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Research paper

Allopurinol attenuates repeated traumatic brain injury in old rats: A preliminary report

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ABSTRACT

Traumatic brain injury (TBI) is an overlooked cause of morbidity, which was shown to accelerate inflammation, oxidative stress, and neuronal cell loss and is associated with spatial learning and memory impairments and some psychiatric disturbances in older adults. However, there is no effective treatment in order to offer a favorable outcome encompassing a good recovery after TBI in older adults. Hence, the present study aimed to investigate the histological and neurobehavioral effects of Allopurinol (ALL) in older rats that received repeated TBI (rTBI). For this purpose, a weight-drop rTBI model was used on old male Wistar rats. Rats received 5 repeated TBI/sham injuries 24 h apart and were treated with saline or Allopurinol 100 mg/kg, i.p. each time. They were randomly assigned to three groups: control group (no injury); rTBI group (received 5 rTBI and treated with saline); rTBI+ALL group (received 5 rTBI and treated with Allopurinol). Then, half of the animals from each group were sacrificed on day 6 and the remaining animals were assessed with Open field, Elevated plus maze and Morris Water Maze test. Basic neurological tasks were evaluated with neurological assessment protocol every other day until after the 19th day from the last injury. Brain sections were processed for neuronal cell count in the hippocampus (CA1), dentate gyrus (DG), and prefrontal cortex (PC). Also, an immunohistochemical assay was performed to determine NeuN, iNOS, and TNFα levels in the brain regions. The number of neurons was markedly reduced in CA1, GD, and PC in rats receiving saline compared to those receiving allopurinol treatment. Immunohistochemical analysis showed marked induction of iNOS and TNFα expression in the brain tissues which were reduced after allopurinol at 6 and 19 days post-injury. Also, ALL-treated rats demonstrated a remarkable induce in NeuN expression, indicating a reduction in rTBI-induced neuronal cell death. In neurobehavioral analyses, time spent in closed arms, in the corner of the open field, swimming latency, and distance were impaired in injured rats; however, all of them were significantly improved by allopurinol therapy.

To sum up, this study demonstrated that ALL may mitigate rTBI-induced damage in aged rats, which suggests ALL as a potential therapeutic strategy for the treatment of recurrent TBI.

1. Introduction

Traumatic brain injury (TBI) is a leading cause of long-term disabilities worldwide and is a public health problem (Cassidy et al., 2004; Cornelius et al., 2013), which has become most popular, especially after the publishment of the findings on chronic traumatic encephalopathy (CTE) in American football players (Omalu et al., 2005). Apart from athletes, such as boxers, fighters, American football players, and soldiers on the battlefield, older adults also have an increased risk for exposure to repetitive TBI (van de Loo et al., 2022). Considering that approximately one-third of the people aged 65 and over living in the community fall at least once a year, it is better understood how high the risk is for such people. More importantly, with respect to the rare admission of older adults for mild head trauma to an institution, based on a large number of

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known and likely unknown cases and interval of injuries (Shultz et al., 2017), we agree with the expression of Goldstein,1990 in TBI which has been referred to as the "*silent epidemic*" (Goldstein, 1990). Over the last several decades, numerous animal models have been developed to address the diverse nature of human TBI. Worldwide, the clinical relevance of these models has been a major point of reflection given the poor translation of TBI interventions to the clinic (Johnson et al., 2015). However, models of repetitive traumatic brain injuries are essential for exploring mechanisms of chronic neurological diseases (Xu et al., 2016) because repetitive injuries are implicated in chronic illnesses which are highly prevalent with aging. Therefore, repetitive TBI animal models with ranging time between injuries, including 5 injuries (1/day) (Kane et al., 2012; Ma et al., 2019; Meehan 3rd et al., 2012; Xu et al., 2016) are inevitably in use to provide a means to study the condition in a rigorous, controlled, and efficient manner in a single study (Shultz et al., 2017).

Additionally, recent evidence demonstrated that mild TBI, the most common form, made the brain more vulnerable to a second mild head injury-hit (Laurer et al., 2001) and that repetitive TBI (rTBI) accelerated brain β-amyloid accumulation, oxidative stress, and cognitive impairment (Uryu et al., 2002). Clinically patients with rTBI suffer from poor concentration, memory impairments, difficulty in tasks involving visuomotor speed, divided attention and executive dysfunctions as well as psychological issues, such as depression, anxiety, anger, and irritability (Collins et al., 1999; Wall et al., 2006; Williams et al., 2010).

In rats, repetitive mild TBI (rmTBI) 1 or 3 days apart results in significant tissue loss on brain imaging compared with a single one (Huang et al., 2013), which was associated with learning and memory impairments (Guskiewicz et al., 2005), mild cognitive impairment, depression and many other psychiatric disturbances (Perry et al., 2016).

TBI process includes two tissue injury pathophysiological mechanisms, named primary and secondary. The latter is reported to contribute to progressive deficits and poor prognosis following primary mechanical tissue damage in TBI. Oxidative stress is reported to play a key role in secondary injury and to cause neurotoxicity including inflammation, cell damage, and neuronal death through a series of reactions (Juurlink and Paterson, 1998; Tyurin et al., 2000). Studies showed that Xanthine oxidase (XO) enzyme activity may increase within 2 h after TBI during oxidative stress and may play an important role in the generation of reactive oxygen species (ROS) (Battelli et al., 1996; Hewinson et al., 2004; Solaroglu et al., 2005). XO catalyzes the conversion of hypoxanthine to xanthine and of xanthine to uric acid and reduces oxygen into ROS (Isik et al., 2006), the excess of which is detrimental to neuronal cells.

On the other hand, Allopurinol, a well-known XO inhibitor, can modulate subsequent ROS production and reduce oxidative stress. Furthermore, it has been reported that Allopurinol reduces pro-inflammatory molecules and oxidative stress in brain infarction in rats, and improves neurocognitive outcomes in experimental studies of ischemia and/or hypoxia. (Maiwald et al., 2019; Palmer et al., 1990; Zhang et al., 2015). Therefore, it could reduce the negative effects of secondary injury and have a favorable impact on the recovery of TBI (Strilchuk et al., 2019). Apart from its beneficial effects on ischemia-induced cerebral injury, the effect of Allopurinol following TBI in rats has not been reported yet. However, provided that TBI increases the risk and severity of an ischemic stroke and that poorer stroke outcomes are revealed in the presence of a prior mild TBI via impairing metabolic and microvascular physiology (Weil et al., 2021), it is essential to assess allopurinol's effect against an experimental TBI.

Additionally, from a clinical standpoint, apart from military operations and sports events, it should be kept in mind that older adults have a great risk for recurrent head injuries as a consequence of repeatedly falling. Moreover, these people are reported to experience worse consequences and prolonged recovery following TBI than younger ones (Colantonio et al., 2009; Fu et al., 2017). Considering that advanced age is particularly at risk of TBI and that TBI is an important cause of morbidity and mortality in the elderly, we aimed to observe the effect of such a widely and safely used drug, Allopurinol in rmTBI in older rats through neuronal injury and ROS mechanisms. To the best of our knowledge, this study will be the first study that investigates Allopurinol after rTBI in older rats.

2. Material and methods

2.1. Animals and husbandry

Thirty-six adult male Wistar strain rats (450–500 g) aged approximately 24–26 months were obtained from the Experimental Animal Laboratory of Dokuz Eylul University. Animals were then consecutively housed three in a cage. All rats were housed in a temperature and humidity controlled animal facility with a 12 h dark and light cycle and they all had free access to food and water throughout the experiments.

