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Ketogenic diet decreases oxidative stress and improves mitochondrial respiratory complex activity

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Abstract

Cerebral metabolism of ketones after traumatic brain injury (TBI) improves neuropathology and behavior in an age-dependent manner. Neuroprotection is attributed to improved cellular energetics, although other properties contribute to the beneficial effects. Oxidative stress is responsible for mitochondrial dysfunction after TBI. Ketones decrease oxidative stress, increase antioxidants and scavenge free radicals. It is hypothesized that ketogenic diet (KD) will decrease post-TBI oxidative stress and improve mitochondria. Postnatal day 35 (PND35) male rats were given sham or controlled cortical impact (CCI) injury and placed on standard (STD) or KD. Ipsilateral cortex homogenates and mitochondria were assayed for markers of oxidative stress, antioxidant expression and mitochondrial function. Oxidative stress was significantly increased at 6 and 24 h post-injury and attenuated by KD while inducing protein expression of antioxidants, NAD(P)H dehydrogenase quinone 1 (NQO1) and superoxide dismutase (SOD1/2). Complex I activity was inhibited in STD and KD groups at 6 h and normalized by 24 h. KD significantly improved Complex II–III activity that was reduced in STD at 6 h. Activity remained reduced at 24 h in STD and unchanged in KD animals. These results strongly suggest that ketones improve post-TBI cerebral metabolism by providing alternative substrates and through antioxidant properties, preventing oxidative stress-mediated mitochondrial dysfunction.

Keywords

Traumatic brain injury, juvenile, ketogenic diet, oxidative stress, mitochondria

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Introduction

Traumatic brain injury (TBI) occurs in two stages. Primary injury is the structural damage caused by mechanical forces either striking the head and/or dynamic movements of the head. Secondary injury includes several interrelated pathological cascades/pathways that evolves within minutes, hours and days after impact and is responsible for the majority of the injuries observed. Various cellular and molecular changes, including ionic imbalance, excitotoxicity, oxidative stress and mitochondrial dysfunction disrupt cellular metabolism leading to neuronal dysfunction and ultimately cell death.[1] There is an enormous burden to develop clinically relevant neuroprotective interventions that preserve and maintain cerebral homeostasis.

TBI has been shown to result in increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) with consequent oxidative and nitrosative damage.[2–4] Mitochondria are thought to be the primary source of free radical production, although TBI upregulates cytosolic pro-oxidants such as NADPH oxidase, xanthine oxidase (XO) and inducible nitric oxide synthase (iNOS).[5,6] Brain tissue is highly

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susceptible to oxidative and nitrosative damage due to its high metabolic activity and high oxygen consumption required for neuronal ionic homeostasis.

Production of free radicals sharply increases following TBI. More recently, focus has been targeted on peroxynitrite ($ONOO^-$) as a mediator of metabolic dysfunction. $ONOO^-$ is formed from the reaction of the superoxide anion (O^{2-}) and nitric oxide (NO).[7] Pathophysiological increases in glutamate after TBI activate NMDA receptors leading to cellular depolarization and influx of intracellular Ca^{2+} that is taken up by the mitochondria.[8] Increased mitochondrial Ca^{2+} has several detrimental actions including inhibition of Complex I leading to O^{2-} production and activation of mitochondrial NOS facilitating further production of $ONOO^-$, inhibiting complex III and further increasing O^{2-} production.[9–12] Breakdown of $ONOO^-$ results in end products with oxidative potential that further inhibit oxidative phosphorylation and deregulates cellular oxidative metabolism.[7]

Cellular oxidative metabolism is not only disrupted by Ca^{2+} and oxidative stress induced mitochondrial dysfunction, but also by injury dependent decreases in glucose metabolism that contribute to observed decreases of ATP.[8,13] This is problematic as the mature brain relies primarily on glucose metabolism for energy production and has led to the search for alternative substrates. In addition to glucose metabolism, the brain retains the ability to metabolize ketone bodies when present.[14] Cerebral metabolism of ketones has been shown to improve cellular energetics, increase glutathione peroxidase activity,[15] reduce cell death[16] and possesses anti-inflammatory and antioxidant capabilities in both *in vitro* and *in vivo* models.[17–20] Prior work from our laboratory has shown that ketones administered after TBI have an age-dependent protective effect.[21] The juvenile (PND35) rat showed improved cellular energetics after TBI[13] that likely contributed to the observed decrease in contusion volume and improved behavioral outcomes.[21,22] It remains unclear what mechanisms underlie the improvement in cellular metabolism and what other mechanisms of ketone action may contribute to the neuroprotection observed after juvenile TBI. Numerous studies have recently reported significant antioxidant effects of ketone metabolism on cerebral oxidative damage.[15,20,23–25] We hypothesize that ketogenic diet improves cellular metabolism after adolescent TBI by reducing oxidative stress thereby attenuating mitochondrial dysfunction.

Materials and methods

All experimental protocols and procedures were approved by the University of California at Los Angeles Chancellor's Committee for Animal Research.

Subjects: 36 Postnatal day 35 (PND35) male Sprague–Dawley rats received either sham surgery or controlled cortical impact (CCI) injury and were randomized to either a standard (STD) (Teklad #7013) or ketogenic (KD) diet (Bioserv #F3666) immediately following surgery. An additional 18 PND70 animals were used to probe potential age-related differences. $N = 6$ for each group (Sham-STD, CCI-STD and CCI-KD).

