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Randhall Bruce Kreismann Carteri

Jejum intermitente profilático promove adaptações mitocondriais influenciando a conectividade metabólica cerebral após um traumatismo cranioencefálico grave

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Trabalho de conclusão de curso de graduação apresentado como requisito parcial para a obtenção do grau de Bacharel em Nutrição, à Universidade Federal do Rio Grande do Sul, Departamento de Nutrição.

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A Comissão Examinadora, abaixo assinada, aprova o trabalho de conclusão de curso elaborado por Randhall Bruce Kreismann Carteri, como requisito parcial para obtenção de Grau de Bacharel em Nutrição

Comissão Examinadora:

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Prof[a]. Dr[a]. Aline Petter Schneider (IPGS)

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Prof[a]. Dr[a]. Zilda Elisabeth de Albuquerque Santos (UFRGS)

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Prof. Dr. Luis Valmor Cruz Portela

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RESUMO

Introdução: O traumatismo cranioencefálico (TCE) está associado a um metabolismo cerebral e conectividade metabólica prejudicados, culminando na neurodegeneração através de vários mecanismos associados à mitocôndria. O jejum intermitente (IF) é uma abordagem dietética reconhecida por causar reprogramação metabólica cerebral, melhorando assim a função cognitiva.

Objetivos: Acessar o potencial profilático de uma estratégia de IF em camundongos para restringir os déficits neuroenergéticos e cognitivos que se seguem ao TCE grave.

Métodos: Camundongos C57BL / 6J foram submetidos à dieta *ad libitum* (ALD) ou protocolo de IF em dias alternados durante 20 dias. Após 48 horas, os camundongos foram submetidos ao impacto cortical controlado (CCI) grave, resultando em três grupos experimentais: SHAM e CCI (ALD) ou IF (IF anterior mais CCI).

Resultados: Cinco dias após a lesão, o CCI apresentou comprometimento da captação de glicose e conectividade metabólica, efeito que foi prevenido pelo IF. Além disso, o IF preveniu a disfunção mitocondrial, o inchamento mitocondrial pelo cálcio e alterações no potencial da membrana mitocondrial induzida pelo CCI. Além disso, a produção aumentada de H_2O_2 mitocondrial induzida por CCI foi atenuada por IF, culminando em viabilidade celular preservada. Esses defeitos metabólicos foram refletidos no comprometimento da memória espacial induzido pelo CCI que foi prevenido pelo IF.

Conclusão: O IF modulou vários mecanismos subjacentes associados à progressão da lesão após TCE grave, prevenindo o comprometimento mitocondrial e cognitivo, e melhorando a conectividade metabólica. Esses resultados expandem a literatura e fornecem novas evidências funcionais e moleculares fortalecendo os efeitos benéficos atribuídos do IF à saúde geral do cérebro e seus benefícios profiláticos ao TCE.

Palavras – chave: Nutrição, bioenergética, cognição, bioquímica.

ABSTRACT

Introduction: Traumatic Brain Injury (TBI) is associated with impaired brain metabolism and metabolic connectivity, culminating in neurodegeneration through several mitochondria –associated mechanisms. Intermittent Fasting (IF) is a recognized dietary approach, which causes brain metabolic reprograming, thereby improving brain metabolism and cognitive function.

Objectives: Access the prophylactic potential of a IF strategy in mice to restrain the neuroenergetic and cognitive deficits that follows severe TBI.

Methods: C57BL/6J mice underwent ad-libitum diet (ALD) or intermitent (alternating day) fasting protocol during 20 days. After 48-hours, mice were assigned to sham or severe controlled cortical impact (CCI) resulting in three experimental groups: SHAM and CCI (ALD) or IF (previous IF plus CCI).

Results: Five days after injury, CCI presented impaired glucose uptake and metabolic conectivity, an effect prevented by IF. Additionally, IF prevented mitochondrial dysfunction, impaired calcium metabolism and mitochondrial membrane potential dynamics induced by CCI. Also, increased CCI-induced mitochondrial H_2O_2 production was attenuated by IF, culminating in preserved cell viability. These metabolic effects were reflected in CCI-induced impairment in spatial memory, which was prevented by IF.

Conclusion: In conclusion, IF modulated several underlying mechanisms associated with injury progression following severe TBI, preventing both metabolic and cognitive impairment. These results expand the literature and provide functional and molecular pieces of evidence strengthening the attributed beneficial effects of IF to overall brain health and its prophylactic benefits to TBI.

Keywords: Nutrition, bioenergetics, cognition, biochemistry.

SUMÁRIO

1. INTRODUÇÃO

Agora reconhecido como um importante problema de saúde pública, o traumatismo cranioencefálico (TCE) comumente resulta em disfunção neurológica persistente (Rosenfeld *et al.*, 2012; Meaney *et al.*, 2014). As alterações fisiopatológicas iniciais resultantes de danos mecânicos primários podem desencadear efeitos deletérios secundários, incluindo neurodegeneração progressiva (Blennow *et al.*, 2012). No entanto, os mecanismos celulares e moleculares envolvidos nessas mudanças progressivas são pouco compreendidos (Meng *et al.*, 2017; Johnson *et al.*, 2018). Portanto, tem sido sugerido que a disfunção metabólica persistente pode estar por trás de algumas das características patológicas do TCE crônico.

Em modelos de TCE experimental, a disfunção mitocondrial tem sido comumente descrita como uma fonte de crise metabólica celular (Gilmer *et al.*, 2009; Hiebert *et al.*, 2015). Acredita-se que isso reflita, em parte, o aumento patológico nas concentrações de cálcio intracelular, que por sua vez é sequestrado pelas mitocôndrias. No entanto, esse tamponamento de cálcio geram uma diminuição da capacidade das mitocôndrias em produzir ATP. O hipometabolismo resultante pode dessincronizar a função celular concomitante ao aumento de espécies reativas de oxigênio (Pandya *et al.*, 2013; Vekaria *et al.*, 2017). Com o aumento do cálcio excedendo a capacidade de sequestro mitocondrial, a destruição seletiva do citoesqueleto pode levar à morte celular por neurodegeneração apoptótica (Johnson *et al.*, 2016).

Assim, foi proposto que o TCE prepara os neurônios para morrer devido à confluência da disfunção mitocondrial e subsequentes processos degenerativos (Ji *et al.*, 2012). Portanto, a mitocôndria se torna um alvo terapêutico em estratégias que visam garantir melhores resultados funcionais (Schon e Przedborski, 2011; Blennow *et al.*, 2012; Gajavelli *et al.*, 2015) onde as intervenções farmacológicas falharam repetidamente. Independentemente do TCE, o jejum intermitente (IF) demonstrou causar reprogramação metabólica cerebral (Hiebert *et al.*, 2015), levando ao pré-condicionamento neuronal (Pani, 2015) influenciado diretamente por alternância cíclica entre glicose e corpos cetônicos como principal substrato energético sistêmico (Mattson *et al.*, 2018). Esse efeito de "Troca metabólica intermitente" (IMS) e suas adaptações podem ser explorados visando prevenir e atenuar mecanismos de neurodegeneração induzidos por diferentes insultos cerebrais (López-Lluch *et al.*, 2006; Amigo *et al.*, 2017). Por conseguinte, roedores mantidos em IF exibem maior resistência neuronal a insultos, incluindo ataques epilépticos e acidente vascular cerebral(Goodrick *et al.*, 1990; Anson *et al.*, 2003).

Aqui, um modelo de TCE grave em roedores foi usado para explorar os potenciais efeitos profiláticos do jejum intermitente após a lesão no contexto da metabolismo mitocondrial, conectividade metabólica e função cognitiva.

1.1. JUSTIFICATIVA

Considerando a escassez de evidências sobre estratégias efetivas para diminuir a progressão do dano cerebral induzido por TCE e que o estado nutricional está intimamente ligado a função cerebral, intervenções nutricionais poderiam ajudar na prevenção, resiliência ou tratamento dos eventos agudos de TCE. Portanto, como a literatura sobre a fisiopatologia e o tratamento do TBI ainda contém lacunas significativas em relações a estratégias nutricionais, o objetivo deste trabalho é investigar os mecanismos associados com a restrição calórica por meio do jejum intermitente (IF) e sua aplicação como estratégia profilática ao dano induzido por um modelo de traumatismo crânio encefálico (CCI, impacto cortical controlado) em camundongos.

1.2. OBJETIVOS

1.2.1 Objetivo geral:

Investigar os mecanismos associados com a restrição calórica por meio do jejum intermitente (IF) e sua aplicação como estratégia profilática ao dano secundário induzido por TCE em camundongos.

1.2.2. Objetivos específicos:

a) Avaliar alterações na composição corporal e padrões de consumo alimentar induzidas por IF antes e após o TCE;
b) Avaliar o metabolismo energético por meio do consumo cerebral de glicose em regiões específicas por microPET, e avaliar a conectividade metabólica cerebral;
c) Avaliar o metabolismo mitocondrial por respirometria de alta resolução, e o potencial de membrana mitocondrial em sinaptossomas;
d) Avaliar a influencia do transportador NCLX no manejo de cálcio em mitocôndrias de sinaptossomas;
e) Avaliar a produção de peróxido de hidrogênio mitocondrial em sinaptossomas;
f) Estimar a viabilidade celular em sinaptossomas;

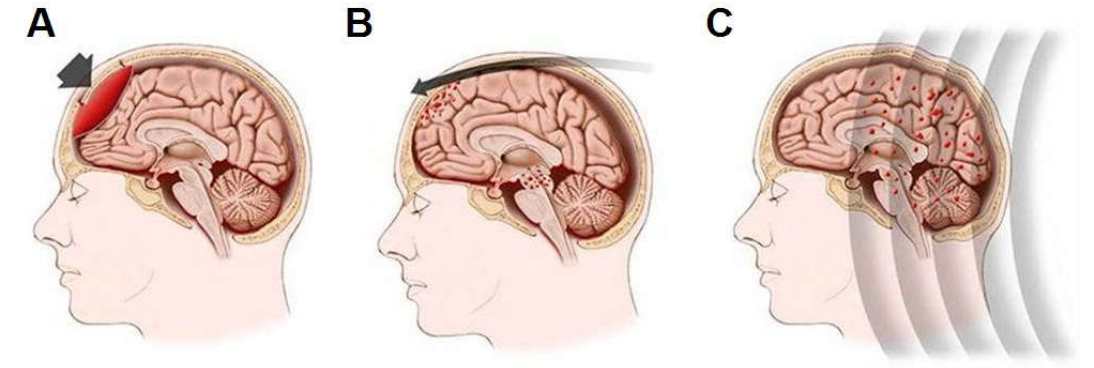
e) Avaliar aprendizado e memória espacial;

2. REVISÃO DE LITERATURA

2.1. Trauma cranioencefálico

O trauma cranioencefálico (TCE) é definido por uma alteração da função normal cerebral, resultante de forças biomecânicas, causada por uma rápida aceleração ou desaceleração do cérebro e decorrente de acidentes com motocicletas ou automóveis; impacto resultante da batida do cérebro devido a quedas, acidentes de motocicletas e automóveis ou em esportes de impacto; uma mudança de pressão e deslocamento de ar devido a explosões; e também pela penetração de projéteis ou de objetos no cérebro (Blennow *et al.*, 2012; Rosenfeld *et al.*, 2012). O TCE é classificado como leve, moderado e severo, podendo levar a morte prematura, alterações cognitivas e neuropsiquiátricas comprometendo, muitas vezes, a qualidade de vida dos indivíduos sobreviventes (Meaney *et al.*, 2014; Levin e Diaz-Arrastia, 2015).

Figura 01 - Forças biomecânicas associadas com o Traumatismo Cranioencefálico.



A) Impacto Cortical direto podendo ser penetrante ou não; B) Aceleração e desaceleração da cabeça; C) Ondas mecânicas ocasionadas por impactos e/ou explosões. Modificado de Blennow et al. 2012.

2.2. Incidência de TCE

A incidência do TCE é crescente em todos os países devido aos acidentes de trânsito. Também, o envelhecimento da população tem dado origem a um aumento das lesões cerebrais

traumáticas, principalmente devido a grande incidência de quedas entres estes indivíduos (Blennow *et al.*, 2012). O Brasil é um dos líderes em incidência de TCE. No ano de 2013, estimou-se cerca de 500 casos para cada 100.000 habitantes. No estado do Rio Grande do Sul, 75 % das causas de TCE são decorrentes de acidentes de trânsito, envolvendo atropelamentos, acidentes de carro e motos. O risco de TCE é especialmente elevado entre os adolescentes, adultos jovens, pessoas com idade inferior a 2 anos e superior a 75 anos. A faixa etária de maior incidência está entre 15 e 24 anos, sendo mais prevalente no sexo masculino. A mortalidade geral relacionada ao TCE grave está em torno de 30-50 %, sendo que 90 % destas ocorrem nas primeiras 48 horas (h) após o insulto (Blennow *et al.*, 2012). Em 2012 o valor total despendido pelo SUS para atendimento de causas externas (que incluem o TCE) foi maior que 1 bilhão de reais com 998.994 internações, com valor médio da internação de R$ 1.079,60 e média de permanência de 5,3 dias. Esses dados sobre custos e valores pagos pelo SUS não incluem custos ambulatoriais e de clínicas de reabilitação. Somam-se a estes, os custos com medicamentos, materiais necessários aos cuidados domiciliares, cuidador, transporte e aqueles indiretos referentes aos dias não trabalhados pelos pacientes e familiares (Fukujima, 2013). Portanto, o TCE e suas consequências são atualmente considerados problemas de saúde pública no Brasil.

2.3. Mecanismos bioquímicos envolvidos no dano após TCE.

Os mecanismos fisiopatológicos associados ao TCE envolvem a lesão primária resultante do dano mecânico ou inercial a substância branca e cinzenta com ruptura das membranas celulares, liberação do seu conteúdo e lesão axonal difusa (Dash *et al.*, 2010; Roozenbeek *et al.*, 2013). O dano secundário se refere à progressão das alterações associadas ao dano primário cerebral resultando em diversas alterações neurológicas podendo resultar em consequências devastadoras ao longo da vida. No dano secundário, a ativação persistente de uma série de cascatas de eventos neurotóxicos, desencadeia dano estrutural, a perda de função e conectividade neuronal, culminando com a morte de células neurais adjacentes ao foco da lesão (Roozenbeek *et al.*, 2013).

A extensão e severidade dos danos secundários são proporcionais à intensidade do trauma e do local do insulto primário. Entre os mecanismos que sustentam o dano secundário estão o aumento excessivo de glutamato extracelular (excitotoxicidade), prejuízos na homeostasia da cálcio (Ca^{2+}), resposta inflamatória persistente, disfunção mitocondrial, deficiência neuroenergética, desbalanço redox, disfunção do sistema vascular, isquemia e

acúmulo de proteínas no axônio (Hemphill *et al.*, 2015). Devido a associação desses mecanismos com a neurodegeneração, o TCE é considerado um fator de risco para diversas doenças neuropsiquiátricas, como ansiedade, depressão, alcoolismo e demências como por exemplo, a doença de Alzheimer (DA) (Blennow *et al.*, 2012; Sivanandam e Thakur, 2012; Johnson e Stewart, 2015). Um único evento de TCE moderado duplica o risco de desenvolver a DA, enquanto que o TCE grave está associado a 5 vezes mais chances [10]. Além disso, grupos específicos que sofrem repetidas lesões na cabeça, como boxeadores, jogadores de futebol americano e veteranos de guerra, têm maior risco de desenvolvimento de demência do que indivíduos normais (Hanten *et al.*, 2013).

