauma treatment was studied in murine experiments. In particular, sodium salt of deoxyribonucleic acid (DNA) was used. This preparation is a drug known as a mixture of peptides with immunomodulatory effect which is widely used for different kinds of therapy. Derinat, a sodium salt of DNA, isolated from the caviar of Russian sturgeon, is a proven immunomodulator for treatment of diseases associatd with reactive oxygen species (ROS), including brain ischemia-reperfusion (IR) injury. Here we show that treatment with Derinat exert neuroprotective, anti-oxidative, and anti-inflammatory effects in experimental model of traumatic brain injury (TBI) in rats. Intraperitoneal injection of Derinat several times over 3 days after TBI showed less pronounced damage of the injured brain area. Immunohistochemical study showed that the Derinat-induced morphological changes of microglia in cerebral cortex and hippocampus 7 days after TBI. TBI-induced accumulation of 8-oxoguanine (8-oxoG), the marker of oxidative damage, was significantly attenuated by Derinat administration, both on 7th and 14th day after TBI. To investigate cellular mechanism of anti-inflammatory effects, the primary cultures of murine microglia supplied with ATP (50 M and 1 mM), as a substance released at injured site, were used to mimic the *in vitro* inflammatory response. Derinate treatment caused an increase of glial levels of mRNAs encoding neurotrophic factor (GDNF) and nerve growth factor (NGF) in the presence of ATP, whereas tissue plasminogen activator (tPA) mRNA was inhibited by ATP with or without Derinat. Interleukin-6 (IL-6) mRNA expression was not affected by ATP but was increased by Derinat. Both mRNA and protein levels of ATP-induced TNFα production were significantly inhibited by Derinat. These results partially contribute to understanding mechanisms of immunomodulatory effects of DNA preparations in traumatic brain injury.

Keywords: traumatic brain injury, microglia, 8-oxoG, ATP, GDNF, NGF, TNFα

Introduction

Traumatic brain injury (TBI) is a result of outside force causing mechanical disruption of brain tissue and delayed pathogenic events that collectively exacerbate the injury. Since TBI is the most common cause of death and disability in young people including sports athletes and soldiers [8, 26], people under 45 years of age in the industrialized countries and represents a growing health problem also in the developing countries [77], or in the aging community [43], it is important to improve early care and functional outcome by use of effective drugs and treatment. Mechanical forces of TBI cause rapid tissue deformation, resulting in primary physical damage [61]. These deformations may directly affect blood vessels, axons, neurons and glial cells in focal, multifocal or diffuse pattern. These

changes initiate dynamic and evolving processes that result in inflammatory, neurochemical and metabolic alterations [9, 63, 84].

The use of animal models is essential for better understanding of the secondary injury processes and for the development of novel therapies. Rat models are the most commonly used pre-clinical models of traumatic brain injury [5, 22, 50, 74, 85]. Weight-drop model uses the gravitational forces of a free-falling weight to produce a largely focal or diffuse brain injury and mimics the real-life conditions of brain injury [61]. In early stage of TBI, for example 1 day after TBI, a global glial reaction has been reported [93]. Among an in interplay between the innate immune system, danger-associated molecular patterns and loss of self-tolerance leading to adaptive autoimmunity [68], astrocytes and microglia, are considered as key players in initiating an inflammatory response after injury, because these cells are capable of secreting various cytokines, chemokines and growth factors, and following injury to the central nervous system (CNS) [40]. In adult rat brains after TBI, astrocytes positive for glial fibrillary acidic protein (GFAP) and complement C3 (marker of bad/disruptive astrocytic A1 phenotype) were significantly increased and microglial phenotype was changed from a ramified appearance with long, thin, highly branched processes to a swollen amoeboid shape [14] or rod-shape [87]. Therefore, detailed analyses of therapeutic drugs on glial cells are needed.

Derinat is isolated from the soft roes of Acipenser gueldenstaedtii (Russian sturgeon), the sodium salt of highly purified native double-stranded DNA (molecular weight; 270-500 kDa), being used in Russia and several other countries in clinic for different pathological conditions [23, 34, 51]. It has been shown that this drug after applying weight-drop model of rat's TBI can normalize splenocyte cytotoxicity and proliferative activity on the 14th day after TBI. Also Derinat prevents development of spatial memory breakdown on 7th day after TBI, normalizes reduced research activity and increased level of anxiety in rats on 7th and 14th days after TBI [20].

In the present study, to investigate on cellular level, we performed immunostaining of the brain and tried to analyze functional changes of microglia, an immune cell population of the central nervous system (CNS) to understand the cellular mechanism of neuroprotection induced by Derinat against TBI.

Materials and methods

The study was approved by the Animal Research Committee of Kyushu University and the Institute of Experimental Medicine, and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animal model of traumatic brain injury and drug treatment

The experiments were conducted using male 12-16-week-old Wisrat rats (RC "Kurchatov Institute" NLA"Rappolovo", St. Petersburg, Russia), kept in a conventional environment. Animals were fed standard diet (GOST R 50258-92, Russia), normal water ad libitum, and were housed in plastic cages. Traumatic brain injury (TBI) (weight-drop/contusion model) was applied according to previously used method [61]: Drop a weight (90-120 g) to the vertex of the head from 120 cm. After trauma, intraperitoneal injection of either saline (control) or natural nucleotide drug, Derinat, 10 mg/kg at 2, 26, 50, or 74 h.

2, 3, 5-triphenyltetrazoliume chloride (TTC) staining to define damaged brain tissue

After 1, 7, and 14 days after trauma animals were anesthetized by pentobarbital sodium (50 mg/kg, i.p.) and perfused transcardially with saline. Fresh brain tissues with 1 mm were stained with TTC to visualize. The damaged areas were quantitated by an image-analysis system (ImageJ, Wayne Rasband, NIH) and calculated as percentage of the whole brain area.