CCI Injury: Anesthesia was induced with 3% isoflurane vaporized in 100% O_2 and then maintained with 2% isoflurane during surgery. The head was positioned in a stereotaxic frame, a midline incision was made, and a 6 mm craniotomy was drilled centered at −4 mm anterior–posterior (AP), 5 mm midlateral (ML) relative to bregma. A CCI injury was produced on the exposed left cortex using an electronically controlled pneumatic piston cylinder (Hydraulics Control, Inc., Emeryville, CA, USA) as previously described.[26] In the present study, the 5 mm diameter flat rod tip was angled at 22.5° away from vertical and compressed the cortex at 1.9 m/s to a depth of 2 mm. After injury, a small piece of gelfoam was placed over the craniotomy site to reduce bleeding and the wound sutured closed. Sham-injured rats received only a craniotomy but no cortical injury.

Diets: Animals were provided with free access to water and either standard rodent chow or ketogenic diet immediately after they regained consciousness. Previous studies have shown that providing rodents with a ketogenic diet results in an increase in the blood concentration of ketone bodies within hours.[16,21,27] The ketogenic diet consists of 8.4% protein, 78.8% fat, 0.8% carbohydrates and 5% fiber. The standard diet consists of 18.6% protein, 6.2% fat, 59.8% carbohydrates and 4.5% fiber.

Tissue collection: Animals were anesthetized with isoflurane (3.0%/100%O_2) and decapitated at 6 and 24 h after injury at which time the ipsilateral cortical area of interest was collected. Tissue for western blots were collected in microcentrifuge tubes and flash frozen in dry ice and stored at −80°C until processed.

Mitochondrial isolation: Rats were anesthetized with isoflurane (3.0%/100%O_2) and euthanized by decapitation and the ipsilateral cortical area of interest was rapidly dissected and homogenized in ice-cold isolation buffer (225 mM mannitol, 25 mM sucrose, 10 mM Hepes, 1 mM EGTA, pH 7.4 at 4°C). The homogenate was centrifuged at 4000 r/min for 3 min. The pellet was discarded and the supernatant centrifuged at 14,000 r/min for 8 min. The pellets were resuspended in 1.5 ml isolation media and 4 µl of 10% digitonin was added. The tubes were gently inverted six times and left to incubate on ice for 4 min and then spun at 14,000 r/min for 8 min. The pellets were resuspended in 1 ml of isolation buffer and 10 mg/ml de-fatted bovine

serum albumin was added. After a final centrifugation at 14,000 r/min for 8 min, the mitochondria were resuspended in 30 µl of EGTA free isolation media.

Protein determination: Protein concentrations were measured using a Lowry DC kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin used as concentration standards.

Western Blots: Tissue homogenates (cytosol) or isolated mitochondria were lysed in RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, pH 7.6 at 4°C) containing a cocktail of protease inhibitors (Calbiochem). Equal amounts of protein were separated by SDS-PAGE (4–12% Bis-Tris gels, Invitrogen) and transferred to PVDF membranes (Invitrogen), blocked in TBST plus 5% non-fat milk and then incubated with the following primary antibodies: 3-nitrotyrosine (3NT) (Abcam, ab52309); 4-hydroxynonenol (4-HNE) (R&D Systems, mab3249), superoxide dismutase (SOD1 and 2) (Abcam, ab16831 and ab13533) and NAD(P)H dehydrogenase quinone 1 (NQO1) (Abcam, ab34173) overnight at 4°C. The membranes were then washed with TBST and incubated for 1 h at room temperature. The washed membranes were then treated with an enhanced chemiluminescence detection reagent (Thermo Scientific). All Blots were developed using ChemiDoc XRS+ Molecular Imager (BioRad) and analyzed using Quantity One software (BioRad). Band densities were normalized to the total amount of protein loaded per lane using Sypro Ruby (BioRad).

Complex I activity: Complex I enzyme activity was quantified in isolated mitochondria according to the manufacturer's instructions (MitoSciences). Briefly, activity is determined by following the oxidation of NADH to NAD^+ and the simultaneous reduction of a dye which leads to increased absorbance at 450 nm.

Complex II-III activity: Complex II-III enzyme activity was quantified in isolated mitochondria according to manufacturer's instructions (MitoSciences). Briefly, the rate of the coupled Complex II + III reaction is measured by monitoring the conversion of cytochrome c in its oxidized form, cytochrome c (oxidized), to its reduced form, cytochrome c (reduced), as a linear increase in absorbance at exactly 550 nm. Addition of rotenone, an inhibitor of Complex I, ensures that all reduction of cytochrome c is via Complex II. Addition of KCN ensures that there is no re-oxidation of cytochrome c by Complex IV.

Statistical analysis: Data are expressed as means ± SEM of n different experiments. Differences between independent groups were assessed using one way ANOVA, with Tukey's test for comparison between groups using SPSS Software. (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant.

