Published in final edited form as:

J Neurosci Methods. 2014 September 30; 235: 234–244. doi:10.1016/j.jneumeth.2014.07.009.

An organotypic brain slice preparation from adult patients with temporal lobe epilepsy

Emmanuel Eugene^{a,b,c,d}, Françoise Cluzeaud^e, Carmen Cifuentes-Diaz^{b,c,d}, Stephane Clemenceau^a, Michel Baulac^a, Jean-Christophe Poncer^{b,c,d}, and Richard Miles^a

^aInstitut du Cerveau et de la Moelle Epinière, INSERM UMR-739, Université Pierre et Marie Curie, CHU Pitié-Salpêtrière, 47, boulevard de l'Hôpital, Paris 75013, France

^bINSERM, UMR-839, Paris 75005, France

^cUPMC Univ Paris, UMR-839, Paris 75005, France

^dInstitut du Fer à Moulin, Paris 75005 France

^eService Microscopie, Centre de recherche biomédicale, CHU Bichat, Université Paris Diderot, 16 rue Henri Huchard, Paris 75870, France

Abstract

Background—A long-term in vitro preparation of diseased brain tissue would facilitate work on human pathologies. Organotypic tissue cultures retain an appropriate neuronal form, spatial arrangement, connectivity and electrical activity over several weeks. However they are typically prepared with tissue from immature animals. In work using tissue from adult animals or humans, survival times longer than a few days have not been reported and it is not clear that pathological neuronal activities are retained.

New Method—We modified tissue preparation procedures and used a defined culture medium to make organotypic cultures of temporal lobe tissue obtained after operations on adult patients with pharmaco-resistant mesial temporal lobe epilepsies.

Results—Organotypic culture preparation and maintenance techniques were judged on criteria of morphology and the generation of epileptiform activities. Short-duration (30–100 ms) interictal-like population activities were initiated spontaneously in either the subiculum, dentate gyrus or the CA2/CA3 region, but not the cortex, for up to 3–4 weeks in culture. Ictal-like discharges, of duration greater than 10 s, were induced by convulsants. Epileptiform activities were modulated by both glutamatergic and GABAergic receptor antagonists.

Comparison with existing methods—Our methods now permit the maintenance in organotypic culture of epileptic adult human tissue, generating appropriate epileptiform activity over 3–4 weeks.

Correspondence: R. Miles richard.miles@upmc.fr tel: +331 5527 4231 E. Eugène emmanuel.eugene@inserm.fr.

Competing interests.

A patent application was submitted in November 2013. Submission number: 13306630.8. Receiving office: European Patent Office. .

Conclusions—We have shown that characteristic morphology and pathological activities are maintained in organotypic cultures of adult human tissue. These cultures should permit studies on the effects of prolonged drug treatments and long-term procedures such as viral transduction.

Graphical abstract

Tissue of the hippocampal formation and temporal cortex (A), resected from patients with epilepsies of the temporal lobe was dissected (B) and sliced at 300 μm (C). Slices were cultured on a transwell semi-porous membrane (D) for up to four weeks using a new defined culture medium. Morphological (E) and electrophysiological (F) data was used to assess the success of organotypic culture procedures.

Keywords

Organotypic culture; human; epilepsy; defined medium; hippocampus

1. Introduction

The use of brain slices in organotypic culture (Crain, 1966) was popularized in the laboratory of Beat Gähwiler (Gähwiler, 1988). With this technique, cellular identity, spatial relations and synaptic connectivity are maintained and neuronal activities similar to those in acute slices are retained. Organotypic slices have been used to examine synaptic interactions between identified cells (Debanne, Gähwiler & Thompson, 1998) and to study long-term effects of stimuli such as axonal lesions (McKinney et al 1997). They are typically prepared from slices of young brain tissue either kept in a roller-tube (Gähwiler et al, 1997) or in a simplified procedure cultured at an air interface with a static medium (Stoppini, Buchs & Müller, 1991).

The organotypic culture technique could be useful in long-term studies on human brain pathologies. Human brain tissue, obtained after operations on patients with focal epilepsies who develop a resistance to anti-epileptic drugs, has been used in work on acutely prepared slices (Kohling et al, 1999; Cohen et al, 2002; Wittner et al, 2009; Huberfeld et al, 2011). However organotypic culture techniques are most successful with peri-natal rodent tissue (P0-P7 in rodents; Gähwiler et al 1997; Stoppini et al, 1991) or pre-natal human tissue (Hansen et al 2010). Applying these techniques to adult tissue from patients with neurological diseases seems to be more difficult. Studies on tissue from older animals (Wilhelmi et al, 2002) or from adult human brain (Jung et al, 2002; Chaichana et al, 2007; Gonzalez-Martinez et al, 2007) have shown an adequate neuronal morphology after several days, but there is little evidence that functional neuronal activity can be retained.

Here we describe how improved techniques, including the use of a defined medium, preserve adequate morphological and electrical properties in organotypic cultures of tissue from the temporal lobe of adult, epileptic patients. We used the spontaneous generation of epileptiform activity as an index of successful culture conditions (Noraberg et al. 2005). In cultures, as in acute slices, both the CA2/3 region (Wittner et al, 2009) and the subiculum (Cohen, et al 2002), but not the temporal cortex, generated a spontaneous interictal-like activity over several weeks. Furthermore ictal-like activity could be induced by convulsants (Huberfeld et al, 2011). These cultures should permit long-term work including tests of novel anti-epileptic drugs or transduction with viral vectors.

2. Materials and Methods

2.1. Temporal lobe tissue from epilepsy patients

Slices for organotypic culture (n=95) were prepared from 28 blocks of hippocampus (n=20) or temporal cortex (n=8) obtained from 21 patients (13 male, 8 female; age 20-55 years) diagnosed with pharmaco-resistant mesial temporal lobe epilepsy associated with hippocampal sclerosis. Patients gave their written, informed consent and protocols were approved by the Comité Consultatif National d'Ethique. Tissue was transported from the operating room to the laboratory in a solution containing sucrose, 248; NaHCO₃, 26; KCl, 1; CaCl₂, 1; MgCl₂, 10; glucose, 10 (in mM) equilibrated with 95% O₂ / 5% CO₂, maintained at 2-10 °C.