This study was conducted in accordance with the guidelines provided by the National Research Council's Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of the Dokuz Eylul University School of Medicine (Protocol number: 22/2019).

2.2. Experimental TBI model

An adult mTBI model (Fujiki et al., 2008; Henninger et al., 2005) was modified for all experiments to induce an injury that closely resembles a mild form of TBI. Briefly, the rats were anesthetized intraperitoneally with Xylazine HCl (Rompun®-Bayer, Turkey) (5–10 mg/kg; intraperitoneal) and Ketamine (Ketalar®-Eczacibasi, Turkey) (50 mg/kg; intraperitoneal). A heating pad and a heating lamp were used to maintain a rectal temperature of 36.5 to 37.58 °C. The rats' heads were shaved and the surgical site was disinfected with Betadine. A midline incision of the scalp was performed. Then, the anesthetized rats were moved to be placed under a laboratory-made weight drop apparatus for subsequent TBI or sham injury for the Control group with the head secured. A 175 g cylindrical weight (10 mm diameter) was freely dropped through the tube onto a cylindrical polyacetal transducer rod (diameter = 10 mm, weight = 32.6 g, length = 15.75 cm) from a height of 42 cm above the sagittal midway of the rat brain. Controls underwent similar procedures including anesthesia without receiving any trauma. After the experiment, the skin was sutured and the rats were given analgesics and were allowed to recover from anesthesia with 100% oxygen on a warming pad and were then returned to their cages. TBI or sham procedures were administered 24 h apart over 5 days. No rats exhibited head fracture, nasal bleeding, or mortality.

2.3. Experimental group and drug administration

Rats were randomly assigned to three groups and each group consisted of 12 rats: the Control group, received identical procedures for anesthesia and analgesia exposure, but were not subjected to brain injury; the rTBI group, received 5 repeated TBI 24 h apart and thereafter treated with saline; rTBI+ALL group, received 5 repeated TBI 24 h apart and treated with 100 mg/kg Allopurinol intraperitoneal each time. Allopurinol dose was used based on prior dose-finding experiments in rats and was in accordance with the reports (Giray et al., 2001; Karageorgos et al., 2006).

After the TBI or sham procedures following euthanasia six rats from each group were sacrificed post-TBI day 6 and tissues were fixed in formalin for histological examination. The rest six rats from each group underwent behaviour tests: Elevated plus maze test on day 7, Open Field test on day 8, and 4 days of behavioral testing training of Morris Water Maze test, thereafter to as days 9–12, and underwent Morris Water Maze test on day 13. On day 19, all the remaining 18 rats were sacrificed and brain tissues were fixed in formalin.

2.4. Behaviour tests

2.4.1. Elevated plus-maze

On D7, rats were placed in the center of a raised plus-shaped 4-arm maze with two open arms and two enclosed arms, starting from facing a closed arm, for 5 min. Anxiety-related behaviour was determined by counting the number of times the animals entered or remained in open or closed arms, as well as the time the animal spent in each arm. The proportion of time spent in the open arms is considered an indication of anxiety (Meconi et al., 2018; Pellow et al., 1985).

2.4.2. Open field

On D8, rats were tested in the open field to assess anxiety-like behaviour and overall locomotion. In this task, the rat is placed in the center of the open field and anxiety behaviour is observed by the amount of time rats spend at the peripheral sides of the open field or decreased time spent in the center edges of the box, avoiding the center of the open field. Total distance travelled, and time spent in each zone were recorded for 5 min. The average velocity of movement was measured alongside these

2.4.3. Morris water maze behaviour test

This is a test of spatial learning and reference memory. It was performed in a tank of 122 cm diameter with the water temperature maintained at 21 °C. The water was tinted with white tempera paint to obscure the platform. A 10 cm × 10 cm platform was hidden 1 cm below the surface of the water. Rats were given a series of daily trials using a random set of start locations around the perimeter of the tank. Each trial lasted until either the rats had found the fixed platform or for a maximum of 1 min. All rats were allowed to rest on the platform for 20 s and each rat was allowed 4 trials per day for 4 days. One day after the last training, the test (probe trial) was performed again with the removed platform and the examiner determined the time of swimming until the rats reached the platform target site. Average distance to find the target platform site and latency times to first target-site crossover were recorded (D'Hooge and De Deyn, 2001; Isik et al., 2016; Vorhees and Williams, 2006).

2.5. Neurological assessment protocol

A neurological assessment protocol (NAP) score was used to quantify the cumulative effects of the repeated injury on the level of consciousness, and basic motor and reflexive capacity in the rats, as performed previously (Meconi et al., 2018). NAP score consisted of four basic neurological tasks: startle reflex, limb extension, balance beam, and rotating beam. NAP was performed before the injury and on days 6, 8,10, 12,14,16, and 18 by two observers who were blinded to the injury status of each animal. Each task was scored as a pass or fail, and a point was awarded for each pass. The maximum score received was 4 (perfect performance), and the scores decreased as the severity of the dysfunction increased. Each task was repeated three times and the average of the performances was considered the valid result. NAP score has been reported to be a useful tool providing a simple, yet sensitive, assessment of neurologic function that can be rapidly applied in the diagnostically important time window immediately after the injury, but, it is limited in that it does not address more subtle cognitive, motor, and emotional changes. No animals were excluded from the analysis.

2.6. Histological examinations

After fixation, brain tissue samples were fixed in formaldehyde for three days. After routine histological procedures, specimens were blocked, and 5 μm sections were taken with a rotary microtome (RM 2255, Leica Instruments, Germany). According to the rat brain atlas of Paxinos and Watson, sections were taken from the prefrontal cortex from plate 8–9, hippocampal regions CA1 and dentate gyrus (DG), from plate 21–22 (Paxinos and Watson, 2002). For stereological and histomorphological evaluations, sections were stained with cresyl violet

Estimated Neuron Density: For the estimation of neuron densities, the images were analyzed by a computer-assisted Image-J analysis program. The number of neurons was counted a square area of ~1.875 μm 2 was used for the analysis of the cresyl violet stain (Tuzun et al., 2012).

2.7. Immunohistochemical examination

Sections were mounted on poly-L-lysine-coated slides. The streptavidin-biotin-peroxidase assay was performed using the primary antibodies against the neuron-specific nuclear protein (anti-NeuN) (Bioss, Bs-1631R), anti-iNOS (Bioss, bs-2072R) and anti-TNFα (Novus, NB600–587). After deparaffinization, sections were treated with trypsin (TA-125-TR, Thermo Fisher) for 15 min and endogenous peroxidase activity was blocked using a 0.3% solution of hydrogen peroxidase in PBS at room temperature for 10 min. Then primary antibodies were applied for 2 h at 4 °C temperature and were washed in PBS. After washing, the secondary antibody (85–9043, Invitrogen) was applied for 30 min, followed by washes in PBS. The peroxidase activity was visualized with DAB (diaminobenzidine) (Roche, Penzberg, Germany). Slides were counterstained with Mayer's hematoxylin, dehydrated and cleared, and analyzed on a light microscope (Özel et al., 2020).

