UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

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"AÇÃO DA MELATONINA SOBRE OS RECEPTORES ESTERÓIDES SEXUAIS NO OVÁRIO, OVIDUTO E ÚTERO E O ESTRESSE OXIDATIVO NOS OVÁRIOS DE RATAS ADULTAS UChB (CONSUMIDORAS VOLUNTÁRIAS DE ETANOL A 10%) DURANTE A OVULAÇÃO"

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) *Luiz Gustavo de Almeida Chuffa*

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Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Celular e Estrutural, na área de Biologia Celular.

Orientador: Prof. Dr. Francisco Eduardo Martinez

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"É preciso a certeza de que tudo vai mudar; É necessário abrir os olhos e perceber que as coisas boas estão dentro de nós: onde os sentimentos não precisam de motivos nem os desejos de razão. O importante é aproveitar o momento e aprender sua duração; Pois a vida está nos olhos de quem sabe ver...Se não houve frutos, valeu a beleza das flores. Se não houve flores, valeu a sombra das folhas. Se não houve folhas, valeu a intenção da semente"

Henfil

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À minha família, por todo o incentivo e carinho incondicional, e pela dedicação absoluta apoiada em gestos capazes de perdurar em uma memória imortalizada.

Ao meu grandioso herói do passado, presente e futuro que conduz meus passos e faz deles motivo para continuar.....Com Eterno Orgulho.....**MEU PAI**
(*In memorian*).

E a aqueles que nem sabem o quanto representaram...

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"Erros são, no final das contas, fundamentos de verdade. Se um homem não sabe o que uma coisa é, já é um avanço do conhecimento saber o que ela não é".

Carl Jung

"Pouco conhecimento faz com que as pessoas se sintam orgulhosas. Muito conhecimento, que se sintam humildes. É assim que as espigas sem grãos erguem desdenhosamente a cabeça para o Céu, enquanto que as cheias as baixam para a terra, sua mãe".

Leonardo Da Vinci

"Quero dizer agora o oposto do que eu disse antes. Eu prefiro ser essa metamorfose ambulante. Do que ter aquela velha opinião formada sobre tudo..."

Raul Seixas

RESUMO

O alcoolismo crônico está associado a distúrbios no sistema reprodutor feminino como disfunção hormonal, alteração na expressão dos receptores esteróides, produção de espécies reativas de oxigênio (ERO), entre outros. A melatonina, hormônio secretado pela glândula pineal, possui função moduladora no ciclo reprodutivo e têm papel importante no combate as ERO. Os estudos envolvendo o alcoolismo crônico e sua interação com a melatonina, em fêmeas, são ainda inconclusivos. O presente trabalho tem como objetivo investigar os efeitos da administração exógena da melatonina sobre os hormônios sexuais, os receptores esteróides sexuais (AR, ER-α, ER-β, PRA e PRB) no ovário, oviduto e útero, além do perfil nutricional e o estresse oxidativo nos ovários de ratas adultas UChB (consumidoras voluntárias de etanol a 10%). Foram utilizadas 60 ratas UChB, distribuídas nos seguintes grupos: UChB Co: sem acesso ao etanol; UChB EtOH: consumo diário de 4 - 5 g etanol/100g de peso corpóreo (PC), ambos recebendo solução veículo. Concomitantemente, os grupos UChB Co+M e UChB EtOH+M receberam injeções diárias de melatonina (100μg/100g PC) via i.p, a partir dos 90 dias de idade, durante 60 dias consecutivos. Aos 150 dias de idade, os animais foram eutanasiados em estro (4a.m) e os materiais coletados e processados. A melatonina aumentou os níveis de progesterona, 6-sulfatoximelatonina e reduziu 17β-estradiol, enquanto a combinação entre etanol + melatonina causou uma queda significativa nesses hormônios. Apesar do receptor androgênico (AR) ovariano não ter sido influenciado pela melatonina, os grupos UChB EtOH e UChB EtOH+M mostraram uma diminuição no AR do oviduto. Ambos os receptores de estrogênio (ER-α e ER-β) no oviduto foram pouco expressos em animais recebendo etanol ou melatonina enquanto somente o ER-β uterino foi reduzido. Por outro lado, receptores de progesterona (PRA e PRB) foram positivamente regulados no ovário por etanol ou etanol + melatonina, enquanto PRA foi negativamente regulado no útero e oviduto, exceto quando o etanol e melatonina foram combinados. Os níveis do receptor de melatonina (MT1R) foram maiores no ovário e útero de ratas tratadas com melatonina, independentemente do consumo de etanol. O peso corpóreo dos animais foi reduzido após interação do etanol e melatonina após 40 dias de tratamento. Em ambos os grupos tratados com melatonina, observou-se redução no consumo energético e líquido. Houve diminuição da quantidade de etanol consumida durante o tratamento e o ciclo estral foi maior em ratas que receberam etanol e melatonina, evidenciado por diestro prolongado. Os níveis de hidroperóxido de lipídio foram maiores nos ovários de ratas UChB EtOH e diminuiu após o tratamento com melatonina. Atividades antioxidantes da superóxido dismutase, glutationa peroxidase e glutationa redutase foram aumentadas nos grupos tratados com melatonina. Conclui-se que a melatonina tem efeito oposto ao etanol sobre os hormônios sexuais. Melatonina e etanol regulam diferencialmente os receptores de esteróides sexuais nos tecidos reprodutivos, atuando principalmente através de seu receptor MT1R. Além disso, a melatonina é capaz de alterar a eficiência alimentar, o ciclo estral, e, contudo, protege os ovários contra o estresse oxidativo resultante do consumo de etanol.

Palavras-chave: melatonina, etanol, lipoperoxidação, receptores de esteróides, ovário.

ABSTRACT

Chronic ethanol intake is associated with female reproductive disturbances including hormonal dysfunction, changes in the steroid receptors expression, production of reactive oxygen species (ROS), among others. Melatonin, an indolamine secreted by pineal gland, plays key roles in the reproductive cycle, besides having an important function in scavenging ROS. Studies focusing chronic alcoholism and its interaction with melatonin, in females, are still inconclusive. This study aims to investigate the effects of exogenous melatonin administration on sex hormones, sex steroid receptors (AR, ER-α, ER-β, PRA and PRB) in the ovary, oviduct and uterus, as well as the nutritional profile and oxidative stress in the ovaries of adult UChB rats (10% (v/v) ethanol voluntary intake). 60 UChB female rats were divided into the following groups: UChB Co: without access to ethanol (used as control); UChB EtOH: drinking daily ethanol at 4 - 5 g ethanol/100g body weight (BW), both receiving vehicle solution. Concomitantly, UChB Co + M and UChB EtOH + M groups received daily injections of melatonin (100μg/100g BW) via i.p, starting from 90 days old and during the next 60 consecutive days. At 150 days of age, all animals were euthanized in estrus (4a.m). Melatonin increased progesterone, 6-sulfatoximelatonin and decreased 17β-estradiol, while the ethanol+melatonin combination caused a significant fall in these hormones. Despite androgen receptor (AR) in ovary has not been influenced by melatonin, ethanol and ethanol+melatonin led to a decrease in oviduct AR. Both estrogen receptors (ER-α and ER-β) were underexpressed by either ethanol or melatonin in oviduct and only uterine ER-β was downregulated. Conversely, progesterone receptors (PRA and PRB) were positively regulated in the ovary by ethanol or ethanol+melatonin, whereas PRA was downregulated in uterus and oviduct, except when ethanol+melatonin were combined. Additionally, melatonin receptor (MT1R) was increased in ovary and uterus of melatonin-treated rats, regardless of ethanol consumption. Body weight gain was reduced with ethanol plus melatonin after 40 days of treatment. In both melatonin-treated groups, it was observed a reduction in food-derived calories and liquid intake toward the end of treatment. The amount of consumed ethanol dropped during the treatment. Estrous cycle was longer in rats that received both ethanol and melatonin, with prolonged diestrus. Following to oxidative status, lipid hydroperoxide levels were higher in the ovaries of ethanol-preferring rats and decreased after melatonin treatment. Additionally, antioxidant activities of superoxide dismutase, glutathione peroxidase and glutathione reductase were increased in melatonin-treated groups. We conclude that melatonin has opposite effect on sex hormones to those of ethanol consumption. Together, melatonin and ethanol differentially regulates the sex steroid receptors in the reproductive tissues, mostly acting “in situ” through its MT1R receptor. Finally, melatonin is able to affect feed efficiency and, conversely, it protects the ovaries against the oxidative stress arising from ethanol consumption.

Key-words: melatonin, ethanol, lipid hydroperoxide, steroid receptors, ovary

LISTA DE ABREVIATURAS E SIGLAS

LHP: Hidroperóxido de Lipídio

SOD: Superóxido Dismutase

GSH: Glutationa Reduzida

GSH-Rd: Glutationa Reductase

GSH-Px: Glutationa Peroxidase

CAT: Catalase

SAT: Substâncias Antioxidantes Totais

ROS: Espécies de Oxigênio Reativas

NSQ: Núcleo supraquiasmático

6-STM: 6-sulfatoximelatonina

FSH: Hormônio Folículo Estimulante

LH: Hormônio Luteinizante

LHRH: Hormônio Liberador de LH

GnRH: Hormônio Liberador de Gonadotrofina

E2: 17β-estradiol

P4: Progesterona

AR: Receptor de Andrógeno

ER: Receptor de Estrógeno

ER-α: Receptor de Estrógeno (isoforma α)

ER-β: Receptor de Estrógeno (isoforma β)

PR: Receptor de Progesterona

PRA: Receptor de Progesterona (isoforma A)

PRB: Receptor de Progesterona (isoforma B)

MT1R: Receptor de Melatonina

ÍNDICE

1. INTRODUÇÃO GERAL

O alcoolismo pode ser conceituado como uma síndrome multifatorial que compromete os aspectos físico, mental e social do indivíduo (Edwards & Gross, 1976; Hingson & Zha, 2009; Spanagel, 2009). Atualmente é considerado um problema de ordem da saúde pública, sendo pertencente ao grupo das principais doenças oriundas dos transtornos mentais (Wojnar et al., 1997), ao lado da esquizofrenia e distúrbio bipolar (Figueira et al., 1999). Embora a prevalência do consumo de álcool seja significativamente menor entre as mulheres (Grant, 1997), a dependência precoce do álcool tem-se mostrado semelhante entre homens e mulheres, uma vez que o impacto do álcool no organismo feminino acarreta rápida progressão das doenças decorrentes do seu uso (Frezza et al., 1990; Urbano-Marquez et al., 1995).

