**JUSSARA MARIA RAGONEZZI MARAGNO CORRÊA**

# INTERAÇÃO BI-DIRECIONAL ENTRE SONO E CRESCIMENTO TUMORAL EM CAMUNDONGOS

Dissertação Apresentada à Universidade Federal de São Paulo – Escola Paulista de Medicina para obtenção do Título de Mestre em Ciências

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Dissertação preparada durante o curso de Pós-graduação em Farmacologia, no Departamento de Farmacologia, Setor de Neurotransmissores, e apresentada à Universidade Federal de São Paulo – Escola Paulista de Medicina, como requisito parcial para a obtenção do Título de Mestre em Ciências.

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*Ao meu pai,* ***João José Maragno*** *(in memorian)*

*Homem honrado e digno,*
*Filho dedicado e respeitoso,*
*Marido fiel e responsável,*
*Pai afetuoso e justo.*

*Grande é a dor da tua ausência,*
*Imensurável é o amor que sinto por ti e*
*Irrevogável são os ensinamentos que me deixaste.*

*Saudades...*

*À minha mãe* ***Ivanilde****, mulher forte e dedicada,*
*Minha base e meu porto seguro.*

*Ao meu marido* ***Eduardo*** *e à minha filha* ***Valentina****,*
*Que juntos me fazem viver plenamente,*
*E por me mostrarem que o amor é paciente, é benigno, não*
*Procura os seus interesses, tudo crê, tudo espera e tudo suporta.*

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*"A ciência humana de maneira nenhuma nega a existência de Deus. Quando considero quantas e quão maravilhosas coisas o homem compreende, pesquisa e consegue realizar, então reconheço claramente que o espírito humano é obra de Deus, e a mais notável."*

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## RESUMO

As intersecções entre o sono e o sistema imunológico têm sido investigadas há anos. Nesse contexto, pesquisas têm sido conduzidas com o objetivo de caracterizar a importância do sono ou o impacto da falta de sono sobre a saúde e a qualidade de vida. Nesse aspecto, sabe-se que existe uma alta prevalência de distúrbios do sono em pacientes com câncer e que a qualidade do sono está comprometida nesses pacientes. Ainda, crescentes evidências sugerem que o prejuízo causado na função imunológica devido aos distúrbios do sono pode contribuir para a progressão tumoral, de modo a afetar o prognóstico e a mortalidade dos pacientes. Nesse contexto, o objetivo geral desse trabalho foi investigar as interações entre sono, sistema imunológico e crescimento tumoral. Para tanto, camundongos BALB/c machos ou fêmeas foram submetidos à privação de sono por 72 horas pelo método da plataforma múltipla imediatamente antes ou após a inoculação do tumor ascítico de Ehrlich. Nossos resultados demonstraram que a privação de sono, quando realizada imediatamente antes da inoculação do tumor, potencializou o crescimento tumoral tanto em camundongos machos quanto em fêmeas. De importância, tal efeito foi decorrente, pelo menos em parte, de uma potencialização induzida pela privação de sono dos prejuízos imunológicos promovidos pelo tumor. Ainda, quando a privação de sono é realizada imediatemente após a inoculação, o efeito potencializador induzido pela privação de sono sobre o crescimento tumoral ocorre de forma tardia nos camundongos machos. Já em camundongos fêmeas, a privação de sono não modifica o crescimento do tumor ascítico de Ehrlich, mas diminui a longevidade desses animais.

## ABSTRACT

The interactions between sleep and immune system have been investigated for years. Within this context, studies have been conducted in order to investigate the importance of sleep and the impact of sleep loss in health. Thus, it is well-known that sleep disorders are highly prevalent in cancer patients and that their sleep quality is impaired. Notwithstanding, a great amount of evidence have suggested that the sleep disorders-induced immunologic impairments could contribute to tumoral growth, affecting the prognostic and mortality of cancer patients. In this way, the aim of the present study was to investigate the interactions among sleep, immune system and Ehrlich ascitic tumor growth. Male or female BALB/c mice were subjected to sleep deprivation for 72h by multiple platforms technique immediately before or after the Ehrlich ascitic tumor inoculation. We verified that previous sleep deprivation potentiated the subsequent tumor growth both in male or female mice. Importantly, the Ehrlich ascitic tumor-induced immunological impairments were exacerbated by sleep deprivation. In addition, when sleep deprivation occurred immediately after tumor inoculation, this potentiating effect (enhanced tumor growth) was delayed in male mice. At last, but not least, in female mice, the same sleep deprivation protocol did not modify Ehrlich ascitic tumor growth but remarkably curtailed life span of these animals.

## ÍNDICE

# 1. INTRODUÇÃO

## *1.1 Neoplasias*

Sob uma perspectiva fisiológica, a auto-reprodução é uma característica fundamental para a célula, possibilitando que uma célula-mãe gere duas células-filhas idênticas, no final de cada ciclo da divisão celular (Porcionatto et al., 2003). Constitui assim, uma condição crítica para sobrevivência, capacitando o organismo a substituir células lesadas ou mortas e proceder ao reparo dos tecidos após inflamação (Cotran et al., 1999). O mecanismo de controle do crescimento celular é dependente de fatores estimulantes e inibidores, assegurando que a formação das células da progênie possua os genomas intactos e que os organismos consigam executar sua manutenção. Os fatores de crescimento são os responsáveis pelo controle da proliferação celular, permitindo que a divisão de células individuais seja coordenada pela necessidade do organismo como um todo. Falhas nos processos envolvidos na divisão celular podem levar à morte da célula por apoptose ou ao seu crescimento desordenado, resultando na formação de uma neoplasia (Cooper, 2002; Porcionatto et al., 2003).

O termo “neoplasia”, do grego, significa literalmente “crescimento novo”. Conceitualmente, se caracteriza como um crescimento anormal da célula, resultante de alterações gênicas que, por sua vez, determinam um descontrole na proliferação celular e uma perda na capacidade de diferenciação, características estas transmitidas para as células filhas (Cotran et al., 1999). Sinônimos como tumor e câncer são utilizados para caracterizar uma

neoplasia. Embora os significados desses termos sejam do domínio do senso comum, uma definição científica, precisa e completa é de difícil obtenção. No entanto, utilizaremos a clássica definição de Rupert Willis, proposta em 1952, segundo o qual “Neoplasia é uma massa anormal de tecido cujo crescimento é excessivo e incoordenado, persistindo mesmo após a retirada do estímulo desencadeador” (Furmanski, 1993).

Historicamente, as primeiras referências clínicas desse processo datam por volta de 1600 a.C., nos relatos de Edwim Papyrus e Ebers Papyrus. Em 1838, Johanes Muller demonstrou que as neoplasias eram compostas por células, embora anormais, possibilitando assim sua avaliação histológica (Sirica, 1989). Com base nessa teoria, Rudolf Virchow, um jovem médico prussiano, postulou em 1863 a doutrina da geração patológica das neoplasias, utilizando-se do seu famoso aforismo *omnis cellula e cellula* (“uma célula sempre se origina de outra célula”), para explicar a origem do câncer (Tamayo, 1987).

A carcinogênese, ou seja, o desenvolvimento de tumores malignos, pode se iniciar de forma espontânea (mutação) ou ser provocada pela ação de agentes carcinogênicos (químicos, físicos ou biológicos). Em ambos os casos, verifica-se a indução de alterações mutagênicas e não-mutagênicas ou epigenéticas nas células (Jakóbisiak et al., 2003). A incidência, a distribuição geográfica e o comportamento de tipos específicos de cânceres estão relacionados a múltiplos fatores, incluindo sexo, idade, raça, predisposição genética e exposição a carcinógenos ambientais. Nesse sentido, os atuais padrões de vida adotados em relação ao trabalho, nutrição e consumo em

geral expõem os indivíduos a fatores ambientais mais agressivos, resultantes de um processo de industrialização cada vez mais evoluído (INCA, 2008).

O número de casos de câncer tem aumentado de maneira considerável em todo o mundo, principalmente a partir do século passado, configurando-se, na atualidade, como um dos mais importantes problemas de saúde pública mundial. De acordo com o UICC *(Union for International Cancer Control)*, o câncer é responsável por cerca de 13% de todas as causas de óbito no mundo e mais de 7 milhões de pessoas morrem anualmente da doença. Acredita-se que em 2010, o número de pessoas mortas devido ao câncer será maior do que ao número total de pessoas mortas pelo HIV/AIDS, tuberculose e malária juntos (Ferlay et al., 2008).

No Brasil, segundo o Instituto Nacional de Câncer, as estimativas para o ano de 2010 (que também são válidas para 2011) apontam para a ocorrência de 489.270 casos novos de câncer, sendo 236.240 para o sexo masculino e 253.030 para o sexo feminino. Estima-se que o câncer de pele do tipo não melanoma será o mais incidente na população brasileira, seguido pelos tumores de próstata e pulmão, no sexo masculino e de mama e colo de útero, no sexo feminino (INCA, 2009).

Nesse cenário, fica clara a necessidade de investimentos no desenvolvimento de ações abrangentes para o controle do câncer, nos diferentes níveis de atuação, como: na promoção da saúde, na detecção precoce, na assistência aos pacientes, na vigilância, na formação de recursos humanos, na comunicação, mobilização social e na pesquisa específica de seus mecanismos fisiopatológicos, fatores de risco e possibilidades terapêuticas.

### *1.2 Neoplasias e Sistema Imunológico*

As respostas imunológicas, natural e adquirida, são os componentes de um sistema integrado de defesa do hospedeiro, no qual várias células e moléculas funcionam em cooperação. Na imunidade natural ou inata, atuam mecanismos de defesa celulares e bioquímicos que já existiam antes do estabelecimento de uma infecção e que estão programados para responder rapidamente às infecções. Seus principais componentes são: barreiras físicas e químicas, células fagocitárias (neutrófilos e macrófagos), células NK (do inglês, *natural killers*), o sistema complemento e as citocinas (Abbas; Lichtman, 2005).

Por outro lado, a imunidade adaptativa ou adquirida é obtida durante a vida de um indivíduo como reação adaptativa à presença de patógenos específicos (Janeway et al., 2006). Esse tipo de resposta imunológica caracteriza-se por uma alta especificidade, sendo capaz de reconhecer antígenos expostos previamente ao organismo e combatê-los com maior intensidade nas exposições subseqüentes desse mesmo microorganismo. É composta pelos linfócitos e seus produtos (Abbas; Lichtman, 2005).

O conceito de vigilância imunológica, introduzido por Paul Ehrlich em 1909 e posteriormente transformado em teoria por Macfarlane Burnet e Lewis Thomas, na década de 50, é definido como a capacidade de o sistema imunológico reconhecer e destruir clones de células transformadas (Dunn et al., 2002; Malmberg and Ljunggren, 2006). Ainda, no que tange a imunogenicidade dos tumores, pressupõe-se que as células tumorais expressem antígenos que são reconhecidos como estranhos pelo sistema imunológico. Tais antígenos, denominados de antígenos de rejeição tumoral, são peptídeos apresentados,

via moléculas de MHC (histocompatibilidade), aos linfócitos T citotóxicos (LT $CD8^+$). Desencadeia-se, assim, uma resposta específica para o tumor, uma vez que esses antígenos não estão presentes em células normais. Com efeito, os LT citotóxicos são importantes para o reconhecimento e a destruição das células tumorais. (Jakóbisiak et al., 2003).

Nos últimos anos, tornou-se evidente que os linfócitos T auxiliares (LT $CD4^+$) também desempenham um papel relevante no combate ao tumor, por meio da ativação de mecanismos da imunidade inata como, por exemplo, a secreção do fator de necrose tumoral (TNF-α) e do interferon-γ (IFN- γ), bem como o recrutamento de macrófagos, granulócitos e células NK para o sitio tumoral. Além disso, os linfócitos T auxiliares potencializam a resposta específica do LT citotóxico, influenciando não apenas na resposta efetora, mas também na formação de LT citotóxicos de memória (Kennedy; Celis, 2008).

Embora a imunidade celular seja, provavelmente, mais importante que os anticorpos na imunidade antitumoral, um grande número de doentes com câncer produz anticorpos contra antígenos tumorais (Abbas; Lichtman, 2005). O potencial para a destruição de células tumorais intermediada por anticorpos tem sido amplamente demonstrado *in vitro*, sendo atribuído à ativação do complemento ou à citotoxicidade celular dependente de anticorpo (ADCC), na qual macrófagos, ou células NK, ligando-se ao anticorpo, intermedeiam a destruição da célula tumoral (Janeway et al., 2006).

A investigação clínica sistemática da fisiopatologia de inúmeras doenças muitas vezes pode se defrontar com barreiras praticamente intransponíveis, tais como aspectos éticos, o curso da doença e a metodologia de investigação. Nesse cenário, o emprego de modelos animais pode superar tais limitações e

fornecer subsídios importantes para o esclarecimento dos mecanismos e tratamento das doenças. Nesse sentido, um modelo animal deve atender as seguintes pressuposições: a) permitir o estudo dos fenômenos biológicos ou de comportamento do animal; b) permitir que o processo patológico espontâneo ou induzido possa ser investigado e c) que o fenômeno, em um ou mais aspectos, seja semelhante ao fenômeno em seres humanos (Fagundes and Taha, 2004). De importância para a presente dissertação, na pesquisa do câncer os modelos animais são necessários para estudo de mecanismos que envolvem o início, a promoção e a progressão do câncer, bem como a caracterização e avaliação de novas técnicas terapêuticas e sua interação com os componentes do sistema imunológico (Rudmann; Durham, 1999; Deng, 2001; Koido et al., 2009; O'Brien; Schedin, 2009; Stover, 2010).

### *1.3 Tumor de Ehrlich*

Na área da Oncologia Experimental, diversos modelos experimentais têm sido propostos tanto para o entendimento da biologia tumoral como para a investigação de novas modalidades terapêuticas. Entre os modelos desenvolvidos ao longo da história, são relevantes aqueles representados pelos tumores experimentais transplantáveis mantidos e propagados em animais de laboratório por meio de transplantes de seus componentes em hospedeiros suscetíveis (Xavier & Kleeb, 2001).

No inicio do século XX, Paul Ehrlich introduziu no mundo científico um tumor experimental, o tumor de Ehrlich, originado espontaneamente como um carcinoma mamário de camundongo fêmea. O tumor de Ehrlich se apresenta

sob duas formas: a ascítica, obtida por meio da inoculação de suspensão de células no peritônio e a sólida, obtida mediante a inoculação dessa mesma suspensão no subcutâneo do dorso ou no coxim plantar de camundongos (Xavier & Kleeb, 2001). O conhecimento do comportamento desse tumor nos animais receptores possibilita sua utilização como modelo para o estudo de diversas situações experimentais.

### *1.4 Sono*

O sono é um processo fisiológico dividido em dois estados fundamentais, o sono NREM (do inglês, *non-rapid eye movements*) e o sono paradoxal ou REM (do inglês, *rapid eye movements*). É uma atividade que ocupa cerca de um terço de nossas vidas e é fundamental para uma boa saúde mental e emocional, além de ser essencial na manutenção de uma vida saudável (Everson et al., 1989).

Fisiologicamente, é considerado como um estado funcional, cíclico e reversível que está presente em todas as faixas etárias e na maioria das espécies animais. Possui algumas características comportamentais, como uma imobilidade relativa e o aumento do limiar de resposta aos estímulos externos (Hoshino, 2008). O organismo tem grande necessidade de manifestar o sono e quando isso não se torna possível podem ocorrer alterações marcantes, incluindo a morte em caso de um período prolongado de privação de sono em animais (Rechtschaffen et al., 1989).

A compreensão moderna do sono originou-se dos primeiros registros da atividade elétrica cerebral em seres humanos. Esses registros, denominados

de eletroencefalograma (EEG), permitiram a identificação de diferentes padrões durante o sono e ainda proporcionaram uma riqueza de informações sobre os potenciais corticais relacionados com mudanças fisiológicas distintas em cada fase do sono. Muitas alterações eletroencefalográficas ocorrem no decorrer de uma noite de sono, indicando uma sucessão bem ordenada e cíclica de freqüências de ondas nas fases de sono. Na primeira fase, ocorre o sono de ondas lentas, caracterizado pela lentificação progressiva da atividade cortical com quatro estágios: estágio 1 (sonolência); estágio 2 (sono “leve”); estágio 3 e 4 (sono de ondas lentas propriamente dito). Esses estágios usualmente ocorrem em seqüência, freqüentemente com flutuações ao longo da noite. A seguir, ocorre o sono REM que tem como principais características o movimento rápido dos olhos e a atonia muscular, além de ser o período em que ocorrem os sonhos (Dement; Kleitman, 1957; Aserinsky; Kleitman, 2003). O sono REM também é denominado sono paradoxal, uma vez que o padrão eletroencefalográfico é semelhante ao da vigília, apesar de ser acompanhado pela atonia muscular que sugeriria um sono profundo (Jouvet et al., 1964). Em humanos, um ciclo de sono completo consiste de uma seqüência de sono NREM e REM, e cada ciclo tem duração em média de 90 a 110 minutos. Em geral, são observados de quatro a seis ciclos durante uma noite de sono. (Zisapel, 2007).

Na década de 70, foi realizada uma detalhada análise das várias fases do sono do rato. Esse estudo revelou a existência de semelhanças com o sono humano muito maiores do que se acreditava até então. Os episódios de sono em roedores freqüentemente se concentram durante o período claro, enquanto que a vigília predomina no período escuro. Os roedores, em média, dormem

cerca de 62% do período claro e 33% do período escuro, sendo que, durante a vigília, os animais realizam suas atividades vitais e sociais (alimentação, procriação, auto-limpeza, interação social e exploração do ambiente). Somando-se os períodos de sono, verifica-se que os roedores dormem cerca de 50% das 24 horas, dividindo-se em sono de ondas lentas e sono paradoxal (Timo-Iaria et al., 1970).