2.8. Semi-quantification of immunostaining data

Semi-quantitative scores were defined as the following: 0 = no immunoreactivity; 1 = very little positive staining and the staining was mild; 2 = positive staining was moderate (between grade 1 and grade 3); 3 = strong positive staining was evenly distributed across the entirety of the images. Each of the sections was graded blindly by two histologists in order to maintain the consistency of the scoring system. The mean values were calculated for immunoscorings (Caglayan Sozmen et al., 2016).

2.9. Statistical analysis

Data were analyzed using SPSS version 22.0 (SPSS, Chicago, IL, USA). Values are expressed as mean ± SD unless otherwise stated. As the data obtained from the study were inconsistent with normal distribution and which have a small sample size, we used the non-parametrical statistical methods. Differences between all groups were examined with the Kruskal Wallis test and the two groups were examined with the Mann–Whitney *U* test, with findings of $p < 0.05$ considered significant. Additionally, for each group, repeated measures in WMT training trials D9–12 were analyzed by using Friedman's test, followed by post hoc analysis with Wilcoxon signed-rank tests. Wilcoxon signed-rank tests were conducted with a Bonferroni correction applied, resulting in a significance level set at $p < 0.008$.

3. Results

3.1. Allopurinol significantly improved the tasks in neurological assessment protocol in rats after TBI

The NAP scores were performed before the brain injury, then after the last injury, and continued every other day until death. NAP showed no significant differences among the groups before the brain injury. Overall, the mean NAP score was lower in the rTBI group compared to the control and rTBI+ALL groups, however, it was not statistically significant ($p > 0.05$) (Fig. 1).

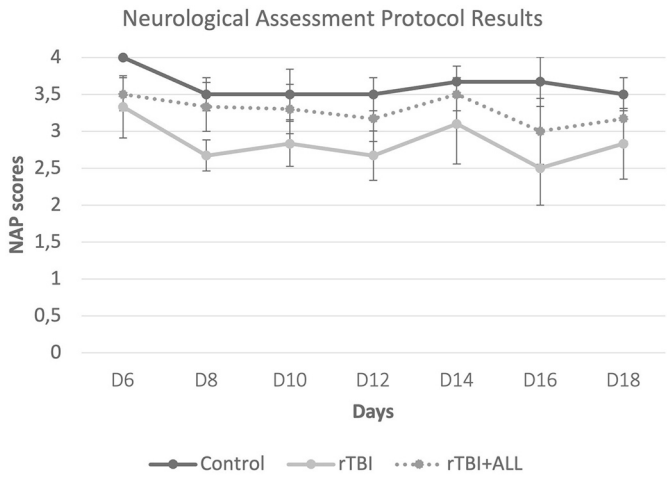


Fig. 1. Neurological assessment protocol results in groups: Neurologic assessment protocol (NAP) testing assessed the startle reflex, limb extension, beam walk, and rotating-beam tasks. Each successfully completed task was given a point so that a score of 4 indicates perfect performance, and a score of 0 indicates severe impairment. NAP was performed after the last injury on the 6th day of time point and every other day thereafter till the 19th day of sacrification timepoint in the remaining half of the animals. Overall, the mean NAP score was lower in the rTBI group compared to the control and rTBI+ALL groups, however, it was not statistically significant ($p > 0.05$). The error bar shows the standard error of the mean. Before the injuries, at the beginning of the experiments, there were no significant differences in NAP scores among the groups (not shown here).

3.2. Anxiety-related behaviour and locomotor activity assessed by Elevated plus-maze and open field

In the elevated plus-maze, the mean speed, or the number of entries to the closed or open arms were similar among all three groups, providing that there was no effect of rTBI on motor impairment (absence of rTBI-induced motor impairment) ($p > 0.05$, for all) (Fig. 2 A, B, C).

However, the groups differed in time spent in closed arms ($p = 0.039$), that the rTBI group spent more time in the closed arms than the rTBI+ALL (287.4 s vs 116.9 s; respectively, $p = 0.019$) as shown in Fig. 2 D, indicating rTBI led to acute/subacute anxiety. The time spent in the open arms was observed more in the rTBI+ALL group than in the others, though, it was not statistically significant ($p = 0.068$) (Fig. 2 E).

In the open field maze, the total distance travelled by rats can be used as a convergent means to assess motor function. The mean velocity of movement and the total distance travelled in the maze were equivalent across groups ($p > 0.05$, for all) further, provided that the mobility was not impaired by the effect of rTBI (Fig. 2 F, G). Besides, the amount of time spent in the corners and the amount of mobile time spent in the central zone differed in the groups ($p = 0.037$ and $p = 0.05$, respectively) (Fig. 2 H, I). When compared to the TBI + ALL group, the rTBI group spent more time in the corners (87.6 s vs 229.5 s, $p = 0.042$) (Fig. 2 H) and the mobile time spent in the central zone was lesser in the rTBI group than in those of the rTBI+ALL group (86.4 s vs 143.6 s, $p = 0.024$) (Fig. 2 I). This provides a significant effect of repetitive injection ALL on the decreation of anxiety.

3.3. Repetitive mTBI induces cognitive deficits in the Morris Water Maze test

TMWM tests were performed for five consecutive days after the last injury and nine days prior to planned death. The latency to find the platform during training days for each group was shown in Fig. 2 J. There was a statistically significant difference in the latency during the training days (D9–12) in the control group ($p = 0.038$) and in the TBI group ($p = 0.027$) (Friedman test). Furthermore, post hoc analysis with Wilcoxon signed-rank tests showed that there was an overall reduction in latency on D12 vs D9 in the control group ($p = 0.028$), and in the TBI group, there was also an overall reduction in the latency on D12 vs D9 ($p = 0.046$); D12 vs D10 ($p = 0.028$), and D11 vs D9 ($p = 0.043$). However, since all the p values were above 0.008, these significances disappeared after post hoc analysis with Wilcoxon signed-rank tests with an applied Bonferroni correction. In the probe trial analysis, there was a difference in the latency to find the platform in the groups ($p = 0.005$). It was longer in rTBI than in control group ($p = 0.005$), and than in rTBI+ALL group ($p = 0.006$) (Fig. 2 K). The latency to find the platform between control and rTBI+ALL groups was similar ($p = 0.898$) (Fig. 2 K). The result suggested that rmTBI has led to spatial learning dysfunction in the rats and the dysfunction improved for the better with ALL treatment.

The swimming speed, platform search time, and the number of entries to the platform zone (crossing numbers) did not exhibit any remarkable difference among the three groups ($p > 0.05$). Motor impairment did not negatively affect the ability of rTBI rats to perform the task (Fig. 2 L).

Additionally, the swimming distance travelled to find the target platform differed among the groups ($p = 0.006$, Kruskal-Wallis), such that it was less in control rats than in the others (vs rTBI $p = 0.011$; vs rTBI+ALL $p = 0.033$). The swimming distance was also lesser in the rTBI+ALL group than in rTBI rats ($p = 0.021$) (Fig. 2 M).

3.4. The number of neurons was significantly increased with Allopurinol treatment which was reduced after rTBI

3.4.1. Neuron counting

In sections stained with cresyl violet, the number of neurons in the PC, CA1, and GD regions was significantly decreased in rTBI group compared to the Control group ($p < 0.05$). In contrast, the number of neurons was significantly higher in the rTBI+ALL group compared to the rTBI group on day 5 ($p < 0.05$) (Fig. 3 A, B). Similar results were obtained by 19 days post-injury ($p < 0.05$) (Fig. 3 A, C).