Na mulher, o consumo abusivo e crônico de álcool está associado a diversas alterações do ciclo reprodutivo, desde a ocorrência de amenorréia, disfunções ovarianas com ciclos anovulatórios, menopausa prematura, metrorragia, além de relatos de maior risco para infertilidade, abortamento espontâneo, além de trazer prejuízos para o desenvolvimento fetal (Becker et al., 1989; Mello et al., 1989; Carrara et al., 1993; Henderson et al., 2007).

Em um trabalho de revisão, Li et al., 1987, menciona a existência de três pares de linhagens de ratos de consumo baixo e elevado de álcool. Os UChA e UChB (UCh= Universidade do Chile), os mais antigos de Helsinki, iniciados por Kalervo Eriksson e os de Lumeng & Li em Indianápolis. Existem atualmente linhagens mantidas através de *outbreeding* (cruzamento não consangüíneo) que fazem consumo preferencial de etanol a 10%, o WHP e WLP - elevado e baixo consumo de etanol (Dyr & Kostowski, 2008). Dos três pares, os únicos que se têm mantido permanentemente mediante *inbreeding* são os UChA e B, e por conseguinte,

são as únicas linhagens de ratos de que se têm conhecimento, podendo ser consideradas linhagens puras para estudos relacionando aspectos genéticos, bioquímicos, fisiológicos, nutricionais e farmacológicos, bem como apetite e tolerância ao álcool, que são importantes fatores do alcoolismo humano. Portanto, os ratos da linhagem UChA e UChB, embora exibam diferenças na preferência ao álcool, são importantes para o estudo de características únicas ligadas ao alcoolismo humano, além da rápida reprodução e o restrito espaço físico ocupado.

A melatonina, hormônio secretado pela glândula pineal durante a fase de escuro do ciclo circadiano, está relacionada com inúmeros processos controladores na fisiologia corpórea, incluindo a digestão e ingestão de líquidos e alimentos, regimento das etapas do sono (bastante desregulado nos indivíduos alcoólicos), o processo inflamatório, mecanismos promotores de situações de estresse, o potencial antioxidante, as atividades do ciclo reprodutivo, entre outros.

Os distúrbios do ritmo circadiano (Danel et al., 2001), devem estar associados, de certa forma, às desordens mentais secundárias que predispõem ao consumo de etanol. O consumo alcoólico, de forma crônica e aguda, parece inibir a secreção de melatonina pela glândula pineal (Danel & Touitou, 2004, 2006; Peres et al., 2011). O etanol pode suprimir indiretamente a função da glândula pineal reduzindo a liberação do neurotransmissor norepinefrina. O bloqueio desse hormônio, deixa de ativar AMPcíclico e N-acetyltransferase, mediadores básicos da síntese de melatonina (Gottesfeld et al., 1996; Schmitz et al., 1996). Por outro lado, indivíduos alcoólicos em período de abstinência, tendem a elevar os níveis de norepinefrina, sem resultar, no entanto, em aumento de secreção de melatonina (Moss et al., 1986). Além disso, o etanol, por sua natureza lipofílica, também interfere diretamente na síntese de melatonina pelos pinealócitos (Schmitz et al., 1996).

O padrão reprodutivo nas espécies está diretamente relacionado ao fotoperíodo, mediante secreção diária e sazonal de melatonina (Barrell et al., 2000). Em humanos, há evidências de que a glândula pineal esteja envolvida na maturação sexual e no ciclo menstrual (Silman, 1991). Em outros mamíferos, a melatonina atua como um cronohormônio modulador da função reprodutiva, devido à habilidade de promover a ciclicidade estral e estimular a atividade das gônadas (Ocal-Irez et al., 1989). Atua influenciando a foliculogênese, a ovulação, a histologia e fisiologia uterina (Zhao et al., 2000). Alterações no ciclo estral e, consequentemente, no útero de ratas são comuns em animais desprovidos de glândula pineal (Teixeira, 1998). Nakamura et al. (2003) evidenciaram, em humanos, folículos pre-ovulatórios contendo elevadas quantidades de melatonina, demonstrando efeito indireto na produção de progesterona. Em ratas desprovidas de pineal houve aumento na duração da fase de estro, diminuição da produção de LH/FSH e 6-sulfatoximelatonina na urina (metabólito da melatonina) (Dardes et al, 2000). Soares Jr. et al. (2003) relacionam o efeito de baixos níveis séricos de melatonina favorecendo aumento de atresia folicular e estradiol, e redução da progesterona e seus receptores. Em contrapartida, a menopausa pode estar associada com redução de melatonina circulante, devido às mudanças nos níveis de gonadotrofinas (Reiter, 1998).

Embora na literatura científica especializada, a melatonina seja relatada como potencializadora da função antigonadotrófica entre as espécies animais, alguns autores não encontraram correlação entre a melatonina e o estradiol na fase folicular (Graham et al., 2001; Luboshitsky et al., 2001). Os receptores de estrógeno (ER) e progesterona (PR) quando ativados, regulam processos fisiológicos fundamentais no útero, tubas uterinas e ovários. O ERβ foi recentemente isolado em ratos (Kuiper et al., 1996) e humanos (Mosselman et al., 1996), e apresenta homologia estrutural com o receptor ERα. Os receptores de progesterona (PR) possuem

as isoformas PRA e PRB (Savouret et al., 1990), ambos codificados pelo mesmo gene (Kastner et al., 1990). Nas células da camada granulosa dos folículos ovarianos, o PRA e PRB são essenciais na foliculogênese e somente PRA atua na ovulação (Mulac-Jericevic et al., 2000; 2003). A melatonina, *in vivo*, pode estimular secreção de progesterona em rata (Fiske et al., 1984), e na mulher (Brzezinski et al., 1992).

A literatura relata que a melatonina pode atuar como um potente agente antioxidante, regulando vários eventos fisiológicos em mamíferos (Okatani et al., 2003; Carrillovico et al., 2004). De acordo com vários autores, a melatonina pode prevenir danos ao fígado provenientes de agentes oxidantes, além de inibir o crescimento de hepatocarcinomas (Gong et al., 2003; Blask et al., 2004). Muitas pesquisas enfatizam a propriedade antioxidante da melatonina, de acordo com o caráter atenuador na peroxidação lipídica, em modelos experimentais alcoólicos (Genç et al., 1998; El-Sokkary et al., 1999).

1.1. ACHADOS PRÉVIOS E SIGNIFICÂNCIA

1.1.1. Síntese e metabolismo da melatonina

O ajuste entre o ambiente, o relógio biológico interno e a produção de melatonina é feito através do sistema nervoso central. Nos mamíferos, a principal influência externa é a alternância do ciclo claro-escuro, sendo a melatonina produzida durante a noite, enquanto na presença de luz ocorre inibição da sua síntese (Hardeland et al., 2006). O feixe de luz é captado por fotorreceptores na retina (cones e bastonetes) e sua informação chega ao NSQ via trato retino-hipotalâmico. A melanopsina, molécula fotorreceptora encontrada no gânglio neural, tem auxiliado no entendimento da percepção luminosa e geração de ritmos biológicos (Rollag et al.,

2003). O NSQ durante a fase de escuro transmite informações ao núcleo paraventricular, e este enviam projeções ao núcleo intermédio lateral da medula torácica alta (Vessely et al., 2002). As fibras pré-ganglionares simpáticas fazem sinapse no gânglio cervical superior e através do plexo carótico, associado à artéria carótida interna, chegam até a pineal inervando-a (Ariens-Kappers, 1960). Nos pinealócitos, os neurotransmissores noradrenalina e ATP atuam em receptores noradrenérgicos α e β, resultando na ativação de adenilato ciclase e aumento de AMPc, e purinérgicos P2Y1, respectivamente (Mortani Barbosa et al., 2000; Ferreira & Markus, 2001), ambos controlando a síntese de NAT (N-acetiltransferase) enzima limitante na conversão de serotonina. A glândula pineal utiliza o aminoácido triptofano (Sumaya et al., 2004) para conversão em serotonina (5- hidroxitriptamina), a qual permanece elevada durante o dia. Durante o escuro, a enzima NAT metaboliza serotonina em N-acetilserotonina, e parte produzida é transformada em melatonina pela ação da HIOMT (hidroxi-indol-O-metil-transferase), dependente de fotoperíodo (Simonneaux & Ribelayga, 2003). Portanto, a melatonina, molécula lipossolúvel, é lançada na circulação à noite.

Atualmente estão descritos três sítios para ligação da melatonina, os receptores MT1 e MT2 ligados a proteína G, e a enzima quinona redutase 2 (QR2/*Mt3*) envolvida no processo de detoxificação (Witt-Enderly et al., 2003). Ambos receptores MT1 e MT2 estão envolvidos na modulação do sistema nervoso central (Hardeland et al., 2006) e tecidos periféricos, essenciais no controle do ritmo circadiano. O ritmo da produção de melatonina em animais, incluindo humanos, diminui com a idade (Touitou & Haus, 2000; Zhao et al., 2002). Em idosos, a melatonina proporciona redução da latência para o sono inicial e aumenta eficiência total do sono, sem interferência durante o sono REM (Zhdanova et al., 2001). Estudo recente mostrou que

o efeito estimulante de agonistas β-adrenérgicos sobre a pineal de ratos adultos é mais eficiente na produção de melatonina em relação aos senis (Nairi-Skandrani et al., 2004).

Existem inúmeros distúrbios do ciclo circadiano em humanos, como os transtornos do sono (dissonias e parassonias), depressão sazonal, tumores hipotalâmicos, e aqueles associados à cegueira, viagens transmeridional (*Jet lag*), troca de turno no trabalho, etc. (Jagota, 2005). A melatonina, devido sua atividade cronobiótica, tem sido utilizada no tratamento destes distúrbios. A melatonina possui uma meia vida de aproximadamente 4-6 horas após administração, sendo convertida em 6- hidroximelatonina pela citocromo P450 1A2 no fígado e, posteriormente conjugada com ácido glicurônico ou sulfato gerando 6-sulfatoximelatonina, sendo este último um marcador eficaz para atividade da melatonina (Arendt, 2006).

1.1.2. Etanol e melatonina: ação sobre os hormônios e receptores esteróides sexuais

O alcoolismo é desenfreado na sociedade moderna e seu consumo crônico está associado com várias disfunções no trato reprodutivo feminino (Carrara et al., 1993; Henderson et al., 2007). Apesar dos efeitos negativos que o etanol exerce no trato reprodutivo feminino, seu complexo mecanismo de ação sobre os hormônios sexuais e receptores esteróides são pouco discutidos e permanece uma questão insolúvel.

