Research Article

Teriflunomide provides protective properties after oxygen-glucose-deprivation in hippocampal and cerebellar slice cultures

https://doi.org/10.4103/1673-5374.310689

Date of submission: September 18, 2020

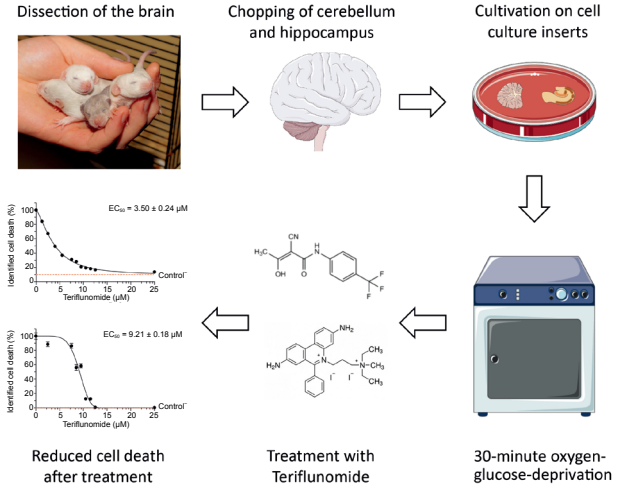
Date of decision: December 14, 2020

Date of acceptance: January 11, 2021

Date of web publication: March 25, 2021

Anna Wolters[1, #], Judith Reuther[1, #], Philipp Gude[2], Thomas Weber[2], Carsten Theiss[1], Heike Vogelsang[2, #], Veronika Matschke[1, *, #]

Graphical Abstract *Post-treatment after an oxygen-glucose-deprivation with teriflunomide shows a protective effect on hippocampal and cerebellar cells in organotypic slice cultures of rats*



Abstract

One of the major challenges in emergency medicine is out-of-hospital cardiac arrest (OHCA). Every year, about 53–62/100 000 people worldwide suffer an out-of-hospital cardiac arrest with serious consequences, whereas persistent brain injury is a major cause of morbidity and mortality of those surviving a cardiac arrest. Today, only few and insufficient strategies are known to limit neurological damage of ischemia and reperfusion injury. The aim of the present study was to investigate whether teriflunomide, an approved drug for treatment of relapsing-remitting-multiple-sclerosis, exerts a protective effect on brain cells in an *in vitro* model of ischemia. Therefore, organotypic slice cultures from rat hippocampus and cerebellum were exposed to oxygen-glucose-deprivation and subsequently treated with teriflunomide. The administration of teriflunomide in the reperfusion time on both hippocampal and cerebellar slice cultures significantly decreased the amount of detectable propidium iodide signal compared with an untreated culture, indicating that more cells survive after oxygen-glucose-deprivation. However, hippocampal slice cultures showed a higher vulnerability to ischemic conditions and a more sensitive response to teriflunomide compared with cerebellar slice cultures. Our study suggests that teriflunomide, applied as a post-treatment after an oxygen-glucose-deprivation, has a protective effect on hippocampal and cerebellar cells in organotypic slice cultures of rats. All procedures were conducted under established standards of the German federal state of North Rhine Westphalia, in accordance with the European Communities Council Directive 2010/63/EU on the protection of animals used for scientific purposes.

Key Words: brain damage; cardiac arrest; cell death; hypoxic chamber; ischemia; organotypic slice cultures; post-treatment; resuscitation

Chinese Library Classification No. R453; R364; R741

Introduction

Cardiac arrest is one of the major global health problems these days. It is estimated that the global incidence of suffering an out-of-hospital cardiac arrest (OHCA) is about 54/100,000 people per year (Berdowski et al., 2010; Myat et al., 2018). Despite worldwide programs for cardiopulmonary resuscitation of bystanders the prognosis of surviving an external heart attack remains low, with a global average survival rate of only 7% (Berdowski et al., 2010). A large number of patients surviving a cardiac arrest show severe

[1]Institute of Anatomy, Department of Cytology, Medical Faculty, Ruhr University Bochum, Bochum, Germany; [2]Department of Anaesthesiology and Intensive Care Medicine, Ruhr University Bochum, St. Josef Hospital, Bochum, Germany

*__Correspondence to:__ Veronika Matschke, Dr.rer.nat., Veronika.Matschke@rub.de.

https://orcid.org/0000-0001-9717-4485 (Veronika Matschke)

#AW und JR contributed equally to the study and share the first authorship. HV and VM contributed equally to the study and share the senior authorship.

How to cite this article: *Wolters A, Reuther J, Gude P, Weber T, Theiss C, Vogelsang H, Matschke V (2021) Teriflunomide provides protective properties after oxygen-glucose-deprivation in hippocampal and cerebellar slice cultures. Neural Regen Res 16(11):2243-2249.*

neurological deficits, where mortality and morbidity after resuscitation mostly depend on the extent of brain injury (Arawwawala and Brett, 2007). Satisfying neurologic outcome is only seen in about 10% of surviving patients (Laver et al., 2004; Mozaffarian et al., 2015).

The brain is very vulnerable to hypoxic damage caused by circulatory arrest because energy reserves to maintain metabolism in poor circulation are very limited (Busl and Greer, 2010; Cronberg, 2017). The concentrations of essential energy sources decrease rapidly after onset of an ischemia causing an anoxic depolarization of the cell (Busl and Greer, 2010) leading to changes in the cellular electrolyte composition and thus a loss of ion gradient, local acidosis and excitotoxic glutamate release (Busl and Greer, 2010). Additionally, rapidly after onset of cardiac arrest, neural ischemic cell death is triggered by reactive oxygen species (ROS) accumulation (Bernard et al., 2002, 2010; Kongpolprom and Cholkraisuwat, 2019). After successful resuscitation and the return of spontaneous circulation, reperfusion injury boosts neuronal death by activating pro-inflammatory cascades leading to necrosis and apoptosis in the brain, inducing remote organ failure (Caplan, 1991; Piantadosi and Zhang, 1996; Busl and Greer, 2010; Minutoli et al., 2016; Cronberg, 2017; Sekhon et al., 2017).

The outcome of hypoxic brain damage is different for every individual and can range from asymptomatic to persistent vegetative states (Piantadosi and Zhang, 1996). One possible explanation is the fact that neurons in some brain areas are more resistant to hypoxic damage than others (Lipton, 1999; Bernard et al., 2010). Neurons of the hippocampus are particularly susceptible to ischemia, followed by neurons of the cerebellum and other brain regions (Lipton, 1999).