Enquanto o alto consumo de energia é um aspecto importante da comunicação neuronal normal (Hyder *et al.*, 2013), o hipometabolismo agudo em áreas cerebrais específicas após o TBI (Nakashima *et al.*, 2007) está intimamente ligado a desfechos neuropsicológicos e neurodegeneração em longo prazo (Scholl *et al.*, 2015; Daulatzai, 2017). Tais patologias do TCE podem surgir de neurônios distantes exibindo comprometimento da conectividade metabólica. A tomografia por emissão de pósitrons com [^{18}F]-fluorodesoxiglucose (FDG-PET) é uma ferramenta clínica estabelecida medindo a taxa de metabolismo cerebral de glicose(Phelps *et al.*, 1979), interpretada como o acoplamento entre a transmissão sináptica e o consumo local de glicose (Magistretti, 2006). Além disso, FDG-PET pode fornecer uma análise integrativa do metabolismo cerebral total, nomeada "conectividade metabólica" (MC; do inglês "*metabolic connectivity*"), que pode ser estimada com a correlação dos valores de captação de glicose entre diferentes regiões anatômicas de interesse (ROI; do inglês "*Region of Interest*"), onde a magnitude da correlação é proporcional à associação funcional (Horwitz *et al.*, 1984). Essa ferramenta pode ser explorada visando compreender os efeitos metabólicos do TCE em diferentes modelos experimentais, tal como na busca de terapias ou também de estratégias de prevenção de piores desfechos.

Nesse contexto, embora se tenha avançado significativamente nos últimos anos na descrição dos mecanismos fisiopatológicos envolvidos com a neurodegeneração pós-TCE, a literatura ainda é escassa no que se refere a como estes podem ser influenciados por manipulações dietéticas antes ou após o TCE.

2.4. Manejo Nutricional do TCE

No manejo do paciente no TCE, a importância da nutrição transcende o fornecimento adequado de nutrientes e calorias para o paciente com injúria cerebral, podendo garantir não

somente a evolução clínica, mas também atenuar a progressão do dano induzido pelo trauma. Entre as variáveis na prescrição de dieta no trauma, a quantidade calórica visa garantir o funcionamento das funções vitais do organismo. Nesse contexto, as diretrizes apontam que a manipulação da ingestão calórica pode influenciar em diversos fatores associados ao desfecho pós-TCE. A base para a prescrição nutricional no TCE é limitada e conflitante, baseada em recomendações associadas ao politrauma. O paciente após TCE enfrenta alto catabolismo (Perel *et al.*, 2006). A nutrição imediata (iniciando 24 horas após o evento) proporciona diminuição na mortalidade, segundo dados de 797 pacientes em 22 centros, onde o aumento de 10 kcal/kg/dia diminuiu o risco de morte, sendo metade quando comparado com pacientes que não foram alimentados. É importante ressaltar que a maioria dos pacientes (62%) não atingiram 25 kcal/kg/dia e os que não foram alimentados apresentaram risco de morte quatro vezes maior (Hartl *et al.*, 2008).

Em ambiente de tratamento intensivo, a restrição calórica é usualmente recomendada até o paciente atingir estabilidade hemodinâmica, ao mesmo tempo em que se busca atingir o total calórico recomendado em um período de sete dias. A aplicação da restrição calórica controlada (entre 60-70% do total calórico recomendado) diminui o risco de morte comparado com restrição mais severa (80-90% do recomendado), porém, as recomendações sobre a ingestão calórica variam entre 10 – 50 kcal/kg/dia (Arabi *et al.*, 2011).

Assim, existe muita dificuldade na estimativa do gasto de energia após o TCE, que aumenta com a lesão em proporção ao grau de resposta inflamatória sistêmica, variando geralmente de aumento de até 100% no TCE e outras injurias graves. Geralmente uma faixa de 25-30 kcal/kg/dia é recomendada, sendo a equação de Harris-Benedict que considera estatura, sexo e idade a mais utilizada. Entretanto, ambos os valores possivelmente subestimam a real necessidade calórica, e também apresentam menor probabilidade de gerar hiperglicemia nas primeiras duas semanas após a injúria (Mcevoy *et al.*, 2009). Se a estimativa for necessária, a equação de Penn State é atualmente considerada a mais precisa, com uma precisão maior que 70% (Frankenfield *et al.*, 2004). Visando retenção de tecido após a lesão, tanto o aporte calórico quanto o proteico é necessário, embora mesmo com o aporte proteico no limite superior (até 1,5 g / kg / dia), a retenção total de massa muscular é muitas vezes impossível na fase aguda da lesão devido ao impacto da resposta inflamatória sistêmica no catabolismo proteico (Jensen *et al.*, 2010)

Outra dificuldade no manejo do paciente com TCE é o controle glicêmico, pois a hiperglicemia persistente em pacientes com TCE é comum e correlaciona-se também com a gravidade da lesão e o desfecho clínico. Em um estudo envolvendo um total de 228 pacientes

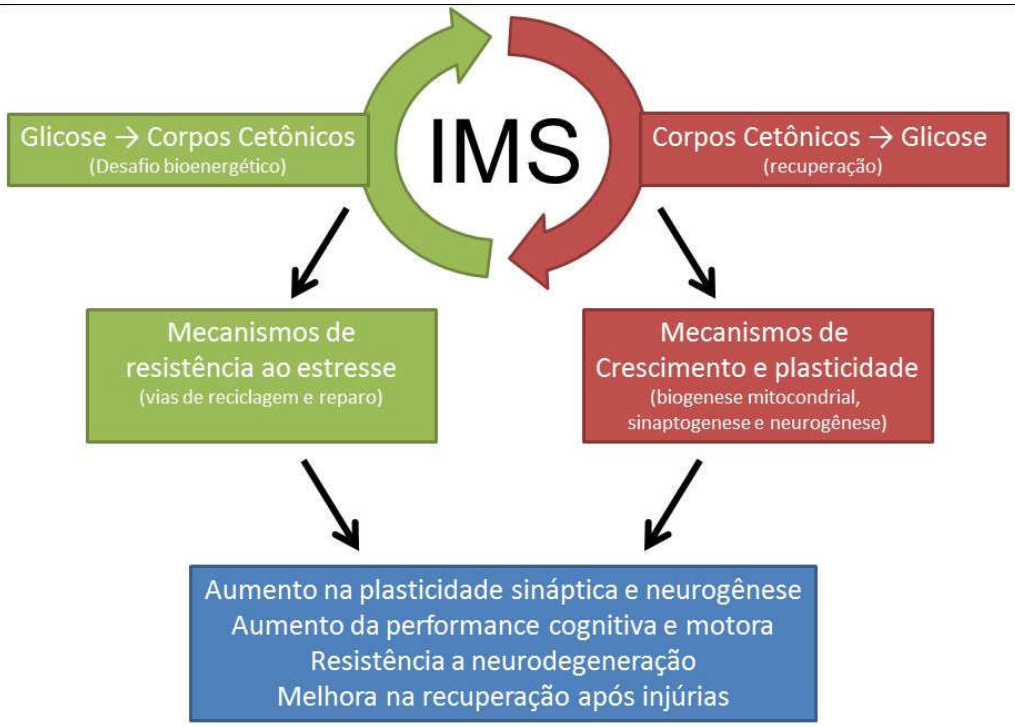
com TCE severo tratados com insulina, durante a primeira semana a glicemia de 90-144 mg / dL foi associada com menor mortalidade e pressão intracraniana (PIC) em comparação com glicemia mantida entre 63-117 mg/dL. No entanto, na segunda semana os grupos reverteram os desfechos, onde o grupo de 63-117 mg / dL demonstrou uma diminuição da incidência de elevação da PIC e reduziu as complicações infecciosas ao contrário do que aconteceu com pacientes mantidos na maior faixa de glicemia (Meier *et al.*, 2008). O grau de severidade também influencia no controle glicêmico e no desfecho. Enquanto a glicemia <108-200 mg / dL poderia reduzir a mortalidade em pacientes com TCE leve, em pacientes com TCE severo, o alvo ideal de glicemia pode ser maior 140-180 mg / dL (Bilotta e Rosa, 2012). Consequentemente, o debate sobre os níveis de glicemia no TCE ainda é controverso e precisa ser explorado mais a fundo. Considerando que a ingestão calórica afeta diretamente a homeostase da glicose, sendo que pacientes alimentados via enteral ou parental comumente apresentam valores de glicemia em torno de 200 mg/dL a restrição de calorias pode promover seus benefícios também por melhoras a homeostase da glicemia (Shi *et al.*, 2016).

2.5. Estratégias nutricionais e pré-condicionamento neuronal: impacto no TCE

É importante considerar que os hábitos alimentares tem papel fundamental na saúde cerebral, podendo exercer efeitos profiláticos em relação a insultos cerebrais. Nesse contexto, a restrição calórica (RC) já demonstrou diversos benefícios em diferentes espécies, entre os principais ao aumento da longevidade, diminuição de fatores associados com desordens neoplásicas, doença renal e maior resistência neuronal a diferentes injúrias cerebrais (Camandola e Mattson, 2017; Mattson *et al.*, 2018). Estudos em roedores também apoiam a noção de que o envelhecimento cerebral e a neurodegeneração estão fortemente ligados ao equilíbrio metabólico e energético (Pani, 2015). De um ponto de vista translacional, a RC é atraente devido ao baixo grau geral de limitação de alimentos associada a esta forma de restrição na dieta (Selman, 2014) e também por seus benefícios já terem sido evidenciado em roedores e humanos em ensaios clínicos (Tinsley e La Bounty, 2015). Diferentes paradigmas de restrição calórica podem ser utilizados em modelos animais, como a alimentação diária limitada, onde os animais recebem uma porção diária de alimentos que geralmente é 30-40% menor que o consumo *ad libitum* (AL) de um grupo controle, resultando em restrição calórica controlada e redução correspondente no peso corporal. No segundo paradigma, os animais são submetidos a jejum intermitente (IF; do inglês "*Intermittent Fasting*") em dias alternados, que geralmente é isocalórico, resultando em diferentes benefícios metabólicos (Xie *et al.*, 2017).

Ambos os paradigmas de restrição calórica afetem a longevidade através de mecanismos comuns, incluindo modificações no peso corporal, níveis de insulina e glicose sérica em jejum, além de melhorar o metabolismo energético e as defesas antioxidantes (Brown-Borg e Rakoczy, 2013), onde a modulação do metabolismo mitocondrial é evidente (Descamps *et al.*, 2005; Qiu *et al.*, 2010).

Figura 02 - O jejum intermitente exerce efeitos com a alternância entre substratos energéticos.



Alternância de substratos (IMS; do inglês "*Intermittent Metabolic Switch*") onde ocorre a alternância da utilização de corpos cetônicos (produzidos pelo fígado durante a restrição alimentar) e utilização de glicose induz mecanismos potencialmente benéficos para a recuperação de injúrias cerebrais. Modificado de Mattson *et al*. 2018

Recentemente, foi proposto que o jejum intermitente causa reprogramação metabólica cerebral (Hiebert *et al.*, 2015) por um efeito nomeado "Troca metabólica intermitente" (IMS; do inglês "*Intermittent Metabolic Switch*") onde ocorre a alternância da utilização de corpos cetônicos (produzidos pelo fígado durante a restrição alimentar) e utilização de glicose (após o acesso alimentar)(Mattson *et al.*, 2018). Os efeitos do IMS levam a mecanismos de adaptação promovendo maior resistência e plasticidade neuronal, além da melhora da função

mitocondrial (López-Lluch *et al.*, 2006; Amigo *et al.*, 2017). Dessa forma, os roedores mantidos em longos períodos de IF exibem maior resistência neuronal a diferentes insultos, incluindo crises epilépticas e acidente vascular cerebral (Goodrick *et al.*, 1990; Anson *et al.*, 2003). Evidências de neuroproteção foram demonstradas com 24 horas de RC em roedores após TCE. Entretanto, o mesmo não foi demonstrado para lesão severa após impacto cortical controlado (Davis *et al.*, 2008). Assim, ainda não existem dados suficientes para se estabelecer se esse efeitos podem ocorrer no TCE severo. Em resumo, os circuitos neuronais e diversos mecanismos respondem aos desafios bioenergéticos intermitentes de forma que aumentam a plasticidade sináptica e a neurogênese, melhoram a função cognitiva e aumentam a resistência neuronal aos estresses metabólicos, oxidativos e excitotóxicos.

3. RESULTADOS

Artigo científico: *Intermittent fasting promotes prophylactic effects improving traumatic brain injury outcomes.*

Nesta seção está o artigo que será submetido ao periódico "*Nature Metabolism*" (as orientações de formatação estão anexadas – ANEXO I).

O hipometabolismo e a disfunção mitocondrial compõem os principais mecanismos associados com a progressão dos danos cerebrais e pior desfecho após o trauma cranioencefálico e o jejum intermitente é uma estratégia que reconhecidamente pode modular positivamente o metabolismo energético frente à diferentes injúrias cerebrais.

Neste estudo, visando investigar os efeitos profiláticos do jejum intermitente no trauma cranioencefálico, foi realizado um protocolo crônico de jejum intermitente e posteriormente induzimos o TCE em camundongos. Avaliamos o metabolismo energético cerebral, a produção de espécies reativas de oxigênio, a homeostasia do cálcio e a viabilidade celular além da função cognitiva.

FULL TITLE: Intermittent fasting promotes prophylactic effects improving traumatic brain injury outcomes

Randhall B Carteri, *MSc*,[1] Afonso Kopzynski,[1] Lizia N Menegassi,[1] Nathan R Strogulski, *MSc*,[1] Marcelo S Rodolphi *MSc*,[1] Samuel Greggio,[3] Gianina T Venturin,[3] Jaderson Costa da Costa,[3] Marco A de Bastiani *MSc*,[2] Douglas H Smith, *PhD*[4] & Luis Valmor Portela, *PhD*[1*]

[1] Laboratory of Neurotrauma and Biomarkers - Department of Biochemistry, Graduate Program in Biochemistry, ICBS, Federal University of Rio Grande do Sul - UFRGS, Porto Alegre, Brazil.

[2] Cell Biochemistry Laboratory - Department of Biochemistry, Graduate Program in Biochemistry, ICBS, Federal University of Rio Grande do Sul - UFRGS, Porto Alegre, Brazil.

[3] Brain Institute of Rio Grande do Sul (BraIns), Pontifical Catholic University of Rio Grande do Sul (PUCRS), Porto Alegre, Brazil

[4] Penn Center for Brain Injury and Repair and Department of Neurosurgery, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA.

Correspondence:

* Dr. Luis V. Portela,

Department of Biochemistry,

ICBS, UFRGS. Rua Ramiro Barcelos, 2600 anexo

90035-003, Porto Alegre, RS, Brazil.

Tel: 55 51 33085558; Fax: 55 51 33085544;

E-mail: roskaportela@gmail.com

Abstract

Traumatic Brain Injury (TBI) is associated with impaired brain metabolism culminating in neurodegeneration through mitochondria–associated mechanisms. Intermittent Fasting (IF) is a recognized dietary approach, which cause brain metabolic reprograming thereby improving function. Therefore, the potential of IF as a preconditioning strategy relative to severe TBI remains to be explored regarding mitochondrial bioenergetics and cognitive function. Here we show that prior IF in mice sustains neuroenergetics connections and avoid memory deficits after severe TBI.

INTRODUCTION

Currently established as a paramount worldwide health concern, traumatic brain injury (TBI) faces uprising scientific attention.[1, 2] TBI pathological process is beyond the primary insult (developed out of the impact force acting on the brain tissue), outcropping an abstruse secondary insult constituting of the amalgamation of several deleterious mitochondria-associated mechanisms,[3-5] attributed as the mechanistic link to impaired calcium (Ca^{2+}) homeostasis,[6] increased degradation of structural proteins,[7, 8] and apoptotic neurodegeneration.[6, 9] Therefore, mitochondria becomes a candidate targeted in strategies aiming to ensure better functional outcomes[1, 10, 11] where pharmacological interventions have repeatedly failed. Regardless TBI, intermittent fasting (IF) has demonstrated to cause brain metabolic reprograming[12,13] directly influencing glucose/ ketone cycling namely "Intermittent metabolic switching" (IMS),[14] and mitochondrial responsiveness to substrates.[15, 16] Accordingly, rodents maintained on IF exhibit increased neuronal resistance to insults including epileptic seizures and stroke.[17, 18] In a rodent model, a 24 h fasting prior to moderate TBI promoted neuroprotection; however when the fasting period was prolonged to 48 h there were no apparent benefits.[19] These results interrogate which is the feasible time-window for the central benefits induced by caloric restriction.