Immunohistochemistry for neuroglia

On 7 and 14 days after trauma animals were anesthetized by pentobarbital sodium (50 mg/kg, i.p.) and perfused transcardially with saline followed by 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS; 80 mM Na_2HPO_4, 20 mM KH_2PO_4, 150 mM NaCl, pH 7.4). Brains were extracted, postfixed in the same fixative and placed in 20% sucrose solution for 24 h at 4 °C. Frozen sections of cortex with 30 μm were made and frozen until use. Frozen sections of 30 μm were sliced by a HM 550 cryostat (Micro-edge Instruments Co., Tokyo, Japan) and incubated for 1 h at room temperature in 5% donkey serum (Jackson Immuno Research, West Grove, PA, USA). Then, the sections were incubated with the following antibodies against: Iba1 (ionized calcium-binding adapter molecule-1, 1:2000, Wako, Osaka, Japan), GFAP (Glial fibrillary acidic protein, 1:800, Millipore), or MAP-2 (1:1000, SIGMA) antibody for 24 h at 4 °C. Subsequently, the cells were rinsed three times for 5 min with PBS and then incubated for 4 h at room temperature in the secondary antibody (IgG-conjugated Alexa Fluor 488 or 594, 1:1000, Molecular Probes, Eugene, OR, USA). To stain the cell nuclei, cells were washed in PBS and treated DAPI for 1 h, then washed with PBS and coverslipped. Digital images were acquired and analyzed using Axioskop2 plus equipped with a CCD camera, AxioCam.

Immunohistochemistry of 8-oxoguanine

In order to analyze the damage of DNA in traumatic brain area, 8-oxoguanine (8-oxoG) was observed immunohistochemically. To detect 8-oxoG in nuclear DNA, section has to be subjected to pre-treatment with 2N HCl for 1 h at room temperature, which allows an efficient denaturation of nuclear chromatin but an extensive degradation of mitochondrial DNA [70]. Free-floating sections pre-treated were incubated in Block Ace, for 30 min at room temperature, and then were incubated with primary antibody (N45.1 mAb 1:100, Japan Institute for the Control of Aging, Japan), which preferentially recognizes 8-oxoG in DNA, in 10% Block Ace, at 4 °C overnight. Alexa Fluor-labeled second antibodies were obtained from Invitrogen (Tokyo, Japan). Digital images were acquired using Axioskop2 plus equipped with a CCD camera, AxioCam. All sections from each experimental animal and group to be compared were processed in parallel.

Microglial cell culture

Mouse microglial cells were isolated from the mixed cultures of cerebrocortical and spinal cord from newborn (1-3 days postnatal) C57BL/6 J mice (8-10 mice, mixture of male and female) (Kyudo, Tosu, Japan), as described previously [67]. In brief, tissue was trypsinized for 3 min and dissociated with a fire-polished pipette. Mixed glial cells were cultured for 9-12 days in Dulbecco's Modified Eagle Medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% Hyclone fetal bovine serum (FBS; Hyclone Labratories, UT, USA), 2 mM L-glutamine, 0.2% Dglucose, 5 mg/ml insulin, 0.37% $NaHCO_3$, 100 U/ml penicillin, 100 mg/ml streptomycin at 37 °C in a 10% CO_2, with medium changes every 3 days. Microglial cells were then separated from the underlying astrocytic layer by gently shaking the flask for 2 h at 37 °C in a shaker-incubator (125 rpm). After unattached cells were removed, microglial cells were isolated as strongly adhering cells. The purity of microglia was > 98%, which was evaluated by staining with Iba1, a marker for microglia/macrophage [36]. Images of Iba1-staining was acquired and analyzed using Axioskop2 plus equipped with a CCD camera, AxioCam.

SYBR green-based real-time quantitative RT-PCR

Cultured microglia cells were plated in 60 mm dishes (10^6 cells/dish) and incubated with adenosine-5'-triphosphate (ATP) to activate microglia and induce inflammatory response. With or without pre-treatment of nucleotide nature drug (1.5, 15, and 150 μg) for 24 h, the cells were subjected to total RNA extraction according to the protocol of the manufacture and purified with QIAamp RNA Blood Mini (Qiagen, Valencia, CA, USA). The amount of total RNA concentration was measured using Smart Spec™ 3000 (Bio Rad, Tokyo, Japan). Total RNA (175 ng) was converted to cDNA by reverse transcription, using random 9 mer (Takara, Otsu, Japan) and RNA PCR kit (Takara). The primers were as follows: GDNF primers (forward: 5'-AAA AAT CGG GGG TGC GTC TTA-3', reverse: 5'-TCAGATACATCCACACCGTTTAG-3'); tissue plasminogen activator (tPA) primers (forward: 5'-GTC AGA TTC CAG TCA GTG TG-3', reverse: 5'- GTT GCT CGT GAT GGT TTT G-3'); NGF primers (forward: 5'-GCA GAC CCG CAA CAT CAC TG-3', reverse: 5'-TCT CCA ACC CAC ACA CTG ACA-3'); IL-6 primers (forward: 5'-CGA GCC CAC CAG GAA CGA AAG TC-3', reverse: 5'-CTG GCT GGA AGT CTC TTG CGG AG-3'); TNFα primers (forward: 5'- TCC CAA CAA GGA GGA GAA GT -3', reverse: 5'- TGG TAT GAA GTG GCA AAT CG -3'); and GAPDH (forward: 5'-TCT ACC CAC GGC AAG TTC AAC-3', reverse: 5'-TCT CGC TCC TGG AAG ATG GT-3'). All primers were purchased from Sigma Aldrich Japan (Tokyo, Japan). PCR amplification was undertaken for Thunderbird Sybr qPCR Mix (Toyobo, Osaka, Japan) in Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems Japan, Tokyo, Japan). Each reaction volume consisted of 12.5 μl Thunderbird Sybr qPCR Mix, 0.05 μl 50 × ROX reference dye, 1 μl mix of forward and reverse primers (0.3 μM each), and 11.45 μl RNAase free water containing cDNA (17.5 ng). PCR was done by 15 sec denaturation at 95 °C, and annealing/extending at 60 °C for 40 cycles. Each mRNA expression level was normalized by GAPDH. The mRNA expression was calculated relative to GAPDH using the $\Delta\Delta C_T$ algorithm.