3.5. Immunohistochemical results

3.5.1. rTBI induces neuronal cell death and oxidative and inflammatory markers in brain tissues which are reduced by Allopurinol treatment

In order to understand the relation between the traumatic brain injury and neuronal damage, oxidative stress, and inflammation, NeuN, iNOS, and TNFα levels in brain tissue (CA1, GD, PC) were determined by immunohistochemical assay. There were decreased NeuN immunopositive cells in brain tissues of the rTBI group when compared with the Control Group ($p \leq 0.05$). The rTBI+ALL group showed decreased positive cells compared to the rTBI group (Fig. 4, Table 1). iNOS and TNFα immunopositive cells in the brain tissues were increased in rTBI group when compared to the Control Group ($p \leq 0.05$). The rTBI+ALL group showed decreased positive cells compared to the rTBI group (Figs. 5, 6, and Table 1).

4. Discussion

This study demonstrated that allopurinol reduces neuronal cell loss, neuroinflammation, and oxidative damage and improves functional outcomes after repeated brain trauma in older rats.

Validation of an experimental model is crucial to reflect the pathogenesis of targeting the disease. In our study, a modified weight-drop rat model of repeated mild TBI (Fujiki et al., 2008; Henninger et al., 2005) with a one-day inter-injury interval over five days was used and the results were evaluated on days 6 and 19 post-injury. The model did not result in a skull fracture, macroscopic structural brain damage, nasal bleeding, or mortality as in other mild rTBI models (Fehily et al., 2019; Blennow et al., 2012). Additionally, in this model, anesthesia administration for all experiments, which were also administered to the control rats, was short to limit the confounding effects of anesthesia on cognitive

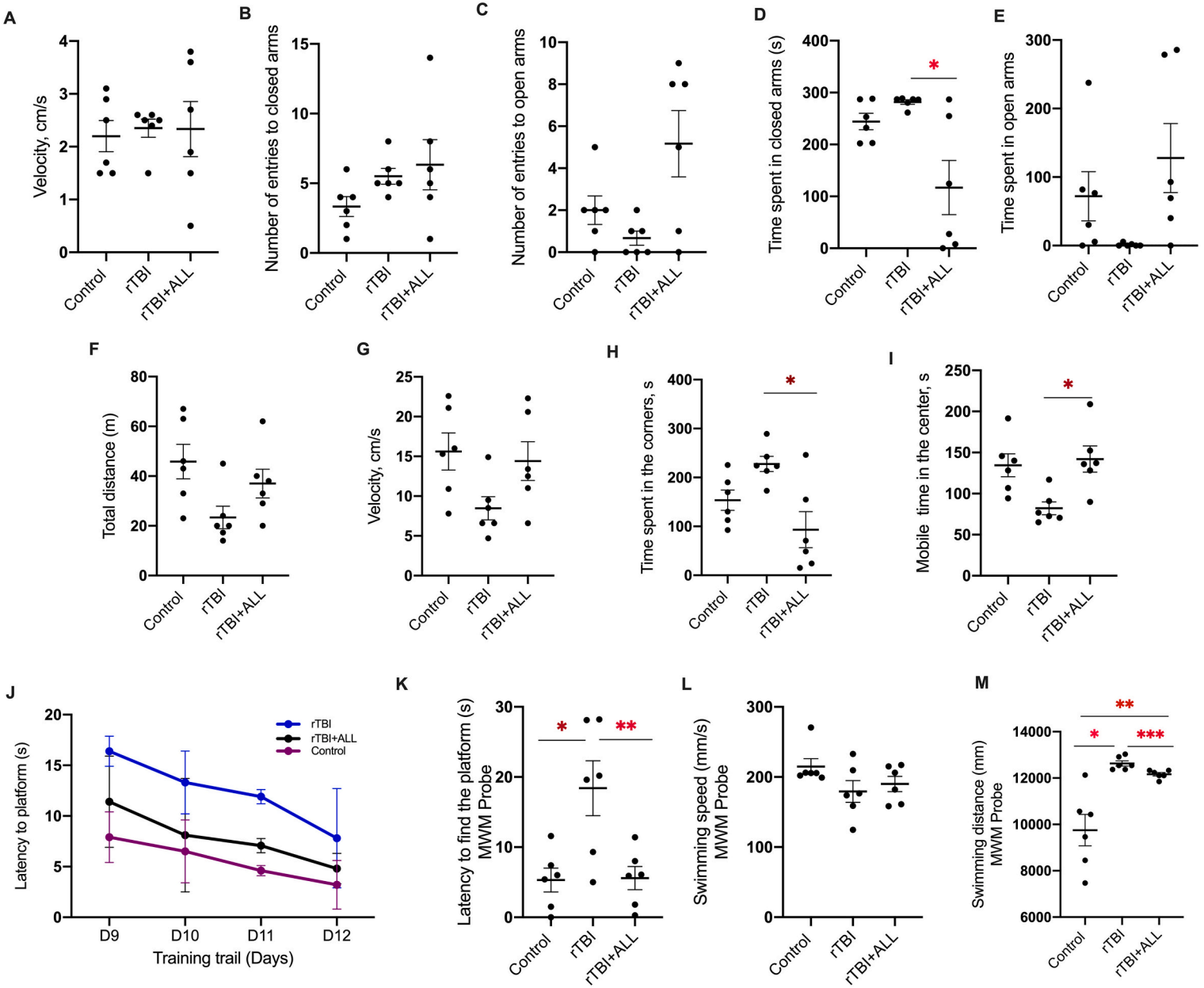

A
Velocity, cm/s
Control
rTBI
rTBI+ALL
B
Number of entries to closed arms
C
Number of entries to open arms
D
Time spent in closed arms (s)
*
E
Time spent in open arms
F
Total distance (m)
G
Velocity, cm/s
H
Time spent in the corners, s
*
I
Mobile time in the center, s
*
J
Latency to platform (s)
rTBI
rTBI+ALL
Control
D9
D10
D11
D12
Training trail (Days)
K
Latency to find the platform (s)
MWM Probe
*
**
L
Swimming speed (mm/s)
MWM Probe
M
Swimming distance (mm)
MWM Probe
**
*
***

Fig. 2. Repeated TBI does not impair mobility but increases anxiety-like behaviour in the elevated plus-maze (EPM) and open field (OF). Anxiety-like behaviour improves with Allopurinol (ALL) treatment. A,B,C. In the EPM, mean velocity, and the number of entries to the closed and open arms were similar across groups ($p > 0.05$). D. rTBI rats spent more time in the closed arms than rTBI+ALL rats ($p = 0.019$). E. rTBI+ALL rats tended to spend more time in the open arms than rTBI rats ($p = 0.068$) F, G. In OF, total distance travelled and mean velocity were similar across the groups. H. TBI rats spent more time in the corner when compared to rTBI+ALL rats ($p = 0.042$) I. rTBI+ALL rats spent more mobile time in the center zone than rTBI rats ($p = 0.024$). J-M. Allopurinol leads to an improvement in memory and spatial learning which were impaired after repeated TBI in the Morris Water test (MWT). J. The line graph shows the latency to find the platform during training days for each group. There was a statistically significant difference in the latency during the training days (D9–12) in the control group ($p = 0.038$) and in the TBI group ($p = 0.027$) (Friedman test). Additionally, there was an overall reduction in latency on D12 vs D9 in the control group ($p = 0.028$), and in the TBI group, also an overall reduction in the latency on D12 vs D9 ($p = 0.046$); D12 vs D10 ($p = 0.028$); D11 vs D9 ($p = 0.043$) was revealed. However, all these statistically significant results disappeared after post hoc analysis with Wilcoxon signed-rank tests with an applied Bonferroni correction. K. In the probe trial (D13), the latency to find the platform site was longer in rTBI rats than in control rats ($^*p = 0.005$), and than in rTBI+ALL rats ($^{**}p = 0.006$). L. The mean swimming speed was similar among the groups ($p > 0.05$). M. The swimming distance to find the target platform site was less in control rats than in rTBI rats ($^*p = 0.011$), and than in rTBI+ALL rats ($^{**}p = 0.033$). Moreover, the swimming distance was less in rTBI+ALL rats than in rTBI rats ($^{***}p = 0.021$). All presented data were shown as mean ± standard error of the mean (SEM).

outcomes (Bennett et al., 2012; Fehily et al., 2019; Rowe et al., 2013; Yates et al., 2017). In addition, in our model, the experimental rTBI was studied in older rats which was the first time it was ever done, to the best of our knowledge.

