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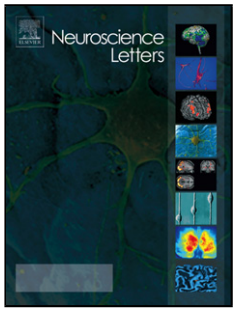

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Heptanoate is neuroprotective *in vitro* but triheptanoin post-treatment did not protect against middle cerebral artery occlusion in rats

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Tel 07-3365-3113Highlights

- Heptanoate pre-treatment is neuroprotective against oxygen glucose deprivation and NMDA *in vitro*

- Heptanoate enhanced basal respiration, ATP turnover and proton leak in cultured astrocytes
- Continuous infusion of triheptanoin (i.v.) initiated 1 h after stroke onset in rats did not alter stroke volume

Abstract

Triheptanoin, the medium-chain triglyceride of heptanoate, has been shown to be anticonvulsant and neuroprotective in several neurological disorders. In the gastrointestinal tract, triheptanoin is cleaved to heptanoate, which is then taken up by the blood and most tissues, including liver, heart and brain. Here we evaluated the neuroprotective effects of heptanoate and its effects on mitochondrial oxygen consumption *in vitro*. We also investigated the neuroprotective effects of triheptanoin compared to long-chain triglycerides when administered after stroke onset in rats. Heptanoate pre-treatment protected cultured neurons against cell death induced by oxygen glucose deprivation and N-methyl-D-aspartate. Incubation of cultured astrocytes with heptanoate for 2 h increased mitochondrial proton leak and also enhanced basal respiration and ATP turnover, suggesting that heptanoate protects against oxidative stress and is used as fuel. However, continuous 72h infusion of triheptanoin initiated 1 h after middle cerebral artery occlusion in rats did not alter stroke volume at 3 days or neurological deficit at 1 and 3 days relative to long-chain triglyceride control treatment.

Abbreviations used: BHB, β-hydryoxybutyrate; CBF, cerebral blood flow; LCT, long-chain triglyceride; MCAo, middle cerebral artery occlusion; NMDA, N-methyl-D-aspartate; OCR, oxygen consumption rate; OGD, oxygen glucose deprivation; TCA, tricarboxylic acid.

Keywords: heptanoate; ischemic stroke; MCAo; mitochondrial function; neuroprotection; triheptanoin

1. Introduction

Ischemic stroke accounts for approximately 87% of stroke incidents [1], and is one of the leading causes of death and disability worldwide. Recent advances in endovascular and pharmacological reperfusion therapies have improved outcomes for some patients dramatically. However, these therapies require early administration and access is variable. Approaches to protect the brain while transporting patients to reperfusion centres offer great promise for even greater reductions in disability associated with stroke.

Growing pre-clinical and clinical evidence suggests that metabolic treatment approaches might be a potential treatment for ischemic stroke due to the metabolic dysfunctions observed in brain. Upon artery occlusion, glucose-dependent ATP generation decreases rapidly, causing both ATP and phosphocreatine levels to decrease in the ischemic core [2-4] and penumbra [4]. Studies using ^{13}C-labeled glucose and acetate found that impairments in neuronal and astrocytic metabolism occurred in both the ischemic core [5] and penumbra of rats subjected to middle cerebral artery occlusion (MCAo) [6]. In addition, a loss of glutamate into systemic circulation and cerebrospinal fluid was observed in both humans [7] and rats [8], which could drain α-ketoglutarate from the tricarboxylic acid (TCA) cycle. This can further exacerbate the underlying metabolic perturbations, as of four- and five-carbon TCA cycle intermediates are needed for the TCA cycle to run efficiently and to produce amino acids [9, 10]. Please note that the four-carbon oxaloacetate is required to allow the two-carbon entry of acetyl-CoA into the TCA cycle.

The entry of carbons into the TCA cycle can be increased by anaplerosis (refilling of four- and five-carbon TCA cycle intermediates. Anaplerosis occurs largely in astrocytes, namely by production of oxaloacetate from pyruvate via pyruvate carboxylase. The carbons from this reaction can be transferred to neurons via several metabolic reactions, including enzymes of the TCA cycle to produce α-ketoglutarate, which is then turned into glutamate and glutamine

in astrocytes. The glutamine-glutamate cycle can transfer carbons to neurons and can replenish their α-ketoglutarate levels. However, after MCAo, the activity of pyruvate carboxylase was found to be decreased [6]. In addition, the entry of glucose-derived carbons into the TCA cycle seems to be impeded, due to reduction in pyruvate dehydrogenase activity [11, 12]. Therefore, a metabolic treatment that bypasses the glycolytic pathway, replenishes the TCA cycle, and thereby improves the entry of glucose-derived carbons into the TCA cycle has been thought to be a potential treatment for ischemic stroke.