Assay of TNFα

Cultured microglial cells were seeded in a 96-well plate at a density of 1.5×10^5 cells per well and were cultured for 1 day, according to our previous work [7, 28]. The cells were treated with 50 μM or 1 mM adenosine triphosphate (ATP) with or without Derinat (150 μg, 1.5 μg) for 12 h. After treatment of ATP, the amount of mouse TNFα released into the culture medium was measured by an ELISA kit (Biosource) with a detection limit of 3 pg/mL. The absorbency at 450 nm was performed by a Microplate Reader (model 450; Bio-Rad).

Statistical analysis

All data are presented as mean ± SEM. The statistical analyses of the results were evaluated by using two-tailed Student's unpaired t-test, one-way ANOVA followed by Dunnett's test or two-way ANOVA. $P < 0.05$ was considered statistically significant.

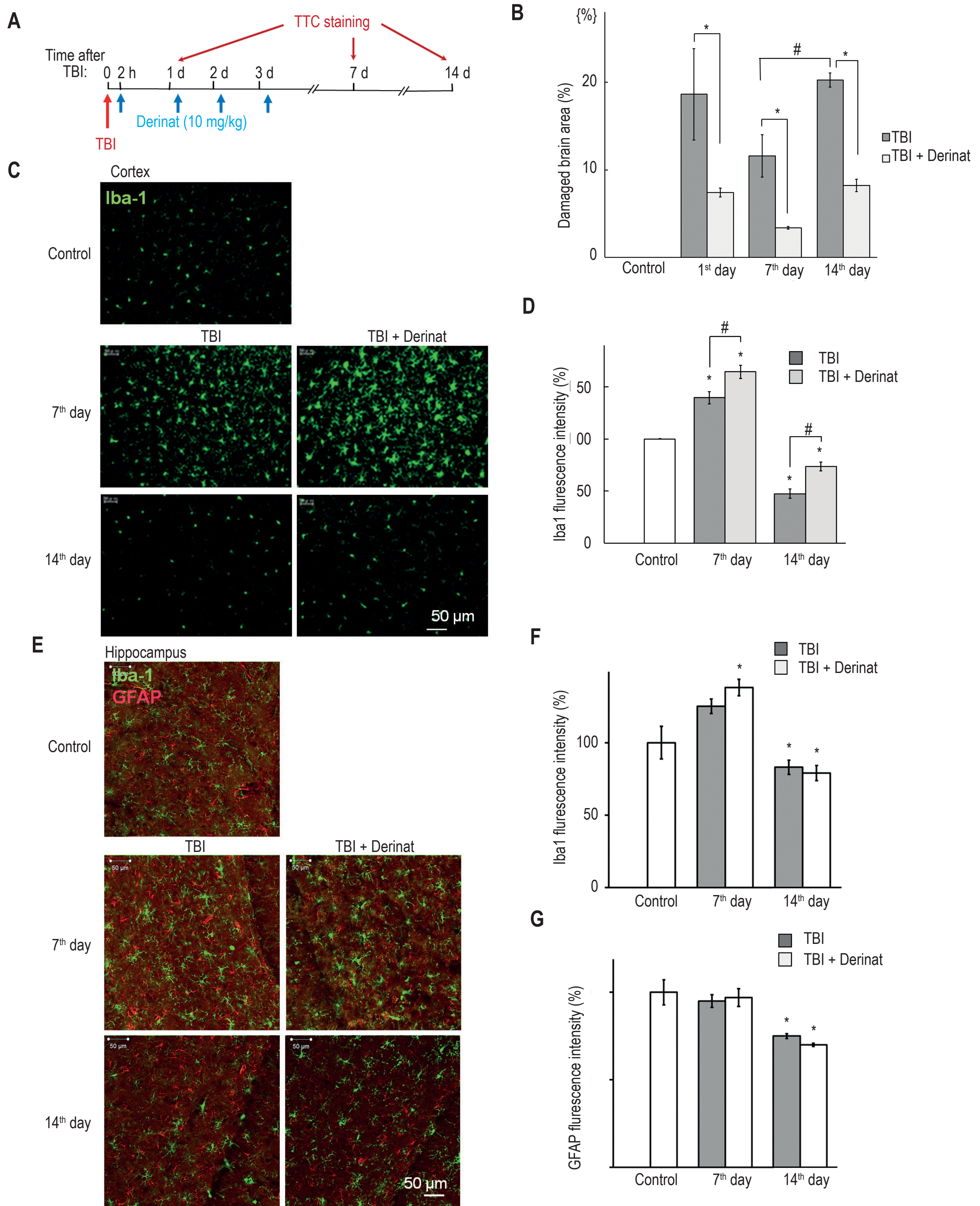

Figure 1. Derinat ameriolates traumatic brain injury (TBI) and activate microglia but not astrocytes

Note. (A) Experimental procedure. (B) Relative volumes of the injured area were calculated in the brain slices at 1st, 7th, and 14th day. (C) Representative images of Iba-1 immunostaining in cortex without TBI (control), 7 days and 14 days after TBI with or without Derinat. (D) Relative fluorescence intensity of Iba1 at day 7th and 14th day after TBI with or without Derinat. *, $p < 0.05$ compared to control. #, $p < 0.05$ compared between with and without Derinat. (E) Representative images of Iba1 (green) and GFAP (red) immunostaining in hippocampus without TBI (control), 7 days and 14 days after TBI with or without Derinat. (F) Fluorescent intensity of Iba1 in hippocampus of rat brain after TBI with and without Derinat. (G) Fluorescent intensity of GFAP in hippocampus of rat brain after TBI with and without Derinat. *$p < 0.05$ compared to control.

