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Research Paper

Combination antioxidant therapy prevents epileptogenesis and modifies chronic epilepsy

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ABSTRACT

Many epilepsies are acquired conditions following an insult to the brain such as a prolonged seizure, traumatic brain injury or stroke. The generation of reactive oxygen species (ROS) and induction of oxidative stress are common sequelae of such brain insults and have been shown to contribute to neuronal death and the development of epilepsy. Here, we show that combination therapy targeting the generation of ROS through NADPH oxidase inhibition and the endogenous antioxidant system through nuclear factor erythroid 2-related factor 2 (Nrf2) activation prevents excessive ROS accumulation, mitochondrial depolarisation and neuronal death during *in vitro* seizure-like activity. Moreover, this combination therapy prevented the development of spontaneous seizures in 40% of animals following status epilepticus (70% of animals were seizure free after 8 weeks) and modified the severity of epilepsy when given to chronic epileptic animals.

1. Introduction

There is burgeoning evidence that oxidative stress plays a key role in acute neurological injury such as prolonged seizures [1], stroke [2] and traumatic brain injury [3], and in neurodegenerative disease such as Parkinson's [4] and Alzheimer's disease [5,6]. Indeed, oxidative stress during and after seizures is instrumental in immediate and longer-term excitotoxic neuronal death [7,8]. Following prolonged seizures, there is evidence that inhibiting oxidative stress not only neuroprotects but also modifies the development of epilepsy [9–11].

Oxidative stress occurs following an imbalance in oxidation state due to excessive production of free radicals or their products [e.g. reactive oxygen species (ROS)], and decreased levels/functions of cellular antioxidant systems [12]. Oxidative stress can therefore be reduced by inhibiting the production of ROS, by the use of exogenous antioxidants or by promoting endogenous antioxidant systems, in particular through the activation of the transcription factor nuclear factor erythroid 2 - related factor 2 (Nrf2) [13]. We have recently shown that activation of Nrf2 through inhibition of Kelch ECH associating protein 1 (KEAP1) can suppress the development of epilepsy in rat temporal lobe epilepsy (TLE) models alone or in combination with an exogenous antioxidant [9,10]. However, we hypothesise that a more effective strategy would be both to activate endogenous antioxidant systems and also to inhibit the production of seizure-induced ROS.

Mitochondria and ROS producing enzymes, such as NADPH oxidase (NOX), are the main intracellular sources of ROS. Previously, mitochondria were proposed to be the major generator of ROS during epileptiform activity [14,15]. However, recent data from our group and others have shown that NOX is the main source for ROS generation during seizure-like activity and has a pivotal role in neurodegeneration and in cell death seen in several animal models of epilepsy [16–20]. The observation that persistent oxidative stress is present in acquired and genetic epilepsies suggest that an effective antioxidant strategy could potentially also have disease modifying effects in established epilepsy [1].

Here we show that NOX inhibition coupled with stimulating cell antioxidant defences through increasing Nrf2 availability is a particularly potent strategy for preventing epileptogenesis and modifying chronic epilepsy.

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Abbreviations

aCSF	Artificial cerebrospinal fluid
AEBSF	4-(2-aminoethyl)-benzenesulfonyl fluoride
ECoG	Electrocorticography
HEt	Dihydroethidium
KA	Kainic acid
KEAP1	Kelch ECH associating protein 1
FCCP	Carbonylcyanide-p-trifluoromethoxyphenyl hydrazine;
NOX	NADPH oxidase
NOX2	NADPH oxidase 2
Nrf2	Nuclear factor erythroid 2-related factor 2
Rh123	Rhodamine123
ROS	Reactive oxygen species
SE	Status epilepticus
TAC	Total antioxidant capacity
TLE	Temporal lobe epilepsy

2. Methods and materials

2.1. Cortical cell cultures

Mixed cortical neurons and glial cells cultures were prepared from postnatal (P0–P1) Sprague-Dawley rat pups (UCL breeding colony) according to a modified protocol described by Haynes [21]. Briefly, the pups were sacrificed by cervical dislocation, and rat brains were quickly removed; neocortical tissue was isolated and submerged in ice-cold HBSS (Ca^{2+}, Mg^{2+}-free, ThermoFisher, Invitrogen, Paisley, UK). The tissue was treated with 1% trypsin for 10 min at 37 °C to dissociate cells. The final neuronal cell suspension was plated on 25 mm round coverslips coated with poly-L-lysine (1 mg/ml, Sigma), and cultured in Neurobasal A medium supplemented with B-27 (ThermoFisher, Invitrogen) and 2 mM L-glutamine.

Neocortical cultures were fed once a week and maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C in a tissue culture incubator.

The cultures were used for experiments at 13–17 days *in vitro* (DIV). Neurons were distinguished from glia by their typical appearance using phase-contrast imaging.