2.2. Preparation of organotypic cultures

Capillaries and damaged tissue were dissected away from the tissue block in the sucrose-based solution in sterile conditions at 2-10 °C, equilibrated with 95% O₂ / 5% CO₂. Slices of the hippocampal formation were cut at thickness 300 µm with a vibrating tissue slicer (HM650V, Microm). They measured 5-6 mm by 10-15 mm and included the dentate gyrus, the CA3 region, the usually sclerotic CA1 region and the subiculum. Slices of temporal cortex, of size ~10-15 × 10-15 mm, were prepared identically.

Slices used for anatomical studies, were fixed overnight in 4% paraformaldehyde at 4°C, with 0.25 % glutaraldehyde added for subsequent electron microscopy. Slices used for culture were placed on an insert in sterile conditions (30mm Transwell, Corning) at the interface between air and a medium containing 20mM HEPES and the antibiotics penicillin (100U/ml) and streptomycin (100µg/ml). They were maintained in an incubator, at 37°C in 95% O₂ / 5% CO₂. After 1 hr, slices on inserts were transferred to a 6-well plate, pre-equilibrated in the incubator and containing the same solution except that HEPES was omitted. The medium was changed 5-6 times per week and antibiotics were omitted after one week.

2.3 Culture medium

Initial studies used the medium of Stoppini et al (1991; minimum essential medium mixed with Hank's medium and including horse serum) or the Neurobasal medium (Brewer et al, (1993). Both visual inspection and electrophysiological records suggested that adult slices of the temporal lobe survived poorly in organotypic culture using these media. We therefore

developed a new organotypic slice culture medium (OSCM) with no serum. The electrical activity and appearance of slices maintained in the medium of Stoppini and of slices cultured in the new medium was compared to improve preparation techniques and medium composition (Results, 3.1). The composition of the serum-free medium is given in Table 1. Inorganic ion concentrations were (in mM): K^+ , 3.3; Na^+ , 149.5; Ca^{++} , 1.3; Mg^{++} , 1.0; Cl^- , 121.5; HCO_3^- , 22.3; $H_2PO_4^-$, 1.1 and SO_4^{--} , 1.0 mM. Trace metal ions included Cu^{++} , 0.01; Fe^{+++} , 0.70 and Zn^{++} , 715 (in μM).

Synchronous neuronal activity involves significant energy consumption (Heinemann et al, 2002; Huchzermeyer et al, 2008) involving the oxidative metabolism of glucose by neuronal and astrocyte mitochondria (McKenna et al 2006). We used an enriched mixture of early and late energy sources and intermediate metabolites of the tricarboxylic acid cycle including d-glucose (4.1 mM), lactate and pyruvate (Nordstrom, 2010; Gallagher et al, 2009), oxaloacetate, acetate and citrate as well as creatine (5 mM), creatine phosphate (0.2 mM) and adenosine tri-phosphate (10 μM) at levels based on previous work (Romijn, de Jong & Ruijter, 1988; Wilhelmi et al, 2002; Nehlig & Coles, 2007). Lactate (4 mM) and pyruvate (0.2 mM) were used at a ratio of 20 based on neuronal metabolism data (Nordstrom 2010).

Lower concentrations of amino acids were used than in many culture media, including MEM, Neurobasal (Brewer et al, 1993) and the R-16 medium of Romijn, de Jong and Ruijter (1988). In particular, levels of L-glutamine (8 μM) and of L-glutamic acid (7.5 μM) were lowered since they may be catabolised into toxic molecules. Oxaloacetate (1 mM), which scavenges free glutamate (Marosi et al, 2009) was included to aid neuroprotection. Antioxidant activity was assured by including reduced glutathione (3.2 μM), superoxide dismutase (0.08 μM), citric acid (570 μM) and putrescine (50 μM). Vitamins, including ascorbic acid (580 μM) and folic acid (1.2 μM) were also present at low concentrations. The pH of the medium was maintained with CO_2/HCO_3 and phosphate buffers, but HEPES was not used (Bonnet, Wiemann & Bingmann, 1998). Equilibrated with 5% CO_2 , the pH of the defined medium was close to 7.2 at 37°C and the osmolarity was near 310 mOsm. Constituents of the culture medium were obtained from Sigma-Aldrich (Lyon, France).

2.4 Multi-unit and field recordings

Extracellular records were made from organotypic slices after 2-30 days in culture. Cultured slices were transferred on the culture insert to an interface recording chamber. Recordings were made in a solution containing 124 NaCl, 26 $NaHCO_3$, 4 KCl, 2 $MgCl_2$, 2 $CaCl_2$, and 10 D-glucose (in mM), maintained at 35-7°C and equilibrated with 5% CO_2 in 95% O_2 .

We attempted to induce ictal-like activity by increasing K^+ from 4 to 10 mM and decreasing Mg^{++} from 2 to 0.2 mM (Huberfeld et al, 2011). Ionotropic glutamate signalling was blocked using 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX, 10 μM) and DL-2-amino-5-phosphonovaleric acid (DL-APV, 100 μM). GABA_A receptor mediated signalling was suppressed by picrotoxin (50 μM) or bicuculline (20 μM). The potassium channel blocker 4-aminopyridine (4AP, 50 μM) was used to induce ictal-like activities in cortical slices. Drugs were obtained from Abcam Biochemicals (Bristol, UK).

Multi-unit activity and field potentials were recorded with up to four extracellular electrodes independently-positioned. Electrodes were made from insulated tungsten wire of diameter 50 μm shaped to a tip of diameter 5-10 μm by electrolysis (ac current passed in a cell of graphite and tungsten in 2 M KNO_2). Extracellular potentials were amplified 1000x using a four channel amplifier (AM systems M1700) with a pass band of 0.1 – 20 000 Hz. They were digitized with a 12 bit A–D converter (Digidata 1200A, Molecular Devices) monitored with the program Axoscope (Molecular Devices) and saved to a computer. Multi-unit spike frequency was displayed with routines written in LabView (Ivan Cohen). Recordings of interictal-like and ictal-like events were defined by multi-unit and field potential activities and analysed using Clampfit (Molecular Devices) and Prism software (Graphpad).

2.5. Immunohistochemistry

Organotypic slices were fixed in 4 % paraformaldehyde, washed in 0.1 M phosphate buffer (PB) and infiltrated with a sucrose-PB solution. They were cryo-protected in a solution containing 15% sucrose and 30% polyethylene-glycol in PB and kept at -20°C . Sections of thickness 40 μm were cut with a cryotome, washed in PB, and stored as floating sections in the cryoprotecting solution at -20°C .