Results

Injury-induced nitrosative and oxidative stress are reduced with ketogenic diet

Consistent with previous findings, the expression of nitrosative damage by 3NT increased with injury. TBI among standard fed animals resulted in increased cytosolic 3NT expression across the entire molecular weight span ($F(4,25) = 9.203$, $p = 0.000$, Figure 1a). When the entire lane was quantified and compared across groups, injury alone significantly increased 3NT expression at

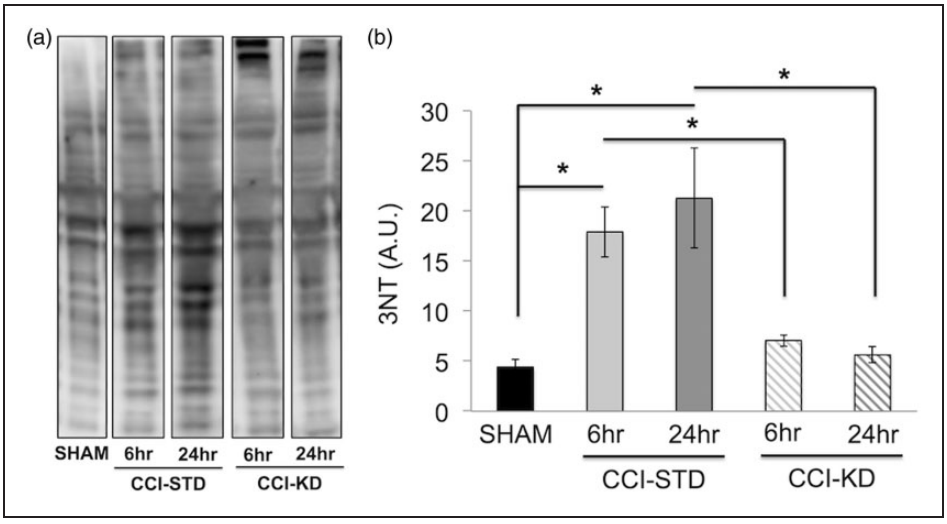

Figure 1. Cytosolic expression of 3NT in PND35 rats on STD or KD diet for 6 or 24 h after TBI. (a) Representative image of 3NT in different treatment groups and time points. (b) Average (± SEM) densitometric ratios for 3NT/total protein, $^*p < 0.05$.

6 and 24 h ($p = 0.001$) post injury by 76% ($p = 0.008$) and 79%, respectively, while KD reduced expression to sham levels at both 6 h ($p = 0.044$) and 24 h ($p = 0.002$) (Figure 1b).

As protein oxidation is a marker of $ONOO^-$ activity, we quantified levels in the mitochondria. Two different patterns of protein oxidation (4HNE) emerged at protein bands near 25 kDa and 27 kDa (Figure 2a) In PND35 animals at 6 h post injury, no changes are observed in the 25 kDa band while there are significant effects of injury and diet ($F(2, 12) = 10.086$, $p = 0.03$) at the 27 kDa band (Figure 2b). Injured animals fed the STD diet had a 452% increase ($p = 0.002$) in 4HNE expression that was reduced by 59% ($p = 0.022$) in ketogenic diet-fed injured animals. At 24 h, there were significant changes at both the 25 kDa ($F(2,12) = 3.923$, $p = 0.049$) and 27 kDa ($F(2,12) = 6.386$, $p = 0.013$) bands (Figure 2(a) and (b)). Injury alone increased 4HNE expression, 83% ($p = 0.043$) and 814% ($p = 0.015$), respectively. Ketogenic diet injured animals had expression levels reduced 30% and 74% ($p = 0.041$), respectively.

A separate group of PND70 animals was also observed as age-related differences in outcome have been previously observed while administering KD after TBI. No significant differences in cytosolic 3NT protein expression were observed between groups: SHAM (269 ± 137), CCI-STD 6HR (350 ± 279), CCI-STD 24HR (119 ± 45), CCI-KD 6HR (599 ± 315) and CCI-KD 24HR (222 ± 130).

Ketogenic diet increases protein expression of cytosolic and mitochondrial antioxidants

One mechanism by which ketogenic diet may improve neurologic outcome is through its antioxidant capabilities. Prior studies have shown that KD is able to activate the Nrf2/ARE system in both acute and chronic settings.[28,29] Activation of the Nrf2 pathway protects cells from oxidative stress-induced cell death by upregulating expression of antioxidant proteins such as NQO1 and cytosolic (SOD1) and mitochondrial (SOD2) superoxide dismutase. These two detoxifying enzymes are of particular interest as they are both superoxide scavengers. Increased expression may both reduce superoxide concentrations and prevent formation of peroxynitrite. Injury alone had no affect on NQO1 ($F(4,25) = 4.262$, $p = 0.009$) or SOD1 ($F(4,25) = 7.845$, $p = 0.000$) expression (Figure 3a), but injured animals on a ketogenic diet had a 2455% ($p = 0.028$) and 700% (0.001) increase, respectively, compared to sham and a 1592% ($p = 0.029$) and 281% ($p = 0.004$) increase, respectively, compared to CCI-STD animals at 24 h post injury (Figure 3(b) and (c).). Mitochondrial SOD2 expression was not changed at 6 h by injury or diet ($F(2,12) = 0.31$,