Nosso grupo de pesquisa mostrou previamente que a ingestão crônica de álcool promove alterações do ciclo estral, desequilíbrio hormonal, aumento da formação de ROS e alteração da morfologia ovariana em ratos alcoolistas UChB (Chuffa et al., 2009, 2011). Estudos com ratos (Hiney et al., 2003; Dees et al., 2005) têm fornecido evidências na qual o etanol induz supressão da secreção de LH devido sua ação no hipotálamo (Figura 1). Além disso, o etanol parece

aumentar 17β-estradiol (E2) quando consumido de forma crônica, mas não aguda, enquanto a progesterona (P4) não varia (Emanuele et al., 2001). Mais diretamente, o consumo de etanol pode afetar a liberação de gonadotrofinas de forma dose e tempo-dependente (Figura 1), no entanto, o seu papel deletério envolvendo os tecidos reprodutivos é ainda controverso. Os ratos UChB são considerados um modelo especial para a compreensão das características ligadas ao alcoolismo, como aquelas observadas em doenças humanas.

A melatonina (N-acetil-5-methoxytryptamine) é uma indolamina produzida pela glândula pineal e secretada de maneira circadiana durante a noite (Masana & Dubocovich, 2001) e tem sido empregada como um agente terapêutico em várias doenças. Contrariamente, o consumo de álcool modula negativamente a síntese de melatonina diária (Peres et al., 2011). Nos mamíferos, a melatonina controla a função reprodutiva através da ativação de receptores dentro do eixo hipotálamo-hipófise-gonadal (Reiter et al., 1982; Malpaux et al., 2001; Figura 1). A melatonina também interage com os hormônios sexuais e parece inibir a esteroidogênese através de mudanças diretas nos níveis de AMP cíclico em células da teca, mas não da granulosa dos folículos ovarianos (Tamura et al., 1998). Nakamura et al. (2003) demonstraram que folículos pré-ovulatórios contem elevados níveis de melatonina, sugerindo um efeito indireto sobre a síntese de E2 e P4. Também foi proposto que a melatonina sérica baixa está ligada ao aumento de E2, bem como redução de P4 e seus receptores (Soares et al., 2003). Recentemente, Adriaens et al. (2006) demonstraram que a melatonina aumenta a produção de P4 e andrógeno nos folículos pré-antrais em camundongos. Até o momento, não há relatos demonstrando os efeitos da ingestão crônica de álcool associados à melatonina exógena sobre os hormônios sexuais durante o processo ovulatório.

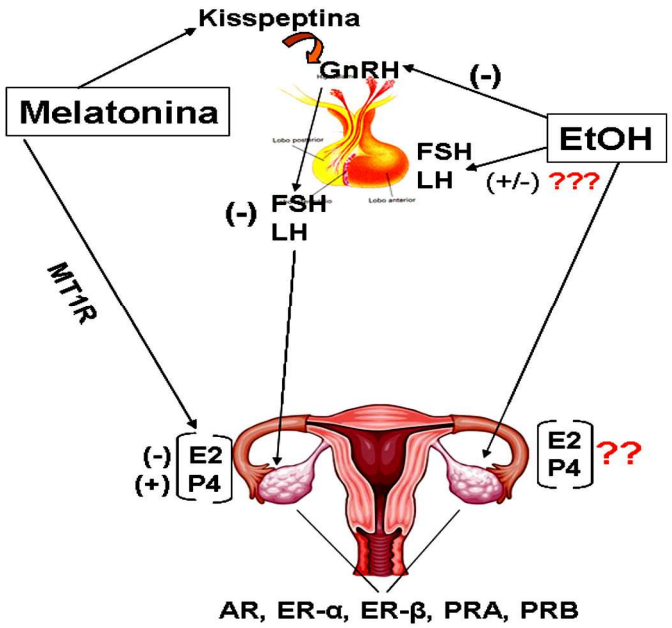

Figura 1. Esquema dos efeitos direto (ovários) e indireto (hipotálamo e hipófise) do etanol e da melatonina, alterando a expressão dos receptores esteróides sexuais. *Ilustração elaborada pelo autor*.

O E2 e a P4 quando ligados em receptores específicos sinalizam uma série de respostas fisiológicas na reprodução. O receptor de estrógeno (ER) pertence à superfamília de receptores nucleares e possui duas isoformas distintas funcionais designadas como ER-α e ER-β (Kuiper et al., 1996). As células da granulosa dos folículos ovarianos expressam maior nível de ER-β que ER-α, enquanto que ER-β é expresso em níveis menores no útero (Okada et al., 2005). A melatonina tem sido apontada como indutora de regulação negativa do ER-α no ovário e útero (Chuffa et al., 2011; Romeu et al., 2011). Por outro lado, o consumo de etanol parece aumentar a expressão tecidual de ER-α, dependendo da quantidade consumida (Wang et al., 2010), que por sua vez, contribui para os efeitos nocivos.

Os receptores de progesterona (PR), uma proteína bem caracterizada regulada pelos estrógenos, é expressa como isoformas PRA e PRB (Kraus et al., 1994). PRA apresenta um

domínio de transativação essencial para o processo de ovulação (Mulac-Jericevic et al., 2003), e funciona como um repressor do PRB e do receptor de andrógeno (Horne et al., 2009). Embora tenha sido sugerido que E2 supra-regula PR, pouco se sabe a respeito da expressão de PRA e PRB durante o consumo de etanol ou administração de melatonina. Foi recentemente descrito que a melatonina aumenta significativamente P4, bem como os níveis de PR ovarianos em proestro (Romeu et al., 2011), enquanto o consumo de etanol parece alterar os níveis de PR indiretamente através da ativação de E2 ou ER (Ma et al., 2006). Porém, os efeitos da melatonina e ingestão de etanol ainda não foram definidos em relação aos receptores esteróides sexuais específicos no ovário, oviduto e útero.

A melatonina sinaliza através de receptores acoplados à proteína G, o MTR1 e MTR2, ou via citoplasmática/nuclear (Benitez-King, 2006). Entre outras ações, MTR1 quando ligado a melatonina causa regulação de ambos ER-α e ER-α mRNA (Molis et al., 1994) e, alternativamente, pode inibir a ligação do complexo E2-ER aos elementos responsivos de estrógeno (ERE) no DNA (Molis et al., 1994; Rato et al., 1999). Sabendo que a melatonina é um potencial agente envolvido no controle da reprodução, o seu efeito(s) associado com etanol ainda não foi identificado através de receptores MT1R.

1.1.3. Sono, alcoolismo, abstinência e a melatonina

O álcool possui propriedade indutora do sono, e quando consumido de forma aguda apresenta tendência de encurtar a latência para o sono inicial, a eficiência total do sono, o sono de ondas lentas (ondas delta) e o sono REM (Castaneda et al., 1998). O álcool parece inibir o sono REM de maneira dose-dependente (Lobo & Tufik, 1997). O álcool desregula o centro neural circadiano durante o desenvolvimento do sistema nervoso central (SNC) (Earnest et al., 2001).

Ratos expostos ao álcool durante o desenvolvimento do SNC exibiram padrões moleculares alterados do ritmo circadiano no adulto (Allen et al., 2004; Farnell et al., 2004). O álcool estimula produção de melatonina diurna (Fonzi et al., 1994), entretanto Danel & Touitou (2006) não encontraram relação entre altas doses de etanol e secreção diária de melatonina em indivíduos saudáveis. Durante a noite, o aumento gradativo da ingestão de etanol reduz inversamente a concentração de 6-sulfatoximelatonina (Stevens et al., 2000) e a produção de melatonina (Rupp et al., 2007). Rosenwasser et al. (2005) sugerem que o consumo crônico de etanol promove dessincronização do ritmo circadiano, onde a elevação dos níveis de melatonina durante o dia (Danel et al., 2006) seja conseqüência de outro sistema hormonal. Os distúrbios do sono, como insônia, em pacientes etilistas crônicos, são decorrentes do atraso da secreção de melatonina (Kuhlwein et al., 2003). O álcool *per se* não aumenta o sono fisiológico, porém quando consumido em doses moderadas tende a modular as fases do sono devido outras influencias circadianas (Rupp et al., 2007).

Os sintomas da abstinência do álcool podem ser confundidos com os de ataque do pânico. Estudos com pacientes etilistas evidenciaram perda do ritmo de secreção de melatonina durante abstinência aguda (Fonzi et al., 1994), observação também confirmada em dois pacientes apresentando quadro de delírio (Mukai et al., 1998). Em alguns pacientes, a abstinência ajustou o ritmo da produção de melatonina, enquanto em outros não houve êxito devido à desorganização temporal circadiana em estruturas relacionadas ao ciclo (Danel et al., 2009).

1.1.4. Fisiologia do apetite: aspectos relacionados ao etanol e melatonina

O etanol é um macro nutriente com densidade energética de aproximadamente 29 kJ (7 Kcal/g), porém não é considerado fonte energética para os seres humanos (Kokavec & Crowe,

2002), uma vez que o piruvato não pode ser formado a partir do etanol. Vários processos bioquímicos mostram que o etanol pode influenciar o balanço energético através de mecanismos que regulam o apetite (Kokavec & Crowe, 2003, 2006). O Neuropeptídeo Y (NPY), sintetizado pelo núcleo arqueado do hipotálamo, além de induzir comportamento alimentar aumentando preferência por carboidratos, possui a função de estimular consumo de etanol e, receptores NPY Y5 quando ativados promovem preferência e consumo voluntário de etanol em roedores (Kelley et al., 2001; Schroeder et al., 2005). O consumo de etanol e sua resistência estão inversamente relacionados com níveis de NPY no cérebro (Badia-Elder et al., 2003; Thiele & Badia-Elder, 2003). Evidências recentes sugerem que exposição crônica ao etanol pode levar a redução nos níveis protéicos de NPY (Roy & Pandey, 2002) e consecutiva supressão gênica pelo núcleo arqueado (Kinoshita et al., 2000). A energia ingerida como caloria oriunda do etanol contribui para aumentar consumo alimentar (Yeomans & Phillips, 2002), modulando os estímulos hormonais relacionados com apetite (Hetherington et al., 2001; Caton et al., 2004). O etanol estimula rede neurais relacionadas com sensibilidade do doce (Lemon et al., 2004), mostrando que essas áreas estão associadas com sistemas de reforço e recompensa. Existe evidências que apontam a dopamina (McQuade et al., 2003), secretada pelo núcleo *accumbens*, exercendo papel inclusive em roedores consumidores voluntários de etanol (Doyon et al., 2003) e os opióides (Herz, 1997) como sendo mediadores de consumo e recompensa pelo etanol. Em humanos, o consumo elevado de etanol possui efeito parcial sobre consumo alimentar, estimulando apetite e atrasando a liberação dos mediadores de saciedade (Caton et al., 2004), além de sua energia resultante não ser incorporada à dieta.