Triheptanoin is a triglyceride containing three seven-carbon heptanoate molecules, which are released after cleavage by lipases in the gastro-intestinal tract and directly taken up into blood. Heptanoate can enter the brain directly or after hepatic metabolism into C_5-ketone bodies [13]. Beta-oxidation of heptanoate and C_5 ketone bodies produces acetyl-CoA and propionyl-CoA, the latter is able to replenish the TCA cycle via the propionyl-CoA carboxylation pathway, which produces the four-carbon succinyl-CoA [14, 15]. In addition to being currently used as the treatment for rare metabolic treatment in children and adults [16, 17], triheptanoin has been shown to be neuroprotective in several animal models of neurological disorders. Triheptanoin pre-treatment (35E% of total caloric intake) for two weeks prior to MCAo significantly reduced infarct area, improved neurological function, prevented increases in extracellular glutamate levels and attenuated reductions in ATP levels as well as mitochondrial complex II and IV activities in mice [18]. Triheptanoin treatment also reduced oxidative stress, prevented the loss of oligodendrocytes and improved motor function in a mouse model of Canavan Disease [19], an autosomal recessive neurodegenerative disorder caused by a deficiency of the enzyme aminoacylase 2. In addition, oral triheptanoin treatment was found to delay motor neuron loss and the onset of symptoms in a mouse Amyotrophic Lateral Sclerosis model [20].

Here, we aimed to investigate the protective effects of heptanoate pre-treatment against cell death induced by oxygen glucose deprivation (OGD) or N-methyl-D-aspartate (NMDA) in cultured neurons. We also assessed changes in mitochondrial function after incubation with heptanoate in cultured astrocyes. In the MCAo model in rats we then determined the effects of triheptanoin in comparison to long-chain triglycerides when administered 1 h after cerebral blood flow reduction. Stroke volume was evaluated at 3 days and neurological deficit at 1 and 3 days.

2. Materials and methods

All chemicals and reagents were obtained from Sigma Aldrich (St. Louis, MO, USA) unless stated otherwise. The final concentrations of chemical and reagents are listed.

2.1 Animals

Male Wistar rats (13-18 weeks old; 270-370 g; ASU Breeding Facility, University of Newcastle, NSW, Australia) were co-housed (a maximum of four rats per cage) under a 12-hour light-dark cycle with free access to food and water and all experiments involving animals were conducted during the light cycle. All experiments involving mice (Animal Resources Centre, WA, Australia) were approved by the animal ethics committee of the University of Queensland (SBMS/128/14) and experiments involving rats were approved by the Animal Care and Ethics Committee of the University of Newcastle (A-2014-431). All experiments were performed in accordance with Australian Code of Practice for the Care and Use of Animal for Scientific Purposes. Every effort was made to minimize animal suffering and all work was conducted according to the ARRIVE guidelines [21].

2.2 Primary cortical neuronal and astrocyte cultures

All culture media and reagents were obtained from Life Technologies (CA, USA), unless stated otherwise. Briefly, bilateral cortices were removed from E15 CD1 mouse embryos, triturated and seeded at a density of 2×10^6 cells per well in a 6-well plate (OGD) or 7.5×10^5

cells per well in a 24-well plate (NMDA) with Neurobasal® media containing 25 mM glucose, 2 mM glutamine, 10 µg/ml gentamicin and serum-free B-27® Supplement. For astrocyte-enriched cultures, cells were seeded at 3 x 10^5 cells/ml in a 175 cm^2 culture flask and cultured in Dulbecco's modified Eagle's medium (DMEM) with Ham's F12 nutrient (1:1) containing 10% foetal bovine serum and antibiotics penicillin and streptomycin (25 U/ml final). Neuronal cells were cultured for 8 or 12 days and astrocyte-enriched cultures for two weeks until confluent at 37°C with 5% CO_2.

2.3 Oxygen glucose deprivation and NDMA-induced cell death

After eight days *in vitro*, neuronal cell cultures were treated with 50 µM or 200 µM of heptanoic acid (pH 7.4; Sigma Aldrich) for 16 h prior to OGD. Culture media were then replaced with Locke's buffer containing 154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 3.6 mM $NaHCO_3$, and 5 mM HEPES (pH 7.4) before being incubated in an anaerobic incubator with 95% N_2, 5% CO_2 and < 1% O_2 at 37°C for 3 or 9 h. In NMDA-induced cell death experiments, neuronal cultures (12 days *in vitro*) were treated with 50 µM of heptanoic acid for 16 h prior to treatment with 25 µM of NMDA (Sigma Aldrich) for 24 h. Cell death was measured using a Cytotoxicity Detection Kit (Roche Applied Science, Mannheim, Germany) and expressed as percentage of lactate dehydrogenase released into the media relative to the total amount of lactate dehydrogenase activity per well.