De importância para essa dissertação, diversos trabalhos abordam as diferenças entre os padrões de sono de homens e mulheres, bem como as diferenças nas frequências nos distúrbios de sono entre os gênereos. Nesse sentido, é amplamente conhecido que a insônia é mais frequente em mulheres. De fato, um estudo realizado entre 1987 a 1995 na cidade de São Paulo revelou que mulheres apresentam maior dificuldade em iniciar o sono (Pires et al., 2007) e maior latência para o sono REM e permanecem mais tempo nos estágios 3 e 4 do sono NREM que os homens (Silva et al., 2008). Por outro lado, a síndrome da apnéia obstrutiva do sono afeta por volta de 32% da população masculina, comprovando a prevalência significativa de distúrbios de sono na população (Santos-Silva et al., 2009; Tufik et al., 2009).

### *1.5 Sono e Sistema Imunológico*

As intersecções entre o sono e o sistema imunológico têm sido investigadas há anos. Nesse aspecto, duas observações gerais norteiam tais estudos: a primeira é o desejo intenso de dormir que acompanha certas infecções e a segunda é que, em situações de privação de sono, há um aumento da suscetibilidade a infecções (Krueger and Fang, 2000; Rogers et al.,

2001; Bryant et al., 2004). Nesse sentido, alguns produtos bacterianos, citocinas e mensageiros químicos do sistema imunológico promovem o sono, levando alguns pesquisadores a sugerirem que o sono poderia contribuir para a defesa do hospedeiro (Bergmann et al., 1996).

Especificamente durante uma infecção, os animais apresentam um aumento no tempo total do sono de ondas lentas e uma diminuição do tempo de sono paradoxal. Porém, as alterações no sono que ocorrem em resposta à infecção dependem da natureza e da via de entrada do microorganismo (Krueger and Fang, 2000). O acúmulo sistêmico e cerebral de citocinas pró-inflamatórias inicia uma complexa resposta fisiológica protetora denominada resposta de fase aguda, na qual o excesso do sono de ondas lentas e a febre estão inclusos (Majde & Krueger, 2005).

As citocinas, que podem ser classificadas como pró-inflamatórias ou anti-inflamatórias, são proteínas produzidas por muitos tipos de células que medeiam as reações imunológicas e inflamatórias, atuando de maneira autócrina, parácrina ou endócrina, por ligação de alta afinidade nos receptores das células-alvo. As células do sistema imunológico são a principal fonte de citocinas, porém sabe-se que neurônios e outras células também as produzem e expressam seus receptores (Abbas & Lichtman, 2005; Palma, 2008).

Algumas citocinas endógenas, como a interleucina-1 (IL-1), o fator de necrose tumoral (TNF) e o interferon (IFN) induzem o sono quando administradas em animais e humanos, sugerindo que essas citocinas contribuem para o aumento de sono durante uma infecção (Toth, 1995). Ainda, essas citocinas possuem inúmeras funções, incluindo proliferação, diferenciação, ativação e sinalização celular. De importância, por serem

produzidas durante as infecções coincidentes com alterações nos padrões de sono, essas substâncias são fortes candidatas a exercerem importante função nos mecanismos fisiológicos de regulação de sono (Mullington et al., 2000). De fato, sabe-se que a regulação fisiológica do sono e a resposta imunológica compartilham moléculas, como as citocinas IL-1 e TNF (Krueger et al., 2001).

Nesse cenário, a privação de sono é considerada um instrumento de estudo útil para elucidar as funções do sono, bem como permitir o melhor entendimento dos mecanismos envolvidos nesse comportamento, inclusive sua relação com o sistema imunológico. Nesse sentido, vários estudos de privação de sono investigam as mudanças no perfil das células imunológicas. Especificamente, células como NK, linfócitos T $CD4^+$ / T $CD8^+$ e linfócitos B apresentam uma diminuição significativa após a privação de sono (Dinges et al., 1994; Boyum et al., 1996; Ozturk et al., 1999). Além de a alteração no número de células, também se observa uma redução na atividade funcional de linfócitos e de células NK (Palmblad et al., 1979; Irwin et al., 1994; Irwin et al., 1996; Born et al., 1997; Bollinger et al., 2008). É importante citar que as modificações, decorrentes da privação de sono, sobre o número e função dessas células imunológicas, podem variar de acordo com a duração e o protocolo de privação de sono (Bryant et al., 2004).

Ainda, pode-se ressaltar que protocolos de privação de sono paradoxal também demonstram uma redução significativa dos linfócitos circulantes (Ruiz et al., 2007; Zager et al., 2007). Além disso, o protocolo de restrição crônica de sono, em que o animal é permitido dormir poucas horas por dia, durante 21 dias, também foi capaz de comprometer a quantidade de linfócitos no sangue (Zager et al., 2007).

Lange e colaboradores (2003) demonstraram que, voluntários privados de sono por uma noite apresentaram uma titulação de anticorpos para hepatite A 50% menor do que os voluntários que dormiram normalmente. Semelhante resultado foi obtido em um estudo anterior, no qual voluntários privados de sono foram imunizados contra o vírus da influenza e apresentaram redução em 50%, na titulação de anticorpos (Spiegel et al., 2002).

Tomados em conjunto, esses estudos sugerem a hipótese de que o sono é crítico para a homeostasia e que sua privação poderia levar a um comprometimento da resposta imunológica.

### *1.6 Sono e Neoplasias*

Nos últimos anos, várias pesquisas foram conduzidas com o objetivo de caracterizar o impacto do sono sobre a saúde, assim como, definir sua relação com as funções fisiológicas e qualidade de vida. Recentemente, estudos abordam a importância de um sono com qualidade e os distúrbios de sono em pacientes portadores de tumores. Nesse aspecto, sabe-se que existe uma alta prevalência de distúrbios do sono em pacientes com câncer (Sela et al., 2005; Savard and Morin, 2001) e que a qualidade do sono está comprometida nesses pacientes (Mystakidou et al., 2007).

Um distúrbio do sono freqüente nos pacientes portadores de câncer é a insônia, que acomete cerca de 30% a 50% dos pacientes, e faz parte de um grupo inter-relacionado de sintomas no qual incluem dor, fadiga, ansiedade e depressão (Theobald,2004). Assim, a insônia causa conseqüências imunológicas que comprometem negativamente a resposta antitumoral (Irwin et

al., 2003). Nesse sentido, Savard e colaboradores (1999) descreveram uma associação entre a qualidade subjetiva do sono e imunossupressão desencadeando um aumento do risco de câncer de colo de útero.

Por outro lado, um único estudo experimental, o qual foi realizado por Bergmann e colaboradores (1996), abordou a relação entre sono e crescimento de um tumor experimental. Nesse estudo, foi observado que a privação total de sono por 10 dias consecutivos reduziu o crescimento do tumor de Walker em ratos. Porém, o acentuado catabolismo, causado pelo protocolo de privação de sono utilizado, pode ter influenciado o resultado desse estudo. Diante desse cenário, tornam-se relevantes novas investigações experimentais, que utilizem diferentes protocolos de privação de sono e neplasias experimentais transplantáveis.

De importância, diferenças entre os gêneros também são verificadas em relação à imunocompetência (Weinstein et al.,1984; Oyeyinka, 1984). De fato, as mulheres na maioria das sociedades não só possuem maior longevidade, como também são mais resistentes a algumas doenças infecciosas e não infecciosas, como por exemplo, o câncer (Nunn et al., 2009). Da mesma forma, as fêmeas de roedores também parecem ser mais resistentes a alguns tipos de tumores (Krzych et al., 1979) e respondem melhor a vários estímulos antigênicos do que os machos (Weinstein et al.,1984). Tais diferenças na magnitude da resposta imunológica entre machos e fêmeas não está completamente elucidada, porém as diferenças hormonais sexuais parecem ser, pelo menos em parte, importantes para esses distintos padrões de resposta imunológica (Oyeyinka, 1984).

## 2. OBJETIVOS

### *2.1 Objetivo Geral*

Crescentes evidências sugerem que o prejuízo causado na função imunológica devido a distúrbios do sono pode contribuir para a progressão tumoral, afetando o prognóstico e a mortalidade dos pacientes. Nesse contexto, o objetivo geral desse trabalho foi investigar as interações entre sono, sistema imunológico e crescimento tumoral em camundongos.

### *2.2 Objetivos Específicos*

- Verificar os efeitos da privação de sono por 72 horas pelo método da plataforma múltipla previamente à inoculação do tumor ascítico de Ehrlich em camundongos machos e fêmeas, sobre o crescimento tumoral
- Verificar os efeitos da privação de sono por 72 horas pelo método da plataforma múltipla concomitantemente ao crescimento do tumor ascítico de Ehrlich em camundongos machos e fêmeas.
- Verificar os efeitos da privação de sono por 72 horas pelo método da plataforma múltipla e do crescimento do tumor de Ehrlich isolados ou em conjunto sobre as populações de linfócitos T $CD4^+$, T $CD8^+$, linfócitos B ($CD19^+$) e células *Natural Killer* ($CD49b^+$) no baço em camundongos machos e fêmeas.
- Caracterizar o padrão de sono de camundongos machos ou fêmeas portadores do tumor ascítico de Ehrlich.

## 3. MATERIAL E MÉTODOS

### *3.1 Sujeitos Experimentais*

Para os protocolos experimentais, camundongos BALB/c com 10-12 semanas, machos ou fêmeas, originários da colônia do biotério da Universidade Federal de São Paulo foram alojados, desde seu desmame, em número de 12 em gaiolas plásticas, medindo 41 cm x 34 cm x 16,5 cm. As gaiolas foram mantidas em nosso biotério, com ventilação e temperatura (20-23°C) controladas por meio de um aparelho de ar condicionado central, em um ciclo claro-escuro de 12/12 horas, iniciando-se o período de claro às 6 horas e 45 minutos e o período de escuro às 18 horas e 45 minutos. Água e comida foram fornecidas *ad libitum.*

Os experimentos foram conduzidos segundo os Princípios Éticos e Práticos do Uso de Animais de Experimentação (Andersen et al., 2004) e respeitando também os princípios estabelecidos pelo *National Institute of Health Guide for the Care and Use of Laboratory Animals* (NIH Publications nº80-23, revisado 1996). Ao final dos protocolos experimentais empregados no presente trabalho, os camundongos foram eutanasiados utilizando o método de decapitação, em uma sala adjacente.

Todos os procedimentos realizados na presente dissertação estão de acordo com as normas estabelecidas pelo Comitê de Ética em Pesquisa da Universidade Federal de São Paulo (nº. do protocolo 1128/08).

### *3.2 Transplante do Tumor de Ehrlich*

O tumor de Ehrlich, neoplasia transplantável originária de um carcinoma mamário de camundongo fêmea foi empregado sob a forma ascítica. A manutenção do tumor foi realizada em nosso laboratório, por meio de transplantes intraperitoneais das células tumorais em camundongos BALB/c fêmeas em intervalos semanais.

Para o transplante do tumor, procedeu-se a coleta do fluido ascítico do camundongo doador para a contagem, na câmara de Neubauer, das células presentes nos quatro quadrantes externos e o teste de viabilidade celular com o corante vital Azul de Tripan 0,4%.

A supensão de células tumorais foi preparada de acordo com estudos prévios (Dagli et al., 1992; Kleeb et al.,1997) e o transplante para os camundongos BALB/c foi realizado por meio da inoculação intraperitoneal de 0,4 mL de suspensão, contendo 5 x $10^7$ células tumorais, com no mínimo de 90% de viabilidade celular (figura 1).

No 10º dia após a inoculação, os animais foram eutanasiados e se procedeu a contagem do número total de células tumorais presentes na cavidade peritoneal, obtido pela multiplicação do número de células por mL pelo volume, em mL, do líquido ascítico obtido. Como controle do procedimento, os animais dos grupos veículos (VEH) receberam a inoculação de PBS (phosphate-buffered saline) – veículo da suspensão do tumor de Ehrlich – em um volume de 0,4 mL.

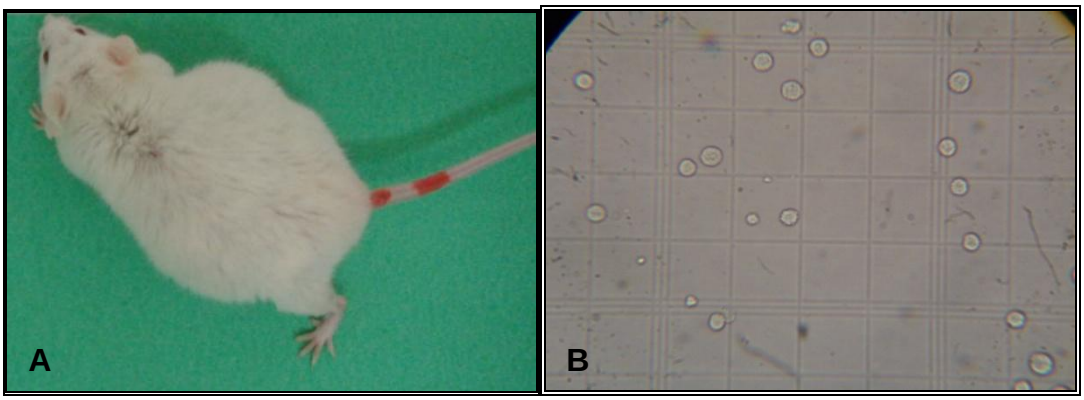


**Figura 1**: **A** - Camundongo portador do tumor ascítico de Ehrlich. **B** – Fotomicrografia das células do tumor ascítico de Ehrlich em suspensão na câmara de Neubauer.

### *3.3 Privação de Sono*

A privação de sono foi realizada conforme proposto por Silva e colaboradores (2004), com pequenas modificações. Os camundongos foram submetidos à privação de sono por 72 horas consecutivas (PS) pelo método da plataforma múltipla modificada. Durante a PS, cinco animais foram colocados em uma caixa de policarbonato (38 x 31 x 17 cm) contendo dez plataformas circulares de 3,5 cm de diâmetro para cada animal, sendo o nível da água 1 cm abaixo da superfície das plataformas (figura 2). Os animais puderam se movimentar livremente no interior da gaiola pulando de uma plataforma para a outra. A atonia muscular, que ocorre caracteristicamente no sono paradoxal, faz com que o animal acorde ao encostar o focinho ou, ainda, o corpo inteiro na água. A temperatura e o ciclo claro-escuro foram controlados, e, além disso, água e ração permaneceram *ad libitum*. Os animais do grupo controle (CTRL) foram mantidos em suas gaiolas moradia na mesma sala onde foram conduzidos os protocolos de privação de sono.

**Figura 2**: Privação de sono - método da plataforma múltipla modificada

### *3.4 Registro dos Parâmetros Eletrofisiológicos de Sono*

Os estados de vigília, sono paradoxal e sono de ondas lentas foram quantificados por meio de uma combinação entre eletrocorticograma (ECG), eletromiograma (EMG) e critérios já estabelecidos (Timo-Iaraia et al., 1970) utilizando o software Somlogica (Embla®, Flaga Medical Devices, Islândia). A duração do sono paradoxal e do sono de onda lentas foi obtida, em minutos, por 20 dias consecutivos. Os animais foram anestesiados com quetamina-diazepam e os procedimentos cirúrgicos esterotáxicos foram realizados para o implante dos eletrodos. Para tanto, o crânio foi exposto e dois orifícios foram abertos (1,5 mm nas laterais em relação à linha central e 2 mm anteriores ao bregma) e no córtex parietal (2 mm nas laterais em relação à linha central e 2,5 mm posteriores ao bregma) de acordo com o atlas de Paxinos & Frankin (2001). Os eletrodos do ECG foram inseridos nesses orifícios de maneira a tocar a dura-máter, evitando-se qualquer dano maior aos tecidos. Finos fios de

níquel-cromo foram implantados nos músculos dorsais do pescoço para avaliação do EMG. Os eletrodos foram soldados a soquetes contendo quatro pinos e cobertos com massa acrílica utilizada em procedimentos dentários. Após 7 dias de recuperação desse procedimento cirúrgico, os animais foram habituados ao sistema de registro por 3 dias e, após esse período, as medidas foram realizadas, como mencionado, em 2 períodos de 10 dias. Assim, foram registrados 10 dias consecutivos após a inoculação de solução veículo e os dez dias subsequentes foram registrados após a inoculação do tumor de Ehrlich. Os sinais de ECG e EMG foram amplificados pelo sistema e filtrados em 0,3-100 Hz (para o ECG) e em 30-300 Hz (para o EMG). A freqüência das amostras foi estabelecida em 200 Hz e os traços avaliados visualmente em períodos de 10 segundos.

### *3.5 Análise Celular por Citometria de Fluxo*

Depois de coletar o fluido ascítico, o baço de cada animal foi removido e lavado em PBS para retirar qualquer resíduo de célula tumoral da cápsula externa do órgão. As células do baço foram suspendidas em RPMI-1640 e centrifugadas a 2500 rpm por 10 minutos e a 4°C. O sobrenadante foi descartado e as células vermelhas lisadas usando o tampão de lise ACK lysing buffer (GIBCO-Invitrogen, Grand Island, NY). Após esse procedimento, as células foram lavadas em PBS e ressuspendidas em PBS com pH 7.4 contendo 2% de FBS (do inglês, *fetal bovine serum*) . Alíquotas contendo $10^6$ células foram marcadas com anticorpos monoclonais anti-mouse conjugados a fluorocromos para os marcadores de superfície $CD3^+$ PE, $CD4^+$ APC, $CD8^+$

FITC, $CD19^+$ PE (células B), $CD49^+$b FITC (células NK) (BD Biosciences, San Jose, CA) e incubadas por 30 minutos a 4°C. Cem mil eventos por amostras foram adquiridos no citômetro de fluxo FACScalibur® (BD Biosciences) e analisados utilizando o software FlowJo® (Tree-Star).

### *3.6 Análise Estatística*

Em um primeiro momento, realizamos o teste de Levene para a constatação de que os dados obtidos seriam paramétricos. Uma vez que o foram, os dados obtidos a partir do protocolo de crescimento tumoral e de citometria de fluxo foram avaliados por meio do teste T para amostras independentes ou ANOVA de duas vias (Snedocor, 1946), seguida do teste de Duncan (Duncan, 1955). Os dados provenientes do registro de sono dos animais foram analisados por meio do Teste T para amostras pareadas. Por fim, os padrões de sobrevida foram analisados pelo método de Kaplan-Meier seguido de “Log rank test”. Em todas as comparações realizadas, a probabilidade de $p<0,05$ foi considerada capaz de determinar diferenças significativas.