Up to date there are no specific drug therapies for neuroprotection against ischemia and reperfusion injury, but there are many well-established drug therapies for the treatment of chronic inflammatory neurologic diseases. Since brain ischemia and reperfusion damage as well as chronic inflammatory diseases partly show common reactions, the question arises whether there is a drug for treatment of inflammatory neurological diseases that could also be beneficial after brain ischemia (Skaper et al., 2018).

Teriflunomide, an oral disease-modifying-therapy drug, integrated in the therapy of relapsing-remitting-multiple-sclerosis (Scott, 2019), may have promising characteristics. The exact mechanism of the numerous effects of teriflunomide is not known yet, but there are several studies concerning leflunomide, being the active metabolite of teriflunomide, trying to elucidate its modes of action. It inhibits the mitochondrial enzyme dihydroorotate dehydrogenase, acts cytostatic to proliferating T- and B-lymphocytes, inhibits the cycloxygenase-2 and tumor necrosis factor-α-mediated nuclear factor kappa-light-chain-enhancer of activated B cells-activation and thereby acts anti-inflammatory in different ways (Cherwinski et al., 1995; Bruneau et al., 1998; Hamilton et al., 1999; Ringheim et al., 2013; Bar-Or, 2014). Furthermore, pre-treatment with teriflunomide diminished pro-inflammatory mediators in activated microglia and astrocytes *in vitro* (Edling et al., 2017).

Since teriflunomide offers the possibility of influencing inflammatory reactions, the question arises whether this drug can also contribute to alleviating cellular brain damage following ischemia. Therefore, the aim of this study was to investigate whether a post-treatment with teriflunomide has a protective effect on cerebellar and hippocampal slice cultures after oxygen-glucose-deprivation (OGD) as an *in vitro* model of ischemia and reperfusion brain injury. A protective effect of teriflunomide could open up new possibilities to reduce the consequences of cardiac arrest on brain damage.

Materials and Methods

Organotypic cell cultures

All procedures were conducted under established standards of the German federal state of North Rhine Westphalia, in accordance with the European Communities Council Directive 2010/63/EU on the protection of animals used for scientific purposes. According to current German and European legislation, the removal of organs or cells from vertebrates for scientific purposes is not considered an animal experiment if the animals have not been subject to surgical interventions or invasive treatments prior to sacrifice. Thus, euthanization of mice intended for the removal of brain tissue does not need an approval or permission by local or governmental authorities.

Organotypic slice cultures from cerebellum and hippocampus of wildtype Wistar rats were prepared, based on the method presented by Stoppini et al. (1991). Therefore, rat pups on their postnatal day 9 (p9) were decapitated, their brains explanted under sterile conditions and kept in Hanks Solution on ice. The cerebelli and the hippocampi of at least six rats of one litter were collected.

The cerebelli and hippocampi were cut into slices (275 µm thickness for cerebellum, 350 µm for hippocampus) by using a McIllwain Tissue Chopper and individually transferred onto Millicell Cell Culture Inserts (hydrophilic PTFE, pore size 0.4 µm; #PICM0RG50, Merck, Darmstadt, Germany), which were preincubated with 1 mL cell culture medium in an incubator at 37°C and 5% CO_2 for 2 hours in six-well plates. Culture medium is based on Dulbeccos Modified Eagle Medium (DMEM; #A14430-01, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 25% heat-inactivated horse serum (#16050-122; Thermo Fisher Scientific), 25 mL Hanks Balanced Salts (NaCl 8 g/L, KCl 0.4 g/L, Na_2HPO_4 0.048 g/L, KH_2PO_4 0.06 g/L, $CaCl_2$•2 H_2O 0.185 g/L, $MgSO_4$ 0.098 g/L, $NaHCO_3$ 0.35 g/L, glucose 1 g/L), glucose 6.5 mg/mL, NGF 2.5 µg/mL (N-0513; Sigma-Aldrich, Darmstadt, Germany), GlutaMax 1 mL (#35050-061; Thermo Fisher Scientific), PenStrep 1 mL (penicillin 10,000 U, Streptomycin 10 mg; #P4333, Sigma-Aldrich) and Phenol Red 10 µg/mL (#P0290; Sigma-Aldrich).

These slices were cultured for 7 days, exchanging half of the cell culture medium for fresh culture medium every 48 hours.

OGD

After seven days in culture (7 div) an OGD was performed. For severely hypoxic conditions slice cultures were inserted into a humidified hypoxic chamber (Hypoxic Chamber Polymer O_2 Control Glove Box; Coy Lab, Grass Lake, MI, USA) at 37°C, < 0.4% O_2, and 5% CO_2. Cell culture medium was removed and replaced by glucose-free-OGD-medium, which has been pregassed for 2 hours in the hypoxic chamber. The cultures were exposed to OGD in the humidified hypoxic chamber for 30 minutes. The OGD was terminated by returning the cultures to an incubator with normoxic conditions (37°C, ~21% O_2 and 5% CO_2) and fresh cell culture media, containing glucose (6.5 mg/mL). Propidium iodide (PI; 5 µg/mL; #P4170; Sigma-Aldrich) was added to the culture medium for identification of dead cells with a permeable membrane, serving as a marker for overall neuronal death. Control slices were kept under normoxic conditions in control-media containing glucose (6.5 mg/mL) at 37°C simultaneously.

Treatment with teriflunomide

Teriflunomide (#SML0936; Sigma-Aldrich) was stored as a stock solution of 50 mM dissolved in dimethyl-sulfoxide (DMSO; #7033; J.T.Baker, Fisher Scientific, Schwerte, Germany) at –20°C.

Teriflunomide was applied in different concentrations (1.25–25 µM) directly after OGD in the reperfusion period to the

culture medium along with glucose and PI. After 24 hours, the medium was switched to cold phosphate-buffered saline (PBS) before fixating the cultures with 4% paraformaldehyde in PBS for 20 minutes at room temperature. After fixation the slices were washed three times with PBS.

The following conditions were investigated in this study: 1) control w/o OGD, w/o treatment; 2) control w/o OGD, w/ treatment (25 µM teriflunomide); 3) OGD w/o treatment; 4) OGD w/treatment (1.25–25 µM teriflunomide). Furthermore, it was investigated whether the solvent DMSO itself had an influence on the slice cultures under control or OGD conditions. Therefore, the cultures were incubated with the highest amount of DMSO (0.2%) after OGD or under control conditions as described above.