2.2. Recording solutions

All experiments were performed at room temperature, unless otherwise mentioned, in an HEPES-buffered salt solution (aCSF), composition (in mM): 125 NaCl, 2.5 KCl, 2 $MgCl_2$, 1.25 KH_2PO_4, 2 $CaCl_2$, 30 glucose and 25 HEPES, pH adjusted to 7.4 with NaOH.

Experiments were carried out in the HEPES buffered salt solution including (aCSF) or excluding $MgCl_2$ (low-Mg^{2+}).

2.3. Imaging of mitochondrial membrane potential ($\Delta\psi m$)

Rhodamine123 (Rh123) (Sigma, UK) (1 μM) was loaded into the culture dishes for 15 min before imaging. Cells were then washed 3 times prior to recordings. An increase of Rh123 signal indicates depolarization of mitochondria. Rh123 signals were normalized to the baseline level (set 0) and maximum signal produced by mitochondrial oxidative phosphorylation uncoupling with carbonylcyanide-p-trifluoromethoxyphenyl hydrazone (FCCP, 1 μM; set to 100). Cells were then washed 3 times prior to recordings. Experiments were repeated at 6 times from 3 different cultures.

2.4. Imaging of intracellular ROS generation

To evaluate rates of ROS production in the cytosol, dihydroethidium (HEt; 5 μM) was present in all solutions throughout the experiments. No pre-incubation was used to avoid accumulation of oxidized products. Experiments were conducted using 3 separate cultures and repeated on 5–6 coverslips.

2.5. Neuronal death

Neurotoxicity was determined following incubation with low-Mg^{2+} for 2 h at 37 °C, by co-staining cells with propidium iodide (20 μM) and Hoechst 33342 (4.5 μM) (Sigma, St. Louis, MO) in a fluorescent live/dead assay. Experiments were conducted on 3 separate cortical cultures and repeated on 7 coverslips for each treatment. In each treated culture coverslip, 5 random fields were counted.

2.6. Live imaging and analysis

Fluorescence images were obtained on an epifluorescence inverted microscope with a 20 × fluorite objective. Excitation light provided by a xenon arc lamp, the beam passing monochromator at 380,490 or 530 nm (Cairn Research, Kent, UK). Emitted fluorescence was detected by a cooled CCD camera (Retiga; QImaging).

An increase of Rhodamine123 signal indicates depolarization of mitochondria. Rhodamine123 signals were normalized to the baseline level (set 0) and maximum signal produced by mitochondrial oxidative phosphorylation uncoupling with carbonylcyanide-p-trifluoromethoxyphenyl hydrazone (FCCP, 1 μM; set to 100). Phototoxicity and photobleaching of cells was minimized by limiting light exposure to the time of acquisition of the images. Fluorescent images were acquired with a frame interval of 10 s. Data were analyzed using Andor software (Belfast, UK).

HEt was excited by illumination at 530 nm. For most of the experiments, we chose to perform measurements of ROS production rates with HEt at a single wavelength, first to avoid photobleaching and phototoxicity from excitation of cells in the range of UV light. Rates of ROS increase were calculated at different time points (2, 10 and 15 min) after exposure to low-Mg^{2+} and were compared with rates recorded during a 1–3 min aCSF exposure period referred to as baseline.

2.7. Animals and surgery

Animal experiments were conducted in accordance with the Animals (Scientific Procedures) Act 1986, and approved by the local ethics committee. Male Sprague-Dawley rats (220–310 g) were individually housed with free access to food and water in 12 h light/dark cycles throughout the study. Animals were acclimatised to the animal house for at least 7 days before experimental use.

Male Sprague–Dawley rats were anaesthetized using isoflurane and placed in a sterotaxic frame (Kopf, CA, USA). At the start of surgery, Buprenorphine (0.2 mg/kg; SC) and Metacam (1 mg/kg; SC) were administered for pain relief. An electrocorticography (ECoG) transmitter (A3028, Open Source Instruments) [30] was implanted subcutaneously. A subdural intracranial recording electrode was positioned above the right hippocampus [2.5 mm lateral and 4 mm posterior of bregma [22]], and a reference electrode was implanted in the contralateral hemisphere (2.5 mm lateral and 6 mm posterior of bregma). The electrodes were fixed to the skull with three skull screws and tissue glue, followed by dental cement. Immediately after surgery, 3–5 ml of warmed Ringer's solution and Amoxicillin (Betamox LA, 100 mg/kg) was administered subcutaneously. Rats recovered for 7–10 days before initiation of the experiment. Rats were housed separately in Faraday cages and ECoG was recorded continuously for up to 18 weeks post-surgery.