Existem algumas abordagens experimentais envolvendo a ação da melatonina sobre os parâmetros nutricionais, tais como: alterando a eficiência alimentar, o ganho de massa corpóreo,

o índice de adiposidade e ambos os gastos e consumo energéticos (Mustonen et al., 2002; Korkmaz et al., 2009). Esses efeitos são controversos e parece estar ligados especificamente a dieta, idade e gênero em questão, entretanto, nenhum estudo avaliou os efeitos associados ao consumo crônico de etanol. A melatonina utilizada de forma progressiva (3 a 15 pg/mL), durante a noite, promove aumento do consumo alimentar e ganho de massa corporal em ratos machos e fêmeas (Angers et al., 2003). Scalera et al. (2008) demonstraram redução no consumo alimentar e aumento dos níveis de leptina em ratos tratados com melatonina. Em coelhos, a melatonina reduziu consumo alimentar, ganho de massa e os níveis de glicose ao longo de quatro semanas.

1.1.5. O papel pró-oxidante do etanol vs. efeito antioxidante da melatonina

O etanol é um agente promotor de estresse oxidativo, principalmente no fígado, devido ação da enzima CYP2E1 P450 microssomal. No consumidor crônico, a atividade da NADPH oxidase aumenta no hepatócito durante o metabolismo do etanol, e consecutivamente os níveis de íon superóxido (O_2^-) e peróxido de hidrogênio (H_2O_2) (Albano et al., 1996; Fang et al., 1998). Similarmente, o ovário é um órgão que sofre ataque por radicais livres, principalmente durante o processo ovulatório (Agarwal et al., 2005). Esta condição é mantida quando as espécies reativas de oxigênio (EROs) como OH, O_2^- e H_2O_2 são produzidos em larga escala ou pouco detoxificados (Zuelke et al., 1997). Pesquisas anteriores utilizando modelos experimentais alcoólicos enfatizam a propriedade antioxidante da melatonina, de acordo com seu caráter atenuador na peroxidação lipídica (Genç et al., 1998; El-Sokkary et al., 1999). De outra forma, atividade antioxidante reduzida no folículo ovariano da mulher geralmente esta acompanhada de infertilidade (Paszkowski et al., 1995). As células da granulosa *per se* é um ambiente favorável a produção de

EROs, sendo oriundos de processos metabólicos diversos inclusive durante toxicidade pelo etanol, os quais podem evoluir para lipoperoxidação de membranas (Figura 2). O estresse oxidativo pode ser a causa da baixa qualidade oocitária, por acentuar o dano e o envelhecimento celular, a deterioração dos lipídios de membrana, induzir apoptose e até mesmo inibir a fertilidade (Noda et al., 1991; Agarwal et al., 2005).

Os efeitos da melatonina envolvendo o ovário de ratas durante o período ovulatório ainda é pouco compreendido, e até o presente momento nenhum estudo abordou seus efeitos associado ao consumo crônico de etanol.

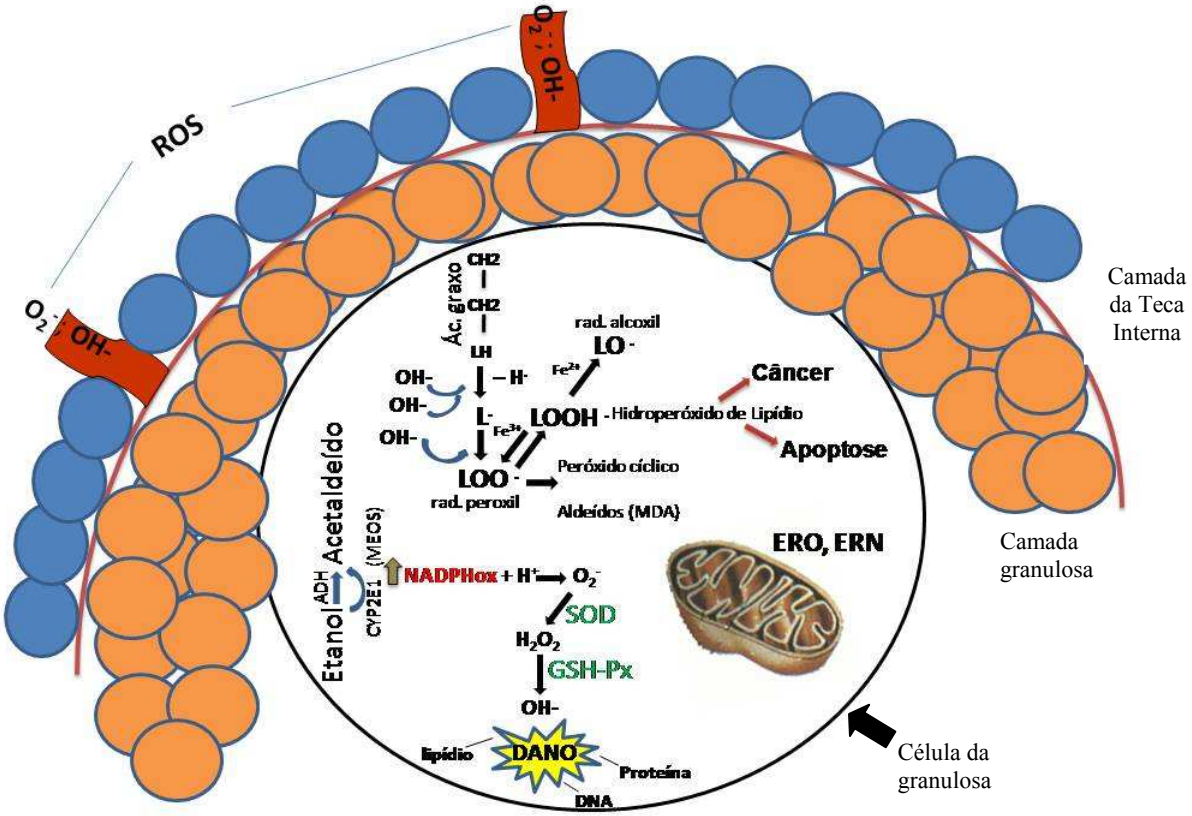

Figura 2. Metabolismo do etanol e propagação da lipoperoxidação de membrana na célula da granulosa. *Ilustração elaborada pelo autor.*

A melatonina exerce efeito antioxidante direto (Claustrat et al., 2005), neutralizando as espécies reativas de oxigênio e nitrogênio (ERN), bem como moléculas ou radicais livres causadores de danos ao DNA, proteínas e lipídios; e também possui efeito indireto promovendo a síntese de enzimas antioxidantes (glutationa peroxidase (GSH-Px), redutase (GSH-Rd), catalase (CAT), Cu, Zn, Mg-superóxido dismutase (SOD)) as quais conferem proteção ao tecido como parte das defesas do organismo (Leon et al., 2005). Além disso, a literatura relata que a melatonina atua como um potente agente antioxidante, regulando vários eventos fisiológicos em mamíferos (Okatani et al., 2003; Carrillo-Vico et al., 2004). Estudos têm demonstrado que a melatonina detoxifica uma ampla variedade de radicais livres e moléculas oxigênio-reativas, incluindo radicais hidroxila, peroxinitrito, íon superóxido, oxigênio singlete, entre outros; atuando como um limpador de produtos oxidativos e evitando a peroxidação lipídica em tecidos reprodutivos (Armagan et al., 2006; Peyrot & Ducrocq, 2008). A melatonina é considerada uma ferramenta útil contra o dano oxidativo decorrente do alcoolismo crônico (Hu et al., 2009). No ovário, elevados níveis de melatonina no fluido folicular e a presença de seus receptores na célula da granulosa sugerem um papel importante e benéfico para a fertilidade feminina (Ronnberg et al., 1990; Woo et al., 2001). Atualmente, a melatonina tem sido bastante utilizada em mulheres com incapacidade para engravidar, com a finalidade de promover melhor qualidade oocitária.

2. HIPÓTESE E RELEVÂNCIA DA TEMÁTICA

É sabido que o consumo abusivo e a dependência alcoólica estão associados a severas alterações estruturais e fisiológicas no ciclo reprodutivo em fêmeas, incluindo a produção de

EROs. A melatonina, utilizada como estratégia terapêutica no tratamento de muitas doenças, minimiza possíveis distúrbios do sono e combate os agentes oxidantes provenientes do alcoolismo crônico. Em contrapartida, de maneira isolada, tanto o etanol quanto a melatonina exercem efeitos diretos ou indiretos sobre a produção de hormônios sexuais, bem como sobre os receptores esteróides sexuais. Tendo em vista que os efeitos do alcoolismo crônico e da melatonina exógena, frente à reprodução feminina, são ainda pouco descritos na literatura e inconclusivos, três questões são levantadas: poderia a combinação entre etanol e melatonina desencadear respostas diferentes e alterar o controle endócrino da reprodução? A melatonina seria capaz de atenuar o estresse oxidativo originado no ovário proveniente do alcoolismo crônico? Em qual (is) circunstância(s) essa associação seria benéfica ou prejudicial à reprodução feminina? Dessa forma, os objetivos do presente projeto vislumbram sobre essas potenciais abordagens.

Tendo em vista que os ratos da variedade UChB foram originados do Wistar há aproximadamente 60 anos atrás (Mardones & Segovia-Riquelme, 1982), surgiu a oportunidade de avaliar o efeito independente da melatonina em ratas Wistar saudáveis (não alcoólicas) durante a ovulação, sobre os mesmos parâmetros daqueles das ratas UChB, com a finalidade de comprovar a eficácia do tratamento (ver ***Capítulos 3 e 4***). No entanto, não houve a intenção de estabelecer qualquer comparação entre os grupos.

3. OBJETIVOS

3.1. OBJETIVO GERAL

Avaliar os efeitos da administração exógena da melatonina sobre os hormônios sexuais e os receptores esteróides sexuais (AR, ER-α, ER-β, PRA e PRB) no ovário, oviduto e útero, além

do perfil nutricional e o estresse oxidativo nos ovários de ratas adultas UChB (consumidoras voluntárias de etanol a 10%).