While the high energy consumption is a major aspect to normal neuronal communication,[20] the acute hypometabolism in brain specific areas after TBI[21] is intimately linked with neuropsychological outcomes and long-term neurodegeneration.[22,23, 24] Such TBI derived pathologies may arise from neurons near to each other or even distant displaying impaired metabolic connectivity. Positron emission tomography with [^{18}F]-fluorodeoxyglucose (FDG-PET) is an established tool measuring cerebral metabolic rate of glucose (CMRglc),[25] interpreted as the coupling between synaptic transmission and local glucose consumption.[26] Additionally, FDG-PET can provide an integrative analysis of whole brain metabolism, namely metabolic connectivity (MC), which can be estimated with the correlation of the glucose uptake values among different brain anatomical regions of interest (ROI), where the magnitude of correlation is proportional to the functional association.[27] Owing that TBI induces

brain metabolic impairment and IF promotes adaptations on several aspects of brain function, the impact of IF on metabolic connectivity coupled with an integrative analysis of nerve terminals mitochondrial bioenergetics in the context of TBI remains to be explored.

Notwithstanding the improvement in understanding the pathological process of TBI, developing preconditioning strategies in TBI is an utmost challenge to health professionals. From a translational point of view, intermittent fasting (IF) is attractive because of the easiness and low cost of its application as well as its reported benefits in both rodents and human clinical trials. [28,29] Therefore, given the potential to promote brain metabolic adaptations, we hypothesized that an IF prophylactic strategy in mice might restrain the neuroenergetic and functional deficits that follows severe TBI.

RESULTS

Intermittent fasting promotes metabolic shift without effects in body mass

Based on the concept of IMS, we implied 10 cycles of 24-hour intervals of food deprivation followed by 24-hour ad libitum food access (IF),[30] as a prophylactic strategy to reduce metabolic impairment after TBI. As expected, the IF protocol here implied induced IMS, while no significant detectable changes in body mass. Total ketones were increased in the IF group (Figure 1A; mean diff. vs SHAM: 0.032; p <0.0001 and mean diff. vs CCI: 0.031; p <0.0001) and decreased levels of blood glucose (Figure 1 B; mean diff. vs SHAM = -40.63; p=0.0174 and mean diff. vs CCI = -40.63; p=0.0174) measured 2 h after a fasting day. When total ketones (Figure 1A; vs SHAM: p <0.743 and vs CCI: p <0.998) and blood glucose (Figure 1B; vs SHAM: p <0.153 and vs CCI: p <0.807) were assessed after an ad-libitum day, no differences were observed. When total ketones and blood glucose were assessed after an ad-libitum day and before CCI injury, no differences were observed among groups (figure 1C; p = 0.720, F = 0.333 and D; p = 0.561, F = 0.591, respectively). Body mass was accessed in five different time-points before CCI, and 24, 48, 72 and 120 h after CCI. After treatment, body mass was not different among groups (Figure 1E) before or after CCI. Also, no difference was

observed in total food consumption before and after CCI (Figure 1F). Therefore, IF promoted the substrates shift and did not influenced body mass.

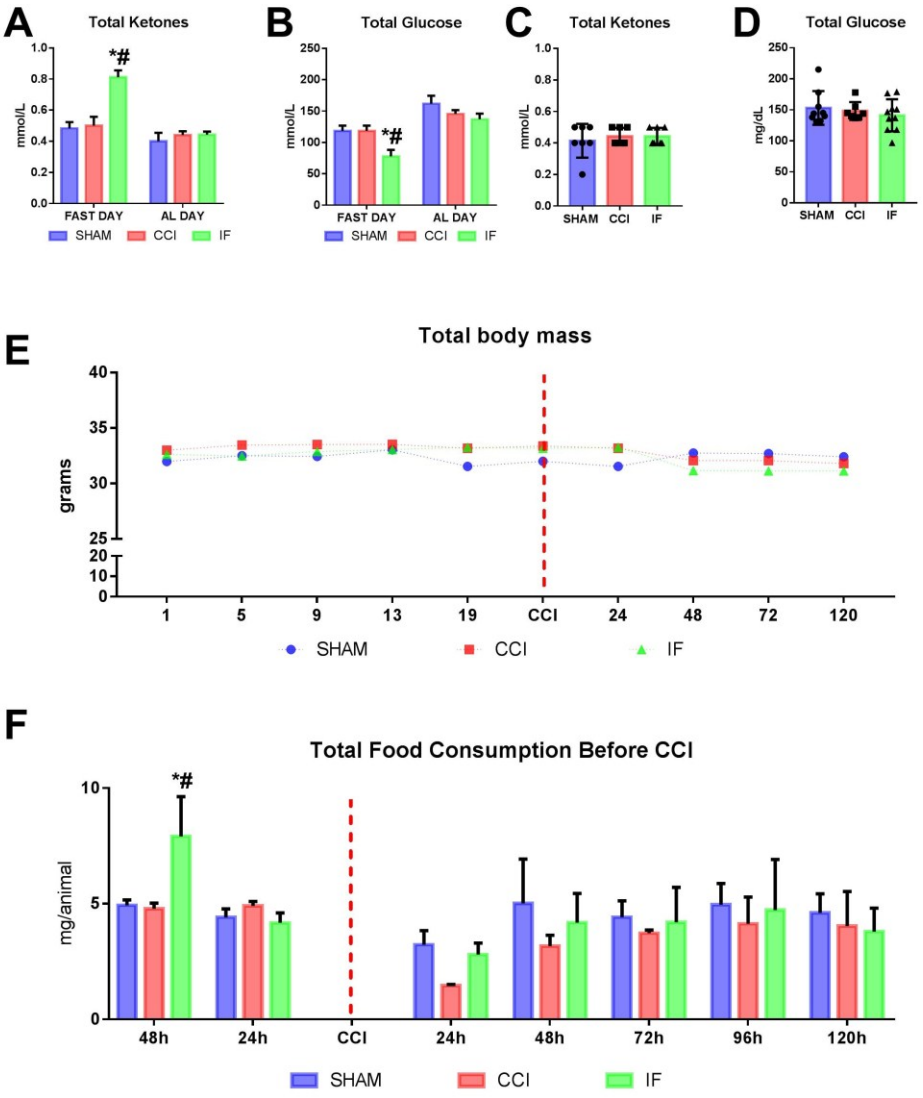


Figure 01. Total ketones bodies and blood glucose, total body mass and total food consumption (n = 7-10). Total Ketones and blood glucose concentration after the last fasting day (A; 72h before CCI) and after following food access day (B, 48h before CCI). Total Ketones and blood glucose concentration 24h before CCI showed no differences (C and D). No changes in body mass were observed (E). Total Food consumed (F) was different 48h before CCI with no differences in the subsequent days *# Denotes significant difference when compared to both SHAM and CCI groups.

Intermittent fasting partially prevents the rupture of brain metabolic connectivity after CCI

Glucose hypometabolism is known to occur after a TBI event.[31, 32] To evaluate the effect of CCI and IF on glucose metabolism, we used FDG-PET to estimate the glucose uptake in specific regions of interest (ROI) (Figure 2 A-G). We observed a decreased FDG uptake in the whole brain 5 days after CCI, and in the regions comprising the synaptosomes preparations (cortex, hippocampi, hypothalamus and cerebellum) as well as in other ROI investigated (see supplementary Table 1). Our prophylactic IF was able to attenuate this effect. Further, we evaluated brain metabolic connectivity through a correlation network and hierarchical clustering of glucose uptake values across all regions evaluated (Figure 2 H-J). It was observed that CCI triggered a distinct association profile and correlation strength compared to control. Indeed, the highly connected functional metabolism displayed by the normal brain was shifted to a brain metabolically poorly correlated and lowly connected due to a severe CCI. Surprisingly, the IF strategy before CCI was able to recover the metabolic integration and association strengths post-injury.

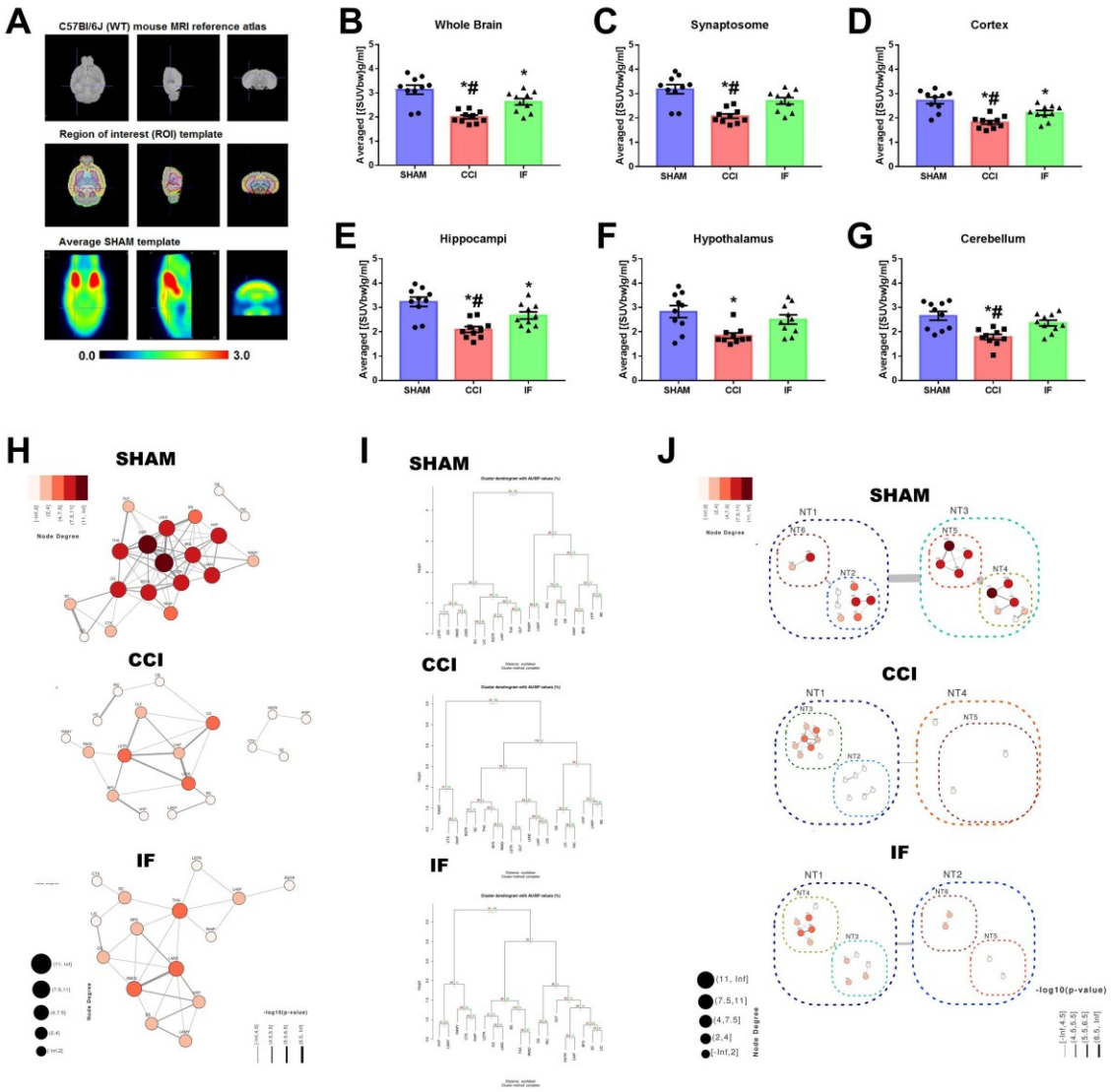


Figure 02. Prior intermittent fasting (IF) prevents the decrease in glucose uptake and metabolic connectivity induced by CCI (n = 7-10). Regions of interest and average SHAM template of FDG-PET (A). Decreased glucose uptake 5 days after CCI was evident ins several brain regions (B-G) and IF attenuated this effect. Brain metabolic connectivity and integration through correlation network and hierarchical clustering of glucose uptake values across all regions evaluated (Figure 2 H-J) showed that CCI triggered a dissociative profile, culminating in impaired metabolic connectivity. Previous IF recovered the metabolic integration and association strengths observed in the healthy brain. *# Denotes significant difference when compared to both SHAM and IF groups.

Intermittent fasting prevents mitochondrial bioenergetics dysfunction 5 days after CCI

Given that neuroenergetic deficits are commonly associated with worst neurological outcomes following TBI we explored whether intermittent fasting promotes alterations in mitochondrial bioenergetics 5 days following CCI. First, we evaluated phosphate consumption in synaptosomes, ipsilateral cortex and hippocampi (Figure 3 A, B and C, respectively) were we found decreased consumption in CCI compared to both SHAM and IF. We used a defined protocol (Figure 3D) of mitochondrial oxygen consumption rate assessment (OCR; Figure 3E) performed in the ipsilateral hemisphere synaptosomes. Intermittent fasting prevented the decrease in OCR induced by CCI in all phosphorylating states, which is reflected by the OxPhos Coupling efficiency (Figure 3 B; SHAM vs CCI: mean diff. = -0.171; p = 0.0062; IF vs SHAM: mean diff. = 0.019; p = 0.932 and IF vs CCI: mean diff. = 0.031; p<0.0001), and Reserve Respiratory capacity (Figure 3 C; SHAM vs CCI: mean diff. = -614; p = 0.0017; IF vs SHAM: mean diff. = 238.9; p = 0.367 and IF vs CCI: mean diff. = 852.9; p = 0.0002). No differences was observed relative to how CI-linked and CII-linked oxidation of substrates influenced total OCR in the phosphorylating state (Figure 3D and E), as is indicated by the succinate effect and rotenone effect (Figure 2 D; p = 0.967, F = 0.326 and E; p = 0.879, F = 0.129, respectively).

Different mitochondrial respiration states are shown in Figure 3E. The OCR in the Leak state was not different among groups. However, when different OxPHOS states were assessed (CI-Linked, CI+CII linked and CII-linked), CCI showed significantly decreased OCR compared to other groups (Figure 6E), while SHAM and IF showed no differences. These functional alterations confirm that CCI impact oxidative phosphorylation and suggest that brain oxygen consumption in response to metabolic substrates is partially diverted from ATP synthesis to production of ROS after CCI.