2.8. Kainic acid-induced status epilepticus

Status epilepticus (SE) was induced using kainic acid (KA) according to a previously described protocol [23]. Briefly, rats were hourly injected intraperitoneally with KA (Tocris Bioscience, Bristol, UK) dissolved in sterile 0.9% saline (10 mg/ml) at a dose of 5 mg/kg until class III, IV, or V seizures were evoked (scored according to a modified Racine's scale [24,25]. Once an animal began showing excessive inactivity or excessive activity (i.e. exaggerated running or jumping), or had more than 10 class IV/V seizures/h, subsequent injections were delayed or reduced to 2.5 mg/kg to avoid excessive toxicity and mortality. The endpoint for KA treatment was considered either when animals have reached class V seizure (i.e. excessive rearing with concomitant forelimb clonus and fall over) or when the total dose of KA reached 45 mg/kg.

Animals were included in the study if there was continuous epileptiform activity for 2 h, during which spike frequencies were more than 2 Hz, and spike amplitude was at least 3 times background. For rats that were not ECoG monitored, duration of SE was measured based on behavioural manifestation. Start of the status was considered when the rat experienced full motor seizure with loss of postural control and falling.

2.9. Drugs administration

Following 2 h of status epilepticus, rats were randomised to treatment with either vehicle (10% DMSO/sterile saline), AEBSF (Sigma, 50 mg/kg), RTA 408 (25 mg/kg) or a combination of both drugs. RTA 408 was synthesized by Reata Pharmaceuticals, Inc. In the multiple dose experiment, control animals were treated with equivalent number and volume of injections of vehicle.

2.10. Electrocorticography analysis

The electrocorticography (ECoG) was acquired from a subdural electrode continuously for 24 h, 7 days per week, using hardware and software from Open Source Instruments (for details of electrode placement please refer to section Surgery). The seizure detection analysis was performed as previously described [10]. Briefly, ECoG was segmented into 4 s epochs and six different metrics calculated (power, intermittency, coastline, coherence, asymmetry, and power between 12 and 30 Hz). Each metric was mapped onto an interval of 0–1, and subsequently compared to a user-generated seizure library, consisting of seizures of at least 3 different animals. Animal specific libraries were generated as soon as at least 3 seizures per animal were detected. Epochs were mapped as seizure-like events, if the Euclidean distance was less than 0.2 of a validated seizure epoch. If a seizure was detected by fewer than three 4 s epochs, the seizure was added to the library. All seizures were validated by a researcher blinded to treatment. Seizures were characterized by the appearance of high frequency (> 2 Hz) spikes with progression of the spike frequency, rhythmic spike wave discharges with amplitudes at least three times that of baseline ECoG that lasted for a minimum of 10 s. All electrographic seizures were confirmed by visual inspection. A selection of > 60% of seizures were also characterised by video monitoring in order to determine the behavioural correlate of the ECoG changes, using a time-locked CCTV camera system, by a researcher blinded to treatment.