The hippocampus and frontal cortices are susceptible to brain areas in TBI (Phillips and Reeves, 2001). Accordingly, in this study, rTBI caused cell loss in the DG and CA1 region of the hippocampus and the prefrontal cortex, and up-regulation of iNOS and TNFα in the hippocampus (CA1, DG) and the prefrontal cortex in the injured rats' brains. Similarly, previous studies reported that upregulation of iNOS expression leads to excessive nitric oxide (NO) production, of which excess reacts with superoxide to form eventually a powerful ROS that is suggested to contribute critically to neuron death in TBI as well as in cerebral ischemia (Abe et al., 1995; Ismail et al., 2020; Lee et al., 2019;).

Having demonstrated these cellular and molecular changes in the injured older rat brains, we showed that rTBI caused a neuronal loss that was significantly reduced by allopurinol treatment. Within this context, allopurinol administration reduced iNOS and TNFα expressions on days 6 and 19 post-injury in the brain regions, suggesting improvement in early and late responses to oxidative stress and neuroinflammation. Our finding in terms of improvement in inflammation after TBI was in accordance with the results of a very recent study in which allopurinol administration prior to simulated aeromedical evacuation following TBI has been suggested to improve late post-TBI systemic inflammation in mice (Singer et al., 2021). Moreover, remarkably, reduced oxidative stress and inflammation were observed in parallel to each other in allopurinol-treated rats, in agreement with previous studies in which allopurinol treatment was reported to reduce infarct volume and inhibit oxidative stress significantly and inflammation in the ischemia-induced cerebral injury rodent models (Isik et al., 2005; Martz et al., 1989; Yamaguchi et al., 2015; Zhang et al., 2015). Given the fact that TBI is an independent risk factor for subsequent stroke, our findings are important to support the potential role of allopurinol to prevent the bidirectional effect between cerebral ischemia and TBI. In accordance with this, a very recent study indicated that allopurinol, a well-known medication that has been in use for many years (Strilchuk et al., 2019), accelerates adenosine triphosphate synthesis and increases the reservoir of the neuroprotective metabolite, adenosine, so that it may help reduce the morbidity associated with TBI and stroke (Frenguelli, 2019).

Apart from the aforementioned histopathological changes, rTBI rats exhibited impairments in the context of behavioral and cognitive changes in this study. In the EPM and OF, injured rats spent more time in the closed arms and the corner, respectively, both of which indicate increased anxiety-like behaviors without impaired mobility following rTBI. In contrast, allopurinol-treated rats exhibited more mobile time in the center zone of OF, indicating improvement of anxiety-like behaviors. These anxiolytic effects of allopurinol may be explained with upgraded adenosinergic transmission via increasing circulating pools of adenosine that is thought to reduce the affinity of dopamine agonists for dopamine receptors (Buie et al., 2006). Adenosine, together with the other final metabolites xanthine, hypoxanthine, uric acid, the nucleosides inosine, and guanosine complete the purinergic system (Schmidt et al., 2007). The anxiolytic effects of allopurinol might also be associated with the ability of guanine-based purines, exerted through XO inhibition, in modulating the glutamatergic excitatory system which plays a significant role in some diseases such as anxiety (Vinadé et al., 2003). Moreover, in light of the evidence that the release of adenosine resulted from the stimulation of guanine in cultured astrocytes (Rathbone et al., 1999), one can not exclude that adenosine does not contribute substantially to the anxiolytic guanosine effect. Moreover, in the Morris Water Maze test results, the fact that the allopurinol-treated rats were similar to the control group in terms of time to find the target platform, and swam less distance than rTBI rats to find the platform, provides the data that allopurinol is capable of ameliorating spatial learning and memory deficits following repeated head injuries in older rats. This may be because guanine-based purines modulate glutamatergic parameters,

