ALEXANDRE ABILIO DE SOUZA TEIXEIRA

Exercício físico como sincronizador do ritmo circadiano no câncer

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS

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Orientador: Prof. Dr. José Cesar Rosa Neto

A Comissão Julgadora dos trabalhos de Defesa da Tese de Doutorado, em sessão pública realizada a **........./......../..........**, considerou o candidato:

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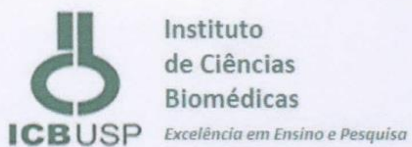

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CERTIFICADO

Certificamos que a proposta intitulada "efeito da doxorrubicina e do câncer no ritmo circadiano ", protocolada sob o CEUA nº 4541221117, sob a responsabilidade de **José Cesar Rosa Neto** *e equipe; Alexandre Abilio de Souza Teixeira* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais do Instituto de Ciências Biomédicas (Universidade de São Paulo) (CEUA-ICB/USP) na reunião de 06/02/2018.

We certify that the proposal "effect of doxorubicin and cancer on circadian rhythm", utilizing 144 Isogenics mice (144 males), protocol number CEUA 4541221117, under the responsibility of **José Cesar Rosa Neto** *and team; Alexandre Abilio de Souza Teixeira* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Biomedical Sciences Institute (University of São Paulo) (CEUA-ICB/USP) in the meeting of 02/06/2018.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: 36 meses   Depto/Setor: Biologia Celular E do Desenvolvimento

Origem: Biotério de Experimentação do Departamento de Biologia Celular e do Desenvolvimento
Espécie: Camundongos isogênicos   sexo: Machos   Idade ou peso: 8 a 10 semanas
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São Paulo, 06 de fevereiro de 2018

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Coordenadora da Comissão de Ética no Uso de Animais
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CERTIFICADO

Certificamos que a solicitação de licença de uso de animais intitulada "***Exercício físico como um sincronizador dos ritmos circadianos no câncer***", registrada sob **nº 95**, nas fls. 36, do livro 3, foi analisada e aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA-ICB/USP) em 25/08/2015.

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Havendo interesse na renovação da proposta, a solicitação deverá ser protocolada pela secretaria da CEUA-ICB/USP *até o último dia de validade da atual proposta*. Após essa data, uma nova proposta deverá ser encaminhada.

CERTIFICATE

We hereby certify that permission for the use of animals was granted to the research proposal "***Physical exercise as a synchronizer of circadian rhythms in cancer***", registered as **Number 95**, in pages 36, of book 3, by the local ETHICS COMMITTEE ON THE USE OF ANIMALS (CEUA-ICB/USP) in 8/25/2015.

Under this license, ***José Cesar Rosa Neto*** (Principal Investigator) and team members *Alexandre Abilio de Souza Teixera* are authorized to make use of animals within the limits of the research proposal presented to this committee and of the Brazilian Federal Law nº 11.794.

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São Paulo, 27 de agosto de 2015.

Prof. Dr. Anderson de Sá Nunes
Coordenador CEUA-ICB/USP

Eliane Aparecida G. M. Nascimento
Secretária CEUA-ICB/USP

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RESUMO

Alterações do ritmo circadiano estão relacionadas ao desenvolvimento e crescimento do câncer e os mecanismos moleculares subjacentes a este efeito ainda precisam ser mais estudados. No entanto, sabe-se que o exercício físico pode sincronizar o ritmo circadiano, atuando na modulação do sistema psiconeuroimunoendócrino. O desenvolvimento do tumor, por sua vez, promove alterações no equilíbrio desse sistema resultando em perturbação do ritmo circadiano. Um programa regular de exercício físico pode ser uma intervenção útil e de baixo custo como estratégia para melhorar a qualidade de vida de pacientes e sobreviventes do câncer. Assim, o objetivo do presente estudo foi avaliar se os mecanismos envolvidos na inibição do crescimento tumoral pelo treinamento físico podem estar associados com o papel cronomarcador do exercício físico. **Métodos:** Foram utilizados camundongos da linhagem C57BL/6J divididos em 4 grupos: controle (CTL); Tumor (LLC); Tumor + Treino no mesmo horário do dia (LLC+T(ZT2)); Tumor + Treino em horários alternados (LLC+T(ZTAlt)). Os animais foram submetidos ao treinamento aeróbio no terceiro dia após a inoculação do tumor. Foram realizadas 14 sessões de treino, compreendendo 5 sessões na primeira e 5 sessões na segunda semana e 4 sessões na terceira semana (60 minutos a 60% da velocidade máxima). Os animais foram eutanasiados com 21 dias após inocular o tumor, a cada 4 horas do dia, até completar 24 horas (ZT0, ZT4, ZT8, ZT12, ZT16 e ZT20). No músculo esquelético gastrocnêmio foram feitas as análises da expressão gênica dos *clock genes* (Clock, Bmal1, Per1, Per2, Per3 Cry1, Cry2, REV ERBα, Rorα e Rorβ), Ampk, Atrogina-1, Murf1 e GAPDH. A determinação das concentrações de TNF-α, IL-1β, MCP-1, IFN-γ, VEGF, IL-10 e IL-4 foram realizadas no músculo gastrocnêmio e no tumor. **Resultados:** O treino reduziu o peso total quando comparado com o grupo LCC. Apenas o grupo LLC+T(ZTAlt) teve redução no peso quando comparado com o grupo CTL. O peso do tumor foi menor no grupo LLC+T(ZTAlt) quando comparado com o grupo LLC. Os músculos EDL e sóleo apresentaram maior peso no grupo LLC+T(ZT2) quando comparado com o grupo LLC. O exercício em diferentes horários reduziu a concentração de glicose quando comparado com o grupo LLC (ZT8). O treinamento físico praticado em horários alternados foi capaz de aumentar a expressão no gene Per1(ZT4) quando comparado com os grupos sedentários. O grupo LLC+T(ZT2) teve aumento da expressão de Per2 quando comparado com o grupo CTL no ZT0 e ZT4, já o grupo LLC+T(ZTAlt) apresentou aumento do Per2 quando comparado com o grupo CTL e LLC apenas no ZT4. Na análise feita no gene Per3, o grupo LLC+T(ZTAlt) apresentou maior expressão no ZT4 quando comparado com os grupos CTL e LLC. A expressão gênica da AMPK foi aumentada no período claro e a Atrogina-1 reduzida, ambos no grupo LLC+T(ZT2). Atrogina-1 estava aumentada no grupo LLC+T(ZTAlt) no ZT20 quando comparado com o grupo CTL. Ainda no músculo gastrocnêmio, a concentração proteica do TNF-α foi maior no grupo LLC(ZT16) quando comparado com os grupos CTL e LLC+T(ZTAlt). Já o VEGF estava aumentado no grupo LLC quando comparado com o grupo LLC+T(ZTAlt). A IL-10 (ZT16) estava aumentada no grupo LCC quando comparado com os grupos CTL e LLC+T(ZT2 e ZTAlt). No mesmo ZT o grupo

LLC+T(ZTAlt) apresentou menor concentração da IL-10 quando comparado com o grupo CTL e LLC+T(ZT2). A IL-4 apresentou aumento da concentração no ZT16 no grupo LLC+T(ZT2) quando comparado com os grupos CTL e LLC+T(ZTAlt). No tumor, TNF-α mostrou efeito do exercício físico em horários alternados, com aumento no grupo LCC+T(ZTAlt) no ZT16 quando comparado com o grupo LLC+T(ZT2). A IL-10 e IL-4 também foram mais expressas no grupo LLC+T(ZTAlt)(ZT4). **Conclusão:** O exercício físico mostrou ser um grande modulador do relógio circadiano no músculo esquelético, promovendo efeitos protetores ao músculo, além de atenuar o crescimento tumoral.

Palavras chave: Ritmo Circadiano, Câncer, Clock Genes, Músculo Esquelético, Inflamação, Exercício Físico.

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ABSTRACT

Background: Changes in circadian rhythm are related to cancer development and growth, however the molecular mechanisms underlying this effect need to be further studied. It is known that physical exercise can synchronize the circadian rhythm, since it acts in the modulation of the psychoneuroimmunoendocrine system. The development of the tumor, in turn, promotes changes in the balance of this system resulting in a disturbance of the circadian rhythm. Thus a regular exercise program can be a useful and a low-cost intervention as a strategy to improve the quality of life of cancer patients and survivors. Therefore, our objective was to evaluate whether the mechanisms involved in the inhibition of tumor growth by physical training can be correlated with the chronological role of physical exercise. **Methods:** C57BL / 6J mice were used, divided into 4 groups: control (CTL); Tumor (LLC); Tumor + Training at the same time of day (LLC + TZT2); Tumor + Training at alternate times (LLC + TZTAlt). The animals were submitted to aerobic training on the third day after tumor inoculation. 14 training sessions were carried out comprising 5 sessions / week (40 to 60 minutes at 60% of maximum speed). The animals were euthanized 21 days after inoculating the tumor, every 4 hours of the day, until completing 24 hours (ZT0, ZT4, ZT8, ZT12, ZT16 and ZT20). In the gastrocnemius muscle, analyzes of the gene expression of the clock genes were performed (Clock, Bmal1, Per1, Per2, Per3, Cry1, Cry2, REV-ERBα, Rorα and Rorβ), Ampk, Atrogin-1, Murf1 and GAPDH. The determination of the concentration of TNF-α, IL-1β, MCP-1, IFN-γ, VEGF, IL-10 and IL-4 were performed in the gastrocnemius muscle and in the tumor. **Results:** The training reduced the total weight when compared with the LCC group, and only the LLC + T(ZTAlt) had a reduction in weight when compared with the CTL group. The tumor weight was lower in the LLC+T(ZTAlt) group when compared to the LLC group. The EDL and soleus muscles presented greater weight in the ZT2 group when compared to the LLC group. Exercise at different times also reduced glucose concentration when compared to the LLC group (ZT8). The physical training practiced at alternated times was able to increase the expression in the Per1 gene (ZT4) when compared with sedentary groups. The group that trained in ZT2 had an increase in Per2 expression when compared with the CTL group in ZT0 and ZT4, whereas the group with alternate training showed an increase in Per2 when compared with the CTL and LLC group only in ZT4. On the Per3 gene, the LLC+T (ZTAlt) group showed a higher expression in ZT4 when compared with the CTL and LLC groups. The gene expression of AMPK was increased in the light period and Atrogin-1 reduced, both in the LLC+T(ZT2) group. Atrogin-1 was increased in the LLC+T(ZTAlt) group in the ZT20 when compared to the CTL group. Also in the gastrocnemius muscle, the protein expression of TNF-α was higher in the LLC group (ZT16) when compared with the CTL and LLC+T(ZTAlt). VEGF was increased in the LLC group when compared to the LLC+T(ZTAlt). IL-10(ZT16) was increased in the LCC group when compared to the CTL and LLC+T groups (ZT2 and Alt). In the same ZT, the LLC+T(ZTAlt) group showed a lower expression of IL-10 when

compared to the CTL and LLC + T(ZT2) group. IL-4 showed an increase in ZT16 expression in the LLC +T(ZT2) group when compared with the CTL and LLC+T(ZTAlt) groups. In the tumor, TNF-α was affected by exercise, being increased in the group that trained at alternate times (ZT16) when compared with the LLC+T(ZT2). And IL-10 and IL-4 were also more expressed in alternate exercise (ZT4). **Conclusion:** Physical exercise proved to be a major modulator of the circadian clock in skeletal muscle, promoting protective effects to the muscle, in addition to attenuating tumor growth.

Keywords: Circadian Rhythm, Cancer, Clock Genes, Skeletal Muscle, Inflammation, Physical Exercise.

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LISTA DE ABREVIATURAS

COVID-19 - Doença do Coronavírus
Clock - Circadian Locomotor Output Cycles Kaput
Bmal1 - Brain and Muscle Arnt-Like Protein 1
Per - Period
Cry – Cryptochrome
CKI - Caseína Quinase 1ε
DNA – Ácido Desoxirribonucleico
RNA – Ácido Ribonucleico
IGFBP-3 - Proteína ligante de IGF-3
PTEN - Homólogo de Fosfatase e Tensina
mTOR - Mammalian Target of Rapamycin
HIF-1α – Fator Induzível por Hipóxia 1 alfa
NK - Natural Killer
IL-6 – Interleucina - 6
TNF-α Fator de necrose tumoral alfa
IL-1β–Interleucina - 1beta
IFN-ᵞ - interferon - gama
MCP-1 - Proteína Quimioatraente de Monócitos 1
VEGF - Fator de Crescimento Endotelial Vascular
IL-10–Interleucina -10
IL-4 – Interleucina - 4
LLC – Carcinoma de Pulmão de Lewis
ZT – Zeitgebers
ATP - Adenosina Trifosfato
TAG - Triacilglicerol
PPARδ - Receptores Ativados Por Proliferador De Peroxissoma delta
EDL - Extensor Longo Dos Dedos
ROREs - Retinoicacid-Related Orphan Receptor Response Elements
Glut4 – Transportador de Glicose 4
LPL - Lipotreina Lipase

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1 INTRODUÇÃO

A sociedade atual apresenta peculiaridades que são responsáveis pelas mudanças no relógio biológico que os seres humanos apresentavam até o fim do século XIX. Neste sentido, vale ressaltar que apesar do aumento da longevidade observado durante o século XX, período este em que praticamente dobrou a expectativa de vida dos seres humanos, a redução do número de pessoas residentes na zona rural, e o rápido aumento dos grandes centros urbanos promoveu mudanças no ritmo biológico da nossa espécie(1–3).

É fato que este problema vem se acentuando no século XXI, com o crescente avanço da tecnologia, ocorre a utilização constante de aparelhos eletrônicos, aumentando a exposição à luz proveniente destes, assim como, o aumento no nível de ruídos e barulhos, além da possibilidade de receber e enviar informações 24 horas por dia tem causado perturbações no ritmo circadiano (1–3). Atualmente, estamos passando por um momento complicado devido a pandemia do COVID-19, com isso muitas pessoas estão trabalhando de suas próprias casas, os horários para o início e o fim das tarefas foram alterados, o tempo na frente dos computadores aumentou, levando a uma jornada maior de trabalho diário, e como consequências, frequentes mudanças nos hábitos e horários alimentares, assim como o aumento da inatividade física, contribuindo para uma alteração ou perturbação no ritmo circadiano.

A crônica exposição à luminosidade dos grandes centros urbanos é capaz de reduzir a produção de melatonina. Na presença de luz, essa informação é transportada da retina pelas células ganglionares da retina intrinsecamente fotossensíveis através do trato retino-hipotalâmico até o núcleo supraquiasmático no hipotálamo, esse sinal de presença de luz é conduzido até a glândula pineal, por meio da qual a produção e secreção rítmica de melatonina são reduzidas, levando ao prejuízo da resposta circadiana, sendo esse um dos principais mecanismos de regulação do nosso ciclo sono-vigília(4).

Não obstante, observa-se que para a manutenção das atividades da nossa sociedade houve um aumento considerável no número de trabalhadores que tem que realizar seu trabalho no turno invertido, ou seja, no período noturno para nós humanos, causando privação de sono constante. Neste caso, esses trabalhadores apresentam grande aumento no número de dias afastados do trabalho, seja por morbidades ou acidentes de trabalhos. Dentre as morbidades, pode ser destacado o aumento no

número de doenças associadas à inflamação crônica de baixo grau, como doenças cardiovasculares, obesidade, diabetes e câncer(5).

A perturbação circadiana constante é sem dúvida nenhuma um fator de risco para o desenvolvimento do câncer(6). Novamente, os estudos envolvendo trabalhadores por turno mostram o alto risco desses sujeitos desenvolverem vários tipos de tumores(7–9). Tem sido mostrado na literatura que durante a progressão do câncer há uma perturbação circadiana, ocorrendo mudanças em muitas tarefas controladas pelo relógio central (núcleo supraquiasmático) ou periférico (tecidos e células) e orquestradas pelos *clock genes*(10).

Existem padrões de comportamentos que são capazes de reajustar a nossa ritmicidade biológica, como por exemplo hábitos alimentares, atividade física e rotinas de trabalho. Hoje discute-se os benefícios de se ter uma rotina rigorosa, com o mesmo horário para as refeições, por exemplo, já que a ingestão de nutrientes é um marcador do ritmo circadiano, e que em nossa sociedade moderna também vem sendo alterado, já que muitas pessoas não são capazes de manter rotina e um padrão alimentar(11).

O treinamento físico é um fator sincronizador do ritmo circadiano, apesar do mecanismo molecular não estar claro, já há o consenso de que as células musculares apresentam padrão de expressão rítmica de suas proteínas, assim da sua atividade(12). Assim, o treinamento físico aeróbio pode ser uma ferramenta não-farmacológica capaz de reduzir os danos causados pela perturbação do ritmo circadiano e de muitos processos biológicos, sendo considerado os possíveis mecanismos pelos quais o treinamento físico é capaz de reduzir o surgimento de diversos tipos de tumores, e também uma redução na capacidade proliferativa dos mesmos(13).

2 REVISÃO DE LITERATURA

2.1 Ritmo Circadiano

A cronobiologia é um campo da biologia que examina a geração de ritmos biológicos em diferentes espécies e em muitas partes do corpo, e sua adaptação aos fenômenos periódicos relacionados ao ciclo claro e escuro ou ao sol e lua(14). Esse ritmo diário previsível e robusto na disponibilidade de alimentos e fatores ambientais levou à evolução de um mecanismo de tempo interno de aproximadamente 24 horas,

denominado de ritmo circadiano, permitindo que os organismos antecipem as mudanças diárias e aperfeiçoem o condicionamento físico(15).

O ritmo circadiano é controlado por relógios circadianos que estão presentes em quase todos os tecidos dos mamíferos. O relógio principal ou central está localizado no núcleo supraquiasmático(SCN), no hipotálamo, uma pequena região do cérebro contendo 10.000-15.000 neurônios(16,17). Desta forma, o relógio central com a colaboração de outros relógios periféricos (tecidos e células) permite a manutenção do ritmo circadiano, e que pode ser alterada, por fatores externos como luz, atividades sociais, alimentação(16,18) e atividade contrátil (exercício físico)(19–21). O relógio circadiano é controlado através da interação entre um ciclo de retroalimentação (feedback loop) dos genes circadianos no núcleo de todas as células do corpo(17).

O funcionamento do relógio circadiano envolve mecanismos de retroalimentação negativa (*the negative feedback loop*). Os genes Clock (*Circadian Locomotor Output Cycles Kaput*) e Bmal1 (*Brain And Muscle Arnt-Like Protein 1*) formam um heterodímero, funcionando como fator de transcrição para a expressão dos genes Period(Per1, 2 e 3), Cryptochrome(Cry 1 e 2), Rev-Erb(α e β) e Ror(α, β e γ)(22). Per e Cry após a tradução, formam oligômeros que são transportados do citoplasma para o núcleo, onde bloqueiam a sua própria transcrição ao inibir a ação do Clock/Bmal1. Outro mecanismo regulatório é induzido pelo complexo heterodimérico Clock/Bmal1 que ativam a transcrição de receptores nucleares órfãos (Rev-Erb e Ror) (23–25). Rev-Erb (α e β) e Ror (α, β e γ) subsequentemente competem pelo ROREs (*Retinoic Acid-Related Orphan Receptor Response Elements*), presentes no promotor de Bmal1. Ror ativa a transcrição de Bmal1(25–27), enquanto Rev-Erb reprime o processo de transcrição(23,27). Assim, a oscilação circadiana de Bmal1 é tanto positivamente quanto negativamente regulada por Rors e Rev-Erbs(9). Em humanos, um ciclo circadiano tem início geralmente nas primeiras horas da manhã com a ativação da transcrição de Per e Cry por Clock/Bmal1. Os níveis de transcrição atingem seu ápice por volta de meio dia e os níveis de proteína citoplasmática atingem seu apogeu cerca de duas horas depois(17,22) (Figura 1).

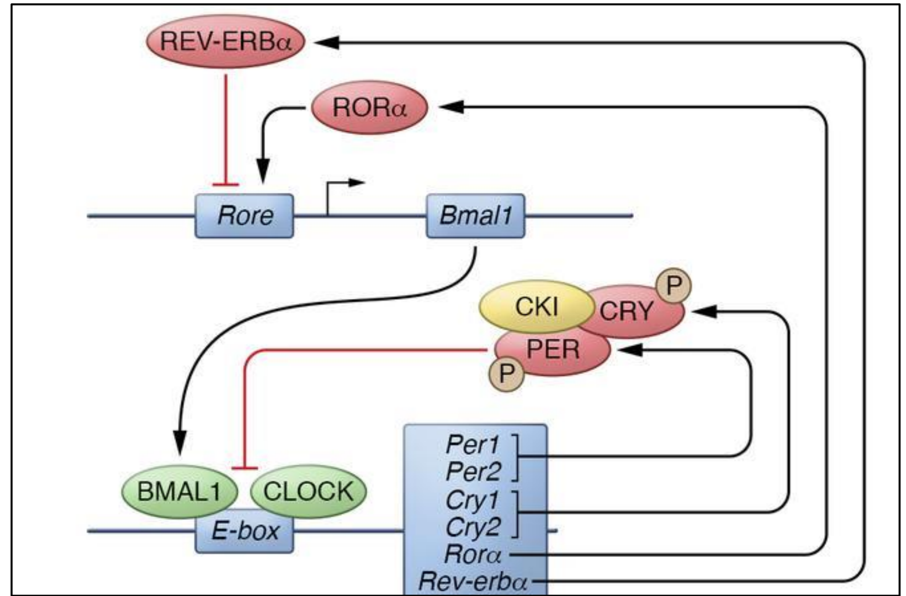


Figura 1. Os heterodímeros dos fatores de transcrição BMAL1 e CLOCK aumentam a expressão de muitos genes alvo. Destes, os produtos proteicos dos genes Período (Per) e Cryptochrome (Cry) fornecem um mecanismo de retroalimentação para inibir a atividade transcricional do Clock-Bmal1. A atividade dos dímeros Per-Cry é regulada no nível pós-transcricional por fosforilação pela caseína quinase 1ε (CKI). Outros alvos genéticos de Clock-Bmal1 incluem os receptores órfãos Ror e Rev-Erb que, respectivamente, promovem e inibem a transcrição de Bmal1. Além desses componentes principais do relógio genético, o Clock-Bmal1 regula a expressão de vários alvos a downstream que são chamados de genes controlados do relógio (CCGs). Adaptado de (22).

2.2 Ritmo Circadiano e Câncer

O relógio circadiano constitui uma maquinaria molecular evolutiva que dita a regulação temporal da fisiologia para manter a homeostase. Oscilações diárias comportamentais e fisiológicas são acionadas por um relógio circadiano endógeno, que por muitas vezes pode sofrer influencias externa (do meio ambiente). Nas sociedades modernas, as mudanças no estilo de vida, levam à interrupção frequente da homeostase circadiana endógena(28). Essa interrupção do ritmo circadiano desempenha um papel fundamental no desenvolvimento e crescimento tumoral. Por outro lado, os processos oncogênicos enfraquecem diretamente os ritmo circadiano (6)

Em humanos, a interrupção circadiana encontrada em trabalhadores por turnos os coloca em risco aumentado de câncer de mama(8) e próstata(9). Além disso, camundongos com uma ablação do relógio central localizado dentro do núcleo supraquiasmático (SCN) exibem aumento do crescimento de xenoenxertos tumorais em comparação com camundongos com ritmo circadiano intacto(29). No geral, existe

uma ligação entre o câncer e a perturbação do ritmo circadiano, embora sua extensão e mecanismos moleculares não sejam totalmente elucidados(30)

Sabe-se que as causas para a formação da massa tumoral podem ser por determinante genético, no entanto, o estilo de vida parece ser o principal contribuinte para isso, sendo que fatores de risco comportamentais e nutricionais são responsáveis por cerca de um terço das mortes por câncer(31). Além disso, diversos trabalhos têm mostrado forte ligação entre o desenvolvimento do câncer e o ritmo circadiano(32–34). Um grande conjunto de evidências conectou os genes que controlam o ritmo circadiano a vários outros fatores reguladores importantes para a transformação celular. Dado o papel pleiotrópico do relógio circadiano na fisiologia, não surpreende que vários *hallmarks* do câncer estejam sob controle do relógio circadiano (Figura 2)(6).

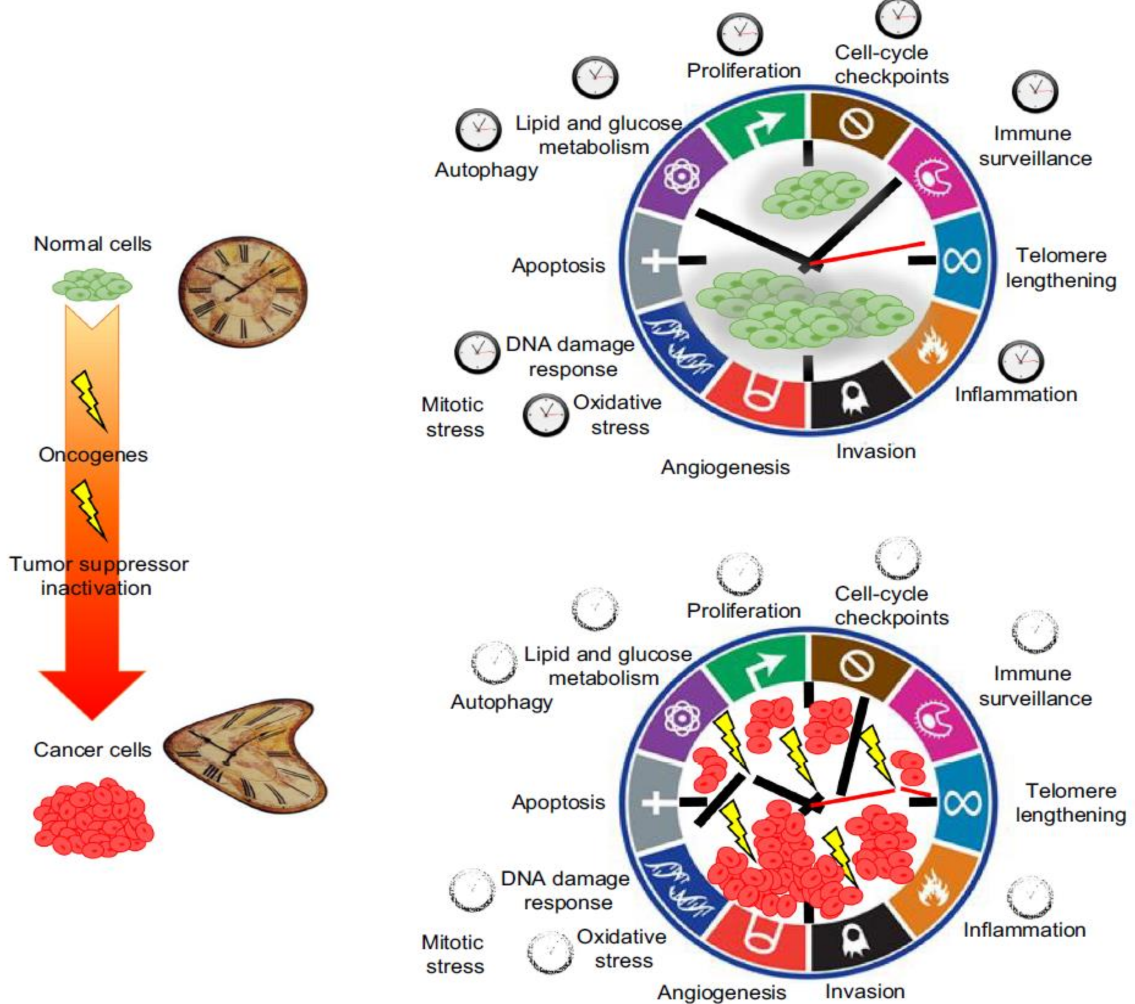


Figura 2. Durante o início do câncer, o ritmo circadiano é interrompido pelos oncogenes; isso sugere que o afrouxamento da função do relógio circadiano pode facilitar a instauração dos *hallmarks* do câncer e desempenhar um papel fundamental na tumorigênese(6).

Portanto, muitas proteínas do relógio circadiano também interagem fisicamente com proteínas que participam de vias relevantes para o desenvolvimento do câncer. Assim, a modulação da função ou da concentração das proteínas que controlam o relógio circadiano pode proteger ou promover o câncer. Primeiramente, as proteínas do relógio circadiano e suas proteínas interativas reconhecem o ambiente celular. Por exemplo, alteração no estado redox da célula pode afetar a afinidade do Clock/Bmal1 para o DNA(35). Consequentemente, alterações no estado redox, co-fatores e modificações pós-traducionais causadas por programas oncogênicos podem alterar a estabilidade, localização ou função das proteínas do relógio(6,36).

Além disso, há outros mecanismos que podem estar relacionados com os componentes do relógio circadiano envolvidos na iniciação ou progressão do câncer. Por exemplo, os componentes do relógio circadiano regulam direta ou indiretamente a expressão de centenas ou milhares de genes em diferentes tipos de células, o que leva a ritmos diários em muitos processos celulares, incluindo metabolismo de nutrientes(36) regulação redox(37), autofagia(38,39), reparo de danos ao DNA(33) e secreção celular(40,41). O ritmo diário desses processos celulares é parte integrante de sua homeostase. A interrupção do ritmo circadiano interrompe esses processos celulares e cria um ambiente celular propício à tumorigênese (isto é, reprogramação metabólica, desequilíbrio redox, inflamação crônica, etc.)(6).

O relógio circadiano também pode regular a expressão de diversos fatores secretados, que exercem funções parácrina ou endócrina. Esses fatores secretados incluem citocinas, hormônios e neurotransmissores, que por sua vez, podem sinalizar através de seus receptores cognatos e vias de sinalização *downstream* para afetar a função do relógio, arrastando ou sincronizando os relógios em diferentes tecidos. Esses fatores endócrinos podem ser biomarcadores da função circadiana em diferentes tecidos. No entanto, alguns tumores podem produzir quantidades excessivas desses hormônios ou citocinas relevantes para o relógio circadiano, o que também pode atrapalhar os relógios circadianos em órgãos distantes(6)

Estudos com seres humanos apontam alterações no ritmo circadiano e aumento da susceptibilidade para o desenvolvimento de câncer e mau prognóstico. Por exemplo, a expressão de todos os três genes Per estão desregulados em células de câncer de mama(42). Expressão Per1 é regulada negativamente na maioria dos pacientes, possivelmente devido à metilação do seu promotor. É importante ressaltar que diversos estudos utilizando modelos animais mostraram relações entre alguns

genes controladores do relógio e tumorigênese(42–46). Especificamente, Per1 e Per2 parecem funcionar como supressores do tumor em camundongos(43). Ausência de Per2 leva ao desenvolvimento de linfomas malignos(44), enquanto que a sua expressão em linhagem de células cancerígenas resulta na inibição do crescimento celular, parada do ciclo celular, apoptose e perda da capacidade clonogênica(44). É interessante notar que os níveis de mRNA de Per2 são regulados negativamente em várias linhagens celulares de linfoma humano e em células tumorais de pacientes com leucemia mieloide aguda(44). A superexpressão de Per1 também pode suprimir o crescimento de linhagem celular de câncer humano(43). Não obstante, os níveis de mRNA de Per1 são regulados negativamente em câncer de pulmão em comparação com os tecidos normais correspondentes(43). Estes resultados apontam diretamente para uma ligação entre desordem do ritmo circadiano e câncer(45,46).

As alterações na expressão gênica circadiana são características compartilhadas de diferentes tipos de tumores. Tais alterações na expressão do gene do relógio nos tumores podem ter relevância funcional. Alguns estudos encontraram alterações na expressão de Rev-Erb e Cry em amostras de tumor que influenciam a sobrevivência de pacientes com glioblastoma, câncer colorretal e gástrico(47–50). Por exemplo, um estudo mostra que a maior expressão de Cry1 correlacionou-se positivamente com o fenótipo agressivo do câncer colorretal, prevendo resultados ruins para os pacientes, além disso, esse estudo mostra que a superexpressão de Cry1 em linhagem de células de câncer colorretal promoveu a proliferação e migração celular(50). Já a expressão de Rev-Erbα estava diminuída em tecidos de câncer gástrico humano e o tempo de sobrevivência dos pacientes foram significativamente associado à expressão do Rev-Erbα, sugerindo que o Rev-Erbα pode ser um fator de prognóstico no câncer gástrico(49).

A interrupção da expressão gênica circadiana pode ser causada não apenas por eventos genéticos. Por exemplo, a acidificação do microambiente do tumor leva a alterações do relógio circadiano(51), e a autofagia, que é altamente regulada em vários tipos de tumor, pode desencadear a degradação do Cry1(38). Em resumo, as análises genéticas e genômicas dos tumores indicaram que as mudanças na expressão dos genes do relógio são mais difundidas do que as mutações nos genes do relógio. Isso levou à hipótese de que programas oncogênicos podem interromper a regulação circadiana, o que, por sua vez, alimenta ainda mais o crescimento do tumor(6).

Os processos oncogênicos podem suprimir o equilíbrio homeostático imposto pelo relógio circadiano para facilitar a instauração dos *hallmarks* do câncer. Em contrapartida, outras linhas de evidência mostram que a disfunção do ritmo circadiano afeta a tumorigênese e que os genes do relógio regulam várias características do câncer. A manipulação farmacológica dos componentes do relógio circadiano pode revelar novas oportunidades para o tratamento do câncer, sendo essa estratégia denominada de cronoterapia do câncer, sendo o tratamento em horários específicos, acompanhando o ritmo circadiano, e buscando otimizar os efeitos antitumorais e diminuir a toxicidade do tratamento(47). Entretanto uma tentativa de manipulação não farmacológica dos componentes do relógio circadiano também pode ser uma estratégia para prevenir ou mesmo atenuar o desenvolvimento do câncer.

2.3 Exercício Físico e Ritmo Circadiano

Os humanos são seres que teoricamente deveriam ser ativos durante o dia e descansar durante a noite(28). Estas oscilações diárias comportamentais e fisiológicas são acionadas por um relógio circadiano endógeno. Este relógio circadiano endógeno muitas vezes pode sofrer influencias externas(52), levando a uma interrupção frequente da homeostase circadiana, proporcionando aumento no risco de várias doenças, incluindo câncer(28). Alguns ciclos ambientais ajustam o oscilador que gera o ritmo circadiano, levando-o a oscilar com o mesmo período do ciclo. Esse processo é conhecido como arrastamento e o ciclo ambiental que arrasta o relógio é chamado de Zeitgeber (Zeitgebers – do alemão: Zeit=tempo, Geber=doador). Esses fatores externos servem ainda como indicadores de tempo, e podemos considerar vários Zeitgebers, sendo a luz o mais importante, que reinicia diariamente o relógio central. Na ausência dos Zeitgebers, os ritmos circadianos estão em curso livre e têm um período com duração diferente de 24 horas(53).

O exercício físico é um robusto Zeitgeber nos músculos esqueléticos, podendo redefinir o relógio circadiano molecular(54). O relógio circadiano tem sido bem documentado no músculo esquelético. McCarthy et al. 2007 foram os primeiros a verificar perfis da expressão rítmica de genes no músculo esquelético, uma abordagem útil para medir genes circadianos, e concluíram que mais de 200 genes exibiram um padrão de expressão rítmica(55). Estes genes foram observados para

abranger uma ampla variedade de processos fisiológicos, incluindo a transcrição, a sinalização celular e o metabolismo de proteínas(55).

Estudos também sugerem que o exercício físico pode alterar o ritmo circadiano, comportamentos e expressão dos clock genes(20) e, além disso, o exercício físico afeta todos os tecidos e sistemas do corpo humano, interagindo em um sistema de modulação psiconeuroimunoendócrino. As alterações no equilíbrio desse sistema, observadas em doenças crônicas, tais como o câncer, promovem perturbação do ritmo circadiano(56).

2.4 Exercício Físico e Câncer

Antigamente, era recomendado aos pacientes após o diagnóstico de câncer o repouso e que atividades extenuantes fossem evitadas, porém nas duas últimas décadas esse dogma foi mudando drasticamente, à medida que estudos de intervenção com exercícios e atividades físicas ganharam ampla aceitação, popularidade, e principalmente efeitos benéficos a saúde(57).

Embora as recomendações sobre atividade física para pacientes com câncer tenham começado a mudar nos últimos anos, a noção de que o exercício voluntário poderia inibir o crescimento de tumores em modelos experimentais de roedores foi demonstrado na década de 1940(57,58). Após esse estudo, o foco na relevância do comportamento do exercício físico estava exclusivamente ligado à prevenção do câncer, e os estudos que examinavam a aplicação do exercício físico em populações com diagnóstico de câncer começou a aparecer no final da década de 80, no qual pacientes com câncer de mama foram submetidos ao treinamento físico como estratégia de ajudar no tratamento quimioterápico(59,60).

Outro fator importante demonstrado nesses últimos anos é que o nível de atividade física está relacionado com a redução do risco de pelo menos 13 tipos diferentes de câncer(61), além de fornecer evidências de que o exercício físico está diretamente relacionado com a redução da recidiva para o câncer de próstata, cólon e mama(62–64).

Para auxiliar no tratamento do câncer, frequentemente são prescritos para pacientes medicamentos para ajudar a minimizar os efeitos secundários da quimioterapia e tratamento relacionados ao próprio câncer, mas a terapia com drogas é vista como solução de curto prazo que resolve apenas temporariamente efeitos

secundários debilitantes(65). Alguns dos sintomas físicos associados ao tratamento são a fadiga, a anemia, a perda de massa muscular e a diminuição do equilíbrio e coordenação(66). Estes sintomas físicos podem trazer diminuição da energia e desempenho físico, reduzindo assim, o estado de humor geral e qualidade de vida(66,67). O aumento dos níveis de atividade física pode melhorar a capacidade física e aspectos psicossociais em pacientes sobreviventes de câncer(68,69). Alguns estudos sugerem que exercício físico de baixa à moderada intensidade é uma opção viável e segura para mediar os efeitos do tratamento médico(65,66,70). Treinamento aeróbio tem a capacidade de atenuar a perda no desempenho físico tipicamente associado com o tratamento do câncer(71,72). Para tanto, um programa regular de exercícios pode ser uma intervenção útil e de baixo custo como estratégia para melhorar a qualidade de vida de pacientes e sobreviventes do câncer(56).

O exercício físico tem um papel importante no processo de reabilitação em pacientes com câncer(73). Uma revisão de dados quantitativos de 82 ensaios controlados com intervenções de atividade física para sobreviventes de câncer mostrou que o exercício físico foi bem tolerado durante e após o tratamento (sem eventos adversos)(74). Esses fatos reforçam que o treinamento físico é seguro durante e após o tratamento do câncer e pode resultar em um aumento da capacidade funcional, qualidade de vida e redução da fadiga relacionada ao câncer em vários grupos de sobreviventes do câncer(75,76). Não obstante, Lira et al. (2008) mostraram que o treinamento aeróbio promove efeito protetor, reduzindo em até 10 vezes o tamanho do tumor(77).

A atividade física regular de intensidade moderada (por exemplo, caminhada rápida) diminui o risco de muitos cânceres, bem como de mortalidade por câncer. Em estudos com animais, o exercício regular inibe o início e a progressão do tumor em ampla gama de modelos de tumor e localizações anatômicas. Uma abundância de evidências apoia uma associação entre atividade física e diminuição do risco de câncer, assim como a mortalidade por câncer(13,78,79). Claramente esta é uma questão importante dada à crescente incidência mundial de câncer e sedentarismo(13). É, portanto, importante elucidar os mecanismos biológicos que explicam os potenciais efeitos antitumorigênicos do exercício físico que podem levar à prevenção do câncer ou pelo menos a uma taxa atenuada de crescimento tumoral.

Sabe-se que o tumor pode derivar de diferentes tipos de células primárias, mas para que ele seja classificado como tal, alguns marcadores *(hallmarks of cancer)*

devem apresentar características específicas, dentre elas: alta e contínua atividade proliferativa, ativação do processo de invasão e/ou metástases, resistência à morte celular, evasão de processos que regulam o crescimento, angiogênese, alteração do metabolismo celular, inflamação, instabilidade genômica e escape do sistema imunitário(34).

Acredita-se que o exercício pode agir em alguns marcadores do câncer, por exemplo, o exercício físico pode ajudar a prevenir o câncer em modelos animais, induzindo redução na concentração do IGF-1(80,81), sendo esse um fator que desencadeia a proliferação celular(82,83). Além disso, o exercício diminuiu os níveis séricos de IGF-1 em homens saudáveis com 60 anos de idade(84). O exercício pode ser um supressor do crescimento tumoral, por aumentar a ativação da p53, resultando em expressão aumentada da p21, IGFBP-3 e PTEN, que por sua vez pode induzir regulação negativa da via de IGF-1 e, assim, contribuir para a prevenção do câncer de pele(85,86). O exercício também pode reduzir a proliferação de células tumorais por meio da estimulação da fosforilação de AMPK e seu substrato raptor, o que reduz a atividade da mTOR(87).

Resistência a morte celular é um fator comum na iniciação e progressão tumoral, o exercício físico mostrou ter um efeito na morte celular e supressão do crescimento do tumor de pâncreas e próstata em um modelo de xenoenxerto de camundongo(88).