Results

Derinat ameliorated the traumatic brain injury (TBI) in rat model

After the TBI, Derinat (10 mg/kg) was intraperitoneal injected at 2, 26, 50, or 74 h. At day 1, 7, and 14, the fresh brains were subjected for TTC staining to measure tissue viability and evaluate infarct size [6, 49] (Figure 1A). Following TBI, TTC staining showed damaged brain area tissue with pale colour. The ratio of damaged brain area at day 1, 7, and 14 after TBI were significantly reduced by Derinat compare to those without Derinat (saline-injected) (Figure 1B). The damaged brain area at day 14 became significantly larger than that at day 7 (Figure 1B).

Derinat induced more activation of microglia but not astrocytes in the TBI area

At the injured brain area, activation of microglia, brain's immune cell population, was expected. Therefore, microglia/invading macrophage were stained by anti-Iba1 antibody followed by green fluorescence-conjugated secondary antibody. After TBI, increased number of Iba1-positive cells with bigger cell bodies was observed (Figure 1C). The fluorescence intensity of Iba1 staining in cortex significantly increased on the day 7, however, on the day 14 it significantly decreased compare to control brains (Figure 1D). With Derinat treatment, the fluorescence intensity of Iba1 even increased, both at day 7 and 14 after TBI, suggesting that Derinat-induced microglial activation contributed to resist TBI.

As for the staining of astrocyte before and after TBI, anti-GFAP staining showed few cells in cortex and did not show much difference with or without Derinat (not shown). On the other hand, in the hippocampus, double immunostaining of Iba1 and GFAP showed substantial number of GFAP-positive cells (Figure 1E). At day 7 after TBI, more microglial cells with more ramified morphology and larger GFAP-positive cell bodies were observed (Figure 1E). Similar to cortex, increased fluorescent intensity of Iba1, especially with Derinat, was observed at day 7 and decreased Iba1-flurescence at day 14 after TBI (Figure 1F), though there was no significant effect

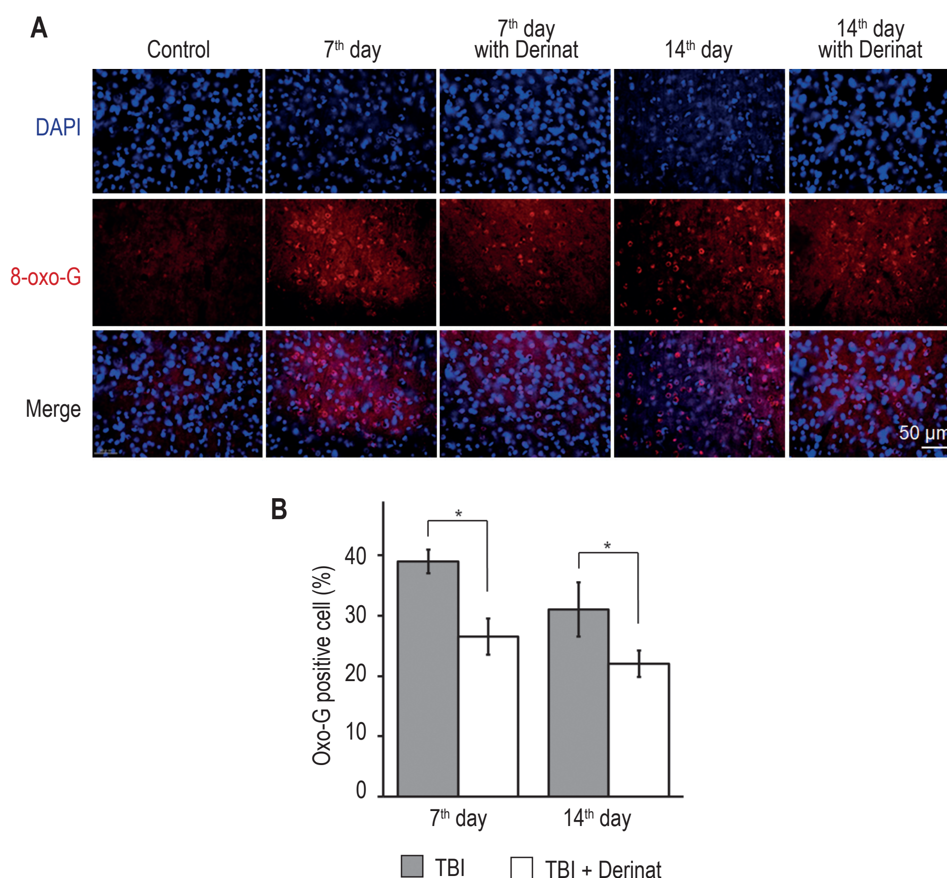

Figure 2. Effect of Derinat on nuclear DNA damage in the cortex of rat brain with TBI

Note. (A) Representative images of DAPI (blue) and 8-oxoG (red) immunostaining without TBI (control), at 7th and 14th day after TBI with and without Derinat. (B) Relative number of 8-oxoG-positive cells in the cortex of rat brain at 7th and 14th day after TBI with and without Derinat. *$p < 0.05$ compared to without Derinat.

of Derinat. On the other hand, no significant change in GFAP-fluorescence intensity was observed at day 7 and decreased GFAP-fluorescence at day 14 after TBI, without any effect of Derinat (Figure 1G).