Immunostaining was performed with an acidic antigen unmasking step (Loup et al. 1998). Slices were washed in PB, embedded in a 30% sucrose solution and submitted to 3 freeze-thaw cycles over dry ice. They were then rinsed in PB, submerged in a 1.5% H_2O_2 solution for 30 min. After rinsing slices were placed 3 hrs in a saturation buffer containing 0.2% milk, 10% goat serum and 0.5% Triton X100 in Tris NaCl blocking buffer (TSA kit, Perkin-Elmer, Courtaboeuf, France). Sections were then incubated with primary antibodies in the same buffer overnight at 4°C . After washing, slices were exposed to secondary antibodies for 4 hrs at 21°C and then to TSA-Cy5 amplification. After washing overnight at 4°C , slices were mounted with an antifade agent (Prolong gold, Invitrogen, St Aubin, France)

The primary antibodies used were: anti-GFAP (chicken; 1:500, Chemicon), anti β -tubulin III (mouse; 1:200, Sigma) and anti Nestin (rabbit; 1:500, Chemicon). Secondary antibodies used together with the TSA-CY5 kit (Perkin Elmer) were: Cy3 conjugated donkey anti-chicken IgY (1:500, Jackson Immunoresearch, Baltimore, USA), anti Mouse-Alexa488 (Jackson Immunoresearch, Baltimore, USA) and biotinylated anti Rabbit (1:1000, Perkin-Elmer).

Images were acquired with a camera (QImaging Retiga EXI) mounted on an inverted microscope (Olympus IX81) using an Optigrid II scanning system (Qioptiq). The optigrid system permitted 3-D image acquisition and subsequent reconstruction (Volocity, Perkin-Elmer, Coventry, UK). Stacks of images of organotypic slices were acquired with a 20x objective of NA 0.85 (30-45 images at interval 0.7 μm with voxel size 0.64 μm) or with a 40x objective of NA 1.3 (40-70 images at 0.4 μm with voxel size 0.32 μm).

2.6. Electron microscopy

For electron microscopy, organotypic slices were fixed at 4°C in 4% para-formaldehyde and 0.25% glutaraldehyde in sucrose solution diluted in Hank's buffer solution. They were washed in PBS and postfixed in 1% osmium tetroxide (Uptima Interchim), dehydrated in

graded ethanols, and embedded in Durcupan (Fluka). Images of semithin sections (500 nm) and ultrathin sections (50 nm) stained with toluidine blue or uranyl acetate and lead citrate, were obtained using a bright field or a transmission electron microscope (CM100, Philips).

2.7. Statistics

The significance of differences in multi-unit and epileptiform activities between tissues cultured in MEM or the new defined medium was tested with the Mann-Witney non parametric test. P values less than 0.05 were considered significant. Activities of different regions of slices were compared with a population comparison test (Fisher and Yates, 1963).

3. Results

3.1. Activity in organotypic slices cultured in an MEM-based or a novel defined medium

We first compared neuronal activity generated by slices maintained in culture under identical conditions with either the MEM-based medium used by Stoppini and colleagues (Stoppini, Buchs & Muller, 1991) or the defined culture medium (Methods). At 6-15 days in culture, multi-unit extracellular discharges were recorded from the subiculum in 1 of 7 organotypic slices (from 5 tissue blocks) cultured with the MEM-based medium (Fig. 1A). Epileptiform activity, defined as bursts of multi unit firing accompanied by field potentials, was never detected. In contrast at 6-15 days in culture with the defined medium, multi-unit firing was detected in the subiculum, the dentate gyrus and the CA2/3 region of 25 of 33 slices (from 19 tissue blocks). Interictal-like epileptiform events, consisting of bursts of multi-unit firing and a field potential of duration 30-300 ms, occurred spontaneously in 19 of 33 slices cultured in the defined medium (Fig. 1B). The probability of recording multi-unit (Fig. 1C) or epileptiform activity (Fig. 1D) was higher for organotypic slices cultured with the defined medium, which was therefore used in all subsequent work.

3.2. Regional variation of activity at 6-10 days in culture

Extracellular records were made of multi-unit and field potential activity generated by the hilus and dentate gyrus, the CA3 region, the CA1 region and the subiculum (n=33 slices, defined medium; Fig. 2). At 6-10 days in culture, multi-unit activity at frequencies ranging between 0.5 and 200 spikes/sec was generated by at least one of these regions in 22 of 23 slices tested. Spontaneous multi-unit activity was recorded from the dentate gyrus in 9 of 13 slices, from the CA2-CA3a region in 7 of 12 slices, from the CA3c region in 4 of 5 slices and from the subiculum in 18 of 23 slices. The sclerotic CA1 region did not generate multi-unit activity.

Epileptiform activity (Fig. 2A, B) consisting of an increase in multi-unit firing with a field potential of duration 30-300 ms, was detected after 6-10 days in culture, in the dentate gyrus (7 of 13 slices), the CA3c region (3 of 5), the CA2-CA3a region (5 of 12) and the subiculum (6 of 22 slices). Spontaneous interictal-like discharges consisted of one or several bursts of duration 30-300 ms recurring at intervals of 0.1-10 s in different regions and slices. Local synchrony was confirmed in records of similar events at short latency from pairs of electrodes separated by 200-300 μ m (not shown). Comparing the timing of events recorded in different regions (Fig. 2B) suggested that activity was initiated either in the dentate gyrus

or the CA3c region (7 of 13 slices), the CA2-3a region (6 of 17 slices) or the subiculum (6 of 22 slices) and then spread to invade nearby regions. In 8 of 13 slices, distinct synchronous events appeared to be initiated in more than one region (Wittner et al, 2009). Activities could spread reciprocally between regions such as CA3 and the dentate gyrus (Scharfman, 2007). When multiple cultured slices were prepared from the same tissue block (n=12) epileptiform activity was always generated by the same regions - CA3, the dentate gyrus or the subiculum. Figs. 2C, D show the proportion of slices from which spontaneous multi-unit and epileptiform activities were generated by different regions.