Every experiment was performed three times for each of the concentrations and conditions.

Immunofluorescence

Slice cultures were stained with 4′,6-diamidin-2-phenylindol (DAPI; #9542, Sigma-Aldrich) for 30 minutes in the dark at room temperature and then washed three times with PBS. The slices were transferred on glass slides and mounted with Fluoroshield (#F6937, Sigma-Aldrich).

Analysis of the slices was performed using a confocal spinning disc microscope (VisiScope Confocal-Cell Explorer, Visitron Systems GmbH, Puchheim, Germany). Slices were viewed with an 20× objective (Nikon PlanFluor 20×, NA 0,5; Nikon Instruments Europe BV, Amsterdam, Netherlands) and captured in its entirety, including z-stacking, to analyze the total fluorescence of each slice. All images subjected to direct comparisons were taken at the same exposure and laser intensity. Total fluorescence values were measured and compared by using ImageJ software 1.51 s (National Institutes of Health, Bethesda, MD, USA). After obtaining the total fluorescence values for the two channels (PI and DAPI) of all samples, the ratio between PI and DAPI values was determined for each sample by dividing the intensity value of the PI channel by the intensity value of the corresponding DAPI channel. The values obtained were then normalized to the mean value of the OGD-condition without treatment with teriflunomide and displayed as percentage. To obtain the half-maximum effective concentration of teriflunomide, a non-linear regression was performed and the half maximal effective concentration (EC_{50}) value was determined.

Statistical analysis

Statistical analyses of the date were performed as described previously (Röderer et al., 2018). In brief, data represent mean values of at least three independent experiments ± standard error of the mean (SEM). Data between two groups were tested for significance using a two-tailed Student's *t*-test and comparison among multiple groups were performed using one-way analysis of variance, while pairwise comparison within groups was performed by Tukey's multiple comparisons *post hoc* test. Results with $P < 0.05$ were considered statistically significant.

Results

OGD leads to cell death in hippocampal and cerebellar organotypic slice cultures

To investigate whether a 30-minute OGD causes cell death in hippocampal and cerebellar organotypic slice cultures, PI-positive cells were examined in the respective slice cultures. Cell death after an ischemic event of 30 minutes and subsequent 24-hour reperfusion was observed in the OGD group in both hippocampal and cerebellar slice cultures. The distribution pattern of dead cells showed up in the hippocampus mainly in CA1 and to a less extend in CA3 and in the cerebellum in form of diffuse cell nests, mainly located in the granular layer (GC), however a few positive signals were also found in molecular layer (**Figure 1A** and **D**). Interestingly, hippocampal slice cultures have shown cell death in CA1 even under control conditions (**Figure 1A–C**), whereas cerebellar cultures have not shown any PI positive signals under these conditions (**Figure 1D–F**). Looking at the identified cell death in hippocampal slice cultures, cell death after ODG is significantly ($P < 0.0001$) about 10 times higher than under control conditions (**Figure 1C**). Since no cell death can be observed in cerebellar slice cultures under control conditions, the cell death after OGD in this region is highly significant ($P < 0.0001$) about 380 times higher (**Figure 1F**).

Teriflunomide displays protective properties in both hippocampal and cerebellar organotypic slice cultures after OGD

To investigate whether teriflunomide shows protective properties on hippocampal as well as cerebellar organotypic slice cultures after ischemic conditions, the drug was supplemented for 24 hours in the culture medium directly after OGD. Different concentrations (cerebellum 2.5, 7.5, 8.5, 9.5, 10.5, 11.5, 12.5, and 25 µM; hippocampus: 1.25, 2.5, 4, 5.5, 7.5, 8.5, 9.5, 10.5, 11.5, 12.5, and 25 µM) were used to determine the EC_{50} of teriflunomide.

To describe the effect of teriflunomide on cell death the value of PI-intensity in the hippocampal cultures after OGD was set to 100%. A continuous decrease in the number of dead cells was observed especially in CA1 (**Figure 2**). The greatest effect on cell survival, measured in terms of the intensity of the PI signal, can already be observed at low concentrations (1.25–5 µM) of teriflunomide (**Figure 2C**). When higher concentrations are applied, the effect on cell survival can only be seen to a small extent. A treatment with 25 µM teriflunomide reduced the PI-intensity nearly to control conditions to 13.72%. The calculated EC_{50} value for the treatment of hippocampal OGD-damaged cells is 3.53 ± 0.14 µM (**Figure 2C**).

To describe the effect of teriflunomide on cell death in the cerebellar slice cultures the value of PI-intensity in the cerebellar cultures after OGD was set to 100%. A decrease in the intensity of PI-signal was observed in the labeling of cerebellar slice cultures mainly in GC layer (**Figure 3**). In contrast to the effect on hippocampal slice cultures, teriflunomide in low concentrations (2.5–7.5 µM) has almost negligible protective effect as measured by PI signal intensity in the examined cerebellar slice cultures after OGD. In the concentration range between 7.5 and 10.5 µM the protective effect of teriflunomide in cerebellar slice cultures following OGD is strongly evident. When using teriflunomide at a concentration of 12.5 µM, the PI signal intensity is almost reduced to the level of the control conditions (**Figure 3C**). The calculated EC_{50} value for the treatment of cerebellar OGD-damaged cells is 9.22 ± 0.15 µM (**Figure 3C**).

The application of 0.2% DMSO alone in hippocampal as well as cerebellar slice cultures had no effect (**Figures 2B** and **3B**). The application of 25 µM teriflunomide in control hippocampal as well as cerebellar slice cultures that did not receive OGD showed no differences compared to control conditions (**Figures 2B** and **3B**). It can therefore be assumed that teriflunomide has not a cytotoxic or beneficial effect on the cultured cells under normoxic conditions.

Discussion

In our study we implemented a model for *in vitro* ischemia by combining organotypic slice cultures of the hippocampus and the cerebellum of the rat with a deprivation of oxygen and glucose (OGD), attempting to mimic an *in vivo* brain tissue reaction to ischemia.