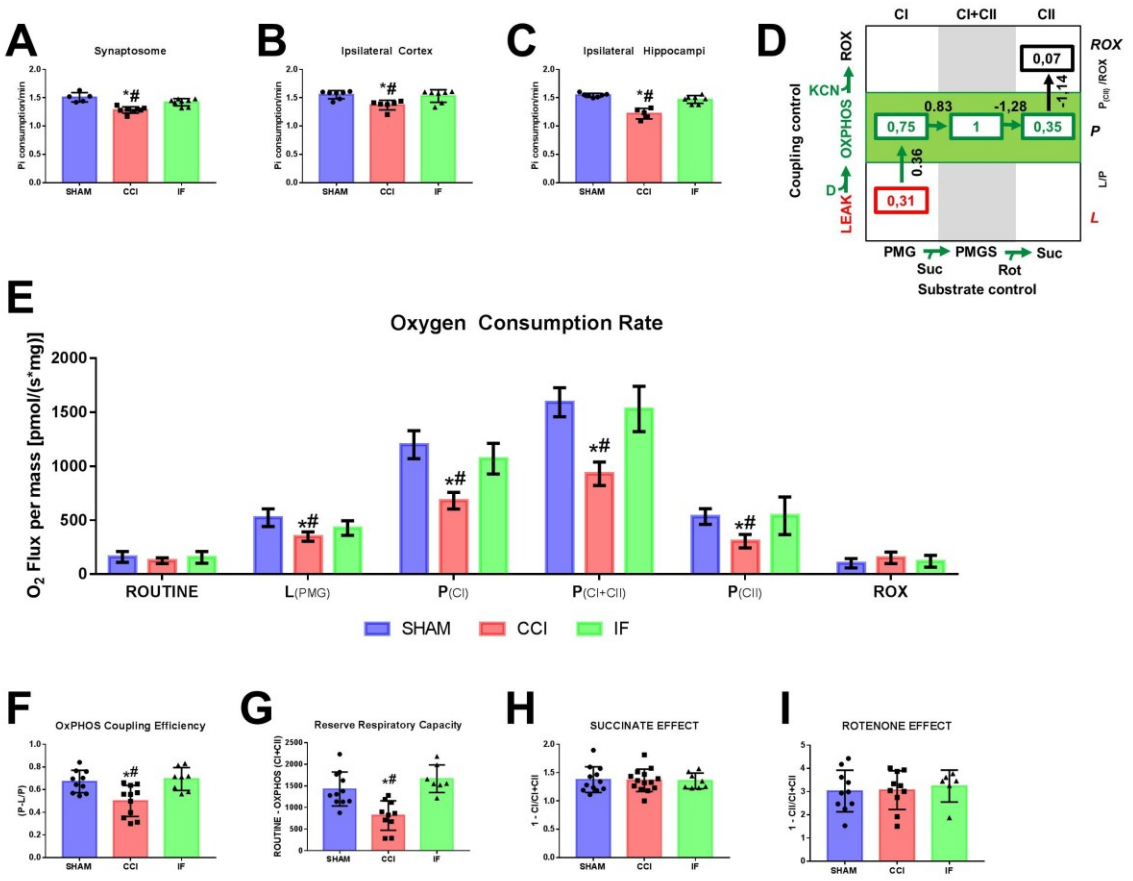


Figure 03. Intermittent fasting (IF) prevents mitochondrial bioenergetics deficits 5 days after CCI. Inorganic Phosphate consumption in Synaptosomes (A), Ipsilateral cortex (B) and hippocampi (C) was decreased by CCI. Synaptosomal oxygen consumption rates at different mitochondrial states (A) after sequential addition of pyruvate, malate, and glutamate (Leak), adenosine diphosphate (ADP; CI), Succinate (Suc), adenosine diphosphate (ADP; CI+CII), Rotenone (CII) and cyanide (ROX); Oxphos coupling efficiency (B) and Reserve Respiratory Capacity (C). IF prevented the impairment in mitochondrial bioenergetics after CCI. *Indicates significant difference compared to SHAM; # Indicates significant difference compared to CCI.

*# Denotes significant difference when compared to both SHAM and IF groups.

Intermittent fasting prevents the rupture of mitochondrial membrane potential ($\Delta\Psi_m$) dynamics after CCI

The $\Delta\Psi_m$ measured through the fluorescence signal emitted by safranin-*O* in synaptosomes is showed in Figure 4. The decrease in dye concentration in the medium parallel with the accumulation of the dye inside the mitochondria, which results in fluorescence quenching.[33]

In the Routine state, were respiration is sustained by endogenous substrates, CCI showed a significant decreased fluorescence (Figure 4A; SHAM vs CCI: mean diff. = -27.7; p <0.0001; CCI vs IF mean diff. = 28; p <0.0001 and IF vs SHAM: p = 0.9987). In the leak state (mitochondrial substrates without ADP) CCI showed a significant decreased fluorescence compared to SHAM (Figure 4A; SHAM vs CCI: mean diff. = -17,3; p = 0.0217; CCI vs IF p = 0.3017 and IF vs SHAM: p = 0.4508), while the variation was not different among groups, indicative of no impairment in the polarization of the mitochondrial inner membrane (elevated $\Delta\Psi_m$). At this point, ADP was added to activate OxPhos and this was associated with a decrease in $\Delta\Psi_m$ (increase in fluorescence in $P_{(CI)}$). This occurs due to protons being shuttled back to the matrix through the FoF1-ATP synthase thereby triggering phosphorylation of ADP to ATP. This is concomitant to the increased OCR via decreased constraining effect of the proton gradient on electron transport. The $\Delta\Psi_m$ of CCI was significantly different compared to both SHAM and IF groups (Figure 4A; SHAM vs CCI: mean diff. = -35.1; p <0.0001; CCI vs IF mean diff. = 35.17*;* p < 0.0001 and IF vs SHAM: p > 0.9999). Surprisingly, the IF showed a significantly higher variation in $\Delta\Psi_m$ compared to both SHAM and CCI groups (figure 4B; SHAM vs CCI: p = 0.0898; IF vs SHAM p = 0.3774 and IF vs CCI p = 0.0032). In saturating concentrations of ADP, CI and CII – linked substrates generated a small decrease in fluorescence due to increased $\Delta\Psi_m$ ($P_{(MAX)}$), the same pattern was observed for fluorescence and variation. After Complex I inhibition by Rotenone (1 μM), the fluorescence decreased, with expected significantly smaller variation in the CCI group. No differences were observed in the ROX state, when cyanide (KCN, 1 μM) was added to inhibit Complex IV.

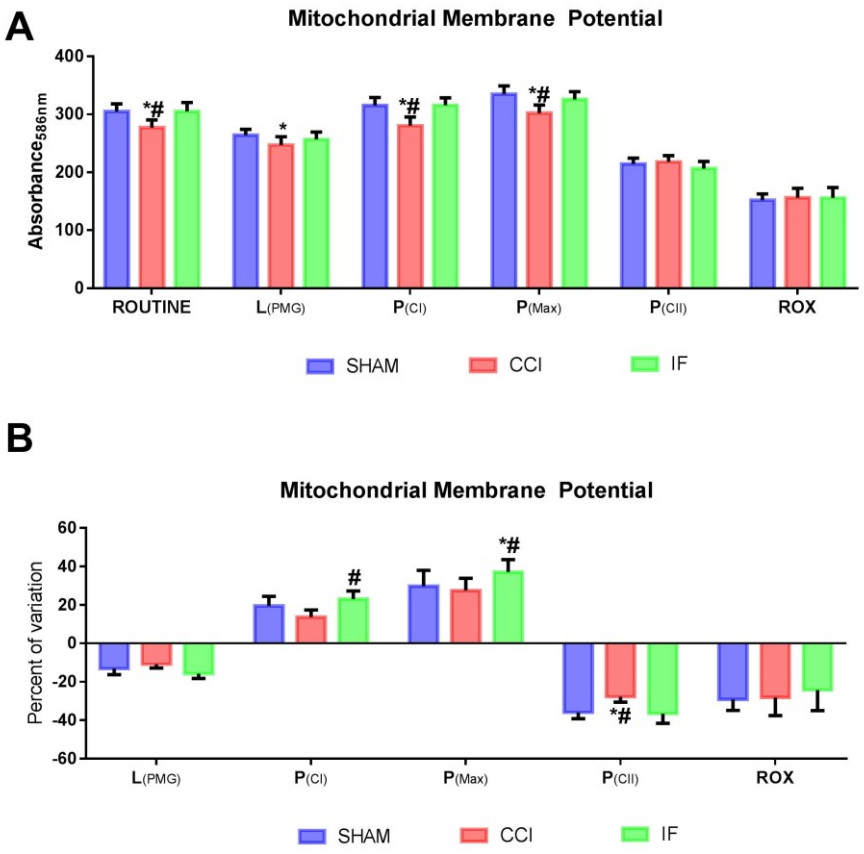


Figure 04. Prior intermittent fasting sustained the mitochondrial membrane potential ($\Delta\Psi_m$) dynamics 5 days after CCI. The fluorescent dye safranin-O is incorporated within synaptosomal mitochondria of the ipsilateral hemisphere then decreasing the fluorescent signal of the medium (A). The $\Delta\Psi_m$ of the CCI was significantly decreased at baseline, after addition of the metabolic substrates after sequential addition of pyruvate, malate, and glutamate (Leak), adenosine diphosphate (ADP; CI), Succinate (Suc), adenosine diphosphate (ADP; CI+CII), Rotenone (CII) and cyanide (ROX). The percentage of variation in the $\Delta\Psi_m$ (B) in the transition from $P_{(CI)}$ to Maximal OxPhos capacity and after CI inhibition ($P_{(CII)}$) confirmed that CCI group does not effectively generates and dissipated $\Delta\Psi_m$ as did SHAM and IF groups. *Indicates significant difference compared to SHAM; # Indicates significant difference between CCI and IF.

Intermittent fasting prevented the impairment in mitochondrial calcium efflux through NCLX channel after CCI

Impaired mitochondrial ATP synthesis and $\Delta\Psi_m$ depolarization are intimately related to Ca^{2+} regulated processes, as persistent high calcium influx impact mitochondrial function and cell integrity by apoptotic signals. [34] Therefore, we evaluated calcium influx and efflux in the ipsilateral synaptosomes. The fluorescence signal (Figure 5A) associated with increased Ca^{2+} and mitochondrial swelling showed no significant differences among groups before and after addition of mitochondrial substrates (PMGSA). Mitochondria from all groups were able to respond to a challenge of Ca^{2+} after the energization with PMGSA (Figure 5A); however the mitochondrial swelling was higher in the CCI group, as demonstrated by the percentage of variation (Figure 5A and B). Additionally, after the addition of Na^+ to stimulate mitochondrial Ca^{2+} efflux, the CCI group showed a weak response, indicating reduced functional capacity of NCLX channel and consequently impaired Ca^{2+} homeostasis. In contrast, SHAM and IF groups displayed an increase in absorbance, which illustrates mitochondrial shrinkage due to Ca^{2+} efflux in exchange with Na^+ (Figure 5A and B). Taken together, these results indicate that CCI induced increased mitochondrial swelling and deficient NCLX (Na^+-dependent) calcium efflux. Remarkably, IF improved NCLX function leading to almost normal extrusion of Ca^{2+} in exchange with Na^+.

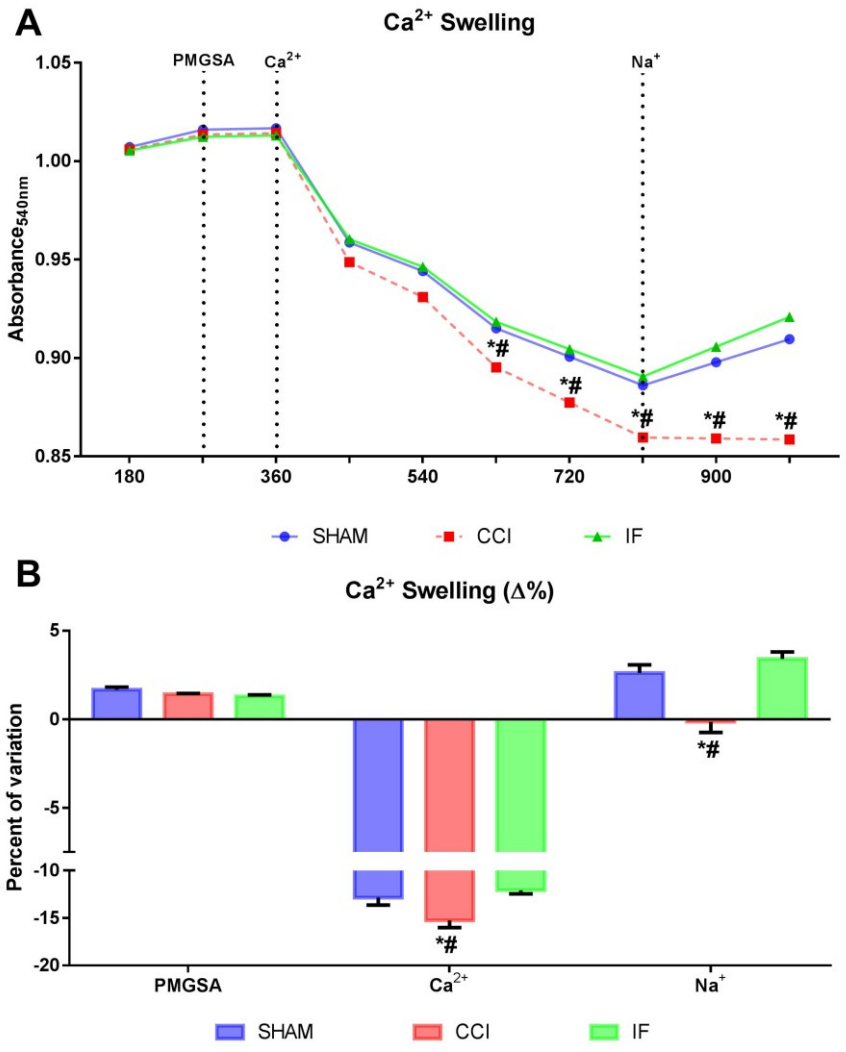


Figure 05. Intermittent fasting (IF) prevented impaired mitochondrial calcium extrusion by the Na+ dependent channel (NCLX) 5 days after CCI. Mitochondrial Ca^{2+} swelling (A and C) stimulated by a calcium challenge after addition of mitochondrial substrates (pyruvate, malate, glutamate, succinate, and ADP; PMGSA) was significantly elevated in CCI group. Stimulation of Ca^{2+} extrusion through the NCLX channel after addition of Na^{+} was not effective in VEH-CCI group (A and B). This effect was prevented by IF. * Denotes significant difference compared to SHAM; # Indicates significant difference compared to IF.

Intermittent fasting attenuated hydrogen peroxide production in different mitochondrial coupling states and preserves cell viability 5 days following CCI

During cellular stress, the increased intracellular ionic charges (e.g. Ca^{2+}) may cause the collapse of the buffering capacity, $\Delta\psi_m$, proton gradient driven ATP synthesis. The mitochondrial oxidation of energy substrates coupled with electron transport system is considered the main site of ROS production including the H_2O_2. Conceptually, the exacerbated uncoupling of the electron transport system associated with decreased antioxidant defenses implies in a presence of detrimental ROS production. Here, the baseline H_2O_2 level 5 days was increased in CCI compared to SHAM, while IF was not significant different compared to CCI and SHAM (Figure 6A). The addition of the CI-linked mitochondrial substrates (PMG) increased H_2O_2 production, significantly higher in the CCI group only. While no differences where observed among groups with the addition of ADP, the addition of succinate culminated in higher O_2 consumption, and evoked a significantly higher H_2O_2 production by the CCI and IF groups. However, IF attenuated hydrogen peroxide production when compared to SHAM and CCI groups. In saturating concentrations of ADP, CI and CII – linked substrates ($P_{(MAX)}$), the CCI group showed significantly higher H_2O_2 production compared to both SHAM and IF. After inhibition of CI and complex VI, H_2O_2 production was higher in both CCI and IF groups compared to SHAM. Remarkably, IF attenuated the increased H_2O_2 production in different mitochondrial respiration states. Given that mitochondrial bioenergetics dysfunction and H_2O_2 levels may affect cell survival, we further explored cell viability (Figure 6B). Remarkably, CCI decreased cell viability and IF prevented this effect (SHAM vs CCI: p <0.0001; IF vs SHAM p = 0.1847 and IF vs CCI p = 0.0004). Therefore, the aforementioned preserved mitochondrial bioenergetics, $\Delta\psi_m$, Ca^{2+} efflux capability, linked with decreased H_2O_2 production mediated by IF parallel with sustained cell viability 5 days after CCI.