3.3. Morphology and ultrastructure of organotypic cultures at 6-10 days in culture

We next compared the morphology of organotypic cultures after 6-10 days with that of freshly fixed temporal lobe tissue. Immunostaining for the neuron-specific microtubule marker β -Tubulin III (Fig. 3A, B), revealed granule cells of the dentate gyrus were dispersed (Houser, 1990). Very few stained neurons were present in the sclerotic CA1 region as in acute tissue (Blümcke, Thom & Wiestler, 2002). Numerous cells were immuno-positive for the astrocyte marker, GFAP (Fig. 3C), but we did not detect a layer of activated astrocytes covering organotypic cultures. The developmentally expressed neuronal marker, Nestin was detected in few isolated precursor neural cells in the CA3 region and the subiculum (Fig. 3C).

We also used electron microscopy to compare the state of intracellular organelles in semi-thin (500 nm) sections of organotypic cultures at 6-10 days with their state in rapidly fixed tissue (Fig. 3D). The nuclei of neurons from cultures tended to have a round shape (Fig. 3E) suggesting that cells were not stressed. Synapses appeared normal with pre-synaptic vesicles, mitochondria and post-synaptic densities (Fig. 3F). Overall, electron microscopy did not reveal signs of widespread neuronal death or damage at 6-10 days in culture.

3.4. Pharmacological sensitivity and effects of convulsants at 6-10 days in culture

Interictal-like activity generated by acute slices of tissue from patients with temporal lobe epilepsies depends on signalling mediated by both glutamatergic and GABA_A receptors (Cohen et al, 2002). We found the glutamate receptor antagonists NBQX (10 μ M) and DL-APV (50 μ M) abolished interictal like activity generated spontaneously by organotypic slices (Fig. 4A; n=3 of 3 experiments). Interictal-like events were also often suppressed (Fig. 4A; 8 of 17) by the GABA_A receptor antagonists picrotoxin (50 μ M) or bicuculline (20 μ M).

Convulsants induce ictal-like discharges in acute slices of human subiculum (Huberfeld et al. 2011). We tested the effects on the activity of organotypic slice activity of a pro-convulsant solution containing 8 mM K⁺ and 0.25 mM Mg⁺⁺. Switching to this solution increased multi-unit activity and enhanced the frequency of synchronous events in all regions, except the sclerotic CA1, in all slices examined (n=18; Fig. 4B). The mean frequency of interictal-like events was increased from 0.2 ± 0.1 Hz to 0.6 ± 0.1 Hz by the pro-convulsant solution, and effects on multi-unit activity and interictal-like events were reversible (not shown).

In 6 slices, recurring ictal-like events with prolonged multi-unit firing and a stereotyped pattern of field potential bursts (Fig. 4C) were generated after prolonged exposure (more

than 90 min) to the convulsant solution. Ictal-like events were of duration 21 ± 10 s and recurred at interval of 58 ± 10 s. They were initiated either in the subiculum ($n=3$) or the dentate region ($n=3$) and typically spread to initiate firing in all areas of the slice except CA1.

3.5. Changes in epileptiform activities with time in culture of organotypic slices

We next asked how activities generated by organotypic slices varied with time in culture. In some culture preparations, cells such as lobster stomatogastric neurones, lose and then regain their specific patterns of activity with time in culture (Turrigiano, LeMasson & Marder, 1995). We asked whether human epileptic slices behaved similarly by comparing population activities recorded at 2-4, 6-10, 13-16 and 20-29 days in culture (Fig. 5A). Records from the CA3 region revealed little or no multi-unit activity at 2-4 days in culture ($n=3$). At 6-10 days spontaneous interictal field potentials emerged at a frequency of 0.2 ± 0.1 Hz ($n=5$). A similar activity was maintained at 13-16 days with a higher frequency of 0.5 ± 0.3 Hz ($n=3$) and at 20-29 days with a reduced frequency of 0.2 ± 0.1 Hz ($n=3$). Changes in the frequency of interictal-like field potentials with time in culture are summarised in Fig. 5B. Frequencies in the presence of the pro-convulsant solution were higher but followed a similar time course as shown in Fig. 5C.

These data suggest that, after an initial loss of activity and synchrony, organotypic slices of human epileptic tissue, maintained in a defined medium, generate epileptiform events in a relatively stable fashion over time. These results differ from some obtained in organotypic cultures of hippocampal tissue from healthy rats (McBain, Boden & Hill, 1989). Using a culture medium with serum, asynchronous multi-unit neuronal activity was transformed over several weeks into epileptiform activity, possibly due to an enhanced synaptic connectivity.

3.6. Temporal cortex from epileptic patients maintained in organotypic culture

We therefore asked whether time in culture could transform asynchronous multi-unit firing into epileptiform activity in cultures of temporal lobe cortex. Acute slices of this tissue, resected from the same patients, do not generate spontaneous interictal-like activity. At 6-10 days in culture, only sparse multi-unit activity was recorded from 1 of 3 slices of temporal lobe cortex cultured in the defined medium (Fig. 6A). Neuronal activity was not notably different in records made at 13-16 ($n=6$) or at 20-29 days in culture (Fig. 6B; $n=4$). Spontaneous epileptiform activity was not detected in any cultured slice from temporal lobe cortex ($n=13$ slices from 9 tissue blocks).

Application of the convulsant solution (K^+ 8 mM and Mg^{++} 0.25 mM) enhanced multi-unit firing ($n=7$) and could induce interictal-like epileptiform activity ($n=4$) in organotypic cultures of temporal cortex (Fig. 6C). Interictal events originated in deep but not superficial cortical layers and spread for distances up to 600 μ m. Similar interictal activity was induced by the GABA_A receptor antagonist picrotoxin (50 μ M; $n=3$; Fig. 6D) and by the convulsant 4-aminopyridine (50 μ M; $n=2/2$; Fig. 6E). This pro-epileptic effect of picrotoxin contrasts with the suppression of interictal events generated by the subiculum. Ictal-like activity was never induced in organotypic slices of temporal lobe by exposure to convulsants. These data

on temporal cortex therefore suggest that long-term organotypic culture does not encourage epileptiform activity. Distinct mechanisms may underlie these activities in different regions.

4. Discussion

These data show that hippocampal formation and temporal lobe tissue from adult epileptic patients can be maintained for up to 4 weeks in organotypic culture. Epileptiform activities generated by cultured slices are similar in duration, form and pharmacological sensitivity (Figs. 3, 5) to those of acute slices of human, epileptic temporal lobe (Cohen et al, 2002; Wittner et al 2009; Huberfeld et al, 2011). The morphology of cultured slices was maintained with two features of the hippocampus of patients with temporal lobe epilepsies - granule cell dispersion (Houser, 1990) and sclerosis of the CA1 region (Blümcke, Thom & Wiestler, 2002) – maintained at 1-4 weeks in culture. Neither light nor electron microscopy revealed (Fig. 3) a strong necrosis due to culture conditions (cf. Romijn, de Jong & Ruijter, 1988).