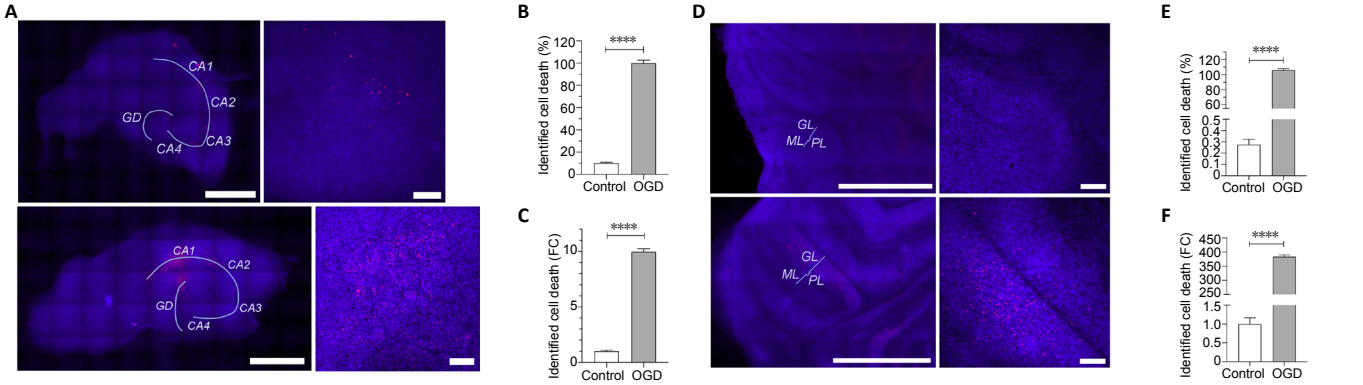


Figure 1 | **A 30-minute OGD induces identifiable cell death in hippocampal and cerebellar organotypic slice cultures.**
(A) Representative images of hippocampal organotypic slice cultures. Shown are control (upper images) and OGD group (lower images). Cell nuclei were stained with DAPI (blue). Identification of dead cells was performed by staining with propidium iodide (red). Pictures were taken using a confocal spinning disc microscope with a 20× objective. Staining showed increased PI-signal in rat hippocampus after 30-minute OGD. PI-positive cells were found mainly in CA1 and the dentate gyrus (gyrus dentatus = GD). Left image: Scale bar: 1000 µm. Right image: Scale bar: 100 µm. (B) Identified cell death in the organotypic slice cultures of the hippocampus in control and OGD group. PI-signal was normalized to DAPI-signal- OGD-treated group was set to 100 % of identified cell death. (C) Identified cell death calculated as fold change [FC] to control conditions. PI-signal was normalized to DAPI-signal. (D) Representative images of cerebellar organotypic slice cultures. Shown are control (upper images) and OGD group (lower images). Cell nuclei were stained with DAPI (blue). Identification of dead cells was performed by staining with propidium iodide (red). Pictures were taken using a confocal spinning disc microscope with a 20× objective. Staining showed increased PI-signal in rat cerebellum after 30-minute OGD. Damage was identified as multiple diffuse cell nests mainly localized in granular layer (GL) and molecular layer (ML). Purkinje cell layer (PL) showed no PI positive signals after OGD. Left image: Scale bar: 1000 µm. Right image: Scale bar: 100 µm. (E) Identified cell death in the organotypic slice cultures of the cerebellum in control and OGD. PI-signal was normalized to DAPI-signal-OGD-treated group was set to 100 % of identified cell death. (F) Identified cell death calculated as fold change [FC] to control conditions. PI-signal was normalized to DAPI-signal. Data are shown as mean ± SEM; ****P < 0.0001 (two-tailed Student's t-test); (B, C) n = 13–26 slice cultures per group, three independent preparations; (E, F) n = 9–18 slice cultures per group, three independent preparations. DAPI: 4′,6-Diamidin-2-phenylindol; OGD: oxygen-glucose-deprivation.

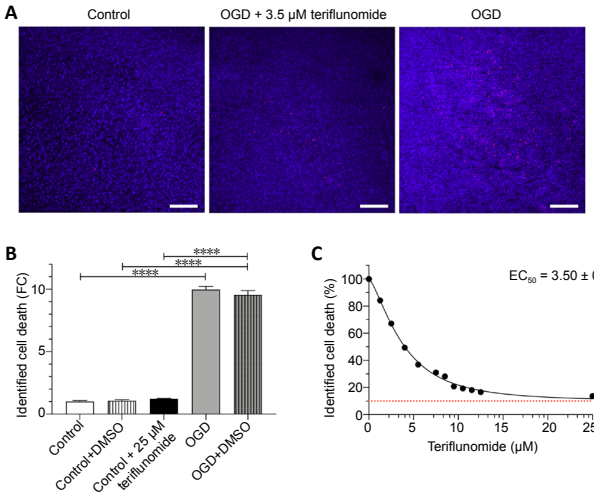


Figure 2 | **Protective effect of teriflunomide on cells of hippocampal organotypic slice cultures after 30-minutes of OGD.**
(A) Representative image of the CA1 area of a hippocampal organotypic slice culture. Shown are the control group (left), an organotypic slice culture after 30 minutes of OGD (middle), and an OGD group after treatment with the half maximal effective concentration (EC_{50}) of teriflunomide [EC_{50} = 3.53 ± 0.14 µM]. The cell nuclei were stained with DAPI (blue). The identification of dead cells with a permeable membrane was performed by staining with propidium iodide (red). Pictures were taken using a confocal spinning disc microscope with a 20× objective. Treatment of the OGD group with teriflunomide leads to a significant reduction of the identified cell death in CA1 and dentate gyrus. Scale bars: 100 µm. (B) Identified cell death in the hippocampal slice cultures after treatment with different concentrations of teriflunomide. PI-signal was normalized to DAPI-signal. Identified cell death was calculated as fold change (FC) to control conditions. The application of DMSO or 25 µM teriflunomide had no effect on the identified cell death under control conditions. After performing OGD, DMSO has no effect on the identified cell death. (C) Concentration dependence of teriflunomide on identified cell death after 30-minute OGD. PI-signal was normalized to DAPI-signal and OGD-treated group was set to 100 % of identified cell death. The identified cell death [%] is significantly reduced with increasing drug concentration of the administered teriflunomide. Data are shown as mean ± SEM; ****P < 0.0001 (one-way analysis of variance with Tukey's multiple comparisons *post hoc* test); n = 3 (B) and 9–26 (C) slice cultures per group, three independent preparations. OGD: Oxygen-glucose-deprivation.