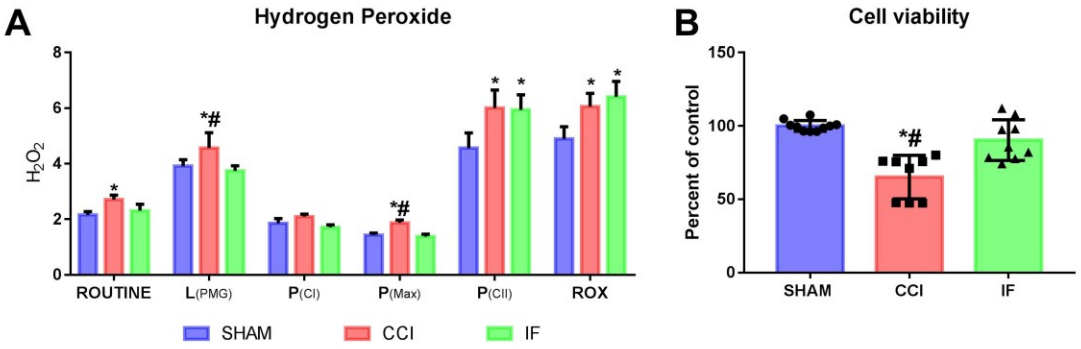


Figure 06. Mitochondrial hydrogen peroxide (H_2O_2) production and cell viability 5 days after CCI. The H_2O_2 levels in synaptosomal mitochondria were significantly increased at baseline, after sequential addition of pyruvate, malate, and glutamate ($L_{(PMG)}$), Succinate and adenosine diphosphate (ADP; $P_{(MAX)}$), Rotenone ($P_{(CII)}$) and sodium azyde (ROX) by CCI. Cell viability (B) was decreased by CCI. Intermittent fasting significantly decreased H_2O_2 production before complex I and IV inhibition and prevented decreased cell viability 5 days after CCI.

* Denotes significant difference when compared to SHAM.

*# Denotes significant difference between CCI and IF groups.

Intermittent fasting attenuates spatial memory deficits five days after CCI

The MWM task is a robust method to evaluate learning and memory deficits in mice submitted to brain trauma. During the acquisition (training) phase of MWM, the SHAM group showed a significant decrease in the time to find the hidden platform when compared to other groups (Figure 7A). In the first training day, SHAM group was different from both CCI and FASTED.

Analyzing the subsequential training days, SHAM group remained different when compared to CCI and IF, albeit CCI and IF were also statistically different, indicating attenuated learning deficit in the IF group. The probe trial indicated that CCI spatial memory deficits, since the time in the target zone (Figure 7B) was significantly higher for CCI compared to both SHAM (Mean difference: 13.99; p = 0.0032) and IF (Mean difference: 10.36; p = 0.038) with no significant difference between SHAM and IF (Mean

difference: -3.633; p = 0.94). The same was observed for Latency to first entry (figure 7C), where CCI was significantly higher compared to both SHAM (25.54; p = 0.0042) and IF (-22.15; p = 0.0114), with no differences between SHAM and IF (3.383; p = 0.8514). The representative tracking plots illustrate the characteristic path performed by a mouse during the probe test (Figure 7C). These results confirms that IF is able to prevent cognitive deficits associated with TBI.

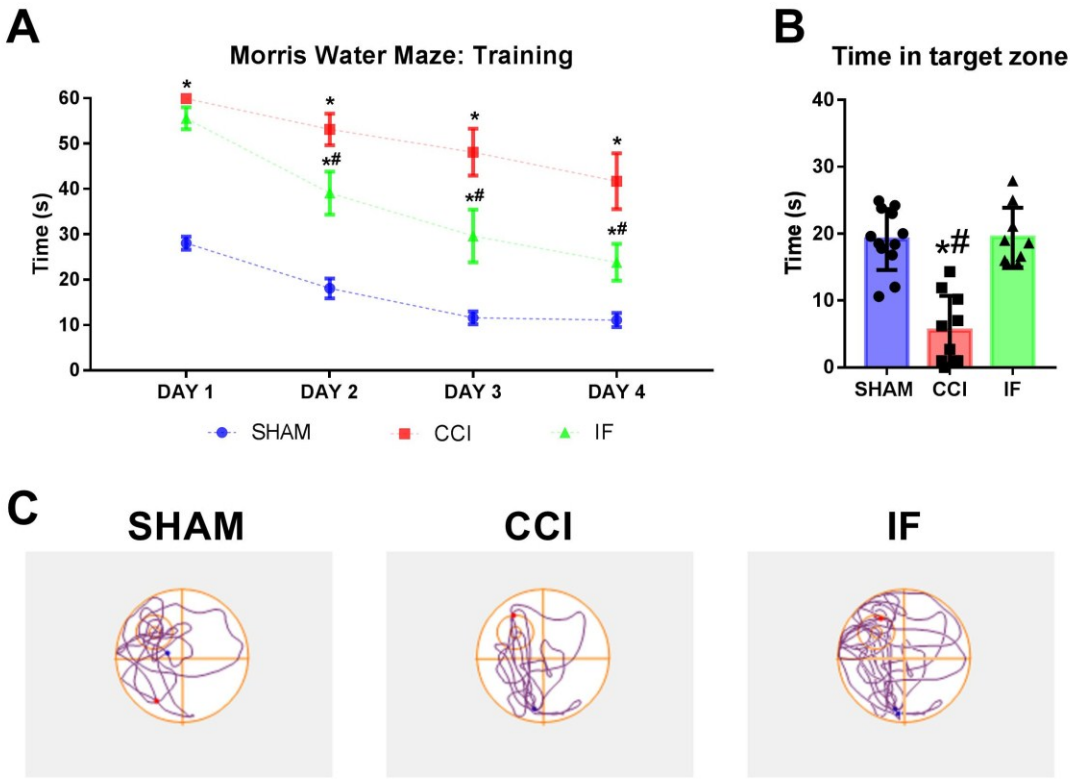


Figure 07. Morris Water Maze parameters. A) Acquisition phase; B) Test phase; C) Video tracking

*# Denotes significant difference when compared to both SHAM and IF groups.

DISCUSSION

Recently, it has been proposed that the benefits associated with IF regimens are related to a "Intermittent metabolic switching"(IMS), which refers to repeating cycles of a metabolic challenges such as dietary restriction and/or exercise, which cause liver

glycogen stores depletion and increased circulating ketone levels, followed by a recovery period characterized by eating, resting and sleeping. Based on this concept, we induced IMS without promoting substantial changes in body mass to prime brain metabolic adaptations before a severe head injury.

The IMS protocol used in our study was a fasting regimen with repeated 24-hour intervals of food deprivation followed by 24-hour ad libitum food access (IF) for twenty-days. As expected, the IF protocol induced IMS, without detectable changes in body mass after 10 cycles. In opposite, Li et al.,[35] showed markedly body mass loss using after 10 to 30 days of IF protocol. We believe that the age of mice used in these studies (8 weeks vs 20 weeks) could account for these differences. However, our study is in agreement with recent literature evaluating weight loss after up to 30 days of IF in comparison to normal diet regimen.[36] Weight loss in rodents following chronic IF still causes divisiveness in the current literature, as different reports indicate that male C57BL/6 mice compensate periods of fasting by increasing their food intake and gaining weight at rates similar to mice fed ad libitum, thus the feeding behavior may be responsible for the lack of differences in body weight.[17, 37, 38] Noteworthy, we limited our analysis to total body mass, which does not imply in changes of body mass composition, an important indicator of IF benefits.[35, 39] Additionally, our IF protocol promoted IMS as indicated by significant differences in total ketones bodies concentration after a fasting day and no differences after a *ad libitum* feeding day, albeit the concentrations of total ketones and glucose soon before TBI induction were not different. This implies that beyond the already demonstration of acute fasting as neuroprotective against TBI, the prolonged IF may also provide neuroprotection through neuroenergetic adaptations. [19] Altogether, our findings integrate new components to IMS with potential relevance to TBI.

Normal brain processes, such as neurotransmission, ionic homeostasis and membrane potentials are metabolically demanding, bolstering the emerging attention to mitochondrial function in the pathogenesis of several brain disorders, including TBI.[12, 40] Precisely, TBI impairs brain metabolism and glucose uptake, in addition to mitochondrial capability to manage high intracellular Ca^{2+} inputs often accompanied by persistent neuroenergetics deficits, increased ROS production and apoptotic signaling, leading to a progressive neurodegeneration and memory deficts.[31, 32, 7, 8, 6, 9] Seemingly, impaired

synaptic activity reduces energy requirements, although astrocyte proliferation and microglial activation seems to be associated with hypometabolism in neurodegenerative disease,[41] which is also a hallmark in severe TBI. Recently, the axiomatic concept relative to FDG-PET representing neuronal glucose consumption specifically at synapses was challenged by Zimmer et al,[42] showing that FDG PET signal is substantially affected by the activation of the majoritary astroglial glutamate transport GLT-1 with ceftriaxone.[43] Remarkably, brain metabolic connectivity is altered by TBI, [43] which could reflect altered astrocytic activity.[44] Our results support these concepts, as TBI decreased whole brain and region specific FDG glucose uptake and disrupted metabolic connectivity, an effect that was prevented by IF. Also, the IF strategy preserved mitochondrial bioenergetics capacity in the synaptic terminals and was capable of reshaping metabolic architecture concomitantly with a partial recover of FDG uptake, which highlight that prophylactic IF strengthens the pre-existent synaptic connections and/or pave the way to build new synaptic connections after TBI at expenses of the trophic metabolic adaptations (IMS).

Although there is robust set of evidences indicating that different dietary restriction paradigms exert prophylactic effects against different brain insults, studies addressed to TBI are scarce.[19, 45] Particularly, studies focusing on synaptic bioenergetics reprograming due to dietary restriction are limited, while the effects on liver and muscle are definitely well known.[38, 46] Therefore, using high-resolution respirometry we explored if mitochondrial metabolism is involved in the prophylactic benefits of IF after TBI. Using this approach, functional aspects of the oxidative phosphorylation system, such as substrate uptake and oxidation in the Krebs's cycle and F1F0-ATP synthase activity was evaluated using the OxPhos Coupling Efficiency, which is equivalent to the Respiratory Control Ratio (RCR; StateIII/StateIV) a classic indicator of mitochondrial bioenergetics functioning.[47, 48] Further, the Reserve Respiratory Capacity (RRC; the difference of between ATP produced by oxidative phosphorylation at basal and those challenged at maximal activity is a reliable indicator of how mitochondria responds to an increased energy demands or to a stressful cellular energy requirement due to a pathological environment.[49] A decreased RRC has been connected with neurodegenerative mechanisms, which is in line with secondary mechanisms of TBI.[50-52]

Our data indicates persistent mitochondrial dysfunction in the synaptic terminals throughout 5 days after injury, as indicated by OxPhos coupling efficiency and RRC, whilst the IF prevented these effects. Mitochondrial dysfunction at synapses as indicated by the RCR has been previously reported 24 h after head injury compared with sham mice.[53] Since RCR and RRC could be affected by particular aspects of the electron transfer system, we investigated the stimulatory effect of both CI and CII - linked substrates to maximal OxPhos capacity. No differences were observed in these parameters, indicating an effect independent of CI or CII function related to ATP synthesis. Therefore, oxygen consumption rates (OCR) in synaptosomes following severe TBI were decreased in different metabolic coupling states, culminating in decreased OxPHOS coupling efficiency and reserve capacity. Importantly, the mitochondrial adaptations primed by IF prevented these bioenergetics defects. Based on these findings, we further explored whether the preserved neuroenergetics is linked with normal $\Delta\psi_m$, Ca2+, handling and ROS production.

Physiologically, during normal respiration in mitochondria, $\Delta\Psi_m$ formation occurs by proton pumping from the matrix to the intermembrane space during ETS activity, driving proton motive force to ATP synthesis. Disruptions of $\Delta\Psi_m$, resulting in decreased ATP synthesis has been observed in response to many apoptotic stimuli.[54] Accordingly, $\Delta\psi_m$ dynamics at day 5 after CCI was significantly impaired relative to other groups, and most importantly, IF group showed similar responses as SHAM group. Given the functional proximity between ATP synthesis and $\Delta\psi_m$ due to electrochemical properties of the mitochondrial internal membrane, we assessed mitochondria calcium handling in a swelling protocol. Mitochondrial function is affected by calcium handling, which involves different pathways of calcium influx and efflux. Mitochondria respond promptly to cytosolic Ca^{2+} oscilations accumulating Ca^{2+} when concentration rises above 0.5 μM. This occurs mainly to the activation of mitochondrial dehydrogenases and the storage of excess Ca^{2+}.[55] Owing that the resting free Ca^{2+} concentration ranges between 100–200 nM in cytosol of most cells, free Ca^{2+} in both cytosol and mitochondria can transiently and abruptly increase by a factor of 10–20 in an tightly coordinated process, intimately dependent on the mitochondrial membrane potential.[56, 57] The outer mitochondrial membrane voltage dependent anion channel (VDAC) conducts Ca^{2+} in

addition to monovalent ions, controlling Ca^{2+} permeation.[58] As for the inner membrane, Ca^{2+} influx into the matrix is mainly regulated by the mitochondrial calcium uniporter (MCU), positively charging the matrix paralleling with $\Delta\psi_m$ depolarization, while the $Ca^{2+}/Na^{+}/Li^{+}$ exchanger NCLX predominantly controls Ca^{2+} efflux to maintain intramitochondrial homeostasis.[58] Although it is not well established if mitochondrial swelling is a strictly inner-membrane phenomenon,[59] a disturbance in calcium homeostasis is linked to neuronal cell death, via the formation of the permeability transition pore (PTP), which is opened by excessive Ca^{2+} accumulation inside mitochondria causing mitochondrial swelling.[60] In this context, ATP is a vital component to induce reversal of swelling, as demonstrated by Albert Lester Lehninger in a classic set of experiments.[61] Notably, higher permeability to Ca^{2+} results in protein release and apoptosis, inducing permeability transition pore opening and cell death.[62] Even though a transitory increase in the Ca^{2+} levels within mitochondrial matrix activates ATP synthesis and other Ca^{2+} sensitive processes in normal cell environment, a persistent Ca^{2+} increase exerts detrimental effects, as increased Ca^{2+} levels parallels with the secondary hypometabolism after TBI.[63-65] Our results indicate that calcium influx is potentially exacerbated after TBI, in addition to impairment in calcium efflux. Actually, mitochondrial NCLX channel is voltage dependent and eletrogenic, and in pathological conditions like TBI the NCLX import Ca^{2+} rather than extrude in a putative reverse mode,[66-68,63] Considering the impairment in mitochondrial ATP production and $\Delta\Psi_m$ combined with the deficient shrinkage in the CCI group, it is plausible to assume that indeed Ca^{2+} overload following TBI ultimately leads to proapoptotic responses. Strikingly, IF prevented impaired mitochondrial Ca^{2+} homeostasis, which potentially benefits mitochondrial metabolism and overall cells survival.

Moreover, the coupling of impaired mitochondrial function, exacerbated ROS production and oxidative damage, is the most validated mechanism of secondary injury after TBI.[69] Consequently, we investigated mitochondrial H_2O_2 production in different coupling states and clearly demonstrated that severe CCI increased overall H_2O_2 production whereas IF attenuated its production. After TBI, mitochondrial dysfunction in cortex and hippocampus parallels with oxidative damage to mitochondria *per se* and cytoskeletal proteins.[70] It is known that calorie restriction up-regulates brain antioxidant

responses and suppress oxidative stress during the aging process. [71, 72] It should be considered that H_2O_2 leads to peroxidation of mitochondrial lipid membranes associated with alterations in the mitochondrial $\Delta\Psi_m$ and neuroenergetics deficits influenced by Ca^{2+} overload properly.[73] In the present study, this relationship is strengthened, as mitochondrial ATP production, $\Delta\Psi_m$, Ca^{2+} overload and H_2O_2 production culminated in spatial memory impairment.