4.1 Culture techniques

Maintenance of adult human tissue in organotypic culture necessitated a different approach from that used with perinatal tissue from rodents. We modified components of the culture medium, using electrical activity and appearance of the cultures to define useful changes. First, the use of a defined medium removed the uncertainty and variability inherent in serum-based culture techniques (Annis, Edmond & Robertson, 1990), and may also reduce astrocyte proliferation (Martin & Wiley, 1995). Second, neurotrophic and transcription factors were not used, perhaps reducing the formation of exuberant synaptic connexions, neurogenesis and network reorganization in culture (Sato et al, 2007; Parent et al, 1997). Third, levels of amino acids including l-glutamate and l-glutamine were reduced, in an attempt to reduce ambient levels of glutamate and GABA (Sandow et al, 2009) and toxic degradation products (Svoboda & Kerschbaum, 2009). We also reduced concentrations of trace elements, hormones and vitamins and used a CO₂ / HCO₃ buffer system rather than HEPES to control pH. Overall the medium (Table 1) was designed to ensure neuronal survival rather than proliferation.

4.2 Spontaneous and induced epileptiform activities: different areas and time in culture

Characteristic electrical activity of acute slices from patients with epilepsies of the temporal lobe was quite faithfully retained for up to 4 weeks in culture. Spontaneous interictal activity was generated in different regions of the hippocampus by about half the cultures and convulsant solutions induced long-duration, patterned, ictal-like events in a significant proportion of slices. The pharmacological sensitivity of epileptiform events was maintained. Antagonists at both glutamatergic and GABAergic receptors blocked interictal events (Cohen et al, 2002). The identity of regions in organotypic slices that generated interictal-like events, the subiculum (Cohen et al, 2002), the CA2/3 region (Wittner, 2009) and the dentate gyrus (Gabriel et al 2004), was similar to that in acute slices. Equally, and as in acute slices, neither the CA1 region nor the temporal cortex (Fig. 6) generated epileptiform activities.

The GABA_A receptor antagonist picrotoxin had opposing effects in temporal cortex where it induced an interictal-like activity and in subiculum and CA3 where it suppressed a similar synchrony. Possibly expression of molecules controlling Cl⁻ homeostasis differs in the two regions. Neuronal activity generated by the temporal cortex did not change greatly with time in culture. Multi-unit activity was sparse at 6–10 days, and epileptiform activity did not emerge with time (cf. McBain, Boden & Hill, 1989). Similarly, spontaneous interictal events generated by regions such as the subiculum did not increase markedly in frequency or develop into ictal-like discharges over time in culture. Thus, neuronal excitability and synaptic connectivity may remain relatively stable in cultures kept in a defined medium without neurotrophic factors.

4.3 Directions

These improved techniques for maintaining organotypic slices should permit long term studies on adult, human epileptic tissue. This preparation could be a useful adjunct to animal models and patient trials in tests on anti-epileptic molecules (Dragunow, 2008). Work on human adult neurogenesis and neuronal migration (Hansen et al, 2010) would benefit from prolonged periods of observation and experiment as could studies on the delayed death of human neurons after ischemia (Rytter et al 2003) or seizure activity. Organotypic human slices may also provide a controlled environment to test strategies of neuronal renewal or replacement in the context of neurodegeneration (Daviaud et al, 2014).

Long-term organotypic cultures also provide enough time for the expression of proteins or other molecules after viral transduction (Karra & Dahm, 2010). This would open the way to test, in human tissue, the use of molecules permitting the optical control of epileptic activity by activation or inactivation of defined neurons (Tønnesen et al, 2009; Wykes et al, 2012). Finally, agents that stimulate neuronal growth were largely omitted from our medium. Including them, would permit work to clarify long term effects of specific growth factors and neurotrophic molecules on synaptic connectivity and epileptic activity (Duveau & Fritschy, 2010; Liu et al 2013).

Techniques described here should be applicable to brain tissue excised from patients with other neurological syndromes. Tissue should be available after operations on patients with epileptic syndromes associated with some developmental disorders including cortical dysplasias (D'Antuono et al, 2004) and some auto-immune epileptic syndromes including Rasmussen's encephalitis (Wirenfeldt et al, 2009). Brain tumors are also routinely removed and have been maintained in organotypic culture over periods up to two weeks (Chaichana et al, 2007; Merz et al, 2013) but analyses have so far been limited to the anatomy and ultrastructure of the cultured tissue.

Acknowledgements

We are grateful for financial support from the INSERM, ERA-net, ERC (322721) and the Agence Nationale de Recherche.