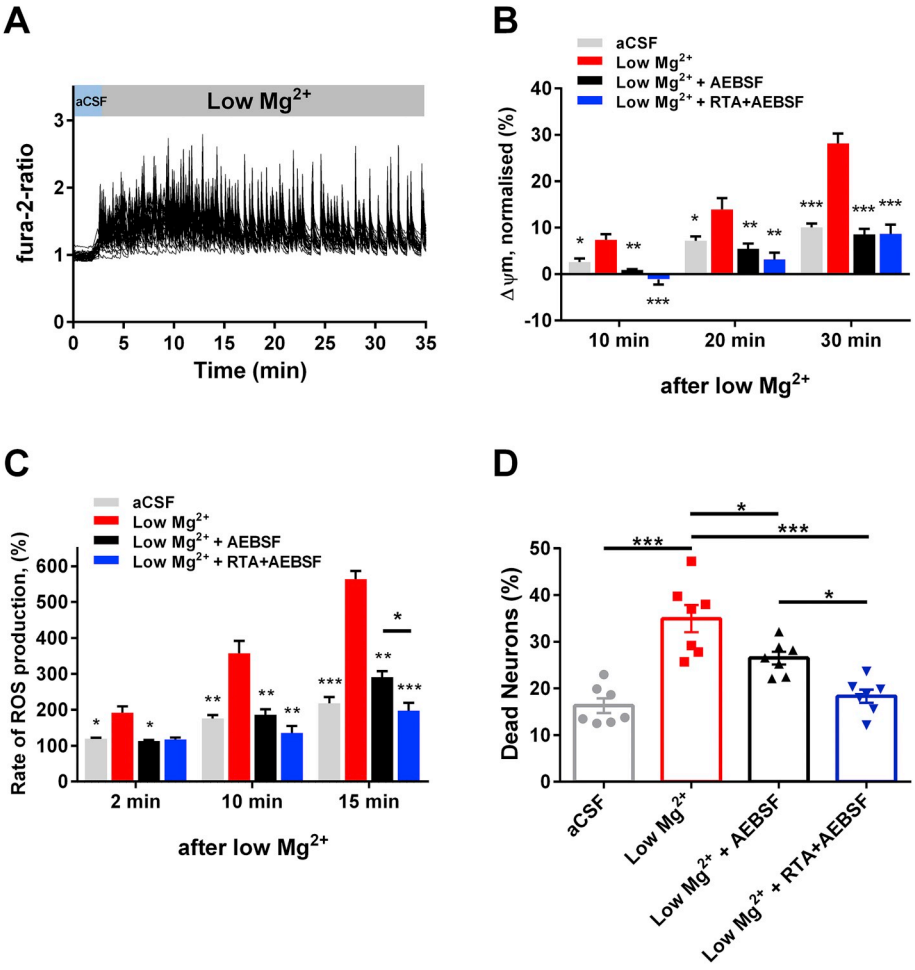

Fig. 1. Combination of antioxidant therapies is neuroprotective *in vitro*.
(A) Representative image of synchronous oscillatory Ca^{2+} signal in neurons indicating seizure like activity induced by replacement of artificial CSF (aCSF) with low Mg^{2+} aCSF. **(B)** Normalised Rhodamine123 fluorescence of neurons 10, 20 and 30 min treated with aCSF (n = 6 experiments (exp.)], low Mg^{2+} (n = 6 exp.), and treated acutely with AEBSF either alone (50 μM; n = 6 exp.) or with RTA (RTA 408, 200 nM, preincubation for 24 h; n = 6 exp., $F(3,20) = 30.201$, $P < 0.001$). **(C)** Normalized rates of ROS generation at different time points in neurons exposed to aCSF (n = 5 exp.), low Mg^{2+} (n = 5 exp.) or neurons treated acutely with AEBSF either alone (50 μM; n = 6 exp.) or with RTA 408 (200 nM, preincubation for 24 h; n = 6 exp., $F(3,18) = 61.299$, $P < 0.001$). Note that the combination therapy has a greater effect at later time point (15 min) than AEBSF only ($P = 0.015$). **(D)** The percentage of neuronal death in cultures following 2 h exposure to aCSF (n = 7), low Mg^{2+} (n = 7), and treatment with AEBSF either alone (50 μM; n = 7 exp.) or with RTA 408 (200 nM, preincubation for 24 h; n = 7 exp., $F(3,24) = 19.699$, $P < 0.001$]. Combination of both treatments has a greater effect versus single treatment ($P = 0.029$). Data (mean ± s.e.m.) were analysed by either repeated measures one-way ANOVA (B and C) or one-way ANOVA (D) followed by Tukey's *post hoc* test. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ versus low Mg^{2+} condition.

2.11. Total antioxidant capacity

Total antioxidant capacity (TAC) was determined using a commercially available assay kit (Abcam ab65329) which utilizes the conversion of Cu^{2+} ions to Cu^{+} through endogenous protein and small molecule antioxidants, standardized to Trolox equivalents. Brain tissue (100 mg of cortex; 50 mg of hippocampus) was homogenized in cold PBS, centrifuged at 4 °C for 10 min at 10,000 × *g* and supernatant was used for analysis of TAC. Plasma was directly used for the assay. Samples were diluted (1:20) in sterile deionized water, and 10 μL of samples and 100 μL of Trolox standard were added to each well of a 96-well plate. The reaction was initiated by adding 100 μL of Cu^{2+} working solution to each well, and mixing the plate on an orbital shaker for 90 min at room temperature shielded from light. Colorimetric activity was measured by optimal density at 570 nm and antioxidant capacity was calculated against the linear Trolox standard calibration curve (1–20 μM Trolox).

2.12. Statistical analysis

All statistical analysis was performed with IBM SPSS 25 or Graph Pad Prism 6.01. All data are expressed as the mean ± s.e.m. Data were analyzed using unpaired Student's *t*-test, Mann-Whitney *U* test, repeated-measures ANOVA, or generalized log-linear mixed model with random effect of animal (autoregressive covariance) and fixed effects of treatment group, week, and the interaction between treatment group and week. The choice of parametric or nonparametric test followed a Kolmogorov–Smirnov test. Sample sizes were chosen based on our previous experiences in the calculation of experimental variability. The numbers of animals used are described in the corresponding figure legends. The significance level was set to an α-error of $P < 0.05$.

3. Results and discussion

3.1. Neuroprotective effects of combination antioxidant therapies in vitro

To investigate the neuroprotective effect of antioxidant therapies *in vitro*, epileptiform activity was induced by omission of magnesium (Mg^{2+}) from culture media of mixed cortical neurons and glial cultures. Epileptiform activity increases mitochondrial depolarization and rate of ROS generation (Fig.1A-C, B: $F(3,20) = 30.201$, $P < 0.001$; C: $F(3,18) = 61.299$, $P < 0.001$, repeated measures one-way ANOVA). We previously reported that Nrf2 activation using a novel cyanoenone triterpenoid, RTA 408, prevented mitochondrial depolarization and reduced the rate of ROS generation in neurons during epileptiform activity [10]. Here, we asked whether NOX inhibition with 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) can also reduce mitochondrial depolarization, rate of ROS generation and neuronal death, and whether its effect is enhanced by RTA 408.