O exercício também está relacionado em melhora da perfusão/vascularização intratumoral em câncer de mama(89,90) e de próstata(91,92), promovendo ativação do HIF-1α pelo exercício e subsequentemente uma regulação positiva de VEGF, associando assim a uma vascularização produtiva do tumor com mudança em direção a redução do processo de metástase em um modelo ortotópico de câncer de próstata(91), esses achados levaram a hipóteses de que o exercício resulta em perfusão tumoral aumentada e assim pode melhorar a administração de drogas e células imunológicas para dentro do tumor(92).

As células inflamatórias dentro do microambiente tumoral têm efeito de promoção ao desenvolvimento do tumor(34), o exercício físico já é bem conhecido pelos seus efeitos antiinflamatórios(93–95). Além do mais, o músculo esquelético é bem conhecido por ser um órgão endócrino capaz de liberar moléculas (principalmente proteínas e pequenos peptídeos, mas também ácidos nucléicos, lipídios e metabólitos) na corrente sanguínea (seja em livre circulação ou dentro de

exossomos e/ou microvesículas) conhecidas como miocinas, atuando sistemicamente e com efeitos antiinflamatórios(96). Algumas miocinas também podem induzir apoptose em tumores específicos, através das miocinas oncostatina M no câncer de mama(97) e osteonectina (proteína secretada ácida e rica em cisteína) no câncer de cólon(98).

Ademais, outra miocina bem descrita e com efeitos imunomodulatórios é a IL-6(99), que por sua vez também apresenta efeitos antitumorigênicos(78). Isso ocorre através da mobilização induzida pelo exercício de células NK e a subsequente infiltração dessas células em tumores mediadas pelo efeito acoplado de liberação na corrente sanguínea da IL-6 dos músculos esqueléticos e a atividade da adrenalina, liberado das glândulas supra-renais(78). Entretanto, esse efeito da IL-6 parece ser exclusivo do exercício físico, pois uma vez que a injeção da IL-6 não conseguiu simular a infiltração de células NK ou a redução do crescimento tumoral e a IL-6 derivada do tumor promove efeitos a favor do crescimento tumoral(100).

Embora os mecanismos pelo qual o exercício físico possa controlar os marcadores do câncer ainda não estão bem elucidados, nota-se que o estilo de vida saudável e a prática regular de exercício físico têm potenciais efeitos antitumorigênicos.

2.5 Exercício Físico, Ritmo Circadiano e Câncer

Como descrito, o exercício físico praticado regularmente é um robusto Zeitgeber do relógio circadiano nos músculos esqueléticos(54) e pode ser uma estratégia não farmacológica para prevenir ou atenuar o desenvolvimento do câncer (94), porém pouco se sabe sobre a relação dos mecanismos envolvidos na prática do exercício físico sobre o câncer e o relógio circadiano.

Alterações no ritmo circadiano estão relacionadas com diversas morbidades, entre elas, alterações no padrão de sono, causando piora na qualidade do sono, distúrbios psiquiátricos, como depressão e ansiedade, aterosclerose, hipertensão, câncer, entre outros(42,101–104).

Estudos em humanos e roedores demonstraram que variáveis como força do músculo esquelético e a capacidade oxidativa apresentam diferenças significativas ao longo do dia(105–108). Por exemplo, estudos têm demonstrado consistentemente um aumento da força no final da tarde em relação à manhã (105), enquanto a capacidade

oxidativa atinge o pico no final da noite(108). Além disso, as concentrações basais de hormônios e metabólitos sistêmicos oscilam ao longo de um período de 24 horas, embora o impacto dessas oscilações no exercício não seja claro(106–109), portanto, o horário da prática do exercício físico pode ser um fator fundamental em uma terapia anticâncer.

Além disso, o exercício físico realizado de maneira voluntária pode afetar a rapidez com que um animal sincroniza a fase de um novo ciclo claro/escuro(110,111), promovendo assim mudanças no relógio circadiano no músculo esquelético(20), e o treinamento aeróbio foi capaz de melhorar o metabolismo e o desempenho através dos mecanismos do relógio circadiano que tinham sido afetados em camundongos mutantes para Clock(112).

Também nota-se a estreita relação dos genes que controlam o ritmo circadiano a vários outros fatores reguladores importantes para a transformação celular, conectando evidências de que vários *hallmarks* do câncer estejam sob controle do relógio circadiano(6), e que o exercício físico pode interferir nesses *hallmarks* atenuando o crescimento tumoral(13).

Posto isso, o nosso objetivo foi avaliar se os mecanismos envolvidos na inibição do crescimento tumoral pelo treinamento físico podem estar correlacionados com o papel cronomarcador do exercício físico.

3 JUSTIFICATIVA

O câncer está entre as principais causas de morbidade e mortalidade no mundo. A alteração do ritmo circadiano também tem sido correlacionada com aumento da susceptibilidade para o câncer em seres humanos. As células cancerígenas apresentam diversas anormalidades metabólicas. Há evidencias mostrando relação íntima entre o ritmo circadiano e o metabolismo. Embora o ritmo circadiano regule várias vias metabólicas, disponibilidade de metabólitos e comportamento alimentar, por sua vez, essas vias podem também regular o ritmo circadiano. O exercício físico tem um papel importante no desenvolvimento do processo de reabilitação em pacientes com câncer. Atividade física para sobreviventes de câncer mostrou ser bem tolerado durante e após o tratamento. Isso apoia a conclusão que o treinamento físico é seguro durante e após o tratamento do câncer e pode resultar em um aumento da capacidade funcional, qualidade de vida e redução da fadiga relacionada ao câncer

em vários grupos de sobreviventes do câncer. Foi observado ainda que o tumor causa um profundo desequilíbrio na homeostasia de tecidos metabólicos, como músculo esquelético, tecido adiposo e fígado, gerando um agravamento do quadro com a instalação da caquexia. A perda da ritmicidade nos processos metabólicos e inflamatórios pode contribuir no prejuízo encontrado na homeostasia do músculo esquelético. Assim, determinar se os marcadores gênicos do ritmo circadiano no músculo esquelético podem ser modulados pelo horário do treinamento físico e se o treinamento pode ser um sincronizador do ritmo circadiano no câncer, é de extrema importância para nortear possíveis tratamentos que envolvam exercício físico em pacientes oncológicos

4 OBJETIVOS

4.1 Objetivo geral

Avaliar se os mecanismos envolvidos na inibição do crescimento tumoral pelo treinamento físico podem estar associados com o papel cronomarcador do exercício físico..

4.2 Objetivos específicos

•Avaliar os efeitos do exercício físico praticado no mesmo horário do dia e em diferentes horários do dia no músculo esquelético;

•Verificar alterações nos *clock genes* causados pelo câncer, e possíveis caos metabólico provocado pelo câncer no músculo esquelético;

•Examinar o perfil inflamatório e analisar a ritmicidade desses fatores (TNF-α, IL-1β, IFN-ᵞ, MCP-1, VEGF, IL-10 e IL-4) no músculo esquelético e no tumor;

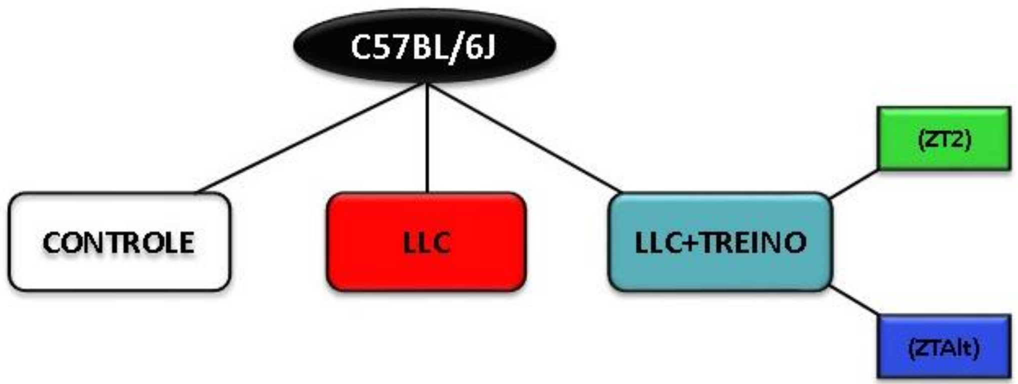
•Investigar quais são as possíveis vias inflamatórias que podem estar associadas ao aumento ou diminuição da massa tumoral nos animais treinados, como possível mecanismo de intervenção futura.

5 MÉTODOS

Foram utilizados camundongos da linhagem C57BL/6J, mantidos em sala com ciclo claro-escuro de 12h/12h e temperatura de 23±2 ºC, sob dieta normal (ração Nuvital da Nuvilab, Colombo, PR) e água *ad libitum* até o início do tratamento (8-10 semanas de idade). Todos os procedimentos desse estudo estão seguindo os princípios éticos de experimentação animal e foram submetidos e aprovados em 25/08/2015 pelo Comitê de Ética em Experimentação Animal da Universidade de São Paulo, registrada sob n° 95, nas fls. 36, do livro 3.

5.1 Desenho experimental

Foram utilizados 4 grupos: controle (CTL); Tumor (LLC); Tumor + Exercício Físico (LLC+T), sendo que os grupos que praticaram exercício físico foram divididos entre os que treinaram no Zeitgeber (ZT) 2 e em horários alternados (ZTAlt). Os horários de treino do grupo alternado foram ZT2, ZT14, ZT6, ZT2, ZT6.

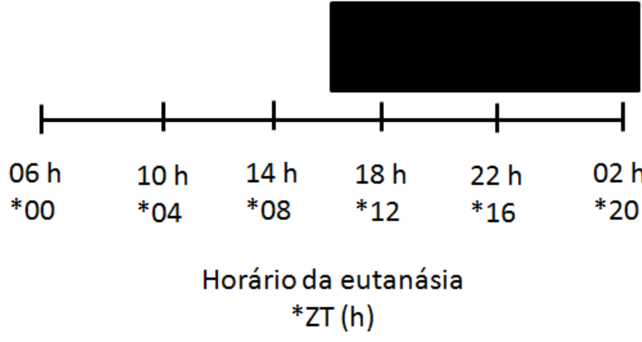


Os animais foram submetidos ao treinamento aeróbio no terceiro dia após a inoculação do tumor. Foram realizadas 14 sessões de treino compreendendo 5 sessões na primeira e 5 sessões na segunda semana e 4 sessões na terceira semana (sessões de 40 a 60 minutos de treino a 60% da velocidade máxima) com dois dias de descanso entre as semanas(113).

Para determinação da velocidade máxima realizamos um teste incremental, no qual os animais realizaram aquecimento de 5 minutos na velocidade de 5 metros/minuto (m/m), e após o quinto minuto a velocidade da esteira foi aumentada em 3 m/mim, a cada minuto, até que os animais chegassem à exaustão. A exaustão

foi caracterizada por alterações biomecânicas na corrida do animal. Este teste foi realizado antes do início do protocolo de treinamento, para a determinação da carga de trabalho inicial.

Para analisar se houve alterações circadianas promovidas pelo tumor e/ou pelo exercício físico, os animais foram eutanasiados a cada 4 horas do dia, até completar 24 horas, sendo as eutanásias realizadas nos seguintes horários: às 6 horas, às 10 horas, às 14 horas, às 18 horas, às 22 horas e às 02 horas. Para a apresentação dos dados esses horários foram descritos como ZT0, ZT4 e ZT8 correspondendo o período claro respectivamente nos horários de 6h, 10h e 14h, e ZT12, ZT16 e ZT20 correspondendo o período escuro respectivamente 18h, 22h e 2h.



5.2 Implante das células de carcinoma de pulmão Lewis (LLC)

As células foram mantidas em Dulbecco's modificado de Eagle' s (DMEM, GIBCO, Invitrogen, NY), suplementados com penicilina (100U/ml), streptomicina (100 µg/ml) e 10% soro fetal bovino (FBS, Atlanta Biological, Lawrenceville, GA). Foram cultivadas e mantidas em garrafas de cultura à 37°C em uma atmosfera humidificada contendo 5% de CO2. Foram injetadas $5x10^5$ de células viáveis subcutaneamente no flanco direito dos camundongos (diluídas em solução salina 0,9%). Os animais

controle receberam o mesmo volume de salina 0,9%. A viabilidade celular foi avaliada por Trypan Blue.

5.3 Composição corporal, ganho de peso e peso dos tecidos

O peso dos animais foi acompanhado semanalmente ao longo do tratamento.

Ao final, os seguintes parâmetros foram mensurados: peso úmido do músculo sóleo, gastrocnêmio, extensor longo dos dedos (EDL), do tumor e peso dos tecidos adiposos retroperitonial, epididimal e subcutâneo e peso seco do EDL. Para a determinação do índice de adiposidade foi utilizada a soma dos tecidos adiposos citados anteriormente

5.4 Determinação da expressão gênica

No tecido muscular esquelético gastrocnêmio foi avaliado os *clock genes*; **Clock, Bmal1, Per1, Per2, Per3 Cry1, Cry2, e REV-ERBα, Rorα, Rorβ, Ampk, Atrogina-1, Murf1 e GAPDH**. O tecido muscular foi homogeneizado em reagente TRIZOL para extração do RNA total (Chomczynski e Sacchi, 1987). O RNA foi quantificado por leitura em espectrofotômetro a 260 nm (A260 nm = 1 corresponde a 44 µg/mL) e o grau de pureza determinado pela razão 260/280 nm (razão igual a 2 indica alto grau de pureza). O cDNA foi sintetizado a partir de 2µg do RNA total extraído utilizando a transcriptase reversa. A expressão gênica foi quantificada por PCR em tempo real (114), utilizando o aparelho StepOnePlus (Applied Biosystems Mortlake, EUA) e SYBER Fast Green como marcador fluorescente. A quantificação da expressão dos genes foi realizada usando o método da Ct comparativa (Ct = threshold cycle; número de ciclo no qual o produto do PCR atinge um limiar de detecção), tendo a expressão da GAPDH como padrão interno (115).

Tabela 1- Sequências dos primer´s do RT-PCR

Gene	Primer Forward	Primer Reverse
Gapdh	CAAGCTCATTTCCTGGTATGACA	GCCTCTCTTGCTCAGTGTCC
Clock	CAAGGCATGTCACAGTTTCAG	AGTTCTTCTTGCTGCCGATG
Per1	GCATCCTCAGGTATTTGGAGAG	GGCTGAAGAGGCAGTGTAGG

Per2	TGAAGAACGCGGATATGTTT	GATGGAGGCCACTTGGTTAG
Per3	AACACGAAGACCGAAACAGAAT	CTCGGCTGGGAAATACTTTTTCA
Cry1	TAGCGGTGGAAATTGCTCT	GAGCTTCTCCCTTGCTTGAGT
Cry2	ACCGATGGAGGTTCCTACTG	TCCCCGGACTACAAACAGAC
Rev-erbα	GTCTAGAGATGCTGTGCGTTTT	AGGCTGCTCAGTTGGTTGTT
Rorα	CGAGGTATCTCAGTCACGAAGA	ATTCCTGACGATTTGTCTCCAC
Rorβ	GCAGCATTAGCAATGGCCTC	GACGGCTGACCGGAATCTATG
Ampk	GCCTGCGTACAATCTTCCTG	AGCCGACTTTGGTCTTTCAA
Atrogina-1	ACAAAGGAAGTACGAAGGAGCG	GGCAGTCGAGAAGTCCAGTC
Murf-1	GTGTGAGGTGCCTACTTGCTC	GCTCAGTCTTCTGTCCTTGGA

5.5 Avaliação dos parâmetros plasmáticos

A glicemia, colesterol total e triacilgicerol foram avaliados no soro através do kit Labtest ©. Seguindo o protocolo da empresa (Ref: 133-1, 76-2 e 87-2).

5.6 Determinação da concentração de TNF-α, IL-1β, MCP-1, IFN-γ, VEGF, IL-10 e IL-4 no tecido muscular esquelético gastrocnêmio e Tumor

O músculo gastrocnêmio e o tecido tumoral (0,1-0,3g) foram homogeneizados em tampão RIPA (0,625% de Nonidet P-40, 0,625% de desoxicolato de sódio, fosfato de sódio 6,25mM, e 1mM de ácido tetra-acético de etilenodiamina a pH 7,4) contendo 10 ug/ml do cocktail com inibidor de protease (Sigma-Aldrich, St. Louis, Missouri). O homogenato foi centrifugado a 12000g durante 30 min à 4° C, o sobrenadante retirado, e a avaliação da concentração de proteína determinada utilizando o ensaio de Bradford (Bio-Rad, Hercules, Califórnia). A avaliação quantitativa de TNF-α, IL-1β, MCP-1, IFN-γ, VEGF, IL-10 e IL-4 foram realizados por ELISA (DuoSet ELISA, R&D Systems, Minneapolis, MN).

5.7 Análise estatística

Os dados entre os grupos e os tempos foram apresentados como média ± desvio padrão da média (DPM) e analisados por Anova Two-Way, seguido por Tukey.

A comparação entre o período claro e escuro foi apresentada como média ± desvio padrão da média (DPM) e analisados por Test-t e Anova one-Way seguido por Tukey. As análises foram realizadas utilizando-se o programa GRAPHPAD PRISM 6.0. Os dados foram significativamente diferentes se $p \leq 0,05$.

6 RESULTADOS

6.1 Peso corporal total inicial, Peso corporal total término do protocolo, Peso do tumor, Peso tecido muscular, índice de adiposidade

Os animais não apresentaram diferença estatística no peso total no início do protocolo (Tabela 2). Ao término do protocolo, após 14 sessões de treinamento físico e 21 dias após inocular o tumor, os grupos que treinaram no ZT2 e ZTAlt apresentaram redução no peso total quando comparado com o grupo LCC, e apenas o grupo LLC+T(ZTAlt) apresentou diferença estatística de peso quando comparado com o grupo CTL (Tabela 2).

O peso do tumor foi menor no grupo LLC+T(ZTAlt) quando comparado com o grupo LLC (Tabela 2 e figura 3). Essa diferença não foi observada no grupo LLC+T(ZT2) (Tabela 2 e figura 3). Os músculos EDL e Sóleo apresentaram maior peso no grupo ZT2 quando comparado com o grupo LLC (Tabela 2) e o índice de adiposidade foi menor nos grupos LLC, LLC+T(ZT2) e LLC+T(ZTAlt) quando comparado com o grupo CTL (Tabela 2).

Tabela 2 - Peso Corporal (PC) e peso dos tecidos de camundongos C57BL/6J com 21 dias de tumor e 14 sessões de treinamento físico.

	CTL	LLC	LLC+ (ZT2)	LLC+T(ZTAlt)
PC inicial (g)	22,57±0,294	22,67±0,198	22,84±0,212	23,01±0,281
PC Final (g)	24,88±0,274	25,20±0,282	24,11 ±0,316#	23,90±0,209*#
Peso Tumor (g)	—	0,8638±0,056	0,8160±0,047	0,6652±0,044#
Peso Gastrocnêmio (g)	0,2726±0,004	0,2702±0,003	0,2612±0,004	0,2582±0,003
Peso EDL molhado (g)	0,0169±,0006	0,0159±0,0003	0,0184±0,0007#	0,0165±0,0006
Peso EDL seco (g)	0,00485±0,00009	0,0047±0,00008	0,00482±0,0001	0,00474±0,0001
Peso Sóleo (g)	0,01517±0,0006	0,01426±0,0003	0,01748±0,0008#	0,01632±0,0007
Peso do fígado (g)	1,218±0,029	1,285±0,027	1,263±0,026	1,239±0,028
Índice de adiposidade (g)	0,409±0,020	0,333±0,010**	0,325±0,014**	0,344±0,012**

Dados representam a média ± desvio padrão da média de 29-30 animais por grupo,*p<0,05 e **p<0,001 *vs*CTL; #p<0,05 e ; ##p<0,001 *vs*,LCC; (ANOVA One-way seguido por Tukey).

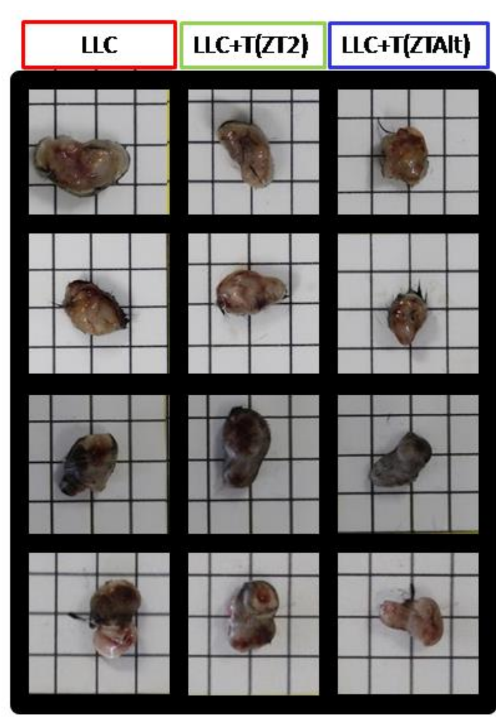


Figura 3. Tecido tumoral dos grupos LLC, LLC+T(ZT2) e LLC+T(ZTAlt).

6.2 Perfil plasmático

As concentrações de glicose, colesterol total e triacilglicerol foram analisadas nos 4 grupos durante os seis tempos (ZT0, 4, 8, 12, 16, 20).

Foi observado que no grupo LLC+T(ZTAlt) a concentração de glicose esteve diminuída significativamente quando comparada com o grupo LLC no ZT8 (figura 4A). Não houve alterações estatisticamente significante da concentração de glicose entre os ZTs nos 4 grupos (figura 4).

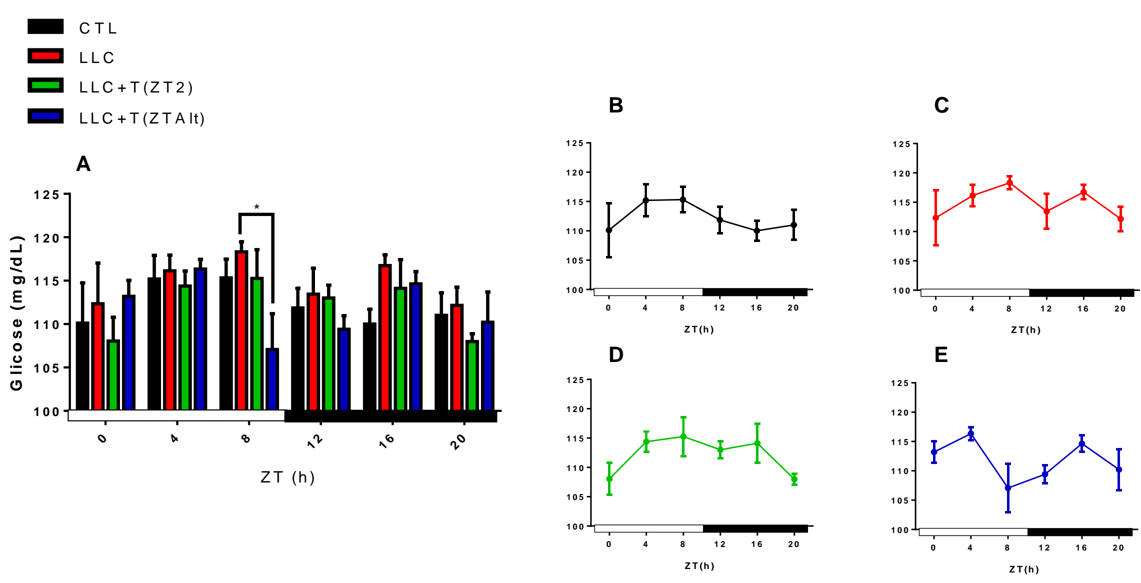


Figura 4. Comparação da Glicemia entre os grupos em cada ZT(A). Efeito do ritmo circadiano da Glicemia nos grupos CTL(B), LLC(C), LLC+T(ZT2)(D) e LLC+T(ZTAlt)(E). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia, após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey) e #p<0,05 entre os tempos (Anova one-way seguido por Tukey).

A concentração de colesterol total apresentou aumento significativo no grupo LLC+T(ZTAlt) e LLC+T(ZT2) quando comparado com o grupo de controle nos tempos ZT12 e 18, respectivamente (figura 5A) . Apenas o grupo LLC+T(ZT2) apresentou variação no colesterol total entre os tempos, com maior amplitude no ZT16 (Figura 5D).

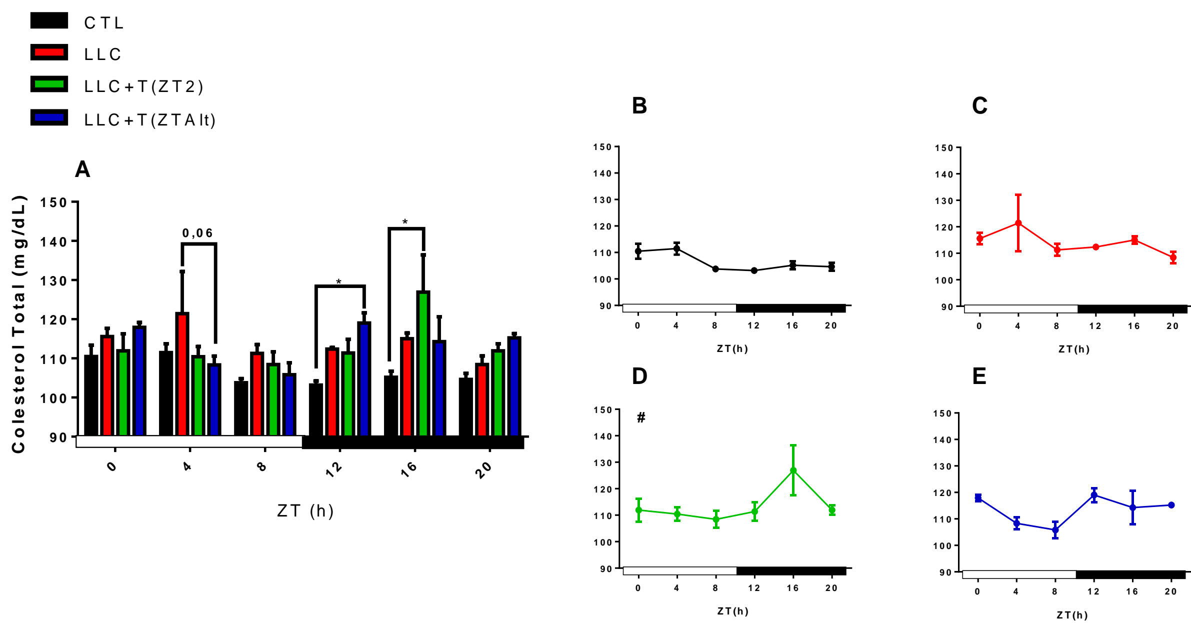


Figura 5. Comparação do Colesterol Total entre os grupos em cada ZT(A). Efeito do ritmo circadiano do Colesterol Total nos grupos CTL(B), LLC (C), LLC+T(ZT2)(D) e LLC+T(ZTAlt)(E). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey) e #p<0,05 entre os tempos (Anova one-way seguido por Tukey).

Quando analisada as concentrações de triacilglicerol (TAG) foi observada oscilação entre os tempos nos 4 grupos com maior amplitude nas primeiras horas do ciclo claro, com exceção do grupo LLC+T(ZT2), que teve maior amplitude da concentração de TAG no ZT16 (figura 6B, C, D e E). Entre os grupos apenas o LLC+T(ZT2) apresentou aumento significante quando comparado com o grupo CTL no ZT16 (figura 6A).

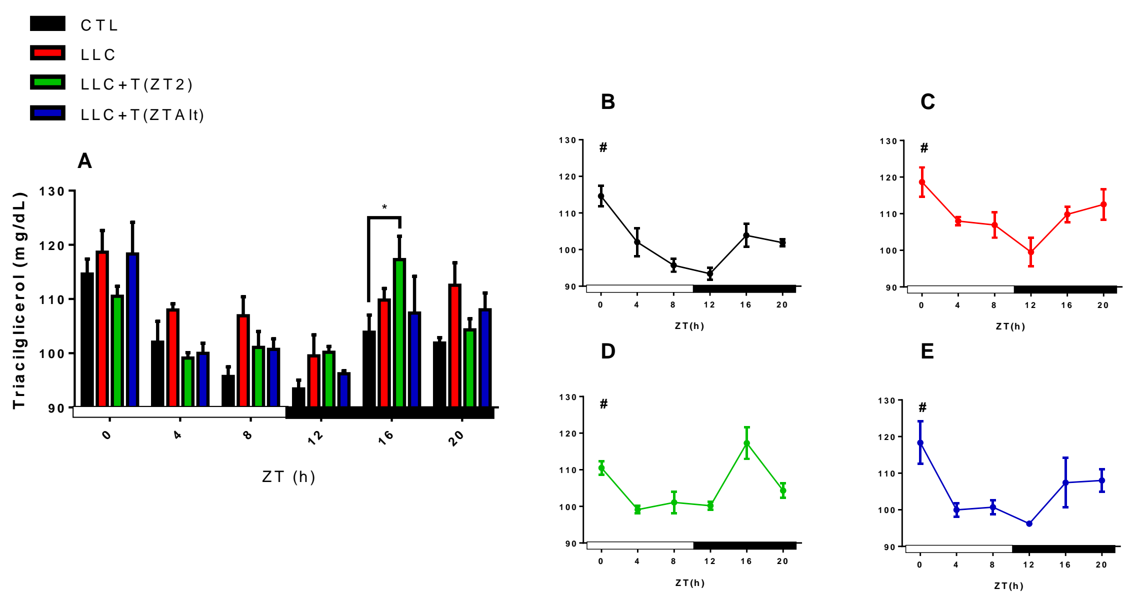


Figura 6. Comparação do Triacilglicerol entre os grupos em cada ZT(A). Efeito do ritmo circadiano da Triacilglicerol nos grupos CTL(B), LLC(C), LLC+T(ZT2)(D) e LLC+T(ZTAlt)(E). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey) e #p<0,05 entre os tempos (Anova one-way seguido por Tukey).

6.3 Expressão gênica dos *Clock genes* no músculo esquelético gastrocnêmio

As análises das expressões dos genes que controlam o relógio circadiano foram realizadas no músculo esquelético gastrocnêmio. No gene Clock, pode ser observado aumento significante da expressão no ZT4 em ambos os grupos que praticaram exercício físico LLC+T(ZT2 e ZTAlt) quando comparado com o grupo CTL (figura 7A). Ainda nos grupos que praticaram exercício físico, foi observado maior oscilação entre os tempos, maior amplitude e a acrofase no ZT4 (figura 7E e F), essas oscilações não foram observadas nos grupos CTL e LLC (figura 7C e D). Quando feito análise da expressão de Clock entre o período claro e escuro, não observou diferença estatística (figura 7B).

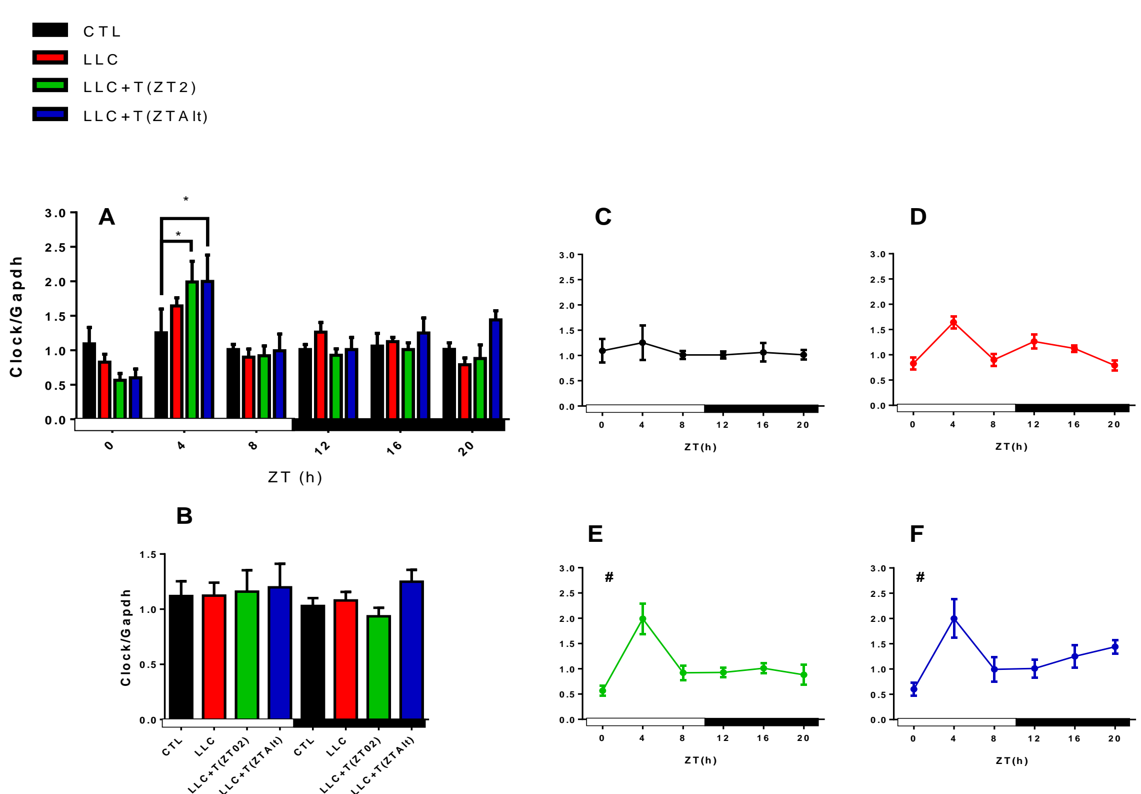


Figura 7. Expressão Gênica de Clock no músculo esquelético gastrocnêmio - Comparação do Clock entre os grupos em cada ZT(A). Comparação entre o período claro e escuro do Clock(B). Efeito do ritmo circadiano do Clock nos grupos CTL(C), LLC(D), LLC+T(ZT2)(E) e LLC+T(ZTAlt)(F). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

Na análise do gene Bmal1 no músculo gastrocnêmio, não houve diferença estatística entre os grupos nos seis ZTs (figura 8A). Também não foi observada diferença estatística da expressão de Bmal1 entre o período claro e escuro (figura 8A), porém observa-se no grupo LLC+T(ZTAlt) maior oscilação entre os ZTs, com maior amplitude e acrofase no ZT20 (figura 8F). Essa ritmicidade não foi observada nos outros grupos (figura 8C, D e E).

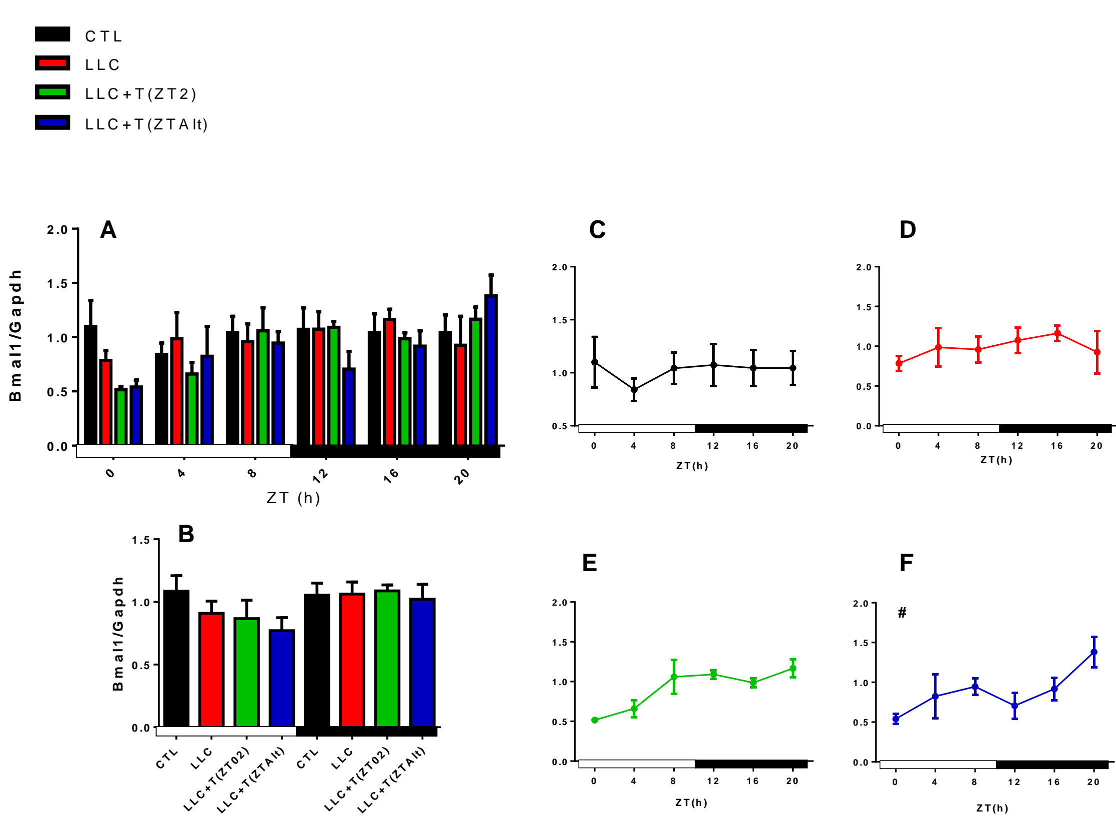


Figura 8. Expressão Gênica de Bmal1 no músculo esquelético gastrocnêmio - Comparação do Bmal1 entre os grupos em cada ZT(A). Comparação entre o período claro e escuro do Bmal1(B). Efeito do ritmo circadiano do Bmal1 nos grupos CTL(C), LLC(D), LLC+T(ZT2)(E) e LLC+T(ZTAlt)(F). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

O treinamento físico praticado em horários alternados, LLC+T(ZTAlt), foi capaz de aumentar a expressão no gene Per1 no músculo gastrocnêmio no ZT4 quando comparado com os grupos CTL e LLC (figura 9A). Quando comparado a expressão de Per1 entre o período claro e escuro, observa-se maior expressão no período claro apenas nos grupos que praticaram exercício físico LLC+T(ZT2 e ZT Alt) (figura 9B). O grupo LLC+T(ZTAlt) também apresentou maior expressão de Per1 quando comparado com CTL no período claro (figura 9B). Além disso, os grupos que treinaram apresentaram maior oscilação entre os tempos, sendo a acrofase observada nos primeiros ZTs do período claro, alterações essas não observadas nos grupos CTL e LLC (figura 9C, D, E e F).

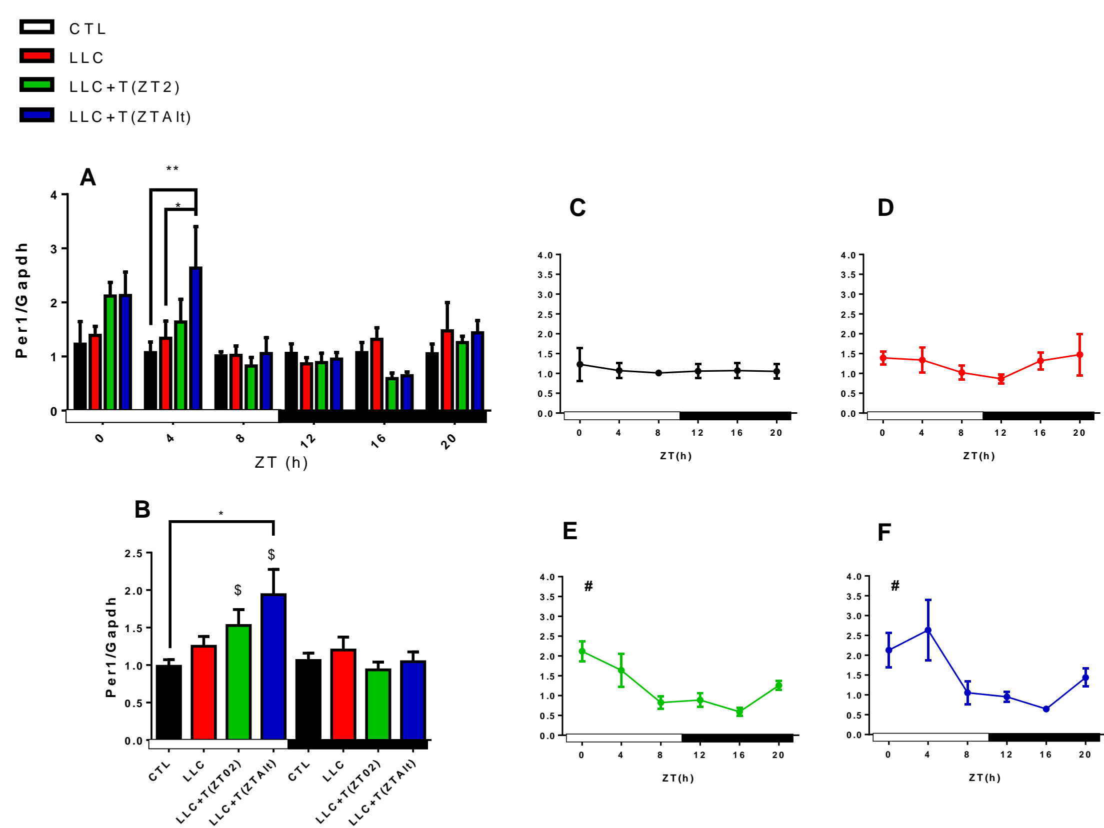


Figura 9. Expressão Gênica de Per1 no músculo esquelético gastrocnêmio - Comparação do Per1 entre os grupos em cada ZT(A). Comparação entre o período claro e escuro do Per1(B). Efeito do ritmo circadiano do Per1 nos grupos CTL(C), LLC(D), LLC+T(ZT2)(E) e LLC+T(ZTAlt)(F). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

O gene Per2 também apresentou aumento de sua expressão promovida pelo exercício físico. O grupo que treinou no ZT2 teve aumento da expressão de Per2 quando comparado com o grupo CTL no ZT0 e ZT4, já o grupo LLC+T(ZTAlt) apresentou aumento do Per2 quando comparado com o grupo CTL e LLC apenas no ZT4 (figura 10A). Quando comparado com o período claro e escuro observa-se uma diferença nos grupos LCC, LCC+T(ZT2 e ZTAlt), no grupo CTL não houve diferença entre os períodos (figura 10B). Os grupos que treinaram também apresentaram maior ritmicidade entre os ZTs (figura 10E e F), com a acrofase no grupo LLC+T (ZT2) no ZT0 e no grupo LLC+T(ZTAlt) no ZT4.