Nuclear DNA damage was inhibited by Derinat

To investigate the oxidative damage, accumulation of 8-oxoG after TBI was immunohistochemically detected (Figure 2). 8-oxoG is the major form of guanine oxidized by hydroxyl radical, and is accumulated in both mitochondrial and nuclear DNA [72]. Hence, 8-oxoG is widely used as an index of DNA oxidative stress. Double-staining of 8-oxoG and 4',6-diamidino-2-phenylindole (DAPI) was performed to identify the nuclear DNA damage (Figure 2A). Accumulation of nuclear 8-oxoG at day 7 and 14 after TBI was attenuated by Derinat treatment compare to untreated animal's brains (Figure 2B).

Effects of Derinat on the ATP-induced morphological and functional changes of microglia *in vitro*

Since morphological activation of microglia by Derinat was observed in the TBI area *in vivo*, functional changes of microglia were analyzed using primary cultured mouse microglia *in vitro*. To mimic the activation of microglia induced by traumatic injury, adenosine trisphosphate (ATP) was used, one of the substances released from damaged neurons and a typical activator of microglia. Two concentrations were used; 50 μm to activate most of the purinergic receptors except P2X7 and 1 mM to activate microglial P2X7 receptor [32, 41].

With both 50 μm and 1 mM ATP, microglial cells showed morphological activation; larger cell bodies with more membrane ruffling (Figure 3A). Treatment of microglial cells with only Derinat, microglial cells looked to be activated in a dose-dependent manner (Figure 3B). With pre-treatment of Derinat (application of Derinat prior to ATP treatment) at the concentration of 1.5, 15, and 150 μM, it looked like more microglial activation with higher concentration in both ATP and Derinat (Figure 3B). With post-treatment of Derinat (application of Derinat after ATP), it looked like even more activation of microglia and rather fewer microglial cells with highest concentration of ATP and Derinat, suggesting a toxic effect of high concentration of the drugs (Figure 3C).

To investigate the functional changes of microglia, several factors released from microglia under pathological conditions were analyzed at mRNA level by semi-quantitative PCR analyses using primary cultured microglia from mouse brain. Activated microglia can be involved in neuroprotection both by blocking pro-inflammatory response and producing more neurotrophic factors and anti-inflammatory cytokines. Again, to activate microglia, low (50 μM)

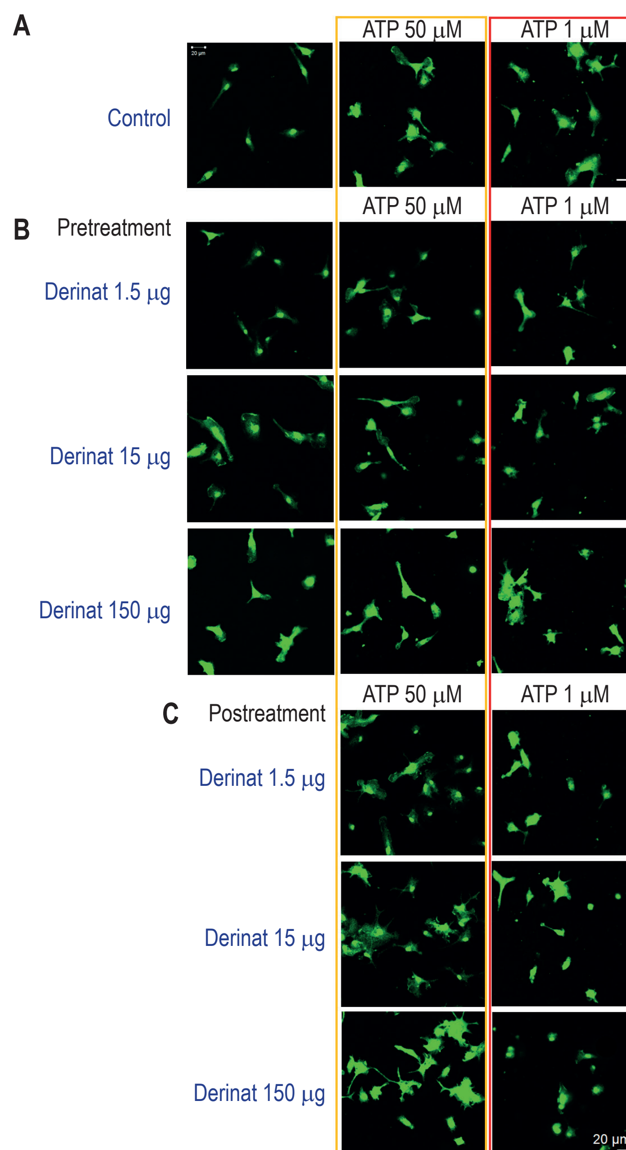

Figure 3. Effect of ATP, pre- and post-treatment of Derinat on Iba1-staining of primary cultured microglia

Note. (A) Effect of ATP (50 μM and 1 mM) on Iba1-staining (green) of primary cultured microglia. (B) Effect of Derinat (1.5, 15, and 150 μg/L) before application of ATP (50 μM and 1 mM). (C) Effect of Derinat (1.5, 15, and 150 μg/L) after application of ATP (50 μM and 1 mM).

or high concentration (1 mM) ATP was applied for 24 h. Considering the therapeutic application, Derinat at low (1.5 μM) or high (150 μM) concentration was then applied and the cells were incubated for 24 h. Glial cell line-derived neurotrophic factor (GDNF) was decreased by 1 mM ATP, which was recovered by both low and high concentration of Derinat (Figure 4A). Tissue plasminogen activator (tPA), a protein involved in the breakdown of blood clots, was decreased by both low and high concentration