3.2. OBJETIVOS ESPECÍFICOS

- Avaliar a dosagem dos hormônios FSH, LH, 17β-estradiol e progesterona através do radioimunoensaio;
- Avaliar a dosagem de melatonina plasmática utilizando a técnica de ELISA;
- Quantificar o metabólito 6-sulfatoximelatonina (6-STM) na urina dos animais por ELISA;
- Quantificar os receptores nucleares (AR, ER-α, ER-β, PRA e PRB) e de membrana (MT1R) no ovário, oviduto e útero por *Western Blot*;
- Mensurar o status nutricional (ingestão de água, etanol e comida), o ganho de massa, a eficiência alimentar total, a glicose, o ciclo estral e a massa dos órgãos genitais internos;
- Avaliar a lipoperoxidação (LHP) ovariana envolvida no estresse oxidativo (ensaio enzimático/espectrofotômetro);
- Avaliar a atividade das enzimas antioxidantes (SOD, GSH-Px, GSH-Rd e Catalase) e os níveis de SAT no tecido ovariano (ensaio enzimático/espectrofotômetro).

4. RESULTADOS

Os resultados foram divididos em quatro capítulos, apresentados na forma de artigos científicos:

Capítulo 1: Effects of chronic ethanol intake and exogenous melatonin treatment on sex hormones and sex steroid receptors in the ovary, oviduct and uterus during rat ovulation, submetido ao periódico J Pineal Res, 2011.

Capítulo 2: Long-term exogenous melatonin treatment modulates overall feed efficiency and protects ovarian tissue against injuries caused by ethanol-induced oxidative stress in adult UChB rats. Alcohol Clin Exp Res, v.35(8), p.1498-508, 2011.

Capítulo 3: Melatonin reduces LH, 17 beta-estradiol and induces differential regulation of sex steroid receptors in reproductive tissues during rat ovulation. Reprod Biol Endocrinol, v.9, p.108, 2011.

Capítulo 4: Long-term melatonin treatment reduces ovarian mass and enhances tissue antioxidant defenses during ovulation in the rat. Braz J Med Biol Res, v.44, p.217-223, 2011.

Capítulo 1

Effects of chronic ethanol intake and exogenous melatonin treatment on sex hormones and sex steroid receptors in the ovary, oviduct and uterus during rat ovulation

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Running title: Melatonin & ethanol on sex steroid receptor

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Keywords: Melatonin, ethanol, sex steroid receptors, ovary, oviduct, uterus.

Abstract

Chronic ethanol intake is associated with female hormonal disturbances and it is well known that melatonin plays key roles in regulating reproductive function. However, the effects of ethanol and melatonin upon sex hormones and steroid receptors remain inconclusive. We evaluated the effects of ethanol consumption and melatonin treatment on sex hormones and sex steroid receptors subunits in ovary, oviduct and uterus of UChB ethanol-preferring rats. Forty adult rats were divided into four groups. UChB Co: drinking water; UChB EtOH: drinking ethanol at 2-5 g/kg/day; both receiving vehicle solution. Concomitantly, melatonin was administered (100μg/100g BW) intraperitoneally to UChB Co+M and UChB EtOH+M rats during 60 days. Melatonin increased progesterone, 6-sulfatoximelatonin and decreased 17β-estradiol, while the ethanol+melatonin combination caused a significant fall in these hormones. Despite androgen receptor (AR) in ovary has not been influenced by melatonin, ethanol and ethanol+melatonin led to a decrease in oviduct AR. Both estrogen receptors (ER-α and ER-β) were underexpressed by either ethanol or melatonin in oviduct and only uterine ER-β was downregulated. Conversely, progesterone receptors (PRA and PRB) were positively regulated in the ovary by ethanol or ethanol+melatonin, whereas PRA was downregulated in uterus and oviduct, except when ethanol+melatonin were combined. Additionally, melatonin receptor (MT1R) was increased in ovary and uterus of melatonin-treated rats, regardless of ethanol consumption. We conclude that melatonin has opposite effect on sex hormones to those of ethanol consumption. Together, melatonin and ethanol differentially regulates the sex steroid receptors in the reproductive tissues, mostly acting “in situ” through its MT1R receptor.

Introduction

Alcoholism is rampant in modern society and its chronic consumption is associated with several dysfunctions of female reproduction, including amenorrhea, blockade of ovulation, early menopause, spontaneous miscarriage and infertility [1,2]. Despite the well-known negative effects of ethanol on female reproductive tract, their complex action mechanism(s) upon sex hormones and steroid receptors are poorly discussed and remain a matter of debate. We have previously reported that chronic ethanol intake promotes estrous cycle disruption, hormonal imbalance, increased ROS formation and altered ovarian morphology in UChB rats [3,4]. Studies using rats [5,6] have provided evidence showing that ethanol-induced suppression in LH secretion is due to its action within hypothalamus. In addition, ethanol seems to increase serum 17 β-estradiol (E2) in the short chronic but not long chronic experiment, whereas progesterone (P4) does not change [7]. More directly, ethanol consumption may affect gonadotropins release in a dose- and time-dependent way, however, its defective role involving the reproductive tissues is controversial. Our ethanol-preferring rats (UChB) are derived from original Wistar rats and have been selectively bred at the University of Chile in the past decades [8]. These animals are considered a special model for the understanding of the alcoholism-linked characteristics such as those observed in human diseases.

Melatonin (*N*-acetyl-5-methoxytryptamine) is an indolamine produced by pineal gland and secreted in a circadian manner at night [9]. It is indisputable that melatonin has been implicated as a therapeutic agent in several cases. Notably, alcohol consumption negatively modulates the daily melatonin synthesis [10]. In mammals, melatonin affects reproductive function through activation of receptor sites within the hypothalamic-pituitary-gonadal axis [11,12]. Melatonin also interacts with sex hormones and is seen to inhibit steroidogenesis via

direct changes in cAMP levels on theca, but not granulosa cells [13]. Nakamura and Kato [14] have previously demonstrated that preovulatory follicles contain high melatonin levels, suggesting an indirect effect on E2 and P4 synthesis. It has also been proposed that low serum melatonin is linked to increased E2 as well as reduced P4 and its receptors [15]. Recently, Adriaens et al. [16] demonstrated that melatonin increased P4 and androgen production in mouse preantral follicles. To date, no reports have been demonstrated the effects of chronic ethanol intake in association with exogenous melatonin on sex hormones during ovulation process.

It is currently assumed that putative estrogen and progesterone-binding specific receptor orchestrates a variety of reproductive physiological responses under a fine tuning of regulation. Estrogen receptor (ER) belongs to the nuclear receptor superfamily and has two distinct functional isoforms designated as ER-α and ER-β [17]. In ovaries, granulosa cells express higher levels of ER-β than ER-α, while ER-β is reportedly expressed at lower levels in uterus [18]. Melatonin has been shown to induce down-regulation of ER-α in ovary and uterus [19,20]. Conversely, ethanol consumption seems to increase ER-α expression depending on the amount consumed [21], which in turn, contributes to the harmful effects.

Progesterone receptors (PRs), one of the well-characterized proteins regulated by estrogens, are expressed as PRA and PRB isoforms [22]. PRA has a transactivation domain essential for ovulation process [23] whereas it functions as a repressor of PRB and androgen receptor [24]. Although it has long been suggested that E2 up-regulates PR, little is known as to whether PRA and B expression can be modulated during either ethanol or melatonin administration. It has been recently described that melatonin significantly increases P4 as well as the number of total PR in ovaries at proestrus [19] while ethanol intake seems to alter PR levels indirectly through the activation of E2 or ER [25]. The effects of long-term melatonin and

ethanol intake are not defined as regarding to specific sex steroid receptors signaling along the ovaries, oviducts and uterus.

Melatonin signals through at least two G protein-coupled receptors, the MTR1 and MTR2 membrane receptors, or via cytoplasmatic/nuclear sites [26]. Among other actions, MTR1-binding melatonin is thought to cause down-regulation of both ER-α protein and ER-α mRNA [27] and, alternatively, it may inhibit the ligation of E2-ER complex to the estrogen response elements (ERE) on DNA [27,28]. Since melatonin is a potential agent targeting the control of reproduction, its long-term effects associated with ethanol have never been identified through MT1R receptors.

To help shed light on the issue, the present study was to investigate the effects of ethanol consumption and melatonin treatment on sex hormones and sex steroid receptors subunits in the ovary, oviduct and uterus of UChB ethanol-preferring rats during ovulation.

Material and Methods

Animals and experimental design

Forty adult female rats (*Rattus norvegicus albinus*), 60 days old (± 250-260 g) were obtained from the Department of Anatomy, Bioscience Institute/Campus of Botucatu, UNESP – Univ Estadual Paulista. They were individually housed in polypropylene cages with laboratory-grade pine shavings as bedding and maintained under controlled room temperature (23±1ºC) and lighting conditions (12L, 12D photoperiod, lights switched on at 6 a.m). Food and filtered water were provided *ad libitum*. All animals were divided into four groups (n=10/group). UChB EtOH group: rats fed 10% (v/v) ethanol *ad libitum* (free choice for water or ethanol) drinking from 2.0 to 6.0 g/kg/day and receiving vehicle solution; UChB Co group: Ethanol-naïve rats without

access to ethanol (used as control) receiving only vehicle; UChB EtOH+M group: rats fed 10% (v/v) ethanol (free choice for water or ethanol) drinking from 2.0 to 6.0 g/kg/day and receiving vehicle+melatonin; UChB Co+M group: without access to ethanol and receiving vehicle+melatonin. When UChB EtOH rats reached 60 days of age, they were given during 20 days, a choice between two bottles containing either water *ad libitum* (1) or 10% (v/v) ethanol (2). After this period, 10 animals per group displaying ethanol consumption higher than 2.0 g EtOH/kg/day (ranging from 4 to 5 g EtOH/kg/day) were finally selected according to Mardones and Segovia-Riquelme [8]. In this study, the preference ratio associated to ethanol-seeking behavior was about 65%. Besides that, to ensure more efficiency and maintenance of constant consumption throughout the experiment, the animals were kept under observation for 10 days when they started with melatonin treatment. Thus, after 90 days old, females received ethanol and/or melatonin during 60 consecutive days (Fig. 1A). After melatonin treatment period, all rats were monitored by vaginal swabs in a dark room using a red dim illumination, and during the early morning of estrus (timing of ovulation) at 4 a.m (or Zeitgeber Time (ZT) 22, corresponding to the environmental circadian time) they were anesthetized and euthanized by decapitation for further analysis. Experimental protocols were accepted by Ethical Committee of the Institute of Bioscience/UNESP, Campus of Botucatu, SP, Brazil (Protocol n^{o} 85/07).