Under basal conditions, Nrf2 is negatively regulated by Keap1 which forms the Keap1-Nrf2 complex, and promotes the proteasomal degradation of Nrf2. Upon treatment with RTA 408 (or other electrophiles), oxidation of cysteine sensors of Keap1 cause conformational changes to the Keap1-Nrf2 complex and drive the dissociation of Nrf2 from Keap1. Nrf2 then translocates into the nucleus, where it binds to the antioxidant response element of target genes, which leads to enhanced expression of downstream antioxidant enzymes, and is a relatively slow process. In contrast, AEBSF is an NADPH oxidase inhibitor. We have previously shown that seizure like activity leads to NADPH oxidase 2 (NOX2) activation [17]. AEBSF inhibition of NOX2 is mediated via inhibition of its assembly [26], which is a rapid process necessary for ROS production [27]. AEBSF was therefore used as an acute treatment, whilst RTA 408 was used chronically.

Neuronal cultures were treated with AEBSF (50 μM, acutely) with or without RTA 408 (200 nM, pre-incubated for 24 h) following exposure to low Mg^{2+} medium. AEBSF almost completely prevented the seizure-like activity induced mitochondrial membrane potential (ΔΨm) depolarization, either alone, or when co-administered with RTA 408 (Fig. 1B). We then showed that, in keeping with a previous report [17], inhibition of NOX activation by AEBSF significantly decreased the rate of ROS generation 2, 10, and 15 min after exposure to low Mg^{2+} (Fig. 1C).

Interestingly, the combination of both drugs had a greater effect (versus AEBSF alone) on reducing ROS generation at a later time point (15 min, $P = 0.015$, repeated measures one-way ANOVA, Tukey's post hoc). We then asked whether inhibition of NOX is neuroprotective. Neuronal cultures exposed to low Mg^{2+} media for 2 h showed a significantly higher extent of neuronal loss when compared to cultures exposed to artificial CSF (aCSF, Fig. 1D). Inhibition of NOX with AEBSF (50 μM) significantly reduced low Mg^{2+} induced cell death (Fig. 1D, $P = 0.023$, one-way ANOVA with Tukey's post-hoc), as was the combination of both drugs (Fig. 1D, $P < 0.001$, one-way ANOVA with Tukey's post-hoc). Furthermore, co-treatment with RTA 408 (200 nM, 24 h preincubation) and AEBSF (50 μM) showed a greater neuroprotective effect compared to AEBSF alone (18.3% ± 1.4 vs. 26.5% ± 1.4, respectively, $P = 0.029$, one-way ANOVA with Tukey's