2.4 Mitochondrial function assessment in cultured astrocytes

After two weeks *in vitro*, microglial cells were shaken off and astrocytes (as prepared in "Primary cortical neuronal and astrocyte cultures" section) were replated at a high density of 1.5 x 10^5 cells/well in polyethylenimine-coated XF^{e}96 Cell Culture Microplates (Seahorse Bioscience, MA, USA) and cultured for three days. Then, cultures were treated with 1 mM sodium pyruvate or 0.2 mM heptanoic acid in XF Assay Medium Modified DMEM (Seahorse Bioscience) containing 2 mM glucose, 0.8 mM lactic acid and 0.4 mM glutamine

for 2 h. Mitochondrial functions were assessed using Seahorse XF^{e}96 Analyzer and XF Cell Mito Stress Kit (Seahorse Bioscience) based on oxygen consumption rates (OCR) at 37°C as previously described [22]. Briefly, OCR was measured prior to and throughout sequential addition of 1.5 µM oligomycin, 1 µM FCCP and 1 µM each of antimycin A/rotenone to stimulate different states of mitochondrial respiration. Various parameters of mitochondrial function including basal respiration, maximal respiration, proton leak, ATP turnover, coupling efficiency and respiratory control ratio uncoupled were quantified as previously described [23] and expressed as percentages relative to 1 mM sodium pyruvate.

2.5 Anesthesia and monitoring

Rats were anesthetized using 5% isoflurane and maintained with 1% to 2% isoflurane in N_2/O_2 (70/30; vol/vol %) through spontaneous breathing. Core temperature was maintained at 37°C using a thermos-coupled rectal probe and warming mat while blood oxygen saturation levels (SpO_2) was monitored throughout surgery. Animals were injected with analgesic 0.05% bupivacaine (2 mg/kg; subcutaneous; Pfizer, Sydney, Australia) prior to incision and were given bupivacaine (0.2 mg/kg) and 3 ml of 0.9% saline (both subcutaneous) post-surgery. All animals were housed individually with free access to mushed standard rodent chow and water.

2.6 Surgical procedures

In the first study (study I), the rats only underwent the jugular vein cannulation procedure described below to investigate whether continuous infusion of 20% LCT or triheptanoin emulsion (i.v.) for 72 h is safe and tolerable. Blood was collected from the left ventricle 72 h post infusion using heparinized syringe and centrifuged at 2,000 g for 10 min at 4°C. Plasma was aliquoted and stored at -80°C until analyzed. In the second study (study II), the rats underwent all the surgical procedures described below to determine whether triheptanoin is neuroprotective in the MCAo model.

2.6.1 Femoral artery cannulation

The right saphenous branch of the femoral artery was cannulated using a laboratory-manufactured catheter which was connected to a blood pressure transducer (CODAN, Forstinning, Germany). Mean arterial pressure, heart rate and respiratory rate were calculated from the arterial pressure tracing and all parameters were monitored throughout surgery. Blood samples (0.1 ml) were taken from the arterial line for blood gas analysis using i-STAT CG8+ cartridges (Abbott, IL, USA) prior to occlusion.

2.6.2 Laser Doppler Flowmetry

The right parietal bone was thinned at 2 mm caudal and 5 mm lateral to bregma and a hollow PEEK® screw (2 mm diameter x 4 mm height; Solid Spot, CA, USA) was inserted. A laser Doppler Flowmetry probe (Moore Instruments, Sussex, UK), which was inserted into the screw for the measurement of cerebral blood flow (CBF) of the middle cerebral artery (MCA) region, was held in place using a caulking material (Silagum, DMG, Hamburg, Germany). Animals with less than 50% decrease in CBF in the penumbra region upon occlusion and those with subarachnoid hemorrhage were excluded from the study.

2.6.3 Jugular vein cannulation

The right jugular vein was cannulated using a laboratory-made catheter which was connected to a syringe pump (Kent Scientific, CT, USA) to allow continuous infusion of 20% LCT or 20% triheptanoin emulsion (both from B. Braun Melsungen, Melsungen, Germany) at a constant rate of 1.36 ml/kg/h to provide 30% of total caloric intake from triheptanoin (Gu et al., 2010).