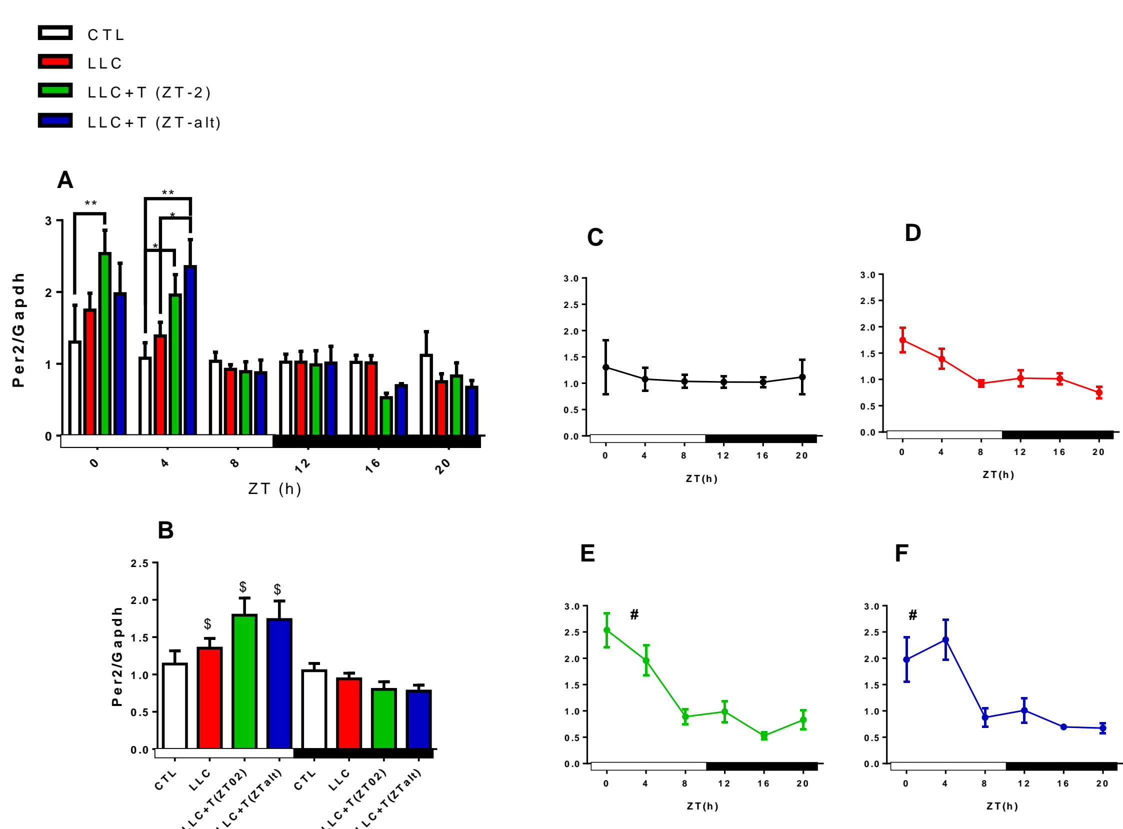


Figura 10. Expressão Gênica de Per2 no músculo esquelético gastrocnêmio - Comparação do Per2 entre os grupos em cada ZT(A). Comparação entre o período claro e escuro do Per2 (B). Efeito do ritmo circadiano do Per2 nos grupos CTL(C), LLC(D), LLC+T(ZT2)(E) e LLC+T(ZTAlt)(F). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

Na análise feita no gene Per3, o grupo LLC+T(ZTAlt) apresentou maior expressão no ZT4 quando comparado com os grupos CTL e LLC (figura 11A), esse mesmo grupo apresentou maior expressão de Per3 no período claro quando comparado com o período escuro, sendo que essas alterações não foram observadas nos outros grupos (figura 11B). Uma maior oscilação do gene Per3 também foi observada no grupo LLC+T(ZTAlt) e LLC+T(ZT2) entre os ZTs (figura 11E e F), com a acrofase no ZT4. Essas oscilações não foram observadas nos grupos CTL e LLC (figura 11C e D).

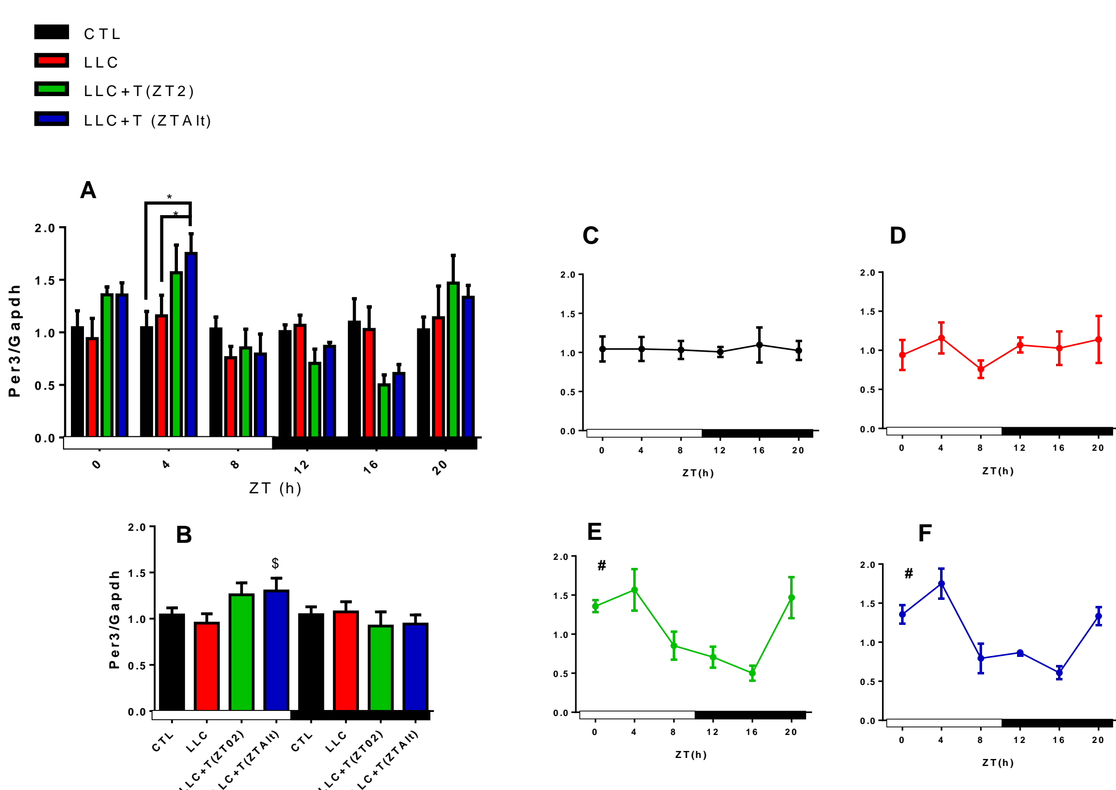


Figura 11. Expressão Gênica de Per3 no músculo esquelético gastrocnêmio - Comparação do Per3 entre os grupos em cada ZT(A). Comparação entre o período claro e escuro do Per3(B). Efeito do ritmo circadiano do Per3 nos grupos CTL(C), LLC(D), LLC+T(ZT2)(E) e LLC+T(ZTAlt)(F). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

No gene Cry1 não observou diferença entre os grupos em nenhum momento (figura 12A). Na comparação entre o período claro e escuro, observou-se no grupo LLC menor expressão do Cry1 no período claro quando comparado com o período escuro (figura 12B). Entretanto, a oscilação do Cry1 entre os ZTs apenas foi observada nos grupos que treinaram, com a acrofase no ZT20 (figura 12E e F).

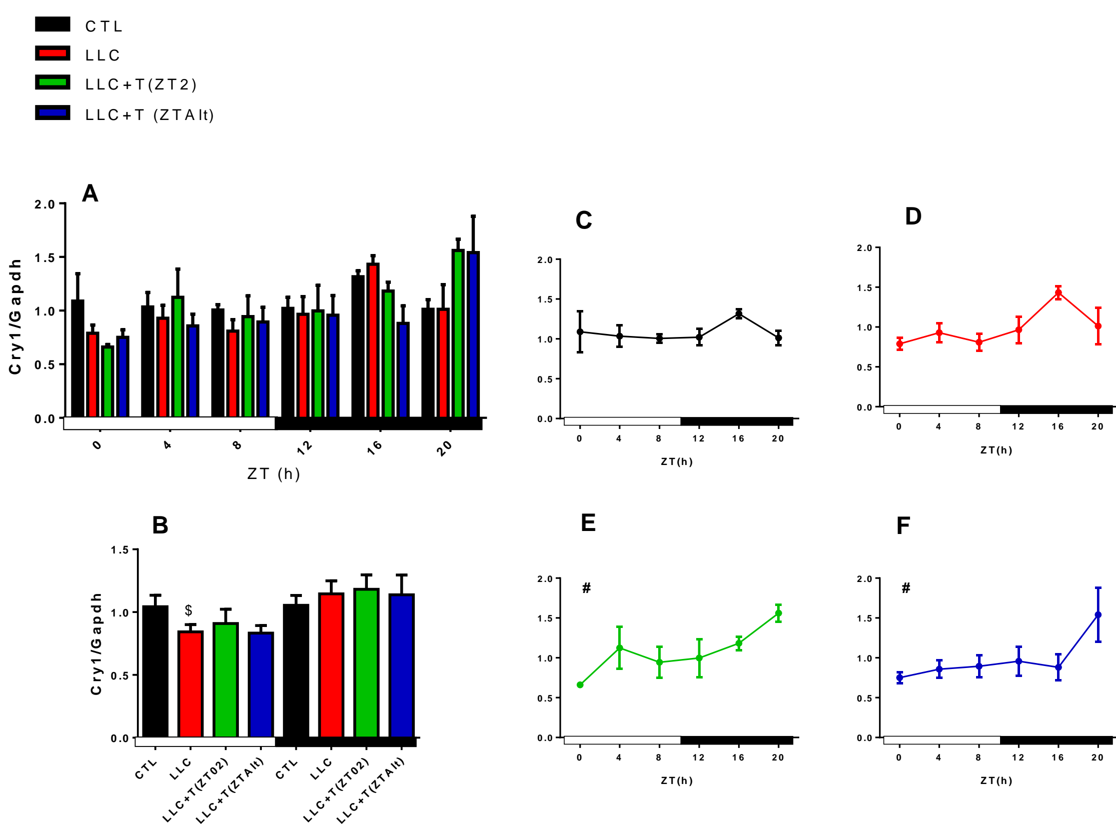


Figura 12. Expressão Gênica de Cry1 no músculo esquelético gastrocnêmio - Comparação do Cry1 entre os grupos em cada ZT(A). Comparação entre o período claro e escuro do Cry1(B). Efeito do ritmo circadiano do Cry1 nos grupos CTL(C), LLC(D), LLC+T(ZT2)(E) e LLC+T(ZTAlt)(F). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

Na análise realizada no gene Cry2 não observou diferença entre os grupos em nenhum dos ZTs (figura 13A). O grupo LLC+T(ZT2) apresentou maior expressão de Cry2 no período claro quando comparado com o escuro, essas diferenças não foram observadas nos outros grupos (figura 13B). Os grupos LLC e LLC+T(ZT2) apresentaram maior oscilação de Cry2 entre os ZTs, com maior amplitude no ZT4 (figura 13D e E).

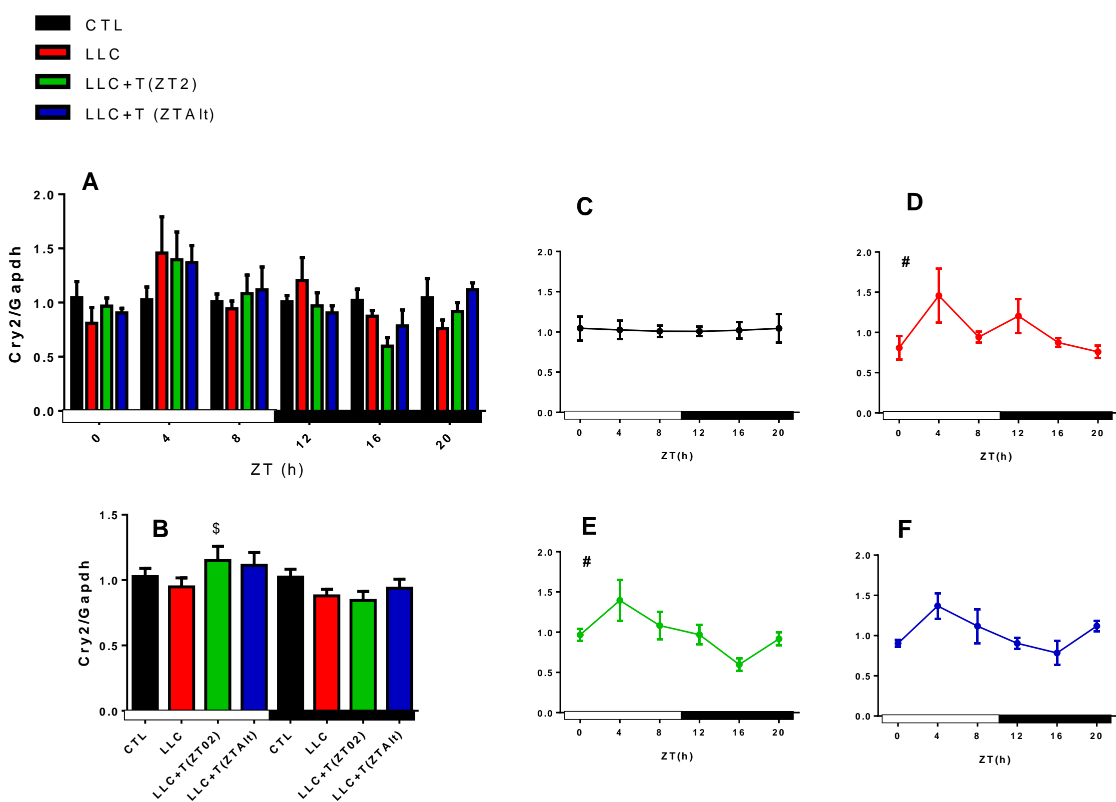


Figura 13. Expressão Gênica de Cry2 no músculo esquelético gastrocnêmio - Comparação do Cry2 entre os grupos em cada ZT(A). Comparação entre o período claro e escuro do Cry2(B). Efeito do ritmo circadiano do Cry2 nos grupos CTL(C), LLC(D), LLC+T(ZT2)(E) e LLC+T(ZTAlt)(F). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

A expressão do Rev-Erbα no ZT12 estava aumentada no grupo LLC quando comparado com o grupo LLC+T(ZT2) (figura 14A). No último tempo analisado, observou maior expressão de Rev-Erbα no grupo LLC+T(ZTAlt) quando comparado com o grupo controle (figura 14A). Na comparação entre o período claro e escuro, apenas o grupo LLC mostrou diferenças estatisticamente significantes (figura14B). Quando analisado a ritmicidade do gene Rev-Erbα, apenas os grupos que treinaram apresentaram maior oscilação entre os ZTs com acrofase no ZT20 (figura 14E e F).

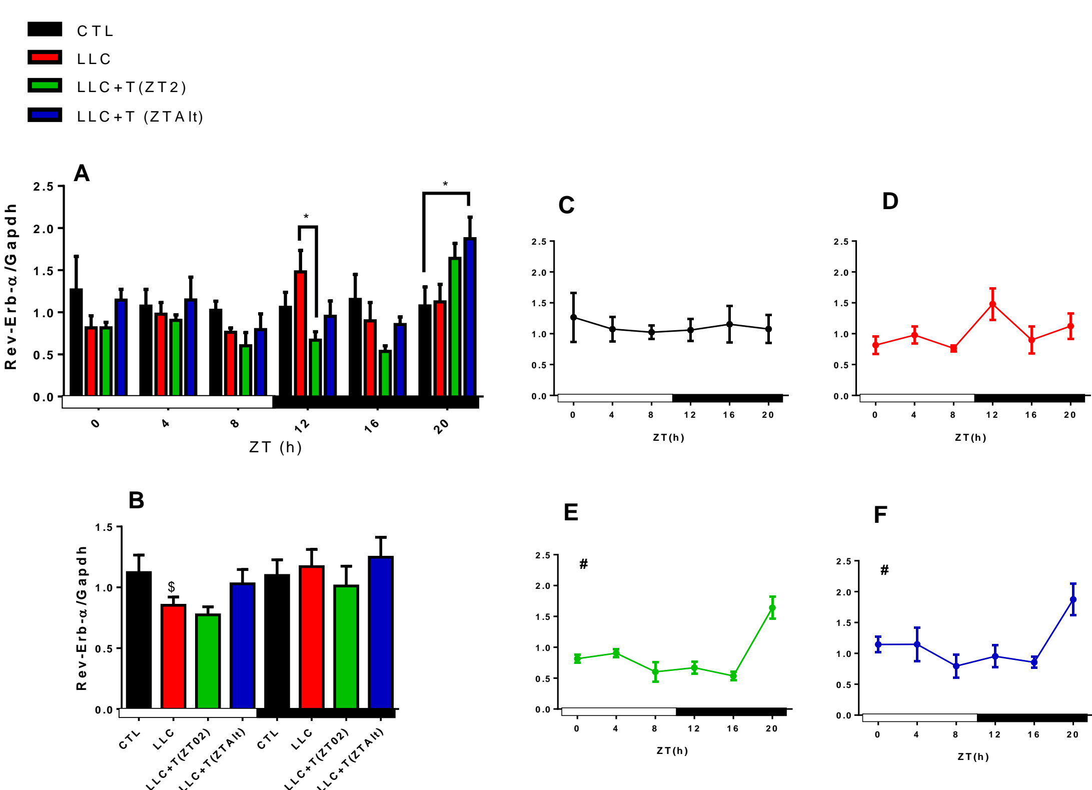


Figura 14. Expressão Gênica de Rev-Erbα no músculo esquelético gastrocnêmio - Comparação do Rev-Erbα entre os grupos em cada ZT(A). Comparação entre o período claro e escuro do Rev-Erbα(B). Efeito do ritmo circadiano do Rev-Erbα nos grupos CTL(C), LLC(D), LLC+T(ZT2)(E) e LLC+T(ZTAlt)(F). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

O gene RORα apresentou diferença estatística no ultimo tempo da fase escuro (ZT20) com diminuição da expressão no grupo LLC quando comparado com o grupo CTL (figura 15A). A expressão de RORα foi maior no período claro quando comparado com o período escuro apenas no grupo LLC+T(ZTAlt). No período escuro a expressão de RORα foi menor nos grupos LLC+T(ZT2 e ZTAlt) quando comparado com o grupo CTL (figura 15B). Não se observou diferença estatística entre os ZTs nos 4 grupos (figura 15C, D, E e F).

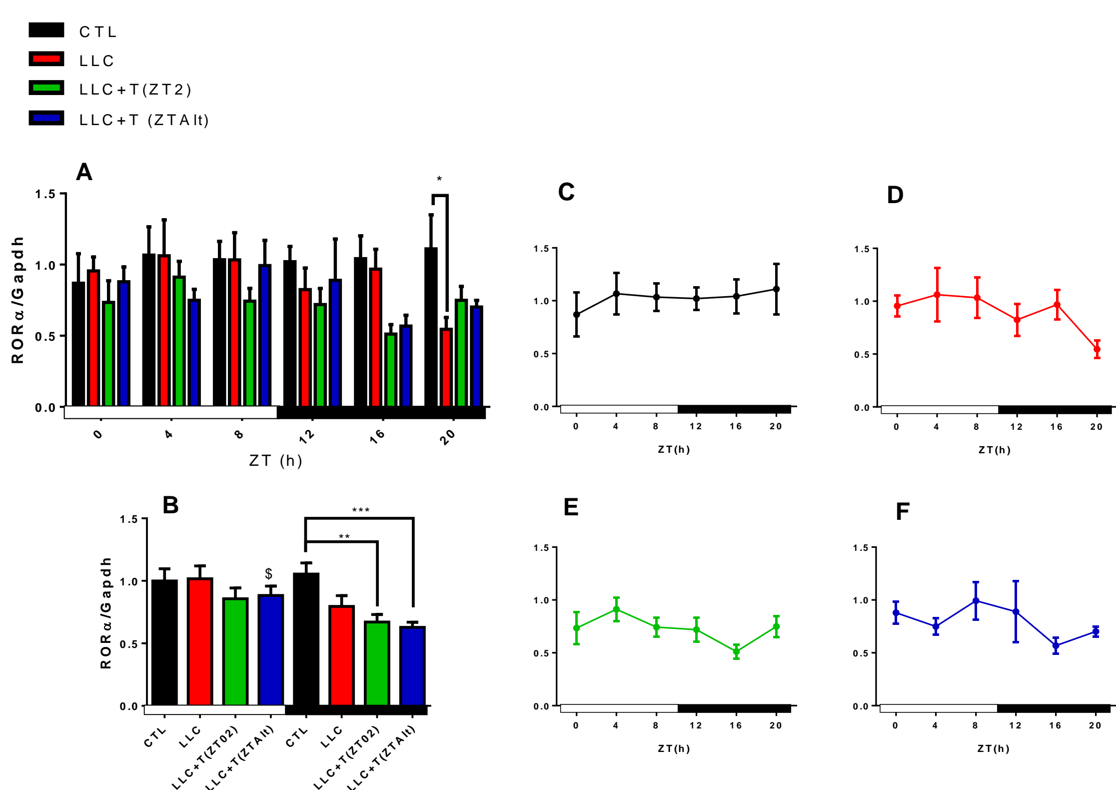


Figura 15. Expressão Gênica de RORα no músculo esquelético gastrocnêmio - Comparação do RORα entre os grupos em cada ZT (A). Comparação entre o período claro e escuro do RORα(B). Efeito do ritmo circadiano do RORα nos grupos CTL(C), LLC(D), LLC+T(ZT2)(E) e LLC+T(ZTAlt)(F). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

O gene RORβ não apresentou diferenças estatísticas entre os grupos, período e tempo (figura 16).

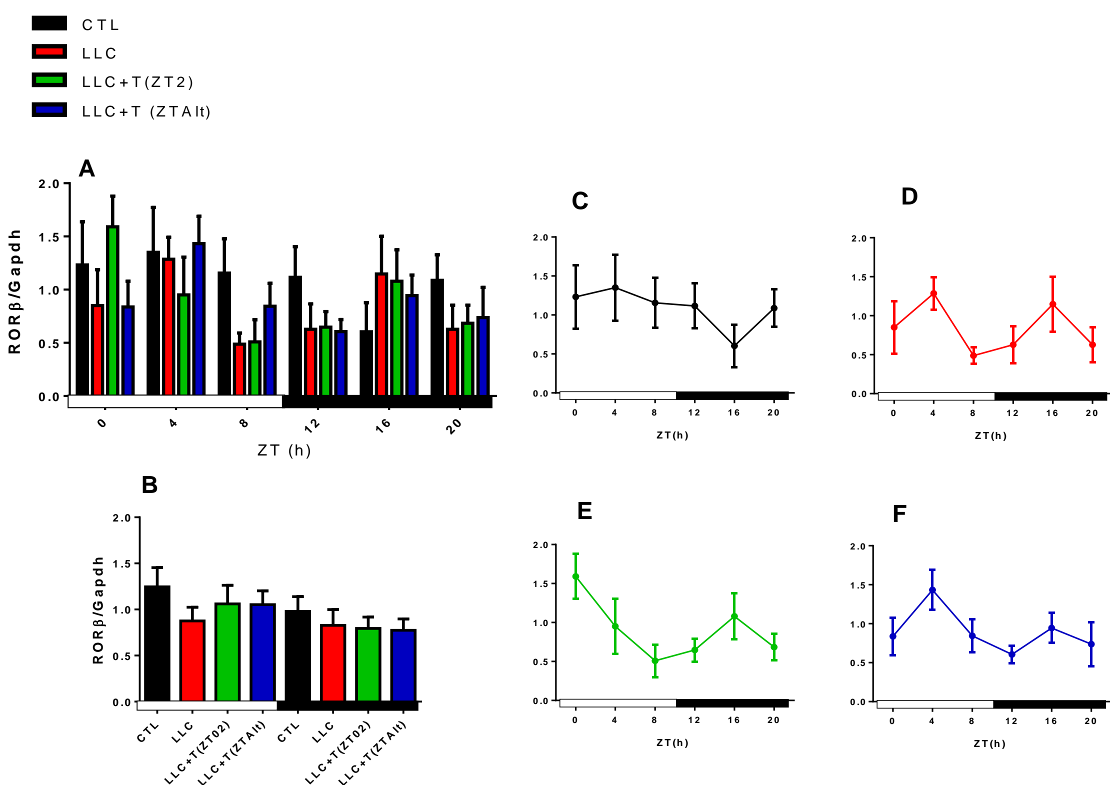


Figura 16. Expressão Gênica de RORβ no músculo esquelético gastrocnêmio - Comparação do RORβ entre os grupos em cada ZT(A). Comparação entre o período claro e escuro do RORβ(B). Efeito do ritmo circadiano do RORβ nos grupos CTL(C), LLC(D), LLC+T(ZT2)(E) e LLC+T(ZTAlt)(F). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

6.4 Expressão gênica da AMPK, Atrogina-1 e Murf1 no músculo esquelético gastrocnêmio

Ainda no músculo esquelético gastrocnêmio foram analisadas a expressão da AMPK, Atrogina-1 e Murf1.

Na expressão gênica da AMPK, não se observou diferença estatística entre os grupos em nenhum dos ZTs (figura 17A). Entretanto quando comparado o período claro e escuro, foi observado aumento da expressão da AMPK no período claro no grupo LLC+T(ZT2) quando comparado com o período escuro (figura 17B). O grupo LLC+T(ZT2) também foi o único grupo que apresentou maior oscilação da AMPK entre os ZTs, com acrofase no ZT0 (figura 17E).

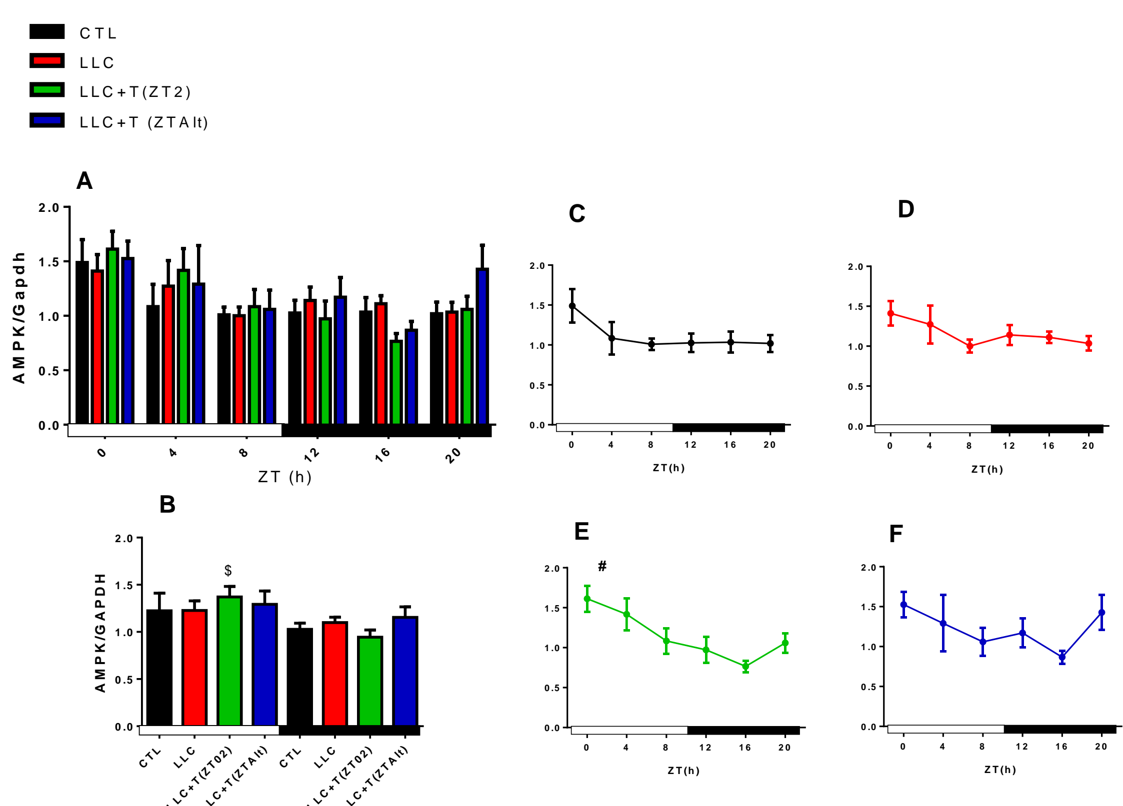


Figura 17. Expressão Gênica de AMPK no músculo esquelético gastrocnêmio - Comparação do AMPK entre os grupos em cada ZT(A). Comparação entre o período claro e escuro do AMPK(B). Efeito do ritmo circadiano do AMPK nos grupos CTL(C), LLC(D), LLC+T(ZT2)(E) e LLC+T(ZTAlt)(F). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

A expressão do gene Atrogina-1 apresentou aumento no grupo LLC+T(ZTAlt) no ZT20 quando comparado com o grupo CTL (figura 18A). Ao contrário da AMPK, a Atrogina-1 apresentou menor expressão no período claro no grupo LLC+T(ZT2) quando comparado com o período escuro (figura18B). A Atrogina-1 apresentou maior oscilação entre os ZTs nos grupos LLC+T(ZT2 e ZTAlt), com a acrofase no ZT20 para ambos os grupos (figura 18E e F).

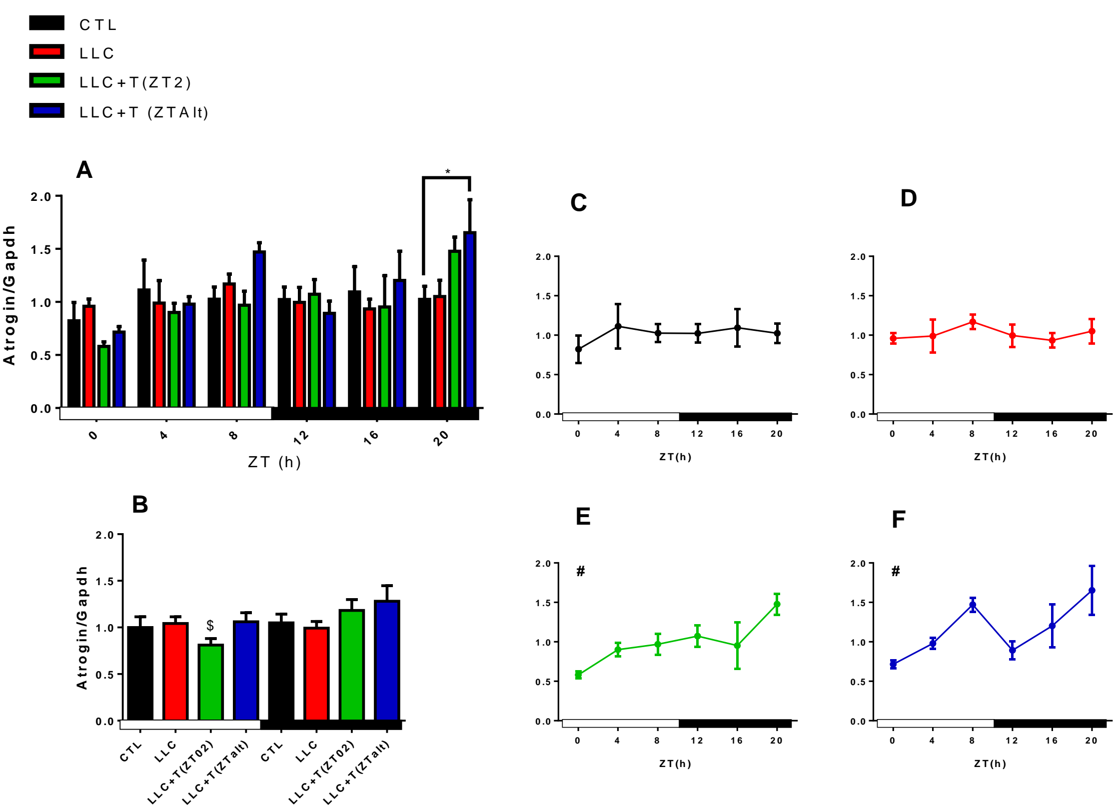


Figura 18. Expressão Gênica de Atrogina-1 no músculo esquelético gastrocnêmio - Comparação do Atrogina-1 entre os grupos em cada ZT(A). Comparação entre o período claro e escuro do Atrogina-1(B). Efeito do ritmo circadiano do Atrogina-1nos grupos CTL(C), LLC(D), LLC+T(ZT2)(E) e LLC+T(ZTAlt)(F). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

O gene Murf-1 não apresentou diferenças estatísticas entre os grupos, período e tempo (figura 19).

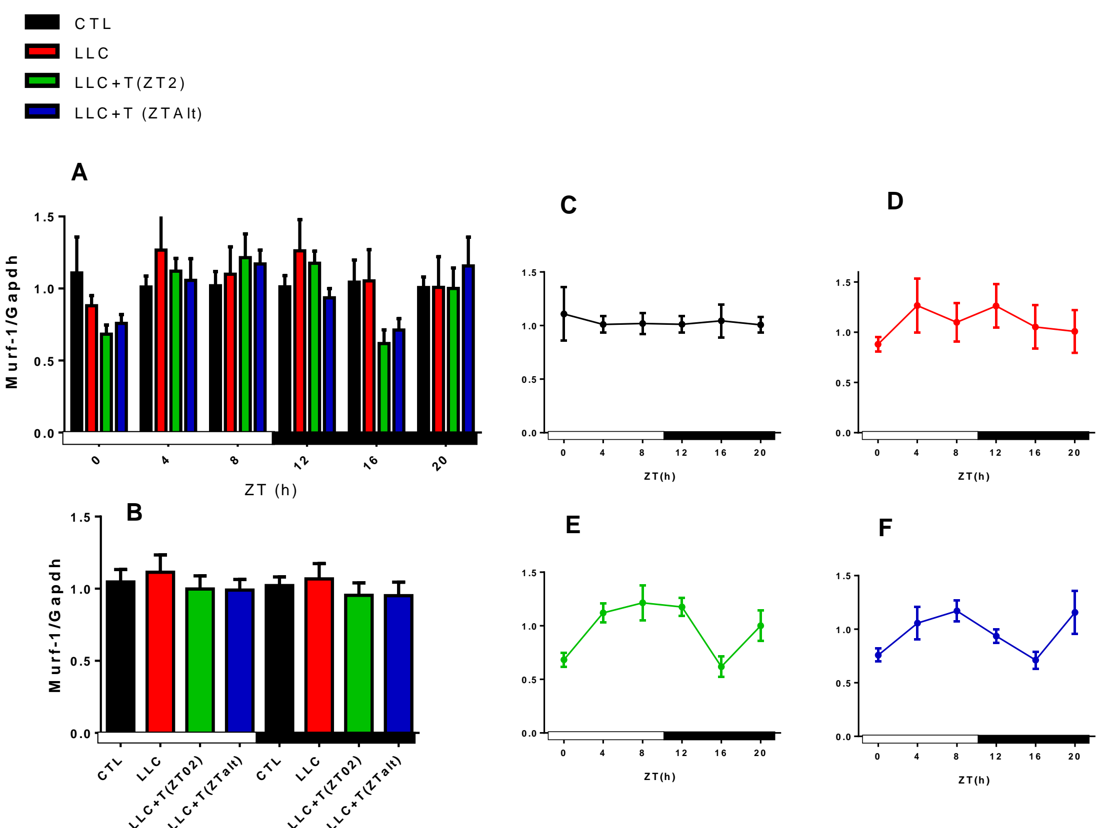


Figura 19. Expressão Gênica de Murf-1 no músculo esquelético gastrocnêmio - Comparação do Murf-1 entre os grupos em cada ZT(A). Comparação entre o período claro e escuro do Murf-1(B). Efeito do ritmo circadiano do Murf-1 nos grupos CTL(C), LLC(D), LLC+T(ZT2)(E) e LLC+T(ZTAlt)(F). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

6.5 Concentração das citocinas TNF-α, IL-1β, MCP-1, IFN-γ, VEGF, IL-10 e IL-4 no músculo esquelético gastrocnêmio

No músculo gastrocnêmio foi feito a análise das concentrações das citocinas TNF-α, IL-1β, MCP-1, IFN-γ, VEGF, IL-10 e IL-4.

Quando analisado o TNF-α, foi observado aumento da concentração no grupo LLC no ZT16 quando comparado com os grupos CTL e LLC+T(ZTAlt) (figura 20A). A comparação entre o período claro e escuro mostrou que o TNF-α apresenta maior concentração no período escuro nos quatro grupos (figura 20B). Uma oscilação entre os tempos foi observada apenas nos grupos LLC e LLC+T(ZT2), com a acrofase no ZT16 para ambos os grupos (figura 20C e D).

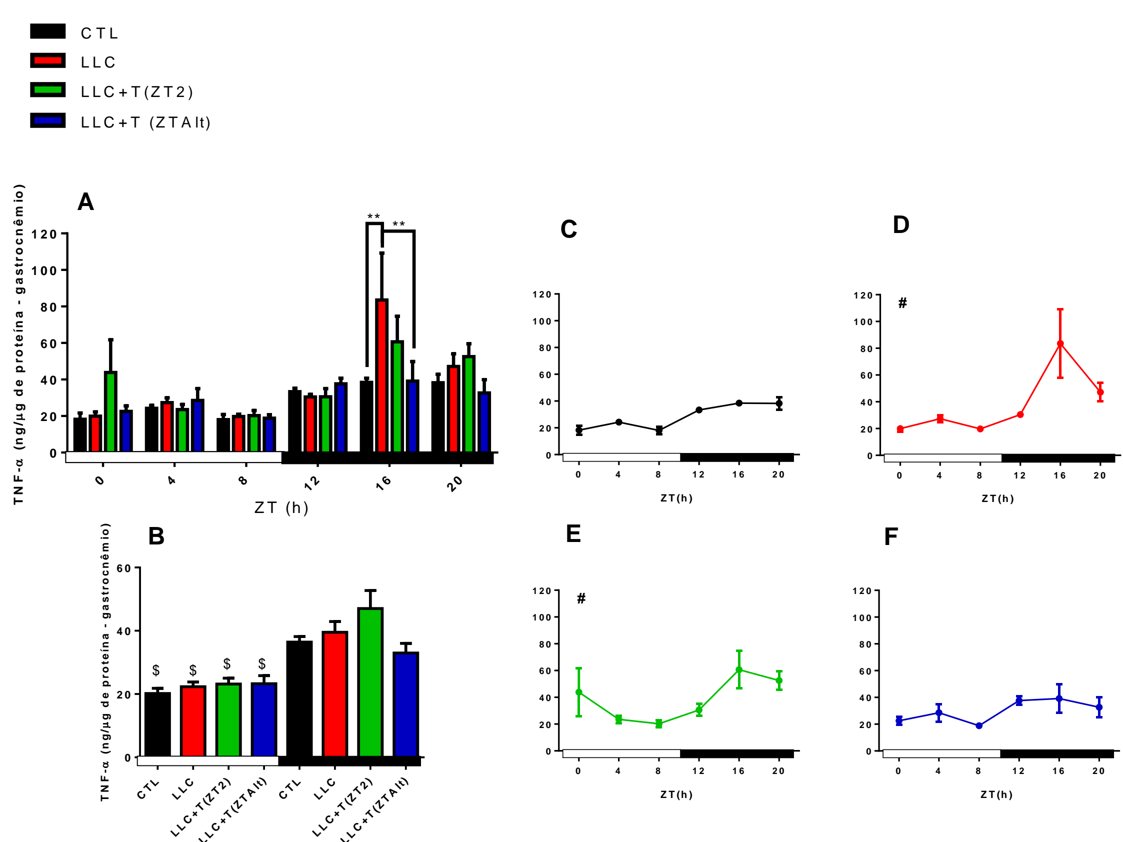


Figura 20. Concentração proteica de TNF-α no músculo esquelético gastrocnêmio - Comparação do TNF-α entre os grupos em cada ZT(A). Comparação entre o período claro e escuro do TNF-α(B). Efeito do ritmo circadiano do TNF-α nos grupos CTL(C), LLC(D), LLC+T(ZT2)(E) e LLC+T(ZTAlt)(F). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

IL-1β no músculo gastrocnêmio não apresentou diferenças estatísticas entre os grupos, período e tempo (figura 21).