# SLEEP DEPRIVATION ENHANCES ASCITIC EHRLICH TUMOR GROWTH IN MICE

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**ABSTRACT**

**STUDY OBJECTIVES:** Whereas increasing evidence suggests a bi-directional interaction between sleep and immune system, there is little experimental work investigating the effects of sleep deprivation on tumor development. The aim of the present study was to investigate the effects of sleep deprivation (SD) on the Ehrlich ascitic tumor (EAT) growth in male or female BALB/c mice. Our study also focused on evaluating whether SD causes changes on immune cell populations from spleen of mice inoculated with EAT. **DESIGN:** Male and female BALB/c mice were deprived from sleep for 72h. At the end of SD procedure, animals were intraperitoneally inoculated with $5 \times 10^7$ EAT cells. Ten days after the inoculation, the number of tumoral cells was quantified and the lymphocytic cell population in spleen was characterized by flow cytometry **RESULTS:** Sleep deprivation potentiated EAT growth irrespective from gender. Moreover, these mice presented decreased frequencies of splenic $CD4^+$, $CD8^+$ and $CD19^+$ cells when compared to control mice equally inoculated with Ehrlich tumor. **CONCLUSIONS:** These results suggest that sleep loss potentiated tumor growth through, at least in part, an immunological suppression.

**KEYWORDS:** Ehrlich ascitic tumor, sleep deprivation, immunologic system, gender.

## INTRODUCTION

Sleep occupies approximately one-third of a person's life. For this reason, sleep disturbances and short sleep duration adversely impact human physical health (Gangwisch et al., 2007) and increase the mortality risk (Dew et al., 2003; Gangwisch et al., 2008; Shankar et al., 2008). The prevalence of sleep disorders is increasing in modern societies, where constant exposure to artificial light and interactive activities combine with social and economic pressures to shorten the time spent asleep (Tufik et al., 2009).

Increasing evidence suggests a bi-directional communication between sleep and the immune system (Irwin, 2002; Ruiz et al., in press). Although the exact mechanisms by which sleep or sleep deprivation (SD) affect systemic immunity are far from ascertained (Maurovich-Horvat et al., 2008), sleep is hypothesized to have a restorative function on immune processes (Moldofsky, 1994; Opp & Imeri, 1999).

It has been demonstrated a high frequency of sleep disturbance as a symptom in cancer patients (Sateia and Lang, 2008). Indeed, the majority of cancer patients report fatigue, sleeping difficulty, being tired and complain of weakness (Ancoli-Israel et al., 2001; Savard and Morin, 2001). In fact, several studies have demonstrated that sleep loss leads to significant alterations in many immune components both in laboratory animal and humans (Irwin et al., 2002; Ruiz et al., 2007; Ruiz et al., in press; Zager et al., 2007). In this way, sleep disturbance adversely modulates the antitumoral immune response (Irwin et al., 2002).

The Ehrlich tumor is a spontaneous murine carcinoma that can grow rapidly in almost any mouse strain, producing ascitic or solid tumors across histocompatibility barriers and represents a classical experimental model for evaluation of tumor development *in vivo* (Carry et al., 1979; Frussa-Filho et al., 1991, 1992; Kleeb et al., 1997, 1999). For the best of our knowledge, no studies have addressed the impact of sleep loss on tumor growth in the Ehrlich tumor model. In order to address this question, we evaluated the effect of SD on Ehrlich ascitic tumor (EAT) growth in males or females BALB/c mice. Additionally, our study also focused on characterizing the lymphocytic cell population in the spleens of these animals.

## MATERIAL AND METHODS

*Subjects*

Three-month-old BALB/c male or female mice (inbred, raised, and maintained in the Centre for Development of Experimental Models in Medicine and Biology of Universidade Federal de São Paulo) were used in the experiments. Animals weighing 30-35 g were housed under controlled temperature (22-23°C) and lighting (12h light, 12h dark; lights on at 6:45 a.m.) conditions. Food and water were available *ad libitum* throughout the experiments. Animals used in this study were maintained in accordance with the National Institute of Health Guide for the care and use of laboratory animals (NIH Publications Nº 80-23, revised 1996) and the experimental procedures were approved by the Institutional Animal Care and Use Committee under the protocol #1128/08.

*Sleep deprivation (SD)*

The experimental groups were submitted to SD for 72 h using the modified multiple platform method (Frussa-Filho et al., 2004; Silva et al., 2004a,b,c; Araújo et al., 2006; Silva et al., 2007; Fukushiro et al., 2007; Calzavara et al., 2008; Patti et al., 2010), which consists of placing 5 mice in a ventilated cage (38 × 31 × 17 cm, Tecniplast, Buguggiate, Italy) containing 14 circular platforms (3.5 cm in diameter) with water 1 cm below the upper surface. At the onset of each paradoxical sleep episode, the animal experiences a loss of muscle tonus and falls into the water, thus awakening. Food and water were available *ad libitum*. The home-cage control group (CTRL) was maintained in

separate cages in the same ventilated cage system as the experimental mice. By housing both groups in the same environment, we are able to control for differences in housing conditions between the 2 groups as well as provide the SD group with satisfactory conditions of the ventilated cages.

We have previously demonstrated that 3-month-old male mice subjected to the above-described protocol displayed a significant reduction in paradoxical sleep, dropping from 55.0 ± 4.6 min (mean ± S.E., baseline) to 2.1 ± 0.6 min on the first day of SD, 2.9 ± 0.7 min on the second day and 2.9 ± 1.0 min on the third day. Slow-wave sleep was also significantly reduced but at a lower magnitude, falling from 558.5 ± 29.8 min (baseline) to 125.5 ± 9.5 min on the first day of the sleep deprivation protocol, 83.9 ± 9.9 min on the second day and 101.5 ± 10.8 min on the third day (Silva et al., 2004).

*Ehrlich Ascitic Tumor (EAT)*

Ehrlich ascitic tumor was maintained in 3-month old female BALB/c mice in ascitic form under week passage. In all experiments, male or female BALB/c mice were intraperitoneally injected with $5 \times 10^7$ viable tumor cells suspended in 0.4 mL of phosphate-buffered saline (PBS) or the same volume of PBS only (vehicle – VEH). The suspension containing tumor cells was prepared according to a previous study (Dagli et al., 1992; Kleeb et al.,1997).The inoculation of EAT or VEH occurred 10 days before measurement of tumor growth (Experiments I and II) or immunological response characterization (Experiments III and IV).

*Flow Cytometric Analyses*

After collection of the ascitic fluid, the spleen of each mouse was removed and vigorously washed in PBS to detach any remaining tumor cell from the external capsule of the organ. A cell suspension of each individual spleen was prepared in RPM-1640 medium and centrifugated at 300 *g* for 10min at 4°C. The supernatant was discharged and red blood cells were lysed with ACK lysing buffer (GIBCO-Invitrogen, Grand Island, NY) following manufacturer instructions. Cells were washed three times in PBS and aliquots containing $10^6$ spleen cells were incubated with anti-mouse CD16/32 (eBioscience Inc., San Diego, CA) for 30 min at $4^oC$ in staining buffer [2% FBS and 0.02% sodium azide in PBS] to block nonspecific Fc receptor binding. Cells were then stained with the following monoclonal antibodies conjugated with fluorochromes for 30 min at 4°C: anti-mouse $CD3^+$ PE, $CD4^+$ APC, $CD8^+$ FITC, $CD19^+$ PE (B cells) or $CD49b^+$ FITC (NK cells) (BD Biosciences, San Jose, CA). A hundred thousand cells per sample were acquired on the FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

*Experimental design*

*Experiments I and II: Effects of sleep deprivation on Ehrlich tumor growth in male or female mice*

Male (Experiment I) or female (Experiment II) BALB/c mice were randomly assigned to one of the following groups: control-Ehrlich ascitic tumor (CTRL+EAT) or sleep deprivation-Ehrlich ascitic tumor (SD+EAT). Groups of mice were kept in their homecages (CTRL condition) or sleep-deprived for 72h (SD condition). Immediately after the end of SD, animals were intraperitoneally

inoculated with 5 x $10^7$ EAT cells. Ten days after the inoculation procedure, animals were euthanized and had their ascitic fluid collected for quantification of the number of tumor cells.

*Experiments III and IV: Effects of sleep deprivation and/or Ehrlich tumor growth in male or female mice: measurement of splenic cell lymphocytic population*

Male (Experiment III) or female (Experiment IV) BALB/c mice were randomly assigned to one of the following groups: control-vehicle (CTRL+VEH), control-Ehrlich ascitic tumor (CTRL+EAT), sleep deprivation-vehicle (SD+VEH) or sleep deprivation-Ehrlich ascitic tumor (SD+EAT). Groups of mice were kept in their homecages (CTRL condition) or sleep-deprived for 72h (SD condition). Immediately after the end of SD, animals were intraperitoneally inoculated with 5 x $10^7$ Ehrlich ascitic tumor (EAT) cells or with vehicle solution (VEH). Ten days after the inoculation procedure, animals were euthanized and had their spleen collected for immunological cellular characterization.

*Statistical analysis*

In experiments I and II, the number of tumor ascitic cells was compared by independent samples T-test. In experiments III and IV, the frequency of cells in spleen was compared using two-way ANOVA followed by Duncan's test. A probability of $p<0.05$ was considered significant for all comparisons made.

## RESULTS

*Experiment I: Effects of sleep deprivation on Ehrlich tumor growth in male mice*

Independent samples T-test revealed that the SD+EAT group displayed an enhancement in the number of EAT cells present in the ascitic fluid when compared to the CTRL+EAT group [$T(37)=3.27$; $p<0.005$], demonstrating that SD potentiated the tumor growth (Figure 1).

*Experiment II: Effects of sleep deprivation on Ehrlich tumor growth in female mice*

The SD+EAT group presented enhanced number of EAT cells in the ascitic fluid when compared to the CTRL+EAT group (n=18) [$T(36)=5.18$; $p<0.001$], as revealed by independent samples T-test, demonstrating, thus, that SD also potentiated the tumor growth in female mice as it did in male animals (Figure 2).

*Experiment III: Effects of sleep deprivation and/or Ehrlich tumor growth in male mice: measurement of splenic cell lymphocytic population*

Regarding T cells, the frequency of splenic $CD4^+$ and $CD8^+$ subpopulations were evaluated into the gated $CD3^+$ cells. Specifically concerning the frequency of $CD4^+$ T cells in spleen, two-way ANOVA revealed significant effects of sleep condition (CTRL or SD) [$F(1,21)=56.61$; $p<0.001$], treatment (vehicle or Ehrlich ascitic tumor) [$F(1,21)=59.67$; $p<0.001$] and sleep condition x treatment interaction [$F(1,21)=6.68$; $p<0.05$] effects. In fact, Duncan's *post hoc* test showed that all groups presented a decrement in the

frequency of $CD4^+$ T cells when compared to the CTRL+VEH group. Besides, the decrement presented by the SD+EAT group had a greater magnitude when compared to the CTRL+EAT group (Figure 3A).

Two-way ANOVA indicated only a significant sleep condition effect [$F(1,21)=4.09$; $p<0.05$] when $CD8^+$ T cells frequency in spleen is analyzed. Thus, the SD+VEH and SD+EAT groups presented a decreased frequency of these cells when compared to their respective control groups (CTRL+VEH and CTRL+EAT, respectively) (Figure 3B).

In respect to the frequency of $CD19^+$ cells (B cells) in spleen, two-way ANOVA revealed only sleep condition significant effect [$F(1,21)=79.92$; $p<0.001$]. Thus, the SD+VEH and SD+EAT groups presented a decreased frequency of $CD19^+$ cells when compared to their respective control groups (CTRL+VEH and CTRL+EAT, respectively) (Figure 3C).

Two-way ANOVA revealed significant sleep condition [$F(1,21)=16.02$; $p=0.001$] and treatment [$F(1,21)=9.52$; $p<0.05$] effects when $CD49b^+$ cells (a marker for NK cells in BALB/c mice) frequency in spleen was analyzed. *Post hoc* analyses showed that the SD+VEH group presented an increased frequency of these cells when compared to the CTRL+VEH group. Conversely, the CTRL+EAT group displayed a decrement in this parameter when compared to the CTRL+VEH group. Moreover, while the SD+EAT group presented a decreased frequency of $CD49b^+$ cells when compared to the SD+VEH group, and an increase in this parameter when compared to the CTRL+EAT group (Figure 3D).

*Experiment IV: Effects of sleep deprivation and/or Ehrlich tumor growth in female mice: measurement of splenic cell lymphocytic population*

Regarding the splenic frequency of $CD4^+$ T cells, two-way ANOVA revealed significant effects of sleep condition (CTRL or SD) [$F(1,21)=27.72$; $p<0.001$], treatment (vehicle or Ehrlich ascitic tumor) [$F(1,21)=51.38$; $p<0.001$] and sleep condition x treatment interaction [$F(1,21)=8.32$; $p<0.05$] effects. Duncan's *post hoc* test showed that the CTRL+EAT and the SD+EAT groups presented a decreased frequency of these cells when compared to the CTRL+VEH group. In addition, the SD+EAT group had its $CD4^+$ T cells frequency diminished when compared to the CTRL+EAT and SD+VEH groups (Figure 4A).

Two-way ANOVA revealed only a significant sleep condition effect [$F(1,21)=9.64$; $p=0.005$] when $CD8^+$ T cells frequency in spleen was analyzed. Thus, the SD+VEH and the SD+ EAT groups displayed a decrement frequency of these cells when compared to their respective control groups (CTRL+VEH and CTRL+EAT, respectively) (Figure 4B).

In respect to the splenic frequency of $CD19^+$ cells, two-way ANOVA revealed only sleep condition [$F(1,21)=147.24$; $p<0.001$] effect. *Post hoc* test showed that the SD+VEH and the SD+EAT groups had their $CD19^+$ cells frequency diminished when compared to their control groups (CTRL+VEH and CTRL+EAT, respectively) (Figure 4C).

Finally, regarding the $CD49b^+$ cells frequency in spleen, two-way ANOVA revealed significant sleep condition [$F(1,21)=9.58$; $p=0.005$] and sleep condition x treatment interaction [$F(1,21)=15.59$; $p=0.001$] effects. Indeed, the SD+VEH

displayed an enhancement of CD49b$^{+}$ cells frequency when compared to all the other groups (Figure 4D).

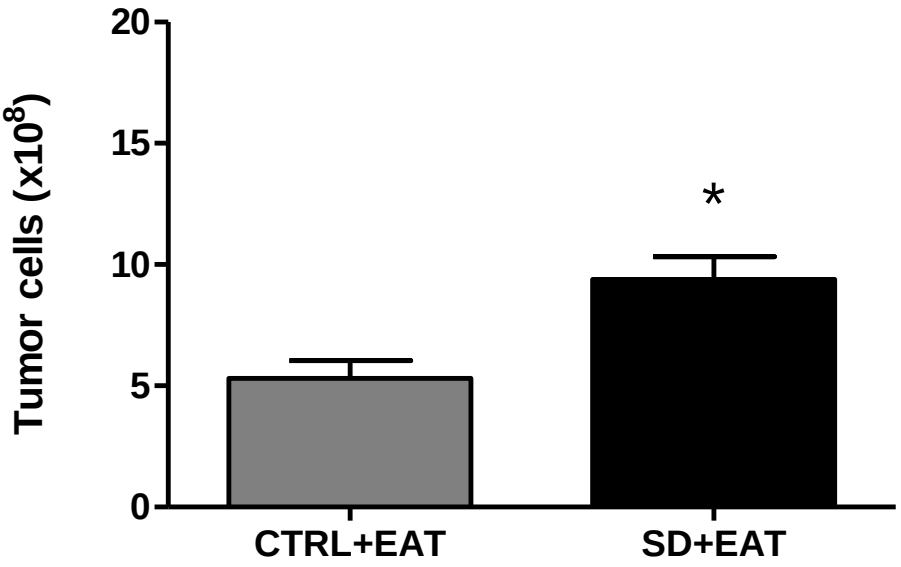


**Figure 1** – Effects of sleep deprivation (SD) on Ehrlich ascitic tumor (EAT) growth in BALB/c male mice. Male mice were sleep deprived for 72h. After the end of the SD, the animals were intraperitoneally injected with 5 x $10^7$ EAT cells. Ten days after the inoculation, the ascitic fluid was collected (n=17 and 22 for the CTRL+EAT and SD+EAT groups, respectively). Data are presented as mean ± SE of tumor cells in the ascitic fluid. *$p<0.05$ when compared to the CTRL+EAT group (Independent samples T-test).

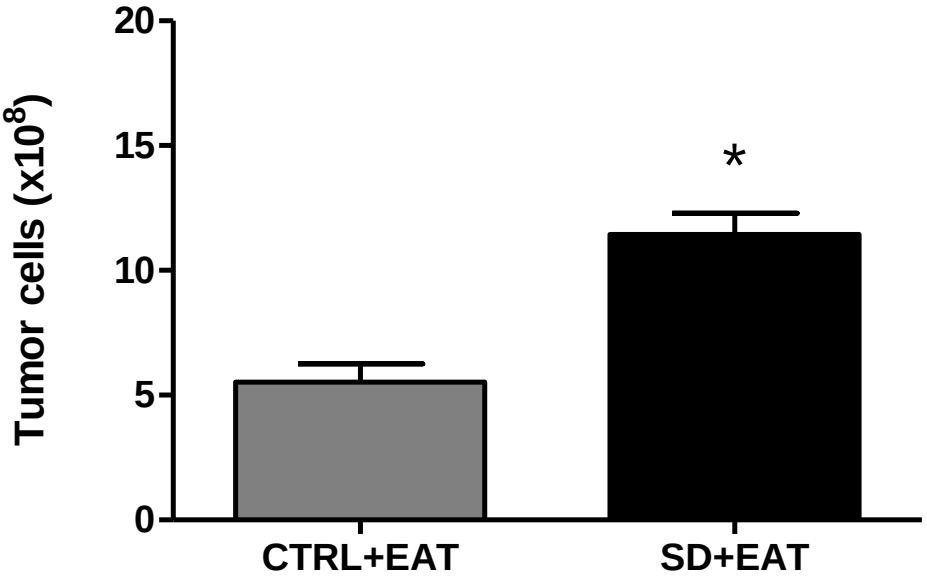


**Figure 2** – Effects of sleep deprivation (SD) on Ehrlich ascitic tumor (EAT) growth in BALB/c female mice. Female mice were sleep deprived for 72h. After the end of the SD, the animals were intraperitoneally injected with 5 x $10^7$ EAT cells. Ten days after the inoculation, the ascitic fluid was collected (n=18 and 22 for the CTRL+EAT and SD+EAT groups, respectively). Data are presented as mean ± SE of tumor cells in the ascitic fluid. *$p<0.05$ when compared to the CTRL+EAT group (Independent samples T-test).