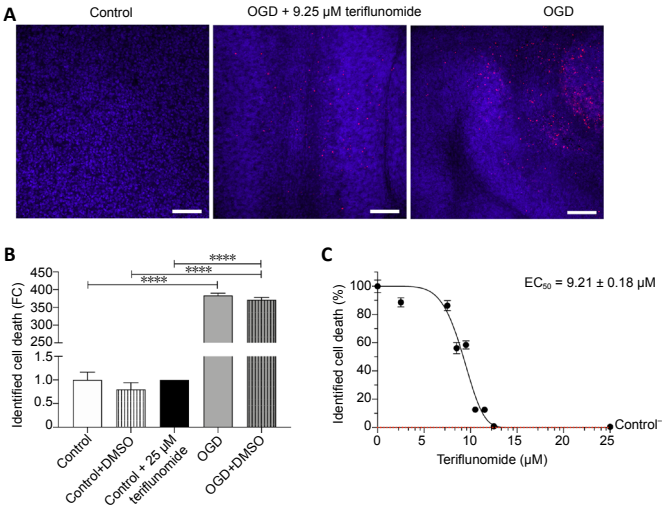


Figure 3 | **Protective effect of teriflunomide on cells of cerebellar organotypic slice cultures after 30-minutes of OGD.**
(A) Representative image of in granular layer and molecular layer of a cerebellar organotypic slice culture. Shown are the control group (left), an organotypic slice culture after 30 minutes of OGD (middle), and an OGD group after treatment with the half maximal effective concentration (EC_{50}) of teriflunomide [EC_{50} = 9.22 ± 0.15 µM]. The cell nuclei were stained with DAPI (blue). The identification of dead cells with a permeable membrane was performed by staining with propidium iodide (red). Pictures were taken using a confocal spinning disc microscope with a 20× objective. The staining showed increased cell death in the rat cerebellar slice cultures after 30-minute of OGD. PI-positive cells were identified as multiple diffuse cell nests mainly in granular and molecular layer. Treatment of the OGD group with teriflunomide leads to a significant reduction of the identified cell death. Scale bars: 100 µm. (B) Identified cell death in the cerebellar slice cultures after treatment with different concentrations of teriflunomide. PI-signal was normalized to DAPI-signal. Identified cell death was calculated as fold change (FC) to control conditions. The application of DMSO or 25 µM teriflunomide had no effect on the identified cell death under control conditions. After performing OGD, DMSO has no effect on the identified cell death. (C) Concentration dependence of teriflunomide on identified cell death after 30-minute OGD. PI-signal was normalized to DAPI-signal and OGD-treated group was set to 100 % of identified cell death. The identified cell death is significantly reduced with increasing drug concentration of the administered teriflunomide. Data are shown as mean ± SEM; ****P < 0.0001 (one-way analysis of variance with Tukey's multiple comparisons post hoc test); n = 3 (B) and 10–21 (C) slice cultures per group, three independent preparations. OGD: Oxygen-glucose-deprivation; PI: propidium iodide.

Several studies showed that organotypic slice cultures are close to *in vivo* tissue conditions (Fedoroff and Richardson, 2001; Humpel, 2015; Li et al., 2016; Doussau et al., 2017), and thus allow experiments on cultures with a preserved three dimensional cytoarchitecture (Stoppini et al., 1991; Fedoroff and Richardson, 2001; Humpel, 2015; Li et al., 2016; Croft et al., 2019), retained neuronal networks (Fedoroff and Richardson, 2001; Humpel, 2015; Li et al., 2016) and upheld cell proportions (Stoppini et al., 1991; Fedoroff and Richardson, 2001; Li et al., 2016). Even small blood vessels e.g. capillaries can be kept in the tissue slice (Moser et al., 2003; Humpel, 2015). Dissociated cells lack integrity as well as complex contacts to each other, which does not depict the natural organism (Fedoroff and Richardson, 2001; Humpel, 2015; Doussau et al., 2017; Croft et al., 2019). Therefore, organotypic slice cultures are more suitable than dissociated cells to investigate complex reactions. A limitation of our model, however, is that diapedesis cannot take place in this system either, since there is no functioning blood circulation as in the whole organism *in vivo*. Further OGD is a widely used and well-suited model to simulate ischemia (Goldberg and Choi, 1993; Strasser and Fischer, 1995; Tasca et al., 2015; Li et al., 2016; Ryou and Mallet, 2018), as they both present circumstances with interrupted supply of oxygen and nutrients. We performed an OGD duration of 30 minutes, since at higher incubation times the damage in the slice cultures was so severe that they were completely destroyed. The question remains open whether this time frame is sufficient to mimic decreased perfusion in living humans.

As cardiac arrests are still one of the major global health problems, leaving many survivors with severe neurological deficits (Busl and Greer, 2010; Sekhon et al., 2017; Welbourn and Efstathiou, 2018), we conducted experiments to contribute to the search for future therapy strategies. Therefore we focused on ischemic damage to the hippocampus and the cerebellum, as both tissue types show a particular susceptibility to hypoxic brain damage *in vitro* models but also *in vivo* in humans (Björklund et al., 2014). There is no specific treatment available yet, which is able to show alleviation of brain damage after a cardiac arrest. Therefore, we searched for potential agents, which might help overcome cell death after ischemia. The study protocol was created in accordance with clinical conditions regarding doses and application time of teriflunomide. We started drug administration immediately after OGD, lasting for 24 hours. In case of clinical use, the emergency physician would also give the medication immediately after successful resuscitation as a posttreatment drug.

Since many studies showed an influence of teriflunomide - an anti-inflammatory drug - on neuronal structures as reviewed by Dekleijin et al. (2020), we considered this drug to be worth investigating if it might help to overcome cell death after ischemia.

Here, we show for the first time a beneficial effect of this compound on organotypic slice cultures after an oxygen-glucose-deprivation by displaying a significant decrease in detectable PI-signal intensity, after application of the drug, compared to cultures subjected to OGD without a post-treatment. Interestingly the EC_{50} values differ between the two brain regions, while the cells of the cerebellum need considerably more teriflunomide to reach its half maximal effect than those of the hippocampus [3.53 µM (hippocampus) *vs*. 9.22 µM (cerebellum)]. Furthermore, in our *in vitro* model of ischemia, we found that the hippocampus is much more vulnerable to OGD than the cerebellum. These findings are consistent with histological studies on brain tissue from patients who did not survive cardiac arrest (Björklund et al., 2014). Björklund et al. (2014) showed that the hippocampus is the most vulnerable brain region and exhibits more damage after cardiac arrest than other parts of the brain, including the cerebellum, which showed an intermediate vulnerability. Several studies found that patients surviving a cardiac arrest suffer from dysfunctions in long-term memory (Sulzgruber et al., 2015; Cronberg, 2017; Caro-Codón et al., 2018). This would be consistent to a possible hippocampal damage acquired during brain ischemia. As a limitation of our study, it must be mentioned that the slice thicknesses differed between the brain regions because of technical limitations. Hippocampal slices were thicker than cerebellar slices (275 µm thickness for cerebellum, 350 µm for hippocampus). This may be of relevance since teriflunomide reached target cells by diffusion. A further limitation also results from the thicker hippocampal slices. Unfortunately, the thickness of the hippocampal slice cultures does not allow complete immunofluorescence staining of the structure with antibodies, so that no differentiation can be made with regard to the vulnerability of the respective cell types.