2.6.4 Middle cerebral artery occlusion

Occlusion of the MCA was performed using very minor modification of the previously described silicone-tipped intraluminal thread occlusion technique [24]. In this study, a silicone tip of 4 mm long with 0.38 mm diameter was used and the right MCA was occluded

for 2.5 h. Sham rats underwent all surgical procedures except that the occlusion thread was not advanced past the internal carotid artery to prevent occlusion of the origin of MCA. Both sham and stroke rats were randomly assigned to receive 20% LCT or 20% triheptanoin emulsion (i.v.) by a blinded experimenter and the continuous 72h infusion was initiated 1 h post-MCAo .

2.7 Plasma metabolite level measurement using GC-MS

The plasma levels of heptanoate and β-hydryoxybutyrate (BHB) from study I were measured using an Agilent Mass Spectrometer (model 5975B) equipped with Agilent Gas Chromatography system (model 6870) and a VF-1 msec capillary column. Samples were extracted, derivatized and measured as previously described [13, 22] with the following modifications. Briefly, 40 nmol of D6-succinic acid (98% D; Cambridge Isotope Laboratory, MA, USA) was added to 40 µL of plasma as internal standards. The samples were derivatized using 105 µL of N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide + 1% tert-butyldimetheylchlorosilane and reacted overnight on a shaker at 37°C.

For the GC-MS, a helium flow of 1 mL/min was used and the starting oven temperature was set at 80°C with pressure of 14.82 psi and flow velocity of 45 cm/s. The temperature was then increased linearly to 220°C and held for 1 min. The derivatized samples were injected into the GC-MS in split mode (1:20) and the selected-ion monitoring mode was used. The m/z ratios for each metabolite are as follow: heptanoic acid (m/z = 187), BHB (m/z = 159) and d6 succinic acid (m/z = 293). The total levels of metabolites in each sample were calculated using the respective standard curves.

2.8 Histological assessment of infarct volume

Rats were euthanized under deep anesthesia with isoflurane 72 h after MCAo and were subjected to transcardial perfusion with 200 ml each of cold 0.9% saline and 4% paraformaldehyde solution before being decapitated. The brains were post-fixed in the skull

in neutral-buffered formalin at 4°C until being dehydrated through ascending concentrations of ethanol, embedded in paraffin and sectioned. Coronal sections (5 µm) were stained using hematoxylin and eosin and imaged at 20x objective using Aperio slide scanner (Aperio Technologies, CA, USA). The infarct area between 3.7 mm to -4.3 mm from bregma (1 mm interval) was determined by an experimenter blinded to treatment group using ImageScope (Leica Biosystems, CA, USA) with a 10x objective and infarct volume was calculated and corrected for oedema as previously described [25]. A second blinded experimenter was employed to determine the infarct area of random samples to ensure that the assessment was reproducible. The infarct areas determined by the two experimenters were less than 10% different.

2.9 Neurological deficit testing

Rats were tested for neurological deficits using forelimb flexion and torso twist tests as previously described [26] at 24 h and 72 h post-MCAo by a blinded experimenter. A score of 0 was assigned when no flexion or twist was observed while scores of 1 and 2 indicated mild and severe flexion or twist respectively. Neurological deficit was reported as the sum of both forelimb flexion and torso twist tests and higher score indicates more severe deficit.

2.10 Statistical analysis

Power analysis was performed based on our previous experience with our stroke model and indicated that 10 rats/group were required to be able to detect >25% difference in stroke volume with $\alpha = 0.05$, $\beta = 0.2$. All graphs show mean ± SEM except for plots depicting individual and median neurological deficit scores. Differences between two groups were compared using unpaired, two-tailed Student's t-test and for experiments with two variables, two-way ANOVA followed by Bonferroni's multiple comparison post hoc test was used except when compared to 0 h (two-way repeated measures ANOVA). A Mann-Whitney test was used to assess neurological deficit and statistical significance was accepted at $p<0.05$.

The number of stars in the figures indicates the significance in Student's t-tests or Bonferroni's multiple comparison tests. All statistical analyses were conducted using GraphPad Prism 7.02 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1 Neuroprotective effects of heptanoate *in vitro*

Pre-treatment with 200 µM of heptanoate reduced cell death by 34 ± 10% after 3 h of OGD in three out of four independent cortical neuronal culture preparations (Mean ± SD; n= 3 independent experiments). A representative experiment with 28% reduction is shown in Fig. 1A ($p=0.01$; n=3 wells each). A lower concentration of heptanoate pre-treatment (50 µM) was also found to reduce neuronal cell death induced by 9 h of OGD by 18% and 22% ($p=0.01$; 18% reduction shown in Fig. 1B; $p=0.02$; all unpaired, two-tailed Student's t-tests; n=3 wells each) in two independent culture preparations. NMDA (25 µM) induced cell death in both vehicle and heptanoate groups ($p<0.0001$; n=4-5 wells each), which was reduced by heptanoate pre-treatment (50 µM) by 20 ± 7% in all four independent culture preparations tested (28% reduction shown in Fig. 1C; $p<0.0001$; two-way ANOVAs; n=4-5 wells each).