Procedures of melatonin administration

Melatonin (M-5250, Sigma-Aldrich Chemicals, St. Louis, MO, USA) were dissolved in few drops of 95% ethanol 0.04mL and diluted in 0.9% NaCl 0.3mL (vehicle) up to desired concentration and then intraperitoneally (i.p) injected [3]. The i.p injections at doses of 100

μg/100 g BW were daily administered during 60 days at evening hours (between 18:30 - 19:00 h after sunset, ZT 13; Fig. 1B).

Urine and reproductive organs collection

In the evening before they were killed, all animals received the last injection of melatonin and they were kept inside metabolic cages (Techniplast, Exton, PA, USA) by 10 h in order to collect individual urine samples. Thereafter, all samples were centrifuged at 10,000 x g for 20 min at 4 ºC and stored at - 20 ºC. On the next day after sacrifice, all reproductive organs (ovaries, oviducts and uterine horns) were entirely dissected and weighed for further assays.

Sex hormones assay

Blood samples were collected from the trunk of decapitated rats into heparinized tubes. Afterwards, plasma was obtained by centrifugation at 1,200 x g for 15 min at 4 ºC and stored at - 20 ºC until assayed by radioimmunoassay (RIA). Plasma FSH and LH were determined by double-antibody RIA with specific kits provided by the "National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases" (NIADDK, Baltimore, MD, USA). The FSH primary antibody was anti-rat FSH-S11, and the standard FSH-RP2. The antiserum for LH was LH-S10 using RP3 as reference. The lower limit of detection for FSH and LH was 0.2 ng/mL and the intra-assay coefficient of variation was 3% and 4%, respectively. Plasma concentrations of E2 and P4 were determined using Estradiol and Progesterone Maia kits (Biochem Immunosystems, Serotec, Italy). The lower detection limit and the intra-assay coefficient of variation were respectively 7.5 pg/ml and 2.5% for E2 and 4.1 ng/ml and 3.7% for P4. All samples were measured in duplicate

and at different dilutions, if necessary. In order to prevent interassay variation, all samples were assayed in the same RIA.

Determination of plasma melatonin and urinary 6-SMT

Melatonin was initially extracted from plasma (n=10 samples/group) using methanol HPLC grade followed by separation into columns Sep-Pak Vac C-18, reverse phase, 12.5 nm (Water Corporation, Milford, Massachusetts, USA). Thereafter, 50 µL of reconstituted samples were assayed with coat-a-count melatonin ELISA kits and measured photometrically at a wavelength of 405 nm. The intra-assay coefficient of variation was 3%. Urinary 6-STM was assayed with solid-phase melatonin sulfate ELISA kits and, finally, read at 450 nm. The intra-assay coefficient of variation was 5.2%. Samples were double assayed at the time to avoid interassay variations. All reagents and microtiter plate were provided by IBL (IBL International, Hamburg, Germany).

Western blotting analysis and protein quantification

After 60 days of melatonin treatment (100µg/100g BW/day), the ovaries, oviducts and uterine horns were rapidly removed and tissue samples of 50 mg were immediately frozen in liquid nitrogen and stored at - 80º C. All tissues were homogenized with RIPA lysis buffer (Pierce Biotechnology, Rockford, IL, USA), 10X (0.5 M Tris-HCl, 1.5 M NaCl, 2.5 % deoxycholic acid, 10 % NP-40, 10 mM EDTA, pH 7.4) and protease inhibitor cocktail (Sigma Chemical Co.) using a homogenizer (IKA® T10 basic Ultra, Staufen, Germany). Aliquots containing 1:10 (v/v) of Triton X-100 were added to homogenates and samples were placed on

dry ice under agitation by 2 h in order to improving extraction. These suspensions were centrifuged at 21,912 x g for 20 min at 4º C and the pellet discarded. Total proteins were measured by the Bradford through colorimetric determination. All proteins were dissolved in 1.5 X sample buffer previously described by Laemmli and used for SDS-PAGE (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein (70 µg) were loaded per well onto preformed gradient gels, 4-12% acrylamide (Amersham Biosciences, Uppsala, Sweden) with a Tris-glycine running buffer system for electrophoresis (60 mA fixed during 2 h). After electrophoresis, total proteins were electro-transferred (200 mA fixed by 1 h 30 min) onto 0.2 µm nitrocellulose membranes in a Tris-glycine-methanol buffer. Kaleidoscope Prestained Standards (Bio-Rad) were used as molecular weight markers. Thereafter, the membranes were blocked with TBS-T solution containing 3% BSA at room temperature (RT) for 60 min and then incubated at 4º C overnight with rabbit primary antibody AR-N20 anti-AR; rabbit clone E115 anti-ERα receptor; rabbit clone 68-4 anti-ERβ; mouse monoclonal [C262] anti-PRA and PRB and rabbit polyclonal anti-MT1R (dilutions of 1:1000; 1:250; 1:500; 1:350; 1:500; 1:500 were carried out at 1% BSA, respectively). This was followed by 3 x 5 min washing in TBS-T solution and then incubated for 2 h at RT with rabbit or mouse HRP-conjugated secondary antibodies (diluted 1:1000 in 1% BSA; Sigma, St. Louis, MO, USA). After sequential washing with TBS-T, signals were enhanced and peroxidase activity was finally detected by mixing 10mL PBS, 8µl H_2O_2 and 0.02g DAB (Sigma Chemicals Co.). Immunoreactive bands were obtained from separate blots of six rats/group using image analysis software (NIS-Elements, Advanced Research, Nikon). β-actin was used as an endogenous control and all results were expressed as mean ± SEM. Immunoblotting concentrations (%) were represented as optical densitometry values (band intensity / β-actin ratio).

Statistical analysis

Values are presented as mean ± SEM. Data of plasma FSH, LH, E2, P4, melatonin, urinary 6-SMT and western blotting analysis were performed by Two-way ANOVA analysis of variance (based on two independent factors - ethanol consumption and melatonin treatment). Significant results were subjected to *post hoc* Tukey's test and statistical significance was set at $p < 0.05$. The statistical software was *GraphPad Instat version 4 and Sigma Plot version 11.0* for graphic design.

Results

Plasma sex hormones, melatonin and urinary 6-STM levels

After 60-day treatment, total FSH levels were reduced ($p < 0.05$) in ethanol-preferring rats compared to those receiving ethanol and melatonin, while plasma LH and melatonin were not influenced by the treatment ($p > 0.05$). Melatonin significantly ($p < 0.05$) increased P4 and decreased E2 levels compared to controls, and conversely when ethanol and melatonin are combined, a significant fall in both P4 and E2 levels was notable in relation to UChB EtOH rats. Additionally, only UChB Co+M animals presented higher 6-STM concentrations than UChB Co at morning of estrus ($p < 0.01$; Fig. 2 A-F).

Analysis of ovarian AR, ER-α, ER-β, PRA, PRB and MT1R levels

Despite total AR levels were unchanged throughout the treatments, melatonin reduced ER-α while ethanol had an opposite effect on this receptor. The interaction between melatonin and ethanol resulted in downregulation of ER-α. In addition, either melatonin or ethanol alone led to a decrease ($p < 0.05$) in ER-β levels compared to controls UChB Co rats (Fig. 3 A-D). Also, it

was observed that ethanol alone and even the interaction between ethanol and melatonin caused significant overexpression of PRA and PRB in relation to UChB Co and UChB Co+M groups, respectively (Fig. 3 A, E, F). Interestingly, as shown in Fig. 3 G, the MT1R levels were significantly higher ($p < 0.05$) in the ovaries of both melatonin-treated groups, regardless of ethanol consumption, which implies that melatonin administration promoted an up-regulation of its own receptors.

Analysis of oviductal AR, ER-α, ER-β, PRA, PRB and MT1R levels

The AR levels in oviduct were decreased ($p < 0.05$) after 60-day exposure to ethanol while interaction between ethanol and melatonin revealed the lowest AR levels compared to UChB Co+M and UChB EtOH groups (Fig. 4 A,B). Total ER-α, ER-β and PRA levels were downregulated by ethanol or melatonin treatment, and otherwise, the interaction between ethanol and melatonin induced a remarkable overexpression of these receptors (Fig. 4 A,C-E). In addition, there was no significant effect of treatment on oviduct PRB levels among the groups, where the expression was maintained relatively constant. Differently from ovaries, MT1R levels were lower ($p < 0.01$) in UChB EtOH than UChB Co rats and become even more reduced after the interaction of ethanol and melatonin (Fig. 4 A,G).

Analysis of uterine AR, ER-α, ER-β, PRA, PRB and MT1R levels

The uterine AR expression showed a significant ($p < 0.01$) increase after melatonin and ethanol-melatonin interactions compared to UChB Co and UChB EtOH groups. Also, AR levels were found to be more positively regulated by melatonin alone compared to ethanol+melatonin-treated group ($p < 0.01$; Fig. 5 A,B). Ethanol alone or in combination with melatonin induced low

ER-α expression, while uterine ER-β was decreased ($p < 0.05$) after treatment with either melatonin or ethanol in comparison to UChB Co and UChB EtOH+M groups (Fig. 5 A,C,D). UChB Co+M and UChB EtOH rats had lower PRA levels than controls ($p < 0.05$), in contrast to PRB levels, which were significantly increased by melatonin and simultaneously decreased by ethanol and ethanol+melatonin treatments. Similarly to those MT1R found in the ovaries, all animals that received melatonin and ethanol+melatonin revealed overexpression of uterine MT1R, regardless of ethanol consumption (Fig. 5 A,E-G).