Different *in vivo* studies showed that ischemia and reperfusion brain injury is mainly caused by inflammatory pathways. A study of Xiang and colleagues (Xiang et al., 2016) on brain injury following cardiac arrest features that one of the key pathways of cerebral injury is inflammation. This is in accordance with findings of Jou and co-workers (Jou et al., 2020) who postulates that inflammation and cytokines play a major role in the pathogenesis of post-cardiac arrest brain injury. Thereby anti-inflammatory agents may offer a beneficial post-treatment strategy. We focused on teriflunomide, an anti-inflammatory drug approved for treatment of relapsing-remitting-multiple-sclerosis since 2013. Teriflunomides numerous modes of action are yet still under discussion. One effect of the active metabolite of teriflunomide, Leflunomide, is the reversible inhibition of the mitochondrial enzyme dihydroorotate dehydrogenase (Cherwinski et al., 1995; Bruneau et al., 1998). This enzyme is particularly active in proliferating cells, which is essential for the *de novo* synthesis of pyrimidines (e.g. cytidine triphosphate, thymidine triphosphate and uridine triphosphate) (Papadopoulou et al., 2012), thus exerting an cytostatic effect due to its importance for DNA- and RNA-synthesis as well as for production of phospholipids and bioactive second messengers (e.g. diacylglycerol, arachidonic acid (Herrmann et al., 2000)), which are important for inflammatory reactions (Gold and Wolinsky, 2011; Papadopoulou et al., 2012). Therefore teriflunomide acts cytostatic to proliferating T- and B-lymphocytes (Ringheim et al., 2013; Bar-Or, 2014). Furthermore is teriflunomide able to reduce the production of pro-inflammatory cytokines, like tumor necrosis factor-α and IL-17, via the Janus-tyrosine-kinases, especially JAK-1 and -3 (González-Alvaro et al., 2009; Papadopoulou et al., 2012). Additionally, Li and colleagues (Li et al., 2013) discovered that teriflunomide can act in a dihydroorotate dehydrogenase-independent way by significantly decreasing the release of pro-inflammatory cytokines and chemokines from activated monocytes. It has been showed that the administration of teriflunomide decreases the proinflammatory cytokine secretion in microglia exposed to human immunodeficiency virus type 1-vector-transduced monocytic cells which is associated with reduced neurotoxicity and enhanced neuronal viability (Ambrosius et al., 2017). A series of experiments conducted by Prabhakara et al. (2018) examined the beneficial aspects of teriflunomide on experimental traumatic brain injury in rats. They demonstrated that teriflunomide is able to decrease inflammation and increases neurogenesis in the hippocampus. In their experiments with live animals they also displayed teriflunomide to be able to decrease the permeability of the blood-brain barrier, preventing edema and thus influx of inflammatory factors and cells, such as microglia and macrophages (Prabhakara et al., 2018). The studies of Wostradowski et al. (2016) suggest teriflunomide has anti-inflammatory properties and reduces microglial proliferation. This fact is confirmed by a recent publication by

Lu et al. (2020). Lu and colleagues demonstrate in a mouse model of transient middle cerebral artery occlusion a positive effect of teriflunomide on neuroinflammation and also milder neurological deficits in mice treated with teriflunomide after transient middle cerebral artery occlusion (Lu et al., 2020). Thus, our results are consistent with new findings of these scientists.

It is known that ischemia and reperfusion injury of the brain is characterized by different phases. In the early phase, predominately oxidative stress disturbs mitochondria followed by activation of inflammasomes and finally complex and cellular inflammatory responses even in remote organs (Eltzschig and Eckle, 2011; Lin and Wang, 2016). It has to be taken into account, that our model does not differentiate between the early and late phases of reperfusion injury since every culture was constantly stopped on the second day after OGD. Nevertheless, we could clearly demonstrate beneficial effects of teriflunomide as a post-treatment drug after OGD. However, it has to be considered that Teriflunomide does not permeate the unimpaired blood-brain-barrier very well. It is estimated that only 1–2% of oral administered Teriflunomide actually reach the CNS (Tallantyre et al., 2008; Limsakun and Menguy-Vacheron, 2010; Wostradowski et al., 2016). So, the delivery to the actual tissue at risk after an ischemia might be problematic.

Conclusion

Organotypic slice cultures as well as oxygen-glucose-deprivation offer a well-established methodology to study the effect of an ischemic event on complex neurological structures. Here, we show for the first time a beneficial effect of teriflunomide on organotypic slice cultures of the hippocampus and cerebellum after an OGD by displaying a significant decrease in detectable PI-signal intensity, after application of the drug, compared to cultures subjected to OGD without a post-treatment. However, hippocampal slice cultures showed a higher vulnerability to ischemic conditions and a more sensitive response to teriflunomide compared to cerebellar slice cultures.

From a clinical point of view, the drug offers promising potential for a targeted treatment of ischemia and reperfusion injury after cardiac arrest. Further studies may proof a potential clinical benefit of teriflunomide in ischemia and reperfusion injury.