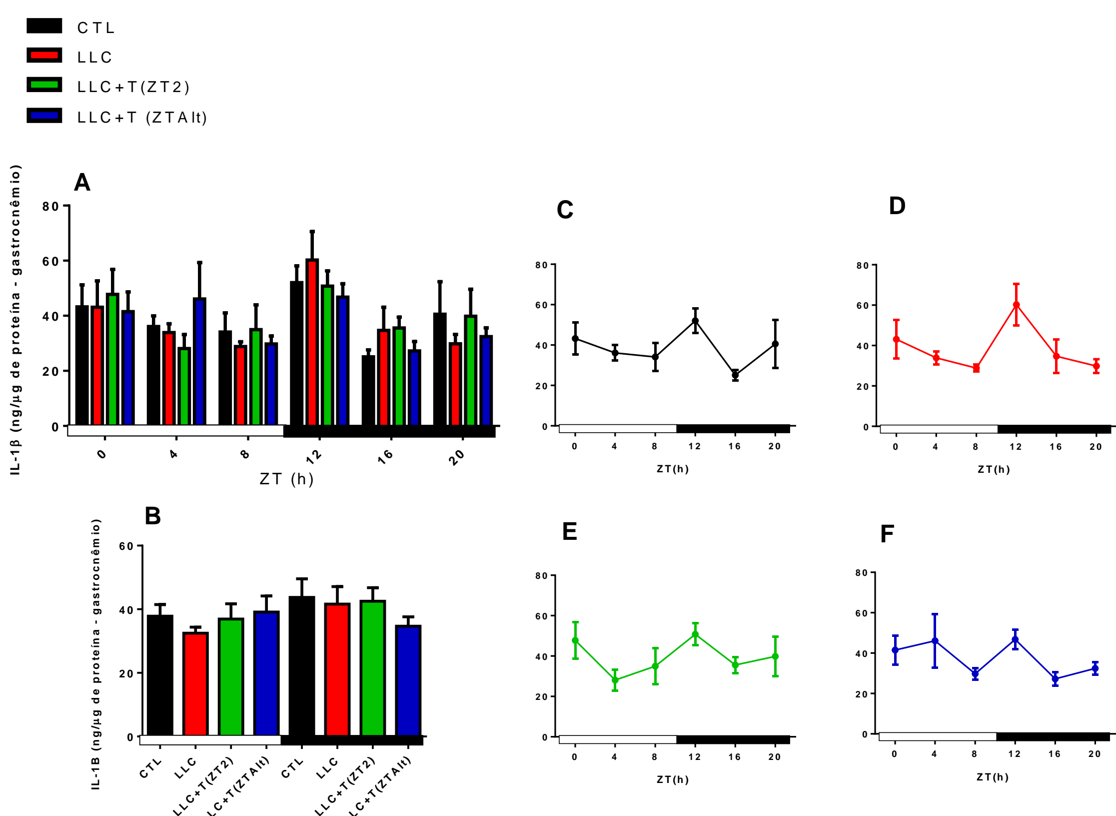


Figura 21. Concentração proteica IL-1β no músculo esquelético gastrocnêmio - Comparação da IL-1β entre os grupos em cada ZT(A). Comparação entre o período claro e escuro da IL-1β(B). Efeito do ritmo circadiano da IL-1β nos grupos CTL(C), LLC(D), LLC+T(ZT2)(E) e LLC+T(ZTAlt)(F). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

A concentração do MCP-1 no músculo gastrocnêmio apresentou aumento nos grupos que praticaram exercício no primeiro ZT do período claro (ZT0) quando comparado com o grupo LLC (figura 22A). Na comparação entre os períodos, o grupo LLC+T(ZTAlt) apresentou maior concentração no período claro quando comparado com o período escuro (figura 22B). Os grupos que treinaram apresentaram maior oscilação entre os tempos, com a acrofase no ZT0 (figura 22E e F).

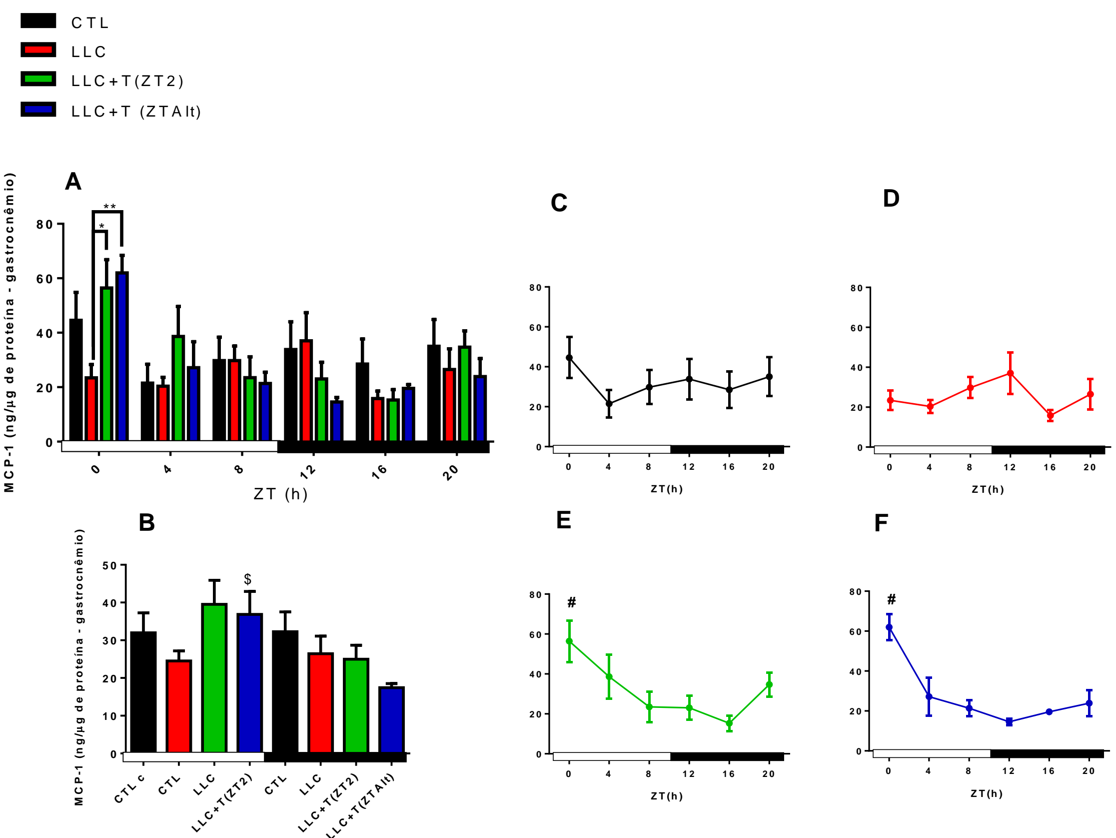


Figura 22. Concentração proteica de MCP-1 no músculo esquelético gastrocnêmio - Comparação da MCP-1 entre os grupos em cada ZT(A). Comparação entre o período claro e escuro da MCP-1(B). Efeito do ritmo circadiano da MCP-1 nos grupos CTL(C), LLC(D), LLC+T(ZT2)(E) e LLC+T(ZTAlt)(F). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

IFN-γ no músculo não apresentou diferenças estatísticas entre os grupos e período (figura 23A e B), porém o grupo LLC+T(ZT2) apresentou maior oscilação entre os tempos, com a acrofase no ZT12 (figura 23E).

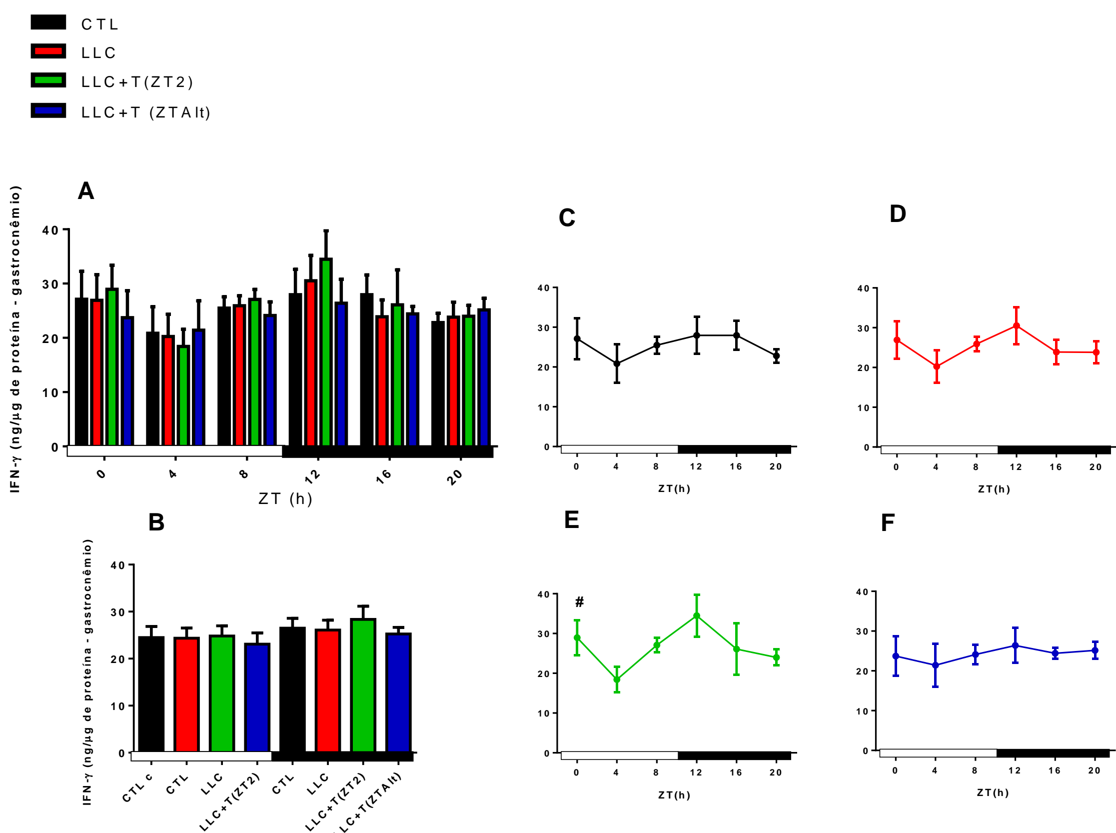


Figura 23. Concentração proteica de IFN-γ no músculo esquelético gastrocnêmio - Comparação do IFN-γ entre os grupos em cada ZT(A). Comparação entre o período claro e escuro do IFN-γ(B). Efeito do ritmo circadiano do IFN-γ nos grupos CTL(C), LLC(D), LLC+T(ZT2)(E) e LLC+T(ZTAlt)(F). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

Na análise feita da concentração do VEGF observou aumento no grupo LLC quando comparado com o grupo LLC+T(ZTAlt) (figura 24A) no ZT16. Não houve diferença entre o período claro e escuro em nenhum grupo (figura 24B). Apenas no grupo LCC foi observado maior oscilação entre os tempos da concentração de VEGF, com a acrofase no ZT16 (figura 24D).

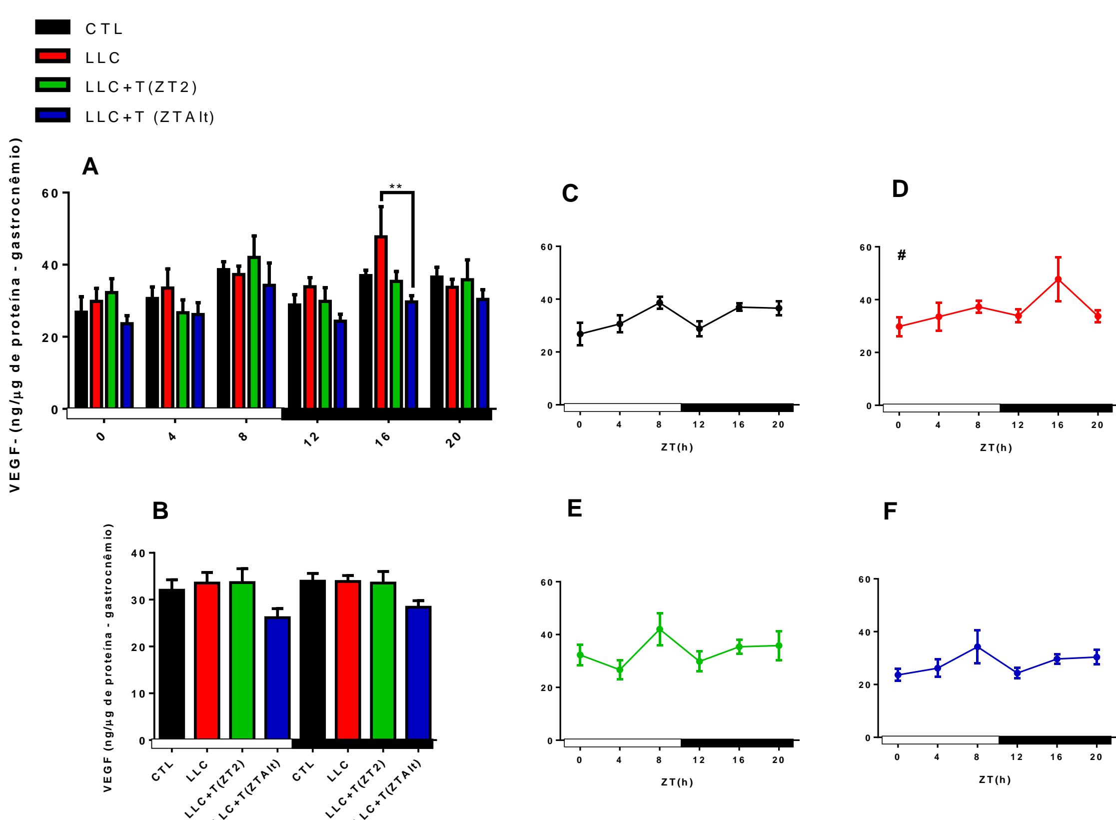


Figura 24. Concentração proteica de VEGF no músculo esquelético gastrocnêmio - Comparação do VEGF entre os grupos em cada ZT(A). Comparação entre o período claro e escuro do VEGF(B). Efeito do ritmo circadiano do VEGF nos grupos CTL(C), LLC(D), LLC+T(ZT2)(E) e LLC+T(ZTAlt)(F). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

A IL-10 apresentou aumento da concentração no ZT16 no grupo LCC quando comparado com os grupos CTL, LLC+T(ZT2 e Alt). No mesmo ZT o grupo LLC+T(ZTAlt) apresentou menor concentração da IL-10 quando comparado com o grupo CTL e LLC+T(ZT2) (figura 25A). Quando comparado a concentração da IL-10 entre o período claro e escuro, observa que a concentração é maior nos 4 grupos no período escuro (figura 25B). Os grupos CTL, LLC e LLC+T(ZT2) apresentaram oscilação entre os tempos da IL-10, com a acrofase no ZT16 (figura 25C, D e E).

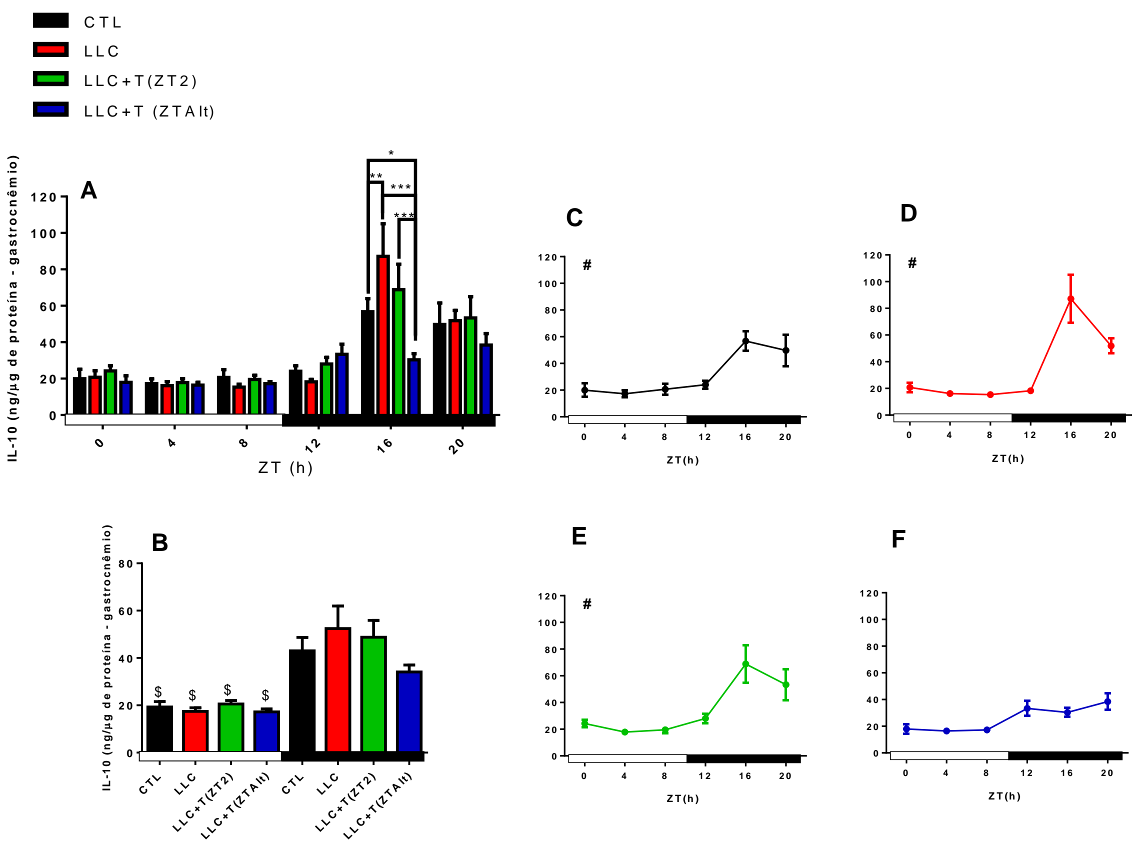


Figura 25. Concentração proteica da IL-10 no músculo esquelético gastrocnêmio - Comparação da IL-10 entre os grupos em cada ZT(A). Comparação entre o período claro e escuro da IL-10(B). Efeito do ritmo circadiano da IL-10 nos grupos CTL(C), LLC(D), LLC+T(ZT2)(E) e LLC+T(ZTAlt)(F). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

A IL-4 apresentou aumento da concentração no ZT16 no grupo LLC+T(ZT2) quando comparado com os grupos CTL e LLC+T(ZTAlt) (figura 26A). Quando comparado a concentração da IL-4 entre o período claro e escuro, observa maior concentração no período escuro nos 4 grupos (figura 26B). Os grupos LLC e LLC+T(ZT2) apresentaram oscilação entre os tempos na concentração da IL-4, com acrofase no ZT16 (figura 26 C, D e E).

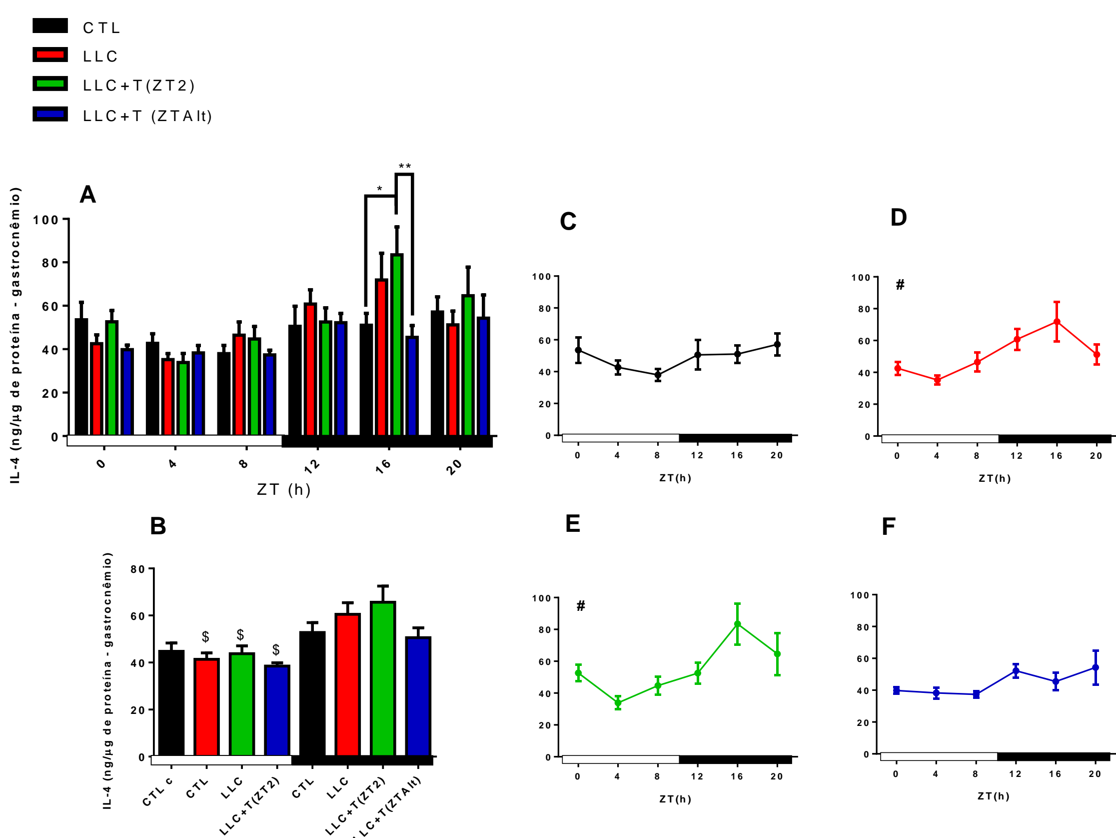


Figura 26. Concentração proteica da IL-4 no músculo esquelético gastrocnêmio - Comparação da IL-4 entre os grupos em cada ZT(A). Comparação entre o período claro e escuro da IL-4(B). Efeito do ritmo circadiano da IL-4 nos grupos CTL(C), LLC(D), LLC+T(ZT2)(E) e LLC+T(ZTAlt)(F). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

6.6 Concentração das citocinas TNF-α, IL-1β, MCP-1, IFN-γ, VEGF, IL-10 e IL-4 no Tumor

No tumor foi realizada a análise das concentrações das citocinas TNF-α, IL-1β, MCP-1, IFN-γ, VEGF, IL-10 e IL-4.

TNF-α mostrou alteração apenas no grupo LLC+T(ZTAlt) no ZT16 com aumento da concentração quando comparado com o grupo LLC+T(ZT2) (figura 27A). Entre o período claro e escuro não foi observado diferença estatística em nenhum grupo (figura 27B). A concentração de TNF-α mostrou oscilação entre os ZTs apenas no grupo LLC+T(ZTAlt), com a acrofase no ZT16 (figure 27E).

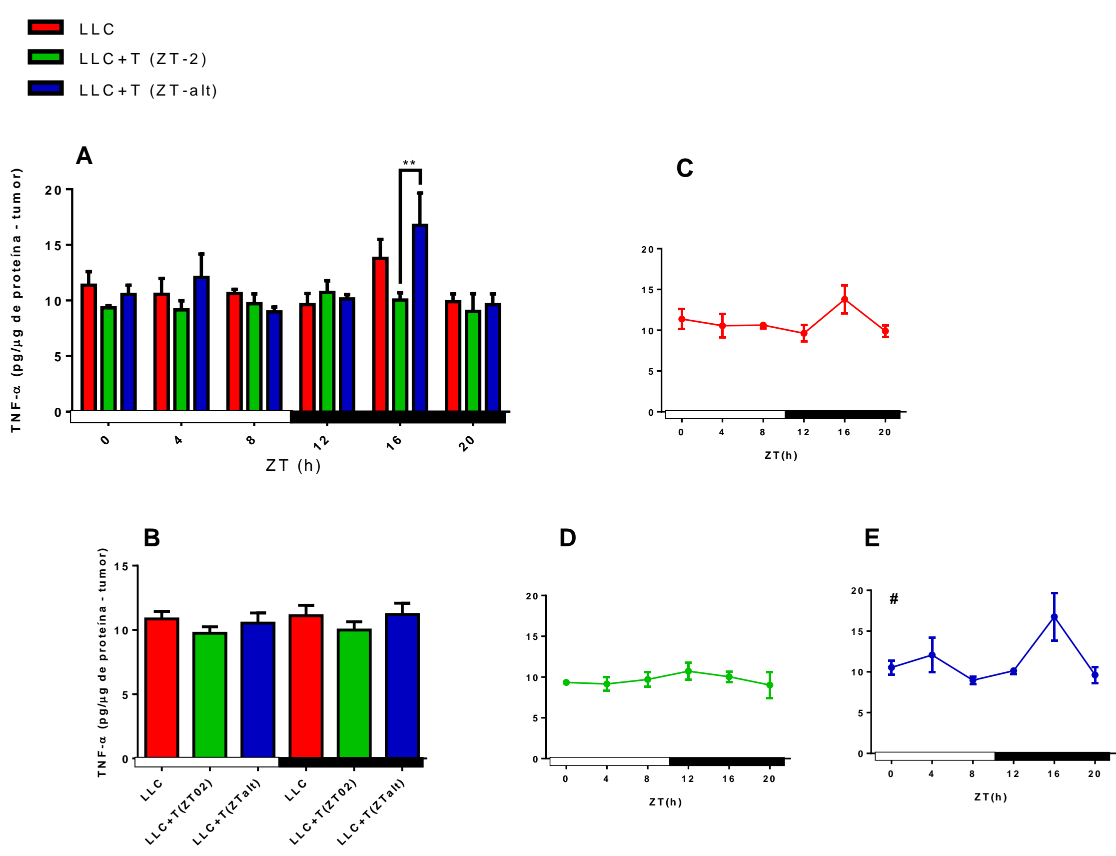


Figura 27. Concentração proteica de TNF-α no tecido tumoral - Comparação do TNF-α entre os grupos em cada ZT(A). Comparação entre o período claro e escuro do TNF-α(B). Efeito do ritmo circadiano do TNF-α nos grupos LLC(C), LLC+T(ZT2)(D) e LLC+T(ZTAlt)(E). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

Na análise feita na concentração da IL-1β no tumor, não houve diferença estatística entre os grupos em nenhum ZT (figura 28A). Entre o período claro e escuro o grupo LLC+T(ZTAlt) apresentou maior concentração no período claro quando comparado com o escuro (figure 28B). Os três grupos apresentaram oscilação da concentração da IL-1β entre os ZTs, com a acrofase no ZT0 (figura 28C, D e E).

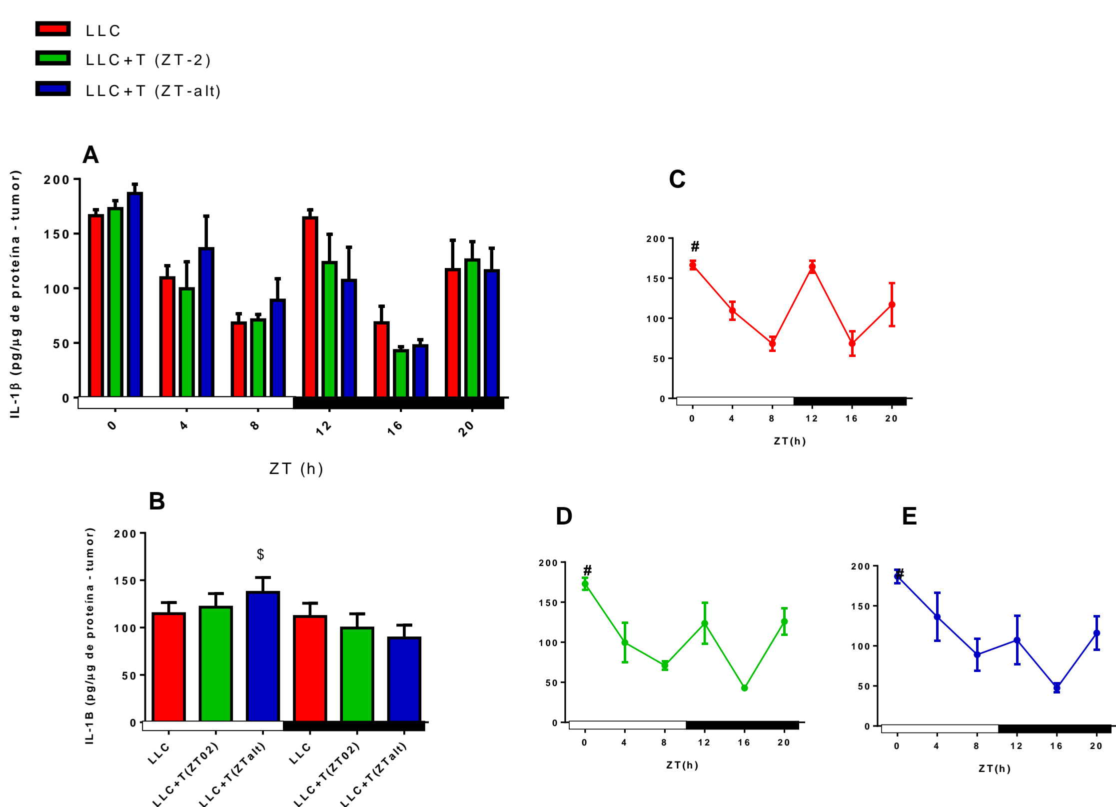


Figura 28. Concentração proteica da IL-1β no tecido tumoral - Comparação da IL-1β entre os grupos em cada ZT(A). Comparação entre o período claro e escuro da IL-1β(B). Efeito do ritmo circadiano da IL-1β nos grupos LLC(C), LLC+T(ZT2)(D) e LLC+T(ZTAlt)(E). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

MCP-1 no tumor apresentou aumento significante no ZT12 no grupo LLC quando comparado com o grupo LLC+T(ZT2) (figura 29A). No grupo LLC, a concentração de MCP-1 apresentou diminuída no período claro quando comparado com o período escuro (figura 29B). Não foi observada diferença estatística entre os ZTs nos três grupos (figura 29C, D e E).

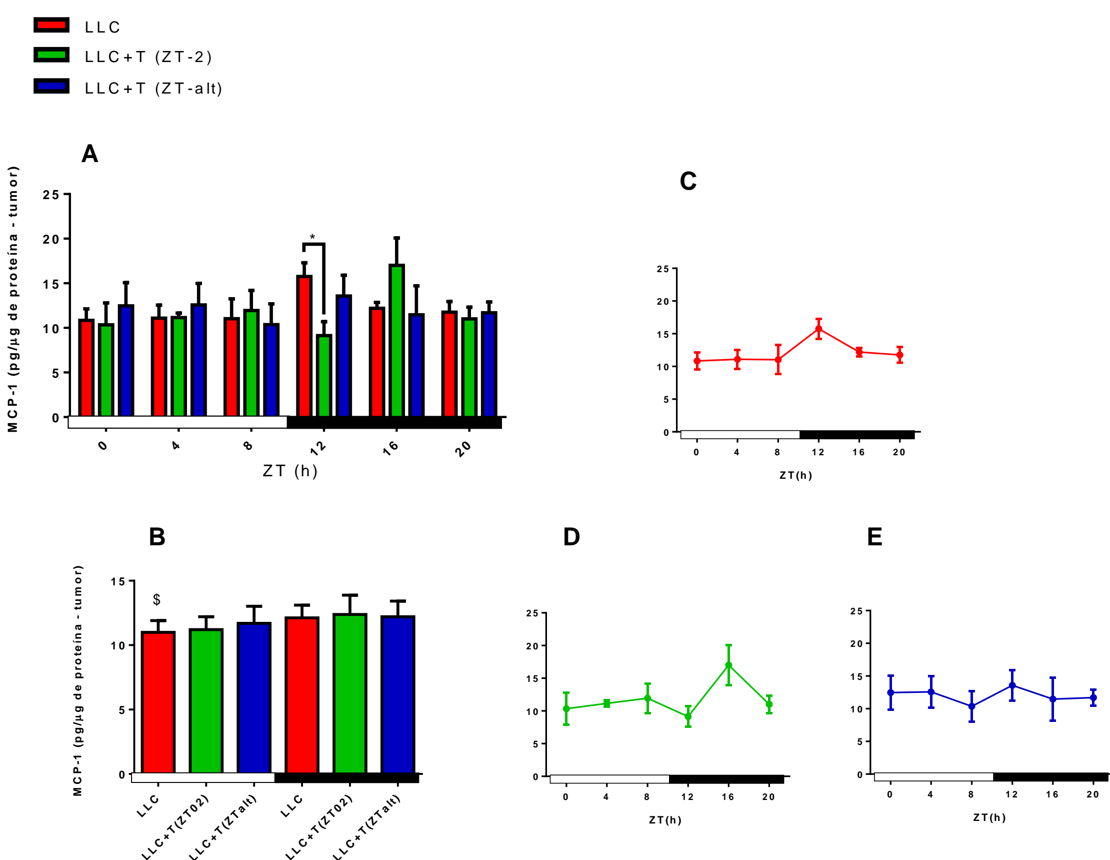


Figura 29. Concentração proteica de MCP-1 no tecido tumoral - Comparação da MCP-1 entre os grupos em cada ZT(A). Comparação entre o período claro e escuro da MCP-1(B). Efeito do ritmo circadiano da MCP-1 nos grupos LLC(C), LLC+T(ZT2)(D) e LLC+T(ZTAlt)(E). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

IFN-γ no tumor não apresentou diferenças estatísticas entre os grupos e tempos (figura 30A, C, D e E). Entre o período claro e escuro, IFN-γ apresentou diferença estatística no grupo LLC, com aumento no período claro (figura 30B).

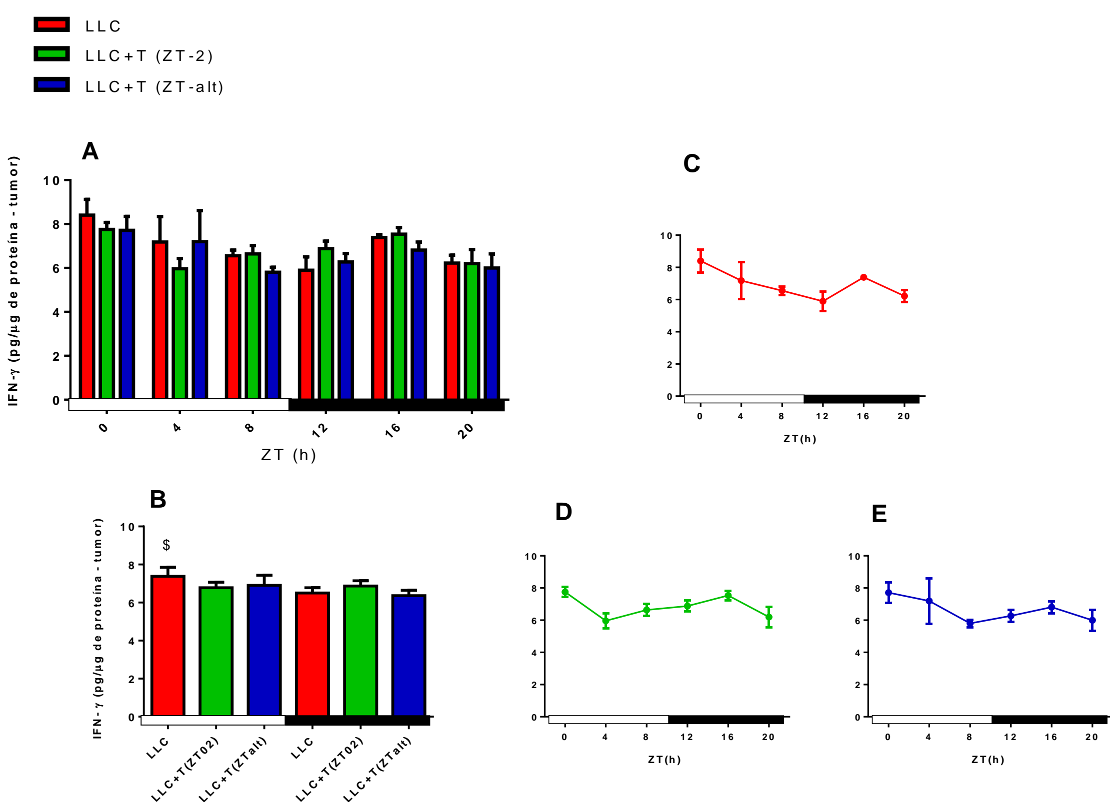


Figura 30. Concentração proteica de IFN-γ no tecido tumoral - Comparação do IFN-γ entre os grupos em cada ZT(A). Comparação entre o período claro e escuro do IFN-γ(B). Efeito do ritmo circadiano do IFN-γ nos grupos, LLC(C), LLC+T(ZT2)(D) e LLC+T(ZTAlt)(E). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

VEGF no tumor não apresentou diferenças estatísticas entre os grupos, período e tempo (figura 31).

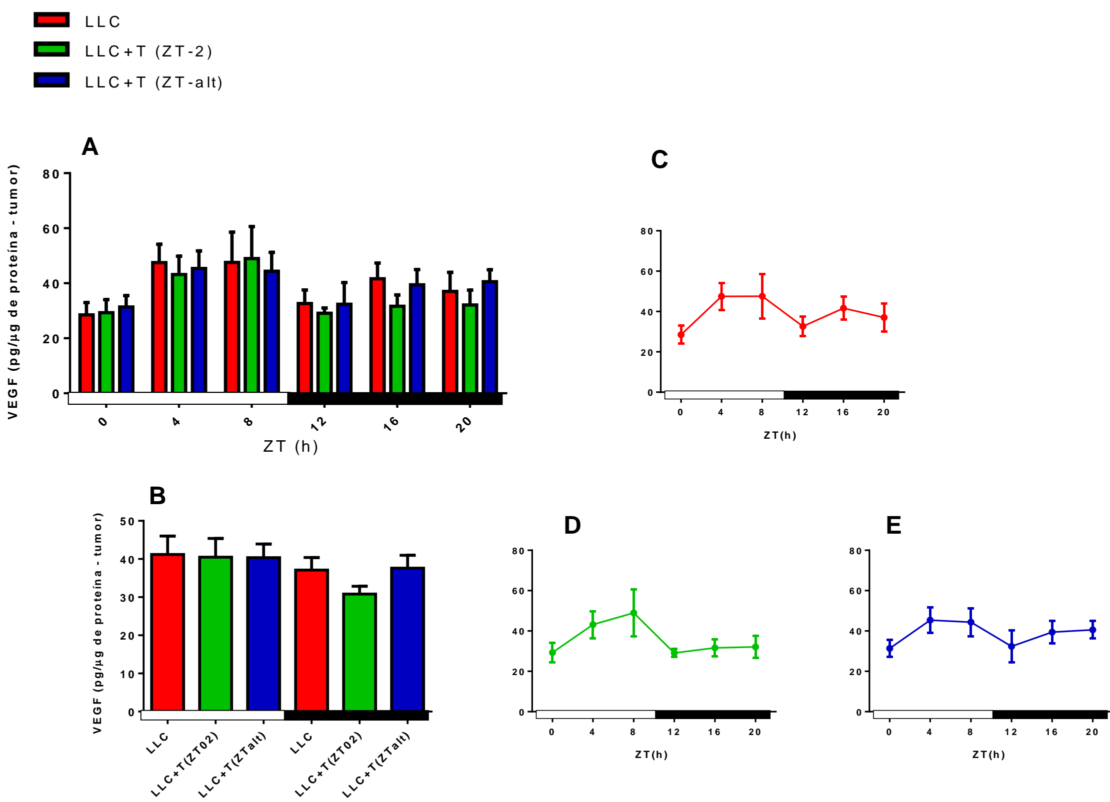


Figura 31. Concentração proteica de VEGF no tecido tumoral - Comparação do VEGF entre os grupos em cada ZT(A). Comparação entre o período claro e escuro do VEGF(B). Efeito do ritmo circadiano do VEGF nos grupos LLC(C), LLC+T(ZT2)(D) e LLC+T(ZTAlt)(E). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

A concentração proteica da IL-10 no tumor mostrou aumentada no grupo LLC+T(ZTAlt) quando comparado com o grupo LLC no ZT4 (figura 32A). Entre o período claro e escuro não houve diferença estatísticas em nenhum grupo (figura 32B). Os 3 grupos apresentaram oscilação entre os ZTs, com a acrofase no ZT0 para o grupo LLC e LLC+T(ZT2) e no ZT4 para o grupo LLC+T(ZTAlt) (figura 32C, D e E).