Discussion

Although melatonin may act as a synchronizer of the reproductive function, the specific cellular and molecular characteristics of melatonin binding sites are so far unknown. It seems obvious that both melatonin and ethanol do not act directly on GnRH neurons [29-31] but, instead, exert indirect actions through *Kiss 1*/GPR54 neural system by inducing low circulating gonadotropins [30]. This disarrangement could lead to the onset of hypogonadotropic hypogonadism as we had previously reported that interaction between melatonin and ethanol caused a loss of ovarian weight [3]. The present study found that melatonin and ethanol are able to reduce P4 and E2 while increasing FSH levels at estrous. Moreover, melatonin alone had induced P4 synthesis in contrast to reduced E2. Chronic ethanol intake has been linked to increased E2 levels by the following: possible dysfunction of hepatocyte metabolism leading to a shift in NAD/NADH ratio, thus disturbing the process of E2-estrone conversion [32]. Otherwise, melatonin was seen to inhibit E2 steroidogenesis by changing cAMP levels through a direct action on the follicles [13] also confirmed by our group [20]. Furthermore, melatonin regulates the expression and activity of aromatase [33], acting as a selective estrogen modulator, and

further contributing to a decrease in E2 levels. The interaction between melatonin and ethanol resulted in reduction of E2, and when E2 levels are low, a negative feedback for FSH secretion does not occur. Taken together, it is believed that the high FSH levels are indirectly related to the melatonin effects rather than ethanol, since ethanol alone promoted an increase of E2 and consequently a fall in FSH levels. These observations suggest the possibility that there may be some interactions among melatonin, ethanol intake and E2 metabolism that should be further deeply explored.

Despite our recent study had proposed that melatonin inhibits LH secretion at estrus, its combination with ethanol, in an UChB rat model, did not caused any disturbance on LH levels. This could be attributed to the responsiveness of each treatment, time of exposure and specific rat line used. Corroborating partially the study, Verkasalo et al. [34] stated that both LH and FSH levels decreased after daily exposure to ethanol. It has long been emphasized that melatonin increases P4 levels [35,19] while ethanol metabolism indirectly reduces P4 after sharing the same cofactor NAD that catalyse the oxidation of 20α-dihydroprogesterone to P4 [36]. Interestingly, when ethanol and melatonin were given, a significant reduction of P4 levels was noted. Presumably, this can be explained by ethanol-induced changes in the redox state leading to either high or low redox potential of the steroids in the liver. Urinary 6-STM levels were raised only in UChB Co+M rats at morning estrus. Since alcohol consumption affects the endogenous melatonin synthesis [10] and it also appears to be inversely associated with the presence of 6-STM [37], we found no significant differences for such levels in animals that received both ethanol and melatonin as treatment. Furthermore, plasma melatonin remained relatively constant in the animals. This is due to the short half-life of melatonin (assessed 7 h after administration), where it is rapidly converted into 6-STM prior to elimination [20].

In ovarian tissue, neither ethanol nor melatonin treatment had effectively influenced AR levels. The present study showed that long-term melatonin is able to reduce ER-α, in contrast to high expression of ER-α after ethanol consumption. Indeed, ER seems to be activated when cAMP is elevated through E2-dependent mechanisms [38]. Notably, melatonin acting via membrane-bound G protein-coupled MT1 receptor may inhibits adenylate cyclase activity, thus decreasing cAMP levels [39], and directly affecting E2-induced ER-α transactivation. Alternatively, ethanol dose-dependently increases ER-α level [40] and potentiates the ERE-binding responsive activities in the presence of E2 [41]. These ethanol-related actions during ovulation can occur directly by altering E2 levels itself or even indirectly through derivatives from ethanol metabolism. Interestingly, the interaction between melatonin and ethanol promoted a down-regulation in ER-α transcription. Since the elevation in ER-α expression is linked to E2-induced carcinogenesis [40], melatonin treatment would reduce ovarian adverse effects arising from chronic ethanol intake. Otherwise, melatonin and ethanol alone caused downregulation of ovarian ER-β. This may be due to different tissue and concentrations of melatonin and ethanol employed in the study. Additionally, PRA and PRB which function as ligand-dependent repressors of ER-mediated transcription [42] were significantly higher at the end of treatments. In this context, we conclude that P4-PR signaling were responsible for attenuating more specifically the ER-β expression. Ethanol alone and the interaction between ethanol and melatonin induced similar overexpression of ovarian PRA and PRB subunits. Because melatonin alone had no effect on PR expression, the main factor activating both PRA and PRB is presumably the ethanol consumption. Ethanol has been pointed to be a toxicant affecting the hormonal balance predominantly through E2 and P4 mediated by their respective receptors [25]. This is partially consistent with our findings, except for P4 levels. Curiously, the MT1R levels were

overexpressed in the ovaries of both melatonin-treated groups. Thus, we have launched first evidence that MT1R remains potentially activated during ovulation process even after chronic ethanol consumption. Also, previous study has indicated that melatonin binding receptor is high during estrus, proestrus and diestrus, in contrast to low levels in metaestrus when E2 and P4 are reduced [43]. Finally, it allows us to conclude that both E2 and P4 might regulate MT1 receptor activity besides the melatonin treatment itself.

We demonstrated for the first time the role of ethanol and melatonin on the expression of oviduct ER and PR subunits. In rat oviduct, the expression of AR was reduced in ethanol-consuming animals and became even more prominent after the combination of ethanol and melatonin. It seems plausible to consider that ethanol increases estrogen levels by promoting the induction of aromatase [44], thus lowering the circulating androgens and latter the AR levels. Furthermore, a direct inhibitory effect of the ER-α/AR heterodimer on both AR and ER-α may adversely affect the transactivation properties [45]. These events could somehow be related to a reduction of AR in the presence of ethanol, besides mentioning our recent report in which melatonin negatively regulated oviductal AR [20]. Ethanol and melatonin alone similarly caused downregulation of oviduct ER-α, ER-β and PRA. Otherwise, the combination between ethanol and melatonin significantly raised these receptors to normal levels. We had previously proved that melatonin reduces ER-α expression in rat oviduct [20], however, the presence of ethanol combined with melatonin was effective in restoring the ER-α and ER-β levels through unknown mechanism(s). Notably, it was reported that ethanol up-regulates ER-α while down-regulates ERE activity depending on the specific cell type and amount of ethanol ingested [46]. Although it has been pointed that the expression of oviduct PRA and PRB is enhanced after melatonin treatment [20], in the present study, ethanol-preferring rats showed increased PRA only after

combination with melatonin. It is likely that the fall in P4 levels might be accentuating PRA expression. Conversely, neither ethanol nor melatonin caused alterations in PRB levels. It seems that oviduct PRB is not quite sensitive to changes after treatments. Nevertheless, the regulation of sex steroid receptors in oviduct is not yet fully clarified. MT1R was downregulated in oviduct after ethanol consumption or in combination with melatonin. Ultimately, oviduct MT1R has a differential responsiveness to ethanol compared with ovary and uterus.

In uterus tissue, melatonin alone upregulated AR expression while ethanol caused its downregulation. Ethanol seems to have a pivotal role in reducing uterine AR even after ethanol-melatonin combinations. The presence of AR in uterine epithelial, stromal and myometrial cells suggests that androgens may exerts direct influence on the development and function of uterus. Although we did not found differences in uterus AR after melatonin administration [20], these ethanol-preferring rats exhibited differential AR expression. In females, ethanol intake was seen to rapidly increase the androgen level even with low ethanol doses during the whole cycle [47,48]. This elevation in circulating androgens may be the main cause of reduced AR expression. Ethanol alone and ethanol-melatonin combination reduced uterine ER-α, whereas only ethanol or melatonin alone induced downregulation of ER-β. Conversely, ethanol-melatonin combination led to an overexpression of ER-β. Since ER-α is abundantly expressed in rat uterus than ER-β [18], ethanol consumption negatively regulated ER-α expression, thus adversely affecting the ER-α-mediated functions during ovulation. It is established that E2 and P4 acts upon the uterus by an interdependent regulation of ER and PR [49,50]. Noticeably, it has been shown that E2 decreases the expression of uterine ER but not PR, while P4 reduces the levels of both receptors [51]. Taking into account that E2 and P4 levels were raised after ethanol intake and melatonin treatment, respectively, it suggests that ethanol has severely influenced both uterine

ER-α and ER-β, in contrast to melatonin which only reduced ER-β levels. Indeed, melatonin has been pointed to reduce the expression of ER while increasing PR [52]. This may partially explain our findings where melatonin administration caused simultaneous downregulation of ER-β and PRA. Moreover, ethanol alone and ethanol-melatonin combination were responsible for decreasing both PRA and PRB levels. PRA plays a role in decidualization process [53] and it is sufficient to mediate the antiproliferative responses to P4 [23]. Importantly, functional polymorphism in PR promoter that results in increased expression of the PRB isoforms is associated with risk of endometrial cancer [54,55]. In this regard, melatonin but not ethanol has a defective effect in uterine tissue. Similarly to the ovaries, the levels of uterine MT1R were increased in melatonin-treated rats, regardless of ethanol intake. As we have proved earlier [20] melatonin after binding to MT1R is able to mediate responses through direct actions in these organs, besides having indirect effects on sex hormones.

In summary, we reported that melatonin has an opposite effect to those observed after ethanol consumption on the E2 and P4 levels, indirectly causing disturbances to ovary, oviduct and uterus. Moreover, melatonin and ethanol alone promoted differential regulation of the sex steroid receptors upon the reproductive tissues, mostly acting “in situ” through its MT1R receptor, especially in ovarian and uterine tissue. Finally, the melatonin-ethanol combination on these receptors is quite variable, enhancing or attenuating their expression depending on the specific tissue. These data represent an important benchmark for furthering the understanding of melatonin-ethanol interface during ovulation process.

Author contributions

LGAC, FEM: collected and analyzed the data and drafted the manuscript conceiving the main idea of the study. FRFS, WJF, GRT: performed the ELISA assays and Western Blotting analysis given substantial interpretation of data. JPAA, LOM, BAF, MM and PFFP: participated in the acquisition of data, in the design of the study and in the intellectual conception. All authors performed the statistical analysis and approved the final version of the manuscript.

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Figure Legends

Figure 1. Detailed schedule for the experimental design. **(A)** Chronological scheme for overall treatment period. (**B)** Schematic protocol used daily for melatonin administration based on Zeitgeber Time (ZT) corresponding to environmental circadian time.

Figure 2. Hormonal profile after 60-day treatment at morning estrus. **(A)** Plasma FSH levels (ng/mL), **(B)** Plasma LH levels (ng/mL), **(C)** Plasma P4 levels (ng/mL), **(D)** Plasma E2 levels (pg/mL), **(E)** Plasma melatonin levels (pg/mL), **(F)** urinary 6-STM levels (ng/mL). ^{a,d} $p < 0.05$ vs. UChB Co and UChB EtOH+M groups, respectively.

Values are expressed as mean ± SEM (N= 10 animals/group). Two-way ANOVA complemented by Tukey's test.