Mitochondrial bioenergetics, calcium buffering capacity, and structural integrity are early compromised following TBI injury in rodents. [40, 70, 74] Following the time-course after injury, several mitochondrial functional parameters appear to recover and remain stable at day 7, but no improvements comparable to uninjured animals are observed.[75] Isolated mitochondria from ipsilateral cortex present impaired metabolism three days after TBI and restored function 5 days after injury.[70] It is plausible that dysfunctional mitochondria are for the most part no longer present in the injured brain 5 days following injury,[70] albeit, density-based gradients often select healthier mitochondrial populations compared to tissue homogenate analysis.[76] Brain mitochondria are heterogeneous, consisting of both synaptic and non-synaptic populations, as isolated synaptic mitochondria consist of pre-synaptic mitochondria located within the synaptosomes, while isolated non-synaptic mitochondria consist of neuronal (axonal, somal, dendritic) and non-neuronal (glial, vascular, etc.) mitochondria.[77, 78] Synaptic mitochondria exhibit increased oxidative damage, decreased bioenergetics and higher vulnerability to calcium imbalance compared to non-synaptic mitochondria.[77, 78] Mitochondrial metabolism after TBI is dependent on the localization of mitochondria in synaptic and non-synaptic sites, as synaptic mitochondria sustain increased damage 24 h following TBI [53]. Appropriate neurotransmission and synaptic plasticity relies on synaptic mitochondrial function, [79] processes that are negatively affected following TBI, [80] where the degree of mitochondrial dysfunction is a decisive factor to subsequent cell death, and functional recovery. [12, 75, 81] Nevertheless, The effects of IF on brain mitochondrial metabolism are still controversial. Although it was initially accepted that increased mitochondrial biogenesis and oxygen consumption were induced in mice fed alternate days,[82] this view was heavily challenged by a wide-range of posterior mechanistic-oriented studies using different approaches, suggesting that IF does not necessarily increase oxygen

consumption *per se*,[46, 83] but leads to an improvement in overall mitochondrial function, through increased in the antioxidant activity/decreased ROS production,[84] increased neurotrophic factors,[85, 86] mitochondrial biogenesis[87] and improved insulin signaling[88] in rodents. Since the pattern of response of specific mitochondrial populations within the brain is differently affected by the injury and microenviroment, this should be considered when comparing different studies, since the coordinated adaptations that can optimize brain metabolism are remarkably complex and are beyond whole-brain basal oxygen consumption. Regardless of the fact that these improvements are not always detectable in normal (i.e. without the presence of a pathologic disorder) conditions, the combination of these mechanisms can explain how IF induced mitochondrial improvement leading to a sustained functionality facing an adverse environment caused by TBI, or in other types of brain injury.[89]

The above mentioned mechanisms are intimately related to TBI induced axonal disruption, characterized in immediate and complete axotomy (i.e. mechanical rupture) and secondary axotomy (progressive degradation and gradual failure linked with metabolic impairment), which are directly associated with decreased cell viability.[8] Cell viability assessment in terms of reductive activity as enzymatic conversion of the tetrazolium compound to water insoluble formazan crystals by dehydrogenases of living cells is widely studied.[90] In the present study, IF prevented the decreased cell viability induced by TBI. Although the reduction of water-soluble salt MTT is widely used as cell viability indicator, many authors express concern regarding this assumptiom.[91, 92] Reduction of MTT is also regarded as indicator of "cell redox activity" or "cell metabolic activity" since different organelles and enzymes can participate in its reduction [93]. In this context, additional indicators of neuronal and glial cells degeneration must be considered.

The potential benefit of IF on learning and memory performance has been attributed to its metabolic effects.[94, 95] In agreement with this conjecture, we challenged mice in the MWM task and observed that a severe CCI injury imposes spatial learning and memory deficits whereas IF prevented these effects, as indicated by progressive decreases in goal latency times with increasing training and the performance in the probe trial similar to sham animals. Several reports indicated enhanced cognitive function and preserved cognitive function after IF paradigms.[37, 96, 97] Impairment in brain bioenergetics

precedes alterations in behavioral performance, albeit this direct causal relationship is currently strictly correlative.[98] The present results expand current literature indicating a cognitive benefit associated to IF and highlights that its prophylactic benefits are consistent with mitochondrial metabolic reprograming providing trophic support to synaptic connectivity.

CONCLUSION

In summary, prophylactic intermittent fasting exerts metabolic benefits to brain after a severe head injury. Remarkably, IF prevented mitochondrial bioenergetics deficits coupled with exacerbated H_2O_2 production, and improved glucose uptake and synaptic connectivity culminating in preserved cognitive function. Taken together, these findings elucidate new trophic mechanisms mediated by IF, which may contributes to preventing the progressive injury associated with TBI. These results expand the literature and provide functional and molecular pieces of evidence strengthening the prophylactic benefits of IF to individuals at increased risk of TBI.

METHODS

Animals and treatment protocol

Male 180 days old C57BL/6J mice were obtained from Foundation for Health Science Research (FEPPS, Porto Alegre/RS, Brazil). Animals (4-5 per cage) were placed into a controlled temperature room (22ºC ± 1) under a 12h light/12h dark cycle (lights on at 7 a.m.) and had free access to food and water. Animals were assigned to two different dietary regimens: a normal, ad libitum diet and an alternate-day fasting, where animals had no food access during 24 h and ad libitum food access in the following day. Animals underwent a total of 10 cycles of food restriction and access. Subsequently, 48h after the last fasting day for the AF group, animals were submitted to a severe CCI injury. After CCI, animals were monitored during 5 days and underwent behavioral analysis. On the 5th day, animals were euthanized and the ipsilateral hemisphere surrounding the impact

injury was dissected for further neurochemical analysis. Body mass was accessed in 5 different time-points before CCI and 24, 48, 72 and 120 h after CCI. Total Ketones and blood glucose were assessed using specific meters and test strips in tail-tip blood samples (FreeStyle Optium Neo and β-Ketone Monitoring System; Abbott, Brazil and On Call® Plus glucose meter; ACON Laboratories, Inc., US, respectively). Blood samples were collected by skilled personnel using the routine tail-tip technique. See the experimental design in the Supplemental figure 01. All experiments were in agreement with the Committee on the Care and Use of Experimental Animal Resources, UFRGS, Brazil number 22436.

Controlled Cortical Impact protocol (CCI)

To induce TBI, mice were placed in the stereotaxic (Kopf Instruments, Tujunga, CA) with a heating bed (37 ± 1 ^{o}C) and maintained with inhalation anesthesia (2.5 % isoflurane) in a mixture of N_2 and O_2 (2:1) during the entire surgical procedure. A 4 mm diameter craniotomy was made in the central part of the left parietal bone to perform the CCI injury using an equipment Benchmark stereotaxic impactor, myNeuroLab®, Leica, St. Louis, MO, USA. The injury was induced by a piston of 3.0 mm diameter on the exposed surface of the intact dura mater. Other lesion parameters were fully adjusted in the equipment, as follows: impact velocity of 5.7 m / s; impact duration was 100 ms, and 2 mm of depth penetration. Soon after injury, the area of the craniotomy was isolated with a concave lamella bonded with dental cement, and the scalp was sutured. After the surgical procedure, anesthesia was discontinued and the animals were placed in a heated box to maintain normal body temperature and were monitored for 2 h post-injury.[99]

microPET-Scan analysis

Non-pharmacologically treated mice were submitted to baseline [^{18}F]FDG uptake evaluation using positron emission tomography scans (microPET), performed at the Brain Institute (Porto Alegre, Rio Grande do Sul, Brazil). The same animals were resubmitted to [^{18}F]FDG uptake scans after 15 days. Prior to both paired scans, animals

were fasted overnight, but had ad libitum access to water. Animals were placed on a controlled temperature environment, and an intraperitoneal injection of 200µL of [^{18}F]FDG, with radioactive activity of 240µCi approximately, was made, allowing conscious drug uptake for 40 min. Animals were then anesthetized (Ketamine/Xylasine 90mg/kg and 7.5mg/kg respectively) and microPET-Scans performed. Images were analyzed based on regions of interest previously defined to determine region specific Standard Uptake Values (SUV). [100] Further, SUV were normalized to body mass.

Metabolic connectivity

In this analysis, we integrated several parameters in *R statistics software*. Pearson's R correlation were calculated among the SUV of brain regions. Networks were built using the "*RedeR*" package. Further, we performed clustering with both "euclidean distance" and "complete linkage agglomeration method"; using the "*pvclust*" package, "10000 bootstraps" and "seed 123" parameters were set for assessing the uncertainty in hierarchical clustering. Additionally, heatmaps were obtained from the correlation plots. Finally, the networks were built using the "RedeR" package.

Preparation of Ipsilateral hemisphere synaptosomes and tissue homogenates

For synaptosome isolation, the homogenized hemisphere was centrifuged at 1330 ×g for 3 min. The supernatant was carefully retained and then centrifuged at 21,200 ×g for 10 min. The resulting pellet was re suspended and carefully layered on top of a discontinuous Percoll gradient (15 and 23%) and centrifuged for 5 min at 30,700 $^{\times}$g, [101, 102] resulting in a synaptosome-enriched band. The synaptosomal fractions were transferred and resuspended in the isolation buffer and centrifuged at 16,900 ^{x}g for 10 minutes. The resulting pellet was resuspended in 2mL isolation buffer and centrifuged at 6,500 ^{x}g for 10 minutes. The final pellet of synaptosome was resuspended in a sucrose and Tris buffer (320mM sucrose, 10mM Tris, pH 7.4) and immediately were used for respirometry, mitochondrial swelling, mitochondrial membrane potential and hydrogen peroxide analysis. The remaining samples were frozen at − 80 °C for protein

quantification. The Pierce™ BCA Protein Assay Kit (Catalog number: 23225) was used for quantification of protein contents of all samples. The assay was performed according to the manufacturer's instructions. Measurements were performed in triplicates (coefficient of variation between duplicates was <3%) and corrected for the absorbance measured in the homogenization buffer only.

Mitochondrial respiratory protocol

Synaptosomal oxygen consumption measurements were performed using a standard respiration buffer (100 mM KCI, 75 mM mannitol, 25 mM sucrose, 5 mM phosphate, 0.05 mM EDTA, and 10 mM Tris-HC1, pH 7.4). Oxygen consumption per tissue mass was measured using the high-resolution Oxygraph-2k system and recorded real-time using DatLab software (Oroboros, Innsbruck, Austria). The results were normalized to the protein content. The experiments were performed at 37°C in a 2-ml chamber, with a modified multi-substrate titration protocol as previously described in detail elsewhere.[47]

Following 5 minutes for establishing ROUTINE respiration values, the multi-substrate titration protocol started. The protocol consisted of sequential addition of pyruvate, malate, and glutamate (PMG; 10, 10, and 20 mM, respectively) to obtain LEAK respiration; Sub sequentially, adenosine diphosphate (ADP, 2.5 mM) was titrated to obtain OxPHOS capacity of Complex I-Linked substrates; Maximal OxPHOS capacity (CI and CII-Linked) was obtained with addition of Succinate (S, 10 mM) in saturating ADP concentrations; Complex II specific capacity was obtained with Rotenone (ROT, 2.0mM) and non-mitochondrial respiration (ROX) after cyanide (KCN, 5 mM).

Respiratory fluxes were corrected automatically for instrumental background by DatLab taking into account oxygen consumption of the oxygen sensor and oxygen diffusion out of or into the oxygraph chamber measured at experimental conditions in incubation medium without biological sample. ROX was extracted from all of the above mentioned states and Tissue-mass specific oxygen fluxes were compared in different substrate and coupling states.

We calculated Flux control factors (FCF), which express the change of flux in a single step of the SUIT protocol, normalized for the high flux as a specific reference state The respiratory control ratio (RCR = P/L) was obtained in the CI-linked substrate state. For statistical analysis RCR was transformed to its respective FCF, which is the OXPHOS coupling efficiency calculated as (P-L)/P = 1-L/P. The FCF for CI-linked substrates stimulating CII-linked respiration was measured as the rotenone effect, 1 – CII/CI&II, in the OxPHOS state in our protocol. The corresponding FCF for the CII-linked substrate (succinate effect) was calculated as 1 – CI/CI&II, determined in the OXPHOS state in the protocol. Finally, Reserve Respiratory Capacity was assessed in the OxPHOS state as (ROUTINE - Maximal OxPHOS capacity).[47, 48]

Mitochondrial Membrane Potential ($\Delta\Psi_m$)

The ΔΨm was measured 10 days after CCI through the fluorescence signal emitted by the cationic dye, safranin-O. Ipsilateral cortex homogenates were incubated in the respiration buffer used for the respirometry protocol, additionally supplemented with 10 μM safranin-*O*. The fluorescence was detected with an excitation wavelength of 495 nm and an emission wavelength of 586 nm (Spectra Max M5, Molecular Devices). An increased in the fluorescence units mirror decreased $\Delta\Psi_m$ whereas decreased fluorescence units indicate increased $\Delta\Psi_m$. Baseline $\Delta\Psi_m$ was measured without addition of substrates and inhibitors in the incubation medium.

Further, PMGS and ADP (2.5 mM) were incubated to modulate the activity of mitochondrial respiratory complex I, II and V, and consequently ΔΨm. Increased ADP utilization after the addition of PMGS mirrors increased oxygen consumption coupled with ATP synthesis by ATP synthase (respiratory Complex V) along with decreased $\Delta\Psi_m$. Addition of the proton ionophore Carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone (FCCP 1 μM), a chemical uncoupler, trigger decrease in the ΔΨm and oxidative phosphorylation while stimulates the maximal mitochondrial oxygen consumption. The addition of cyanide (KCN, 5 mM), an inhibitor of mitochondrial respiratory complex IV (cytochrome oxidase), was used to decrease ATP synthesis and disrupt mitochondrial membrane potential. Moreover, we used the data achieved through

the manipulation of mitochondrial respiratory rates with the components PMGS, ADP, FCCP and KCN to calculate the percentage of change in the $\Delta\Psi_m$. Each component has a percentage of variation relative to previous one. For instance, PMGS relative to baseline, and ADP relative to PMGS. Data are reported as arbitrary fluorescence units (AFUs) and were normalized to protein content.[103]

Mitochondrial H_2O_2 production

The pattern of mitochondrial production of hydrogen peroxide (H_2O_2) was assessed in ipsilateral brain hemisphere synaptosomes through the Amplex Red oxidation method (n=10 per group). The same substrates, uncoupler and inhibitors used in the respirometry protocol were incubated sequentially in the respiration buffer supplemented with 10 μM Amplex Red and 2 units/mL horseradish peroxidase to assess H_2O_2 generation. The baseline H_2O_2 level was measured without the presence of substrate in the incubation medium. Also, PMGS was used as the substrate to stimulate mitochondrial respiration, ADP to analyze the functional capacity of mitochondria to produce ATP, and the proton ionophore FCCP (Carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone, a reversible inhibitor of mitochondrial oxidative phosphorylation) to estimate the maximal mitochondrial electron transport system capacity. Cyanide (KCN, 5 mM) was used to assess non-mitochondrial H_2O_2 production (ROX). Fluorescence was monitored at excitation (563 nm) and emission (587 nm) wavelengths with a Spectra Max M5 microplate reader (Molecular Devices, USA).[103]

Mitochondrial calcium handling

To assess mitochondrial calcium buffering capacity, namely calcium influx, and efflux, we measured the mitochondrial refractance spectrophotometrically at 540 nm (Spectra Max M5, Molecular Devices). As the mitochondria need to maintain homeostasis of its internal environment, the fluxes of ions across the inner membrane should be tightly controlled. This is particularly important because the dual physiological and detrimental roles of Ca^{2+} in the mitochondrial matrix.[34] Ipsilateral cortex

homogenates (50 uL) from 10 days treated mice were added to standard swelling incubation medium (100 mM KCl, 50 mM Sucrose, 10 mM HEPES and 5 mM KH_2PO_4) and the basal mitochondrial refractance was monitored during 3 min in microplates. Mitochondrial substrates, 3.5 mM pyruvate, 4.5 mM malate, 4.5 mM glutamate, 1.2 mM succinate (PMGSA) and 100 uM of adenosine diphosphate (ADP) were added to energize mitochondria and support ATP synthesis. Thus, the mitochondrial refractance coupled with oxidative phosphorylation was monitored during 5 min. Afterward, calcium (Ca^{2+}, 20 mM) was added to induce large-amplitude swelling driven by the colloid osmotic force of Ca^{2+} influx at proteins localized in the mitochondrial matrix. The changes in the absorbance caused by the Ca^{2+} influx were monitored for 10 min. After calcium-induced mitochondrial swelling, we accessed mitochondrial Ca^{2+} efflux as an indicator of shrinkage. Then, Na^{+} (30 mM) was added to the medium and the absorbance was monitored for additional 10 min. For a mitochondrial enriched suspension, the swelling and shrinkage causes a decrease and increase in the absorbance respectively, which are both discernible even to the naked eye.[104] Finally, we normalized all results for protein content and calculated the percentage of variation in the mitochondrial refractance after the addition of substrates (PMGSA) relative to basal; after addition of Ca^{2+} relative to (PMGSA); and after addition of Na+ relative to Ca^{2+}.