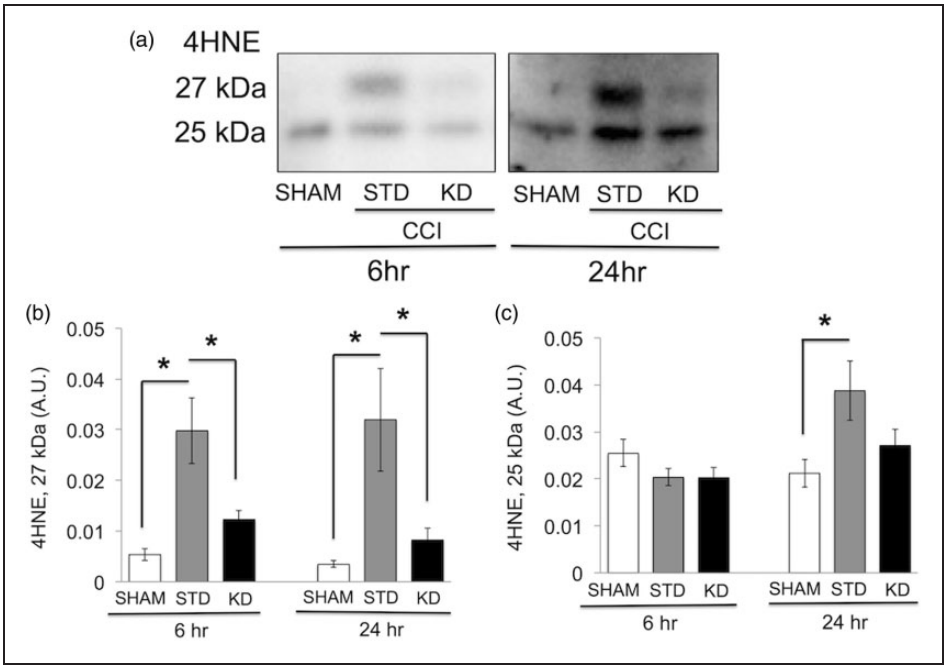

Figure 2. Mitochondrial expression of 4HNE in PND35 rats on STD or KD diet for 6 or 24 h after TBI. (a) Representative image of 4HNE. (b) Average (± SEM) densitometric ratios for 4HNE, 27 kDa/total protein, $*p < 0.05$. (c) Average (± SEM) densitometric ratios for 4HNE, 25 kDa/total protein, $*p < 0.05$.

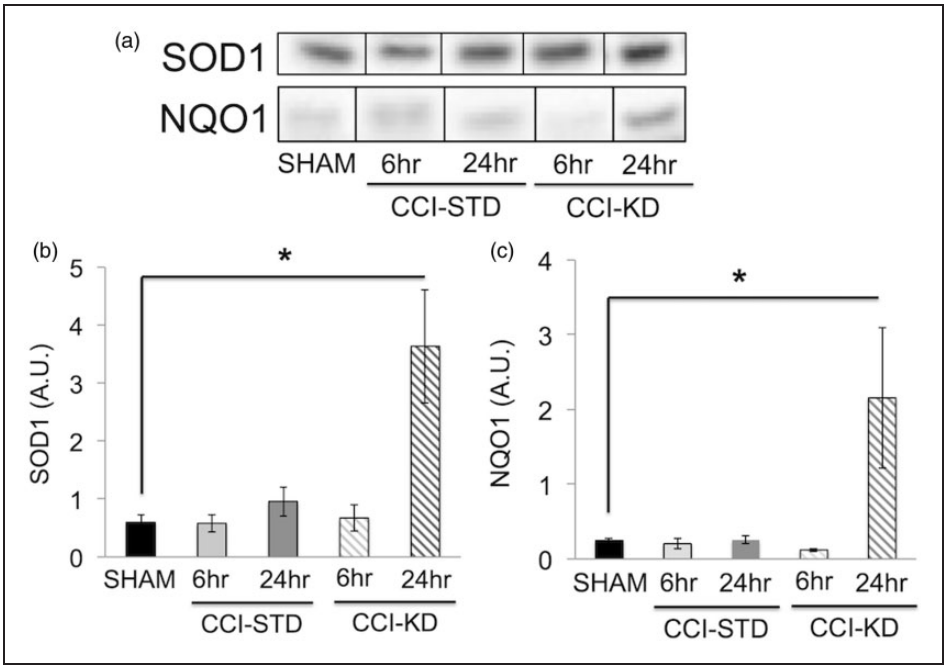

Figure 3. Cytosolic expression of NQO1 and SOD1 in PND35 rats on STD or KD diet for 6 or 24 h after TBI. (a) Representative image of NQO1 and SOD1. (b) Average (± SEM) densitometric ratios for SOD1/total protein, $^*p < 0.05$. (c) Average (± SEM) densitometric ratios for NQO1/total protein, $^*p < 0.05$.