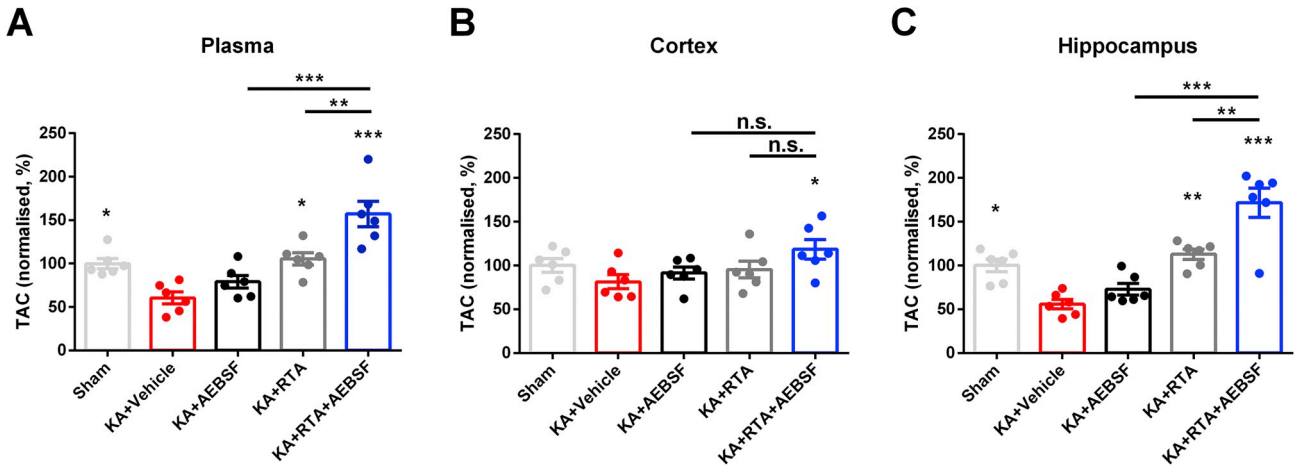

Fig. 2. Combination antioxidant therapy increases total antioxidant capacity following status epilepticus.
Total antioxidant capacity normalized to Trolox standard in plasma **(A)**, cortex **(B)** and hippocampus **(C)** of rats 24 h following KA induced SE (2 h). A-C: n = 6, data are expressed as mean ± s.e.m, (A: $F(4,25) = 16.625$, $P < 0.001$; B: $F(4,25) = 2.389$, $P = 0.078$; C: $F(4,25) = 22.594$, $P < 0.001$), one way-ANOVA followed by Tukey's post hoc. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. KA + Vehicle group. n.s. $P > 0.05$.

post-hoc).

3.2. Effects of antioxidant therapies on total antioxidant capacity in vivo

Thus, the combination of NOX inhibition and Nrf2 activation could completely prevent the metabolic and excitotoxic consequences of seizure-like activity *in vitro*. We next determined the effect of this combination antioxidant therapy on total antioxidant capacity following *in vivo* status epilepticus (SE). Combined non-enzymatic (small molecules and proteins) antioxidant capacity, termed Total Antioxidant Capacity (TAC), can be considered as a cumulative index of antioxidant status [28]. Following 2 h of kainic acid (KA) induced SE, rats were randomised to a single administration of either vehicle, AEBSF 50 mg/kg, RTA 408 25 mg/kg, or a co-treatment of both drugs. TAC was measured in plasma, cortex and hippocampus of sham operated animals as well as treated animals 24 h post SE. Following SE, TAC was significantly reduced in plasma and the hippocampus of vehicle treated animals (60% ± 7 (P = 0.033, one-way ANOVA followed by Tukey's post hoc) and 56% ± 5 (P = 0.021, one-way ANOVA followed by Tukey's post hoc), respectively) in comparison to sham operated animals (no SE). Inhibition of NOX by AEBSF had no significant impact on TAC in plasma, cortex or hippocampus (Fig. 2A–C, KA + Vehicle vs. KA + AEBSF A: P = 0.593, B: P = 0.925, C: P = 0.706, one way-ANOVA followed by Tukey's post hoc). RTA 408 completely restored TAC in both plasma and hippocampus (Fig. 2A, C; A: P = 0.012, C: P = 0.002, one way-ANOVA followed by Tukey's post hoc). Moreover, in rats treated with the combination of RTA 408 and AEBSF, TAC was increased to supranormal levels, and was 2–3 times higher in plasma and hippocampus compared to vehicle group (Fig. 2A, C; A: 157% ± 15 vs. 60% ± 7, P < 0.001, one-way ANOVA with Tukey's post hoc; C: 172% ± 17 vs. 56% ± 5 , P < 0.001, one-way ANOVA with Tukey's post hoc). Interestingly, despite TAC levels in vehicle treated animals was not significantly reduced in the cortex (compared to sham group), the combination of RTA 408 and AEBSF significantly increased the TAC in the treated animals (Fig. 2B; P = 0.047, KA + Vehicle vs. KA + RTA + AEBSF vs., one way-ANOVA followed