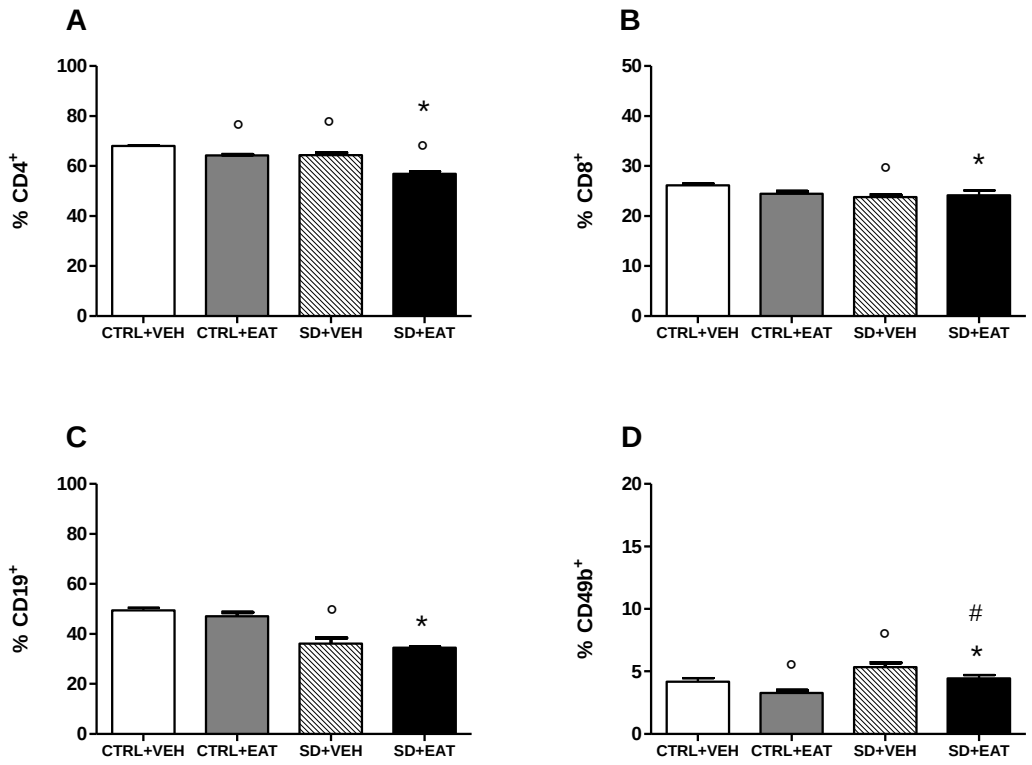


**Figure 3** – Effects of sleep deprivation (SD) associated or not with Ehrlich ascitic tumor (EAT) on splenic lymphoid cell types of male BALB/c mice. Mice were kept in their homecages (CTRL) or were sleep deprived for 72h. After the end of the SD, the animals were intraperitoneally injected with 5 x $10^7$ EAT cells or with vehicle solution (VEH). Ten days after the inoculation, the spleens were collected for flow cytometry analyses (n=5, 7, 6 and 7 for the CTRL+VEH, CTRL+EAT, SD+VEH and SD+EAT groups, respectively). Data are presented as mean ± SE of $CD4^+$ (**A**) and $CD8^+$ (**B**) subpopulations gated into the $CD3^+$ cells, $CD19^+$ B lymphocytes cells (**C**) and $CD49b^+$ NK cells (**D**). °p<0.05 when compared to the CTRL+VEH group; *p<0.05 compared to the CTRL+EAT and $^{\#}$p<0.05 compared to the SD+VEH (Two-way ANOVA and Duncan's test).

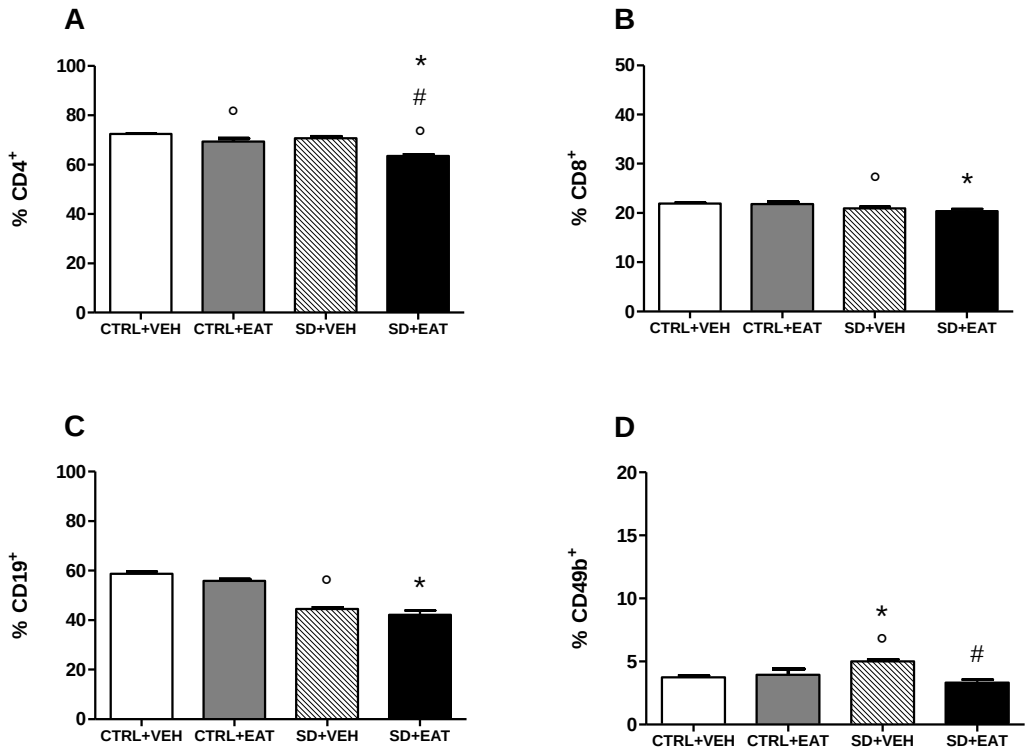


**Figure 4** – Effects of sleep deprivation (SD) associated or not with Ehrlich ascitic tumor (EAT) on splenic lymphoid cell types of female BALB/c mice. Mice were kept in their homecages (CTRL) or were sleep deprived for 72h. After the end of the SD, the animals were intraperitoneally injected with 5 x $10^7$ EAT cells or with vehicle solution (VEH). Ten days after the inoculation, the spleens were collected for flow cytometry analyses (n=7, 5, 7 and 6 for the CTRL+VEH, CTRL+EAT, SD+VEH and SD+EAT groups, respectively). Data are presented as mean ± SE of $CD4^+$ (**A**) and $CD8^+$ (**B**) subpopulations gated into the $CD3^+$ cells, $CD19^+$ B lymphocytes cells (**C**) and $CD49b^+$ NK cells (**D**). °p<0.05 when compared to the CTRL+VEH group; *p<0.05 compared to the CTRL+EAT and $^{\#}$p<0.05 compared to the SD+VEH group (Two-way ANOVA and Duncan's test).

## DISCUSSION

The major finding of our study is that 72-hour deprivation from sleep prior to inoculation of the EAT in mice promoted a potentiating effect on tumor cell growth. This effect occurred regardless the gender. Thus, it is possible to state that SD exerted an enhancement of the number of ascitic tumor cells when compared to mice that were allowed to sleep causing a detrimental consequence on tumor development.

In Experiments I and II, we verified that SD potentiated the tumor growth in both male and female mice in an equally manner. It has been reported by Bergmann and colleagues (1996) that sleep deprivation impaired Walker 256 rat tumor growth. These authors, besides employing a subdermal allogenic carcinoma, subjected rats to a 10-day sleep deprivation protocol. In Bergmann's study, the tumor reached peak size more quickly in sleep-deprived rats and the final result might have been confounded by the pronounced catabolism (loss of body weight) that occurred as the result of the duration of sleep deprivation. From our knowledge, Bergmann's paper is the only study that has investigated the relationship between sleep and an experimental tumor model. However, the differences in the SD protocol, species employed in the study, type of tumor and inoculation site, make a direct comparison of Bergmann's study and the present one impossible.

It is well accepted that T lymphocytes play a crucial role in the host's immune response to cancer (Shiku, 2003). Specifically, the $CD4^+$ lymphocyte subset augments both humoral and cellular immune responses (Kennedy and Celis, 2008). It has been previously demonstrated that EAT reduces the

frequency of T $CD4^+$ cells in spleens of Swiss CD1 mice from the first to seventh day after tumor inoculation (Segura et al., 1997). We achieved similar results 10 days after tumor inoculation either in male or female, although with smaller differences between control and EAT groups than previously observed (Segura et al., 1997). The fact that Swiss mice belong to a outbred strain and BALB/c mice belong to a inbred mice strain might explain such differences. In addition, SD potentiated the EAT-induced decrease of $CD4^+$ T cell population. Dinges and colleagues (1994), comparing the effects of 40h and 64h of sleep deprivation, showed that $CD4^+$ cells and NK cells decreased in number after 40h of sleep deprivation but that the number of NK cells increased after 64h of sleep deprivation in humans. Supporting these findings, patients with chronic insomnia presented decreased numbers of $CD4^+$ and $CD8^+$ cells (Savard et al., 2003).

In addition to $CD4^+$, $CD8^+$ cytotoxic T lymphocytes can recognize tumor-associated antigens (van der Bruggen et al., 1991; Wolfel et al., 1995; Ikeda et al., 1997) and adoptive transfer of tumor-specific $CD8^+$ T cells is able to eradicate certain types of tumors (Hanson et al., 2000). This suggests that these cells play a critical role in the cell-mediated immunity responsible for tumor elimination (Sheeja and Kuttan, 2007). In our study, SD (but not EAT) led to a decrement in the frequency of splenic $CD8^+$ T cell subset in both in male and female mice. Thus, while $CD4^+$ T cell population was decreased by both SD and EAT, $CD8^+$ cells were diminished only by SD. In this regard, splenic $CD4^+$ lymphocytes but not of $CD8^+$ cells from mice bearing plasma cell tumors displayed a diminished proliferating response to mitogens (Ruzek et al., 1995). Futhermore, Boyum and colleagues (1996) have demonstrated that SD *per se*

is able to induce a decrement in $CD8^+$ subset in humans. Although studies investigating cellular immunity against tumors mostly focused on $CD8^+$ T cells, it became evident that $CD4^+$ T cells also play a critical role in the development of effective anti-tumor immunity (Kennedy and Celis, 2008). While $CD4^+$ T cells can have direct anti-tumor effects through secretion of cytokines, such as TNF-α (Schattner et al., 1996; Thomas and Hersey, 1998), they also contribute for anti-tumor effects by providing the required T-cell help for generating and augmenting tumor-specific cytotoxic T-lymphocyte responses not only in primary but also in $CD8^+$ memory T-cell formation (Kennedy and Celis, 2008). Collectively, the current results suggest that as long as there is an interplay relationship between $CD4^+$ and $CD8^+$ T cells, the negative modulatory effects of SD on the splenic $CD4^+$ T cells frequency could have contributed, at least in part, to a possible ineffective function of splenic $CD8^+$ T cells in mice bearing EAT.

Concerning splenic $CD19^+$ cells (B cells), SD promoted a decrease in the frequency of these cells in male and female mice, irrespective from the presence of EAT. Of note, SD is reported to reduce the number and the function of these cells (Boyum et al.; 1996) as well as the antibody titers to hepatitis A (Lange et al., 2003) and influenza virus (Spiegel et al., 2002) after vaccination. In parallel, a recent study has demonstrated that B cells can cross-present antigens via MHC class I thus activating $CD8^+$T cells (Heit et al., 2004). This finding suggests that the diminished frequency of $CD19^+$ cells could have also led, at least in part, to an impaired activation of $CD8^+$ T cells, as it also seems to be the case of $CD4^+$.T cells. Additionally, a possible explanation for the lack of effects of EAT on $CD19^+$ cells population could be the fact that, although EAT is

a highly immunogenic tumor (Viñuela et al., 1991), ten days could not have been enough for the production of a humoral response.

Previous reports have shown that SD can induce enhancement of both the number (Born et al., 1997) and function of NK cells (Dinges et al., 1994). Our study is in line with these findings since we verified that SD *per se* enhances the frequency of splenic $CD49b^+$ cells (a marker for NK cells in BALB/c mice) in both male and female mice. Conversely, EAT *per se* decreased the frequency of these cells only in male mice. The diminished frequency of $CD49b^+$ cells in male mice could be a result of cellular migration. In fact, as NK cells can enter the site of tumor growth (Flannery et al., 1981), their number in blood or lymphoid organs could be diminished owing to their sequestration at the tumor site (Olinescu et al., 1983). On the other hand, the absence of modifications in the frequency of $CD49b^+$ cells induced by EAT *per se* in female mice could be explained by the fact that EAT is a female mammary carcinoma, leading to a subtler immunological response than that produced by male mice. It should be noted, however, that in SD-mice, EAT was able to reduce the frequency of splenic $CD49b^+$ cells in both in male and female mice.

Taken together, the lymphocytic cell population evaluated seems to work cooperatively. In fact, $CD4^+$ T cells are critical for the function of $CD8^+$ T cells. Besides, as mentioned above, $CD19^+$ cells can also contribute to the activation o $CD8^+$ T cells, an important antitumoral defense. In this scenario, SD promoted significant modifications in the frequency of these cells types, altering their delicate functional balance and resulting, at last, in a potentiation of EAT growth in a similar manner in male and female mice.

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# SLEEP AND TUMOR GROWTH: BI-DIRECTIONAL INTERACTIONS IN MICE

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**ABSTRACT**

**STUDY OBJECTIVES:** Recently, we have demonstrated that 72h of sleep deprivation (SD) prior to Ehrlich ascitic tumor (EAT) inoculation induces a remarkable potentiating effect on tumor growth. Thus, the aim of the present study was to investigate the effects of the same protocol SD, but concomitantly to EAT inoculation, on tumor growth in male BALB/c mice. Our study also focused on evaluating whether EAT *per se* induces alterations in sleep pattern, as well as the influence of this tumor, SD and the association of both on splenic lymphocytic cell population. **DESIGN:** Male BALB/c mice were intraperitoneally inoculated with EAT cells and immediately after were sleep deprived for 72h. Ten or 15 days after the inoculation, the number of tumoral cells was quantified and the lymphocytic cell population in spleen was characterized by flow cytometry. Moreover, EAT influence in sleep pattern was verified. **RESULTS:** SD potentiated EAT growth exclusively 15 days after the inoculation. In addition, mice inoculated with EAT and deprived from sleep presented decreased frequencies of splenic $CD4^+$, $CD8^+$ and $CD19^+$ cells when compared to control mice equally inoculated with EAT. In respect to sleep pattern, EAT enhanced the total time of sleep as well as the slow-wave sleep time. **CONCLUSIONS:** These results suggest a bi-directional interaction between sleep and tumor development in that tumor development increases sleep duration and sleep loss enhances tumor development.

**KEYWORDS:** Ehrlich ascitic tumor, sleep deprivation, immunologic system, male mice

## INTRODUCTION

Voluntary sleep curtailment in response to the rigors of a competitive world is common in present day society. Historically, sleep deprivation (SD) was used as a tool to investigate the functions of sleep (Hui et al., 2007). In recent years, SD has become one of the most common albeit under-recognized public health hazards of modern civilizations (Honkus, 2003; Tufik et al., 2009).

It has been demonstrated a high frequency of sleep disturbance as a symptom in cancer patients (Sateia and Lang, 2008). Indeed, the majority of cancer patients report fatigue, difficulty sleeping, being tired and complain of weakness (Ancoli-Israel et al., 2001; Savard and Morin, 2001). Alternatively, it is thought that sleep disorders or disruption of sleep pattern lead to alterations in immune functions that may adversely affect host resistance to infectious disease (Everson, 1993), increase cancer risk (Savard et al., 1999), and alter the progression of inflammatory disease (Crofford et al., 1997). Moreover, it is a common perception that we are more susceptible to infectious when we sleep-deprived and, on the other way round, many infectious seem to cause increased somnolence (Bryant et al., 2004).

Recently, we have reported that a previous sleep loss potentiated subsequent Ehrlich ascitic tumor growth (EAT) (Maragno-Corrêa et al., submitted data), an experimental tumor model, characterized by a spontaneous murine carcinoma that can grow rapidly in almost any mouse strain, producing ascitic or solid tumors across histocompatibility barriers (Carry et al., 1979; Frussa-Filho et al., 1991, 1992; Kleeb et al., 1997, 1999). Additionally, we also verified that the SD prior to EAT inoculation induced a decrease in the

frequency in splenic lymphocytic population, which could have contributed, at least in part, to the potentiation of tumor growth.