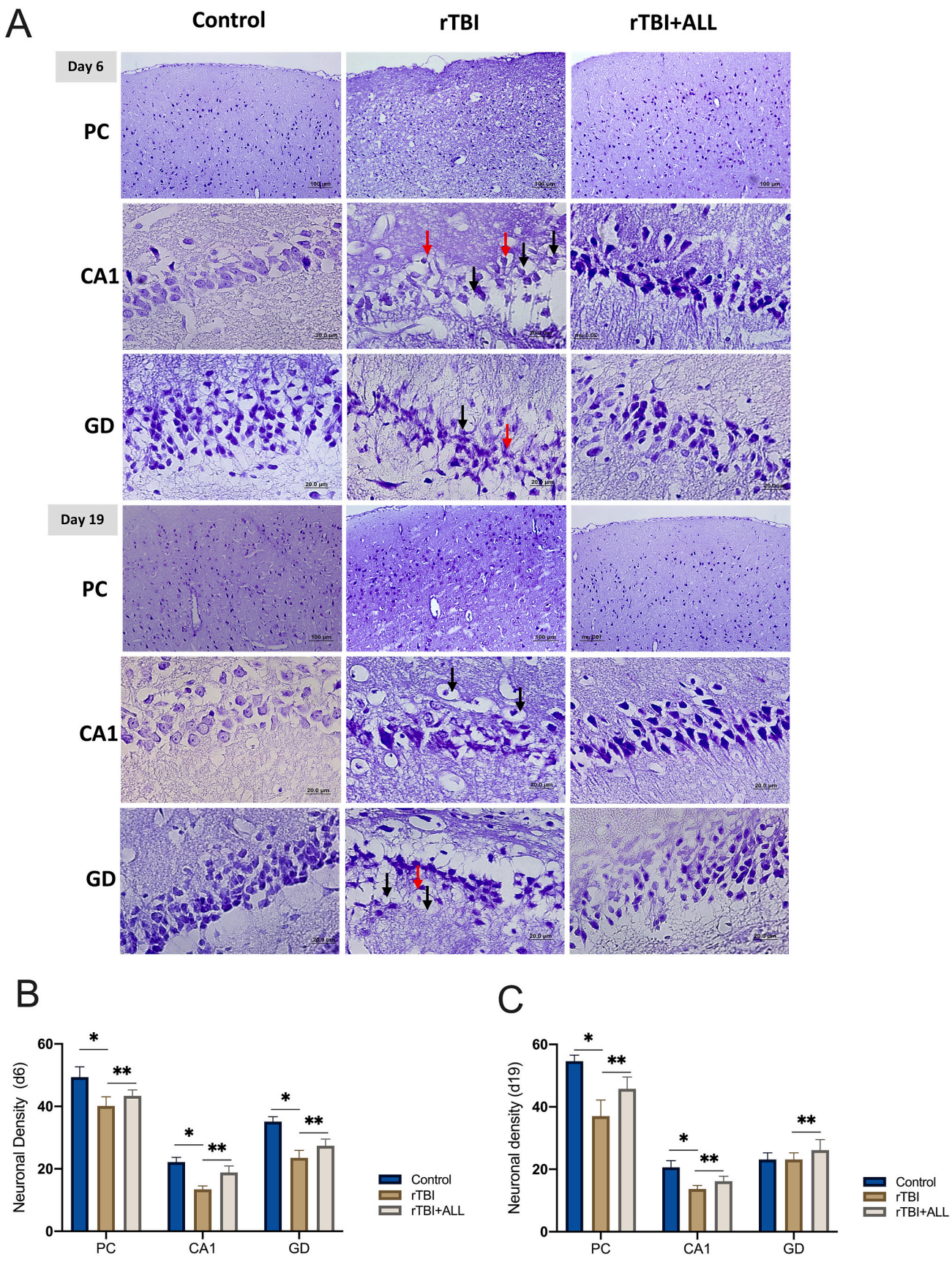


Fig. 3. Effects of Allopurinol treatment on the neuron density in the PC, CA1, and GD on day 6 and day19 in rats. A: Representative coronal section pictures are stained by cresyl violet staining. Red arrows show darkness and small cell, yellows arrows show edema. B: Graph shows neuron density on day 6. C: Graph shows neuron density on day 19. *: Significantly decreased, **: Significantly increased. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

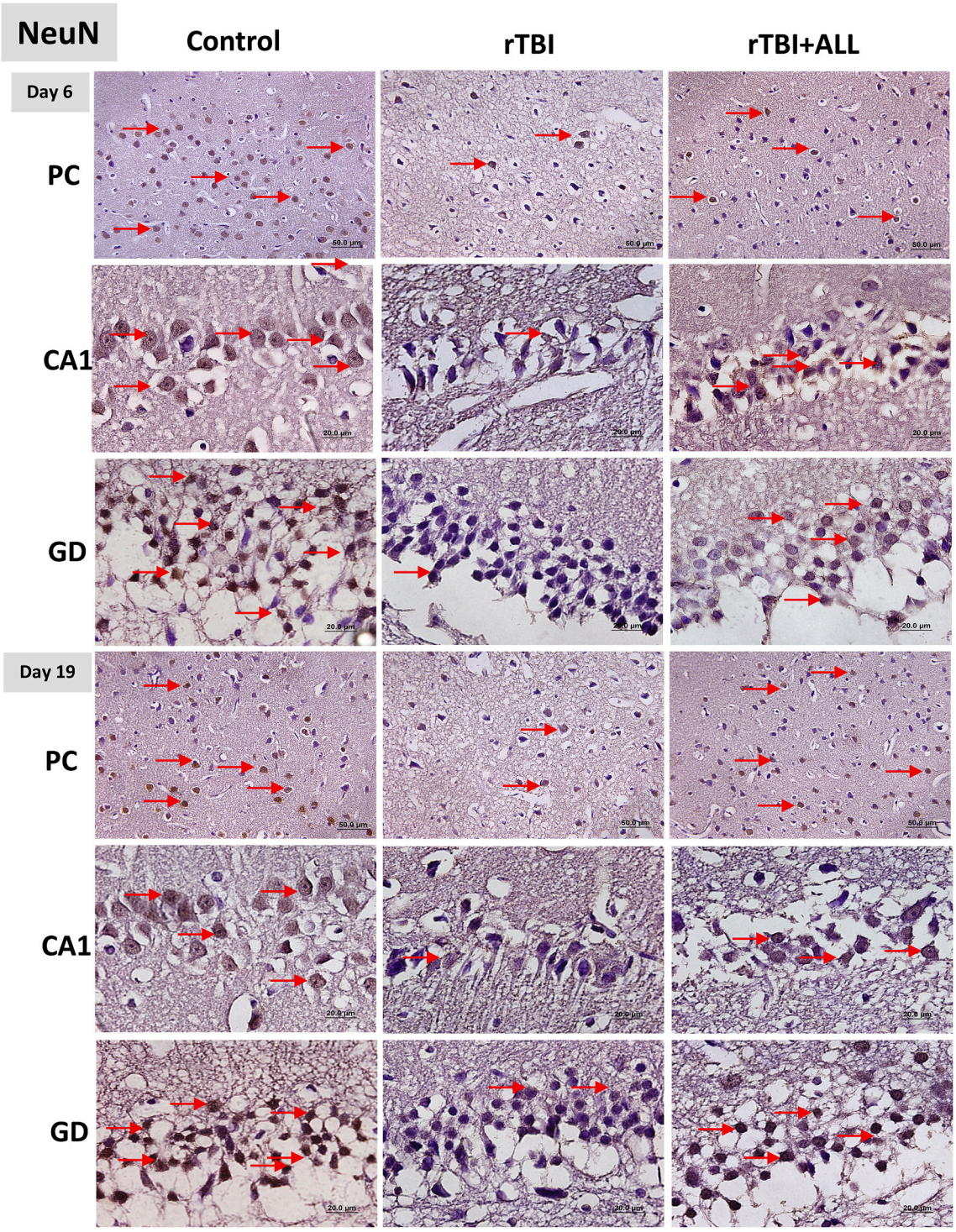


Fig. 4. Effect of Allopurinol treatment on neuron cell loss. The Anti-NeuN antibody was employed to identify neurons 6 and 19 days after injury and in the PC, CA1, and GD regions of the hippocampus. The NeuN (+) cell number in the control and Allopurinol treated group was significantly higher than that in the rTBI group. Red arrows show NeuN positive cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Scoring criteria of Immunohistochemistry (IHC) assay with specific antibodies used in this study.

Group	NeuN			TNF			iNOS		
Day 6	PC	CA1	GD	PC	CA1	GD	PC	CA1	GD
Control	2.28 ± 0.48	2.57 ± 0.53	2.42 ± 0.53	0.42 ± 0.53	0.42 ± 0.53	0.28 ± 0.48	0.57 ± 0.53	0.42 ± 0.57	0.57 ± 0.53
rTBI	0.42 ± 0.53$^{\#}$	0.57 ± 0.53$^{\#}$	0.57 ± 0.53$^{\#}$	2.57 ± 0.53*	2.42 ± 0.53*	2.28 ± 0.48*	2.42 ± 0.53*	2.42 ± 0.53*	2.42 ± 0.53*
rTBI+ALL	1.42 ± 0.53$^{\#\#}$	1.42 ± 0.53$^{\#\#}$	1.57 ± 0.53$^{\#\#}$	1.42 ± 0.54**	1.28 ± 0.48**	1.28 ± 0.48**	1.28 ± 0.48**	1.42 ± 0.53**	1.57 ± 0.53**
Day 19									
Control	2.33 ± 0.51	2.50 ± 0.54	2.50 ± 0.54	0.50 ± 0.54	0.33 ± 0.51	0.33 ± 0.51	0.50 ± 0.54	0.66 ± 0.51	0.66 ± 0.51
rTBI	0.42 ± 0.53$^{\alpha}$	0.57 ± 0.53$^{\alpha}$	0.71 ± 0.53$^{\alpha}$	2.28 ± 0.48	2.42 ± 0.53	2.28 ± 0.48	2.42 ± 0.53	2.28 ± 0.48$^{\beta}$	2.28 ± 0.48$^{\beta}$
rTBI+ALL	1.25 ± 0.70$^{\alpha\alpha}$	1.0 ± 0.75	1.50 ± 0.53$^{\alpha\alpha}$	1.25 ± 0.70$^{\beta\beta}$	1.00 ± 0.75$^{\beta\beta}$	1.00 ± 0.75$^{\beta\beta}$	1.12 ± 0.22$^{\beta\beta}$	1.37 ± 0.51$^{\beta\beta}$	1.50 ± 0.53$^{\beta\beta}$