References

- Annis CM, Edmond J, Robertson RT. A chemically-defined medium for organotypic slice cultures. *J. Neurosci. Meth.* 1990; 32:63–70.
- Bonnet U, Wiemann M, Bingmann D. CO₂/HCO₃ withdrawal from the bath medium of hippocampal slices: biphasic effect on intracellular pH and bioelectric activity of CA3-neurons. *Brain Res.* 1998; 796:161–70. [PubMed: 9689466]
- Blümcke I, Thom M, Wiestler OD. Ammon's horn sclerosis: a maldevelopmental disorder associated with temporal lobe epilepsy. *Brain Pathol.* 2002; 12:199–211. [PubMed: 11958375]
- Brewer GJ, Torricelli JR, Evege EK, Price PJ. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J Neurosci Res.* 1993; 35:567–76. [PubMed: 8377226]
- Chaichana KL, Capilla-Gonzalez V, Gonzalez-Perez O, Pradilla G, Han J, Olivi A, Brem H, Garcia-Verdugo JM, Quiñones-Hinojosa A. Preservation of glial cytoarchitecture from ex vivo human tumor and non-tumor cerebral cortical explants: A human model to study neurological diseases. *J Neurosci Methods.* 2007; 164:261–70. [PubMed: 17580092]
- Cohen I, Navarro V, Clemenceau S, Baulac M, Miles R. On the origin of interictal activity in human temporal lobe epilepsy in vitro. *Science.* 2002; 298:1418–21. [PubMed: 12434059]
- Crain SM. Development of organotypic bioelectric activities in central nervous tissues during maturation in culture. *Int Rev Neurobiol.* 1966; 9:1–43. [PubMed: 5337726]
- D'Antuono M, Louvel J, Köhling R, Mattia D, Bernasconi A, Olivier A, Turak B, Devaux A, Pumain R, Avoli M. GABA_A receptor-dependent synchronization leads to ictogenesis in the human dysplastic cortex. *Brain.* 2004; 127:1626–40. [PubMed: 15175227]
- Daviaud N, Garbayo E, Schiller PC, Perez-Pinzon M, Montero-Menei CN. Modeling nigrostriatal degeneration in organotypic cultures, a new ex vivo model of Parkinson's disease. *Experimental Neurology.* 2014; 248:429–440. [PubMed: 23899655]
- Debanne D, Gähwiler BH, Thompson SM. Long-term synaptic plasticity between pairs of individual CA3 pyramidal cells in rat hippocampal slice cultures. *J Physiol.* 1998; 507:237–47. [PubMed: 9490845]
- Dragunow M. The adult human brain in preclinical drug development. *Nat Rev Drug Discov.* 2008; 7:659–66. [PubMed: 18617887]
- Duveau V, Fritschy JM. PSA-NCAM-dependent GDNF signaling limits neurodegeneration and epileptogenesis in temporal lobe epilepsy. *Eur J Neurosci.* 2010; 32:89–98. [PubMed: 20597970]
- Fisher, RA.; Yates, E. Statistical Tables for Biological Agricultural and medical research. Hafner pres; London: 1963.
- Gabriel S, Njunting M, Pomper JK, Merschhemke M, Sanabria ER, Eilers A, Kivi A, Zeller M, Meencke HJ, Cavalheiro EA, Heinemann U, Lehmann TN. Stimulus and potassium-induced epileptiform activity in the human dentate gyrus from patients with and without hippocampal sclerosis. *J Neurosci.* 2004; 24:10416–30. [PubMed: 15548657]
- Gähwiler BH. Organotypic cultures of neural tissue. *Trends Neurosci.* 1988; 11:484–9. [PubMed: 2469173]
- Gähwiler BH, Capogna M, Debanne D, McKinney RA, Thompson SM. Organotypic slice cultures: a technique has come of age. *Trends Neurosci.* 1997; 20:471–7. [PubMed: 9347615]
- Gallagher CN, Carpenter KL, Grice P, Howe DJ, Mason A, Timofeev I, Menon DK, Kirkpatrick PJ, Pickard JD, Sutherland GR, Hutchinson PJ. The human brain utilizes lactate via the tricarboxylic acid cycle: a ¹³C-labelled microdialysis and high-resolution nuclear magnetic resonance study. *Brain.* 2009; 132:2839–49. [PubMed: 19700417]
- González-Martínez JA, Bingaman WE, Toms SA, Najm IM. Neurogenesis in the postnatal human epileptic brain. *J Neurosurg.* 2007; 107:628–35. [PubMed: 17886564]
- Hansen DV, Lui JH, Parker PR, Kriegstein AR. Neurogenic radial glia in the outer subventricular zone of human neocortex. *Nature.* 2010; 464:554–561. [PubMed: 20154730]
- Heinemann U, Buchheim K, Gabriel S, Kann O, Kovacs R, Schuchmann S. Cell death and metabolic activity during epileptiform discharges and status epilepticus in the hippocampus. *Prog Brain Res.* 2002; 135:197–210. [PubMed: 12143341]

- Houser CR. Granule cell dispersion in the dentate gyrus of humans with temporal lobe epilepsy. *Brain Res.* 1990; 535:195–204. [PubMed: 1705855]
- Huberfeld G, Menendez de la Prida L, Pallud J, Cohen I, Le Van Quyen M, Adam C, Clemenceau S, Baulac M, Miles R. Glutamatergic pre-ictal discharges emerge at the transition to seizure in the human epileptic temporal lobe. *Nature Neurosci.* 2011; 14:627–34. [PubMed: 21460834]
- Huchzermeyer C, Albus K, Gabriel HJ, Otáhal J, Taubenberger N, Heinemann U, Kovács R, Kann O. Gamma oscillations and spontaneous network activity in the hippocampus are highly sensitive to decreases in pO₂ and concomitant changes in mitochondrial redox state. *J. Neurosci.* 2008; 28:1153–1162. [PubMed: 18234893]
- Jung S, Kim HW, Lee JH, Kang SS, Rhu HH, Jeong YI, Yang SY, Chung HY, Bae CS, Choi C, Shin BA, Kim KK, Ahn K. Brain tumor invasion model system using organotypic brain-slice culture as an alternative to in vivo model. *J Cancer Res Clin Oncol.* 2002; 128:469–76. [PubMed: 12242510]
- Karra D, Dahm R. Transfection techniques for neuronal cells. *J Neurosci.* 2010; 30:6171–7. [PubMed: 20445041]
- Köhling R, Qü M, Zilles K, Speckmann E. Current-source-density profiles associated with sharp waves in human epileptic neocortical tissue. *Neuroscience.* 1999; 94:1039–50. [PubMed: 10625046]
- Liu G, Gu B, He XP, Joshi RB, Wackerle HD, Rodriguiz RM, Wetsel WC, McNamara JO. Transient inhibition of TrkB kinase after status epilepticus prevents development of temporal lobe epilepsy. *Neuron.* 2013; 79:31–8. [PubMed: 23790754]
- Loup F, Weinmann O, Yonekawa Y, Aguzzi A, Wieser HG, Fritschy JM. A highly sensitive immunofluorescence procedure for analyzing the subcellular distribution of GABAA receptor subunits in the human brain. *J. Hist & Cytochem.* 1998; 46:1129–1139. 1998.
- Marosi M, Fuzik J, Nagy D, Rákos G, Kis Z, Vécsei L, Toldi J, Ruban-Matuzani A, Teichberg VI, Farkas T. Oxaloacetate restores the long-term potentiation impaired in rat hippocampus CA1 region by 2-vessel occlusion. *Eur. J. Pharmacol.* 2009; 604:51–57. [PubMed: 19135048]
- Martin FC, Wiley CA. A serum-free, pyruvate-free medium that supports neonatal neural/glia cultures. *J Neurosci Res.* 1995; 41:246–58. [PubMed: 7650760]
- McBain CJ, Boden P, Hill RG. Rat hippocampal slices in vitro display spontaneous epileptiform activity following long-term organotypic culture. *J Neurosci Meth.* 1989; 27:35–49. [PubMed: 2563782]
- McKenna MC, Waagepetersen HS, Schousboe A, Sonnewald U. Neuronal and astrocytic shuttle mechanisms for cytosolic-mitochondrial transfer of reducing equivalents: current evidence and pharmacological tools. *Biochem. Pharm.* 2006; 71:399–407. [PubMed: 16368075]
- McKinney RA, Debanne D, Gähwiler BH, Thompson SM. Lesion-induced axonal sprouting and hyperexcitability in the hippocampus in vitro: implications for the genesis of posttraumatic epilepsy. *Nat Med.* 1997; 3:990–6. [PubMed: 9288725]
- Merz F, Gaunitz F, Dehghani F, Renner C, Meixensberger J, Gutenberg A, Giese A, Schopow K, Hellwig C, Schäfer M, Bauer M, Stöcker H, Taucher-Scholz G, Durante M, Bechmann I. Organotypic slice cultures of human glioblastoma reveal different susceptibilities to treatments. *Neuro Oncol.* 2013; 15:670–81. [PubMed: 23576601]
- Nehlig A, Coles JA. Cellular pathways of energy metabolism in the brain: is glucose used by neurons or astrocytes? *Glia.* 2007; 55:1238–50. [PubMed: 17659529]
- Norberg J, Poulsen FR, Blaabjerg M, Kristensen BW, Bonde C, Montero M, Meyer M, Gramsbergen JB, Zimmer J. Organotypic hippocampal slice cultures for studies of brain damage, neuroprotection and neurorepair. *Curr Drug Targets CNS.* 2005; 4:435–52.
- Nordström CH. Cerebral energy metabolism and microdialysis in neurocritical care. *Childs Nerv Syst.* 2010; 26:465–72. [PubMed: 19937248]
- Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH. Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. *J. Neurosci.* 1997; 17:3727–3738. [PubMed: 9133393]
- Romijn HJ, de Jong BM, Ruijter JM. A procedure for culturing rat neocortex explants in a serum-free nutrient medium. *J. Neurosci. Meth.* 1988; 23:75–83.