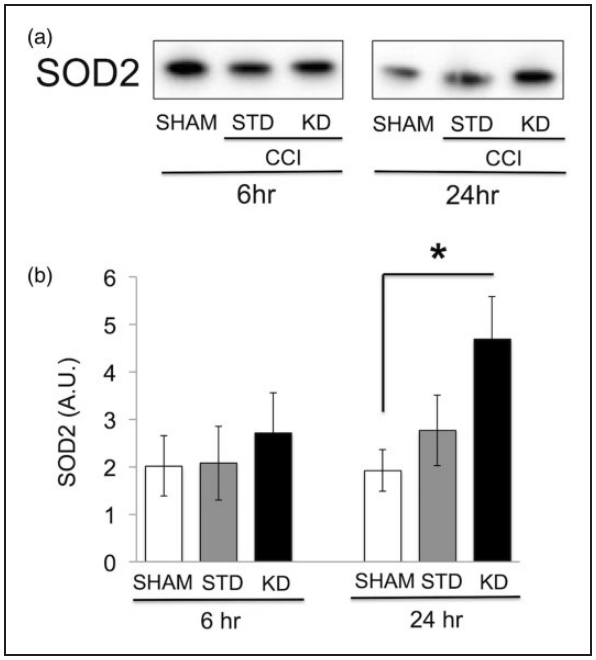

Figure 4. Mitochondrial expression of SOD2 in PND35 rats on STD or KD diet for 6 or 24 h after TBI. (a) Representative image of SOD2. (b) Average (± SEM) densitometric ratios for SOD2/total protein, $^*p < 0.05$.

$p = 0.739$); however, by 24 h ($F(2,12) = 3.826$, $p = 0.051$), KD increased SOD2 expression by 144% ($p = 0.046$), (Figure 4).

Similar to prior results, no differences were seen between groups in protein expression for SOD1 and NQO1 in PND70 animals. SOD1: SHAM (0.13 ± 0.02), CCI-STD 6HR (0.11 ± 0.03), CCI-STD 24HR (0.07 ± 0.01), CCI-KD 6HR (0.08 ± 0.01), CCI-KD 24HR (0.09 ± 0.02); NQO1: SHAM (0.10 ± 0.01), CCI-STD 6HR (0.10 ± 0.02), CCI-STD 24HR (0.06 ± 0.01), CCI-KD 6HR (0.07 ± 0.01), CCI-KD 24HR (0.08 ± 0.02).

Ketogenic diet preserves mitochondrial Complex II-III activity inhibited by TBI

It is well established that there is mitochondrial dysfunction following and that mitochondria are a source of the ROS/RNS produced after injury. This dysfunction and ROS/RNS production has largely been attributed to the inhibition of Complex I, based on the use of different respiratory substrates in *ex vivo* mitochondrial polargraphic measurements. In addition to Complex I, Complex III is susceptible to oxidative modification, particularly by peroxynitrite. Inhibition of Complex III by peroxynitrite is known to generate superoxide. Both Complex I and II-III activity were measured at 6 and 24 h to temporally define their relative contributions to the ATP decline observed in PND35 animals.[13] At 6 h post injury, both Complex I ($F(2,15) = 4.658$, $p = 0.027$) (Figure 5) and Complex II–III ($F(2,25) = 4.254$, $p = 0.034$) (Figure 6) activity

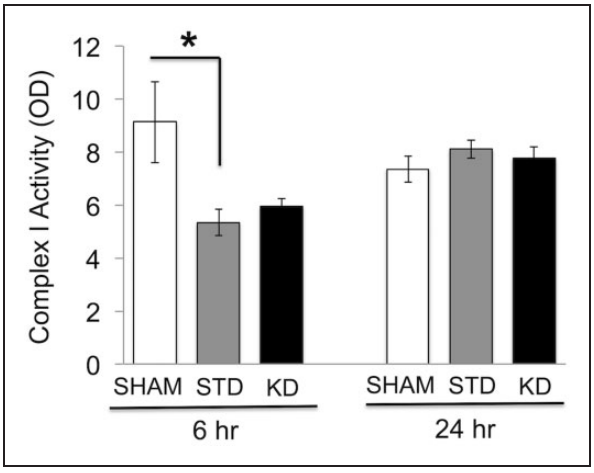

Figure 5. Mitochondrial Complex I activity in PND35 rats on STD or KD diet for 6 or 24 h after TBI. Average (± SEM) activity for Complex I, $*p < 0.05$.

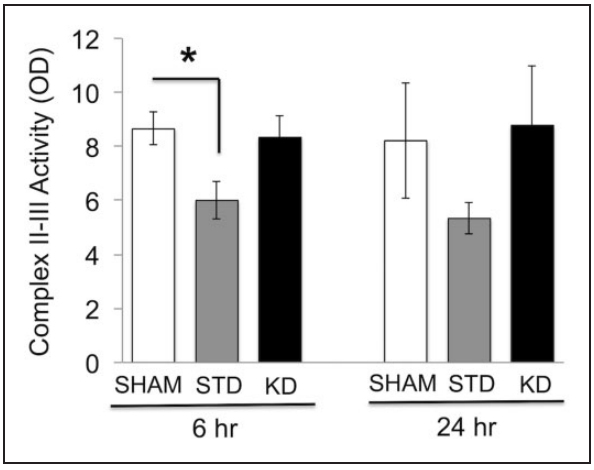

Figure 6. Mitochondrial Complex II/III activity in PND35 rats on STD or KD diet for 6 or 24 h after TBI. Average (± SEM) activity for Complex II/III, $*p < 0.05$.

were significantly reduced 42% ($p = 0.031$) and 31% (0.043), respectively, in standard chow fed TBI animals compared to sham. While not significant, Complex I activity was still inhibited by 35% in KD animals in contrast to 38% improvement in Complex II–III activity compared to STD-TBI animals. At 24 h, Complex I activity returned to sham levels in both the STD-TBI and KD-TBI groups. Complex II–III activity remained inhibited by 34% in STD-TBI animals compared to sham and no difference in activity between sham and KD-TBI groups.