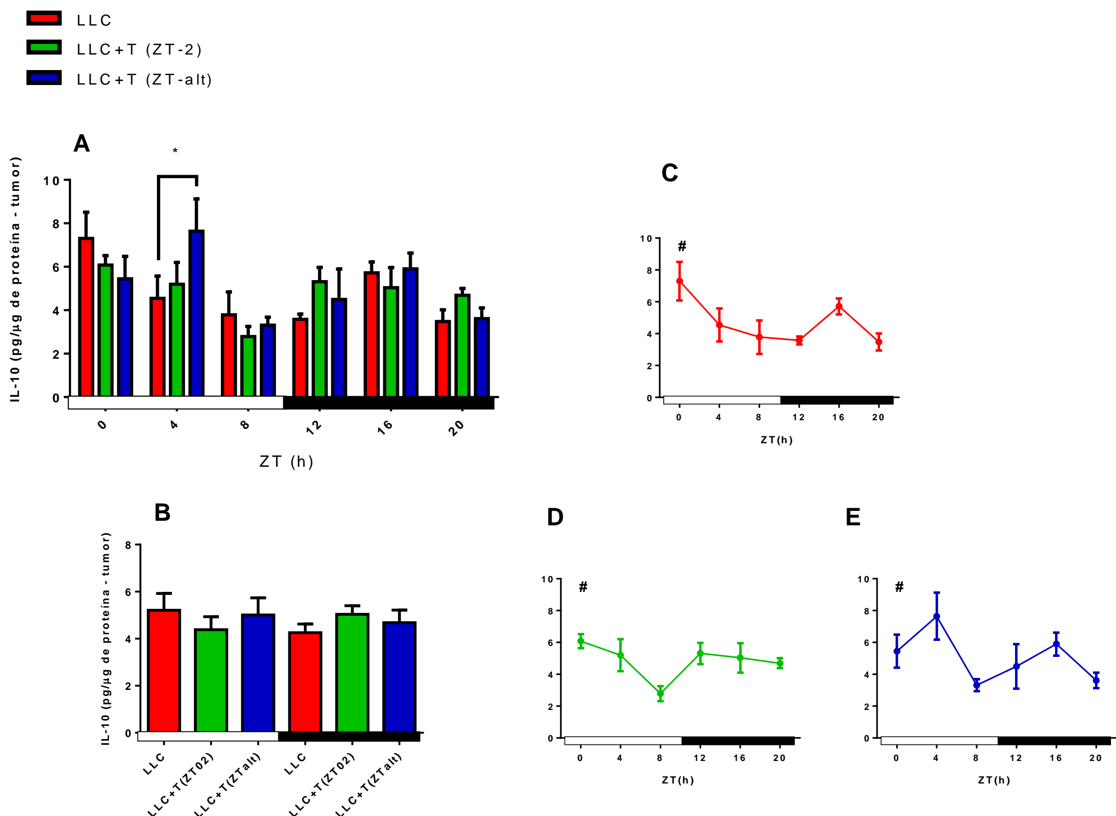


Figura 32. Concentração proteica da IL-10 no tecido tumoral - Comparação da IL-10 entre os grupos em cada ZT(A). Comparação entre o período claro e escuro da IL-10(B). Efeito do ritmo circadiano da IL-10 nos grupos LLC(C), LLC+T(ZT2)(D) e LLC+T(ZTAlt)(E). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

A IL-4 mostrou mais expressa no grupo LLC+T(ZTAlt) no ZT4 quando comparado com os grupos LLC e LLC+T(ZT2)(figura 33A). O grupo LCC apresentou maior concentração da IL-4 no período claro quando comparado com o escuro (figura 33B). Entre os ZTs apenas foi observado oscilação no grupo LLC+T(ZTAlt), com a acrofase no ZT4 (figura 33E).

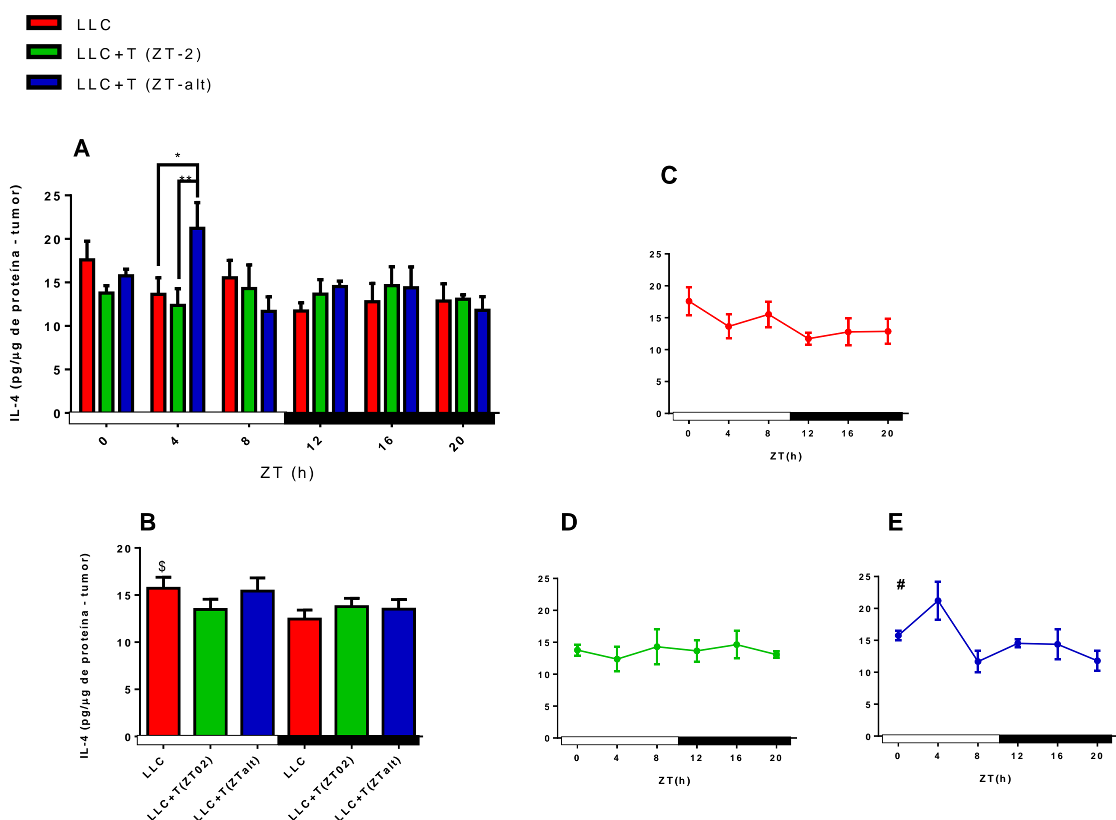


Figura 33. Concentração proteica da IL-4 no tecido tumoral - Comparação da IL-4 entre os grupos em cada ZT(A). Comparação entre o período claro e escuro da IL-4(B). Efeito do ritmo circadiano do IL-4 nos grupos LLC(C), LLC+T(ZT2)(D) e LLC+T(ZTAlt)(E). O tumor foi inoculado em camundongos da linhagem background C57BL/6J entre 8 a 10 semanas de vida. No terceiro dia após a inoculação do tumor iniciou o treinamento aeróbio a 60% do teste máximo. Dados representam a média ± desvio padrão da média *p<0,05, **p<0,01, ***p<0,001 entre os grupos (Anova two-way seguido por Tukey), #p<0,05 entre os ZTs e $p<0,05 entre os períodos (Anova one-way seguido por Tukey).

7 DISCUSSÃO

Os mecanismos envolvidos na inibição do crescimento tumoral e melhora do metabolismo muscular, com consequente desfecho para a obtenção de uma melhora na qualidade de vida em pacientes com tumor que aderem ao treinamento físico, podem estar correlacionados com o papel cronomarcador do exercício físico. Por meio de análises dos *clock genes* no músculo gastrocnêmio, foi possível verificar a oscilação circadiana dentro das 24 horas e como o exercício físico modula esses genes durante o crescimento tumoral.

Em suma, os resultados mostraram que o exercício físico foi capaz de atenuar o crescimento tumoral no grupo que treinou em horário alternado e teve efeito protetor no músculo no grupo que treinou sempre no mesmo horário do dia (tabela 2 e figura 3). No músculo esquelético gastrocnêmio, o exercício em horários alternados promoveu mudança na expressão dos genes controladores do relógio, principalmente no ZT4 (figuras 7, 9, 10 a 11). No grupo que treinou no ZT2, foram observadas mudanças nos genes Clock e Per2 (figuras 7 e 10). O tumor apenas causou alterações nos genes Rev-Erbα e Rorα (figuras 14 e 15). Outro dado interessante observado no grupo que treinou no ZT2 foi à relação da expressão gênica AMPK e Atrogina-1, enquanto no período claro a AMPK estava aumentada, a Atrogina-1 esteve diminuída quando comparada com o período escuro (figuras 17 e 18), dados esses que corroboram para o peso dos músculos sóleo e EDL, que foram maiores quando comprado com o grupo LLC (tabela 2). Ainda foi observado que nos animais sedentários (não treinados) não apresentaram oscilação dos *clock genes.* Já com a adoção do treinamento físico, seja ele em horários alternados ou no mesmo horário do dia observou um estabelecimento de ritmicidade no músculo esquelético. Nas análises da concentração proteica no músculo gastrocnêmio, o grupo com tumor sedentário apresentou maior concentração de TNF-α, VEGF e IL-10 no meio do período escuro (figuras 20, 24 e 25). Nesse mesmo tempo, o grupo que treinou no ZT2 teve aumento da concentração da IL-4 (figura 26). No tecido tumoral, as citocinas IL-10 e IL-4 (figuras 32 e 33) tiveram maiores concentrações no grupo que treinou em horários alternados no meio do período claro.

7.1 Efeito do exercício físico e do tumor no ritmo circadiano da glicose, colesterol total e triacilglicerol

O ritmo circadiano é um processo biológico que ocorre aproximadamente a cada 24 horas. Os processos comportamentais e fisiológicos apresentam ritmicidade que variam de acordo com a espécie animal e também com o próprio tipo celular. Esta ritmicidade permite antecipar e adaptar-se às mudanças ambientais diárias. O sistema circadiano é importante na regulação do ritmo diário do metabolismo, incluindo o metabolismo da glicose (116,117).

Perturbação deste controle circadiano ou de sua coordenação em relação ao ciclo ambiental/comportamental, como mudanças frequentes nos horários de comer, alterações genéticas, jet lag, doenças como câncer resultam em alterações circadianas, sendo uma dessas alterações nas concentrações glicêmicas, aumentando, por exemplo, o risco de desenvolver diabetes tipo 2(116). Estudos com animais mostraram que a deleção de Bmal1 em vários tecidos, causaram diferentes efeitos na homeostase da glicose(117–120). A deleção de Bmal1 no tecido muscular promoveu redução da expressão de glut4(121). A deleção de Bmal1 no músculo esquelético também altera o metabolismo lipídico, reduzindo o peso da massa adiposa e da colesterolemia, reduziu também o acumulo de gordura ectópica no músculo, quando os animais foram alimentos com dieta hiperlipídica. Esses dados demonstram como Bmal1 pode alterar tanto o metabolismo de glicose quanto o metabolismo de lipídeos(122).

Além do mais, para gerar energia e manter o equilíbrio redox (ATP) as células utilizam glicose. As células e os tecidos não cancerígenos ou normais dependem principalmente da fosforilação oxidativa, que ocorre na mitocôndria e é o processo mais energeticamente eficiente. Somente na ausência de oxigênio, as células não cancerígenas deslocam seu metabolismo para a glicólise. O metabolismo das células cancerígenas difere das células não cancerígenas(123), porém, a natureza exata da diferença do metabolismo dessas células continua a ser elucidada e debatida. A glicose é um dos principais metabolitos utilizados pelas células cancerígenas para gerar ATP, manter o estado redox e criar biomassa(123,124). A inatividade física é conhecida por estar associada a alterações da homeostase metabólica, causando diminuição na sensibilidade à insulina, alterações no perfil lipídico e acumulo de

gordura visceral(125) e o exercício físico é conhecido por ser capaz de prevenir doenças crônicas, como a diabetes tipo 2, por diminuir a concentração glicêmica(126).

Nas análises deste estudo, observa-se que o grupo LLC teve maior concentração de glicose no ZT8 quando comparado com o grupo que treinou em horários alternados, coincidentemente, esse grupo de treino alternado também apresentou um menor peso no tecido tumoral. É digno de notar, que o mesmo grupo de animais que apresentou redução na glicemia é o mesmo que apresentou menor massa tumoral, assim podemos supor que houve competição entre os tecidos pela glicose, atenuando parcialmente o crescimento tumoral, mas esta observação depende de comprovação.

Além do relógio circadiano controlar a fosforilação oxidativa(127) e a homeostase redox(128), esse mecanismo também é capaz de controlar o metabolismo lipídico(129). Estudos epidemiológicos sugerem que o risco de câncer é maior para as pessoas com níveis elevados de colesterol(130) e/ou níveis de TAG(131,132). Além do câncer as alterações lipídicas podem levar a outras patologias, incluindo diabetes tipo 2 e doenças cardiovasculares(116). Nosso trabalho mostrou que o TAG apresenta grande oscilação circadiana nos 4 grupos, no entanto, no colesterol total essa oscilação só foi observada no grupo que treinou no ZT2. Tanto o colesterol total como o TAG estavam aumentado no grupo LLC+T(ZT2) no meio do período escuro (ZT16), enquanto o grupo com treino alternado apresentou aumento de colesterol total no ZT12. O grupo apenas com tumor teve uma tendência de aumento de colesterol no meio do período claro.

Ensaio de lipidômica realizada em humanos saudáveis mostram variação significativa de picos de diferentes espécies de lipídeos medidos entre indivíduos, sugerindo a existência de cronotipo metabolômico(133). Para que os ácidos graxos esterificados dentro do TAG em circulação entrem em algum tecido, deve haver a atividade da lipotreina lipase (LPL), no qual essa enzima exibe um padrão diurno em vários tecidos, atingindo um pico durante a fase ativa no tecido adiposo(134). A quebra do TAG em 3 moléculas de ácidos graxos e 1 de glicerol e a mobilização desses ácidos graxos livres a partir do tecido adiposo também apresentam uma ritmicidade, os quais são aumentados durante a fase clara de roedores alimentados *ad libitum*(116,135).

7.2 Efeito do exercício físico e do tumor no ritmo circadiano da expressão gênica dos C*lock Genes,* Ampk e Atrogina-1 no músculo esquelético gastrocnêmio

O músculo esquelético representa o maior órgão do corpo humano compreendendo aproximadamente 40% da massa corporal total e contêm 50-75% de todas as proteínas do corpo, desempenhando um papel central no metabolismo de proteínas de todo o corpo, servindo como o principal reservatório de aminoácidos na ausência de ingestão de nutrientes e permitindo a manutenção da síntese de proteínas em outros tecidos do corpo(136). No geral, as funções do músculo esquelético são críticas para a saúde sistêmica, e a redução da massa e das funções musculares podem levar ao desenvolvimento de muitas doenças crônicas(12).

A manutenção do ritmo circadiano está emergindo como um novo fator importante na saúde humana, com interrupções ligadas ao envelhecimento, bem como ao desenvolvimento de muitas doenças crônicas, incluindo diabetes, doenças cardiovasculares, depressão e câncer(137,138).

Estudos têm mostrado que o exercício pode modificar o ritmo do relógio circadiano no músculo esquelético(20,139,140), porém o momento ideal da prática do exercício físico para a saúde e o para sincronizar o ritmo circadiano interrompido ainda não foram totalmente elucidados(54).

A natureza do treinamento físico envolve sessões repetitivas de exercícios que desafiam a homeostase de todo o corpo, levando a adaptações generalizadas em células, tecidos e órgãos(141,142). Embora as adaptações biomecânicas e metabólicas tenham sido extensivamente estudadas no músculo esquelético, coração, tecido adiposo e sistema imune, pouco sabe sobre a interação do exercício físico com o crescimento tumoral(143).

Diversos estudos ligaram a interrupção da função do relógio circadiano à tumorigênese(28–30,32,144,145). Os estudos até o momento indicam que Clock e Bmal1 podem exercer funções supressoras no desenvolvimento do tumor(146). Em humanos, os polimorfismos de nucleotídeo nos genes Clock e/ou Bmal1 estão associados ao aumento da suscetibilidade ao câncer de próstata, mama, ovário e pâncreas(147–152).

Além disso, estudos mostraram que o exercício pode impactar no comportamento circadiano(110,153). Evidências sugerem que genes responsivos ao exercício, incluindo AMPK(154–156), HIF-1α(139) e PGC1α(157) influenciam a

expressão dos genes controladores do relógio, revelando um potencial mecanismo através do qual o exercício serve como marcador no ritmo circadiano. Por exemplo, a atividade da AMPK é aumentada em resposta ao exercício físico e o aumento da atividade da AMPK altera a estabilidade das proteínas Per e Cry e por consequência afeta a expressão dos genes do relógio circadiano central(154,158). Especificamente, a ativação de AMPK resulta em estabilidade reduzida de Cry1(158), ainda as proteínas Cry1 e Cry2 reduzem a capacidade do exercício por meio da repressão PPARδ(159), sugerindo então que a atividade da AMPK influencia diretamente o relógio molecular, revelando um mecanismo pelo qual o exercício atua como marcador de tempo circadiano. O exercício também induz a expressão de HIF-1α, que influencia a saída do relógio molecular através da ligação direta aos promotores do gene do relógio no núcleo(139). Assim, o HIF-1α pode mediar parcialmente os efeitos do exercício como um indicador de tempo (160).

O efeito mecânico do exercício físico no músculo esquelético pode contribuir para alterações no relógio circadiano, embora a regulação relacionada ao exercício da expressão dos genes controladores de relógio seja provavelmente multifatorial (160,161).

Esses dados talvez ajudem a explicar alguns dos achados deste estudo, pois o exercício físico foi capaz de mudar a expressão de alguns dos *clock genes*. Ademais, diversos estudos têm demonstrado que a ativação da AMPK inibe o crescimento tumoral(162,163). Nosso trabalho mostra que a prática do exercício físico na mesma hora do dia foi capaz de aumentar a expressão de AMPK no período claro quando comparado com o período escuro. Outro dado interessante nesse grupo que treinou sempre no mesmo horário do dia foi a diminuição da expressão da Atrogina-1 no período claro, mostrando que o exercício físico pode ter um efeito protetor no músculo esquelético, pois o gene Atrogina-1 está associado a um importante mecanismo de degradação muscular(164), além disso, esse grupo que treinou no ZT2 apresentou maior peso nos músculos EDL e sóleo quando comparado com o grupo que apenas possuía o tumor.

Um aspecto importante do câncer é a perda exacerbada do músculo esquelético, conhecida como caquexia do câncer(165), e um dos fatores indutores de proteólise que está relacionado com a caquexia do câncer é o TNF-α(166). Corroborando o exposto deste trabalho, as concentrações de TNF-α no músculo esquelético no ZT16 se mostraram aumentadas apenas no grupo LLC, e esse

aumento não foi observado nos grupos que praticaram exercício físico, mostrando mais uma vez o efeito protetor do exercício físico.

Portanto o treinamento praticado na mesmo hora do dia mostrou promover um efeito protetor no músculo esquelético. Esses efeitos não foram observados no grupo que treinou em horários alternados, entretanto, esse grupo apresentou menor massa tumoral. Uma das hipóteses para esse resultado é que talvez o treinamento praticado em horários alternados tenha causado mudanças no ritmo circadiana no tumor, essas mudanças no relógio circadiano no tumor podem ter contribuindo para atenuar o seu desenvolvimento, mas esta observação depende de comprovação.

7.3 Efeito do exercício físico e do tumor no ritmo circadiano da concentração das citocinas no músculo esquelético gastrocnêmio

As citocinas apresentam como principal função a regulação do sistema imunológico. No entanto, as citocinas também promovem efeitos na proliferação, diferenciação, migração, sobrevivência e apoptose celulares, permitindo assim que tenham um desempenho não apenas no sistema imunológico, mas exercendo um papel no controle homeostático de vários tecidos, órgãos e sistemas(167).

O músculo esquelético é reconhecido como órgão endócrino por secretar uma série de citocinas e proteínas, denominadas miocinas. As miocinas são sintetizadas e secretadas pelos miócitos em resposta à contração muscular, exercendo uma função autócrina na regulação do metabolismo muscular, bem como possuem atuação parácrina/endócrina em órgãos e tecidos distantes, como osso, tecido adiposo, cérebro e fígado(168), porém o efeito das miocinas no tumor ainda não está claro.

Essas descobertas estimularam um interesse considerável no significado biológico e na regulação da produção de citocinas no músculo durante o exercício e como resultado da sepse, envelhecimento, caquexia do câncer e doenças inflamatórias crônicas(167), assim, muitos benefícios do exercício físico a saúde pode estar relacionada com a secreção das miocinas(125).

A inflamação é uma resposta imunológica desencadeada em organismos vivos em resposta ao perigo associado a patógenos e lesões. A inflamação pode variar ao longo de 24 horas. Por exemplo, na sepse, a inflamação mostra uma grande variação diurna(169). A secreção das citocinas também apresentam um ritmo circadiano em diversos tecidos e células imunes(170,171).

A concentração do TNF-α é elevada na caquexia do câncer e alguns estudos têm mostrado que pode exercer efeitos catabólicos no músculo esquelético(172,173). A IL-10 é considerada uma citocina antiinflamatória e a relação IL-10/TNF-α é amplamente utilizada como um índice que reflete o estado anti-inflamatório (174,175). Em nosso trabalho, foi observado que as citocinas TNF-α, IL-10 e IL-4 no músculo apresentaram maior concentração no período escuro. Além disso, no ZT16, é observado um aumento de TNF-α e IL-10 no grupo com tumor sem exercício quando comparado com os grupos controle e exercício no horário alternado.

Interessantemente, o grupo que treinou no ZT2, apresentou aumento da IL-10 no ZT16 quando comparado com o grupo que treinou em horário alternado e IL-4 também estava aumentado nesse grupo, quando comparado com os grupos controle e treinamento em horários alternado. O treinamento físico pode aumentar a síntese de proteína do músculo esquelético, diminuir os efeitos catabólicos da caquexia e controlar a inflamação(176). Nos últimos anos o treinamento físico aeróbio tem sido considerado uma excelente ferramenta não farmacológica para reduzir a inflamação crônica de baixo grau(177), que é um fator de risco significativo para o desenvolvimento de câncer(178).

7.4 Efeito do exercício físico e do tumor no ritmo circadiano da concentração das citocinas no tecido tumoral

O microambiente tumoral contém uma variedade de células estromais não tumorais, incluindo fibroblastos, células endoteliais, miócitos e células inflamatórias, tais como células supressoras derivadas de mielóide, células T reguladoras, macrófagos e células dendríticas(179,180), além de proteínas da matriz extracelular e fatores promotores de tumor (por exemplo, citocinas, fatores de crescimento, etc)(181). As células imunológicas, quando orquestradas com as células cancerígenas e células estromais circundantes, formam um microambiente tumoral inflamatório(182).

A inflamação pode moldar o microambiente tumoral para um estado mais maligno e direcionar os sinais promovendo o crescimento tumoral. Estudos transcriptômicos e metabolômicos do microambiente tumoral indicam concentrações aumentada das citocinas inflamatórias e quimiocinas em tumores primários e lesões

metastáticas, o que está correlacionado com um número aumentado de infiltrados inflamatórios e um pior prognóstico clínico(183,184).

A neutralização ou silenciamento genético da sinalização inflamatória em modelos pré-clínicos diminui o crescimento e a progressão do tumor(185).

A interação das células tumorais com as células estromais leva à produção de uma matriz de mediadores que fornecem uma estrutura no qual as células tumorais crescem, invadem e promovem a metástase(179,180,186). Estes mediadores incluem metaloproteinases da matriz, fatores de crescimento, citocinas e quimiocinas, tais como a MCP-1.

Neste trabalho, o grupo sedentário apresentou maior concentração de MCP-1 no tumor no ZT12 quando comparado com o grupo que treinou sempre no mesmo horário. Entretanto, poucos trabalhos mostram o efeito do exercício físico sobre a MCP-1 no tecido tumoral. Um estudo realizado em 2014 mostrou que 12 semanas de exercício físico foi capaz de diminuir o número de pólipos, porém não houve diminuição de MCP-1(187). Outro estudo realizado pelo mesmo grupo analisaram os níveis circulantes de MCP-1 em um modelo de câncer de mama C3(1)SV40Tag e observaram diminuição significativa no MCP-1 plasmático após 20 semanas de exercício físico que foi associada a redução no número e volume do tumor(188).

Dentro do microambiente tumoral, a IL-10 mostrou ser um paradoxo em termos de resposta imunológica, provavelmente resultante de sua ação pleiotrópica em uma variedade de células do sistema imunológico. De fato, na maioria dos estudos de imunidade antitumoral, a IL-10 endógena é considerada o principal fator que contribui para a imunossupresão do microambiente tumoral(189–191). No entanto, paradoxalmente, evidências crescentes demonstram que IL-10 pode induzir efeitos antitumorais de uma maneira dependente do sistema imunológico(192–194). Nossos achados mostram que o exercício físico praticado em horários alternados mostrou aumento das citocinas IL-10 e IL-4 no tecido tumoral no ZT4, sendo que esse mesmo grupo teve menor peso tumoral quando comparado com o grupo sedentário. Sabe-se que o exercício físico é bem conhecido por promover efeitos anti-inflamatórios, uma vez que a IL-6 derivada do músculo esquelético inibe a produção de TNF-α e estimula a produção das citocinas anti-inflamatórias , como a IL-10(177). No tecido tumoral há poucas evidencias sobre o efeito do exercício físico e seu efeito anti-inflamatório.

As citocinas no microambiente tumoral ainda apresentam algumas controvérsias, e mais estudos são necessários para elucidar o seu papel no

desenvolvimento e crescimento tumoral. Por exemplo, estudos tem mostrado que os macrófagos no microambiente tumoral classificados como tipo M1 podem secretar citocinas pró-inflamatórias, como IL-1β, IL-6, IL-8, IL-18, TNF-α e IFN-γ, e exercem efeito antitumoral(195–197), porém o crosstalk das citocinas produzidas durante a contração muscular sobre o microambiente tumoral ainda precisam ser elucidadas.

Este trabalho mostrou que a concentração de TNF-α está aumentada no meio do período escuro no grupo que treinou em horários alternados quando compara com o grupo que treinou todos os dias no mesmo horário, além de ser o único grupo que houve oscilação circadiana no tumor entre os horários analisados. O TNF-α e seu receptor podem promover alterações circadianas, sendo que um estudo mostrou que o estimulo de LPS em camundongos promoveu aumento nas concentrações de TNF-α e consequentemente uma mudança de fase causada pelo aumento do TNF-α(198,199).

Contudo, as citocinas no microambiente tumoral ainda apresentam algumas controvérsias, e mais estudos são necessários para elucidar o seu papel no desenvolvimento e crescimento tumoral.

8 CONCLUSÃO

Neste trabalho, o exercício físico em horários alternados foi mais eficiente em reduzir a massa tumoral, porém o exercício físico praticado no mesmo horário do dia promoveu maior efeito protetor para o músculo esquelético, assim como preveniu a perda de massa muscular. Além disso, o exercício em horários fixos apresentou efeito cronomodularor, aumentando a expressão gênica de AMPK e reduzindo a expressão de Atrogina-1 no período claro quando comparado ao período escuro

Ainda no músculo gastrocnêmio, o exercício físico foi responsável pela oscilação dos *clock genes* dentre os horários analisados, elevando a expressão de Clock, Per1, Per2 e Per3, principalmente no período claro. Já no período escuro, o grupo que treinou no mesmo horário do dia apresentou aumento das citocinas anti-inflamatórias. No tecido tumoral, este efeito anti-inflamatório foi observado no grupo que treinou em horários alternados e durante o período claro. De modo geral, o exercício físico se mostrou um grande modulador do relógio circadiano no músculo esquelético, além de promover efeitos protetores ao músculo e mitigar o crescimento tumoral.

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Review article

Aging with rhythmicity. Is it possible? Physical exercise as a pacemaker

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A R T I C L E I N F O

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A B S T R A C T

Aging is associated with gradual decline in numerous physiological processes, including a reduction in metabolic functions and immunological system. The circadian rhythm plays a vital role in health, and prolonged clock disruptions are associated with chronic diseases. The relationships between clock genes, aging, and immunosenescence are not well understood. Inflammation is an immune response triggered in living organisms in response to the danger associated with pathogens and injury. The term 'inflammaging' has been used to describe the chronic low-grade-inflammation that develops with advancing age and predicts susceptibility to age-related pathologies. Equilibrium between pro-and anti-inflammatory cytokines is needed for healthy aging and longevity. Sedentary and poor nutrition style life indices a disruption in circadian rhythm promoting an increase in pro-inflammatory factors or leads for chronic low-grade inflammation. Moreover, signals mediated by pro-inflammatory cytokines, such as tumor necrosis factor-alpha and interleukin-6, might accentuate of the muscle loss during aging. Circadian clock is important to maintain the physiological functions, as maintenance of immune system. A strategy for imposes rhythmicity in the physiological systems may be adopted of exercise training routine. The lifelong regular practice of physical exercise decelerates the processes of aging, providing better quality and prolongation of life. Thus, in this review, we will focus on how aging affects circadian rhythms and its relationship to inflammatory processes (inflammaging), as well as the role of physical exercise as a regulator of the circadian rhythm, promoting aging with rhythmicity.

1. Introduction

Aging is characterized by a gradual decline in numerous physiological processes, including a reduction in metabolic function [1], increase in fat mass, reduction in lean mass [2], a decline in immune function leading to increased susceptibility to disease [3], and reduced cognitive performance [4,5]. Moreover, sleep and circadian rhythm activity measured under natural environmental conditions showed significant age-related alterations. Both sleep and activity rhythms become fragmented and attenuated in the elderly. The degeneration of the circadian timing system likely contributes to the age-related changes in sleep and the circadian rhythm [6–8].

Circadian rhythms exert control in several biological processes, such as sleep-wake cycles, body temperature, food intake, secretion of hormones and enzymes, glucose homeostasis, and regulation of the cell cycle. The control of circadian rhythms is regulated by central and peripheral clocks [9].

The term circadian rhythm was defined by Halberg et al. in 1959 to describe the association between the endogenous oscillation of many physiological factors and the earth's daily rotation [10,11].The circadian rhythm can be controlled by endogenous oscillators, which govern the rhythmic pattern of several physiological and behavioral functions [12]. Some environmental cycles adjust the oscillator, which generates the circadian rhythm, causing it to oscillate with the same cycle period [13]. The circadian timing system is vulnerable to aging, showing alterations with advanced age. The major circadian changes observed in aging include the reduction of the amplitude and earlier timing of the phase of daily rhythms. These alterations were observed in sleep-wake cycles, melatonin production, and body temperature [14].

Furthermore, the immune system exhibits rhythm in the control of its tasks. The first evidence of rhythmicity in the immune response was reported in the 1950s and showed that the host defense against pathogens (endotoxin) and mortality were dependent on the time of the day that the endotoxin was injected in mice [15]. Another study showed that the complex interactions between biological and physiological rhythms affect the immune response. For example, shift workers

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exhibited an elevated risk in the development of different diseases characterized by chronic inflammation, such as cancer, cardiovascular diseases, type 2 diabetes, and obesity [16]. Hence, several researchers have tried to understand the role of clock genes and the central clock in innate and adaptive immune systems, and we review this topic below.

Thus, in this review, we focus on the circadian rhythm by aging and its relationship to inflammatory processes (inflammaging), as well as the role of physical exercise as a modulator of the circadian rhythm, promoting aging with rhythmicity.

2. Mechanisms of the circadian rhythm

The circadian rhythm is controlled by circadian clock that are present in almost all mammalian tissues. The central clock is located in the suprachiasmatic nucleus of the hypothalamus (SCN), a small region of the brain containing 10,000–15,000 neurons [17]. The SCN clock can operate autonomously without any external input from stimuli but can be entrained by environmental stimuli, such as light. Clocks outside the SCN are referred to as peripheral clocks, but they are synchronized by the central clock to temporally ensure physiological coordination. In addition, peripheral clocks in liver or skeletal muscle can be synchronized by the availability of metabolites, feeding time, or physical exercise [18]. Peripheral clocks are present in almost all mammalian tissues, including the liver, heart, lungs, kidneys, and immune cells, in which circadian rhythms are maintained [19].

The circadian clock is controlled by interactions between the feedbacks looping of circadian genes in the nucleus of all cells of the body. The fundamental mechanism of rhythm maintenance is similar in central and peripheral clocks [19].

As master regulators, Circadian Locomotor Output Cycles Kaput (CLOCK) and Brain and Muscle ARNT-like protein 1 (BMAL1) form heterodimers, which interact to form a transcriptional activator complex that stimulates the transcription of the Period (PER1, PER2, and PER3) and Cryptochrome (CRY1and CRY2) genes (negative feedback). In turn, CRY and PER form a complex, which acts as the negative arm to inhibit the transcriptional activity of CLOCK/BMAL1 heterodimers [17,19,20]. Another regulatory mechanism is induced by the CLOCK/BMAL1 heterodimeric complex that activates the transcription of nuclear orphan receptors REV-ERBs (α and β) and RORs (α, β, and γ isoforms) [21,22]. REV-ERB and ROR subsequently compete for retinoic acid-related orphan receptor response elements present in the BMAL1 promoter. ROR activates the transcription of BMAL1 [23–25], whereas REV-ERB suppresses its transcription [17,19,20]. Thus, the circadian oscillation of BMAL1 is both positively and negatively regulated by RORs and REV-ERBs [19].

We observe the deregulation on central and peripheral clocks on the aging that affect directly on quality of life and life expectancy. Thus, we detailed bellow the consequences of aging on circadian rhythm and the early aging that may be observed in adults with disruption of circadian rhythms.

3. Aging and circadian rhythms disruption

The aging process can be characterized as the progressive impairment of homeostasis at genomic, cellular, tissue, and whole organism levels, which reduces survival and fertility while increasing the risk of disease and death [23].

The circadian rhythm plays a vital role in health, and prolonged clock disruptions are associated with negative health consequences. With advancing age, the circadian system undergoes significant changes that affect behavioral rhythms, temperature regulation, and hormones release, and these changes in circadian rhythms are related to poor quality sleep [14]. These changes in sleep quality and total sleep time are directly related to higher daytime sleepiness, poorer quality of life of the elderly, and cognitive impairment [14].

One of the factors observed with advancing age is a change in sleep architecture. Studies show that in humans, changes in sleep can begin in the fourth decade of life, leading to a decrease in total sleep time of up to 30 min every 10 years [26]. When compared with young adults, older adults experience more night awakenings, have longer latencies to fall asleep, and spend less time in stage 3 and rapid eye movement sleep [14,24].

Aging is related to the declined secretion of hormones, such as melatonin. Melatonin is a hormone produced by the pineal gland. During daylight, intense light blocks pineal melatonin production. However, as light intensity declines with dusk, the retina sends information through the retino-hypothalamic tract to the SCN in which this information is transmitted to the pineal gland to initiate melatonin production and release [25,27]. Melatonin release regulates core body temperature, promotes sleep onset, modulates the activity of intrinsically photosensitive retinal ganglion cells that provide timekeeping signals to the SCN [14,28], and controls the circadian oscillator system [29].

Furthermore, a decline in the secretion of melatonin is related to multiple alterations in the immune system that can be taken to the proinflammatory side [25,30], and studies have shown the relationship between melatonin and age-associated neuropathologies, such as Alzheimer's or Parkinson's disease [25].

In addition, the expression of the clock genes are found beyond of the central clock (SNC), as in a number of extra-SCN sites in the mouse brain, including the hippocampus, amygdala and the paraventricular, arcuate and dorsomedial nucleus of the hypothalamus, therefore the amplitude and phase of expression in these regions might be altered with the aging [31,32]. In the SCN, Clock, Bmal1 and Per2 have been showed decreases in the rhythm amplitude or its expression levels with the age advanced [31,33].

As previously discussed, the circadian clock consists of input pathways perceived mainly by the SCN and other brain areas; this signaling via SCN will synchronize peripheral tissues. Similar to the differential physiological impacts of aging on organ and tissue function, aging also appears to affect peripheral clocks to several degrees [34].

In peripheral tissue may be disturbance in the oscillations of the genes that control the circadian clock affected by aging. For example, the circadian clock regulates many genes involved in the responses to oxidative stress and cellular redox [35,36], and this mechanism can changes with aging [37].

Whereas, the aging is characterized by disruption on circadian rhythms, the opposite is true too. The chronic deraignment on circadian clock reduces the longevity on *Drosophila* [38,39]. In humans, the shift-workers show loss of circadian rhythms and consequently higher risk to develop metabolic abnormalities and early aging observed by shorter of leukocytes telomere length [40].

In accordance, the other life habits that they are able to induce the loss of circadian rhythm and cause the early aging. Obesity is the most well established risk factor able to induce the early aging. Thus, the obese young adults with higher insulin concentrations showed telomere shortening, an important parameter to show the non-chronological age [41]. Furthermore, when metabolic syndrome was present during the pregnancy the child showed 14% of reduction on telomere length [42]. Regarding to vascular aging, the obesity accelerate the early vascular aging in young obese [43,44].

Drugs, in special chemotherapy and radiotherapy are able to induce shorter of telomere and reduction on activity in fibroblast. The telomere dysfunction leads to reduction on life expectancy and genomic instability on somatic cell [45]. In this sense, doxorubicin is able to induce early senescence on human cardiac progenitor cells [46].

Moreover, the doxorubicin induces the senescence of mesenchymal stem cells with reduction on telomere length and activity, and induces the senescence associated secretory phenotype (SASP) that it is a trigger to immunesenescence [47]. Our group recently demonstrated that the doxorubicin treatment in vivo and in vitro disrupt the clock gene expression with concomitantly immunesupression [48].

The immune system modifications throw the life cause direct effect on aging process. Thus we discuss bellow the role of immune system and the circadian rhythms in aging.

4. Immune system, circadian rhythms and aging

Many parameters of the immune system show rhythmicity, including circulating white blood cells, hormones (cortisol and catecholamine), and cytokine production [49]. In humans, the peak of mature leukocytes (except for effector CD8T lymphocytes) occurs at night, whereas the secretion of cortisol, catecholamine, and pro-inflammatory cytokines reaches the maximum concentration during the day [49].

Furthermore, the activity of immune cells is tightly regulated by circadian rhythms. The response of lipopolysaccharide (LPS) is stronger at the end of the rest period or beginning of the active period than in the early rest period [50]. Moreover, recently was demonstrated that the sepsis causes disruption on circadian rhythms associated with sepsis severity [51]. In addition, the phagocytosis ability of intraperitoneal macrophages shows circadian rhythms. Specifically, their phagocytic activity is enhanced at the end of the rest period in comparison with all other times of the day [52]. CRY1 and CRY2 double knockout caused several disturbs on many aspects of immunity and the mice showed increase on autoimmune diseases [53].

The relationships between clock genes, aging, and immunosenescence are not fully clarified, but there is the emergency in clarify this complex interplay to improve the immunity in elderly. Monocytes and macrophages show great rhythmicity with a high amplitude of clock gene expression [50]. Our group observed that the control of clock genes in macrophages is very well-controlled [48].

On the past year, we observed the intrinsic pathway between circadian rhythm, clock gene on the function of group 3 innate lymphoid cells (ILC3) and gut homeostasis [54,55]. Godinho-Silva and colleagues showed that conditional depletion of BMAIL1, a master a circadian activation on hematopoietic cells affected severely the ILC3 causing hyperinflammation, gut disbiosis and disorder on lipid metabolism, and the environmental cues as light-dark cycles, feeding schedule and microbiota impact in clock machinery in ILC3 and consequently on intestinal homeostasis [55]. In accordance, the BMAL1 conditional deletion on ROR-γ T cells showed the disruption on ILC3 with increased pro-apoptotic and inflammatory pathways [56]. Noteworthy, that the gastrointestinal tract show a concentration 400 times higher of melatonin that the pineal gland, what it exemplify the interplay between circadian clock and gut homeostasis [57]. This may explain the gut derangement during in the aging process.

The rhythmicity of the immune response in aging is impaired, but the cause of this is unclear. It was observed that the elderly showed dysregulation of circadian rhythms in T helper lymphocytes [58]. Furthermore, the neuroendocrine-immune axis lost circadian rhythm expression in elderly people [59,60]. Another cause of immunosenescence can be related with reduction in melatonin secretion since this hormone is able to induce the production of macrophages, granulocytes, and lymphocytes [30].

Many alterations are found in the immune system during aging. For example, humans lose the better response to immunization, with a decrease in the effectiveness of vaccines and an increase in autoimmunity diseases and inflammaging (see below) [59].

The role of clock genes in innate immunity is more clear, but few studies have shown the role of clock genes in adaptive immunity [61]. T-cells express clock genes, and this clock machinery is highly associated with cytokine responses [62]. Interestingly, in the last year, Nobis and colleagues showed that the effectiveness of vaccines is dependent on the time of day. A more pronounced response occurred after the vaccination in the middle of the day, and the deletion of BMAL1 in dendritic cells or CD8 lymphocytes abrogated this circadian rhythmicity response to vaccination [63].