$^{\#,\ \alpha}$: Significantly decreased in rTBI Group vs Control, $^{\#\#,\ \alpha\alpha}$: Significantly increased in rTBI +ALL Group vs rTBI, $^{*,\beta}$: Significantly incresead in rTBI Group vs Control, $^{**,\beta\beta}$:Significantly decreased in rTBI +ALL Group vs rTBI.

such as glutamate uptake by synaptic vesicles, and are shown to be able to affect learning and memory (Schmidt et al., 2007). Also, purines stimulate the differentiation and process outgrowth from primary cultures of hippocampal neurons. They are also reported to exert trophic effects in vivo following brain injury because high concentrations of guanine were shown to remain extracellularly for up to a week after the injury (Rathbone et al., 1999). Additionally, provided that caspase activation, a pivotal marker for apoptosis (programmed cell death) was reduced with allopurinol treatment, it is suggested to reduce brain injury.

From a clinical perspective, there is still no cure for TBI, and it is a global challenge for all ages, including older adults. Therefore, agents that may mitigate TBI-related negative health consequences related to the neurodegenerative process triggered by recurrent head trauma (Sharp et al., 2014; McKee et al., 2014) are of great importance for clinical geriatric practice, considering that falls are a geriatric syndrome. From a geriatrics perspective, it is clear that older adults with falls may be at risk for chronic traumatic encephalopathy (CTE), which is defined as permanent behavioral and pathological consequences following exposure to repeated mild TBI in a subset of individuals (Gold et al., 2018). Given that repetitive mild TBI is reported to model CTE in human tau mice (Gold et al., 2018; Ojo et al., 2013), allopurinol might be a future therapeutic option as well as for CTE. Furthermore, patients with a TBI history tend to develop the pathophysiological feature of neurodegenerative disorders such as Alzheimer's disease (AD) (Gupta and Sen, 2016). In this respect, it is also worth noting that treating behaviorally and cognitively impaired aged rats with allopurinol improves the performance of these animals in learning and memory tasks and reduces anxiety, such that it might have a role in the management of patients with AD. There is substantial evidence demonstrating uric acid's neuroprotective properties in experimental and clinical studies of neurodegenerative disease (Paganoni and Schwarzschild, 2017; Tuven et al., 2017). Nevertheless, with the evidence that TBI survivors have a major risk of developing neurodegenerative diseases, allopurinol requires further investigation.

As secondary damage is an essential determinant of a patient's outcome, we aimed to assess ALL effects on this stage in older rats in the present study. Our findings are encouraging for further studies on whether observing ALL has similar effects in juvenile or young mouse brains. Besides, previous studies have shown moderate TBI causes both similar and different characteristics of neuronal death in the cortex and in the hippocampus of the juvenile and young adult mice brains (Zhao et al., 2018). Moreover, moderate TBI induces acute synaptic degeneration in the hippocampal dentate gyrus (Gao et al., 2016) and mild TBI induces it in the cerebral cortex (Gao and Chen, 2011). ALL also needs to be further investigated from the perspective of preventive effects on synaptic degeneration (Wu et al., 2018). With no effective treatment for TBI and the disorders caused by it, studies will continue to expand our knowledge of TBI's pathophysiological events in order to potentially serve as the basis to define established novel treatments.

The strengths of this report are that it is the first study evaluating beneficial structural and functional effects of allopurinol following rTBI against secondary brain injury. Moreover, repeated TBI studies have utilized young adult animals, even though age effect is accounted for being one of the most important variables, and repeated injuries are reported to have long-term health effects in the elderly population. Provided that a month for the rats is approximately equivalent to 2.5 human years (Andreollo et al., 2012), the rat's age in this study is roughly equal to 65 years in adulthood. On the other hand, the present study also had several limitations. There were no younger adult animals to compare those and the aged rats, which certainly warrants investigation in further studies. Moreover, all rats were male. Thus, the issue of whether pathological and behavioral changes might differ in gender remains open to be evaluated. Nevertheless, with aging, the potential positive impact of estrogen is minimalized (Frick, 2009). Also, although evidence indicates that observations in mild recurrent head injuries reflect those seen in humans (McCrory et al., 2017), this is yet to be investigated in older humans with repeated head trauma. Additionally, as weight drop models for TBI have limitations regarding biomechanical data (O'Connor et al., 2011), the investigation of allopurinol for head injury is needed to be performed with other TBI models, such as cortical impact injury (CCI) which was reported to produce TBI similar to those seen in humans (Gao and Chen, 2013; Romine et al., 2014). However, since human TBI is a heterogenous disease no single animal model is entirely successful in mimicking the whole spectrum of findings observed in human TBI (O'Connor et al., 2011). The sample size for behavioral testing was low, another limitation of the study. To determine the effect of the drug in non-TBI old male rats, including a sham group receiving only ALL was needed, however, we could not have additional animals because of the pandemic restrictions.

In conclusion, our results suggest that allopurinol might be a promising therapeutic option for TBI in older individuals due to its antioxidant, anti-inflammatory, neuroprotective, and cognitive enhancer properties and improving effect on anxiety-like behaviors. These findings encourage us for further studies to investigate whether it could be a treatment strategy."