Discussion

The current study shows that the ketogenic diet may confer neuroprotection through more than its actions as an alternative substrate for glucose. Mitochondrial dysfunction is common after TBI and plays a large role in diminished cerebral metabolism following injury. No one has yet explained how providing an alternative substrate results in either maintained or increased ATP production while the mitochondria are in an impaired state. We show that TBI specifically inhibits Complex I and II/III activity, leaving no route for substrate entry, and only Complex II/III inhibition is ameliorated by KD while oxidative stress is significantly reduced in both the cytosol and mitochondria. This establishes a mechanistic pathway where KD first reduces oxidative stress and reverses mitochondrial impairment at which time respiratory substrates are again able to enter through Complex I and II (Figure 7).

Ketones decrease markers of oxidative damage in the juvenile brain

Ketone metabolism has been shown to result in cellular changes that could potentially contribute to its neuroprotective properties.[17] The results of the current study demonstrate a KD mediated reduction in oxidative damage within both the cytosol and mitochondria and is consistent with previous findings of decreased oxidative damage in the presence of ketones.[24,25,30] There are likely multiple mechanisms through which KD can reduce oxidative stress. While KD has been shown to increase antioxidant enzyme activity after chronic long-term administration,[15,28] this is unlikely to be the cause of decreased oxidative damage at 6 h in our KD-CCI animals. Rather it appears scavenging abilities of ketone bodies are responsible. Haces et al. has shown *in vitro*, both beta-hydroxybutyrate (ßOHB) and acetoacetate (AcAc) are able to directly scavenge OH, while AcAc is able to scavenge hypochlorous acid (HOCl), $ONOO^-$ and singlet oxygen ($^1O_2$).[20] Addition of ßOHB and AcAc to either dissociated neurons exposed to glutamate or mitochondria exposed to calcium inhibited O_2^- accumulation and improved Complex I-driven state III respiration, respectively.[25] The short duration of these experiments strongly suggest the rescue observed was due to scavenging abilities of ketone bodies. While Deng-Bryant et al. did not show quantifiable levels of ßOHB by 6 h in the cortex,[13] it is likely that AcAc is present as it crosses the blood brain barrier at 2–3× the rate of ßOHB.[31] It is currently unknown what concentration of ketones is necessary to scavenge free radicals in the brain. Haces et al.[20] showed that supraphysiologic concentrations of ketones were able to directly scavenge, but did not test any concentrations under 5 mM. Further studies using a hypoglycemic model demonstrated that 500 mg/kg ßOHB resulted in a plasma concentration of 0.371 mM and significantly reduced markers of