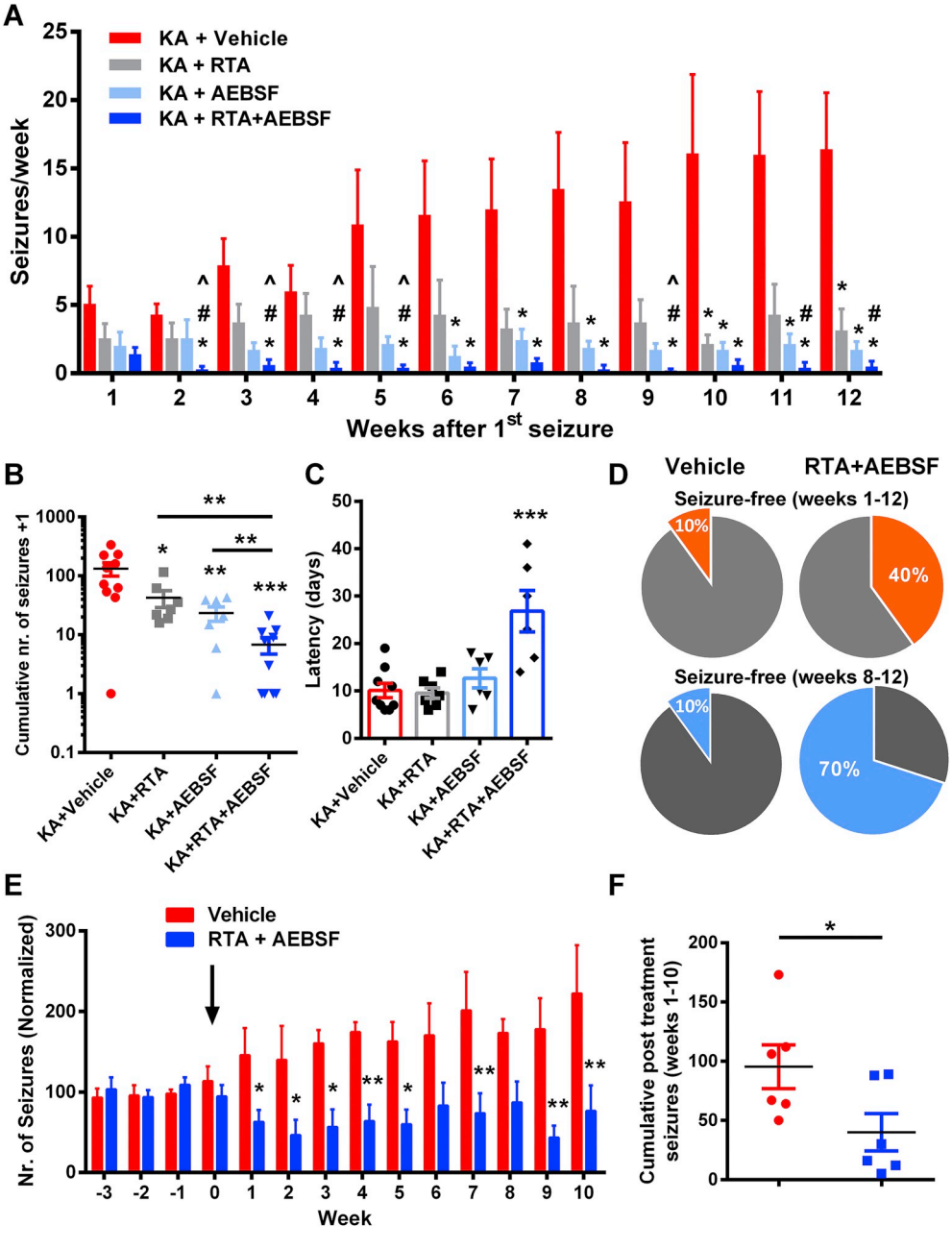

Fig. 3. Combination of NOX inhibition and Nrf2 activation suppresses the development of epilepsy following status epilepticus in rats.
(A) Bar charts of seizure frequency (seizures \week; mean ± s.e.m) of animals following KA induced status epilepticus (2 h), treated immediately after termination of SE (Diazepam 5 mg/kg) with single administration of either vehicle (10% DMSO/saline; n = 10), RTA 408 25 mg/kg (n = 7), AEBSF 50 mg/kg (n = 7) or RTA 408 25 mg/kg and AEBAF 50 mg/kg (n = 10). F (3,300) = 8.005, P < 0.001 by generalized log-linear mixed model followed by sequential Bonferroni *post hoc* test. *P < 0.05, vs. Vehicle; #P < 0.05, RTA 408 + AEBSF vs. RTA 408; ^P < 0.05, RTA 408 + AEBSF vs. AEBSF. **(B)** Cumulative number of seizures of animals in A. Data are plotted on a logarithmic scale after incrementing each total seizure count by 1 to avoid zero values. *P < 0.05, **P < 0.01, ***P < 0.001, Student's *t*-test **(C)** Combination therapy increases the latent period, F(3,24) = 11.197, P < 0.001, by one-way ANOVA followed by Tukey's post hoc, ***P < 0.001 vs. KA + Vehicle group. **(D)** The pie charts illustrate percentage of animals seizure free for the whole study and for the last 5 weeks following KA induced status epilepticus treated with either vehicle (left) or with combination of RTA 408 (25 mg/kg) and AEBSF (50 mg/kg) (right). **(E)** Normalized seizure frequency (seizures\week; mean ± s.e.m) of animals following KA induced SE (2 h) treated with either vehicle (n = 6; red) or with combination of RTA 408 (25 mg/kg) and AEBSF (50 mg/kg) (n = 6; blue) for 3 days, 12 weeks after SE. F(1,100) = 6.737, P = 0.011 by generalized log-linear mixed model on weeks 1–10, followed by sequential Bonferroni post hoc test). *P < 0.05, **P < 0.01 **(F)** Cumulative number of post-treatment seizures (mean ± s.e.m) for the two groups in E. P = 0.022 Student's *t*-test.

by Tukey's post hoc).

Using a dose of AEBSF that had a non-significant effect on TAC in hippocampus, the effect of RTA 408 on TAC was increased by approximately 50%, suggesting a synergistic effect of the combination therapy.