3.2 Effects of heptanoate on mitochondrial function in cultured astrocytes

Changes in mitochondrial function were assessed following two-hour incubation with 0.2 mM of heptanoate in cultured astrocytes using an extracellular flux analyzer to measure OCR. The average basal respiration with 0.2 mM heptanoate was 7.1 ± 3.4 pmol oxygen/min/µg protein (n=6 independent cultures). Heptanoate increased basal respiration by 27% ($p=0.002$; Fig. 1D), proton leak by 21% ($p=0.0002$; Fig. 1D) and ATP turnover by 55% ($p=0.0003$; Fig. 1D) relative to 1 mM of sodium pyruvate. Supplementation with heptanoate did not alter maximal respiration ($p=0.13$; Fig. 1D), coupling efficiency ($p=0.08$; Fig. 1E) or

respiratory control ratio uncoupled (p=0.84; Fig. 1E; all unpaired, two-tailed Student's t-tests; n=6 independent culture preparations).

3.3 Safety and tolerability of continuous triheptanoin emulsion infusion

Safety and tolerability of continuous triheptanoin emulsion infusion was first investigated in a small group of naïve rats in study I. Body weights were not significantly different before and after 72 h of infusion or between 20% LCT (0 h: 321 ± 35 and 72 h: 313 ± 29 g) and triheptanoin treatment (0 h: 323 ± 48 and 72 h: 303 ± 55 g; two-way repeated measures ANOVA; both n=4 rats). We did not observe significant changes in blood glucose levels within 20% LCT and triheptanoin groups neither between the two groups at 0, 1, 3 and 72 h post infusion (two-way repeated measures ANOVA; n=3 rats/group; Table 1). The levels of blood electrolytes (Fig. 2A) and hematocrit (Fig. 2B) were also not altered by continuous infusion and did not differ between the two emulsions (all two-way repeated measures ANOVAs; n=4 rats/group). Body weight, blood glucose and electrolytes were not altered 72 h post infusion, suggesting that continuous infusion of both 20% LCT and triheptanoin emulsions for 72 h via the jugular vein is safe and tolerable in rats.

3.4 Plasma metabolite levels

In study I, plasma heptanoate levels were increased by 40-fold after triheptanoin emulsion (LCT: 6.6 ± 0.9 and triheptanoin: 264.0 ± 90.1 µM; p=0.001; n=4 rats/group) infusion, while the plasma levels of BHB were similar in both groups (LCT: 1.57 ± 0.43 and triheptanoin: 1.44 ± 0.19 mM; p=0.64; both unpaired, two-tailed Student's t-tests; n=3 rats/group).

3.5 Physiological parameters

In study II, three rats were excluded by a blinded experimenter for the following reasons: less than 50% reduction in CBF upon occlusion (one LCT; one triheptanoin) and subarachnoid hemorrhage (one triheptanoin-treated rat). Physiological parameters including body temperature, arterial partial pressure of oxygen and carbon dioxide as well as arterial blood

pH were not significantly different between groups immediately before the occlusion at 0 h (Supplementary Table 1; all two-way ANOVAs; sham: n=5 rats/group; stroke: n=10-11 rats/group). Other physiological parameters including SpO_2%, heart rate, respiratory rate and mean arterial pressure were also not significantly different between or within groups at 0 h or during reperfusion at 2.5 h after MCAo (Supplementary Table 2; all two-way repeated measures ANOVAs; sham: n=5 rats/group; stroke: n=9-11 rats/group).

Body weights, blood sodium, potassium and ionized calcium levels were not different between groups at 0 h or 72 h post occlusion (Table 2; all two-way repeated measures ANOVAs; sham: n=5 rats/group; stroke: n=10-11 rats/group). Apart from blood potassium levels, which increased in all groups at 72 h post-MCAo, body weights, blood sodium and ionized calcium levels were significantly decreased at 72 h post occlusion in most groups (Table 2; all two-way repeated measures ANOVAs; sham: n=5 rats/group; stroke: n=10-11 rats/group). Blood glucose levels and hematocrit were unaltered between and within groups (Table 2; all two-way repeated measures ANOVAs; sham: n=5 rats/group; stroke: n=10-11 rats/group) at both 0 h and 72 h.