Cell viability assay

Cell viability assay was performed 10 days after CCI through the colorimetric [3(4,5-dimethylthiazol-2- yl)- 2,5-diphenyl tetrazolium bromide] (MTT, Sigma) method.[105] Animal's cortex were incubated with 0.5 mg/ml of MTT, at 37°C during 45 min. The formazan product generated during the incubation was solubilized in dimethyl sulfoxide and measured at 560 and 630 nm. The results were expressed as percentage of control.

Morris Water Maze task

We additionally investigated the cognitive function of animals using a spatial memory paradigm. [106, 107] Thus, one independent group of animals (n=10 per group) was treated with testosterone for 10 days after CCI and, in the last five-days, animals were challenged in the Morris Water Maze task (MWM). The apparatus was a black, circular pool (110 cm diameter) with a water temperature of 21 ± 1 °C. During 4 days training, the mice learned to escape from the water by finding a hidden black platform submerged in a fixed place of the tank. Each animal was submitted to four trials per day. If the animal failed to find the platform in 60 s, it was manually placed on the platform and allowed to rest for 20 s. Each trial was separated by at least 10 min to avoid hypothermia. The result is the average of four trials per animal/day/group and was used as an indicator of learning. The second phase of the MWM task, namely test phase (1 day), is an indicator of memory retention. The test phase was performed without the submerged platform 24 h after the 4 days training, and the time spent in the target quadrant was measured. Videos were obtained and analyzed using the N-Maze program.[107]

Statistical Analysis

Results were calculated and expressed as the mean ± S.E.M. To analyze the differences between groups, we used one-way analysis of variance (ANOVA) followed by a post-hoc Tukey test; or Kruskal-Wallis test when necessary. All procedures were performed using GraphPad Prism 6.0 software. The differences were considered statistically significant at $p < 0.05$.

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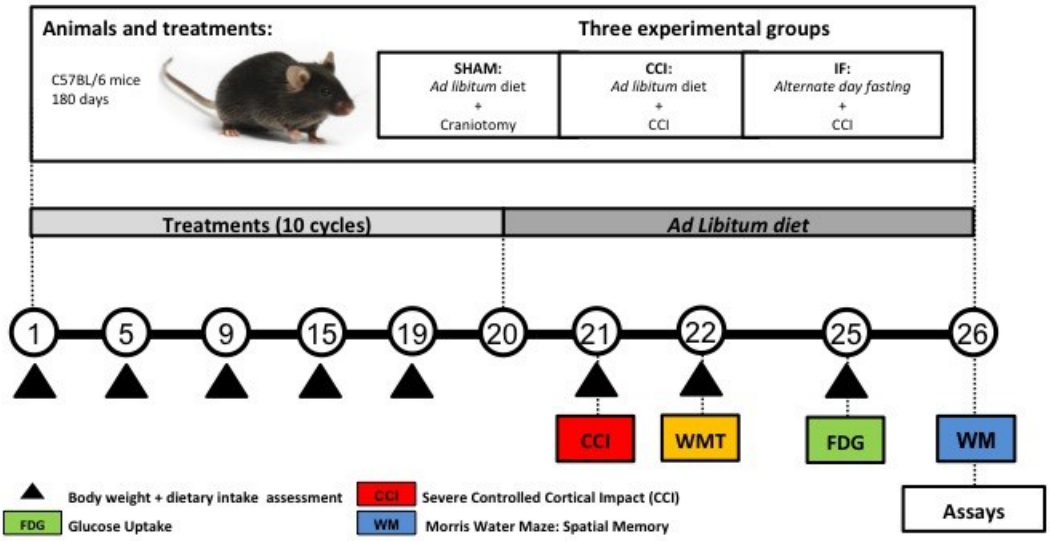

Supplemental Figure 01. Study design and protocols. CCI: controlled cortical impact; WMT: Morris Water Maze training; FDG: FDG-PET glucose uptake; WM: Morris Water Maze probe trial.

Supplementary Table 01. Standard uptake values (SUV) of FDG in the different regions of interest (ROI).

Tukey's multiple comparisons test	Mean Diff,	95,00% CI of diff,	P Value
Contralateral Striatum			
CCI vs. SHAM *	-1,313	-1,849 to -0,7772	<0,0001
IF vs. SHAM *	-0,6593	-1,195 to -0,1235	0,0111
IF vs. CCI *	0,6537	0,118 to 1,189	0,0119
Ipsilateral Striatum			
CCI vs. SHAM *	-1,172	-1,708 to -0,6363	<0,0001
IF vs. SHAM	-0,5041	-1,04 to 0,0316	0,0701
IF vs. CCI*	0,6679	0,1322 to 1,204	0,0099
Cortex			
CCI vs. SHAM *	-0,8987	-1,434 to -0,363	0,0003
IF vs. SHAM	-0,5056	-1,041 to 0,03019	0,0691
IF vs. CCI	0,3931	-0,1426 to 0,9289	0,1969
Contralateral Hippocampus			
CCI vs. SHAM *	-1,116	-1,652 to -0,5805	<0,0001
IF vs. SHAM *	-0,5983	-1,134 to -0,06256	0,0242
IF vs. CCI	0,5179	-0,01785 to 1,054	0,0607
Ipsilateral Hippocampus			
CCI vs. SHAM *	-1,136	-1,672 to -0,6006	<0,0001
IF vs. SHAM *	-0,5599	-1,096 to -0,02414	0,0381
IF vs. CCI *	0,5764	0,04067 to 1,112	0,0314
Thalamus			
CCI vs. SHAM *	-1,321	-1,856 to -0,7848	<0,0001
IF vs. SHAM *	-0,6677	-1,203 to -0,132	0,0099
IF vs. CCI *	0,6528	0,1171 to 1,189	0,0121
Cerebellum			
CCI vs. SHAM *	-0,8691	-1,405 to -0,3333	0,0005
IF vs. SHAM	-0,2994	-0,8352 to 0,2363	0,3882
IF vs. CCI *	0,5696	0,0339 to 1,105	0,0340
Basal forebrain and septum			
CCI vs. SHAM *	-0,8744	-1,41 to -0,3386	0,0004
IF vs. SHAM	-0,2658	-0,8015 to 0,27	0,4741

IF vs. CCI *	0,6086	0,07284 to 1,144	0,0213
Hypothalamus			
CCI vs. SHAM *	-0,9899	-1,526 to -0,4542	<0,0001
IF vs. SHAM	-0,3228	-0,8585 to 0,2129	0,3333
IF vs. CCI *	0,6672	0,1314 to 1,203	0,0100
Contralateral Amygdala			
CCI vs. SHAM *	-0,8649	-1,401 to -0,3292	0,0005
IF vs. SHAM	-0,2478	-0,7835 to 0,2879	0,5225
IF vs. CCI *	0,6171	0,08136 to 1,153	0,0192
Ipsilateral Amygdala			
CCI vs. SHAM *	-0,7091	-1,245 to -0,1733	0,0056
IF vs. SHAM	-0,1772	-0,7129 to 0,3586	0,7172
IF vs. CCI	0,5319	-0,003836 to 1,068	0,0522
Brain stem			
CCI vs. SHAM *	-1,062	-1,598 to -0,5262	<0,0001
IF vs. SHAM	-0,2876	-0,8234 to 0,2481	0,4175
IF vs. CCI *	0,7743	0,2386 to 1,31	0,0021
Superior colliculi			
CCI vs. SHAM *	-1,35	-1,886 to -0,8147	<0,0001
IF vs. SHAM *	-0,84	-1,376 to -0,3043	0,0007
IF vs. CCI	0,5104	-0,02535 to 1,046	0,0657
Olfactory bulb			
CCI vs. SHAM *	-0,9205	-1,456 to -0,3847	0,0002
IF vs. SHAM	-0,4667	-1,002 to 0,06902	0,1021
IF vs. CCI	0,4538	-0,08199 to 0,9895	0,1155
Contralateral Midbrain			
CCI vs. SHAM *	-1,462	-1,998 to -0,9268	<0,0001
IF vs. SHAM *	-0,5909	-1,127 to -0,05514	0,0265
IF vs. CCI *	0,8716	0,3359 to 1,407	0,0004
Ipsilateral Midbrain			
CCI vs. SHAM	-1,405	-1,94 to -0,8688	<0,0001
IF vs. SHAM	-0,5028	-1,039 to 0,03295	0,0711
IF vs. CCI	0,9018	0,366 to 1,437	0,0003

Whole Brain			
CCI vs. SHAM *	-1,128	-1,664 to -0,5923	<0,0001
IF vs. SHAM	-0,4992	-1,035 to 0,03651	0,0738
IF vs. CCI *	0,6288	0,0931 to 1,165	0,0165

* Denotes significant difference

4. CONCLUSÃO

Em resumo, conclui-se que o modelo utilizado de jejum intermitente exerceu um efeito profilático no TCE severo experimental. Observou-se modulação dos componentes da conectividade metabólica e da função mitocondrial preservando a bioenergética cerebral, prevenindo a combinação de déficits neuroenergéticos e neurodegeneração apoptótica. Notavelmente, o jejum intermitente evitou a disfunção mitocondrial induzida pelo TCE, sustentando a captação de glicose e conectividade metabólica, síntese de ATP mitocondrial, homeostase de Ca^{2+} e potencial de membrana, juntamente com produção atenuada de H_2O_2, culminando em função cognitiva preservada. Em conjunto, esses achados elucidam os mecanismos subjacentes modulados pelo jejum intermitente, que podem contribuir para prevenir a lesão progressiva associada ao TCE. Esses resultados expandem a literatura e fornecem evidências funcionais e moleculares que fortalecem os efeitos benéficos atribuídos do jejum intermitente à saúde geral do cérebro e seus benefícios profiláticos em injúrias cerebrais como o TCE.

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Figures divided into parts should be labelled with a lower-case bold a, b, and so on, in the same type size as used elsewhere in the figure. Lettering in figures should be in lower-case type, with only the first letter of each label capitalized. Units should have a single space between the number and the unit, and follow SI nomenclature (for example, ms rather than msec) or the nomenclature common to a particular field. Thousands should be separated by commas (1,000). Unusual units or abbreviations should be spelled out in full or defined in the legend. Scale bars

should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. In legends, please use visual cues rather than verbal explanations, such as "open red triangles".

Authors are encouraged to consider the needs of colorblind readers (a substantial minority of the male population) when choosing colors for figures. Many colorblind readers cannot interpret visuals that rely on discrimination of green and red, for example. Thus, we ask authors to recolor green-and-red heatmaps, graphs and schematics for which colors are chosen arbitrarily. Recoloring primary data, such as fluorescence or rainbow pseudo-colored images, to color-safe combinations such as green and magenta, turquoise and red, yellow and blue or other accessible color palettes is strongly encouraged.

Unnecessary figures should be avoided: data presented in small tables or histograms, for instance, can generally be stated briefly in the text instead. Figures should not contain more than one panel unless the parts are logically connected; each panel of a multipart figure should be sized so that the whole figure can be reduced by the same amount and reproduced on the printed page at the smallest size at which essential details are visible.

When a manuscript is accepted for publication, we will ask for high-resolution figure files, possibly in a different electronic format. This information will be included in the acceptance letter. See below for details of digital image production and submission.

Supplementary information

Supplementary information should be submitted with the manuscript and will be sent to referees during peer review. Supplementary information is not copy-edited by *Nature Metabolism*, so authors should ensure that it is clearly and succinctly presented, and that the style and terminology conform

with the rest of the paper. The following guidelines detail the creation, citation and submission of supplementary information. Please note that modification of supplementary information after the paper is published requires a formal correction, so authors are encouraged to check their supplementary information carefully before submitting the final version.

Nature Research recognizes the utility and value of Supplementary data. However, the proliferation of such data has in many cases made the reading of a manuscript confusing and unwieldy. In response to our authors, referees and readers, we have therefore decided to limit the volume of Supplementary Information, specifically Supplementary Figures in research papers. The number of Supplementary Figures should not exceed the number of main figures in the article, and each Supplementary Figure should fit one printable 8.5 x 11 inch page. While we would encourage authors to be selective in including as other types of supplemental data, such as Supplementary Tables and Videos, no restrictions will currently apply to these data types.

In general, only Supplementary data that supports the main conclusions of the paper should be included. Supplementary data should be presented in a logical order and related to a main text figure, wherever possible. Any supplementary data that extend beyond the conceptual scope of the main article may be removed at the editor's discretion.

Designate each item as Supplementary Table, Figure, Video, Audio, Notes, Data, Discussion or Equations. Number Supplementary Tables and Figures as, for example, "Supplementary Table 1". This numbering should be separate from that used in tables and figures appearing in the main printed article. Supplementary Notes should not be numbered and can have an optional title. Please provide a title for Supplementary Tables and a title and a caption for Supplementary Figures,

Supplementary Video and Supplementary Notes. The latter should only be used in consultation with the editors for specific elements best presented in Supplementary Information, such as standalone descriptions related to methods (for example algorithm description, compound synthesis and characterization). Please note that Supplementary Methods will no longer be allowed.

Refer to each piece of supplementary information at least once within the text of the main article, at the appropriate point(s). Be sure to include the word "Supplementary" each time one is mentioned. Please do not refer to individual panels of supplementary figures.

Where there is supplementary information to be included exclusively in the online version of a paper published in *Nature Metabolism*, please follow these guidelines, or publication may be delayed.

Figure files should be submitted as web-ready files through *Nature Metabolism* online submission system.
With the exception of spreadsheet, audio and video files, please submit the supplementary information as a single combined PDF, if possible. If necessary, we can accept any of these formats:

- .txt | Plain ASCII text
- .gif | GIF image
- .htm, .html | HTML document
- .doc, .docx | MS Word document
- .jpg | JPEG image
- .swf | Flash movie
- .xls, .xlsx | MS Excel spreadsheet
- .pdf | Adobe Acrobat file
- .mov | QuickTime movie
- .ppt, .pptx | MS Power Point slide
- .wav | Audio file

- Compressed Archive File (.zip),
- Encapsulated Postcript (.eps),
- MPEG animation (.mpg),
- PostScript (.ps),
- Rich Text Format (.rtf),
- Systems Biology Markup Language (.sbml, .xml, .owl),
- TAR archive file (.tar),
- TIFF image (.tif),
- WordPerfect document (.wpd).

File sizes should be as small as possible, with a maximum size of 30 MB, so that they can be downloaded quickly. The combined total size of all files must not exceed 150 MB. Video files should use a frame size no larger than 320 x 240 pixels.