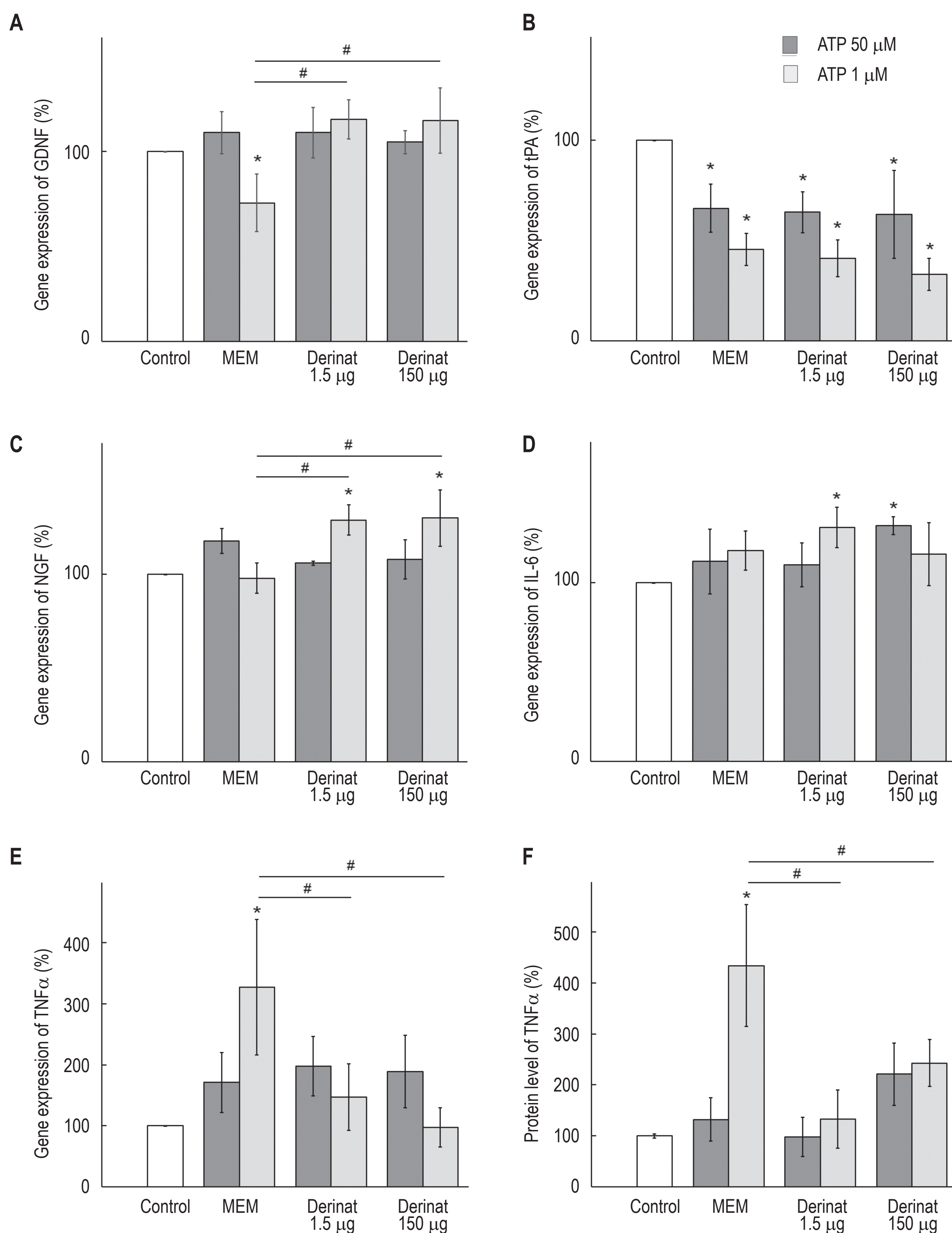

Figure 4. Effects of Derinat on the expression level of neurotrophic factors, tissue plasminogen factors (tPA), and cytokines in primary cultured mouse microglia

Note. (A) Relative expression level of GDNF mRNA. (B) Relative expression level of tPA mRNA. (C) Relative expression level of GDNF mRNA. (B) Relative expression level of NGF mRNA. (D) Relative expression level of IL-6 mRNA. mRNAs were analyzed by semi-quantitative RT-PCR. (E) Relative expression level of TNFα mRNA. (F) Protein level of TNFα assessed by ELISA. MEM: Modified Eagle Medium. *, $p < 0.05$ compared to control. #, $p < 0.05$ compared to without Derinat.

of ATP. With post-treatment of Derinat even inhibited the tPA mRNA level (Figure 4B). Nerve growth factor (NGF) was not affected by ATP, however, application of Derinat, both low and high concentration, increased NGF mRNA significantly (Figure 4C). As for interleukin (IL)-6, ATP with low and high concentration did not affect, however, 1 μM ATP-induced IL-6 mRNA was augmented by low concentration of Derinat, while 50 μM ATP-induced IL-6 mRNA was augmented by high concentration of Derinat (Figure 4D). One of the typical pro-inflammatory cytokines, tumor necrosis factor (TNF)-α, was significantly augmented in mRNA by 1 mM ATP, which was significantly prevented by both low and high concentration of Derinat (Figure 4E). To confirm the inhibitory effect of Derinat on TNFα, the protein level of TNFα was also investigated by ELISA. Similar to mRNA analyses, production of TNFα protein was significantly augmented by 1 mM ATP, but it was completely prevented by both low and high concentration of Derinat (Figure 4F).

Discussion

The present study suggests that Derinat may have immunomodulatory and neuroprotective effects. Previously, the TBI model was successfully created and the effects of Derinat on behavioral reactions were investigated at days 1, 7, and 14 [20]. In the current study, it became again obvious that Derinat administration significantly reduced the proportion of injured areas in all three days at 1, 7, and 14. The previous studies have reported that the most prominent amount of injury was observed on the first day after the TBI, and that the amount of injury decreased on the 7th day. In the present study as well, the proportion of damaged areas tended to decrease on day 7 compared to day 1, but it is interesting that the proportion of areas increased again on day 14. In the future, it will be useful to investigate the effects of Derinat in long-term TBI treatment by preparing a group with a longer days after TBI until sacrifice. In addition, though the administration period of Derinat was limited untill 74 hours after the TBI in this experiment, designing a plan to administer Derinat for a longer period of time after the TBI will be interesing to see whether more therapeutic effects are observed.