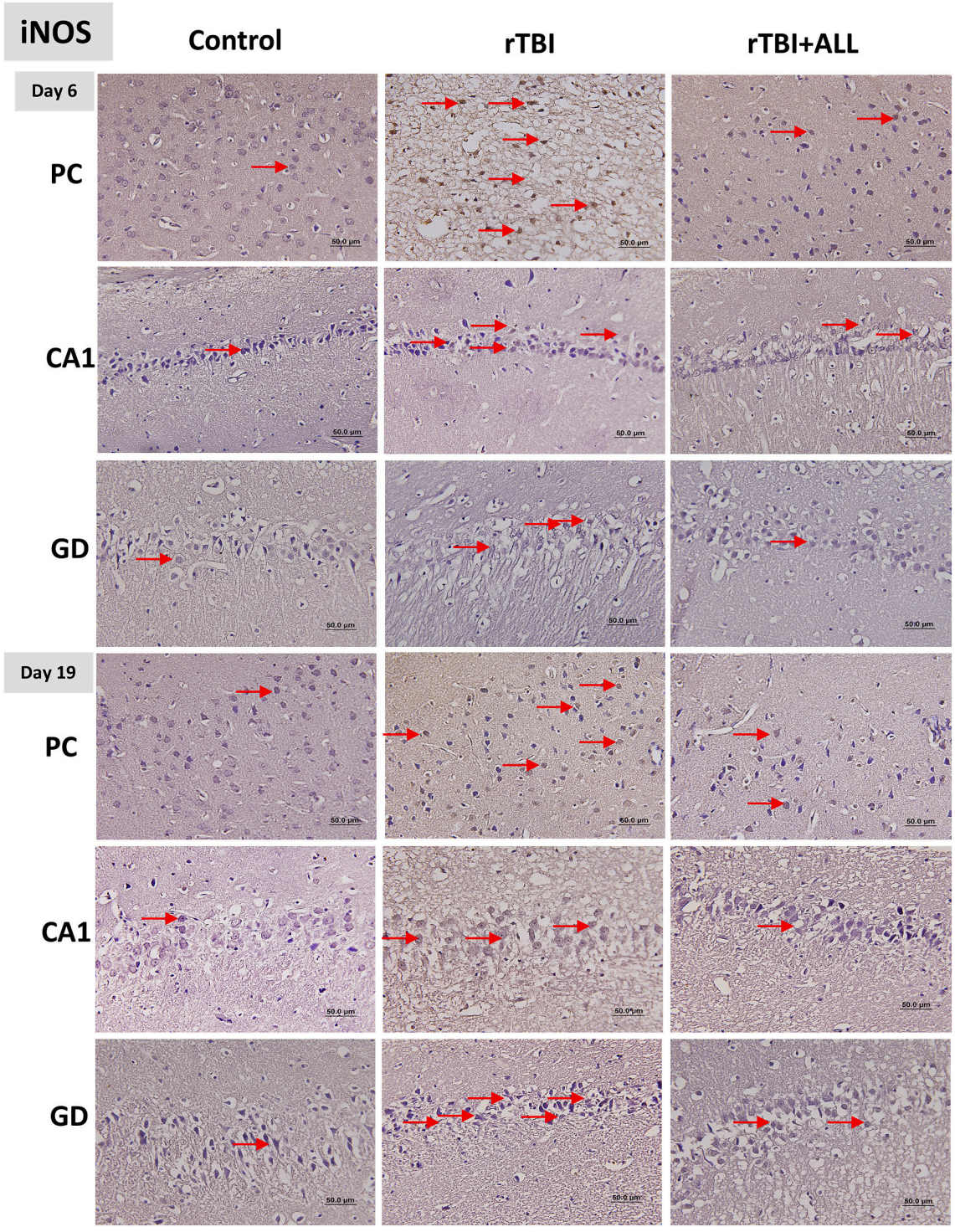


Fig. 5. Effect of Allopurinol treatment on the iNOS in the PC, CA1, and GD on day 6 and day19 in rats. When compared with the Control and rTBI+ALL groups, the number of iNOS positive cells increased in PC, CA1, and GD regions in the rTBI group. In the rTBI+ALL group, the number of positive cells were decreased compared to the rTBI group. Red arrows show iNOS positive cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

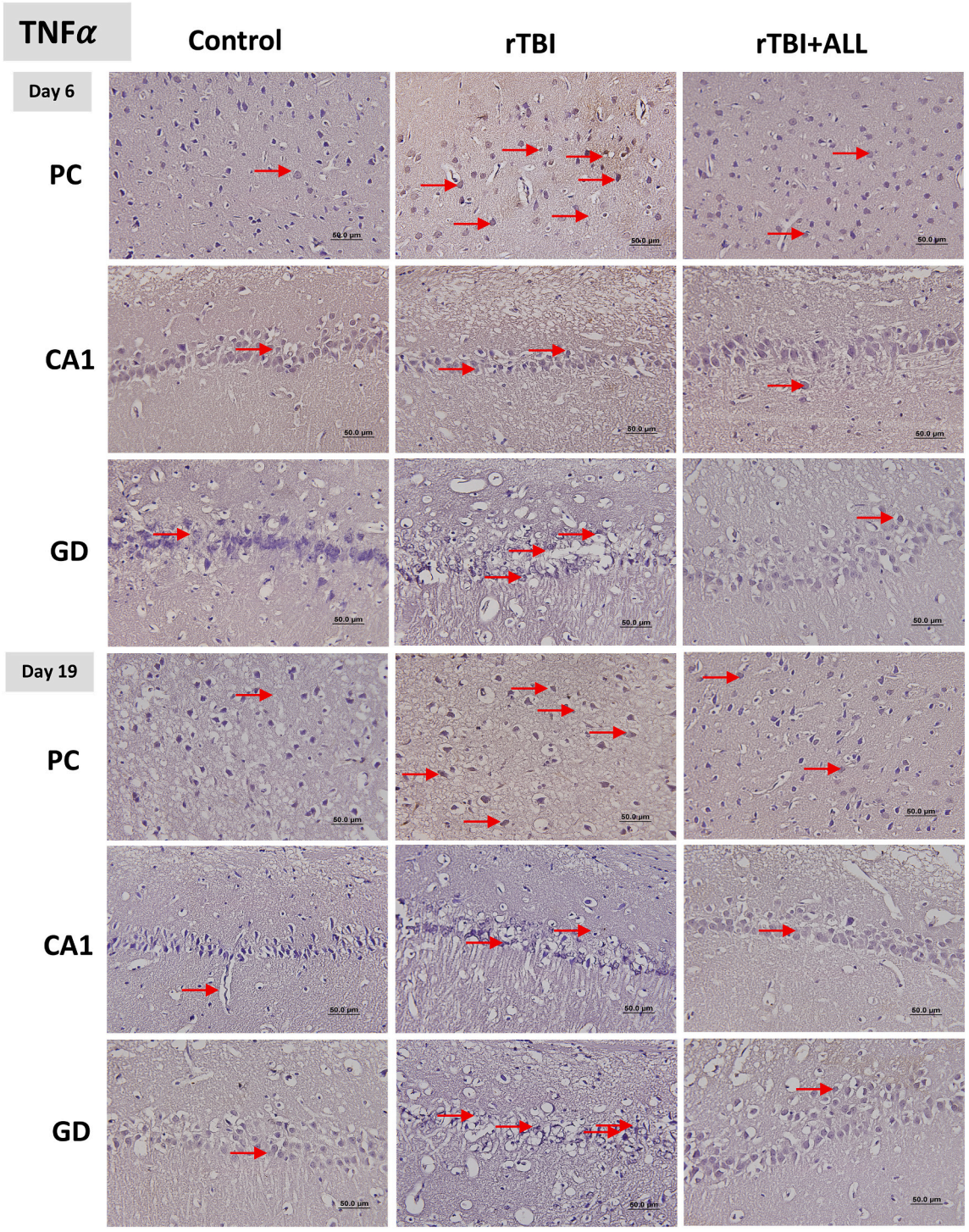


Fig. 6. Effect of Allopurinol treatment on the TNFα in the PC, CA1, and GD on day 6 and day19 in rats. When compared with the group Control and rTBI+ALL groups, the number of TNFα-positive cells increased in PC, CA1, and GD regions in the rTBI group. In the rTBI+ALL group, the number of positive cells were decreased compared to the rTBI group. Red arrows show TNFα-positive cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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CRediT authorship contribution statement

Derya Kaya: Conceptualization, Methodology, Writing – original draft. **Serap Cilaker Micili:** Methodology, Software, Visualization, Investigation. **Ceren Kizmazoglu:** Methodology, Data curation. **Ali Osman Mucuoglu:** Methodology, Data curation. **Sibel Buyukcoban:** Software, Validation. **Nevin Ersoy:** Data curation. **Osman Yilmaz:** Supervision. **Ahmet Turan Isik:** Supervision, Writing – review & editing.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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