Several studies have investigated changes in immune-cell numbers after SD, but the results are inconsistent, thereby indicating that the degree or moment of SD is important in determining its effect on immune function (Bryant et al., 2004). In this scenario, we aimed to verify whether SD condition occurring concomitantly with tumor development would also have a potentiating effect on EAT growth. Additionally, we also aimed to examine possible modifications in splenic lymphocytic population in this setting. Finally, we also investigated the effects of EAT on mice's sleep pattern.

## MATERIAL AND METHODS

*Subjects*

Three-month-old BALB/c male mice (inbred, raised, and maintained in the Centre for Development of Experimental Models in Medicine and Biology of Universidade Federal de São Paulo) were used in the experiments. Animals weighing 30-35 g were housed under controlled temperature (22-23°C) and lighting (12h light, 12h dark; lights on at 6:45 a.m.) conditions. Food and water were available *ad libitum* throughout the experiments. Animals used in this study were maintained in accordance with the National Institute of Health Guide for the care and use of laboratory animals (NIH Publications Nº 80-23, revised 1996) and the experimental procedures were approved by the Institutional Animal Care and Use Committee under the protocol #1128/2008.

*Sleep Patterns Recording*

The recordings of electrocorticographic (ECoG) and electromyographic (EMG) activities of male BALB/c mice were run, thus permitting assessment of the sleep-wake cycle after the inoculation of vehicle solution (VEH) or EAT cells for 3 days in the same animal. Anesthesia was induced by i.p. administration of ketamine-xylazine. Two pairs of electrodes (steel screws) were implanted in the fronto-parietal medial derivation (1 pair on each side of the skull) for ECoG recording. One additional pair of nickel-chrome electrodes was also implanted in the dorsal muscle of the mouse's neck for EMG recording. The electrodes were soldered to a connector, which was fixed to the animal cranium with acrylic dental cement. After surgery, mice were given pentabiotic and

diclofenac, and were allowed to recover from surgery for 2 weeks. After the recovery period, mice were subjected to an i.p. injection of vehicle solution in their homecages and their sleep pattern was recorded for 72h. Ten days after the vehicle administration, mice received an i.p. inoculation of EAT and their sleep pattern was again recorded for 72h. The animals were subjected to undisturbed sleep recording using the Somnologica software (EMBLA Medical digital polygraph, Reykjavik, Iceland). The sleep pattern was visually and manually scored by a single blinded researcher, thus ensuring consistency of the data. The following sleep parameters were considered: total sleep time (percentage of sleep time during the recording), slow-wave sleep time (SWS) (percentage of all periods of deep sleep during the recording), and paradoxical sleep time (percentage of all periods of paradoxical during the recording). SWS was classified by EEG voltage of 20–30 μV (usually 200–400 μV, peak to peak) and low frequency activity (delta waves, 1–4 Hz). Paradoxical sleep presented EEG voltage below 20 μV with high and regular theta activity (6–10 Hz), especially in the fronto-parietal medial EEG derivation, and muscle atonia in EMG recording.

*Sleep deprivation (SD)*

The experimental groups were submitted to SD for 72 h using the modified multiple platform method (Frussa-Filho et al., 2004; Silva et al., 2004a,b,c, 2007; Araújo et al., 2006; Fukushiro et al., 2007; Calzavara et al., 2008; Patti et al., 2010), which consists of placing 5 mice in a ventilated cage (38 × 31 × 17 cm, Tecniplast, Buguggiate, Italy) containing 14 circular platforms (3.5 cm in diameter) with water 1 cm below the upper surface. At the onset of

each paradoxical sleep episode, the animal experiences a loss of muscle tonus and falls into the water, thus awakening. Food and water were available *ad libitum*. The home-cage control group (CTRL) was maintained in separate cages in the same ventilated cage system as the experimental mice. By housing both groups in the same environment, we are able to control for differences in housing conditions between the 2 groups as well as provide the SD group with satisfactory conditions of the ventilated cages.

We have previously demonstrated that 3-month-old male mice subjected to the above-described protocol displayed a significant reduction in paradoxical sleep, dropping from 55.0 ± 4.6 min (mean ± S.E., baseline) to 2.1 ± 0.6 min on the first day of SD, 2.9 ± 0.7 min on the second day and 2.9 ± 1.0 min on the third day. Slow-wave sleep was also significantly reduced but at a lower magnitude, falling from 558.5 ± 29.8 min (baseline) to 125.5 ± 9.5 min on the first day of the SD protocol, 83.9 ± 9.9 min on the second day and 101.5 ± 10.8 min on the third day (Silva et al., 2004).

*Ehrlich Ascitic Tumor (EAT)*

Ehrlich ascitic tumor was maintained in 3-month old female BALB/c mice in ascitic form under week passage. In all experiments, male BALB/c mice were intraperitoneally injected with $5 \times 10^7$ viable tumor cells suspended in 0.4 mL of phosphate-buffered saline (PBS) or the same volume of PBS only (vehicle – VEH). The suspension containing tumor cells was prepared according to a previous study (Dagli et al., 1992; Kleeb et al.,1997). The inoculation of EAT or VEH occurred 10 or 15 days before measurement of tumor growth

(Experiments II and III) and 10 days before immunological response characterization (Experiment IV).

*Flow Cytometric Analyses*

After collection of the ascitic fluid, the spleen of each mouse was removed and vigorously washed in PBS to detach any remaining tumor cell from the external capsule of the organ. A cell suspension of each individual spleen was prepared in RPM-1640 medium and centrifugated at 300 *g* for 10min at 4°C. The supernatant was discharged and red blood cells were lysed with ACK lysing buffer (GIBCO-Invitrogen, Grand Island, NY) following manufacturer instructions. Cells were washed three times in PBS and aliquots containing $10^6$ spleen cells were incubated with anti-mouse CD16/32 (eBioscience Inc., San Diego, CA) for 30 min at 4$^o$C in staining buffer [2% FBS and 0.02% sodium azide in PBS] to block nonspecific Fc receptor binding. Cells were then stained with the following monoclonal antibodies conjugated with fluorochromes for 30min at 4°C: anti-mouse $CD3^+$ PE, $CD4^+$ APC, $CD8^+$ FITC, $CD19^+$ PE (B cells) or $CD49b^+$ FITC (NK cells) (BD Biosciences, San Jose, CA). A hundred thousand cells per sample were acquired on the FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

*Experimental design*

*Experiment I: Characterization of sleep pattern in ascitic Ehrlich tumor-bearing mice*

Four mice were intraperitoneally injected with vehicle solution (VEH) and their sleep pattern was recorded during 3 consecutive days (D1 to D3). Ten

days after D1, animals were intraperitoneally inoculated with 5 x $10^7$ EAT viable cells and their sleep pattern was recorded for additional 3 days (D11 to D13).

*Experiments II and III: Effects of concomitant sleep deprivation on Ehrlich ascitic tumor growth in mice*

Mice were randomly assigned to one of the following groups: Ehrlich ascitic tumor-control (EAT+CTRL) or Ehrlich ascitic tumor-sleep deprivation (EAT+SD). Groups of mice were intraperitoneally inoculated with 5 x $10^7$ Ehrlich ascitic tumor cells (EAT). Immediately after the inoculation, animals were kept in their homecages (CTRL condition) or sleep-deprived for 72h (SD condition). Ten (Experiment II) or 15 (Experiment III) days after the inoculation procedure, animals were euthanized and had their ascitic fluid collected and the number of tumor cells was evaluated.

*Experiment IV: Effects of concomitant sleep deprivation and/or Ehrlich ascitic tumor growth in mice: measurement of splenic cell lymphocytic population*

Mice were randomly assigned to one of the following groups: vehicle-control (VEH+CTRL), Ehrlich ascitic tumor-control (EAT+CTRL), vehicle- sleep deprivation (VEH+SD) or Ehrlich ascitic tumor-sleep deprivation (EAT+SD). Groups of mice were intraperitoneally inoculated with 5 x $10^7$ EAT cells or with vehicle solution (VEH). Immediately after the inoculation, animals were kept in their homecages (CTRL condition) or sleep-deprived for 72h (SD condition). Ten days after inoculation procedure, animals were euthanized and had their spleen collected and the splenic lymphocytic cell population was characterized by flow cytometry.

*Statistical analysis*

In the Experiment I, the sleep parameters were analyzed using paired samples T-test. In the Experiments II and III, the number of tumor ascitic cells was compared by independent samples T-test. In the Experiment IV, the frequency of splenic lymphocytic cells was compared using two-way ANOVA followed by Duncan's test. A probability of $p<0.05$ was considered significant for all comparisons made.

## RESULTS

*Experiment I: Characterization of sleep pattern in Ehrlich ascitic tumor-bearing mice*

In the light photoperiod, concerning total sleep time and slow-wave sleep time, paired samples T-test revealed an increment in these parameters on D13 (third day after EAT inoculation) when compared to D3 (third day after the injection of vehicle) [$T(3)=3.57$ and 3.22; $p<0.05$, respectively] (Figure 1A and 1B, respectively) No differences in paradoxical sleep time were observed (Figure 1C).

Paired samples T-Test did not reveal any significant differences concerning sleep parameters in the dark photoperiod.

*Experiment II: Effects of concomitant sleep deprivation on the 10$^{th}$ day of Ehrlich ascitic tumor growth in mice*

Independent samples T-test did not reveal any significant statistical differences between the EAT+CTRL and the EAT+SD groups for the number of EAT [$T(12)=0.72$; $p=0.49$] (Figure 2).

*Experiment III: Effects of concomitant sleep deprivation on the 15$^{th}$ day of Ehrlich ascitic tumor growth in mice*

In contrast with Experiment II, independent samples T-test revealed that the EAT+SD group displayed an enhancement in the number of Ehrlich ascitic tumor cells present in the ascitic fluid when compared to the EAT+CTRL group [$T(17)=3.04$; $p<0.05$] (Figure 3).

*Experiment IV: Effects of concomitant sleep deprivation and/or Ehrlich ascitic tumor growth in mice: measurement of splenic cell lymphocytic population*

Concerning T cells, the frequency of splenic $CD4^+$ and $CD8^+$ subpopulations were evaluated into the gated $CD3^+$ cells. Regarding the frequency of $CD4^+$ T cells in spleen, two-way ANOVA revealed significant treatment (vehicle or Ehrlich ascitic tumor) [$F(1,21)=22.40$; $p<0.001$] and sleep condition (CTRL or SD) [$F(1,21)=26.76$; $p<0.001$] effects. In fact, Duncan's *post hoc* test showed that the VEH+SD and EAT+CTRL groups displayed a decreased frequency of cells when compared to VEH+CTRL. Besides, the EAT+SD group also presented a decreased in this parameter when compared to VEH+SD and EAT+CTRL groups (Figure 4A).

Two-way ANOVA revealed significant treatment [$F(1,21)=19.34$; $p<0.001$] and sleep condition [$F(1,21)=35.66$; $p<0.001$] effects when $CD8^+$ T cells frequency in spleen is analyzed. *Post hoc* analyses revealed that the VEH+SD and EAT+CTRL groups displayed a decreased frequency of cells when compared to VEH+CTRL. Still, the EAT+SD group also presented a decreased in this parameter when compared to VEH+SD and EAT+CTRL groups (Figure 4B).

In respect to the frequency of $CD19^+$ cells (B cells) in spleen, two-way ANOVA revealed treatment [$F(1,21)=12.06$; $p<0.01$] and sleep condition [$F(1,21)=133.11$; $p<0.001$] effects. In fact, the VEH+SD group displayed a decreased frequency of cells when compared to VEH+CTRL. Additionally, the EAT+SD group also presented a decreased in this parameter when compared to VEH+SD and EAT+CTRL groups (Figure 4C).

Two-way ANOVA revealed significant treatment [$F(1,21)=40.97$; $p<0.001$], sleep condition [$F(1,21)=6.46$; $p<0.05$] and treatment x sleep condition interaction [$F(1,21)=6.30$; $p<0.05$] effects when $CD49b^{+}$ cells (a marker for NK cells in BALB/c mice) frequency in spleen is analyzed. *Post hoc* analyses showed that the VEH+SD presented an enhancement of the frequency of these cells when compared to VEH+CTRL. Conversely, the EAT+CRTL and EAT+SD presented a decrement in frequency when compared to VEH+CTRL and VEH+SD groups (Figure 3D).

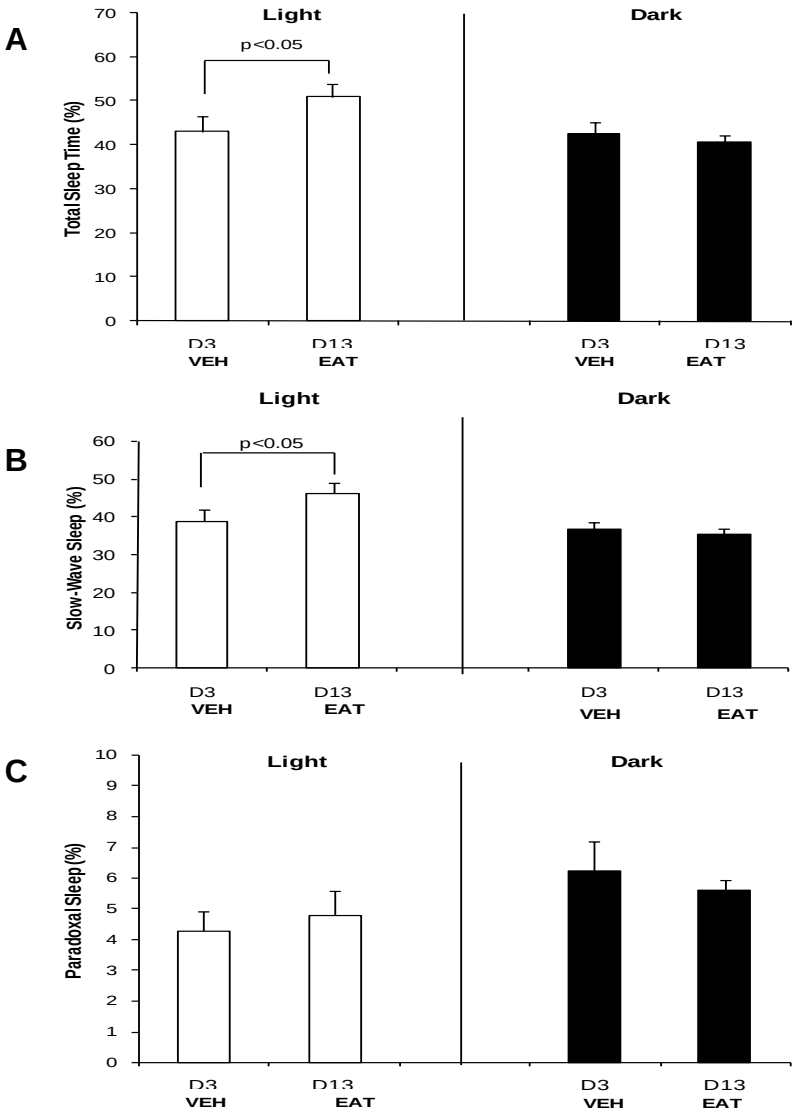


**Figure 1** – Characterization of sleep pattern in mice bearing Ehrlich ascitic tumor (EAT). Four BALB/c mice were intraperitoneally injected with vehicle solution (VEH) and their sleep pattern was recorded during 3 consecutive days (D1 to D3). Ten days after D1, animals were intraperitoneally inoculated with 5 x $10^7$ EAT cells and their sleep pattern was recorded for additionally 3 days (D11 to D13). Data are presented as mean ± SE of total sleep time (A), slow-wave sleep time (B), and paradoxical sleep time (C) (Paired samples T-test).

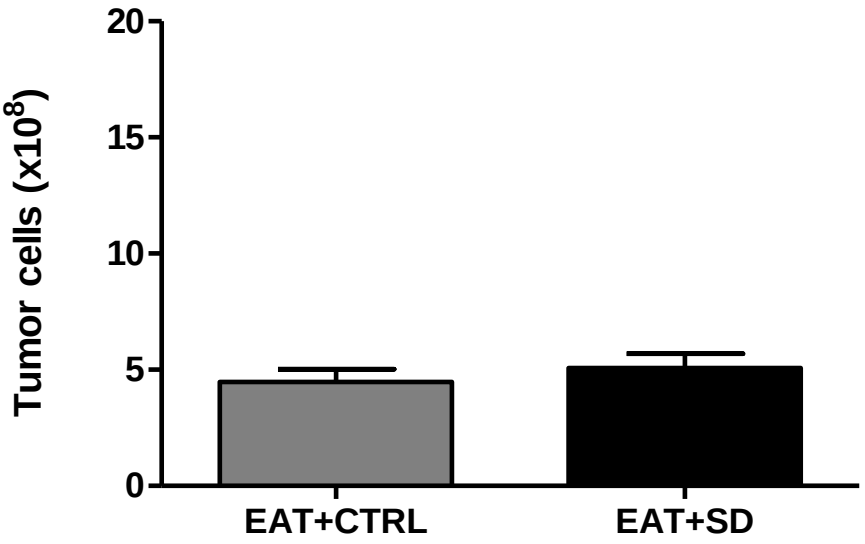


**Figure 2** – Effects of concomitant sleep deprivation (SD) on the 10$^{th}$ day of Ehrlich ascitic tumor (EAT) growth in BALB/c mice. Mice were intraperitoneally injected with 5 x 10$^{7}$ EAT cells. Immediately after inoculation, animals were sleep deprived for 72h. Ten days after inoculation, the ascitic fluid was collected (n=7 for both groups). Data are presented as mean ± SE of tumor cells in the ascitic fluid. No statistical differences were found between groups (Independent samples T-test).