A perspective very important in this moment, during the COVID-19 pandemic, it is evaluate the clock genes expression in dendritic cells and lymphocytes that may explain the impair on vaccination response making possible that induction of regulation of clock genes expression in immune cells, so mitigate the effects of immunosenescence on efficacy of vaccination.

The immunosenescence can be better characterized and marked in cells of the adaptive immune system [64,65]. One of the main changes in the adaptive immune system is the replacement of naive T and B cells by memory cells [65,66]. The changes in the aging adaptive immune system occur in the T cell compartment [67], this increase in the number of memory T cells and, at the later, that of B cells may be due to a continuous chronic antigenic stimulation of inflammatory process [68,69]. Therefore, with advancing age, a deterioration of immunity can be observed, while some cells remain unchanged or others tend to have greater activity or hyperactivity [70–72].

Moreover, the immunosenescence should be defined by senescence phenotype on lymphocytes, with increase on end stage of differentiation of CD4 and CD8 T cells and B lymphocytes, and reduction on naïve circulating lymphocytes. Moreover, the chronological or non-chronological aging present inflammaging that will be discussed in the next topic.

5. Clock genes deregulation and inflammaging on elderly

Inflammation is an immune response triggered in living organisms in response to the danger associated with pathogens and injury. Inflammation may vary over 24 h. For example, in sepsis, inflammation shows a large diurnal variation [73]. On the other hand, studies have shown the relationship between clock genes and inflammatory processes. For example, CLOCK can directly interact with the p65 subunit of NF-kB to enhance its transcriptional activity on the promoters of inflammatory genes [74]. Additionally, the deletion of CRY proteins augments both basal and inducible inflammatory responses, as CRY1 and CRY2 knockout mice show increased expression of TNF-alpha [75].

However, BMAL1 may promote an anti-inflammatory effect when in a heterodimer with CLOCK, thus preventing the interaction of CLOCK with the p65 subunit of NF-kB, and BMAL1 deficiency may result in chronic inflammation [74]. Moreover, NF-kB activation is under the control of a feedback loop represented by the clock genes BMAL1/CLOCK and their transcriptional regulators RORα (positive) and REV-ERBα (negative). Recently, Zhao et al. (2019) showed that the forced increased expression of REV-ERBα reduced the secretion of pro-inflammatory cytokines induced by LPS via the inhibition of TLR-4-NF-kB activation [76].

Interestingly, cytokines are able to modify clock genes. TNF-α in synovial cells culture reduces the expression of PER2 and REV-ERBα [77]. Moreover, this cytokine increased the expression and association between BMAL1 and RORα [78]. The underlying mechanisms involved in the alteration of clock genes after cytokine stimuli are not well understood, but this is a two-way road.

A balance between pro- and anti-inflammatory cytokines is needed for healthy aging and longevity. A disruption in circadian rhythm may alter this balance, favoring an increase in pro-inflammatory factors or causing chronic low-grade inflammation. In the last two decades, the term 'inflammaging' has been used to describe the chronic low-grade inflammation that develops with advancing age and predicts susceptibility to age-related pathologies [79].

Chronic inflammation is associated with many age-related physiologic or pathophysiologic processes and diseases. Even in healthy aging, serum concentrations of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, IL-12, IL-15, IL-17, IL-18, TNF-α, and IFN-γ) are significantly increased in comparison with younger individuals [80]. In the same way that the concentrations of pro-inflammatory cytokines are increased in the elderly, anti-inflammatory cytokines are also increased (IL-1Ra, IL-4, and IL-10) [80]. This balance between pro- and anti-inflammatory cytokines occurs in an attempt to protect tissues. The imbalance between the

action of pro- and anti-inflammatory cytokines is related to unhealthy aging and the development of some age-related pathological conditions [81].

Other factors that contribute to chronic low-grade inflammation are physical inactivity/sedentary [82] that contributes for augmented visceral adipose tissue. The prevalence of physical inactivity and sedentarism is quite high in the aging population [83] (see next topic).

The different adipose tissue depots exert impact to inflammation in aging, and are related to metabolic alterations and immune cell infiltration, particularly in visceral adipose tissue. For example, the macrophages trapped within fat depots are able to release pro-inflammatory cytokines, such as IL-6 and TNF-α [84]. Further, a recent study in mice showed that in the absence of obesity, visceral adipose tissue possesses a pronounced anti-inflammatory phenotype during aging, which is further enhanced by exercise [85].

In addition, studies have been proposed that obesity could be considered an accelerated model of aging. The hallmarks of aging may be compared with the physiological stresses induced by obesity. This compare is related cause a range of cellular and whole-body deteriorations that maintenance the pathophysiology of adipose tissue accumulation and dysfunction [65,86,87].

Regarding the clock genes, obesity and inflammation, the ROR family (RORα and RORγ) are very important on immunometabolism response. RORγ, for instance, is essential to T lymphocyte differentiation on Th17 subset [88]. Moreover, ROR family is very expressed in metabolic peripheral organs (liver, skeletal muscle, kidney and adipose tissue) and regulates the lipid metabolism, in special de lipogenesis, triacylglycerol formation and storage and cholesterol metabolism [89,90]. RORα or RORγ knockout mice showed resistance to weight-gain and metabolic syndrome in high fed diet model, while the obesity induces the increased of RORα or RORγ on liver and adipose tissue [90]. Moreover, recently, Hams et al. (2020) showed that humans and mice obese showed raise mRNA expression of RORα. In accordance, the myeloid cells the specific deletion of RORα on myeloid cells is sufficient to reduction of inflammation, insulin resistance and weight gain on high fatty diet fed mice [91].

In summary, the immunosenescence founded in obese and elderly people show many similarities. The low-grade inflammation, reduction on anti-viral response and inflammaging are close associated with increase on visceral adiposity that is very common in obese and aging process. Moreover, the physical inactivity induces the fat accumulation on visceral adipose tissue. On the other hand, the high physical fitness induces the reduction and protection against the fat accumulation in this depot [92]. Thus, the visceral adiposity is in the center of comorbidities related with, obesity poor aging (Fig. 1), and physical activity is able to delay this process as will be explained in the next topics.

6. Inflammaging and absence of contractile activity/inactivity physical

The promotion of catabolic signals mediated by pro-inflammatory cytokines, such as TNF-α and IL-6, induces muscle loss during aging. Chronic low-grade inflammation is related to low muscle regeneration capacity through satellite cells in older adults, which contributes to muscle loss and sarcopenia [93,94].

Nevertheless, when and where the aging-related physiological and biochemical changes? These questions remain unclear. However, these dysfunctions, such as changes in circadian rhythm, hormonal changes, worsening sleep quality, and chronic inflammation associated with a sedentary lifestyle, contribute to a substantial loss of life quality. In addition to the factors listed above, including chronic low-grade inflammation and hormonal changes, a sedentary lifestyle and the consumption of low-quality food, such as processed foods, also contribute to sarcopenia [95].

All of these changes are part of the life cycle, but the question is how to delay these changes as much as possible for prolonged and healthy aging. International clinical practice guidelines for sarcopenia (ICFSR): screening, diagnosis, and management published in 2018 recommend physical activity as the primary treatment of sarcopenia [96].

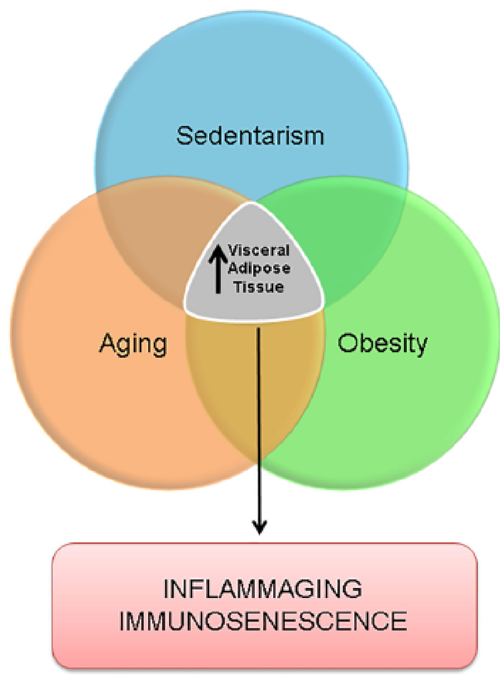

Fig. 1. Aging, obesity and sedentarism have in common the increase in visceral adipose tissue that can lead to a state of inflammaging and immunosenescence.

Clock genes are important in the maintenance of the physiological functions of skeletal muscle. Approximately 17% of the skeletal muscle transcriptome shows circadian rhythms, including clock genes, as well as genes related to metabolism, cell cycle, cytoskeletal organization, stress response, and differentiation, and the overexpression of CLOCK caused a significant modification in the pattern of these gene expression profiles [97]. Moreover, the deletion of BMAL-1 in the whole body or specifically in skeletal muscle induces early aging with the development of sarcopenia and reduction in metabolism, oxidative stress responses, and physical activity [98]. However, it is necessary to elucidate how clock genes may be related to physical activity and the preservation of muscle mass in aging.

Physical exercise is associated with a lower rate of developing chronic diseases, such as cardiovascular disease, type 2 diabetes mellitus, sarcopenia, obesity, and others. In addition, lifelong exercise is related to increased longevity. It is not so recent that studies showed the relationship between physical exercise and inflammation [99].

In healthy populations, exercise training has been shown to increase the level of anti-inflammatory cytokines (e.g., IL-10), reduce overall TNF-α expression, decrease pro-inflammatory adipokines, and reduce the expression of Toll-like receptors on monocytes and macrophages [100,101]. Furthermore, a 12-month program of moderate-intensity physical activity (combination of aerobic, strength, balance, and flexibility exercises, 40–60 min, 3 times/week) results in reduced systemic concentrations of IL-6 in elderly individuals [102]. However, IL-6 could be a dual effect, when produced by skeletal muscle during contractile activity, IL-6 is known by the anti-inflammatory effect. It has been consistently demonstrated that the plasma concentration of IL-6 increases during exercise. This increase is followed by the appearance of

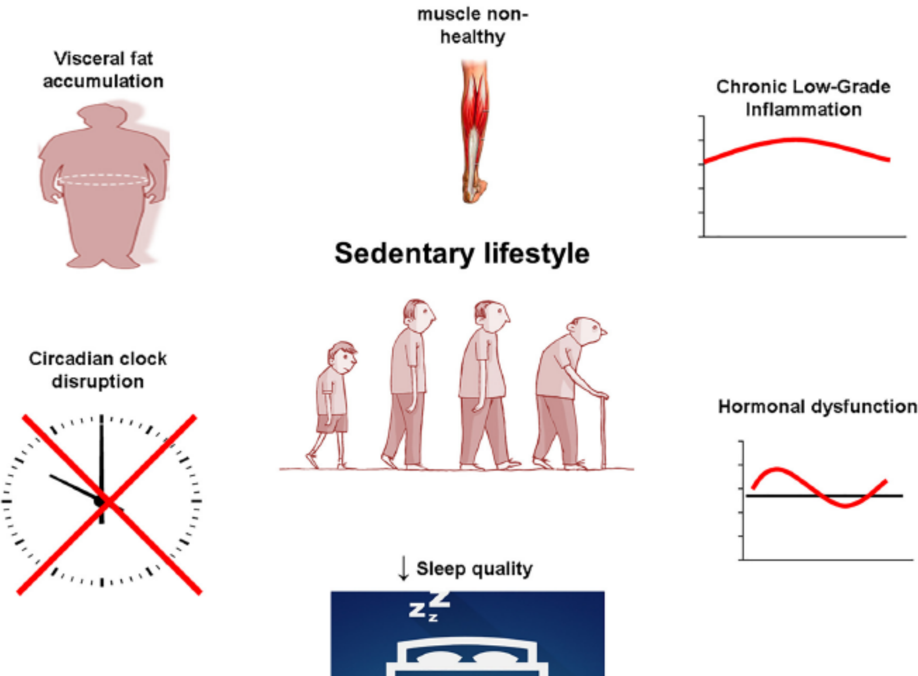

Fig. 2. A sedentary lifestyle is related to the accumulation of visceral fat, chronic low-grade inflammation, hormonal dysfunction, worsening sleep quality, skeletal muscle non-healthy, and circadian clock disruption.

IL-1ra and IL-10 the anti-inflammatory cytokine [103–105], and this anti-inflammatory profile.

Recently, Minuzzi et al. (2019) showed that lifelong training helps to maintain the balance of pro- and anti-inflammatory cytokines, together with IL-10 levels close to those found in young adults [106], and this will be discussed in next section.

7. Exercise as a time-conditioning effector

The lifelong regular practice of physical exercise decelerates the processes of aging, providing better quality and prolongation of life. Lifelong exercise is characterized by regular exercise throughout many years (20 or more).

Interestingly, immunosenescence observed in aging can be enhanced by obesity and sedentarism. This can be explained by similar conditions as the development of chronic low-grade inflammation. The therapy for react in this situation is increases physical fitness status, with enrollment of exercise training [65,92]. Thus, lifelong athletes show decreased inflammaging and immunosenescence [106,107].

Moreover, the age-related dysfunction in muscle is caused, at least in part, by an increase in inflammatory cytokines observed during inflammaging [108]. Thus, the inhibition of pro-inflammatory markers can help to preserve muscle mass. It is interesting because aging modified the profile of myokines secreted by skeletal muscle, which induced defect in the communication between skeletal cells and immune cells and initiated the vicious inflammatory cycle [108].

Minuzzi et al. (2018) showed that maintaining high levels of aerobic fitness during the natural course of aging may help prevent the accumulation of senescent T-cells [107]. Moreover, lifelong training helps to maintain the balance of pro- and anti-inflammatory cytokines, together with IL-10 levels close to those found in young adults [106]. In the Minuzzi's study, the master athletes trained approximately 4 h per week, with intensity the training sessions classified as moderate.

Physical exercise is a well-known anti-inflammatory therapy and induces a "health profile of myokine secretion". For example, lifelong participation in exercise training delays the senescence process. Furthermore, one bout of exercise is able to induce the release of myokines in the circulation. Lavin et al. showed that in lifelong athletes who trained in aerobic modality, one bout of resistance training induces higher anti-inflammatory myokines than in sedentary elderly people. Thus, this study showed a better adaptive response after one acute bout of exercise in lifelong athletes [108]. The schedule of exercise training routine in the Lavin's study show that master athletes trained approximately ~5 days for week, 7 h per week, with intensity the training sessions classified as moderate.

The same group of researchers analyzed women with an average age of 72 who trained in the last 48 years and found that lifelong training did not benefit type MHC I and IIa muscle fiber size but promoted adaptations of the contractile function that increased the strength of type I fibers and preserved the energy of type IIa fibers through different contractile mechanisms [109]. In addition, a greater intensity of training throughout life provided increased protection against the infiltration of adipose tissue in muscle [110].

Another study showed that more than 50 years of aerobic exercise fully preserved capillarization and aerobic enzymes regardless of intensity and suggested that skeletal muscle metabolic fitness may be easier to maintain with lifelong aerobic exercise [111]. In addition, other benefit of lifelong exercise is the reduction in the decline of maximum oxygen consumption (VO_2max). This concept describe show the subject is able to transfer oxygen from the atmosphere to working muscles [112]. This is the measure of cardiorespiratory ability, and this function is severely reduced with aging [113]. However, lifelong

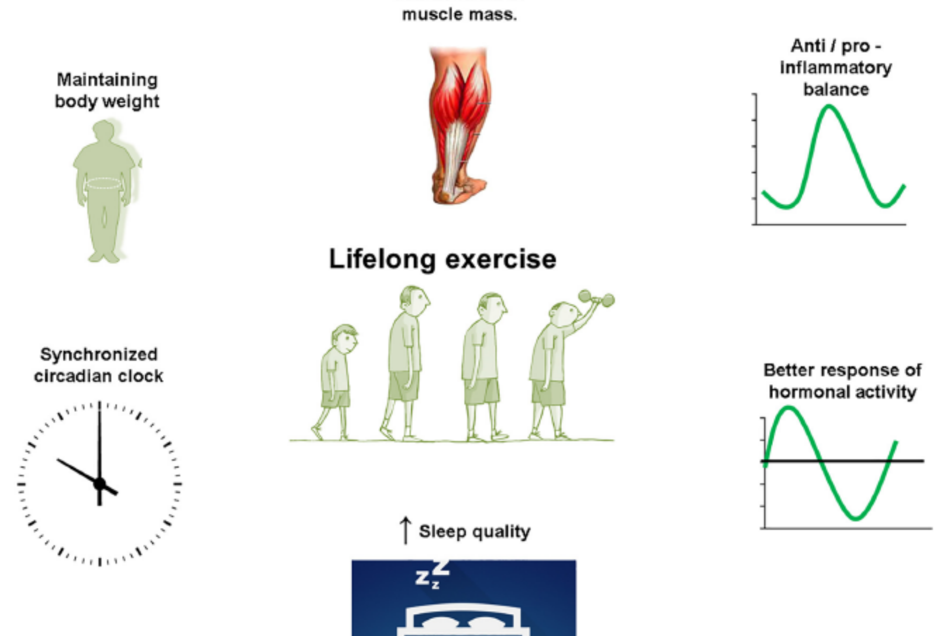

Fig. 3. Lifelong exercise is related to the maintenance of body weight, the anti/pro-inflammatory balance, better response of hormonal activity, improvement in sleep quality, maintenance of muscle mass, and maintenance of the circadian rhythm throughout life.

exercise has attenuated the decline in VO_2max that is related to aging [114,115].

Our group has recently found a relationship between the genes that control the circadian rhythm in EM CD4+ T-cells (but not in EM CD8+ T-cells) and the production of anti-inflammatory cytokines in individuals who trained throughout their lives (Teixeira et al. *data not published*). Taken together, many scientific evidences suggestion the exercise as a "Time-conditioning Effector" [116]. News avenues are open for better understating of the essential role of lifelong exercise as "the best" synchronizator of clock genes, promoting efficient signaling between immunological cells, skeletal muscle, brain, adipose tissue, and others tissue/organs (Figs. 2 and 3).

8. Conclusion

Thus, lifelong exercise prevents an exacerbates of pro-inflammatory processes, promotes better immune function, prevents the decline in VO_2max related to aging, improves the hormonal profile, and improves the quality of sleep. Together, this helps to maintain the circadian rhythm throughout life and can assist in healthy aging, increasing the quality of life through regular physical exercise. However, the interactions between clock genes as central players of this game should be proved by molecular and physiological approaches that show the role of them in the slow aging in lifelong athletes.

Declaration of competing interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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The present study was conducted in tribute of the 15-year anniversary of the death of Professor Luis Fernando Bicudo Pereira Costa Rosa (GG), an eminent exercise immunology researcher in Brazil, whose ideas, erudition, critical sense and honesty continue to inspire us. In special with this interaction between neuro-immune-endocrine axis influenced by physical activity.

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RESEARCH PAPER

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Doxorubicin modulated clock genes and cytokines in macrophages extracted from tumor-bearing mice

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ABSTRACT

Circadian rhythm is essential for cellular regulation of physiological, metabolic, and immune functions. Perturbations of circadian rhythms have been correlated with increased susceptibility to cancer and poor prognosis in the cancer treatment. Our aim is to investigate the role of doxorubicin (DOX) treatment on clock genes expression and inflammation in intraperitoneal macrophages and the anti-tumoral response. Methods: Macrophages were extracted from intraperitoneal cavity of mice without or with Lewis lung carcinoma (LLC) and treated with DOX totaling four groups (CTL, LLC, LLC+DOX and DOX) and analyzes of clock genes in six time points (ZT02, ZT06, ZT10, ZT14, ZT18 AND ZT22). Intraperitoneal macrophages cell culture was stimulated with LPS and DOX and clock genes and inflammatory profile were analyzed. In tumor were analyzed macrophages markers. Results: The expression of F4/80 (ZT22) and CD11c (ZT06) tumor tissue was significantly differed between LLC and LCC +DOX groups. In the intraperitoneal macrophages, DOX increased Clock (ZT10), Rev-Erbα (ZT18 and ZT22) and Per2 expressions (ZT18); in the LLC+DOX group was increased Bmal1 (ZT10), Per2 (ZT18) and NF-kB (ZT22) expressions; IL-6 expression increased in the LCC group (ZT02). In intraperitoneal macrophages cell culture stimulated with DOX and LPS after 24 h decreased Clock and Per1. DOX causes depression after 6 and 24 h in TNF-α content and Per2 gene expression after 24 h IL-1β expression was reduced also. Conclusion: DOX treatment in vivo disrupted cytokine and clock genes expression in intraperitoneal macrophages suppressing immune response. Moreover, macrophages cultured with DOX had decreased expression of LPS-stimulated inflammatory cytokines.

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Introduction

Cancer is a chronic disease promoted by uncontrolled cell proliferation that is often fatal.[1] In the etiology of cancer, a cell type presents marked genomic instability leading to the formation of clones with a high degree of heterogeneity which generates an environment rich in several growth and chemoattraction factors that recruits different type cells. Thus, the tumor and surroundings can be considered a highly complex organ.[1] Within this complexity, immune cells have an important role in recognition, death, or maintenance of tumor.

Macrophages are plastic immune cells that may be residents or derived from circulating monocytes and play an ambiguous role in cancer.[2] Macrophages have the potential to kill tumor cells yet are also associated with sustained proliferative signals and tumor immune evasion by the production of growth factors and anti-inflammatory cytokines.[3] There is evidence that clock genes regulate the inflammatory response in macrophages, in particular, via the anti-inflammatory role of BMAL-1 (brain and muscle Arnt-like protein 1) and pro-inflammatory activation by CLOCK (circadian locomotor output cycles kaput).[4–6]

Circadian rhythms are biological pulses that fluctuate around 24 h and can be biochemical, physiological, or behavioral events required to regulate the cell cycle.[7] The mechanism underlying circadian rhythmicity is composed by a set of interlocking transcription/translation feedback loops that results in cascades of gene expression within 24 h.[8] In the core of this mechanism in mammals is the heterodimeric transcription factor complex of Clock and Bmal1. These proteins activate the transcription of the Period (Per) genes and Cryptochrome (Cry) genes via E-box enhancer elements in their promoters.[9]

Changes in circadian rhythms have been correlated with increased susceptibility to cancer and poor prognosis in humans.[10–14] The expression of Per family genes is deregulated in breast cancer cells;[10] however, Per1 and Per2 appear to function as tumor suppressors in mice.[12] Moreover, Per2 acts as a liver tumor suppressor from initiation to progression,[15] whereas its expression in cancer cell lines results in inhibition of cell growth, cell cycle arrest, and apoptosis.[11] It is interesting to note that levels of Per2 mRNA are down regulated in various human lymphoma cell lines and tumor cells of patients with myeloid leukemia.[11] The overexpression of Per1 can also suppress growth of human

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cancer cell lines. In addition, Per1 mRNA levels are down regulated in lung cancer compared to the corresponding normal tissues.[12] These results point directly to a link between circadian rhythm disorders and cancer,[13] even though the pattern that may explain this link is not clear.

Recently, the role of chemotherapeutic drugs such as 5-fluorouracil and adriamycin was investigated in modulation of the immune response in tumor-associated macrophages (increased in M1 or classically activated) with increased anti-tumoral ability.[16] Doxorubicin (DOX) is an anthracycline developed from a metabolite of the bacterium Streptomyces peucetius var. Caesius.[17] This drug is widely used for the treatment of solid tumors[18] and is effective against tumor cells, but toxic to healthy tissues. Patients receiving chemotherapy commonly present sleeping disorders[19] and alteration in circadian rhythms. Thus, our aim is to investigate the role of DOX treatment in clock gene expression in intraperitoneal macrophages and the correlation between clock genes and inflammation in macrophages and the antitumoral response.

From the data obtained, we characterized the perturbation of the circadian rhythm by DOX treatment (intraperitoneal application of DOX [2.5 mg/kg], twice a week, for 2 weeks). Moreover, we verified macrophage markers in tumors and then analyzed possible alterations of clock gene expression and cytokine expression in the intraperitoneal macrophages. Finally, we analyzed the relationship between clocks genes and inflammation in primary culture cell of intraperitoneal macrophages treated with DOX.

Methods

Animal procedure

Male C57BL/6J mice were maintained in a room with a light-dark cycle of 12 h (light cycle: zeitgeber (ZT) 0–12, dark cycle: ZT 12–24) and a temperature of 23±2°C under normal diet (Nuvital ration of Nuvilab, Colombo, PR) and water ad libitum. The mice started treatment at 8 to 10 weeks of age. All study procedures followed the ethical principles of animal experimentation and were submitted and approved by the Ethics Committee on Animal Experimentation of the University of São Paulo registered under n° 4541221117.

The mice were divided into four groups: control (CTL), tumor (LLC), tumor + doxorubicin (LLC+DOX), and doxorubicin (DOX). Mice were inoculated subcutaneously with Lewis lung carcinoma (LLC and LLC+DOX groups). After 7 days of tumor inoculation, the animals were submitted to doxorubicin chemotherapy (LLC+DOX and DOX groups) (2.5mg/kg body weight intraperitoneally). In order to analyze whether there were circadian changes promoted by the tumor and/or Doxorubicin, animals were euthanized every 4 h, until completing 24 h, with euthanasia at 8 o'clock (Zeitberg, ZT 02), 12 o'clock (ZT 06), 16 o'clock (ZT 10), 20 o'clock (ZT 14), 24 o'clock (ZT 18), and 4 o'clock (ZT 22).

Subcutaneous implantation of tumor cells

Lewis lung carcinoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Invitrogen, NY) supplemented with penicillin (100U/ml), streptomycin (100µg/ml), and 10% fetal bovine serum (Atlanta Biological, Lawrenceville). Cells were cultured and maintained in tissue culture flasks at 37°C in a humidified atmosphere containing 5% CO2. 5 × 105 viable cells were injected subcutaneously into the right flank of the mice (diluted in 0.9%saline). Control animals received the same volume of saline at 0.9%. Cell viability was assessed by Trypan Blue.

Body composition, weight gain, and tissue weight

Weight was monitored throughout the treatment. In the end, the following parameters were measured: wet weight of the soleus, gastrocnemius, and extensor digitorum longus (EDL) muscle, and weight of retroperitoneal, epididymal, and subcutaneous adipose. For the determination of the adiposity index and learn mass index, the sum of the adipose retroperitoneal, epididymal, and subcutaneous adipose and soleus, gastrocnemius, and EDL muscles were used.

Enzyme-linked immunosorbent assay (ELISA)

Tumor tissue samples (80–100 mg) were carefully homogenized in RIPA buffer (0.625% Nonidet *P*-40, 0.625% sodium deoxycholate, 6.25 mM sodium phosphate, and 1mM EDTA at pH 7.4) containing 10µg/ml of protease inhibitor cocktail (Sigma-Aldrich®, St. Louis, Missouri, USA). The supernatant of the homogenate was utilized to measure total protein concentration via a Bradford assay (Bio-Rad®, Hercules, CA, USA) and to determine the expression of IL-6, MCP-1, IL-10, and VEGF by ELISA (DuoSet ELISA®, R&D Systems, Minneapolis, MN, USA).

RNA isolation, reverse transcription, and real-time PCR

The expression of tumor and macrophage genes related to circadian rhythms was assessed by qRT-PCR with SYBR Green. Total RNA was extracted as described by Chomczynski and Sacchi 1987,[20] quantified in a spectrophotometer (260 nm), and cDNA was synthesized from the total RNA using reverse transcriptase. The sequences of the primers are shown in Supplementary Table 1.Gene expression was quantified by the comparative method using the expression of GAPDH as a standard.[21]

Cell viability (MTT)

After the stimulation period, macrophages were incubated with MTT (bromide 3-(4,5-dimethylthiazol-2-il)-2,5-diphenyltetrazolium) solution (0.5 mg/ml) for 4 h. The formazan formed was resuspended with isopropanol/HCl (11 M) and the absorbance measured (595 nm) was utilized to calculate the cell viability in relation to the absorbance of the control group (100%). Cells were treated with 0.5 µM of doxorubicin.

Extraction and culture of intraperitoneal macrophages

Macrophages were extracted from the intraperitoneal cavity of C57BL6 mice with RPMI medium and counted in

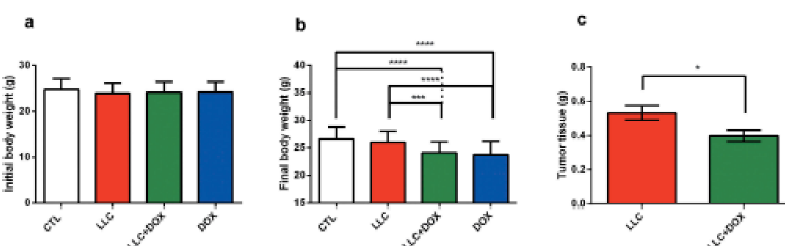

Figure 1. Initial body weight (a), Final body weight (b) and Weight tumor (c). The tumor was inoculated into mice of the C57BL/6J background between 8 and 10 weeks old. After 7 days of tumor inoculation began treatment with doxorubicin (2.5 mg/kg twice weekly intraperitoneal). Data represent mean ± standard error of the mean of 22 to 26 animals per group. *p < 0.05, **p < 0.01 e ***p < 0.001 (Anova one way seguido por Tukey).

a hemocytometer using the Trypan Blue exclusion method. 2.5 × 105/cm^2 viable macrophages were plated, and after 2 h macrophages were collected for further analysis.

The macrophages were also extracted from the intraperitoneal cavity of C57BL6 mice without tumors with RPMI medium and counted in a hemocytometer using the TRIPAN Blue exclusion method. 2.5 × 105/cm^2 viable macrophages were exposed to PBS (control) or LPS (2.5 μg/ml, E. coli O111:B4) DMSO, or doxorubicin (0.5 μM). After 2, 6, and 24 h, the medium and macrophages were collected for further analysis.

Statistical methods

Normal distribution and variance homogeneity were tested, and the appropriate statistical test (two-way ANOVA) was employed. Statistical analysis was performed with the program GraphPad Prism 6.0, with significance being <5% ($p < .05$). Data are expressed as means±standard error of the mean.

Results

Doxorubicin induced weight loss regardless of tumor presence

Doxorubicin induced weight loss, with reduction on adipose and skeletal muscle mass, regardless of tumor presence. LLC mice did not show a reduction in these parameters 21 days after the inoculation. The tumor was significantly reduced by doxorubicin administration (Figure 1).

Correlation between tumor weight and clock genes expression

LCC group showed correlation between tumor weight and clock genes, CLOCK, BMAL-1 and CRY 1 showed negative correlation with tumor weight and PER-1, Rev-erb-α and Rev-erb-β showed positive correlation. Besides, the group treated with DOX did not showed correlation (Table 1).

M1 marker is highly expressed in DOX-treated tumor at 6 h

We observed that the expression of F4/80 did not differ between LLC and LLC+DOX groups during the light period (ZT 02, 06 and 10) but significantly differed at the end of the dark period (Figure 2a). CD11c expression significantly differed between the groups only during the middle of the light period (Figure 2b). We did not observe any difference in CD206 expression between the groups within the period (Figure 3c).

Table 1. Correlation of LLC (A) and LLC+DOX (B) groups between tumor weight and clock genes expression.

(A) Correlation of LLC group between the tumor weight and clock genes expression			(B) Correlation of LLC+DOX group between the tumor weight and clock genes expression		
	R	P ≤		R	P ≤
Clock	−0.6052	0.0017**	Clock	−0.2221	0.3333
Bmal1	−0.5555	0.0039**	Bmal1	−0.1348	0.5497
Per1	0.5071	0.0096**	Per1	−0.0077	0.9728
Per2	−0.3031	0.1409	Per2	−0.3288	0.1352
Cry1	−0.5940	0.0017**	Cry1	−0.0997	0.6588
Cry2	−0.3863	0.0565	Cry2	−0.2996	0.1756
Rev-erb-α	0.4333	0.0304*	Rev-erb-α	0.0037	0.9870
Rev-erb-β	0.6445	0.0005**	Rev-erb-β	0.0078	0.9726

Correlation of tumor weight and clock genes with the group without treatment doxorubicin (table A) and with treatment doxorubicin (table B). Pearson correlation coefficients were used (*p < 0.05 and **p < 0.01).

Expression of the clock genes in intraperitoneal macrophages

Clock gene expression was discrete in the light period in the CTL, LLC, and LLC+DOX groups, with a great increase in the DOX group at the end light period (ZT 10). During the dark period, the expression of Clock continued low, increasing only at the last time point for all groups (Figure 3a). Bmal1 gene expression increased at the end of the light and dark periods in the LLC+DOX group (ZT 10 and 22). At the others ZTs, there was no difference in Bmal1 expression among the groups (Figure 3b).

The Per1 gene showed no change among the groups at any time (Figure 4a). Per2 gene expression increased at the beginning of the light period in the LCC group when compared with control. In the dark period, Per2 expression increased first in the LCC+DOX group (ZT14) and then in the LCC +DOX and DOX groups (ZT18) (Figure 4b). Per3 gene expression was only altered at the beginning of the dark period, in which there was an increase in Per3 expression for the CTL group when compared with the LLC+DOX group (Figure 4c).

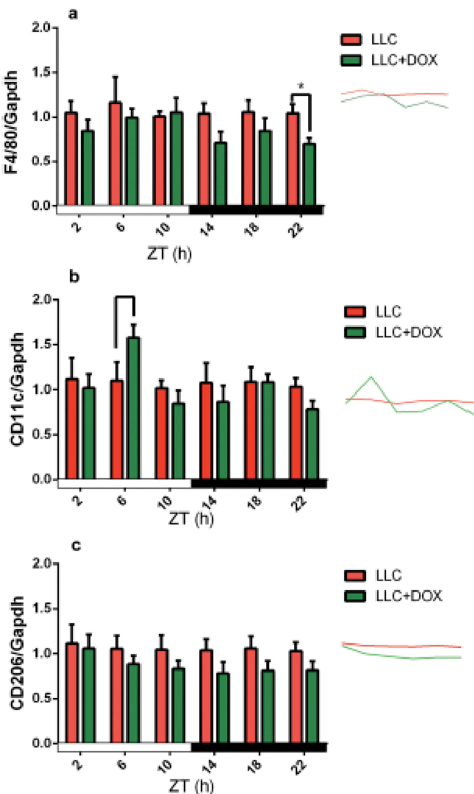

Figure 2. Gene Expression of F4/80 (a), CD11c (b) and CD206 (c) follows circadian rhythms in Tumor Tissue.

The tumor was inoculated into mice of the C57BL/6J background between 8 and 10 weeks of age. After 7 days, treatment with doxorubicin began (2.5 mg/kg twice weekly intraperitoneal). Data represent means ± standard error of the mean of 4 to 6 animals per group. *p < .05, **p < .01, and ***p < .001 (two-way ANOVA followed by Tukey's posttest).

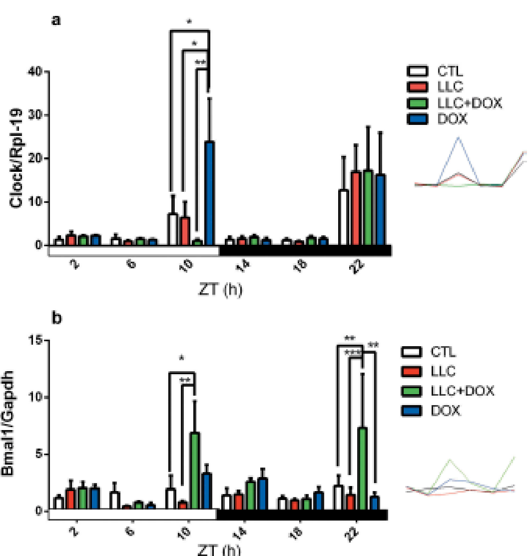

Figure 3. Gene expression of CLOCK AND Bmal1 follows circadian rhythms in intraperitoneal macrophages.

The tumor was inoculated into mice of the C57BL/6J background between 8 and 10 weeks of age. After 7 days, treatment with doxorubicin began (2.5 mg/kg twice weekly intraperitoneal). Data represent means ± standard error of the mean of 4 to 6 animals per group. *p < .05, **p < .01, and ***p < .001 (two-way ANOVA followed by Tukey's posttest).

Cry1 gene expression increased at the end of the light period for CTL, LLC, and DOX, and this increase was not observed in the LLC+DOX group (Figure 5a). Cry2 expression changed over time, but no differences were observed among the groups (Figure 5b).

Rev-Erbα interestingly showed increase in gene expression in the groups treated with doxorubicin in the middle and at the end of the dark period (Figure 6a). Rev-Erbβ expression increased in the CLT group at the beginning of the light period (Figure 6b).

Gene expression of NF-kB, IL-6, and IL-10 follows circadian rhythms in intraperitoneal macrophages

Gene expression of NF-kB increased at the end of dark period in the LLC+DOX group (Figure 7a). IL-6 expression increased in the LCC group at the beginning of the light period (Figure 7b). IL-10 expression was similar to NF-kB expression and increased only in the LLC+DOX group at the end of the dark period (Figure 7c).

Clock gene expression and MCP-1, TNF-α, il-1β, IL-6, and IL-10 protein expression in primary intraperitoneal macrophage cultures

Primary intraperitoneal macrophages were cultured stimulated with doxorubicin and LPS. These macrophages were collected after 2, 6, and 24 h to analyze the clock gene expression and MCP-1, TNF-α, IL-1β, IL-6, and IL-10 protein expression.

No differences was found in Clock and Per1 gene expression after 2 and 6 h of stimulus but after 24 h was observed a decrease of these genes in the DOX+LPS group (Figure8 A and B). Doxorubicin has been shown to lead a decrease in Per2 gene expression after 6 and 24 h (Figure 8c). The Per3 gene was affected by LPS after 24 h (Figure 8d).

After 24 h, doxorubicin increased Cry1 gene expression (Figure 8e). The expression of Cry2, Rev-Erbα, and Rev-Erbβ genes did not differ among the groups (figure 8f, G, and H). We showed that doxorubicin causes an immunodepression after 6 and 24 h in TNF-α expression and after 24 h in IL-1β expression (Figure 9).

Discussion

Chemotherapy treatment reduced body weight regardless the presence of the tumor yet can also reduce the tumor mass in LLC tumor-bearing mice. The tumor weight presented a correlation with clock genes expression in tumor tissue of the LLC group. The group treated with doxorubicin did not show correlation with tumor weight and clock genes expression, besides that, it had a shorter survival, although this

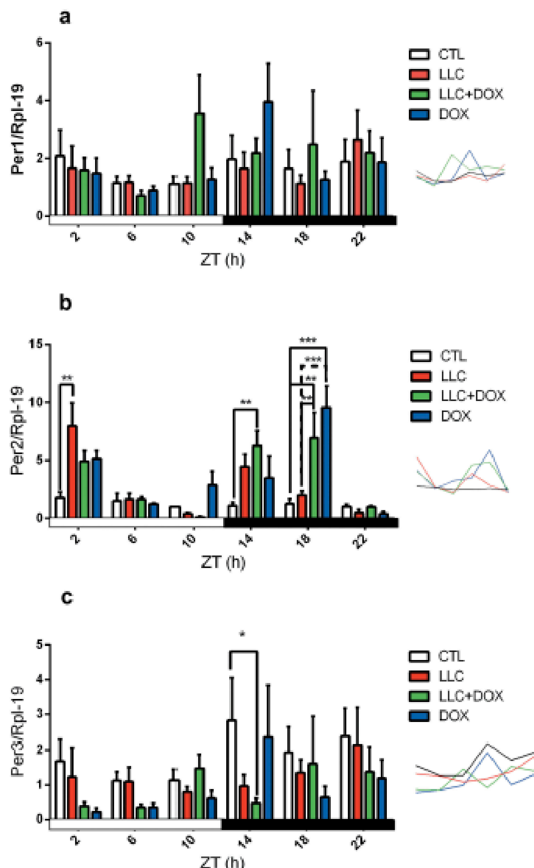

Figure 4. Gene expression of Per1 (a) Per 2 (b) and Per 3 (c) follows circadian rhythms in intraperitoneal macrophages.

The tumor was inoculated into mice of the C57BL/6J background between 8 and 10 weeks of age. After 7 days, treatment with doxorubicin began (2.5 mg/kg twice weekly intraperitoneal). Data represent means ± standard error of the mean of 4 to 6 animals per group. $*p < .05$, $**p < .01$, and $***p < .001$ (two-way ANOVA followed by Tukey's posttest).

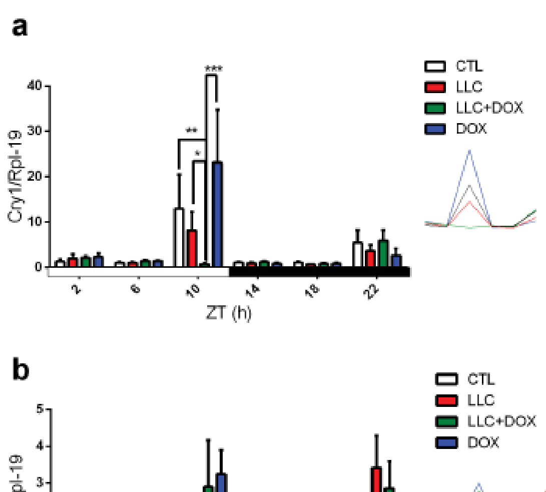

Figure 5. Gene expression of Cry1 (a) Cry2 (b) follows circadian rhythms in intraperitoneal macrophages.