Acknowledgments: *The authors gratefully acknowledge Anke Lodwig, Claudia Grzelak, and Anja Harbecke (Institute of Anatomy, Department of Cytology, Medical Faculty, Ruhr University Bochum, Bochum, Germany) for technical assistance, as well as Aja Lenz (Institute of Anatomy, Department of Cytology, Medical Faculty, Ruhr University Bochum, Bochum, Germany) for secretarial work. We acknowledge support by the Open Access Publication Funds of the Ruhr-Universität Bochum.*
Author contributions: *PG, TW, CT, HV, and VM contributed to the study conception and design. Material preparation, data collection and analysis were performed by AW, JR and VM. The first draft of the manuscript was written by AW, JR, HV, and VM and PG, TW, and CT commented on previous versions of the manuscript. All authors read and approved the final manuscript. HV and VM contributed equally to the study and share the senior authorship.*
Conflicts of interest: *The authors declare that they have no conflict of interest.*
Financial support: *None declared.*
Institutional review board statement: *According to current German and European legislation, the removal of organs or cells from vertebrates for scientific purposes is not considered an animal experiment if the animals have not been subject to surgical interventions or invasive treatments prior to sacrifice. Thus, euthanization of mice intended for the removal of brain tissue does not need an approval or permission by local or governmental authorities.*
Copyright license agreement: *The Copyright License Agreement has been signed by all authors before publication.*
Data sharing statement: *Datasets analyzed during the current study are available from the corresponding author on reasonable request.*
Plagiarism check: *Checked twice by iThenticate.*
Peer review: *Externally peer reviewed.*
Open access statement: *This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.*

References

Ambrosius B, Faissner S, Guse K, von Lehe M, Grunwald T, Gold R, Grewe B, Chan A (2017) Teriflunomide and monomethylfumarate target HIV-induced neuroinflammation and neurotoxicity. J Neuroinflammation 14:51.

Arawwawala D, Brett SJ (2007) Clinical review: Beyond immediate survival from resuscitation- Long-term outcome considerations after cardiac arrest. Crit Care 11:235.

Bar-Or A (2014) Teriflunomide (Aubagio®) for the treatment of multiple sclerosis. Exp Neurol 262:57-65.

Berdowski J, Berg RA, Tijssen JGP, Koster RW (2010) Global incidences of out-of-hospital cardiac arrest and survival rates: Systematic review of 67 prospective studies. Resuscitation 81:1479-1487.

Bernard SA, Gray TW, Buist MD, Jones BM, Silvester W, Gutteridge G, Smith K (2002) Treatment of comatose survivors of out-of-hospital cardiac arrest with induced hypothermia. N Engl J Med 346:557-563.

Bernard SA, Smith K, Cameron P, Masci K, Taylor DM, Cooper DJ, Kelly AM, Silvester W (2010) Induction of therapeutic hypothermia by paramedics after resuscitation from out-of-hospital ventricular fibrillation cardiac arrest: A randomized controlled trial. Circulation 122:737-742.

Björklund E, Lindberg E, Rundgren M, Cronberg T, Friberg H, Englund E (2014) Ischaemic brain damage after cardiac arrest and induced hypothermia-a systematic description of selective eosinophilic neuronal death. A neuropathologic study of 23 patients. Resuscitation 85:527-532.

Bruneau JM, Yea CM, Spinella-Jaegle S, Fudali C, Woodward K, Robson PA, Sautès C, Westwood R, Kuo EA, Williamson RA, Ruuth E (1998) Purification of human dihydro-orotate dehydrogenase and its inhibition by A77 1726, the active metabolite of leflunomide. Biochem J 336:299-303.

Busl KM, Greer DM (2010) Hypoxic-ischemic brain injury: Pathophysiology, neuropathology and mechanisms. NeuroRehabilitation 26:5-13.

Caplan LR (1991) Reperfusion of Ischemic Brain: Why and Why Not! In: Thrombolytic therapy in acute ischemic stroke (Hacke W, del Zoppo GJ, Hirschberg M, eds). Berlin, Heidelberg: Springer.

Caro-Codón J, Rey JR, Lopez-de-Sa E, González Fernández Ó, Rosillo SO, Armada E, Iniesta ÁM, Fernández de Bobadilla J, Ruiz Cantador J, Rodríguez Sotelo L, Irazusta FJ, Rial Bastón V, Merás Colunga P, López-Sendón JL (2018) Long-term neurological outcomes in out-of-hospital cardiac arrest patients treated with targeted-temperature management. Resuscitation 133:33-39.

Cherwinski HM, Cohn RG, Cheung P, Webster DJ, Xu YZ, Caulfield JP, Young JM, Nakano G, Ransom JT (1995) The immunosuppressant leflunomide inhibits lymphocyte proliferation by inhibiting pyrimidine biosynthesis. J Pharmacol Exp Ther 275:1043-1049.

Croft CL, Futch HS, Moore BD, Golde TE (2019) Organotypic brain slice cultures to model neurodegenerative proteinopathies. Mol Neurodegener 14:1-11.

Cronberg T (2017) Neuroprognostication of cardiac arrest patients: outcomes of importance. Semin Respir Crit Care Med 38:775-784.

De Kleijn KMA, Martens GJM (2020) Molecular Effects of FDA-Approved Multiple Sclerosis Drugs on Glial Cells and Neurons of the Central Nervous System. Int J Mol Sci 21:4229.

Doussau F, Dupont JL, Neel D, Schneider A, Poulain B, Bossu JL (2017) Organotypic cultures of cerebellar slices as a model to investigate demyelinating disorders. Expert Opin Drug Discov 12:1011-1022.

Edling A, Woodworth L, Agrawal R, Mahan A, Garron T, Hagan N, Siders B (2017) Teriflunomide Impacts Primary Microglia and Astrocyte Functions In Vitro (P2.348). Neurology 88:P2.348.

Eltzschig HK, Eckle T (2011) Ischemia and reperfusion-from mechanism to translation. Nat Med 17:1391-1401.

Fedoroff S, Richardson A (2001) Protocols for neural cell culture, 3rd ed. Totowa, NJ: Humana Press.

Gold R, Wolinsky JS (2011) Pathophysiology of multiple sclerosis and the place of teriflunomide. Acta Neurol Scand 124:75–84.

Goldberg MP, Choi DW (1993) Combined oxygen and glucose deprivation in cortical cell culture: Calcium-dependent and calcium-independent mechanisms of neuronal injury. J Neurosci 13:3510–3524.

González-Alvaro I, Ortiz AM, Domínguez-Jiménez C, Aragón-Bodi A, Díaz Sánchez B, Sánchez-Madrid F (2009) Inhibition of tumour necrosis factor and IL-17 production by leflunomide involves the JAK/STAT pathway. Ann Rheum Dis 68:1644-1650.

Hamilton LC, Vojnovic I, Warner TD (1999) A771726, the active metabolite of leflunomide, directly inhibits the activity of cyclo-oxygenase-2 in vitro and in vivo in a substrate-sensitive manner. Br J Pharmacol 127:1589-1596.

Herrmann ML, Schleyerbach R, Kirschbaum BJ (2000) Leflunomide: An immunomodulatory drug for the treatment of rheumatoid arthritis and other autoimmune diseases. Immunopharmacology 47:273-289.