3.6 Triheptanoin did not alter infarct volume and neurological deficit

Infarct volumes were assessed using hematoxylin and eosin staining at 72 h post-MCAo. High variability in stroke volume was observed in both groups and there was no significant difference in infarct volume between LCT and triheptanoin groups (mean ± SD; LCT: 46.6 ± 32.9 and triheptanoin: 60.6 ± 32.5 mm^3; p=0.37; unpaired, two-tailed Student's t-test; n=10-11 rats/group; Fig. 3A). The sum of two neurological tests in stroke rats, forelimb flexion and torso twist, were not statistically different between LCT and triheptanoin groups at both 24 h (median scores; LCT: 1 and triheptanoin: 0; p=0.37) and 72 h post occlusion (median scores; LCT: 0 and triheptanoin: 1; p=0.41; both Mann Whitney tests; p=10-11 rats/group; Fig. 3B). Interestingly, small intracerebral hemorrhages were observed in the majority of stroke rats

from both emulsion groups (LCT: 8 out of 11 and triheptanoin: 8 out of 10 rats). All were located in the ipsilateral hemisphere within or in close proximity to the infarct.

4. Discussion

In previous studies triheptanoin was anticonvulsant and neuroprotective in several animal models of neurological disorders [18-20]. Pre-treatment with triheptanoin appeared promising in a mouse model of MCAo [18]. Here we found that pre-treatment with heptanoate protected against cell death induced by oxygen glucose deprivation and NMDA in cultured neurons, indicating heptanoate is neuroprotective *in vitro*. We also showed that basal respiration and ATP turnover were increased in cultured astrocytes treated with 0.2 mM heptanoate relative to 1 mM sodium pyruvate. These findings suggest that heptanoate is oxidized directly by astrocytes, consistent with a previous study which demonstrated that other medium-chain fatty acids, specifically octanoate and decanoate were utilized by cultured astrocytes [22]. However, we did not observe any neuroprotective effects of triheptanoin when administered (i.v.) commencing 1 h after stroke in rats in this study.

The protective effects of heptanoate against oxygen glucose deprivation- and NMDA-induced cell death in cultured neurons indicate that heptanoate can be neuroprotective. The observed changes in mitochondrial function in cultured astrocytes provide direct evidence that it can be oxidized by astrocytes. This finding is consistent with another study which also showed that heptanoate is oxidized in mouse brain mostly by astrocytes following i.v. infusion by tracing the fate of ^{13}C-labeled heptanoate [15]. Although it is unclear to which extent heptanoate and C_5 ketones reached penumbral tissue, heptanoate logP is between 2.1 and 2.4 indicating that it will easily diffuse into the brain and also the penumbra. When radioactively labelled octanoate, a medium chain fat with similar properties to heptanoate (logP = 3), was injected in rats (i.v.), labelled octanoate and metabolites were found in the brain within 0.5-2 min [27]. Taken together, our study is consistent with others indicating that heptanoate can be

utilized as fuel in the brain. It is still unclear to what extent neurons can metabolize heptanoate.

In addition, we found that heptanoate increased mitochondrial proton leak in cultured astrocytes, which is similar to effects of decanoate described earlier [22]. The mechanisms underlying increased proton leak are unknown, although studies have shown that fatty acids are able to activate uncoupling proteins responsible for mitochondrial proton leakage [28, 29]. Further studies are necessary to determine to which extent heptanoate alters the levels of uncoupling proteins. Mild uncoupling has been shown to be beneficial by reducing the production of free radicals in the mitochondria [30, 31]. Furthermore, the finding that ATP turnover was enhanced after heptanoate treatment indicates that ATP synthesis was not compromised despite mild proton leak in cultured astroyctes.

Continuous intravenous infusion with triheptanoin emulsion appeared to be safe and tolerable in normal rats in this study. The changes in body weights and blood electrolytes at 72 h post-MCAo were independent of emulsion type and stroke. These changes could be attributed to post surgery trauma, which have been observed in patients following major surgery [32].

Intriguingly, continuous infusion with LCT and triheptanoin emulsion led to small intracerebral hemorrhage in the majority of the rats in the MCAo groups. This has not previously been seen in other studies using this method [24, 33, 34]. We do not understand the exact cause of hemorrhage. Since both groups were equally affected, we speculate that glycerol, which has been shown to be greatly increased (1.7 mM) following triheptanoin infusion [13], could contribute to the hemorrhage. High concentration of glycerol given systemically induce intraventricular and subarachnoid hemorrhage in neonatal rabbit models [35, 36]. These findings suggest that care should be taken if considering administering lipid-based parenteral nutrition to stroke patients. As intra-duodenal infusion avoids high glycerol

levels [14, 15] and is similar to oral administration used successfully by Schwarzkopf, [18], this requires further investigation.