3.3. Effect of combination antioxidant therapy on epileptogenesis

We next asked if NOX inhibition in combination with Nrf2 activation can suppresses the development of epilepsy following SE in rats and is superior to either intervention alone in a randomised, control trial. We induced SE in rats using repeated low doses of KA, according to a previously reported protocol [23]. Following 2 h of SE, rats were randomly assigned to treatment with single dose of either vehicle, RTA 408 (25 mg/kg), AEBSF (50 mg/kg) or co-treatment with both drugs. The electrocorticogram (ECoG) was recorded from all animals for up to 12 weeks following the first spontaneous seizure. Vehicle treated animals developed a progressive increase in seizure frequency over time. Rats treated with single administration of either AEBSF (50 mg/kg) or RTA 408 (25 mg/kg) had a decrease in seizure frequency (Fig. 3A), however, this effect was significant only at later time points (6–12 and 10–12 weeks after the first spontaneous seizure, respectively). Importantly, seizure frequency in rats treated with both drugs was dramatically decreased from 2 to 12 weeks after the first seizure (Fig. 3A).

The cumulative number of seizures was significantly decreased in all treatment groups (Fig. 3B, KA + Vehicle vs. KA + RTA $P = 0.0249$, KA + Vehicle vs. KA + AEBSF $P = 0.008$, KA + Vehicle vs. KA + RTA + AEBSF $P < 0.001$, Student's *t*-test). As predicted, the combination therapy was significantly more effective than either intervention alone.

Moreover, rats treated with vehicle, RTA 408 or AEBSF alone had no difference in the latency period (time from the day of KA treatment to the first electrographically recorded seizure), whilst rats co-treated with RTA 408 and AEBSF had an increased latency period by 265% (from 10 ± 2 days to 27 ± 4 days; Fig. 3C, $P < 0.001$, Student's *t*-test).

Four of 10 rats co-treated with both drugs had no seizures for the whole 12 week study period compared to only 1 of 10 treated with vehicle. Since RTA 408 has a delayed effect and to avoid the confounder of acute drug effects, we determined the seizure frequency 2 months after drug administration. From weeks 8–12, 7 of 10 animals treated with combination therapy were seizure free, compared to only 1 of 10 animals treated with vehicle (Fig. 3D). For the combination therapy, odds ratio for being seizure free (weeks 8–12) compared to vehicle treatment was 21 (95% CI = 1.8–248).

In those rats, in whom we examined neuronal damage in the hippocampus, the combination therapy resulted in marked neuroprotection (Suppl Fig.1) similar to that observed by us for each therapy individually [10,29].

3.4. Effect of combination antioxidant therapy on chronic epilepsy

To test the therapeutic efficacy of the antioxidant therapy on chronic epilepsy, we performed a randomized, blinded trial in a model of TLE induced by systemic KA injection. Rats were implanted with wireless electrocorticography (ECoG) transmitters 10–12 weeks after the induction of SE by intraperitoneal KA. After recording baseline seizure activity for 3 weeks, 12 epileptic animals were randomized to treatment with either vehicle, or a combination of RTA 408 (25 mg/kg) and AEBSF (50 mg/kg), as a single daily dose over 3 days. ECoG recordings were continued for a further 11 weeks. During the baseline period, there was no significant difference in seizure frequency between the two groups (vehicle median = 5.9 (IQR 5.7–6), RTA + AEBSF median = 5.8 (IQR 5.5–6.3); Mann Whitney *U* test, $P = 0.125$).

The combination antioxidant therapy significantly decreased the normalized (to baseline) seizure frequency compared to vehicle treated animals following treatment (generalized log-linear mixed model on weeks 1–10, treatment group*week interaction effect: $F(1,100) = 6.737$, $P = 0.011$; Fig. 3E). In addition, the cumulative number of seizures experienced per animal post treatment was significantly decreased (Fig. 3F, $P = 0.022$ Student's *t*-test).

Epileptogenesis is associated with well recognised structural changes which will have occurred by the time we are treating the established epilepsy. These (such as cell loss) will not be reversed by our treatment, possibly explaining the lesser effect of late treatment compared to early treatment.

4. Conclusion

Combination therapy inhibiting ROS generation via NOX and increasing antioxidant defences via Nrf2 activation prevents seizure induced mitochondrial depolarization, ROS generation and neuronal cell death *in vitro* and was more effective than either intervention alone. Further, we showed that a combination of both NOX inhibition and Nrf2 activation is the most effective mean of increasing antioxidant capacity following KA-induced SE *in vivo*, and prevents the development of epilepsy and also modifies chronic epilepsy.

Conflicts of interest

The authors declare no competing interests exist.

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Author contributions

Taweeq Shekh-Ahmad: Conceptualization, Data curation, Formal analysis, Investigation, Writing-original draft, Writing-review and editing, Performed experiments, Analyzed data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101278.

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