In the TBI, microglia can produce neuroprotective factors, clear cellular debris and orchestrate neurorestorative processes that are beneficial for neurological recovery after TBI. However, microglia can also become dysregulated and can produce pro-inflammatory and cytotoxic mediators [13] that hinder CNS repair and contribute to neuronal dysfunction and cell death. The dual role of microglial activation in promoting beneficial and detrimental effects on neurons is often accounted for by their polarization state, such as M1-like and M2-like, and functional responses after injury [52]. As an example, modulating microglia polarization through SIRT1-mediated deacetylation of the HMGB1/NF-κB pathway and atteuation of the inflammatory response by omega-3 polyunsaturated fatty acid was reported [10]. Recently a focused study identified several physiologic processes within microglia after TBI, demonstrating the capability of longitudinal transcriptional profiling to uncover potential cell-specific targets for the treatment of TBI [59].

Previous studies have reported that microglial activation was observed by examining Iba1 expression after TBI [25, 30, 75], and indeed microglial activation in the cortex of animal model was suggested by fluorescent staining of microglia/invading macrophage with anti-Iba1 antibody (Figure 1). Compared with the control group, the non-administered group showed the Ib-1 staining with stronger fluorescence on the 7th day, while it was attenuated on the 14th day (Figure 1D). Microglia are largely responsible for perpetuating the injury-induced inflammatory response but in the developing brain they play beneficial roles in both normal and disease states. Following closed head injury in the neonate rat, depletion of microglia with intracerebral injections of liposomes containing clodronate was associated with an increase in neurodegeneration in the early post-injury period (3 days), suggesting that activation of microglia may be beneficial and important for removal of dying neurons in the immature TBI [29]. In the present study, using adult rats we observed more morphologically activated microglia in reduced TBI area by Derinat, indicating activation of microglia may play beneficial role. On the contrary, chronic phase removal of neurotoxic microglia after TBI using colony stimulating factor 1 receptor (CSF1R) inhibitors markedly reduced chronic neuroinflammation and associated neurodegeneration [31]. As for the transition time from beneficial to toxic role of microglia, it may be necessary to define the time-dependent changes more precisely. It may be interesting to see the effects of Derinat with microglial depletion in the future.

GFAP is an astrocyte-specific substance and is used as an activity marker for astrocytes in immunostaining [16, 58, 64, 73]. Although TBI increased GFAP, no effect of Derinat administration on astrocytes in cerebral cortex was observed in this experiment due to the low expression of GFAP (data not shown). On the other hand, in the hippocaumpus neither Iba1 nor GFAP staining showed any effect

by Derinat administration (Figure 1G). It has been reported that astrocytic edema is a secondary and fatal symptom of TBI, and that inhibition of vasopressin 1a receptor suppresses GFAP elevation and is useful for prevention and treatment of cerebral edema [62]. In addition, there is a report that cytotoxic cerebral edema occurs after TBI due to the increase of NF-κB in astrocytes [38]. Therefore, we need to further continue to investigate the action of Derinat on astrocytes.

As in our previous studies, 8-oxo-G was used as a DNA damage marker [24, 60, 70, 72, 90], and TBI changes guanine to 8-oxo-G under oxidative stress and accumulates it [48, 54]. From our results, Derinat suppressed DNA damage on both the 7th and 14th days (Figure 2). Further research on the mechanism how Derinat suppress oxidative stress is needed.

In vitro study using mouse primary cultured microglia, the administration of Derinat altered the morphology of microglia compared to the control group (Figure 3). The fluorescence intentisy of Iba-1 staining became stronger and the size of microglial became larger depending on the concentration of Derinat. It is important to know which concentration of Derinat *in vitro* study corresponds to the one *in vivo*, to understand the mechanism of therapeutic effects induced by Derinat.

To activate microglia and induce an inflammatory response, ATP was used, because it is one of the substances produced by TBI [37] and a typical microglial activator [71]. In TBI, it has been reported that microglia converge to the injured site rapidly and autonomously without cell movement [69], and this chemotactic reaction can be mimicked by administration of ATP [19]. When Derinat was administered before and after the addition of ATP, Derinat before the addition of ATP did not significantly change the state of microglia (Figure 3B). On the other hand, post-treatment of Derinat affected the morphology of microglia in a dose-depenent manner (Figure 3C). At least this result suggest that Derinat affect microglia morphologically under the injured condition and enlarged and morphologically activated microglia does not necessarily suggest inflammatory or harmful condition.

Not only morphological changes, functional changes were investigated with primary cultured microglia. Certain microglial receptors have been implicated in TBI pathology, including fractalkine receptor (CX3CR1), purinergic receptor (P2Y12R), Toll-like receptor (TLR4), scavenger receptors, tumor necrosis factor receptor (TNF1R), interleukin receptor (IL-1R), complement receptors, and peroxisome proliferator-activated receptor (PPAR) [92]. Also, the transient receptor potential cation channels (TRP channels), TRPV1 and TRPA1, are polymodal receptors that are activated by a variety of stimuli associated with TBI, leading to the release of neuropeptides such as substance P (SP). SP augments many aspects of the classical inflammatory response via activation of microglia and astrocytes [15]. Though we have not analyzed whether or not Derinat affects the expressions of these receptors, it may be useful to see in the future. It was also suggested that TBI may increase the microglial-derived microparticles with pro-inflammatory mediators, interleukin-1 and microRNA-155, being sufficient to initiate neuroinflammation [45]. It will be also interesting if the DNA substance like Derinat can increase the microglial-derived microparticles.