The tumor was inoculated into mice of the C57BL/6J background between 8 and 10 weeks of age. After 7 days, treatment with doxorubicin began (2.5 mg/kg twice weekly intraperitoneal). Data represent means ± standard error of the mean of 4 to 6 animals per group. $*p < .05$, $**p < .01$, and $***p < .001$ (two-way ANOVA followed by Tukey's posttest).

group did not show statistically significant differences in the expression of the clock genes in the tumor, there is a clear relationship between the expression of the clock genes and tumor weight. We suggest this relation between clock genes, tumor weight and tumor staging should be clarified in clinical practice.

The tumor tissue showed circadian alteration in the expression of macrophage surface markers as a reduction of F4/80 at the end of the dark period (ZT 22) and increased CD11c in the middle of the light period (ZT 6). Moreover, the mRNA levels of IL-10 and IL-6 were altered by chemotherapy in peritoneal macrophages. The clock genes are deep modified in the circadian expression of these macrophages. In culture, doxorubicin inhibits cytokine secretion after LPS stimulation in peritoneal macrophages and the expression of clock genes are also altered.

Macrophages have a dual role in immunity against tumor cells. The tumor-associated macrophages (TAM) can help the tumor sustain growth and evade or induce tumor suppression. The immunometabolic signature of the TAMs determines this process. In breast cancer, the presence of M2 is associated with a great reduction in the efficacy of doxorubicin response.[22] Moreover, triple-negative breast cancer is characterized by a high proportion of TAM leading to reduced chemotherapy efficacy.

In our model, doxorubicin treatment was efficient in alteration the proportion of M1 macrophages observed in the LLC tumor, by increasing CD11c mRNA expression. Thus, increased immune cells surveillance can be associated with the reduction of tumor growth. Doxorubicin has been shown to reduce the myeloid-derived suppressor cells and consequently increase effective T lymphocytes.[16,23] Furthermore, the induction of ATP levels caused by doxorubicin induces the chemoattraction of myeloid cells and differentiation of the myeloid cells in antigen-presenting cells.[24]

This increase in CD11c and F4/80 mRNA in the tumors of doxorubicin-treated mice shows the increased chemotaxis ratio of monocytes and differentiated in macrophages in tumor microenvironment.[25] This could explain the difference between the response of TAMs and intraperitoneal macrophage in the mice treated with doxorubicin. Was observed an

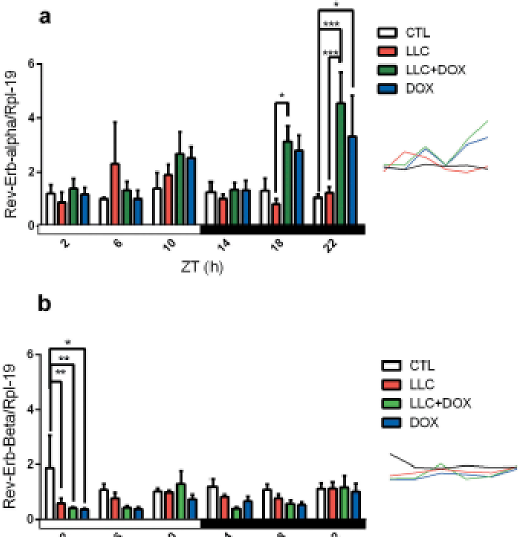

Figure 6. Gene expression of Rev-Erbα (a) Rev-Erbβ (b) follows circadian rhythms in intraperitoneal macrophages.
The tumor was inoculated into mice of the C57BL/6J background between 8 and10 weeks of age. After 7 days, treatment with doxorubicin began (2.5 mg/kg twice weekly intraperitoneal). Data represent means ± standard error of the mean of 4 to 6 animals per group. *p < .05, **p < .01, and ***p < .001 (two-way ANOVA followed by Tukey's posttest).

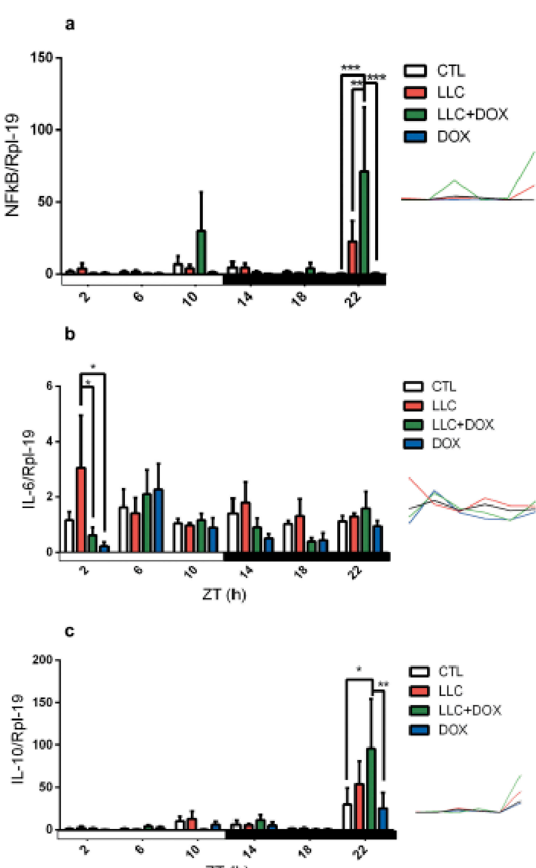

Figure 7. Gene expression of Nf-kB (a) IL-6 (b) IL-10 (c) follows circadian rhythms in intraperitoneal macrophages.
The tumor was inoculated into mice of the C57BL/6J background between 8 and10 weeks of age. After 7 days, treatment with doxorubicin began (2.5 mg/kg twice weekly intraperitoneal). Data represent means ± standard error of the mean of 4 to 6 animals per group. *p < .05, **p < .01, and ***p < .001 (two-way ANOVA followed by Tukey's posttest).

increase in IL-6 mRNA in tumor-bearing mice, with a reduction in mice treated with DOX in intraperitoneal macrophages. Moreover, tumor-bearing mice treated with DOX showed increased IL-10 mRNA expression in intraperitoneal macrophages. The increase in IL-10 is observed in M2 macrophages polarization.[26]However, the concomitant increase in NF-kB and IL-10 mRNA indicate an increased TLR response. The activation of TLR response in macrophages induces the ERK and MAPkinase pathways, and these routes lead to increased NF-kB and IL-10 mRNA. Moreover, the induction of NF-kB is a positive signal for IL-10 or IL-10 transcription. Thus, these results show an elevation of TLR downstream signaling.[27] Furthermore, the intraperitoneal macrophages stimulated by LPS and treated with DOX showed the inhibition in TNF-α and IL-1β cytokines production and released after 24 h of LPS stimulation.

The effect of doxorubicin on immune cells stimulated with LPS is controversial. The increased ROS production in bone marrow-derived monocytes stimulated with LPS induced a robust increase in the inflammasome pathway.[28] Conversely, Asmis et al. 2006 showed that mice treated with Dox had reduced resident intraperitoneal macrophages associated with a reduction in cytokine production.[29] Our results show that incubation with a non-lethal dosage of DOX in intraperitoneal macrophages reduced cytokine production.

Finally, the mRNA expression of clock genes was deregulated in macrophages. The immune response and cytokine production in immune cells are influenced by clock gene expression. The CLOCK, BMAL-1, and Rev ErB family are the main clock genes related to the immune response.[30]

CLOCK is required for the transactivation of NF-kB by of the p65 isoform acetylation; thus the activation of CLOCK is important for stabilization of binding between NF-kB and a promoter region.[6] Since BMAL-1 dimerizes with CLOCK and impairs the cross-activation of NF-kB, BAML-1 has anti-inflammatory effects.[4] Intraperitoneal macrophages that did not express BMAL-1 showed increased cytokine production after LPS stimulation in comparison with wild type. Oishi et al. 2017 showed that the deletion of BMAL allowed higher acetylation of NF-kB, which resulted in higher production of pro-inflammatory cytokines together with the reduction in ReV-Erb, which realized a positive feedback loop with BMAL-1.[31]

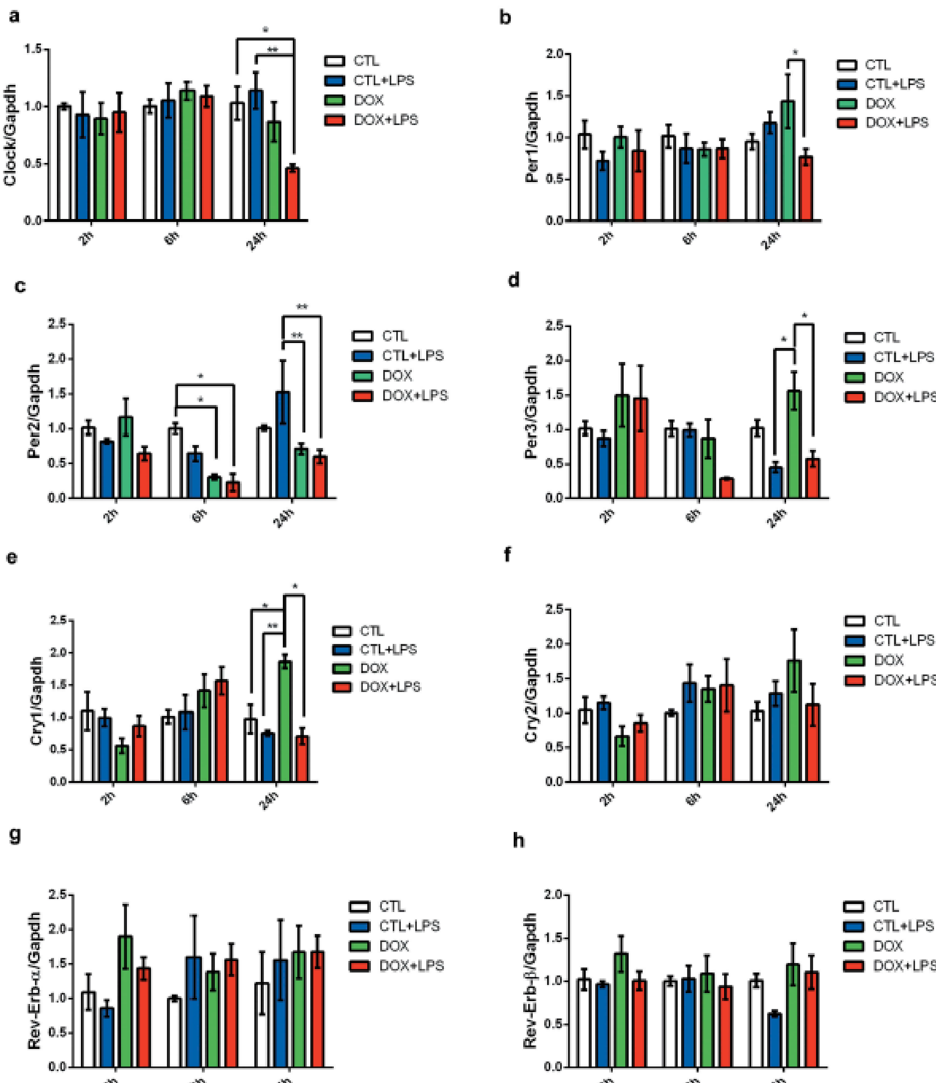

Figure 8. CLOCK Gene Expression in intraperitoneal macrophages comparing the circadian rhythm at each time between the groups (a) comparing the circadian rhythm of intra-group (b). Per1 Gene Expression in intraperitoneal macrophages comparing the circadian rhythm at each time between the groups (c) comparing the circadian rhythm of intra-group (d). Per2 Gene Expression in intraperitoneal macrophages comparing the circadian rhythm at each time between the groups (e) comparing the circadian rhythm of intra-group (f). Cry2 Gene Expression in intraperitoneal macrophages comparing the circadian rhythm at each time between the groups (g) comparing the circadian rhythm of intra-group (h).

$2.5 \times 10^5/cm^2$ viable macrophages were exposed to PBS (control) or LPS (2.5 μg/mL, E. coli 0111:B4) DMSO or doxorubicin (0.5 μM). After 2 h, 6 h and 24 h the macrophages were collected for further analysis. Data represent mean ± standard error of the mean of 4 to 6 animals per group. $*p < 0.05$, $**p < 0.01$ e $***p < 0.001$ (Anova two-way seguido por Tukey).

Interestingly, our results showed higher expression of NF-kB in intraperitoneal macrophages in ZT22 in the LLC+DOX group, but in the same ZT, IL-10 expression increased in LLC +DOX. BMAL-1 and ReVErb alpha also presented higher expression. Elevation in the clock genes could explain the anti-inflammatory effects of doxorubicin treatment. Moreover, the treatment with DOX in intraperitoneal macrophages showed reduced IL-1b and TNF-α associated with the reduction of mRNA expression of CLOCK 24 h after the LPS stimulation.

Recently, Korkmaz et al. 2018 showed that the deletion of BMAL-1 in a breast cancer tumor cell line induces the apoptosis by doxorubicin. Thus, the deletion of BMAL-1 presented an anticancer effect, yet this modified cell line showed increased invasion properties.[32] Few studies established the

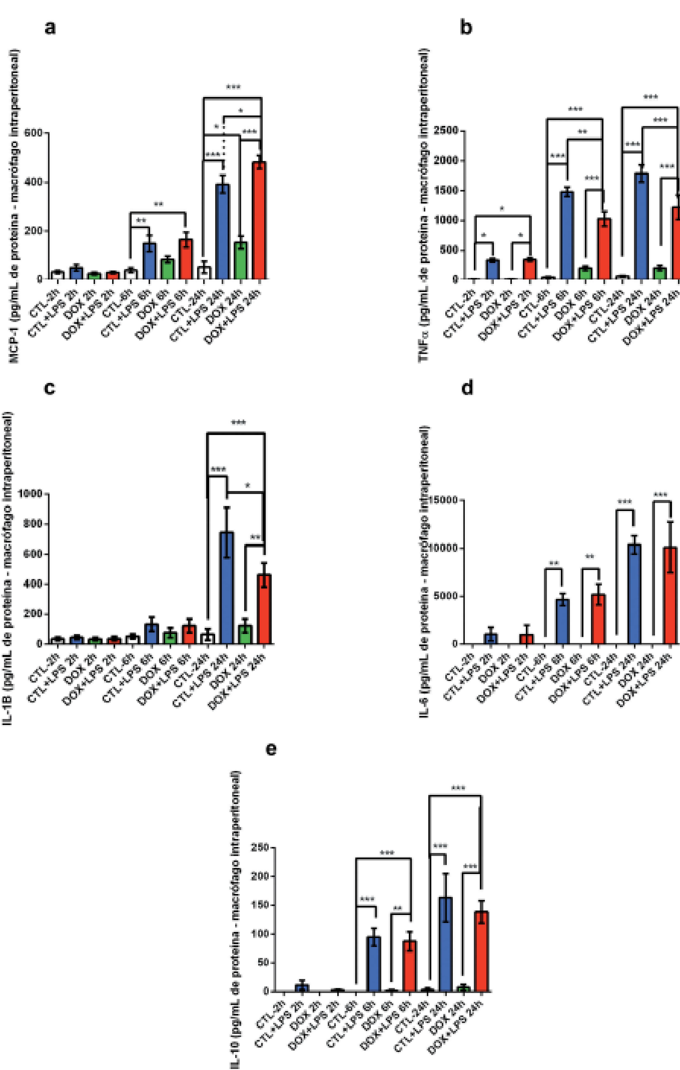

Figure 9. MCP-1 (a), TNF-α (b), IL-1β (c), IL-6 (d) and IL-10 (10) protein expression in intraperitoneal macrophages.
2.5x10^5/cm^2 viable macrophages were exposed to PBS (control) or LPS (2.5 μg/mL, E. coli O111:B4) DMSO or doxorubicin (0.5 μM). After 2 h, 6 h and 24 h the culture medium was collected for further analysis. Data represent mean ± standard error of the mean of 4 to 6 animals per group. *$p < 0.05$, **$p < 0.01$ e ***$p < 0.001$ (Anova two-way seguido por Tukey).

relationship between the molecular clock genes and tumors. BMAL-1 KO mice showed increased tumor burden in a pulmonary tumor model.[33] Furthermore, the CLOCK or BMAL-1 deletion induced early aging phenotypes.[34]

Thus, we conclude that the doxorubicin treatment in vivo disrupts cytokine expression and clock genes expression in intraperitoneal macrophages to suppress the immune response. Moreover, the LPS-stimulated macrophages cultured with DOX had decreased expression of inflammatory cytokines. The LLC tumor only did not disrupt circadian rhythms in macrophages.

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Disclosure of potential conflicts of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Inflammopharmacology

ORIGINAL ARTICLE

Short-term treatment with metformin reduces hepatic lipid accumulation but induces liver inflammation in obese mice

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Abstract
The study aimed to evaluate the metabolic and inflammatory effects of short-term treatments (10 days) with metformin (MET) on the NAFLD caused by a high-fat diet (HFD) in C57BL/6 mice. After the treatment, histological liver slices were obtained, hepatocytes and macrophages were extracted and cultured with phosphate buffered saline, LPS (2.5 μg/mL) and MET (1 μM) for 24 h. Cytokine levels were determined by ELISA. NAFLD caused by the HFD was partially reduced by MET. The lipid accumulation induced by the HFD was not associated with liver inflammation; however, MET seemed to promote pro-inflammatory effects in liver, since it increased hepatic concentration of IL-1β, TNF-α, IL-6, MCP-1 and IFN-γ. Similarly, MET increased the concentration of IL-1β, IL-6 in hepatocyte cultures. However, in macrophages culture, MET lowered levels of IL-1β, IL-6 and TNF-α stimulated by LPS. Overall, MET reduced liver NAFLD but promoted hepatocyte increase in pro-inflammatory cytokines, thus, leading to liver inflammation.

Keywords Obesity · Metformin · Inflammation · Liver

Introduction

The liver is a complex metabolic organ, and a classical example of the tight interaction between the immune system and metabolism. The liver's metabolic role is coordinated by hepatocytes, the most abundant cell type, but the cells responsible for the initial immune responses to stimuli, Kupffer cells (KC) and hepatic satellite cells (HSc), only constitute 15% of the liver cell population. (Nati et al. 2016). Pathological conditions promote the migration of immune cells, which, besides changes to the proportion and subtypes of cells in the liver, could also lead to the maintenance of a chronic inflammatory state (Fader et al. 2015; Mikami et al. 2014).

Nonalcoholic fatty liver disease (NAFLD), characterized by an accumulation of fat droplets in hepatic parenchyma, is the most common chronic liver disease (Tiniakos et al. 2010). NAFLD has been highly associated with other metabolic diseases, such as obesity, insulin resistance, type 2 diabetes and cardiovascular diseases (Love-Osborne et al. 2008). It was also observed that long-term NAFLD can progress into harmful kinds of liver disease, like nonalcoholic steatohepatitis (NASH), cirrhosis, hepatocellular carcinoma and liver failure (McCullough 2004).

The western diet, rich in fatty acids, can trigger the process of apoptosis, necrosis, oxidative stress, and lipid peroxidation in the liver (Casas et al. 2014; Yadav and Ramana 2013). Although the most accepted hypothesis for the NAFLD pathogenesis points to the excess of free fatty acids (FFA) caused by peripheral insulin resistance as a trigger

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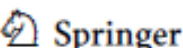

for higher triacylglycerol incorporation (Adams et al. 2005; Teixeira et al. 2016) and impaired oxidation functions (Berlanga et al. 2014), it is well known that inflammation could promote metabolic alterations in the liver.

In fact, low grade inflammation (LGI), observed in obesity and insulin-resistant patients (Brestoff and Artis 2015; McNelis and Olefsky 2014), seems to have an important role in the progression of NAFLD (Xu et al. 2015a). LGI is responsible for a low sustained exacerbation of cytokines, chemokines, adipokines and acute phase proteins, which increase the recruitment of immune cells and also seem to impair insulin sensitivity and oxidative metabolism in the liver (Liu et al. 2016; Nati et al. 2016).

Until the present moment, no drug has been approved as a treatment for NAFLD. Nevertheless, Metformin (MET) activates AMPK, reducing glucose production in the liver and improving glucose and fatty acid uptake, as well as their oxidation in skeletal muscle (Viollet et al. 2009). Moreover, MET has been described as being able to induce an anti-inflammatory response by (1) decreasing the levels of inflammatory cytokines in macrophages activated by lipopolysaccharide (LPS) (Kelly et al. 2015; Kim et al. 2014), (2) impairing monocyte–macrophage differentiation (Vasamsetti et al. 2015) and (3) increasing M2 (anti-inflammatory) macrophages activation (Chen et al. 2015).

Although the use of MET as a treatment for type 2 diabetes is well established, its actions over hepatic illnesses have not been widely investigated. Thus, the aim of this study was to evaluate the effects of short-term metformin treatments (10 days) on NAFLD caused by a high-fat diet in mice.

Materials and methods

Animal procedure

Male C57BL/6 J mice maintained in standard conditions were fed with a high-fat diet (HFD, caloric content: 59% fat, 15% proteins, 26% carbohydrate) (Reeves et al. 1993) or a standard diet (SD, caloric content: 9% fat, 15% protein, 76% carbohydrate) (Reeves et al. 1993) for 12 weeks. In the last 10 days, the mice received phosphate-buffered saline (PBS) or metformin (300 mg/kg of body weight) daily by oral administration (gavage), based on previous studies (Souza-Mello et al. 2010; Spruss et al. 2012). The body weight was evaluated weekly for 12 weeks, then the mice were fasted (4 h), culled, and the samples of blood (total cholesterol: Labtest®, Lagoa Santa, MG, Brazil) and tissue (liver, adipose tissue and skeletal muscle) were collected. Adipose tissue index was calculated as sum of epididymal, subcutaneous, and retroperitoneal adipose tissue weight. The experimental protocols were approved by the Ethics Committee for Animal Experimentation, Institute of Biomedical Sciences, registered under the number 050 in the fls. 05 of book 03.

Histological analyses

Liver biopsies (50 mg) were obtained carefully excised using scissors following by fixation 4% paraformaldehyde (w/v), pH 7.4. The samples were dehydrated in absolute ethanol, diaphanization in xylol and for last, embedded in paraffin (Paraplast X-TRA, SIGMA-ALDRICH). The sections of 5 µm were mounted onto slides Starfrost® (Knittel Glass). Deparaffinized and hydrated sections were stained with hematoxylin (H) and counterstained with eosin (E) and photographed under light microscopy (Olympus).

Picro sirius red

The 5-µm sections previously deparaffinized were hydrated during 5 min in running water followed for 1 h at room temperature of staining by Picro Sirius red dye. The slides were mounted with Permount (Tuluene Solution, Fischer Scientific).

Oil red O

For Oil Red O (ORO) staining, the fresh liver samples were cryo-preserved with isopentane and freezing into liquid nitrogen. Before cryostat sections set to − 21 °C were obtained, the samples were covered with Tissue-Tek. Sections of 7 µm were hydrated in phosphate buffer during 5 min and fixed for 30 min at room temperature in buffered formalin (4%), ph 7.4. The staining protocol was performed adapted from VanSaun (2009). After fixation, the slides were rinsed in running water for 10 min followed by 60% isopropanol for 5 min. The working solution of ORO was directly applied into each section during 1 h at room temperature. The working solution was prepared from stock solution, consisting of 5 g ORO (Sigma, Aldrich, St. Louis, MO) in 100% isopropanol, at 3:2 with distilled water. After staining, slides were immersed in 60% isopropanol following by 5 min with distilled water. Mayer's hematoxylin was used to counterstaining, tris-buffered saline during 1 min and distilled water by 5 min. After, the slides were mounted with glycerol.

All images were acquired using Image ProPlus v.5.2 (Media Cybernetics, Bethesda, MD, USA) digital camera system coupled to a light microscope (Olympus).

Glucose tolerance tests

The glucose tolerance tests were performed at the seventh (before metformin treatment) and 11th week (after 8 days

with metformin treatment), in which blood samples were collected 0 (basal), 15, 30, 60 and 90 min after the D-glucose (2 g/kg body weight) intraperitoneal injection (Bergmeyer and Bernet 1974). The levels of plasma glucose were measured using an Accu-Chek® performa glucometer (ROCHE®, São Paulo, SP, Brazil), and the difference of glycemia before and after glucose administration over time was used to calculate the area under the curve (AUC).

Enzyme-linked immunosorbent assay (ELISA)

Liver retroperitoneal adipose tissue and gastrocnemius muscle samples (80–100 mg) were carefully homogenized in RIPA buffer (0.625% Nonidet P-40, 0.625% sodium deoxycholate, 6.25 mM sodium phosphate, and 1 mM EDTA at pH 7.4) containing 10 μg/mL of protease inhibitor cocktail (Sigma-Aldrich®, St. Louis, Missouri, USA). The homogenate supernatant was utilized to measure total protein concentration by Bradford assay (Bio-Rad®, Hercules, CA, USA) and to determine expression of IL-1β, TNF-α, IL-6, IL-12, MCP-1, IFN-γ, IL-1Ra, IL-10, IL-4, adiponectin and FGF-21 by ELISA (DuoSet ELISA®, R&D Systems, Minneapolis, MN, USA).

RNA isolation, reverse transcription, and real-time PCR

The expression of hepatic genes related to metabolic and inflammatory factors was assessed by qRT-PCR with a SYBR Green marker. For this reason, total RNA was extracted as described by Chomczynski and Sacchi (1987), quantified in a spectrophotometer (260 nm), and cDNA was synthesized from the total RNA using reverse transcriptase. The sequences of the primers are shown in Supplementary Table 1; gene expression was quantified by the comparative method using the expression of GAPDH or RPL-19 as standard (Livak and Schmittgen 2001).

Western blotting (WB)

The total protein extracted (25 μg) from the liver, retroperitoneal adipose tissue and gastrocnemius muscle was diluted in Laemmli buffer, subjected to electrophoresis in a SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. The membranes were incubated with antibodies against Total AMPK (1:1000), Phosphorylate AMPK (1:1000), PPAR-γ (1:1000), Total ACC (1:1000), Phosphorylate ACC (1:1000), FAS (1:1000) and FABP4 (1:1000) (Cell Signaling Technologies, USA) or β-tubulin (1:1000) (Santa Cruz Biotechnology®, USA) followed by anti-IgG peroxidase-conjugated antibody, and then incubated with the peroxidase substrate (ECL kit, Biorad®, USA) and exposed to X-ray film.

Table 1 Effect of 10-day metformin treatment on body weight (BW), tissue weight AND total cholesterol

	SD	SD M	HFD	HFD M
Initial BW (g)	23.35 ± 0.57 (n = 12)	23.70 ± 0.3019 (n = 12)	23.69 ± 0.26 (n = 12)	23.14 ± 0.79 (n = 12)
Tenth week BW (g)	29.860 ± 0.84 (n = 12)	29.74 ± 0.9590 (n = 12)	40.46 ± 0.79^{a,b} (n = 12)	40.50 ± 1.61^{a,b} (n = 12)
Final BW (g)	29.46 ± 0.75 (n = 12)	28.77 ± 0.7969 (n = 12)	42.39 ± 0.91^{a,b} (n = 12)	37.92 ± 1.55^{a,b,c} (n = 12)
Liver weight (g)	0.990 ± 0.023 (n = 12)	1.014 ± 0.043 (n = 12)	1.30 ± 0.058^{a,b} (n = 12)	1.14 ± 0.018 (n = 12)
Adiposity index (g)	1.762 ± 0.214 (n = 12)	1.578 ± 0.2194 (n = 12)	5.109 ± 0.327^{a,b} (n = 12)	4.702 ± 0.245^{a,b} (n = 12)
Retroperitoneal adipose tissue (g)	0.308 ± 0.033 (n = 12)	0.281 ± 0.039 (n = 12)	0.782 ± 0.061^{a,b} (n = 12)	0.7625 ± 0.029^{a,b} (n = 12)
Brown adipose tissue weight (g)	0.115 ± 0.011 (n = 12)	0.1130 ± 0.007 (n = 12)	0.166 ± 0.016^{a,b} (n = 12)	0.144 ± 0.012 (n = 12)
Gastrocnemius muscle (g)	0.2970 ± 0.011 (n = 12)	0.2951 ± 0.006 (n = 12)	0.3034 ± 0.005 (n = 12)	0.2878 ± 0.009 (n = 12)
Total cholesterol (mg/dL)	139.0 ± 15.27 (n = 8)	173.4 ± 14.07 (n = 8)	204.5 ± 16.07^{a} (n = 7)	182.8 ± 10.38 (n = 8)

C57BL/6 mice were fed with a standard diet (SD) or a high-fat diet (HFD), and either treated with metformin (SDM; HFDM) or PBS as placebo

The data are presented as the mean ± SEM

$^{a}p < 0.05$ versus SD

$^{b}p < 0.05$ versus SDM

^{c}HFD versus HFDM (Two-way ANOVA followed by Bonferroni correction)

A
AUC (glycemia - mg/dL)
50000
40000
30000
20000
10000
0
***
SD
HFD

B
Glycemia (mg/dL)
600
400
200
0
0 15 30 45 60 75 90
Time (min)
SD
HFD

C
AUC (glycemia - mg/dL)
50000
40000
30000
20000
10000
0
***
SD
SDM
HFD
HFDM

D
Glycemia (mg/dL)
600
400
200
0
0 15 30 45 60 75 90
Time (min)
SD
SDM
HFD
HFDM

E
Glucose uptake without stimulating insulin (umol/g of tissue)
10
8
6
4
2
0
SD
SDM
HFD
HFDM

F
Glucose uptake with stimulating insulin (umol/g of tissue)
15
10
5
0
**
SD
SDM
HFD
HFDM

G
Palmitate oxidation without stimulating insulin (umol/g of tissue)
80
60
40
20
0
*
**
**
SD
SDM
HFD
HFDM

H
Palmitate oxidation with stimulating insulin (umol/g of tissue)
60
40
20
0
*
SD
SDM
HFD
HFDM

Fig. 1 Variation in glycemia in the glucose tolerance test in the seventh (**a**) and 11th week (after 8 days with metformin treatment) (**c**), respective area under curve (AUC) (**b**, **d**), glucose uptake of tissue soleus without stimulating insulin (**e**), with stimulating insulin (**f**) Palmitate Oxidation without stimulating insulin (**g**) and Palmitate Oxidation with stimulating insulin (**h**) of mice submitted to standard diet (SD) or high fat diet (HFD) for 12 weeks and treated with PBS or metformin (M) (300 mg/kg of body weight) for 10 days. The data are mean ± SEM of 8 animals, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. (Two-way ANOVA followed by Bonferroni)

Insulin Response in Isolated Soleus Muscles

Soleus muscles were carefully isolated from the culled mice, weighed (8–10 mg), and attached to stainless steel clips to maintain resting tension. The muscles were pre-incubated in Krebs–Ringer bicarbonate buffer containing 5.69 mM glucose and 1% bovine serum albumin, pH 7.4, and pre-gassed (95% O_2, 5% CO_2) with agitation (100 oscillations/min). After these procedures, the muscles were transferred to fresh vials containing the same buffer containing 0.3 µCi/mL D-[U-14C]-glucose and 0.20 µCi/mL 2-deoxy-D-[2,6-3H]-glucose in the presence or absence of 7 nM insulin. After the incubation period, the samples were processed to measure the uptake of 2-deoxy-D-[2,6-3H]-glucose, the incorporation of D-[14C]-glucose, the synthesis of [14C]-glycogen, and the decarboxylation of D-[14C]-glucose, according to the methods described by (Challiss et al. 1983; Espinal et al. 1983; Leighton et al. 1985).

Measurement of palmitic acid oxidation in skeletal muscle

Oxidation of [U-14C] palmitic acid was evaluated as previously described (Hirabara et al. 2006; Leighton et al. 1985). Skeletal muscle cells were incubated in DPBS containing 25 µM palmitic acid, 0.2 µCi/mL D-[U-14C]palmitic acid and incubated for 2 h in the absence or presence of etomoxir (10 µM) or bromopalmitate (25 µM). Phenylethylamine, diluted 1:1 v/v in methanol, was added into a separate compartment for $14CO_2$ adsorption.

Isolation and culture of hepatocytes

Hepatocytes were isolated according to the method described by (Edwards et al. 2013), in which mice anesthetized by inhalation of isoflurane were subjected to liver perfusion technique in situ (de Morais et al. 2012) with collagenase. After perfusion, the liver was filtered and the hepatocytes obtained were counted by TRIPAN Blue exclusion method. $1 \times 10^5/cm^2$ viable cells were plated and cultivated in standard medium containing LPS (2.5 µg/mL, *E. coli* O111:B4), and/or metformin (1 µM). After 24 h, the medium was collected for ELISA and a cell viability assay was performed.

Cell viability (MTT)

After the stimulation period, hepatocytes were incubated with MTT (bromide 3-(4,5-dimethylthiazol-2-il)-2,5-diphenyltetrazolium) solution (0.5 mg/mL) for 3 h. The formazan formed was resuspended with isopropanol/HCl (11 M) and the absorbance measured (595 nm) was utilized to calculate the cell viability in relation to the absorbance of the control group (100%). For the treatment of the cells, a dose of 1 µM of metformin was used

Extraction and culture of intraperitoneal macrophages

Macrophages were extracted from the intraperitoneal cavity of C57BL6 mice with RPMI medium and counted in a hemocytometer using TRIPAN Blue exclusion method. $2.5 \times 10^5/cm^2$ viable macrophages were exposed to PBS (control) or LPS (2.5 µg/mL, *E. coli* O111:B4) DMSO or metformin (1 µM) for 24 h. Then, the medium and macrophages were collected for further analysis.

Statistical methods

Normal distribution and variance homogeneity were tested and the appropriate statistical test (two-way ANOVA) was employed. Statistical analysis was carried out with the program GraphPad Prism 5.0, with significance being < 5% ($p < 0.05$). Data are expressed as mean ± standard error of the mean (SEM).

Results

The high fat diet group (HFD) markedly increased the body weight gain of mice (Table 1). This body weight gain caused by the HFD was associated with an increase of liver weight and adiposity (Table 1). As expected by the increase of body weight, the HFD also increased the concentration of total cholesterol (Table 1).

After seven weeks of HFD feeding, an increase of glycemia was observed in the glucose tolerance test (GTT) (Fig. 1b), and this effect could be confirmed by the increased AUC (Fig. 1a). After eight days of metformin (M) treatment, the high fat diet group treated with metformin (HFDM)

A

IL-1β (ng/g of retroperitoneal AT protein)

SD SDM HFD HFDM

B

TNF-α (ng/g of retroperitoneal AT protein)

SD SDM HFD HFDM

C

IL-6 (ng/g of retroperitoneal AT protein)

SD SDM HFD HFDM

D

IL-12 (ng/g of retroperitoneal AT protein)

SD SDM HFD HFDM

E

MCP-1 (ng/g of retroperitoneal AT protein)

SD SDM HFD HFDM

F

IFN-γ (ng/g of retroperitoneal AT protein)

SD SDM HFD HFDM

G

IL- 1RA (ng/g of retroperitoneal AT protein)

SD SDM HFD HFDM

H

IL-10 (ng/g of retroperitoneal AT protein)

SD SD M HFD HFD M

I

IL- 4 (ng/g of retroperitoneal AT protein)

SD SDM HFD HFDM

J

Adiponectin (ng/g of retroperitoneal AT protein)

SD SDM HFD HFDM

◂**Fig. 2** Protein expression in retroperitoneal adipose tissue by ELISA of **a** IL-1β, **b** TNF-α, **c** IL-6, **d** IL-12, **e** MCP-1, **f** IFN-γ, **g** IL-1RA, **h** IL-10, **i** IL-4 and **j** Adiponectin of mice submitted to standard diet (SD) or high-fat diet (HFD) for 12 weeks and treated with PBS or metformin (M) (300 mg/kg of body weight) for 10 days. The data are mean ± SEM of 8 animals, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (Two-way ANOVA followed by Bonferroni)

presented a lower glycemia in the glucose tolerance test (Fig. 1 C/D).

Although the HFD or the metformin treatment had no effect on the weight of the gastrocnemius muscle (Table 1), when compared to the HFD group (Fig. 1f), this drug increased the glucose uptake in skeletal muscle of mice fed with a standard diet. Additionally, increased palmitate oxidation in skeletal muscle in both situations, without insulin stimulus when compared to all the other groups (Fig. 1g) and with the insulin stimulus when compared to the SD (Fig. 1h).

Despite the total weight loss of the HFDM group when compared to the HFD group, when weighed the adiposes tissues we did not observe significant differences between these groups (Table 1). In the retroperitoneal adipose tissue, the protein content of IL-1β, TNF-α, IL-6, IL-12, MCP-1, IFN-γ, IL-1RA, IL-10, IL-4 and adiponectin was analyzed (Fig. 2). This analysis allowed us to observe that IL-1β (Fig. 2a), IL-6 (Fig. 2c) and MCP-1 (Fig. 2e) were increased in the HFDM when compared to standard diet groups. Similarly, the TNF-α (Fig. 2b) and IFN-γ (Fig. 2f) were increased in SDM when compared to SD, while IL-12 (Fig. 2d) was higher in both groups treated with metformin, when compared to their respective controls.

These results show that metformin increased the concentration of some inflammatory cytokines, however, the same effects of metformin were observed on anti-inflammatory cytokine concentrations. The levels, of IL-1RA (Fig. 2g) and IL-10 (Fig. 2h), were increased in the adipose tissue from HFDM, when compared to SD. Similarly, the concentrations of IL-4 and adiponectin (Fig. 2i, j, respectively) were also increased by metformin in both diets, when compared to their respective controls.

In histological slides of the liver stained with H&E, Oil Red and Sirius red, we observed that the lipid accumulation caused by the HFD, was partially reduced in the MET treatment (Fig. 3). The gene expression of AMPK (Fig. 4a), ACC (Fig. 4b) and FAS (Fig. 4c) in the liver was not significantly different. The protein expression of pAMPK (Fig. 5a) increased in the HFDM when compared with HFD, and pACC (Fig. 5b) presented no significant difference. This hepatic steatosis induced by the HFD was not associated with liver inflammation in the HFD. However, metformin seemed to induce liver inflammation, as observed by the higher hepatic concentration of IL-1β (Fig. 6a), TNF-α (Fig. 6b), IL-6 (Fig. 6c), MCP-1 (Fig. 6e) and IFN-γ (Fig. 6f) in HFDM mice. Besides its inflammatory effects, metformin also increased the hepatic concentration of IL-10 (Fig. 6h) and adiponectin (Fig. 6j). Nevertheless, the concentration of FGF-21 (Fig. 6k) was decreased in the liver of mice treated with metformin, and the levels of IL-12 (Fig. 6d), IL-1RA (Fig. 6g) and IL-4 (Fig. 6i) were not modulated by the diet nor the -treatment. In the protein expression analyzed by WB, we observed that NF-κB is increased in the groups treated with metformin (Fig. 6l).

Although metformin increased the levels of IL1β in the livers of HFD mice, the hepatic mRNA expression of IL-1β decreased in HFDM when compared with SD. (Figure 7e). However, other inflammatory genes such as IKB-α (Fig. 7c), NF-κB (Fig. 7d), NLRP-3 (Fig. 7f) and Caspase-1 (Fig. 7g), as well as the receptors OCT-1 (Fig. 7a) and OCT-3 (Fig. 7b), were not altered by the diets nor the treatments.

In cultured isolated hepatocytes, the expression of IL-1β, IL-6 and TNF-α stimulated with LPS was analyzed and then treated with 1 μM of metformin. We observed that metformin, exclusively, increased the concentration of IL-1β (Fig. 8a) and IL-6 (Fig. 8b), and metformin plus LPS increased the concentration of TNF- α (Fig. 8c). However, in intraperitoneal macrophages, we observed lower levels of IL-1β (Fig. 8d), IL-6 (Fig. 8e) and TNF-α (Fig. 8f) in those cells stimulated with LPS and then treated 1 μM of metformin.

Discussion

We observed that the metformin treatment (300 mg/kg of metformin) improved glucose tolerance, weight loss, increased palmitate oxidation in skeletal muscle, alleviated the lipid accumulation in the liver, with a decrease in lipid storage and fibrosis. Furthermore, we observed the different expression of cytokines that occur in different proportion dependent of the tissue, or cell type. A pro-inflammatory profile was found in liver, and we observed that this effect was induced by increased in pro-inflammatory cytokines produced by hepatocytes, while the pro and anti-inflammatory cytokines were increased in adipose tissue, and finally the metformin avoided the pro-inflammatory response in macrophages stimulated with LPS.

Metformin is a well-established drug used for the treatment of type 2 diabetes mellitus (T2D) for decades and appears to also be useful against obesity (Nasri and Rafieian-Kopaei 2014). Specifically, metformin inhibits gluconeogenesis by decreasing the endogenous glucose production by liver (Nasri and Rafieian-Kopaei 2014). Furthermore, this drug improved both insulin sensitivity and β-cell function (Bi et al. 2013; Patane et al. 2000). An improved glucose tolerance was observed in our model.