Humpel C (2015) Organotypic brain slice cultures: A review. Neuroscience 305:86-98.

Jou C, Shah R, Figueroa A, Patel JK (2020) The role of inflammatory cytokines in cardiac arrest. J Intensive Care Med 35:219-224.

Kongpolprom N, Cholkraisuwat J (2019) Long term survival and functional neurological outcome in conscious hospital survivors undergoing therapeutic hypothermia. Indian J Crit Care Med 23:20-26.

Laver S, Farrow C, Turner D, Nolan J (2004) Mode of death after admission to an intensive care unit following cardiac arrest. Intensive Care Med 30:2126-2128.

Li L, Liu J, Delohery T, Zhang D, Arendt C, Jones C (2013) The effects of teriflunomide on lymphocyte subpopulations in human peripheral blood mononuclear cells in vitro. J Neuroimmunol 265(1-2):82-90.

Li Q, Han X, Wang J (2016) Organotypic hippocampal slices as models for stroke and traumatic brain injury. Mol Neurobiol 53:4226-4237.

Limsakun T, Menguy-Vacheron F (2010) Pharmacokinetics of oral teriflunomide, a novel oral disease-modifying agent under investigation for the treatment of multiple sclerosis. Neurology 74:A415.

Lin L, Wang X (2016) Ischemia-reperfusion injury in the brain: mechanisms and potential therapeutic strategies. Biochem Pharmacol Open Access 5:213.

Lipton P (1999) Ischemic cell death in brain neurons. Physiol Rev 79:1431-1568.

Lu Z, Zhang D, Cui K, Fu X, Man J, Lu H, Yu L, Gao Y, Liu X, Liao L, Li X, Liu C, Zhang Y, Zhang Z, Wang J (2020) Neuroprotective action of teriflunomide in a mouse model of transient middle cerebral artery occlusion. Neuroscience 428:228-241.

Minutoli L, Puzzolo D, Rinaldi M, Irrera N, Marini H, Arcoraci V, Bitto A, Crea G, Pisani A, Squadrito F, Trichilo V, Bruschetta D, Micali A, Altavilla D (2016) ROS-mediated NLRP3 inflammasome activation in brain, heart, kidney, and testis ischemia/reperfusion injury. Oxid Med Cell Longev 2016:2183026.

Moser KV, Schmidt-Kastner R, Hinterhuber H, Humpel C (2003) Brain capillaries and cholinergic neurons persist in organotypic brain slices in the absence of blood flow. Eur J Neurosci 18:85-94.

Mozaffarian D Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, de Ferranti S, Després JP, Fullerton HJ, Howard VJ, Huffman MD, Judd SE, Kissela BM, Lackland DT, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Matchar DB, McGuire DK, et al. (2015) Heart disease and stroke statistics-2015 update: A report from the American Heart Association. Circulation 131:e29-322.

Myat A, Song KJ, Rea T (2018) Out-of-hospital cardiac arrest: current concepts. Lancet 391:970-979.

Papadopoulou A, Kappos L, Sprenger T (2012) Teriflunomide for oral therapy in multiple sclerosis. Expert Rev Clin Pharmacol 5:617-628.

Piantadosi CA, Zhang J (1996) Mitochondrial generation of reactive oxygen species after brain ischemia in the rat. Stroke 27:327-331.

Prabhakara KS, Kota DJ, Jones GH, Srivastava AK, Cox CS, Olson SD (2018) Teriflunomide modulates vascular permeability and microglial activation after experimental traumatic brain injury. Mol Ther 26:2152-2162.

Ringheim GE et al. (2013) Teriflunomide attenuates immunopathological changes in the dark agouti rat model of experimental autoimmune encephalomyelitis. Front Neurol 4:169.

Röderer P, Klatt L, John F, Theis V, Winklhofer KF, Theiss C, Matschke V (2018) Increased ROS level in spinal cord of Wobbler mice due to Nmnat2 downregulation. Mol Neurobiol 55:8414-8424.

Ryou M gwi, Mallet RT (2018) An in vitro oxygen–glucose deprivation model for studying ischemia–reperfusion injury of neuronal cells. In: Methods in Molecular Biology 1717:229-235.

Scott LJ (2019) Teriflunomide: A review in relapsing-remitting multiple sclerosis. Drugs 79:875-886.

Sekhon MS, Ainslie PN, Griesdale DE (2017) Clinical pathophysiology of hypoxic ischemic brain injury after cardiac arrest: A “two-hit” model. Crit Care 21:90.

Skaper SD, Facci L, Zusso M, Giusti P (2018) An inflammation-centric view of neurological disease: Beyond the neuron. Front Cell Neurosci 12:72.

Stoppini L, Buchs PA, Muller D (1991) A simple method for organotypic cultures of nervous tissue. J Neurosci Methods 37:173-182.

Strasser U, Fischer G (1995) Quantitative measurement of neuronal degeneration in organotypic hippocampal cultures after combined oxygen/glucose deprivation. J Neurosci Methods 57:177-186.

Sulzgruber P, Kliegel A, Wandaller C, Uray T, Losert H, Laggner AN, Sterz F, Kliegel M (2015) Survivors of cardiac arrest with good neurological outcome show considerable impairments of memory functioning. Resuscitation 88:120-125.

Tallantyre E, Evangelou N, Constantinescu CS (2008) Spotlight on teriflunomide. Int MS J 15:62-68.

Tasca CI, Dal-Cim T, Cimarosti H (2015) In vitro oxygen-glucose deprivation to study ischemic cell death. Methods Mol Biol 1254:197-210.

Welbourn C, Efstathiou N (2018) How does the length of cardiopulmonary resuscitation affect brain damage in patients surviving cardiac arrest? A systematic review. Scand J Trauma Resusc Emerg Med 26:77.

Wostradowski T, Prajeeth CK, Gudi V, Kronenberg J, Witte S, Brieskorn M, Stangel M (2016) In vitro evaluation of physiologically relevant concentrations of teriflunomide on activation and proliferation of primary rodent microglia. J Neuroinflammation 13:250.

Xiang Y, Zhao H, Wang J, Zhang L, Liu A, Chen Y (2016) Inflammatory mechanisms involved in brain injury following cardiac arrest and cardiopulmonary resuscitation. Biomed Rep 5:11-17.

C-Editors: Zhao M, Li CH; T-Editor: Jia Y