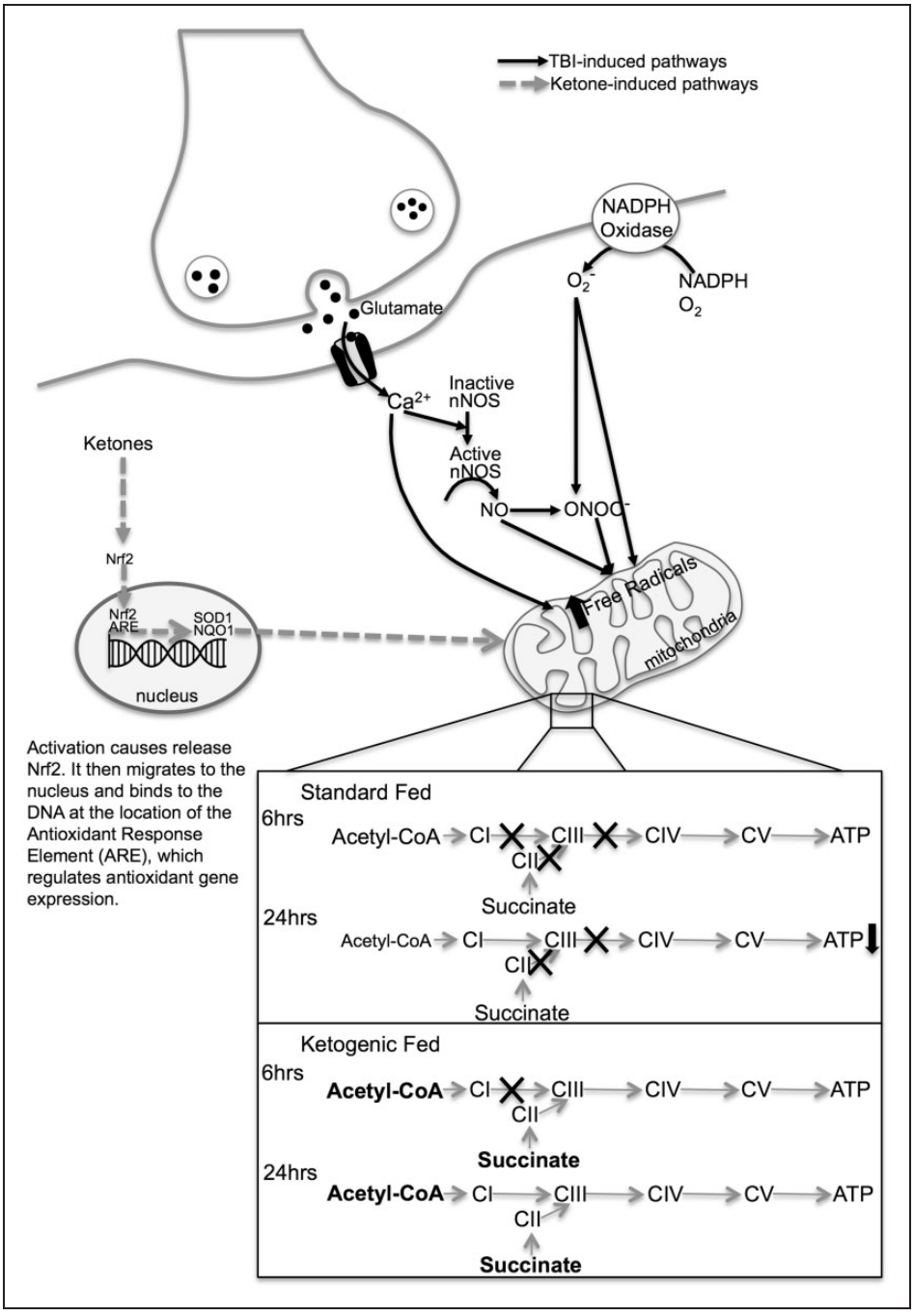

Figure 7. Representative mechanism of ketogenic diet actions. Injury-induced activation of NMDA receptors causes an influx of Ca^{2+} into the cell. Increased concentrations of Ca^{2+} and injury lead to increased cytosolic ROS/RNS production that inhibit mitochondrial function. Ca^{2+} is also taken up by the mitochondria and inhibits Complex I resulting in further ROS production. Despite mitochondrial inhibition, ATP concentrations are initially maintained through increased lactate production and phosphocreatine breakdown. By 24 h, both decreased glycolysis and mitochondrial function lead to decreased ATP function. KD decreases oxidative stress at 6 h and maintains ATP by increasing succinate and improving Complex II/III activity allowing flux of succinate through Complex II. KD is able to prevent decreases in ATP at 24 h from diminished glycolysis by increasing Acetyl-CoA production.

oxidative stress in the brain.[20] Work done by Gueldry et al. showed treatment results with an increase of plasma ßOHB from 0.38 mM to 0.996 mM, and microdialysate measurements showed an increase of ßOHB in the brain from 14.7 µM to 54.7 µM in adult rats.[32] Similarly, we have previously shown that KD increases plasma ßOHB from 0.12 mM to 0.21 mM at 6 h and 1.39 mM at 24 h.[13] While this evidence is

circumstantial, collectively it strongly suggests that KD is providing a high enough cerebral concentration of ketones to scavenge free radicals. Entry of ketones into the brain is also age dependent. Expression of monocarboxylate transporters necessary for uptake of ketones into the brain decrease significantly with age[33] and thus the time to enter the brain is increased compared to PND35 animals.[13] This "lag" is the likely cause of no differences seen in reducing 3NT and increasing antioxidant protein expression in KD-treated PND70 animals.

KD continued to ameliorate oxidative damage at 24 h after injury. In addition to the ability to directly scavenge free radicals, KD induced expression of antioxidant proteins, SOD1/2 and NQO1 both of which are O_2^- scavengers and are under genomic control of Nrf2 signaling.[34,35] Nrf2 has been shown to be activated by both acute and chronic administration of ketone bodies and diet.[28,29] Beyond both direct and indirect ROS scavenging, KD is able to upregulate expression of mitochondrial uncoupling proteins (UCP) through increased free fatty acids activating the peroxisome proliferator activating receptor (PPAR). UCPs uncouple the electron transport chain from ATP production through the translocation of protons, the consequence of which is decreased generation of mitochondrial ROS.[30] The effectiveness the KD reducing oxidative stress is due to its ability to activate multiple protective antioxidant pathways.

Ketogenic diet improves mitochondrial activity

It is well established that both hypoglycolysis and mitochondrial dysfunction occur following TBI that result in decreased overall cerebral metabolism. KD has been shown to increase both glucose metabolism and ATP production in PND35 animals.[13] Largely, these improvements have been attributed to ketone bodies being able to act as an "alternative fuel", ultimately bypassing glycolysis and providing Acetyl-CoA to enter the citric acid cycle and facilitate ATP production. One large element left out of this equation is the mitochondria – if they are still inhibited by injury, no amount of Acetyl-CoA whether derived from glucose and/or ketone bodies should improve ATP generation. This suggests that ketone bodies' mechanism of neuroprotection is through other mechanisms than just their ability to act as a substrate.

Excitotoxicity following injury leads to increased cytosolic ROS/RNS and NO production that can inhibit mitochondria and large influxes of calcium into the cell.[8] The calcium is taken up by the mitochondria which in turn inhibits Complex I, promoting the production of O_2^-, and activation of mitochondrial NOS. The increased O_2^- and NO production leads to formation of $ONOO^-$ that in turn further inhibits Complex I and also Complex III, another major source of O_2^- production.[36] $ONOO^-$ decomposition products are extremely toxic and responsible for oxidative modification of proteins (including the respiratory chain complexes) and lipids, leading to uncoupling, membrane potential collapse, activation of the permeability transition pore and ultimately complete bioenergentic failure.