As a beneficial role of microglia, upregulation of microRNA-124 which is a brain specific miRNA that is highly expressed in microglia, was reported to induce M2 polarization of microglia through inhibiting TLR4 pathway, improving hippocampal neurogenesis and functional recovery after brain injury. The M2 polarization of microglia might be a strategy to improve the outcome of TBI [91]. More recently, astrocyte-derived exosomes enriched with miR-873a-5p has been reported to inhibit neuroinflammation via microglia phenotype modulation after TBI [53]. The effect C-C chemokine receptor 5 (CCR5) may be a therapeutic target for recovery after TBI as well [39]. In the future, effects of Derinat on these factors may help understand the mechanisms of nucleotide drugs for TBI.

In our study, GDNF has been used to study neuroprotective effects after TBI [18, 35, 78, 79, 88]. NGF is also drawing attention in investigating the neuroprotective effect after TBI [55, 56, 94]. Increased expression of GDNF and NGF has a neuroprotective effect, which has a positive effect on the treatment of TBI. It has been reported that intranasal administration of NGF improves cognitive function after TBI, although the mechanism of action is unknown [56]. As mentioned in the Introduction, similar memory improvement was clarified by administration of Derinat [20]. As TBI treatments, many means have been proposed, such as administration of transplantation of bone marrow stromal cells [78], utilization of ginsenoside contained in ginseng [35], administration of histone diacetylase inhibitor [55], granulocyte colony stimulating factor (G-CSF) [79], quercetine [21], erythropoietin, and a pleiotropic cytokine produced in the kidney and CNS [76]. Since an increase in GDNF, NGF, or brain-derived neurotrophic factor (BDNF) has been reported for these treatments as well, the

administration of Derinat can also be an alternative therapy.

tPA promotes neural remodeling in the CNS and also has a thrombolytic effect, so it is used as a standard treatment for ischemic stroke at an early stage [27, 42, 47, 66]. On the other hand, it has also been reported that upregulation of tPA after TBI may cause loss of cerebrovascular self-regulation due to neurotoxicity induced by overactivation of NMDA receptors and intracranial hemorrhage [2, 3, 4, 33, 65, 66]. In the present study, the expression level of tPA was decreased by ATP treatment, and no effect of Derinat was observed. Using the controlled cortical impact model [61], administration of tPA by intranasal treatment to rats 7 days after TBI treatment improved cognitive function and increased BDNF [66]. It has also been reported that treatment with the tPA variant (tPA S481A) can limit the neurotoxicity caused by NMDA receptor activation by suppressing the upregulation of the ERK isoform of mitogen-activated protein kinase (MAPK) [4, 33]. Since Derinat had no effect on tPA *in vitro*, TBI treatment by simultaneous administration of tPA containing mutants and of Derinat *in vivo* may be considered in the future.

Increased IL-6 has multiple effects such as increased glucose uptake, increased fatty acidation, and anti-inflammatory effect [81]. Decreased IL-6 expression reduces post-TBI cerebral edema [89]. There are reports that plasma IL-6 levels are associated with the development of acute respiratory distress syndrome (ARDS) in patients with severe TBI [1]. On the other hand, it has been pointed out that there is a contradiction on the relationship between TBI and IL-6 [46]. In the present study, the low concentration of Drinat (1.5 μM) in the presence of 1 mM ATP or high concentration of Derinat (150 μM) in the presence of 50 μM ATP increased IL-6 mRNA compared to the control group. Though favorable and unfavorable effects of IL-6 are expected from previous studies, the outcome of the effect of Derinat is likely to be favouralble. Indeed, microglia in the mammalian brain can be manipulated to adopt a neuroprotective and pro-regenerative phenotype that can aid repair and alleviate the cognitive deficits arising from brain injury in IL-6-dependent manner [86]. On the other hand, it has also been reported that the -174 C/G gene polymorphism of IL-6 causes a significant difference in short-term results in patients with severe TBI [17]. Therefore, the therapeutic effect of IL-6 under various conditions will be required in the future.

TNFα is an important factor involved in various stages of inflammatory reaction and nerve damage in TBI, and is released by microglia and astrocytes [12, 83]. In the present study, both real-time PCR and ELISA have shown that Derinat administration significantly reduced TNFα levels. Candidates for TBI treatment include the use of the selective TNFα inhibitor, etanercept [11, 82, 83], because activated subtype of microglia (similar to M1 type of macrophage) produces higher levels of TNFα [12]. Regarding the inhibitory effect of Derinat on TNFα, additional studies on the specificity of Derinat on a microglial subtype might be interesting.

Many substances have been listed as biomarkers for TBI [80]. In investigating the damage caused by TBI, the effects of substances such as c-tau, ferritin, -actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and porphobilinogen deaminase (PBGD) [80] were investigated. NIX, also called BNIP3L (Bcl-2/E1B-19K-interacting protein 3-like), belonging to the Bcl-2 family and being considered a proapoptotic protein [57], and neutral sphingomyelinase inhibitors [44], are also likely to play a neuroprotective role in TBI through autophagy and apoptosis pathways or by attenuating the release of microparticles and phosphorylation of p38 MAPK and ERK1/2. The effects of Derinat on these signaling may also helpful to understand the protective actions in the future.

Conclusion

Injection of sodium salt of DNA isolated from the soft roes of Russian sturgeon followed by experimental TBI attenuates the area of brain damage via modulation of immune response of microglia and subsequently inhibiting the accumulation of oxidative stress. The future subject will be to investigate more precise mechanism with molecular or genetic level. Furthermore, it will be useful if similar sodium DNA from other natural sources are also effective. If so, these nucleic acid drugs might become potential therapeutic drugs or supplemental subjects against oxidative stress-induced disorders in the future.

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