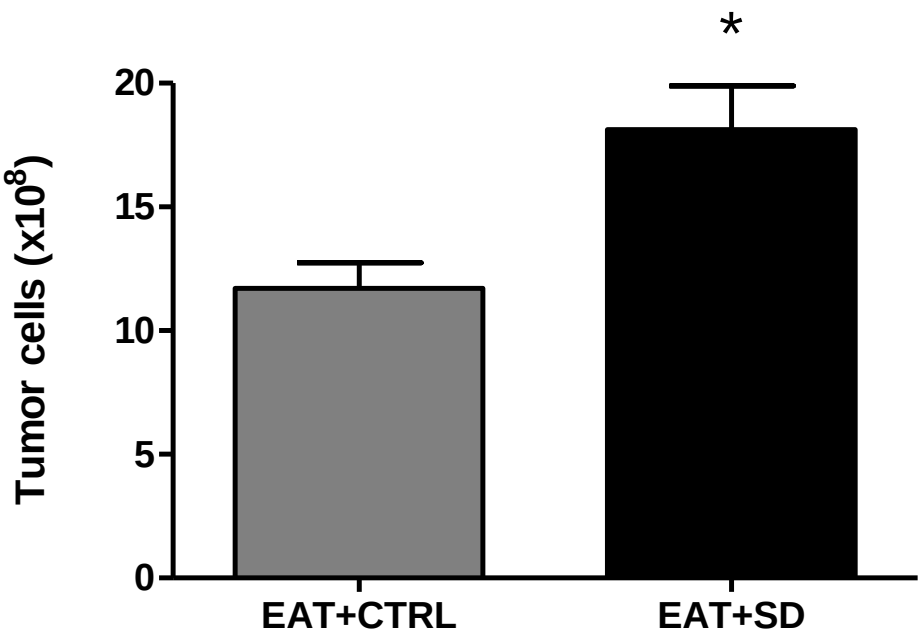


**Figure 3** – Effects of concomitant sleep deprivation (SD) on the 15$^{th}$ day of Ehrlich ascitic tumor (EAT) growth in BALB/c mice. Mice were intraperitoneally injected with 5 x 10$^7$ EAT cells. Immediately after inoculation, animals were sleep deprived for 72h. Ten days after inoculation, the ascitic fluid was collected (n=7 and 10 for EAT+CTRL and EAT+SD, respectively). Data are presented as mean ± SE of tumor cells in the ascitic fluid. *p<0.05 when compared to the EAT+CTRL group (Independent samples T-test).

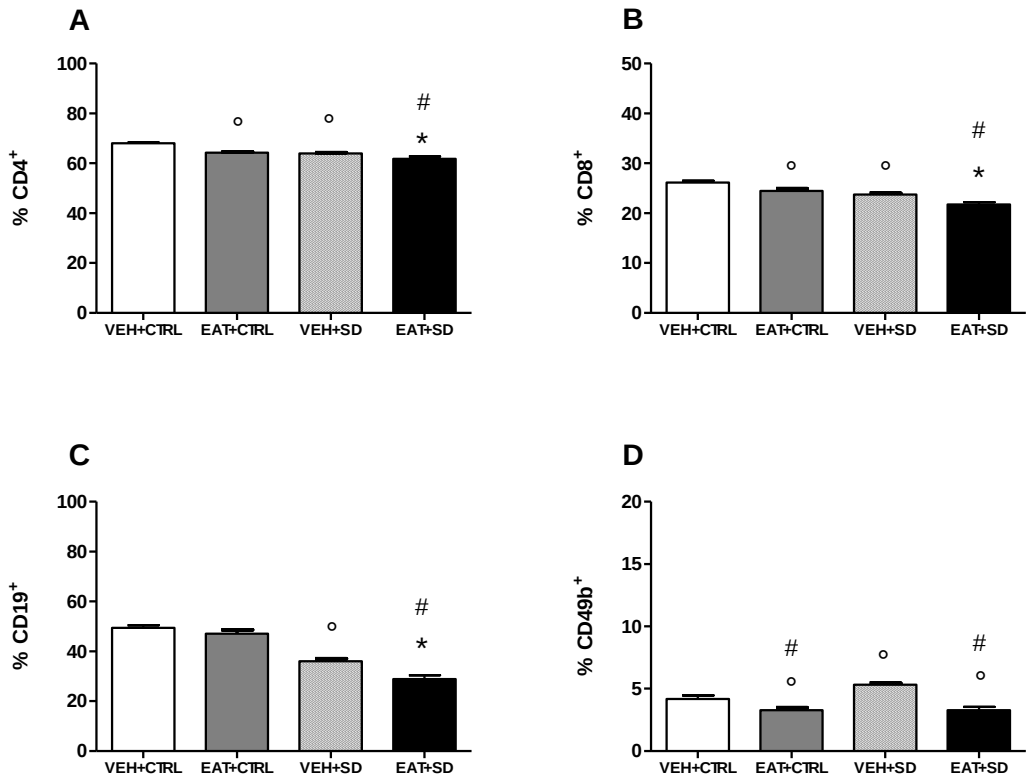


**Figure 4** – Effects of concomitant sleep deprivation (SD) on the Ehrlich ascitic tumor (EAT) growth in BALB/c mice: *measurement of splenic cell lymphocytic population.* Mice were intraperitoneally injected with 5 x $10^7$ EAT cells or vehicle solution (VEH). Immediately after the inoculation, animals were kept in their homecages (CTRL) or were sleep deprived for 72h. Ten days after the inoculation, the spleens were collected for flow cytometry analyses (n=5, 7, 6 and 7 for VEH-CTRL, EAT+CTRL and EAT+SD, respectively). Data are presented as mean ± SE of $CD4^+$ (**A**) and $CD8^+$ (**B**) subpopulations gated into the $CD3^+$ cells, $CD19^+$ B lymphocytes cells (**C**) and $CD49b^+$ NK cells (**D**). °$p<0.05$ when compared to the VEH+CTRL group; *$p<0.05$ compared to the EAT+CTRL and $^{\#}p<0.05$ compared to the VEH+SD (Two-way ANOVA and Duncan's test).

## DISCUSSION

The major finding of the present study is that concomitant SD with EAT inoculation remarkably potentiated tumor growth. In fact, this SD protocol did not induce significant alterations in tumor growth on the 10$^{th}$ day, but potentiated its growth on the 15$^{th}$ day after the inoculation procedure. The EAT inoculation induced an increment in the total sleep time as well as in slow-wave sleep of mice, suggesting that sleep *per se* is important to the antitumoral mechanisms.

As mentioned previously, high frequency of sleep disturbances is reported by cancer patients (Sateia and Lang, 2008). In this way, our data demonstrate that EAT inoculation altered sleep pattern by increasing both total sleep time and slow-wave sleep. These results are in line with the findings that support the observation that sleep is increased during immunological challenges (Bryant et al., 2004). In this concerning, sleep is hypothesized to have a restorative function on immune processes (Moldofsky, 1994; Opp and Imeri, 1999). In turn, disordered sleep and sleep loss are thought to impair host defense mechanisms (Benca and Quintas, 1997; Dinges at al., 1995).

Recently, we have demonstrated that previous SD potentiated subsequent EAT growth in mice 10 days after the tumor inoculation (Maragno-Corrêa et al, submitted data). Interestingly, the submission of the same protocol of SD (72h) immediately after the EAT inoculation postponed the potentiating effect on tumor growth, since the number of EAT cells was enhanced only 15 days after the inoculation procedure (and not 10 days after it, as was verified when SD occurred immediately prior to the tumor inoculation). A possible

explanation to this delayed SD-induced potentiating effect could be related to the influence of the moment when SD occurred in the mice's capability of development an antitumoral response. Specifically, when mice were sleep-deprived immediately before the tumor inoculation, their immune response was already impaired by SD, facilitating the tumor development. On the other hand, when mice were sleep-deprived immediately after the tumor inoculation, the tumor development occurred concomitantly to the progressive impairments induced by SD in the immune response, delaying the tumor growth. Importantly, host immune response to tumor antigens are not immediate (Leach et al., 1996), the early contact between host and tumor is critical in determining the future directions of the immune response elicited by tumors antigens (Segura et al., 2000).

Several studies have demonstrated that sleep loss leads to significant alterations in many immune components both in laboratory animal and humans (Irwin et al., 2002; Ruiz et al., 2007; Ruiz et al., in press; Zager et al., 2007). In this way, we have also verified that SD-induced potentiating effect is associated with a reduced frequency of splenic lymphocyte cells, responsible for antitumoral defense (Maragno-Corrêa et al, submitted data). Corroborating these results, in the present study, while EAT reduced the splenic frequency of T $CD4^+$ and $CD8^+$cells, SD potentiated such decrement. In this way, the $CD4^+$ lymphocyte subset augments both humoral and cellular immune responses (Kennedy and Celis, 2008) and $CD8^+$ cytotoxic T lymphocytes can recognize tumor-associated antigens (van der Bruggen et al., 1991; Wolfel et al., 1995; Ikeda et al., 1997) and adoptive transfer of tumor-specific $CD8^+$ T cells is able to eradicate certain types of tumors (Hanson et al., 2000). Moreover, SD *per se*

diminished the splenic frequency of $CD19^+$ cells and this decrement had a greater magnitude in EAT bearing mice. Although $CD19^+$ cells are well-known for their role in humoral response, a recent study has demonstrated that B cells can cross-present antigens via MHC class I, thus activating $CD8^+$T cells (Heit et al., 2004). Finally, although SD increased the frequency of $CD49b^+$ cells (a marker for NK cells in BALB/c mice) in spleens, both EAT and the association of EAT and SD decreased such frequency. Taken together, the lymphocytic cell population evaluated seems to work cooperatively. In fact, $CD4^+$ T cells are critical for the function of $CD8^+$ T cells. Moreover, $CD19^+$ cells can also contribute to the activation o $CD8^+$ T cells, an important antitumoral defense. In this scenario, SD promoted significant modifications (reduced frequency of $CD4^+$, $CD8^+$ or $CD19^+$ cells and increased frequency of $CD49b^+$ cells) in the frequency of these cells subsets, altering their delicate functional balance.

Collectively, our results demonstrate a bi-directional interaction between sleep and tumor development. While tumor growth increases sleep duration, sleep deprivation in animals bearing tumor potentiates tumor growth, though at least in part, modifications of the delicate functional balance of lymphocytic cells.

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# SLEEP DEPRIVATION ANTECIPATES MORTALITY IN FEMALE MICE BEARING EHRLICH ASCITIC TUMOR

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**ABSTRACT**

**STUDY OBJECTIVES:** Recently, we have demonstrated that 72h sleep deprivation (SD) prior to Ehrlich ascitic tumor (EAT) inoculation induces a remarkable potentiating effect on tumor growth in both male and female mice. Additionally, the same period of SD – given immediately after EAT inoculation – inducds the same potentiating effect (although postponed) in male mice. The aim of the present study was to investigate the effects of this latter SD protocol (concomitant to EAT inoculation) on tumor growth in female BALB/c mice. Our study also focused on evaluating whether EAT *per se* induces alterations in female mice's sleep pattern. Splenic lymphocytic cell population and life span were also quantified. **DESIGN:** Female BALB/c mice were intraperitoneally inoculated with EAT cells. Immediately after the inoculation procedure, animals were sleep-deprived for 72h. Ten or fifteen days after the inoculation procedure, the number of tumoral cells was quantified. Moreover, the lymphocytic cell population in spleen was characterized by flow cytometry and the effect of SD on EAT-induced mortality was quantified. Finally, EAT influence in sleep pattern was determined. **RESULTS:** Although SD did not potentiate EAT growth, it remarkably curtailed life span. Still, mice subjected to EAT and SD presented decreased frequencies of splenic $CD4^+$, $CD8^+$ and $CD19^+$ cells in relation to control mice equally inoculated with EAT. In respect to sleep pattern, EAT significantly enhanced the paradoxical sleep time. **CONCLUSIONS:** Concomitant SD induces a curtailment of life span in female mice bearing EAT.

**KEYWORDS:** Ehrlich ascitic tumor, sleep deprivation, immunologic system, female mice.

## INTRODUCTION

While the prevalence of sleep disorders is increasing in modern societies (Tufik et al., 2009) evidence has been accumulating that sleep disturbances or disruption lead to alterations in immune functions that may adversely affect host resistance to infectious disease (Everson, 1993), and increase cancer risk (Savard et al., 1999). On the other hand, the majority of cancer patients report fatigue, difficulty sleeping, being tired and complain of weakness (Ancoli-Israel et al., 2001; Savard and Morin, 2001).

Ehrlich ascitic tumor (EAT) is an experimental tumor model, characterized by a spontaneous murine carcinoma that can grow rapidly in almost any mouse strain, producing ascitic or solid tumors across histocompatibility barriers (Carry et al., 1979; Frussa-Filho et al., 1991, 1992; Kleeb et al., 1997, 1999). Recently, we have demonstrated that 72h of SD prior to EAT inoculation induces a remarkable potentiating effect on tumor growth in both male and female mice (Maragno-Corrêa et al., submitted data). Additionally, the same period of SD – given immediately after EAT inoculation – induced a long-term potentiating effect 15 days after the EAT inoculation in male mice.

Several studies support the observation that sleep is increased during immunological challenges (Bryant et al., 2004). In this way, we have demonstrated that EAT inoculation altered sleep pattern by increasing both total sleep time and slow-wave sleep in male mice. In this concerning, sleep is hypothesized to have a restorative function on immune processes (Moldofsky, 1994; Opp and Imeri, 1999). In turn, disrupted sleep and sleep loss are thought

to impair host defense mechanisms (Benca and Quintas, 1997; Dinges at al., 1995).

In this vein, we aimed to verify whether SD condition occurring concomitantly with tumor development would also have a potentiating effect on EAT growth in female mice and if this possible potentiating effect would have an influence in mice's life span. Additionally, we also aimed to examine modifications in splenic lymphocytic population in this setting. Finally, we investigated possible alterations in female mice sleep pattern induced by EAT.

## MATERIAL AND METHODS

*Subjects*

Three-month-old BALB/c female mice (inbred, raised, and maintained in the Centre for Development of Experimental Models in Medicine and Biology of Universidade Federal de São Paulo) were used in the experiments. Animals weighing 30-35 g were housed under controlled temperature (22-23°C) and lighting (12h light, 12h dark; lights on at 6:45 a.m.) conditions. Food and water were available *ad libitum* throughout the experiments. Animals used in this study were maintained in accordance with the National Institute of Health Guide for the care and use of laboratory animals (NIH Publications Nº 80-23, revised 1996) and the experimental procedures were approved by the Institutional Animal Care and Use Committee under the protocol #1128/08.

*Sleep Patterns Recording*

The recordings of electrocorticographic (ECoG) and electromyographic (EMG) activities of male BALB/c mice were run, thus permitting assessment of the sleep-wake cycle after the inoculation of vehicle solution (VEH) or EAT cells for 3 days in the same animal. Anesthesia was induced by i.p. administration of ketamine-xylazine. Two pairs of electrodes (steel screws) were implanted in the fronto-parietal medial derivation (1 pair on each side of the skull) for ECoG recording. One additional pair of nickel-chrome electrodes was also implanted in the dorsal muscle of the mouse's neck for EMG recording. The electrodes were soldered to a connector, which was fixed to the animal cranium with acrylic dental cement. After surgery, mice were given pentabiotic and

diclofenac, and were allowed to recover from surgery for 2 weeks. After the recovery period, mice were subjected to an i.p. injection of vehicle solution in their homecages and their sleep pattern was recorded for 72h. Ten days after the vehicle administration, mice received an i.p. inoculation of EAT and their sleep pattern was again recorded for 72h. The animals were subjected to undisturbed sleep recording using the Somnologica software (EMBLA Medical digital polygraph, Reykjavik, Iceland). The sleep pattern was visually and manually scored by a single blinded researcher, thus ensuring consistency of the data. The following sleep parameters were considered: total sleep time (percentage of sleep time during the recording), slow-wave sleep time (SWS) (percentage of all periods of deep sleep during the recording), and paradoxical sleep time (percentage of all periods of paradoxical during the recording). SWS was classified by EEG voltage of 20–30 μV (usually 200–400 μV, peak to peak) and low frequency activity (delta waves, 1–4 Hz). Paradoxical sleep presented EEG voltage below 20 μV with high and regular theta activity (6–10 Hz), especially in the fronto-parietal medial EEG derivation, and muscle atonia in EMG recording.

*Sleep deprivation (SD)*

The experimental groups were submitted to SD for 72 h using the modified multiple platform method (Frussa-Filho et al., 2004; Silva et al., 2004a,b,c, 2007; Araújo et al., 2006; Fukushiro et al., 2007; Calzavara et al., 2008; Patti et al., 2010), which consists of placing 5 mice in a ventilated cage (38 × 31 × 17 cm, Tecniplast, Buguggiate, Italy) containing 14 circular platforms (3.5 cm in diameter) with water 1 cm below the upper surface. At the onset of

each paradoxical sleep episode, the animal experiences a loss of muscle tonus and falls into the water, thus awakening. Food and water were available *ad libitum*. The home-cage control group (CTRL) was maintained in separate cages in the same ventilated cage system as the experimental mice. By housing both groups in the same environment, we are able to control for differences in housing conditions between the 2 groups as well as provide the SD group with satisfactory conditions of the ventilated cages.

We have previously demonstrated that 3-month-old male mice subjected to the above-described protocol displayed a significant reduction in paradoxical sleep, dropping from 55.0 ± 4.6 min (mean ± S.E., baseline) to 2.1 ± 0.6 min on the first day of SD, 2.9 ± 0.7 min on the second day and 2.9 ± 1.0 min on the third day. Slow-wave sleep was also significantly reduced but at a lower magnitude, falling from 558.5 ± 29.8 min (baseline) to 125.5 ± 9.5 min on the first day of the SD protocol, 83.9 ± 9.9 min on the second day and 101.5 ± 10.8 min on the third day (Silva et al., 2004).

*Ehrlich Ascitic Tumor*

Ehrlich ascitic tumor (EAT) was maintained in 3-month old female BALB/c mice in ascitic form under week passage. In all experiments, female BALB/c mice were intraperitoneally injected with $5 \times 10^7$ viable tumor cells suspended in 0.4 mL of phosphate-buffered saline (PBS) or the same volume of PBS only (vehicle – VEH). The suspension containing tumor cells was prepared according to a previous study (Dagli et al., 1992; Kleeb et al.,1997). The inoculation of EAT or VEH occurred 10 or 15 days before measurement of

tumor growth (Experiments II and III) and 10 days before immunological response characterization (Experiment IV).