Triheptanoin emulsion increased plasma heptanoate levels and although brain heptanoate and C_5 ketone levels were not measured in this study, it is known that heptanoate enters the brain directly or as C_5-ketone bodies based on a previous study which infused ^{13}C-labeled heptanoate (i.v.) in conscious mice [15]. In our study, triheptanoin treatment for 72 h did not alter stroke volume and neurological deficit when administered 1 h after stroke onset relative to LCT emulsion. This was unexpected given the promising neuroprotective effects found *in vitro* in this study and following pre-treatment *in vivo* [18]. There are several limitations to our study. The variability in stroke volume was higher than in our previous studies, which were used to determine sample size. On the other hand there was no protective trend, therefore we concluded that there was no protective effect. The high variability does not appear to be explained by our >50% threshold for cerebral blood flow reduction with a LDF probe positioned over the penumbral region (not infarct core). Additional earlier and later time points may have shed more light on the effects of triheptanoin. In addition to the high variability, it is also possible that LCT is neuroprotective and may have masked the effects of triheptanoin. Thus, future *in vitro* experiments should include long-chain fatty acids. We considered including another group of animals receiving other extra fuel or no extra fuel (i.v.) after stroke onset in the initial experimental design. However, animals do not feed well after experimental stroke and tend to lose signinficant body weight. Therefore we felt that the latter was unethical.

Although triheptanoin post-treatment did not alter infarct volume and neurological functions in this study, this does not imply that there is no benefit of triheptanoin or other metabolic approaches, several of which appear promising. The lack of effect of triheptanoin in our model may be specific to the rat, as so far all neuroprotective effects were described in mice.

In addition, neuroprotective effects have been observed in animal models of ischemic stroke following ketogenic state induction [37, 38] and supplementation of TCA cycle intermediates or their derivatives including pyruvate [39-42], α-ketoglutarate [43], fumarate [44, 45] and oxaloacetate [46-49]. Therefore, the use of metabolic treatments in ischemic stroke remains a potential avenue, although further research is required.

5. Conclusions

Here we found that heptanoate pre-treatment was protective against oxygen glucose deprivation- and NMDA-induced cytotoxicity in cultured neurons. Heptanoate increased basal respiration and ATP turnover in cultured astrocytes, indicating that heptanoate can be utilized by astrocytes. Despite the promising neuroprotective effects found *in vitro*, triheptanoin treatment for 72 h did not alter stroke volume or neurological deficit when administered 1 h after stroke onset relative to LCT emulsion. There were also noticeably frequent small haemorrhages seen in both triheptanoin and control emulsion treated animals. This result is in contrast to a previous report in a mouse model of experimental stroke using triheptanoin pretreatment. However, due to the high variability in this study, further investigation is required before any conclusion can be drawn about the neuroprotective effects of triheptanoin post-MCAo. Also, other metabolic approaches remain promising as potential treatments for ischemic stroke. Our results suggest that any future studies using lipid emulsions should seek to also determine potential effects of such emulsions on haemorrhage rates, as well as effects on infarct volumes and long term behavioural outcomes.

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Declaration of conflicting interests

KB has filed for a US patent on triheptanoin as a treatment for seizures and has obtained a patent for the treatment of Amyotrophic Lateral Sclerosis (both licensed to Ultragenyx Pharmaceutical Inc.). All other authors declare no conflict of interest.

Supplementary material

Additional supporting information can be found online for this article.

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Titles and legends to figures

Figure 1. Neuroprotective effects of heptanoic acid *in vitro* and effects on mitochondrial functions in cultured astrocytes. Representative examples of the protection of heptanoic acid pre-treatment against neuronal cell death (expressed as % lactate dehydrogenase (LDH) release) induced by (A) 3 h and (B) 9 h of oxygen glucose deprivation (OGD; n=3 wells each) as well as (C) NMDA using primary cortical neuronal cultures (n=4-5 wells each) are shown. Cultured astrocytes grown in 2 mM glucose were incubated with 0.2 mM heptanoic acid or 1 mM sodium pyruvate for two hours. Using an extracellular flux analyzer, various mitochondrial function parameters, including (D) basal respiration, maximal respiration, proton leak, ATP turnover, (E) coupling efficiency and respiratory control ratio (RCR) uncoupled, were determined based on oxygen consumption rates. These parameters were then calculated and expressed as percentages relative to 1 mM sodium pyruvate. (A, B, D, E) All unpaired, two-tailed Student's t-tests, stars indicate t-test significances. (C) Two-way ANOVA followed by Bonferroni's multiple comparison test, stars indicate Bonferroni's post hoc test significances, n=5-6 independent culture preparations.