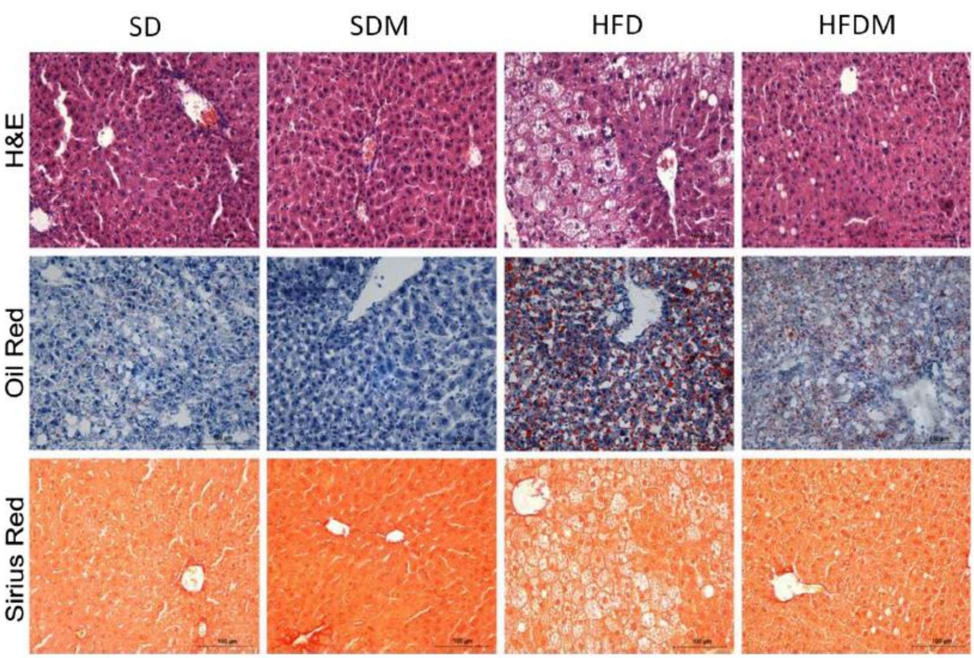

Fig. 3 Histological slices of livers colored by hematoxylin and eosin (H&E), Oil Red and Sirius Red at ×40 magnification. Livers of mice submitted to standard diet (SD) or high fat diet (HFD) for 12 weeks and treated with PBS or metformin (M) (300 mg/kg of body weight) for 10 days. $N = 4$ per group

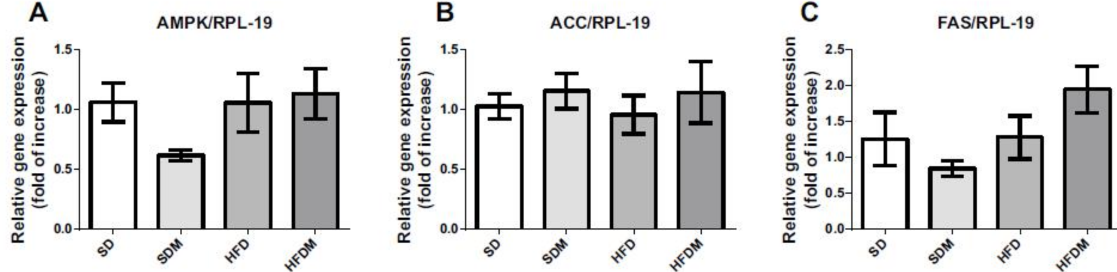

Fig. 4 mRNA expression in liver of **a** AMPK, **b** ACC and **c** FAS of mice submitted to standard diet (SD) or high-fat diet (HFD) for 12 weeks and treated with PBS or metformin (M) (300 mg/kg of body weight) for 10 days. The data are mean ± SEM of 8 animals, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. (Two-way ANOVA followed by Bonferroni)

Moreover, the treatment increased palmitate oxidation in the skeletal muscle of mice submitted to a HFD and treated with metformin. Kitzmann et al. showed that skeletal muscle cells derived from moderately obese T2D patients exhibited decreased beta-oxidation and increased lipid accumulation in response to palmitate overload (Kitzmann et al. 2011). Other studies showed that metformin induced decreases in palmitate uptake (Bogachus and Turcotte 2010) and suppression of lipid accumulation in skeletal muscle, by promoting an increase in fatty acid oxidation (Wang et al. 2014). In fact, some studies show the relation between the abnormalities in skeletal muscle, such as fat accumulation, insulin resistance and loss of skeletal muscle, with the pathogenesis and severity of hepatic steatosis (Flannery et al. 2012; Kitajima et al. 2013; Moon et al. 2013).

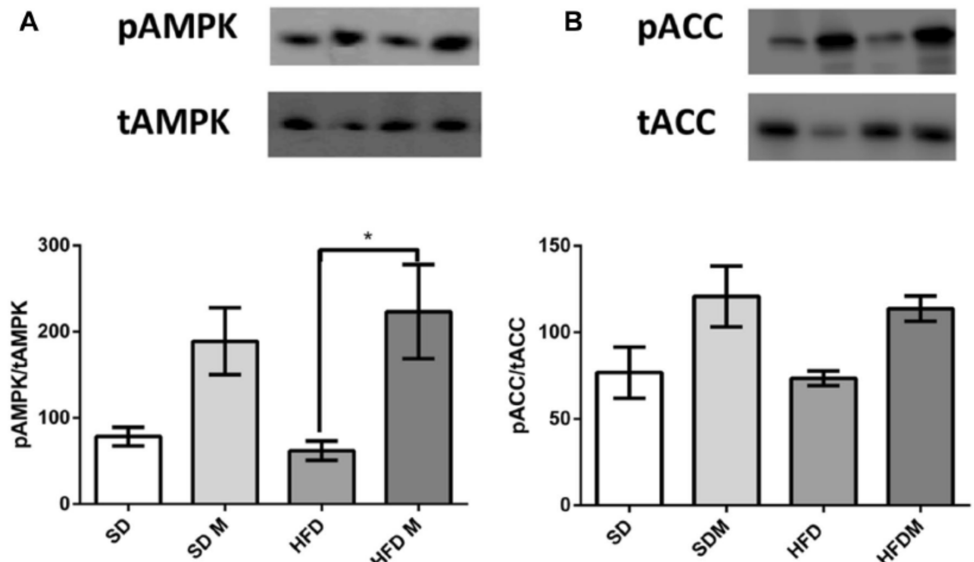

Fig. 5 Protein expression in liver by WB of **a** AMPK and **b** ACC of mice submitted to standard diet (SD) or high-fat diet (HFD) for 12 weeks and treated with PBS or metformin (M) (300 mg/kg of body weight) for 10 days. The data are mean ± SEM of 6 animals, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. (Two-way ANOVA followed by Bonferroni)

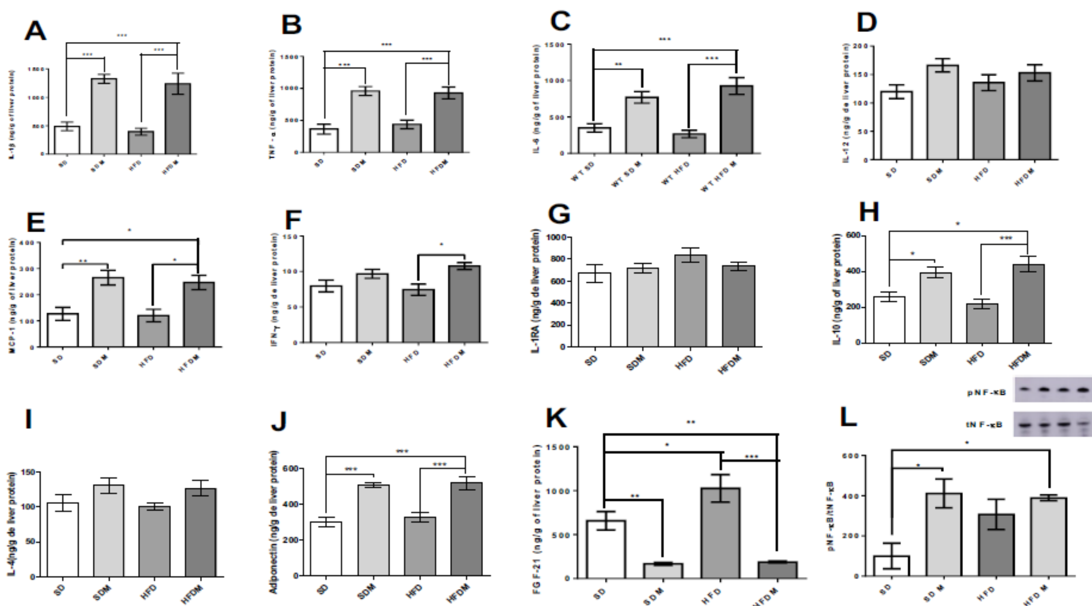
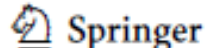

Fig. 6 Protein expression in liver by ELISA of **a** IL-1β, **b** TNF-α, **c** IL-6, **d** IL-12, **e** MCP-1, **f** IFN-γ, **g** IL-1RA, **h** IL-10, **i** IL-4, **j** Adiponectin and **k** FGF-21 of mice submitted to standard diet (SD) or high-fat diet (HFD) and Protein expression in liver by WB of phosphorylated and total NF-κB p65 (L) for 12 weeks and treated with PBS or metformin (M) (300 mg/kg of body weight) for 10 days. The data are mean ± SEM of 8 animals in ELISA and 4 animals in WB, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ (Two-way ANOVA followed by Bonferroni)

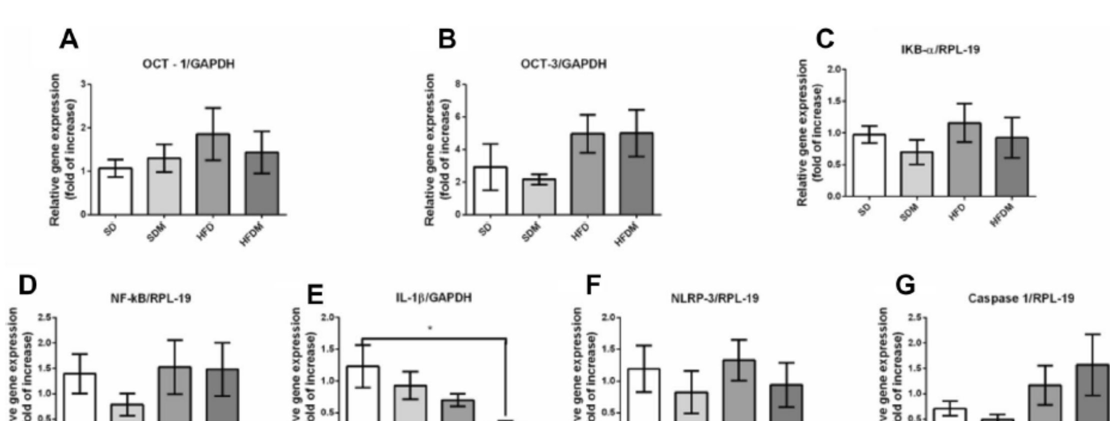

Fig. 7 mRNA expression in liver of **a** Organic Cation Transporter 1 (OCT-1), **b** Organic Cation Transporter 3 (OCT-3), **c** nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκB-α), **d** nuclear factor kappa light chain enhancer of activated B cells (NF-κB), **e** Interleukin 1β (IL-1β), **f** leucine-rich repeat-containing family protein-3 (NLRP-3) and **g** Caspase 1 of mice submitted to standard diet (SD) or high-fat diet (HFD) for 12 weeks and treated with PBS or metformin (M) (300 mg/kg of body weight) for 10 days. The data are mean ± SEM of 8 animals, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$. (Two-way ANOVA followed by Bonferroni)

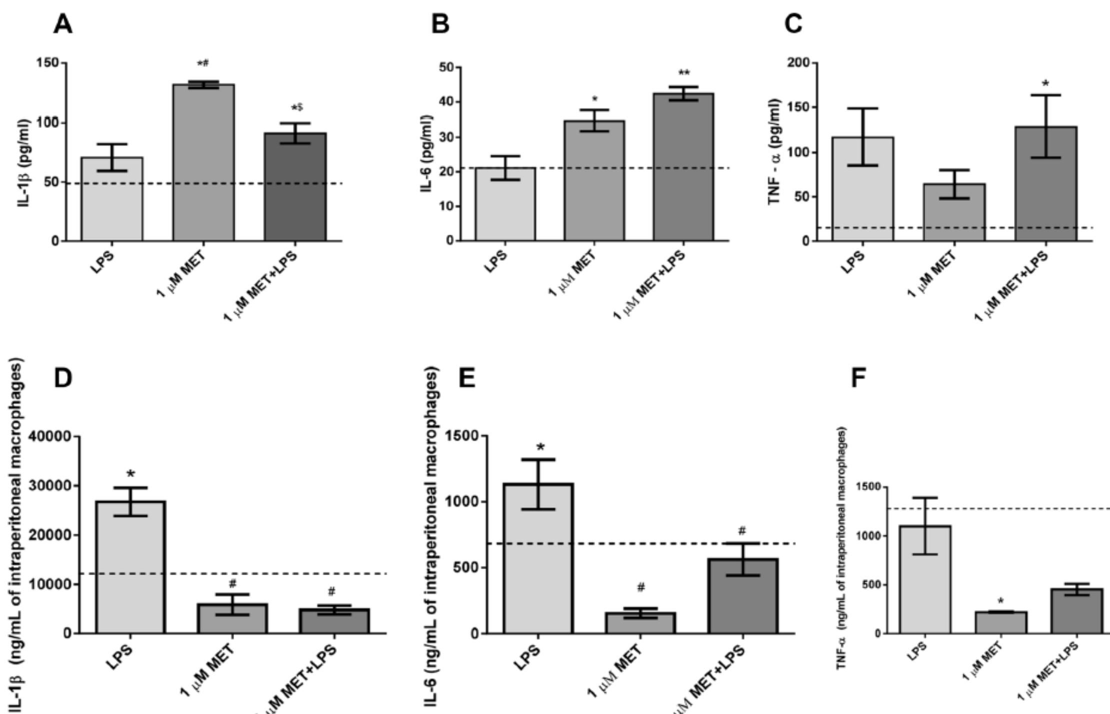

Fig. 8 Protein expression in hepatocytes by ELISA of **a** IL-1β, **b** IL-6 and **c** TNF-α stimulated with 2.5 ng/mL LPS and/or treated with 1 μM of metformin and protein expression in intraperitoneal macrophages by ELISA of **d** IL-1β, **e** IL-6 and **f** TNF-α stimulated with 2.5 ng/mL LPS and/or treated with 1 μM metformin. The data are mean ± SEM of 4 animals, the dotted line represents the control group, $*p < 0.05$ versus control group, $^{\#}p < 0.05$ versus LPS group and $^{\$}p < 0.05$ versus 1 μM MET group. (Two-way ANOVA followed by Bonferroni)

The improvement in glucose homeostasis showed in the HFDM could be explained by decreased in weight gain, associated with increased in adiponectin content and huge increased in anti-inflammatory cytokines (IL-1RA, IL-10 and IL-4) induced by metformin treatment in HFD mice.

We observed a decrease in NAFLD in mice submitted to a HFD and treated with metformin confirmed by histological analysis. AMPK protein expression in the liver was increased in the HFDM group. Also, the phosphorylated AMPK (THR 172) had suppressed expression of lipogenesis-associated genes like fatty acid synthase, and phosphorylated ACC, thus deactivating them (Foretz and Viollet 2011; Viollet et al. 2009). Although the ACC did not increase significantly in the HFDM group, there was a trend observed. ACC is an enzyme that controls the synthesis of malonyl-CoA, which is a precursor for the biosynthesis of fatty acids and a potent inhibitor of mitochondrial fatty acid oxidation by inhibiting CPT1. Inhibition of ACC by AMPK leads to a decrease in malonyl-CoA content, and a subsequent decrease in fatty acid synthesis and an increase in fatty acid oxidation. Thus, excessive storage of triglycerides in the liver is reduced (Foretz and Viollet 2011; Viollet et al. 2009). The association between the increase in peripheral fatty acid oxidation and the increase of AMPK in the liver led to the decrease of NAFLD by metformin.

Furthermore, the chronic utilization of low doses of metformin also presents an anti-inflammatory effect in humans, animal models, and in cell culture (Koh et al. 2014; Woo et al. 2014; Xu et al. 2015b).

In our study, 300 mg/kg of metformin during 10 days promoted a dual inflammatory and anti-inflammatory effect in the retroperitoneal adipose tissue and intraperitoneal macrophages, respectively. Kim et al. 2014, showed that metformin presented an anti-inflammatory action in intraperitoneal macrophages, partially through pathways involving the activation of AMPK and activating transcription factor-3 (ATF-3). A mechanism model shows that ATF-3 inhibits the translocation of NF-kB subunit involved in the transcription of pro-inflammatory genes such as TNF-α and IL-6 (Kim et al. 2014). The potential, anti-inflammatory mechanism through which metformin operates is by the inhibition of NF-κB through AMPK-dependent and independent pathways (Salminen et al. 2011). Metformin can also increase nitric oxide production and inhibit the poly [ADP ribose] polymerase 1 pathway through AMPK activation, leading to suppression of the inflammatory response. (Saisho 2015).

However, in the liver, metformin caused an inflammatory effect, which was further confirmed with the hepatocyte isolation. It is interesting once a recent study has shown a relationship between inflammation and the progression of NAFLD to steatohepatitis (Wan et al. 2016). However, we observed that the metformin treatment was able to promote a reduction in lipid toxicity and in accumulation in the liver. In according with our findings, the recent meta-analysis showed that the metformin administration reduced the serum levels of glucose and lipids with the improvement of biochemistry parameters, but induced lobular inflammation after metformin treatment in humans (Said and Akhter 2017).

In conclusion, the short-term metformin treatment reversed lipid accumulation in liver by central and peripheral oxidation, improved the glucose homeostasis, ameliorated the inflammation in adipose tissue and intraperitoneal macrophages, but leads to an inflammatory response in liver through an increase in cytokine production by hepatocytes.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interests regarding the publication of this paper.

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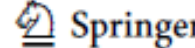

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Aerobic Exercise Modulates the Free Fatty Acids and Inflammatory Response During Obesity and Cancer Cachexia

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ABSTRACT: White adipose tissue (WAT) is no longer considered a tissue whose main function is the storage of TAG. Since the discovery of leptin in 1994, several studies have elucidated the important role of WAT as an endocrine organ, the source of the adipokines. The low-grade inflammation observed in obese and cancer cachexia patients is explained, at least partially, by the exacerbated release of proinflammatory adipokines. Despite of the recent progress in the characterization of the various adipokines and lipokines produced by WAT, little is known about the mechanisms regulating the secretion of these molecules in different physiological and pathological circumstances. Chronic exercise is a nonpharmacological therapy employed in several chronic diseases and shows an anti-inflammatory effect through the regulation of the cytokine network. In this review, we address the potential mechanisms by which the aerobic physical exercise modulate the production and release of inflammatory adipokines, as well as the inflammation-lipolysis axis in WAT, with special focus in the therapeutic role of exercise in obesity-associated insulin resistance and cancer cachexia.

KEY WORDS: white adipose tissue, inflammation, obesity, cancer cachexia, aerobic exercise

I. INTRODUCTION

White adipose tissue (WAT) has been envisaged for decades as a tissue that stores energy as lipids in situations of positive energy balance and releases this substrate in periods of increased metabolic demand. However, the underlying biology and metabolism of adipose tissue have been shown to be much more complex (for a review, see Thompson et al.[1]). Currently, it is recognized that WAT produces and releases several proteins denominated as adipokines. These proteins have important functions regulating metabolic processes in several tissues, both healthy and diseased. For instance, in addition to leptin, an adipokine that acts on the hypothalamus, regulating food intake and energy expenditure, adipose tissue secretes, among others, adiponectin, which regulates glucose uptake and liver fatty acid oxidation[2,3] acylation stimulation protein (ASP), which regulates fatty acid uptake and incorporation into triacylglycerol[4] and several pro- and anti-inflammatory cytokines that modulate immune function.[5] Recently, in addition to proteins, adipose tissues have been associated with the production and release of fatty acid–derived nonsteroid lipid hormones, denominated as lipokines. One of these lipokines, C16:1 n7-palmitoleate, was demonstrated to act in the muscle and liver, improving cell sensitivity to insulin and blocking hepatic fat accumulation.[6]

In mammals, adipose tissue has been classified into two main distinct types: WAT is the primary site of energy storage, and brown adipose tissue

(BAT), which is associated with heat production.[7] Recently, human BAT was found to be induced by cold exposure, which enhances energy expenditure and decreases body fat mass, indicating that BAT is an interesting target for obesity treatment.[8]

In obesity, WAT mass is increased, and in cachectic cancer patients, an opposite effect is observed; obesity and cancer cachexia share some similarities regarding WAT biologies. For example, in both conditions, WAT plays a fundamental role in the inflammatory state, contributing to regional as well as to systemic inflammation.[9,10] Adipokine secretion by adipocytes or vascular stromal cells may be responsible for some metabolic disarrangements in these conditions that lead to sustained growth of tumor.[11] Physical exercise, a therapeutical strategy recognized in the prevention and treatment of obesity, cancer, and associated diseases, has been shown to modulate the endocrine function of WAT.[12–14] In the present review, we address the mechanisms by which the physical exercise modulate adipose tissue production and release of inflammatory adipokines and the vicious cycle of inflammation, lipolysis, with a special focus on the putative therapeutic role of exercise in obesity and cancer cachexia.

II. IMMUNE CELLS AND ADIPOSE TISSUE

Extensive research over the years has elucidated the biological function of several adipokines and lipokines released by WAT and their implication in the regulation of the immune system. In addition to the release of classical cytokines such as interleukin (IL)-6 and -10 and tumor necrosis factor (TNF)-α, other adipokines have been demonstrated to act upon and modulate immune cell functions.[15,16] This may be illustrated, for instance, by the existence of leptin receptor in several immune cells including monocytes, macrophages, and lymphocytes.[17] Leptin upregulates the production of proinflammatory cytokines by monocytes[18] and induces the maturation and survival of lymphocyte T cells.[19] On the other hand, adiponectin secreted by WAT has an opposite effect to leptin in monocytes, stimulating the release of anti-inflammatory cytokines.[20]

A. Inflammation-Induced Adipose Tissue Lipolysis

A great number of proinflammatory adipokines induce lipolysis, including IL-1β, IL-6, TNF-α, and the interferon family.[21] Suganami et al. showed that free fatty acids release at the lipolysis in adipocytes activates monocytes, creating a paracrine loop between lipolysis and local inflammation.[22] However, several members of the Toll-like receptor (TLR) family are expressed in adipocytes and recognize the free fatty acids (FFAs) as ligands inducing the production of proinflammatory adipokines (Fig. 1).

This featured is shared by the two antagonic syndromes: obesity and cachexia. In these diseases, patients show high levels of FFAs and proinflammatory mediators in serum.[23] Initially, TNF-α was considered the primary mediator of inflammation-lipolysis-insulin resistance.[24,25] Despite the success of anti-TNF-α therapy in animals models, with antibodies that neutralize this protein[26–28] and the success of studies with TNF-α knockout mice, which attenuated the insulin resistance and improved glucose metabolism in the obese mice and rats,[29] in clinical practice the results are disappointing: chronic administration of anti-TNF-α agents failed to improve insulin resistance in humans.[30]

B. Obesity, Cancer, and Inflammation

In spite of the well-established association between obesity and low-grade inflammation, the triggering factor (or factors) linking these two conditions have yet to be identified. Several pieces of evidence point to an important role of lipids as mediators of excessive fat accumulation and chronic inflammatory processes. The activation of members of the TLR family, an evolutionarily ancient pattern-recognition class of receptors that facilitate the detection of pathogens,[31] triggers a potent immune response that encompasses the production of cytokines and chemokines by immune cells and adipocytes, as well as the upregulation of costimulatory molecules that contribute to the induction of adaptative immune responses.[32,33] Since the 1970s, studies have shown that obese animals presented increased basal lipolysis.[34]

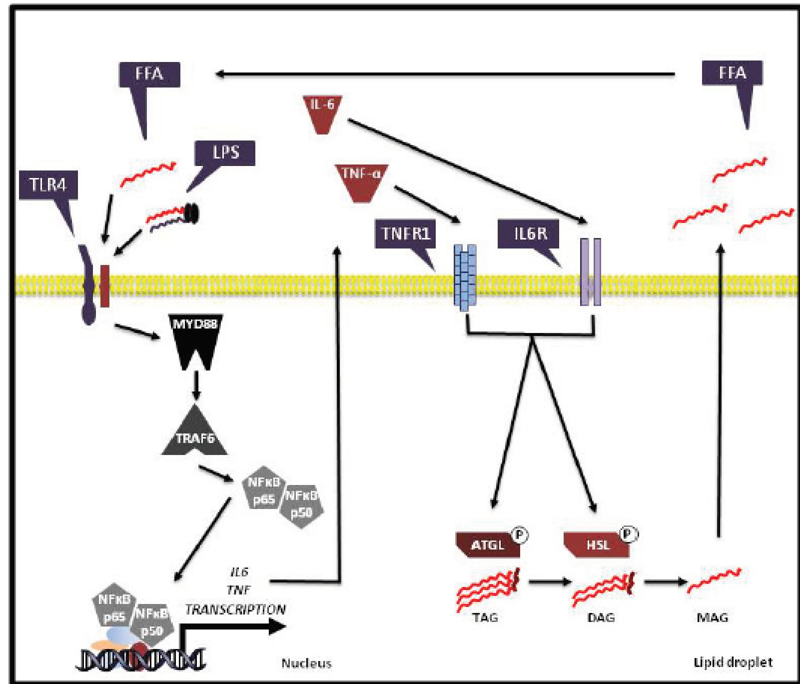

FIG. 1: The lipolysis-inflammation cycle occurs via elevation in free fatty acids (FFAs), which increase adipose tissue lipolysis. The excess of fatty acids causes an increase in the paracrine effect and increased inflammation. The inflammatory cytokines increased the lipolysis; these cytokines increase the ATGL and HSL activity, which increases the FFA in intersticial of adipose tissue, generating the vicious cycle.

FFA concentrations are augmented in the serum in obese patients in relation to eutrophic weight subjects. One elegant study[35] showed that over 24 hours, FFA concentrations in obese adolescents were higher than in nonobese adolescents, mainly in the afternoon and at night. Chronic elevation in FFA levels promotes metabolic disturbances such as insulin resistance, intolerance to glucose, and hyperglycemia[36,37] as well as low-grade inflammation.[38] Moreover, as previously stated, the increased in proinflammatory cytokines caused by augmented FFA release further enhances lipolysis, which contributes to the establishment of the lipolysis-inflammation cycle (Fig. 1). In addition, obesity and type 2 diabetes mellitus have been associated with ectopic fat deposition in non-adipose tissue, such as skeletal muscle, which plays a central role in the etiology of insulin resistance.[39] Ectopic lipids principally accumulate as triacylglycerols deposited within lipid droplets in the skeletal muscle and liver.

The previously mentioned TLR differential activation depends on the type of fatty acid. Thus, saturated long-chain fatty acids (SLCFAs), such as palmitate, stearic acid, and the laureates (saturated medium-chain fatty acids), increased TLR-4 expression in different cell types (e.g., microglia, adipocytes, skeletal muscle, liver, macrophages, and hypothalamus).[40,41] However, FFA cannot to directly bind to TLR-4.[42] Recently, a protein was proposed to be the endogenous ligand causing TLR-4 activation and FFA, fetuin-A (FETA). It is produced by the liver and released in circulation.[43] In cancer patients, FETA in circulation is not associated with diagnosis or disease severity,[44] and it is not related to colon rectal cancer.[45] It is necessary to study the circula-

tion levels of FETA in cancer cachexia states further.

Conversely, mice containing a loss-of-function–related mutation in TLR4 are partially protected from high-fat diet-induced inflammation and insulin resistance.[33] These findings clearly suggest that TLRs represent a potential linking factor between lipid metabolism and innate immune responses.

III. ADIPOSE TISSUE AND CANCER

Several studies have found a positive association between obesity and some types of cancer. A recent estimate suggests that approximately 20% of all cancer-related deaths worldwide are associated with obesity.[46] Furthermore, associated studies have indicated that body mass index (BMI) is an important risk factor for the development of breast, endometrial, esophageal, and renal cancer.[47] Although the mechanisms linking obesity with cancer are poorly understood, the evidence suggests that the enhanced secretion of adipokines during obesity may have a role in accelerating tumor development.[48] For example, leptin, whose plasma levels are elevated in obese subjects, was demonstrated to stimulate the proliferation of neoplastic cells.[49] On the other hand, adiponectin, which is reduced in obese individuals, was found to inhibit tumor growth and angiogenesis.[50] Corroborating on a possible role of adiponectin in the inhibition of carcinogenesis, reduced plasma levels of this adipokine have been associated with an increased risk of breast cancer.[51] In addition to leptin, proinflammatory cytokines were shown to trigger cancer by stimulating the proliferation of neoplastic cells.[48]

Cachexia is not restricted to cancer patients. Others diseases, such as AIDS, congestive heart failure, diabetes, and others may induce this syndrome. One important aspect of cancer-associated cachexia is the exacerbated loss of WAT and skeletal muscle, which are due to increased fat mobilization, lipolysis, and proteolysis, respectively (Fig. 2).[52,53]

The mechanisms of exacerbated lipolysis found in cachexia have not been completely elucidated. Actually, it is believed that exacerbated lipolysis is associated with increased mRNA and that protein levels of hormone-sensitive lipase, a key enzyme of lipolysis, are markedly increased in the adipose tissue of patients with cancer-associated cachexia.[54]

The increased beta adrenergic response could be caused by augment on sympathetic nervous system tonus, via increased in inflammatory cytokine in the paraventricular nucleus,[53] yet elevation in circulating catecholamines concentration is not observed in cancer cachexia patients.[55] The stimulated lipolysis observed in cachexia could be promoted by increased in number and activity of adrenergic receptors in WAT,[54] especially the β1-adrenergic receptor.[52,56]

In addition to the increased hormone sensitivity lipase (HSL) activity and expression associated with cancer cachexia,[54] adipose triglyceride lipase (ATGL) seems to play a fundamental role in the syndrome.[57] Tumor-bearing mice showed increased in ATGL protein levels in WAT,[57] whereas mice with ATGL deletion had a protective effect in WAT and skeletal muscle loss by decreased in FFA mobilization.[58]

Other mechanisms driving exacerbated lipolysis found in cachexia are associated with pro-inflammatory cytokines, especially TNF-α and IL-6, which directly promoted lipolysis in adipose tissue.[59] Our group has demonstrated a marked increase in TNF-α protein levels in retroperitoneal WAT of Walker 256 tumor-bearing cachectic rats.[60] These findings contrast with results from patients with gastrointestinal tumor and cachexia in whom differences in the expression of IL-6 and TNF-α mRNA in white adipose tissue were observed.[53] Taken together, these findings suggest that stimulation of adipose-tissue proinflammatory adipokines in cachexia are tumor- and adipose-depot–specific.

Adipose tissue in cachexia oversecretes chemokines and proinflammatory cytokines and is markedly infiltrated by immune cells.[13] The triggering factors driving the marked infiltration of immune cells in adipose tissue of cachetic rats are unknown, but it is possible that lipolysis-derived fatty acids might have an important role in this process. An increase in lipolysis-derived fatty acids levels activates TLR-4 signaling, stimulating the NF-kB pathway, and thus increasing the expression of several cytokines and chemokines in immune cells and adipocytes. Therefore, by activating TLR-4, lipolysis-derived fatty acid

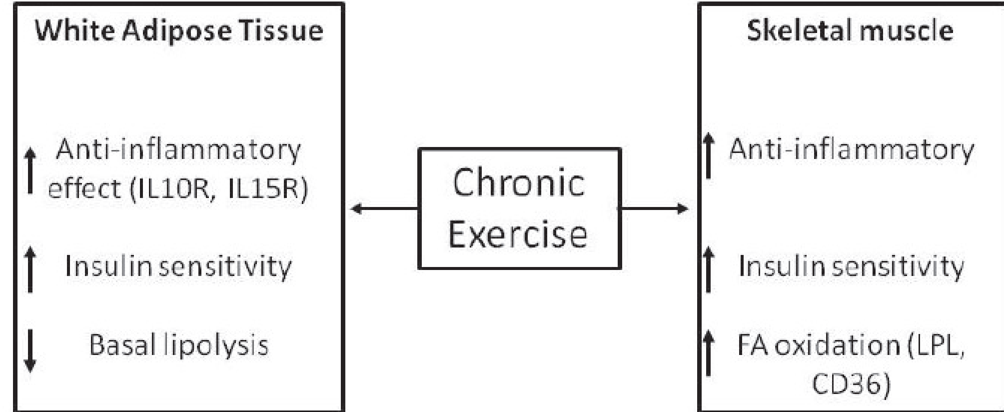

FIG. 2: Moderate chronic aerobic exercise increases fatty acid oxidation, increases the uptake of FFA in circulation by peripheral tissues by improved in insulin sensitivity, and increases the antiinflammatory cytokines in skeletal muscle as well as receptors of these cytokines in adipose tissue.

would potentiate the inflammatory response and the release of pro-inflammatory adipokines by adipocytes that in turn would enhance lipolysis and fatty acids release, creating a positive feedback system (Fig. 1).

IV. MODERATE AEROBIC TRAINING AS THERAPY

It is well established that physical exercise is one of the most recommended strategies to prevent and treat obesity and some types of cancer, lead to the sedentary life style being considered like disease.[61,62]

In cachectic patients, skeletal muscle and adipose tissue loss is accentuated and associated with poor prognosis and deep impact on patient quality of life.[63] Moderate aerobic training (MAT, 65–70%vo_2max) is able to maintain cardiovascular fitness and decrease fatigue and muscle strength loss; MAT could be a non-pharmacological treatment option for decrease side effects of cachexia (as reviewed in Lira et al.[64]). However, the effects of MAT in cancer cachexia are not well established in human patients.

On the other hand, the excess of adipose mass in obesity is associated with insulin resistance, metabolic syndrome, and low-grade inflammation.[61] The beneficial effects of MAT in obese patients are associated with decreased in low-grade inflammation,[65,66] improved lipid profile,[67] and glucose sensitivity.[68]

Both obesity and cachexia lead to metabolic, and inflammatory disturbances, and these alterations are attenuated by MAT. FFA has a central role in this interaction between inflammation and metabolism in obesity and cachexia, via TLR-4 signaling. Our hypothesis, therefore, is that MAT leads to a decrease in FFA by increasing oxidation, thus reducing activation of TLR4 and, consequently, promoting the increased in anti-inflammatory cytokines production in skeletal muscle.[69] As a result, systemic inflammation is downregulated.

A. Aerobic Exercise Driver the FFAs and Oxidation in Pathologic Conditions

FFAs in circulation are increased in obesity and cachexia because basal lipolysis is increased and FFA uptake by skeletal muscle is decreased under these conditions. Acute aerobic exercise increases nonesterified fatty acids in the circulation by increasing sympathetic nervous activity through the release of adrenaline,[70,71] which activates hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL).

Deletion of these enzymes impairs exercise performance.[72,73] In addition, we showed that acute exhaustive exercise increases the TLR4 activation in the adipose tissue.[74]

Three or four months after chronic aerobic exercise, there is a conspicuous decrease in FFA in obese humans, which is induced by reduced basal lipolysis.[75,76] In rats, after 8 weeks, this effect is observed in rats fed a high-fat diet.[77]

Accordingly, it has been recently demonstrated that myocites require fatty acid receptors for the uptake of FFAs. The fatty acid translocase (CD36) is increased in mice subjected to aerobic training, and it is an important mediator of physical performance.[78,79] However, the other receptor of long-chain fatty acids, the G protein-coupled receptor GPR120, is not necessary for the uptake of FFA by skeletal muscle during exercise.[80]

MAT increases fatty acid oxidation in skeletal muscle by increasing in carnitine palmitoyl transferase I (CPT-1) and citrate synthase activity in human skeletal muscle.[81] Additionally, physical exercise and fasting can increase the blood factor (FGF21) concentration, which activates the noradrenaline secretion, stimulating the HSL in the WAT.[82,83]

However, in cancer cachexia, few studies have evaluated aspects that involve the energy metabolism. Recently, it has been found that cancer cachexia impairs the oxidative metabolism in the skeletal muscle.[84–86] Mitochondrial function in skeletal muscle is compromised by cancer cachexia. Incubation of C2C12 myotubes with the serum of mice bearing the Lewis Lung carcinoma showed alteration in electron flux, with a decrease in ATP production and elevation in reactive oxygen species.[87] Although aerobic exercise appears to modulate FFA levels during obesity and cancer cachexia, the mechanisms by which the chronic physical training improve the clinic conditions remain unknown.

B. Anti-inflammatory Effect of Exercise Training in Adipose Tissue

Another mechanism by which MAT acts is the increased of anti-inflammatory response. The similarity found in inflammation of adipose tissue in cachetic cancer and obese patients indicates that an anti-inflammatory therapy could be adopted for both diseases.

The modulatory effects of MAT in adipose tissue can be associated with beneficial effects in protection from low-grade inflammation. In rats, after 8 weeks of aerobic training, the IL-10/TNF-α ratio is increased in the mesenteric adipose tissue, indicating an anti-inflammatory effect of endurance training.[42] Likewise, we and others groups have demonstrated that MAT decreases the mRNA and protein expression of IL-6, TNF-α, and MCP-1 in adipose tissue of obese rats subjected to aerobic exercise training.[14,88–90] The same effect was observed in tumor-bearing rats with cachexia associated with cancer. MAT decreased the inflammation in adipose tissue and maintained adipose tissue mass.[60]

Interleukin-6, TNF-α, interferon-α, and other pro-inflammatory cytokines are increased in the serum of cachetic and obese patients, with the contribution of the adipose tissue. These cytokines are responsible for increased in lipolysis and proteolysis, which are considered pro-cachetic factors.[91]

First, MAT increases the release of anti-inflammatory cytokines, in special of IL-10, and of IL-1ra by the muscle, and these myokines lead to the development of an anti-inflammatory environment in different tissues, such as adipose tissue, liver, and brain (Fig. 2).[92–95]

Moreover, MAT promotes a reduction in the number of inflammatory immune cells in adipose tissue. In humans, exercise increases M2 macrophage counts, characterized by increased CD163+ cell number in the subcutaneous adipose tissue.[96] Additionally, in mice it was observed that exercise promotes a reduction of CD8 lymphocyte in the adipose tissue, the suppression of macrophage infiltration, and an increase in the polarization of M2 in the epididymal adipose tissue.[97] In addition, the IL6 cytokine acts as an important molecule that potentiates the polarization of M2 macrophages via IL4 cytokine; once the IL4 is increased, the inhibition of pro-inflammatory macrophages (M1) occurs. These findings are also dependent on the AMPK-PI3K-Akt axis.[98]

In addition, MAT decreased the TLR4 mRNA and protein in adipose tissue[97,99] with consequent

blunting of the TLR-4 to ligands (FFA). Therefore, aerobic training acts as a key modulator of the metabolic-immuno-inflammatory axis.

Finally, we propose that the effect of MAT in the vicious cycle inflammation downregulation and lipolysis consists of an interaction of the metabolic effects of MAT (increased uptake and oxidation of FFA) associated with decreased TLR-4 signaling, which leads to a healthier cycle (Fig. 3).

V. CONCLUSION

In conclusion, the virtuous cycle generated by aerobic exercise (Fig. 3) is an important nonpharmacological treatment that decreases low-grade inflammation and improves the fatty acid metabolism—common symptoms of obesity and cancer cachexia. Likewise, future studies are needed to reveal the molecular mechanisms and their consequences related to the anti-inflammatory action of MAT, which may lead to the prescription in clinical practice of adequate MAT for cachetic subjects.

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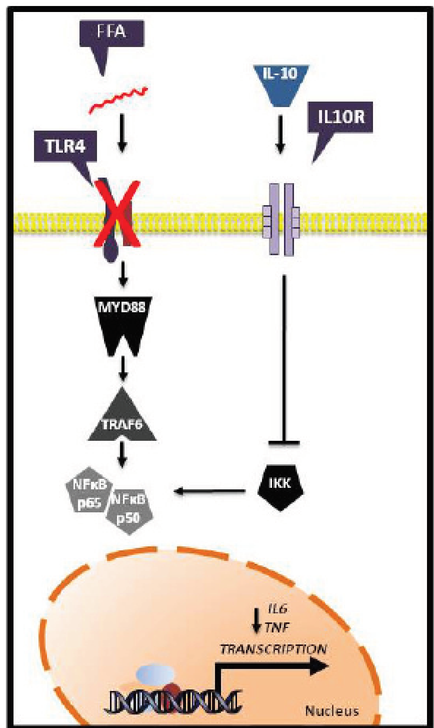

FIG. 3: The beneficial effects of exercise, the "virtuous cycle." Moderate aerobic training (MAT) increases fatty acid uptake and oxidation in skeletal muscle and reduces TLR4 expression, thereby reducing the receptor and ligant. Moreover, anti-inflammatory cytokine expression was elevated in the tissue and circulation.

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