*Flow Cytometric Analyses*

After collection of the ascitic fluid, the spleen of each mouse was removed and vigorously washed in PBS to detach any remaining tumor cell from the external capsule of the organ. A cell suspension of each individual spleen was prepared in RPM-1640 medium and centrifugated at 300 *g* for 10min at 4°C. The supernatant was discharged and red blood cells were lysed with ACK lysing buffer (GIBCO-Invitrogen, Grand Island, NY) following manufacturer instructions. Cells were washed three times in PBS and aliquots containing $10^6$ spleen cells were incubated with anti-mouse CD16/32 (eBioscience Inc., San Diego, CA) for 30 min at $4^oC$ in staining buffer [2% FBS and 0.02% sodium azide in PBS] to block nonspecific Fc receptor binding. Cells were then stained with the following monoclonal antibodies conjugated with fluorochromes for 30 min at 4°C: anti-mouse $CD3^+$ PE, $CD4^+$ APC, $CD8^+$ FITC, $CD19^+$ PE (B cells) or $CD49b^+$ FITC (NK cells) (BD Biosciences, San Jose, CA). A hundred thousand cells per sample were acquired on the FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

*Statistical analysis*

In the Experiment I, the sleep parameters were analyzed using paired samples T-test. In the Experiments II and III, the number of tumor ascitic cells was compared by independent samples T-test. In the Experiment IV, the frequency of cells in spleen was compared using two-way ANOVA followed by

Duncan's test. Finally, in the experiment V, longevity was analyzed using Kaplan-Meier followed by Log rank test. A probability of $p<0.05$ was considered significant for all comparisons made.

*Experimental design*

*Experiment I: Characterization of sleep pattern in the ascitic Ehrlich tumor-bearing mice*

Four mice were intraperitoneally injected with vehicle solution (VEH) and their sleep pattern was recorded during 3 consecutive days (D1 to D3). Ten days after D1, animals were intraperitoneally inoculated with $5 \times 10^7$ Ehrlich ascitic viable cells (EAT) and their sleep pattern was again recorded during three days more (D11 to D13).

*Experiments II and III: Effects of concomitant sleep deprivation on the $10^{th}$ or on the $15^{th}$ days of Ehrlich tumor growth in mice*

Mice were randomly assigned to one of the following groups: Ehrlich ascitic tumor-control (EAT+CTRL) or Ehrlich ascitic tumor- sleep deprivation (EAT+SD). Groups of mice were intraperitoneally inoculated with $5 \times 10^7$ Ehrlich ascitic tumor cells (EAT). Immediately after the inoculation, animals were kept in their homecages (CTRL condition) or sleep-deprived for 72h (SD condition). In the $10^{th}$ or in the $15^{th}$ day after inoculation procedure, animals were euthanized and had their ascitic fluid collected and the number of tumor cells was evaluated.

*Experiment IV: Effects of concomitant sleep deprivation and/or Ehrlich tumor growth in mice: measurement of immunological response*

Mice were randomly assigned to one of the following groups: vehicle-control (VEH+CTRL), Ehrlich ascitic tumor-control (EAT+CTRL), vehicle- sleep deprivation (VEH+SD) or Ehrlich ascitic tumor-sleep deprivation (EAT+SD). Groups of mice were intraperitoneally inoculated with 5 x $10^7$ Ehrlich ascitic tumor cells (EAT) or with vehicle solution (VEH). Immediately after the inoculation, animals were kept in their homecages (CTRL condition) or sleep-deprived for 72h (SD condition). In the $10^{th}$ day after inoculation procedure, animals were animals were euthanized and had their spleen collected for immunological cellular characterization.

*Experiment V: Effects of concomitant sleep deprivation and/or Ehrlich tumor growth in mice: longevity study*

Mice were randomly assigned to groups: vehicle-control (VEH+CTRL, n=10), Ehrlich ascitic tumor-control (EAT+CTRL, n=9), vehicle-sleep deprivation (VEH+SD, n=7) or Ehrlich ascitic tumor-sleep deprivation (EAT+SD, n=9). The animals were intraperitoneally inoculated with 5 x $10^7$ Ehrlich ascitic tumor cells (EAT) or with vehicle solution (VEH). Immediately after the inoculation, animals were kept in their homecages (CTRL condition) or sleep-deprived for 72h (SD condition). The life-span was measured from the day of inoculation until the day of death of tumor-bearing mice.

## RESULTS

*Experiment I: Characterization of sleep pattern in female mice bearing Ehrlich ascitic tumor*

In the light photoperiod, paired samples T-Test revealed that the amount of paradoxical sleep was enhanced on D13 when compared to D3 [$T(3)=3.13$; $p<0.05$] (Figure 1C). No differences in total sleep time and slow-wave sleep time were observed (Figures 1A and B).

On the other hand, paired samples T-test did not reveal any differences concerning sleep parameters in the dark photoperiod.

*Experiment II: Effects of concomitant sleep deprivation on the 10$^{th}$ day of Ehrlich tumor growth in mice*

Independent samples T-test did not reveal any significant statistical differences between the EAT+CTRL and the EAT+SD groups for the number of EAT cells (Figure 2).

*Experiment III: Effects of concomitant sleep deprivation on the 15$^{th}$ day of Ehrlich tumor growth in mice*

Independent samples T-test did not reveal any significant statistical differences between the EAT+CTRL and the EAT+SD groups for the number of EAT cells (Figure 3).

*Experiment IV: Effects of concomitant sleep deprivation and/or Ehrlich tumor growth in mice: measurement of immunological response*

Concerning T cells, the frequency of splenic $CD4^+$ and $CD8^+$ subpopulations were evaluated into the gated $CD3^+$ cells. Regarding the frequency of $CD4^+$ T cells in spleen, two-way ANOVA revealed significant treatment (vehicle or Ehrlich ascitic tumor) [$F(1,22)=37.03$; $p<0.001$] and sleep condition (homecage or sleep deprivation) [$F(1,22)=12.62$; $p<0.01$] effects. In fact, Duncan's *post hoc* test showed that the EAT+CTRL group displayed a decreased frequency of cells when compared to VEH+CTRL. Besides, the EAT+SD group also presented a decreased in this parameter when compared to VEH+SD and EAT+CTRL groups (Figure 4A).

Two-way ANOVA revealed only significant effect of sleep condition [$F(1,22)=15.98$; $p=0.001$] when $CD8^+$ T cells frequency in spleen is analyzed. Indeed, the EAT+SD group displayed a decrement in this parameter when compared to the EAT+CTRL group (Figure 4B).

In respect to the frequency of $CD19^+$ cells (B cells) in spleen, two-way ANOVA revealed sleep condition [$F(1,22)=208.66$; $p<0.001$] and sleep condition x treatment interaction [$F(1,22)=9.30$; $p<0.05$] effects. In fact, all the groups presented a decrement in this parameter when compared to the VEH+CTRL

group. Additionally, the VEH+SD and EAT+SD groups also presented such decrement when compared to EAT+CTRL group (Figure 4C).

Two-way ANOVA revealed significant treatment [$F(1,22)=23.31$; $p<0.001$] and treatment x sleep condition interaction [$F(1,22)=34.28$; $p<0.001$] effects when $CD49b^{+}$ cells (a marker for NK cells in BALB/c mice) frequency in spleen is analyzed. Duncan's *post hoc* analyses showed that the VEH+SD presented an enhancement in frequency of these cells when compared to the VEH+CTRL and the EAT+CTRL groups. Conversely, the EAT+SD presented a decrement in frequency when compared to the VEH+CTRL, EAT+CTRL and VEH+SD groups (Figure 4D).

*Experiment V: Effects of concomitant sleep deprivation and/or Ehrlich tumor growth in mice: longevity study*

The Kaplan-Meier estimator was used to estimate the proportion surviving (life span) by a given time. Mice that did not die until the end of the study were considered censored data. Data containing uncertainty as to when exactly an event happened are termed as censored data. Proportion and survival curves were compared using the log rank test.

The log rank test, as expected, detected differences between the survival curves of EAT+CTRL when compared to the VEH+CTRL and VEH+SD groups ($p<0.001$ and 0.001, respectively – data not shown graphically). Conversely, no statistical differences were found in the survival curves of the VEH+CTRL and VEH+SD groups ($p=1.0$). Importantly, the EAT+SD presented life span diminished when compared to EAT+CTRL ($p<0.001$, Figure 5).

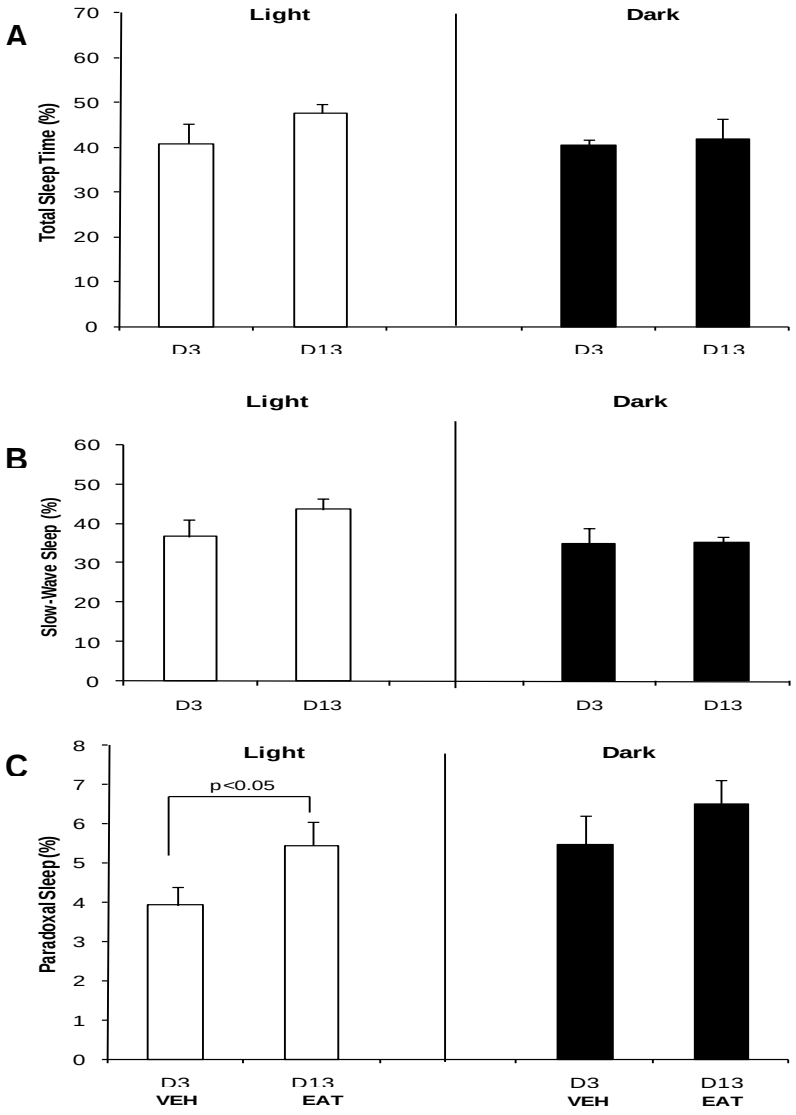


**Figure 1** – Characterization of sleep pattern in female mice bearing Ehrlich ascitic tumor. Four female BALB/c mice were intraperitoneally injected with vehicle solution (VEH) and their sleep pattern was recorded during three consecutive days (D1 to D3). Ten days after D1, animals were intraperitoneally inoculated with 5 x $10^7$ Ehrlich viable ascitic tumor cells (EAT) and their sleep pattern was again recorded during three days more (D11 to D13). Data are presented as mean ± SE of total sleep time (**A**), slow-wave sleep time (**B**), and paradoxical sleep time (**C**) (Paired samples T-test).

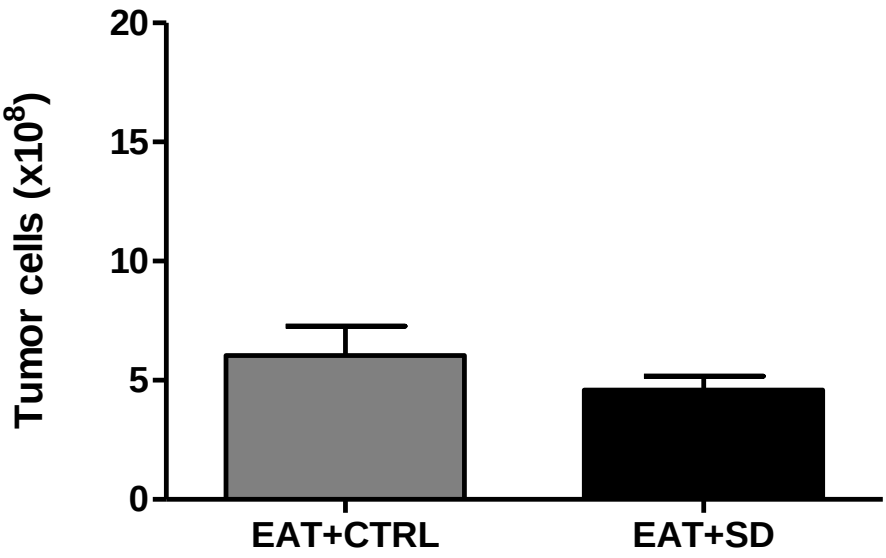


**Figure 2** – Effects of concomitant sleep deprivation on the 10$^{th}$ day of Ehrlich ascitic tumor growth in BALB/c female mice. Female mice were intraperitoneally injected with 5 x 10$^{7}$ Ehrlich viable ascitic tumor cells (EAT). Immediately after inoculation, animals were sleep deprived for 72h (SD). Ten days after the inoculation, the ascitic fluid was collected (n=5 and 7 for EAT+CTRL and EAT+SD, respectively). Data are presented as mean ± SE of tumor cells in the ascitic fluid. No differences were found between groups (Independent samples T-test).

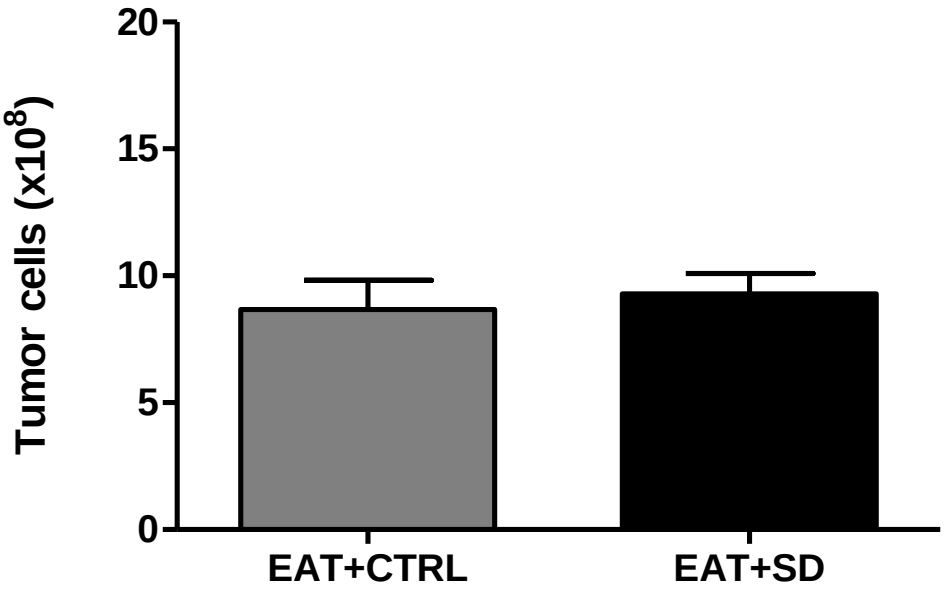


**Figure 3** – Effects of concomitant sleep deprivation on the 15$^{th}$ day of Ehrlich ascitic tumor growth in BALB/c female mice. Female mice were intraperitoneally injected with 5 x 10$^7$ Ehrlich viable ascitic tumor cells (EAT). Immediately after inoculation, animals were sleep deprived for 72h (SD). Fifteen days after the inoculation, the ascitic fluid was collected (n=10 for both groups). Data are presented as mean ± SE of tumor cells in the ascitic fluid. No differences were found between groups (Independent samples T-test).

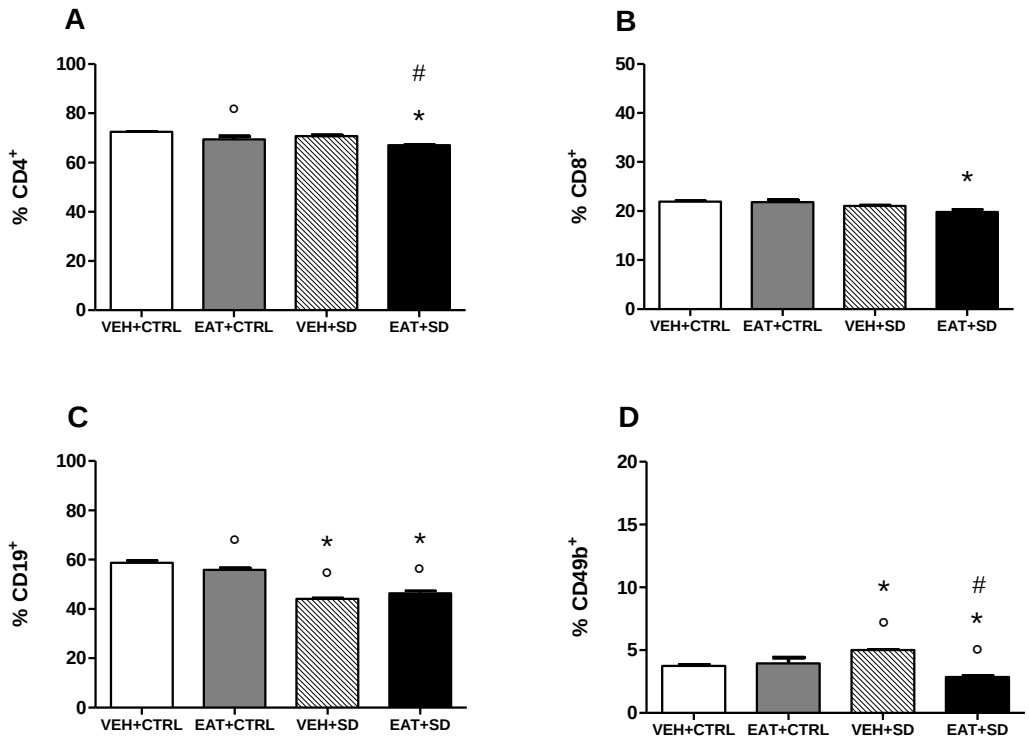


**Figure 4** – Effects of concomitant sleep deprivation on the Ehrlich ascitic tumor growth in BALB/c mice: *measurement of splenic cell lymphocytic population*. Mice were intraperitoneally injected with 5 x $10^7$ Ehrlich viable ascitic tumor cells (EAT) or vehicle solution (VEH). Immediately after the inoculation, animals were kept in their homecages (CTRL) or were sleep deprived for 72h (SD). Ten days after the inoculation, the spleens were collected for flow cytometry analyses (n=7, 5, 7 and 7 for VEH+CTRL, EAT+CTRL, VEH+SD and EAT+SD, respectively). Data are presented as mean ± SE of $CD4^+$ (**A**) and $CD8^+$ (**B**) subpopulations gated into the $CD3^+$ cells, $CD19^+$ B lymphocytes cells (**C**) and $CD49b^+$ NK cells (**D**). °$p<0.05$ when compared to the VEH+CTRL group; *$p<0.05$ compared to the EAT+CTRL and $^{\#}p<0.05$ compared to the VEH+SD (Two-way ANOVA and Duncan's test).