Mitochondria are inhibited up to several days after injury depending on severity of injury and age at time of injury.[37,38] Inhibition has been observed with both Complex I and II linked respiratory substrates.[38] In our model, we used enzymatic activity of Complex I and II–III to establish a temporal profile to determine which inhibited complex(es) may be responsible for mitochondrial dysfunction and decreased ATP production. Based on the work from Maalouf et al. and the known ability of ketones to reduce oxidative stress, it was hypothesized that ketone bodies would reduce both cytosolic and oxidative stress and improve mitochondrial function. At 6 h post-injury, Complex I was inhibited with little improvement by KD. There may be several explanations for this and we propose a dual mechanism by which KD is ineffective in improving Complex I activity. First, calcium entry into the mitochondria is unaffected by the presence of ketone bodies,[25] and therefore any calcium-mediated inhibition of Complex I will not be improved by KD. Second, the enhanced and sustained inhibition of Complex I due to oxidative modification (induced by calcium) is likely happening prior to ketone bodies entering the brain due to route of administration. This is supported by evidence where isolated mitochondria exposed to calcium alone have impaired Complex I-driven state III respiration, but in the presence of calcium and ketone bodies, respiration is improved compared to insult alone, but not back to control levels. The presence of ketone bodies also did not affect calcium uptake by the mitochondria.[25] This suggests that (a) calcium is inhibitory in a KD independent manner and (b) early ketone administration is immediately able to scavenge Complex I ROS production induced by calcium inhibition. The improvement in Complex I activity seen at 24 h is most likely due to return of calcium homeostasis and enzyme turnover/re-activation.[39] Unlike Complex I, Complex II–III activity was significantly improved in CCI-KD animals at 6 h post-injury. It is unlikely that ketone bodies were present at an early enough time point to prevent inhibition of either or both of these complexes, but instead suggests the inhibition is reversible. At 24 h, Complex II–III activity continues to be inhibited and is likely contributing to the decrease in ATP production observed.

Mechanisms of ketogenic improvement

As mentioned previously, it is thought that much of the KD's improvement in cellular metabolism and neuroprotection is through its ability to act as an alternative substrate. Here, we show rather that it first acts in an antioxidant manner to reverse mitochondrial dysfunction. Both Complex I and II–III are inhibited in CCI-STD mice at 6 h post-injury. Increased production of lactate is a reflection of impairment of oxidative phosphorylation as well as an attempt to maintain ATP concentrations and cellular membrane potential through increased glycolytic output.[13] While Complex I activity returns to sham levels by 24 h, Complex II–III activity remains inhibited. $ONOO^-$ has been shown to not only inhibit Complex II–III, but also Complex V[40] and suggests that the observed decline in ATP production in PND35 animals[13] is due in part to impaired Complex III and/or V activity. In addition to inhibition of mitochondrial complexes, decomposition products of $ONOO^-$ increase the amount of lipid peroxidation leading to thiol linkages and pore formation in the inner membrane ultimately uncoupling the mitochondria.

Although Complex I activity is inhibited in CCI-KD animals, Complex II–III activity is not. This will continue to allow electron flow through the respiratory chain and production of ATP. KD not only has antioxidant properties, but may provide substrates beyond Acetyl-CoA. The reaction of AcAc with Succinyl-CoA produces succinate, and animals either fed a KD or infused with ßOHB show a significant increase in succinate concentrations.[41,42] Other groups have also shown that KD increases Complex II activity (succinate dehydrogenase activity).[43] By increasing Complex II activity and its substrate, KD is able to maintain mitochondrial membrane potential and ATP production and prevent bioenergetic failure. At 24 h post-injury, KD is likely to exert its affects through three mechanisms: (1) continued direct and indirect ROS/RNS scavenging, (2) increased Complex II activity and (3) increased acetyl-CoA and succinate.

Summary

While many studies have shown improved outcome and cerebral metabolism with ketogenic diet, its mechanisms of action remain unclear. Previous TBI studies have focused on the ketogenic diet's ability to act as an alternative substrate as the explanation for improvement. The success of the diet, however, is due to it acting through multiple mechanisms and pathways. Here we show that at earlier time points, KD's protective affects are modulated through its antioxidant capabilities while at later points its role as an alternative substrate come more heavily into play. Also addressed is how this diet would fare in other age groups. Improvements in oxidative stress and antioxidant protein expression shown in the PND35 animals were not observed in PND70 animals and are likely due to age-related difference in the ability to uptake and metabolize ketones due to decreases in enzymes necessary to metabolize ketone bodies and transporters to take up ketones into the brain. This would suggest that specific ketone-based therapies have a limited population of use. Different routes of administration, however, such as intravenous, may overcome these differences. Prins et al. showed that a 3-h intravenous infusion of ßOHB increased cerebral ßOHB uptake and prevented an injury-induced 20% decrease of ATP in the ipsilateral cortex. Future work comparing age differences using rapid and comparable methods of ketone body administration are needed to determine the full extent of its use as a neuroprotective agent.

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Authors' contributions

TG designed and conducted experiments as well as wrote the manuscript. MLP designed experiments, supervised and trained technical personnel that measured the evidence of oxidative damage. She also revised the manuscript written by TG. TCG was involved in manuscript organization and revisions. DAH contributed to the original design of the experiments and was involved in manuscript organization and revisions.

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