- Rytter A, Cronberg T, Asztély F, Nemali S, Wieloch T. Mouse hippocampal organotypic tissue cultures exposed to in vitro “ischemia” show selective and delayed CA1 damage that is aggravated by glucose. *J Cereb Blood Flow Metab.* 2003; 23:23–33. [PubMed: 12500088]
- Sandow N, Zahn RK, Gabriel S, Heinemann U, Lehmann TN. Glutamine induces epileptiform discharges in superficial layers of the medial entorhinal cortex from pilocarpine-treated chronic epileptic rats in vitro. *Epilepsia.* 2009; 50:849–58. [PubMed: 19175401]
- Sato K, Akaishi T, Matsuki N, Ohno Y, Nakazawa K. Beta-Estradiol induces synaptogenesis in the hippocampus by enhancing brain-derived neurotrophic factor release from dentate gyrus granule cells. *Brain Res.* 2007; 1150:108–20. [PubMed: 17433270]
- Scharfman HE. The CA3 “backprojection” to the dentate gyrus. *Prog Brain Res.* 2007; 163:627–37. [PubMed: 17765742]
- Stoppini L, Buchs PA, Muller D. A simple method for organotypic cultures of nervous tissue. *J Neurosci Methods.* 1991; 37:173–82. [PubMed: 1715499]
- Svoboda N, Kerschbaum HH. L-Glutamine -induced apoptosis in microglia is mediated by mitochondrial dysfunction. *Eur J Neurosci.* 2009; 30:196–206. [PubMed: 19614980]
- Tønnesen J, Sørensen AT, Deisseroth K, Lundberg C, Kokaia M. Optogenetic control of epileptiform activity. *Proc Natl Acad Sci U S A.* 2009; 106:12162–7. [PubMed: 19581573]
- Turrigiano G, LeMasson G, Marder E. Selective regulation of current densities underlies spontaneous changes in the activity of cultured neurons. *J Neurosci.* 1995; 15:3640–52. [PubMed: 7538565]
- Wilhelmi E, Schöder UH, Benabdallah A, Sieg F, Breder J, Reymann KG. Organotypic brain-slice cultures from adult rats: approaches for a prolonged culture time. *Altern Lab Anim.* 2002; 30:275–83. [PubMed: 12106005]
- Wirenfeldt M, Clare R, Tung S, Bottini A, Mathern GW, Vinters HV. Increased activation of Iba1+ microglia in pediatric epilepsy patients with Rasmussen’s encephalitis compared with cortical dysplasia and tuberous sclerosis complex. *Neurobiol Dis.* 2009; 34:432–40. [PubMed: 19285133]
- Wittner L, Huberfeld G, Clémenceau S, Er ss L, Dezamis E, Entz L, Ulbert I, Baulac M, Freund TF, Maglóczky Zs, Miles R. The epileptic human hippocampal CA2 region generates spontaneous interictal like activity in vitro. *Brain.* 2009; 132:3032–46. [PubMed: 19767413]
- Wykes RC, Heeroma JH, Mantoan L, Zheng K, MacDonald DC, Deisseroth K, Hashemi KS, Walker MC, Schorge S, Kullmann DM. Optogenetic and potassium channel gene therapy in a rodent model of focal neocortical epilepsy. *Sci Transl Med.* 2012; 4:161. [PubMed: 23147003]

Highlights

- An organotypic culture preparation of brain tissue from epileptic patients. (77)
- A novel defined culture medium. (33)
- Spontaneous and induced epileptiform activity maintained for 2-4 weeks. (72)

Fig. 1.

Comparison of activity after culture in MEM or a defined medium.

(A) Extracellular activity recorded from a slice cultured for 9 days in a MEM-based medium including serum. (B) Extracellular activity of a slice cultured for 9 days in a defined medium with no serum. Multi-unit activity was more frequent and epileptiform events (arrowhead) occurred. (C) The probability of recording multi-unit activity from slices cultured in the MEM-based and the defined medium. (D) Proportion of slices maintained in the MEM-based medium and the defined medium from which interictal-like events were recorded. Significance values from the Fisher and Yates (1963) with * indicating $p < 0.05$.

Fig. 2.

Electrical activity generated at 6-10 days by organotypic slices.