Figure 2. Blood electrolytes and hematocrit in normal rats after 72 h of 20% long-chain triglycerides or triheptanoin emulsion infusion. (A) Blood electrolytes, including sodium (Na^+), potassium (K^+) and ionized calcium (iCa^{2+}) as well as (B) hematocrit (Hct), were measured before and after 72 h of continuous infusion of 20% long-chain triglyceride (LCT) or triheptanoin emulsion (i.v.) in rats. (A, B) Both two-way repeated measures ANOVAs, n=4 rats/group.

Figure 3. Triheptanoin treatment did not alter stroke volume and neurological deficits when administered 1 h after stroke onset in rats. (A) Brain sections were stained using hematoxylin and eosin and infarct volume at 72 h post-MCAo was assessed from bregma 3.7 to -4.3 mm in rats given 20% long-chain triglycerides (LCT) or triheptanoin (TRIH) emulsion

infusion (i.v.). (B) Neurological deficits were assessed at 24 h and 72 h post occlusion using forelimb flexion and torso twist tests and the sum of both tests are shown (n=10-11 rats/group). (A) Unpaired, two-tailed Student's t-test, n=10-11 rats/group. (B) Mann Whitney tests, n=10-11 rats/group.

Table 1. Blood glucose levels at 0, 1, 3 and 72 h after 20% long-chain triglycerides or triheptanoin emulsion infusion in normal rats.

Time post infusion (h)	Blood glucose - mM (Mean ± SD)		p-Values
	20% LCT	20% TRIH	
0	8.4 ± 0.5	8.0 ± 0.9	>0.99
1	7.4 ± 0.7	7.2 ± 1.0	>0.99
3	6.6 ± 0.6	6.6 ± 0.4	>0.99
72	8.6 ± 0.6	9.4 ± 1.0	>0.99

Two-way repeated measures ANOVA was used. p-Values of Bonferroni's multiple comparison tests are shown; n=3 rats/group. LCT, long-chain triglycerides; TRIH, triheptanoin.

Table 2. Body weights, blood electrolytes, hematocrit and blood glucose levels at 0 h and 72 h post-MCAo in rats given 20% long-chain triglycerides or triheptanoin emulsion infusion.

	Time post-MCAo (Mean ± SD)		p-Values
	0 h	72 h	
Body weight			
Sham LCT	312.0 ± 22.6	283.4 ± 26.1	<0.0001
Sham TRIH	323.0 ± 23.7	286.6 ± 27.8	<0.0001
Stroke LCT	323.5 ± 22.8	289.8 ± 19.3	<0.0001
Stroke TRIH	324.4 ± 15.9	285.0 ± 15.2	<0.0001
Na (mM)			
Sham LCT	141.4 ± 2.6	136.2 ± 1.2	0.04
Sham TRIH	143.0 ± 1.8	135.6 ± 1.4	0.002
Stroke LCT	142.5 ± 3.4	135.2 ± 2.4	<0.0001
Stroke TRIH	142.4 ± 2.1	136.1 ± 1.8	0.0002
K (mM)			
Sham LCT	3.7 ± 0.5	5.0 ± 0.3	0.03
Sham TRIH	3.4 ± 0.3	5.6 ± 0.9	<0.0001
Stroke LCT	3.6 ± 0.4	5.0 ± 0.7	0.0002
Stroke TRIH	3.7 ± 0.5	5.1 ± 0.7	0.0003
iCa (mM)			
Sham LCT	1.39 ± 0.08	1.18 ± 0.10	0.01
Sham TRIH	1.31 ± 0.09	1.15 ± 0.05	0.08
Stroke LCT	1.32 ± 0.15	1.16 ± 0.15	0.005
Stroke TRIH	1.36 ± 0.10	1.15 ± 0.10	0.0006
Hematocrit			
Sham LCT	0.40 ± 0.02	0.48 ± 0.11	0.18
Sham TRIH	0.36 ± 0.04	0.40 ± 0.03	0.95
Stroke LCT	0.37 ± 0.06	0.43 ± 0.08	0.16
Stroke TRIH	0.38 ± 0.03	0.41 ± 0.03	>0.99
Glucose (mM)			
Sham LCT	11.0 ± 2.8	11.9 ± 0.5	>0.99
Sham TRIH	9.6 ± 1.2	12.9 ± 3.1	0.13
Stroke LCT	10.5 ± 3.3	11.3 ± 1.3	>0.99
Stroke TRIH	10.6 ± 1.6	11.0 ± 1.7	>0.99

Two-way repeated measures ANOVAs were used and p-values of Bonferroni's multiple comparison tests (versus 0 h) are shown. Sham: n=5 rats/group; stroke: n=10-11 rats/group. LCT, long-chain triglycerides; MCAo, middle cerebral artery occlusion; TRIH, triheptanoin.