All panels of a figure or table (for example, Fig. 1a, b and c) should be combined into one file; please do not send as separate files. Image files should be just large enough to view when the screen resolution is set to 640 x 480 pixels. Remember to include a brief title and legend (preferably incorporated into the image file to appear near the image) as part of every electronic figure submitted, and a title as part of every table.

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In addition, if your paper has been accepted, in principle, for publication, the revised manuscript must comply with the formatting requirements as specified by the editor and detailed above, with the length and figure limits appropriate to the content type, and with the following requirements.

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As *Nature Metabolism* is read by scientists from diverse backgrounds, many of whom are not native English speakers, it is essential that the findings are reported in an accessible language. Manuscripts are subject to substantial editing, in consultation with the authors, to achieve this goal. You are welcome to discuss proposed changes with the editors, but *Nature Metabolism* reserves the right to make the final decision about matters of style and the size of figures.

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Chemical structures

Chemical structures should be produced using ChemDraw or a similar program. All chemical compounds must be assigned a bold, Arabic numeral in the order in which the compounds are presented in the manuscript text. Figures containing chemical structures should be submitted in a size appropriate for direct incorporation into the printed journal. Authors using ChemDraw should make use of our journal template or use the preferences below, submitting the final files at 100% as .cdx files. Creating molecules within or copying them into the template will ensure that most of our journal style points are followed. For more information, please also review our Chemical Style Guide.

Drawing settings: chain angle, 120° bond spacing, 18% of width; fixed length, 14.4 pt; bold width, 2.0 pt; line width, 0.6 pt; margin width 1.6 pt; hash spacing 2.5 pt.

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Stereo diagrams should be presented for divergent 'wall-eyed' viewing, with the two panels separated by ~5.5 cm. In the final accepted version of the manuscript, the stereo images should be submitted at their final print size.

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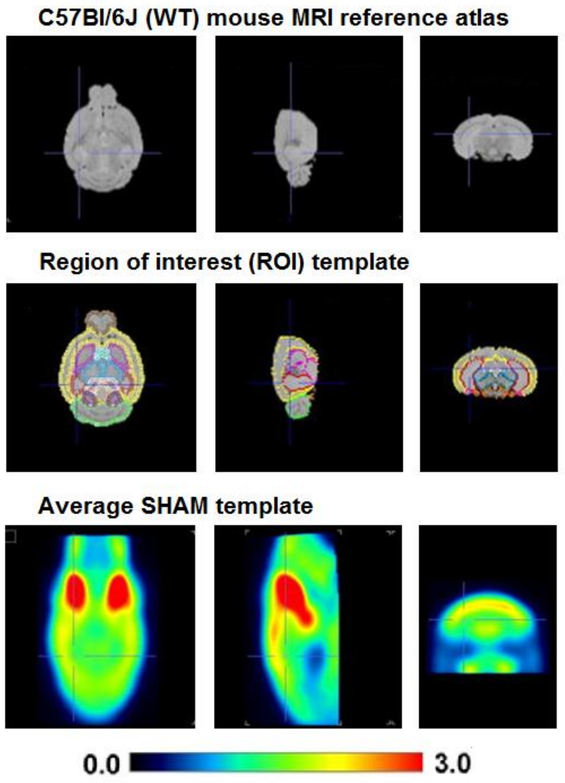
A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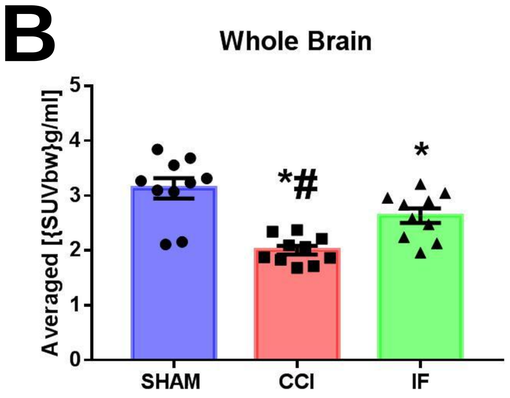
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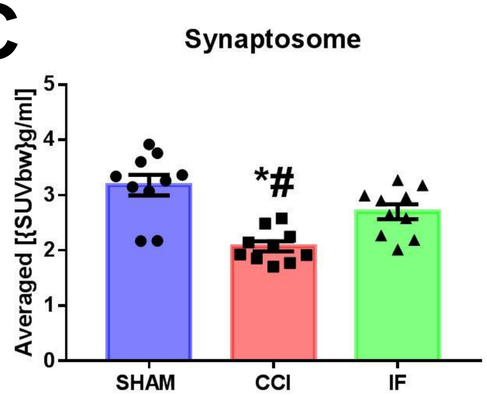
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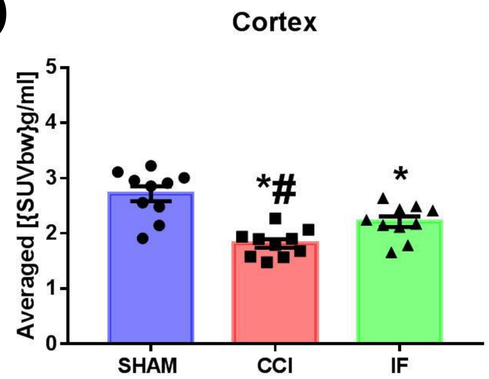
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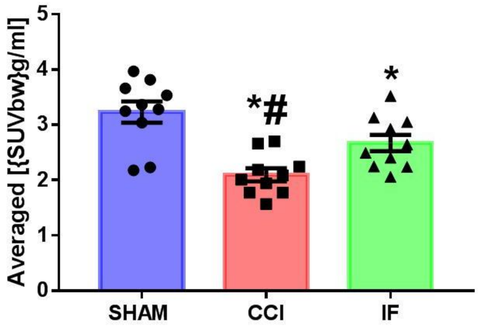
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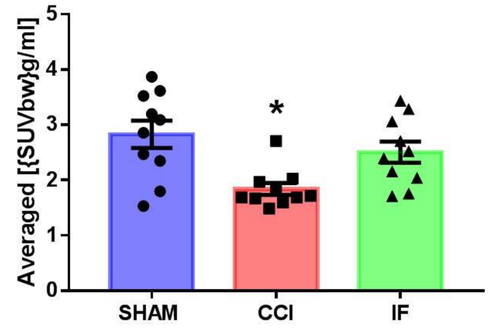
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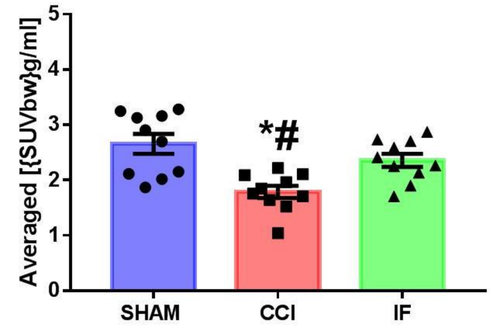
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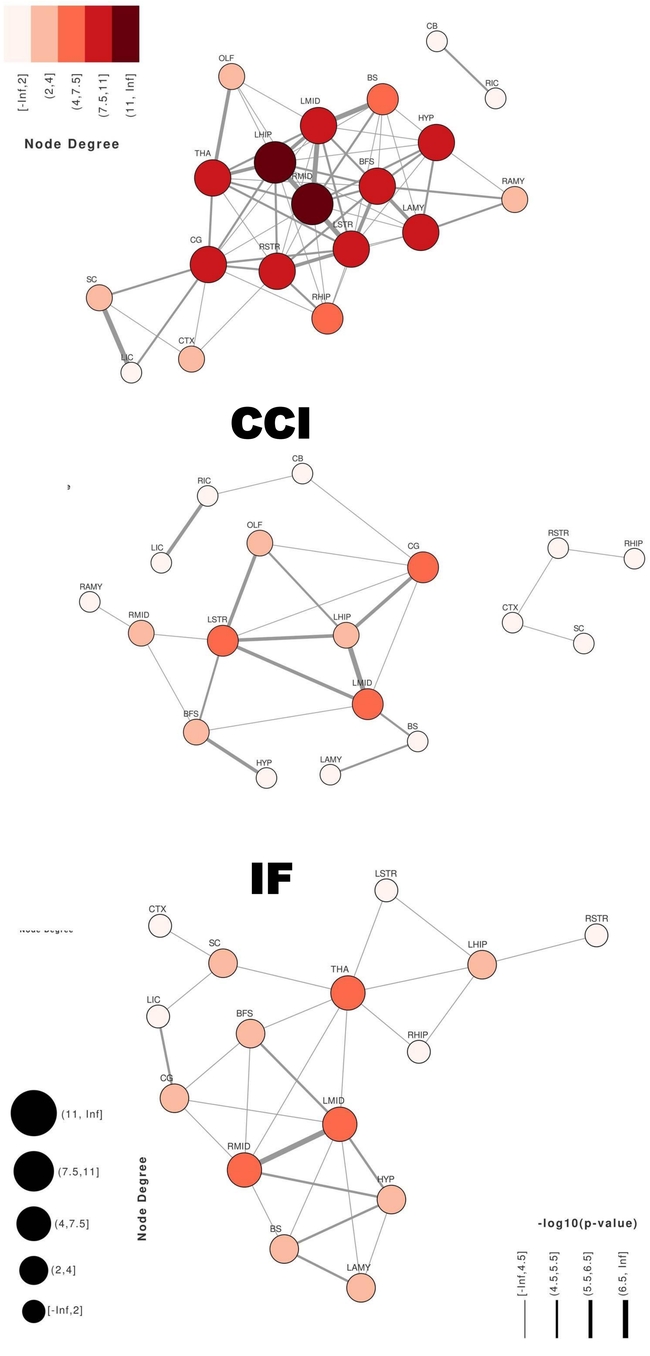
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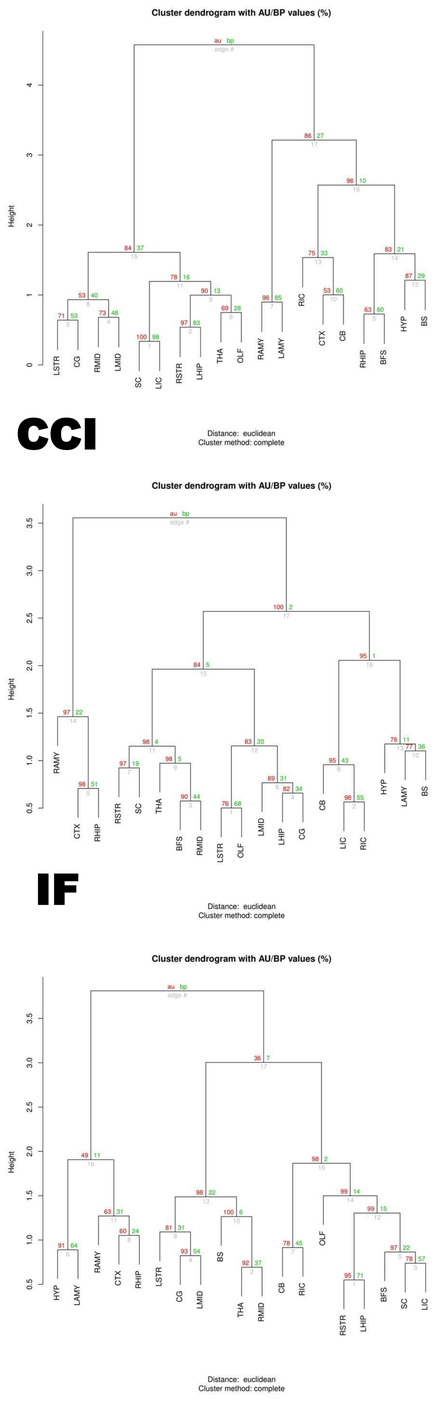
Node Degree
CCI
IF
-log10(p-value)

I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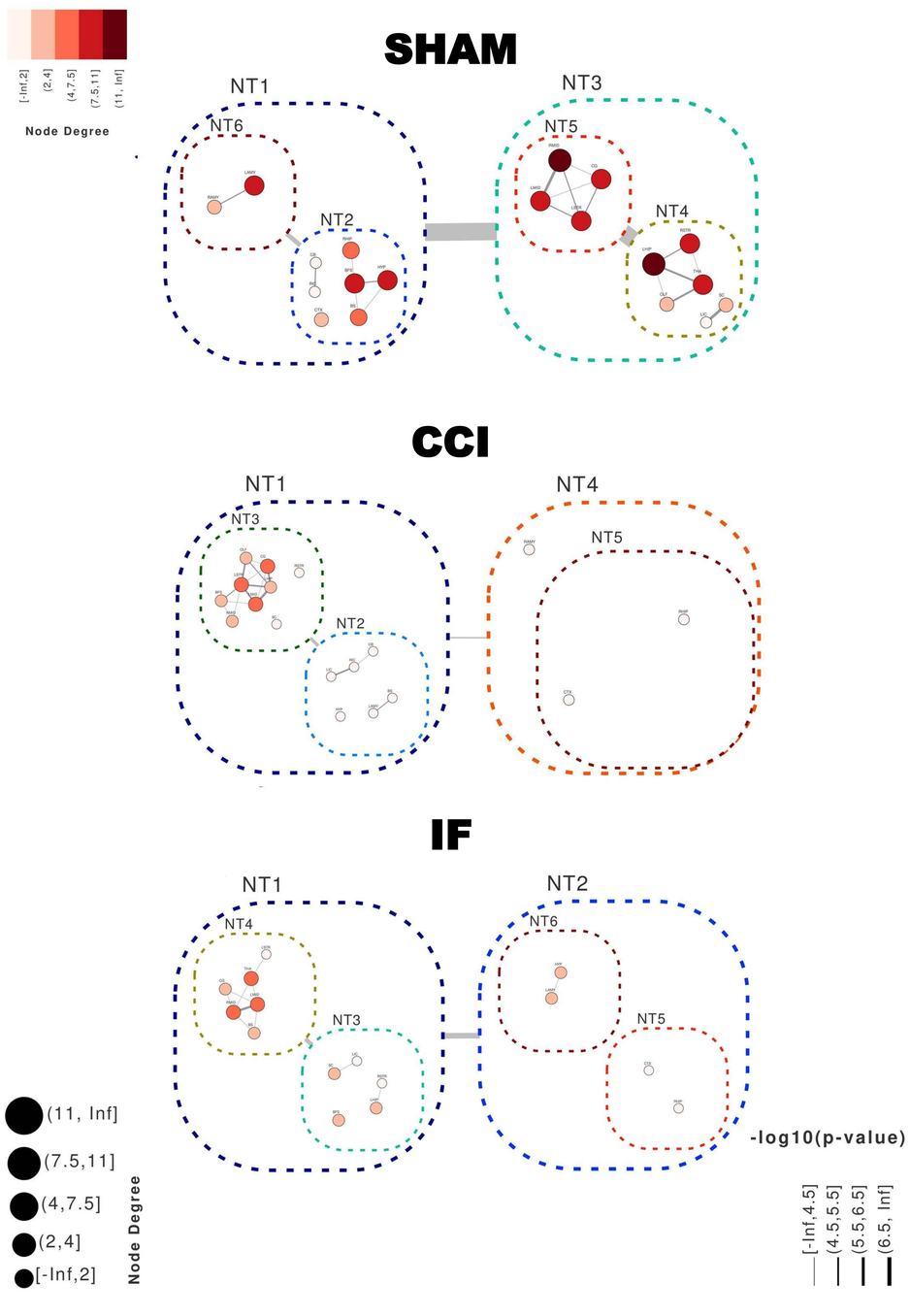
CCI
Distance: euclidean
Cluster method: complete
IF

J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NT2
NT3
NT4
NT5
NT6
CCI
IF
Node Degree
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