(A) Interictal-like activity recorded from the dentate gyrus at 6-10 days in organotypic culture. From the top, activities generated by the dentate gyrus (DG), the CA3c region (CA3c) the CA2/CA3a region (CA3a) and the subiculum (sub). (B). Expanded view of the initiation of an interictal-like event suggests it was generated in the dentate gyrus and propagated to the CA3 and CA2 regions. (C) Probability of recording multi-unit activity (MUA) from different regions at 6-10 days. (DG, dentate gyrus; CA2/CA3a region; CA3a; CA3c region; CA3c; Sub, subiculum). (D) Probability of recording interictal-like discharges (IID) from the same regions.

Fig. 3.

Anatomy of organotypic slices at 6-10 days in culture.

(A) Immunostaining with an antibody against β -tubulin-III revealed few positive cells (arrow) in the CA1 region. (B) Immunostaining with an antibody against β -tubulin-III showed dispersion of granule cells (arrows) in the dentate gyrus. (C) Combined immunostaining of the CA3 region for GFAP (red) and Nestin (white) reveals a marked activation of astrocytes (arrowheads) and presumed progenitor cells (asterisk). (D, E) Electron micrographs of semithin sections of the dentate gyrus from tissue fixed just after resection (0div) and after 9 days in organotypic culture (9div) shown at low (D) and higher (E) magnification. Scale bars 100 μ m (D) and 10 μ m (E). Nuclei, outlined in red, are more round and less invaginated after 9 days in culture. (F) Transmission electronmicrograph of a synapse from the dentate gyrus at 9 days in culture. Pre-synaptic vesicles (arrow head), a

mitochondrion (arrow), an active zone with invaginated presynaptic membrane (double arrow) and the post-synaptic density (asterisk) are visible. Scale bar 50 nm.

Fig. 4.

Effects of antagonists and pro-convulsant solution on activity at 6-10 days in culture.

(A) Spontaneous interictal-like activity generated by the CA3 region of organotypic slices was abolished by 50 μ M picrotoxin and after returning in control, was blocked by the glutamate receptor antagonists NBQX (10 μ M) and DL-L APV (100 μ M). (B) A pro-convulsant solution containing 8 mM K^+ and 0.25 mM Mg^{++} increased the frequency of interictal-like events. (C) In a different slice, the pro-convulsant solution induced patterned seizure-like discharges of duration 10-45 s.

Fig. 5.

Variation in electrical activity with time in culture.

(A) Sparse multi-unit activity recorded from the CA3 region at 2 days in culture (2div), and interictal-like discharges with multi-unit firing and a field potential generated at 9 (9div), 16 (16div) and 24 (24div) days in organotypic culture. (B) The mean frequency of spontaneous interictal-like discharges was higher at 15 than at 9 or 22 div, but not significantly (Mann-Whitney test). (C) In the presence of a pro-convulsant solution, 8 mM K^+ and 0.25 mM Mg^{++} , the mean frequency of interictal-like events was increased with similar changes between 9, 16 and 24 div.

Fig. 6.

Electrical activity of temporal cortex in organotypic cultures.

(A) Temporal cortex slices, cultured in the defined medium, generated sparse multi-unit discharges at 9 div and at 24 div (B). (C) At 9 div, epileptiform events of duration 4-8 s were induced by a pro-convulsant solution containing 8 mM K^+ and 0.25 mM Mg^{++} (D) Similar epileptiform events were induced by 50 μ M picrotoxin and by (E) 50 μ M 4-aminopyridine.

Table 1

Composition of the defined culture medium.

Group	Compound	Concentration (μM)
Inorganic salts	Calcium chloride	1.3×10^3
	Ferric (III) citrate	0.02
	Ferric (III) sulphate	0.68
	Potassium chloride	3.3×10^3
	Cupric(II) sulphate	0.01
	Magnesium sulphate	0.99×10^3
	Magnesium chloride	45.3
	Sodium chloride	116.9×10^3
	Sodium dihydrogen phosphate	1.1×10^3
	di-Sodium hydrogen phosphate	75.0
	Sodium hydrogen carbonate	22.3×10^3
	Zinc sulphate	715.0×10^{-3}
	Manganese chloride	1.0×10^{-3}
Amino acids	L-Glutamine	8.0
	L-Alanine	7.5
	L-Arginine	119.8
	L-Asparagine	7.5
	L-Aspartic acid	7.5
	L-Cysteine	15.0
	L-Glutamic acid	7.5
	L-Cystine	22.5
	Glycine	37.5
	L-Histidine	30.2
	L-Isoleucine	92.1
	L-Leucine	91.3
	L-Lysine	74.8
	L-Methionine	24.9
	L-Phenylalanine	47.2
	L-Proline	22.5
	Taurine	8.0
	L-Serine	37.5
	L-threonine	97.6
	L-tryptophan	9.6
	L-tyrosine	47.0
	L-valine	97.9
Vitamins	Biotin (vitamin B7)	0.6
	D-Calcium panthotenate (B5)	1.0
	Choline choride	10.7
	Folic acid (B9)	1.2
	Myo-inositol	12.0
	Nicotinamide (B3)	3.7
	Pyridoxal (B6)	2.1
	Riboflavin (B2)	0.1
	Thiamine (B1)	1.4
	Cobalamin (B12)	0.08
	Ascorbic acid (C)	580.0
	α -Tocopherol (E)	2.3
	α -Tocopherol acetate (E)	2.1
Hormone	Insulin	0.6
	T3	3×10^{-3}
	17- β Oestradiol	0.8×10^{-3}
	Corticosterone	0.06
	Progesterone	0.01
Energy sources	D-Glucose	4.1×10^3
	Sodium lactate	4.0×10^3
	Creatine	5.0×10^3
	Phosphate creatine	200
	Sodium pyruvate	215
	Adenosine tri-phosphate	10

Group	Compound	Concentration (μM)
Other	Sodium hypoxanthine	2.2
	Thymidine	0.2
	Lipoic acid	0.08
	Linoleic acid	0.02
	Putrescine	50.0
	Oxaloacetic acid	1×10^3
	Heparin	50.0
	GABA	1.0
	Glycerol	50.0
	Citric acid	573.0
	Acetic acid	570.0
	Sodium β -hydroxybutyrate	1×10^3
	Mannitol	5×10^3
	Transferrin	5.0
	Bovine serum albumin fraction V	40.0
	Glutathione, reduced	3.2
	Selenium	0.01
	Superoxide dismutase	0.08