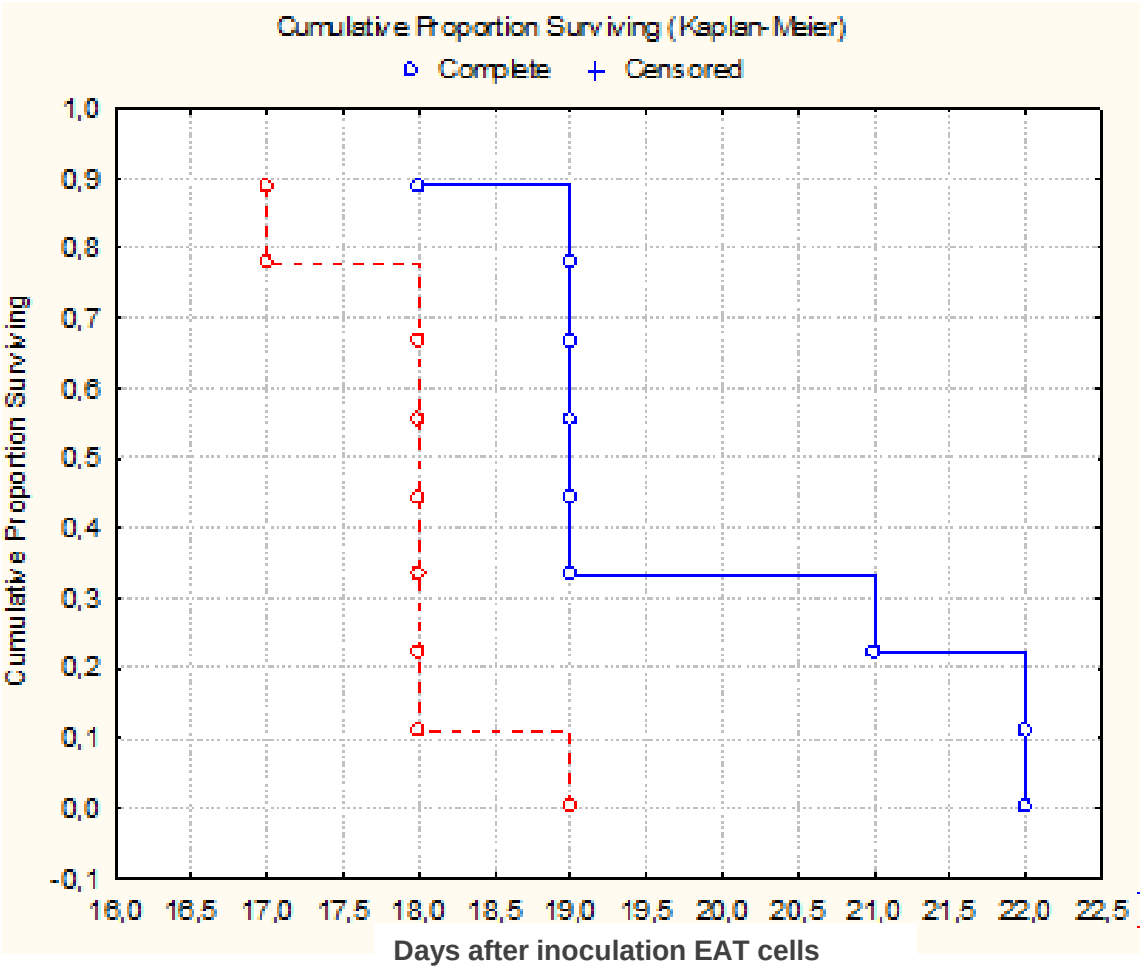


**Figure 5** – Effects of concomitant sleep deprivation on Ehrlich ascitic tumor growth in BALB/c mice: longevity study. Mice were intraperitoneally injected with 5 x $10^7$ Ehrlich viable ascitic tumor cells. Immediately after the inoculation, animals were kept in their homecages (CTRL) or were sleep deprived for 72h (SD). The life-span was measured from the day of inoculation until the day of death of tumor-bearing mice. The EAT+CTRL group is presented by the blue line and the EAT+SD is presented by the red line (Kaplan-Meier and Log rank test, $p<0.001$).

## DISCUSSION

The major finding of the present study is that concomitant SD with EAT inoculation remarkably curtailed life span in female mice. Curiously, this SD protocol did not induce significant alterations in tumor growth 10 or 15 days after tumor inoculation procedure. Moreover, the EAT inoculation did not induce alterations in total sleep time or slow-wave sleep, but enhanced paradoxical sleep.

As mentioned in the Introduction section, a high frequency of sleep disturbance as a symptom is reported by cancer patients (Sateia and Lang, 2008). The current data demonstrate that EAT inoculation altered sleep pattern by increasing paradoxical sleep. In this regard, we have recently demonstrated that EAT enhanced both total sleep time and slow-wave sleep (but not paradoxical sleep) in male mice (Maragno-Corrêa et al., submitted data). This discrepancy could be attributed to the sex differences in sleep pattern even though few studies in animals have directly examined the influence of sex on sleep. Indeed, two studies in rats found that males display significantly more paradoxical sleep during both the day and the night than females (Yamaoka, 1980; Fang and Fishbein, 1996), whereas another study in Swiss-Webster mice reported a slight diurnal sex difference in paradoxical and non-paradoxical sleep amount (Bright and Fishbein, 1987). In this regard, even though hormonal fluctuation is a factor that has been associated with sleep variations in both humans and rats (Hachul de Campos et al., 2006; Andersen et al., 2008), little information is available about how they affect sleep (Paul et al., 2009). Additionally, from a clinical

perspective, women and men sleep differently and recover differently from sleep deprivation (Paul et al., 2008).

We have recently verified that SD previously to the EAT inoculation potentiated subsequent tumor growth in the same magnitude in both male and female (Maragno-Corrêa et al, submitted data) and that when SD is applied concomitantly with EAT inoculation, it had a postponed potentiating effect on tumor growth in male mice (Maragno-Corrêa et al., submitted data). The present data demonstrate that, in female subjects, this last protocol (SD immediately after EAT inoculation) did not modify tumor growth 10 or 15 days after its inoculation. A possible explanation for these data could be that, as it was verified in male mice, the SD-induced potentiating effect would have a delayed pattern. In other words, SD would also potentiate EAT growth but in an even more delayed manner that occurred in male mice. In fact, female murine are more resistant to the growth of some tumors (Oyeyinka, 1984). However, if it was the case, the EAT growth should be evaluated 20 days after its inoculation. Nevertheless, SD induced a curtailment in life span of animals. Indeed, 20 days after tumor inoculation, all animals bearing EAT and subjected to SD had died, demonstrating that even though no significant differences were observed in EAT cells number, SD shortened longevity of these animals.

Several studies have demonstrated that sleep loss leads to significant alterations in many immune components both in laboratory animal and humans (Ruiz et al., 2007; Ruiz et al., in press; Zager et al., 2007). In this way, we have also verified that SD-induced potentiating effect on tumor growth is associated with a reduced frequency of splenic lymphocyte cells, responsible for antitumoral defense (Maragno-Corrêa et al, submitted data). Corroborating

these results, in the present study, while EAT *per se* reduced the splenic frequency of $CD4^+$ T cells, SD potentiated such decrement. In addition, the association of EAT and SD also decreased the splenic frequency of $CD8^+$ T cells. In this way, the $CD4^+$ lymphocyte subset augments both humoral and cellular immune responses (Kennedy and Celis, 2008) and $CD8^+$ cytotoxic T lymphocytes can recognize tumor-associated antigens (van der Bruggen et al., 1991; Wolfel et al., 1995; Ikeda et al., 1997) and adoptive transfer of tumor-specific $CD8^+$ T cells is able to eradicate certain types of tumors (Hanson et al., 2000). Moreover, EAT *per se* diminished the splenic frequency of $CD19^+$ cells and this decrement had a greater magnitude in sleep-deprived mice, irrespective from tumor inoculation. Although $CD19^+$ cells are well-known for its role in humoral response, a recent study has demonstrated that B cells can cross-present antigens via MHC class I, thus activating $CD8^+$T cells (Heit et al., 2004). Finally, although SD increased the frequency of $CD49b^+$ cells (a marker for NK cells in BALB/c mice) in spleens, the association of EAT and SD decreased such frequency. Taken together, the lymphocytic cell population evaluated seems to work cooperatively. In fact, $CD4^+$ T cells are critical for the function of $CD8^+$ T cells. Besides, $CD19^+$ cells can also contribute to the activation o $CD8^+$ T cells, an important antitumoral defense. In this scenario, SD promoted significant modifications (reduced frequency of $CD4^+$, $CD8^+$ or $CD19^+$ cells and increased frequency of $CD49b^+$ cells) in the frequency of these cells subsets, altering their delicate functional balance.

As mentioned above, SD concomitantly with EAT inoculation did not induce significant modifications in the tumor growth either 10 or 15 days after its inoculation in female mice. However, this protocol of SD shortened the life span

of these animals. Besides, it was also verified that female mice bearing EAT displayed a significant enhancement of paradoxical sleep time. In this way, it could be proposed that this increment in paradoxical sleep stage is critical for female's antitumoral response. In parallel, Silva and colleagues (2004) have reported that the same sleep deprivation method applied in the present study deprived animals from slow-wave sleep in a magnitude of about 80% and almost suppresses paradoxical sleep with a reduction of 96.4%. In this context, this deprivation of paradoxical sleep could have significantly contributed to impairment in female host's defense and led to the anticipation of EAT-induced females' death. Supporting the idea that paradoxical sleep is critical for female, Paul and colleagues (2006) have reported that female mice spent more time awake during 24h baseline recording at the expense of non-rapid eye movement sleep, preserving the time spent in paradoxical sleep.

In conclusion, our results indicate that SD when applied immediately after EAT inoculation did not modify tumor growth either 10 or 15 days after tumor inoculation. Importantly, SD induced a curtailment in the life span of female mice that could be attributed, at least in part, immunological deficits induced by a suppression in the paradoxical sleep of these animals.

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# 5. CONCLUSÕES

## *5.1 Conclusões Específicas*

1. A privação de sono por 72 horas previamente à inoculação do tumor ascítico de Ehrlich potencializou com a mesma magnitude o crescimento tumoral tanto em camundongos machos quanto em fêmeas;

2. Em camundongos machos, a privação de sono por 72 horas concomitantemente ao crescimento do tumor ascítico de Ehrlich (ou seja, imediatamente após a inoculação do tumor), não modificou o crescimento desse tumor 10 dias após a sua inoculação, mas o potencializou aos15 dias;

3. Em camundongos fêmeas, a privação de sono por 72 horas concomitantemente ao crescimento do tumor ascítico de Ehrlich (ou seja, imediatamente após a inoculação do tumor), não modificou o crescimento desse tumor quer 10 ou 15 dias após a sua inoculação. Contudo, tal protocolo de privação de sono promoveu uma diminuição na sobrevida desses animais;

4. A privação de sono *per se*, independentemente do momento de sua ocorrência (imediatamente antes ou após a inoculação tumoral), promove uma diminuição nas populações esplênicas das células T $CD4^+$, $CD8^+$ e $CD19^+$ (linfócito B) e um aumento na população esplênica das células $CD49b^+$ (células NK) em camundongos machos. Por outro lado, o tumor ascítico de Ehrlich *per se* diminui as populações esplênicas das células T $CD4^+$ e $CD49b^+$. Finalmente, a privação de sono (independente do momento) potencializou a diminuição das populações esplênicas das células T $CD4^+$, $CD8^+$ e $CD19^+$ nos animais portadores do tumor;

5. A privação de sono *per se*, independentemente do momento de sua ocorrência (imediatamente antes ou após a inoculação tumoral), promove uma diminuição nas populações esplênicas das células T $CD8^+$ e $CD19^+$ (linfócito B) e um aumento na população de células $CD49b^+$ (células NK) em camundongos fêmeas. Por outro lado, o tumor ascítico de Ehrlich *per se* diminuiu a população esplênica das células T $CD4^+$. Finalmente, a privação de sono (independente do momento) potencializou a diminuição das populações esplênicas das células T $CD4^+$, $CD8^+$ e $CD19^+$ nos animais portadores do tumor;

6. A inoculação do tumor ascítico de Ehrlich promoveu um aumento do tempo total de sono e do sono de ondas lentas, sem modificar o tempo de sono paradoxal nos camundongos machos. Por outro lado, a inoculação desse tumor promoveu um aumento no tempo de sono paradoxal nos camundongos fêmeas.

### *5.2 Conclusões Gerais*

Tomados em conjunto, nossos resultados sugerem que a privação de sono, quando realizada imediatamente antes da inoculação tumoral, potencializa o crescimento do tumor ascítico de Ehrlich tanto em camundongos machos quanto em fêmeas e que tal efeito está intimamente relacionado a uma potencialização induzida pela privação de sono dos prejuízos imunológicos promovidos pelo tumor. Ainda, quando a privação de sono é realizada imediatemente após a inoculação, o efeito potencializador induzido pela privação de sono sobre o crescimento tumoral ocorre de forma lentificada nos camundongos machos. Já em camundongos fêmeas, a privação de sono não

modifica o crescimento do tumor ascítico de Ehrlich, mas diminui a longevidade desses animais.

Finalmente, nossos resultados revelam que o desenvolvimento do tumor ascítico de Ehrlich potencializa de forma específica em camundongos machos ou fêmeas a necessidade de sono, caracterizando a interação bidirecional entre o sono e o câncer.

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São Paulo, 15 de agosto de 2008.
**CEP 1128/08**

Ilmo(a). Sr(a).
Pesquisador(a) JUSSARA MARIA RAGONEZZI MARAGNO CORREA
Co-Investigadores: Roberto Frussa Filho; Monica Levy Andersen; Francieli Ruiz da Silva; Adriano Zager
Disciplina/Departamento: Farmacologia da Universidade Federal de São Paulo/Hospital São Paulo
Patrocinador: CAPES/AFIP/CNPq (edital universal).

## PARECER DO COMITÊ DE ÉTICA INSTITUCIONAL

Ref: Projeto de pesquisa intitulado: **"Efeitos da privação do sono paradoxal sobre o desenvolvimento do tumor de Ehrlich e sobrevida em camundongos portadores dessa neoplasia".**

CARACTERÍSTICA PRINCIPAL DO ESTUDO: Estudo experimental crônico em camundongos.
RISCOS ADICIONAIS PARA O PACIENTE: Não se aplica.
OBJETIVOS: Verificar os efeitos da privação de sono paradoxal sobre o crescimento do tumor de Ehrlich e a sobrevida dos animais portadores dessa neoplasia.
RESUMO: Serão utilizados camundongos BALB/c machos e fêmeas adultos que serão submetidos à privação de sono paradoxal por 72 horas (realizada por meio do método das plataformas múltiplas) ou ao procedimento de controle dessa situação (permanência nas gaiolas moradia) antes ou após inoculação do tumor de Ehrlich. O crescimento tumoral será avaliado pela contagem do número total de células presentes na cavidade peritoneal e a sobrevida dos animais será determinada , em dias, a partir da inoculação do tumor até o óbito de cada animal.
FUNDAMENTOS E RACIONAL: O conhecimento do comportamento do tumor de Ehrlich experimental nos animais receptores tem possibilitado sua utilização, com precisão, como modelo para o estudo de diversas situações.
MATERIAL E MÉTODO: Descritos os procedimentos experimentais de domínio da equipe.
DETALHAMENTO FINANCEIRO: CAPES, AFIP, CNPq(Edital Universal).
CRONOGRAMA: 24 meses.
OBJETIVO ACADÊMICO: Mestrado.
ENTREGA DE RELATÓRIOS PARCIAIS AO CEP PREVISTOS PARA: **10/8/2009** e **10/8/2010**.

O Comitê de Ética em Pesquisa da Universidade Federal de São Paulo/Hospital São Paulo **ANALISOU** e **APROVOU** o projeto de pesquisa referenciado.

1. Comunicar toda e qualquer alteração do projeto.
2. Comunicar imediatamente ao Comitê qualquer evento adverso ocorrido durante o desenvolvimento do estudo.


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3. Os dados individuais de todas as etapas da pesquisa devem ser mantidos em local seguro por 5 anos para possível auditoria dos órgãos competentes.

Atenciosamente,

**Prof. Dr. José Osmar Medina Pestana**
Coordenador do Comitê de Ética em Pesquisa da
Universidade Federal de São Paulo/ Hospital São Paulo