Figure 3. (A) Representative western blotting analysis of androgen receptor (AR), estrogen receptor (ER-α and ER-β), progesterone receptor (PRA and PRB) and melatonin receptor (MT1R) in ovarian tissue of rats receiving 10% (v/v) ethanol and melatonin (100 µg/100 g B.W). Indicated concentrations of each total protein (70 µg extracted from a pool of 6 organs/group) were used to detect specific proteins in the blots (upper panel). **(B – G)** Densitometry values for AR, ER-α, ER-β, PRA, PRB and MT1R levels were studied following normalization to the house-keeping gene (β-actin). All results are expressed as mean ± SEM (N= 6 animals/group). ^{a} $p < 0.05$ vs. UChB Co; ^{b} $p < 0.05$ vs. UChB Co+M; ^{c} $p < 0.05$ vs. UChB EtOH and ^{d} $p < 0.05$ vs. UChB EtOH+M.

Figure 4. (A) Representative western blotting analysis of androgen receptor (AR), estrogen receptor (ER-α and ER-β), progesterone receptor (PRA and PRB) and melatonin receptor (MT1R) in oviduct tissue of rats receiving 10% (v/v) ethanol and melatonin (100 µg/100 g B.W). Indicated concentrations of each total protein (70 µg extracted from a pool of 6 organs/ group) were used to detect specific proteins in the blots (upper panel). **(B – G)** Densitometry values for AR, ER-α, ER-β, PRA, PRB and MT1R levels were studied following normalization to the house-keeping gene (β-actin). All results are expressed as mean ± SEM (N= 6 animals/group). [a] $p < 0.05$ vs. UChB Co; [b] $p < 0.05$ vs. UChB Co+M; [c] $p < 0.05$ vs. UChB EtOH and [d] $p < 0.05$ vs. UChB EtOH+M.

Figure 5. (A) Representative western blotting analysis of androgen receptor (AR), estrogen receptor (ER-α and ER-β), progesterone receptor (PRA and PRB) and melatonin receptor (MT1R) in uterine tissue of rats receiving 10% (v/v) ethanol and melatonin (100 µg/100 g B.W). Indicated concentrations of each total protein (70 µg extracted from a pool of 6 organs/ group) were used to detect specific proteins in the blots (upper panel). **(B – G)** Densitometry values for AR, ER-α, ER-β, PRA, PRB and MT1R levels were studied following normalization to the house-keeping gene (β-actin). All results are expressed as mean ± SEM (N= 6 animals/group). [a] $p < 0.05$ vs. UChB Co; [b] $p < 0.05$ vs. UChB Co+M; [c] $p < 0.05$ vs. UChB EtOH and [d] $p < 0.05$ vs. UChB EtOH+M.

Figure 1

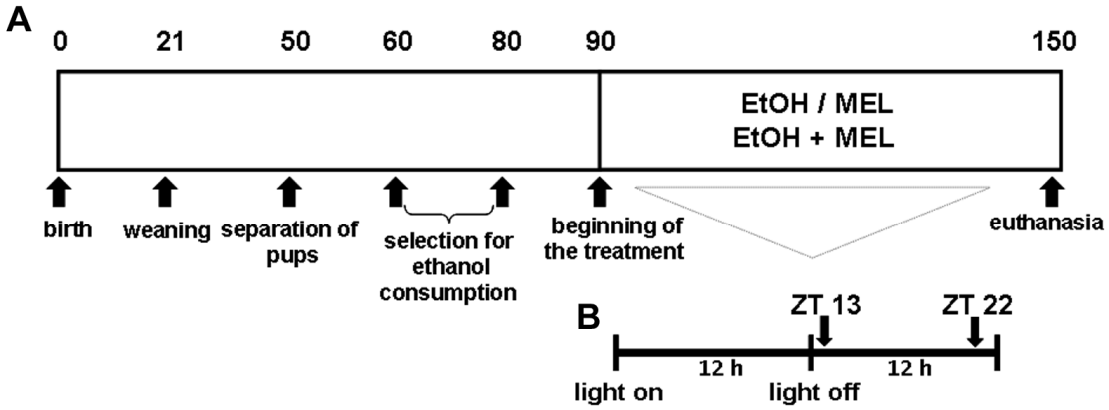

A
0
21
50
60
80
90
150
EtOH / MEL
EtOH + MEL
birth
weaning
separation of pups
selection for ethanol consumption
beginning of the treatment
euthanasia
B
ZT 13
ZT 22
12 h
12 h
light on
light off

Figure 2

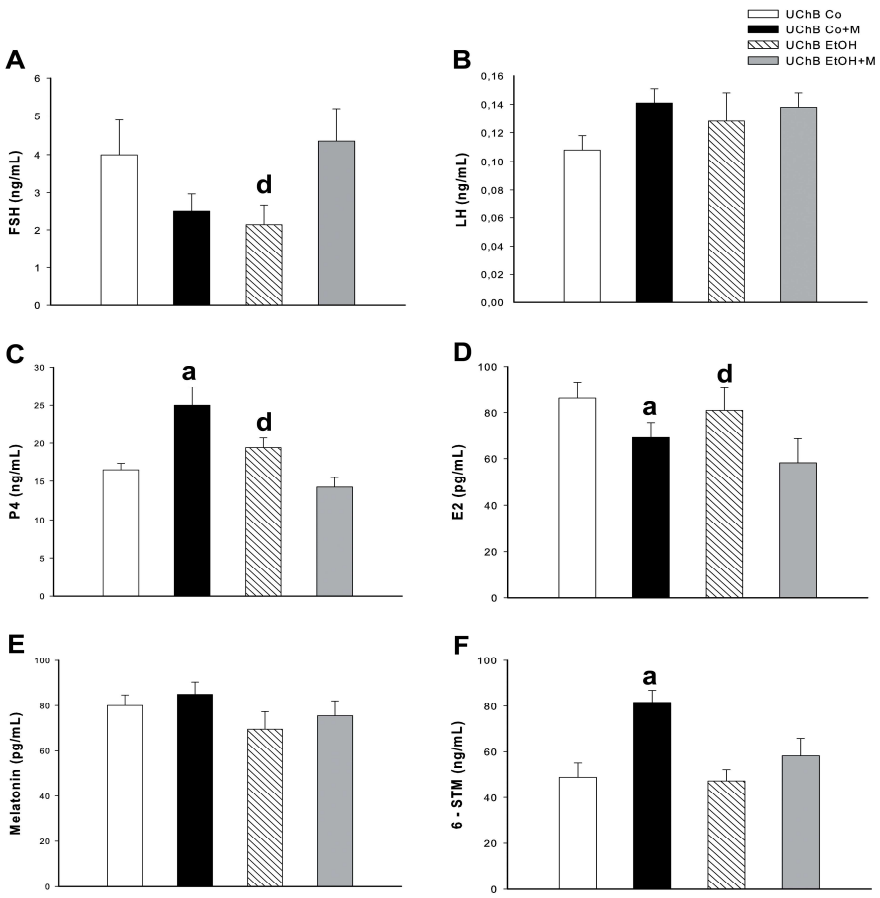

UChB Co
UChB Co+M
UChB EtOH
UChB EtOH+M
A
FSH (ng/mL)
d
B
LH (ng/mL)
C
P4 (ng/mL)
a
d
D
E2 (pg/mL)
a
d
E
Melatonin (pg/mL)
F
6 - STM (ng/mL)
a

Figure 3

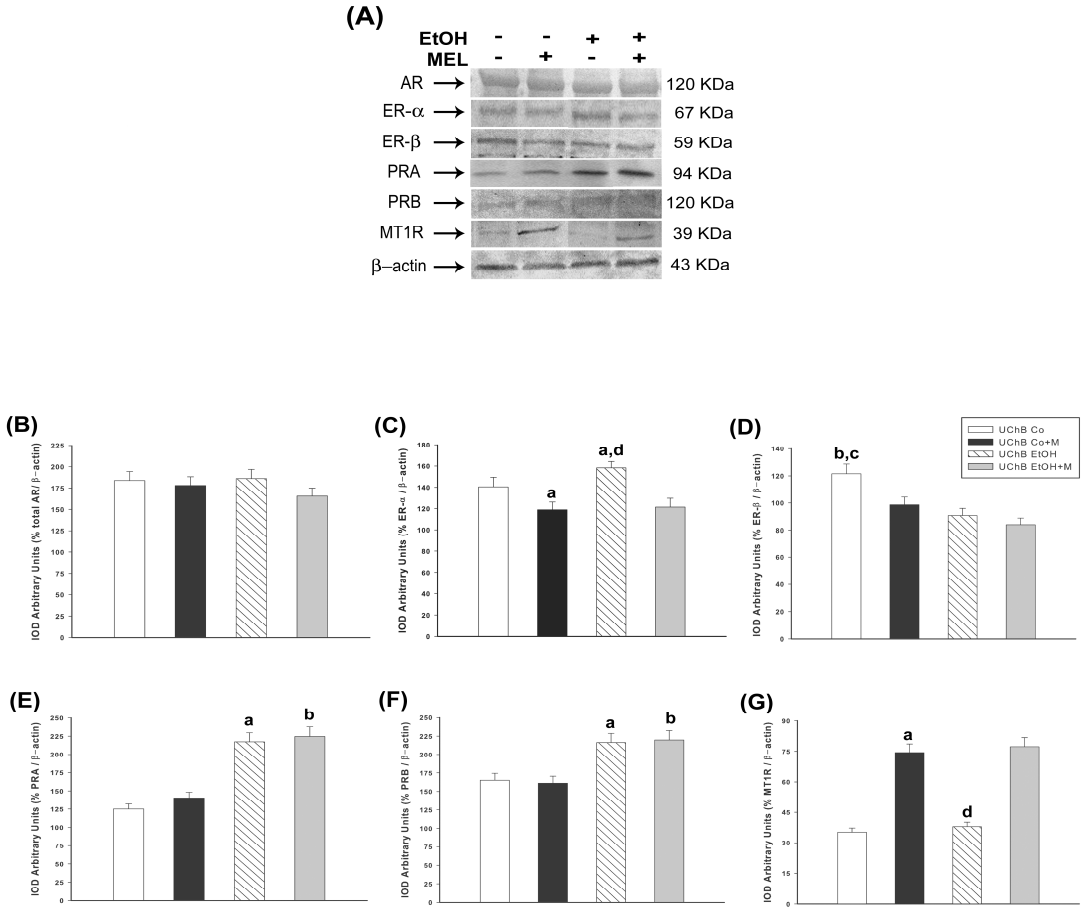
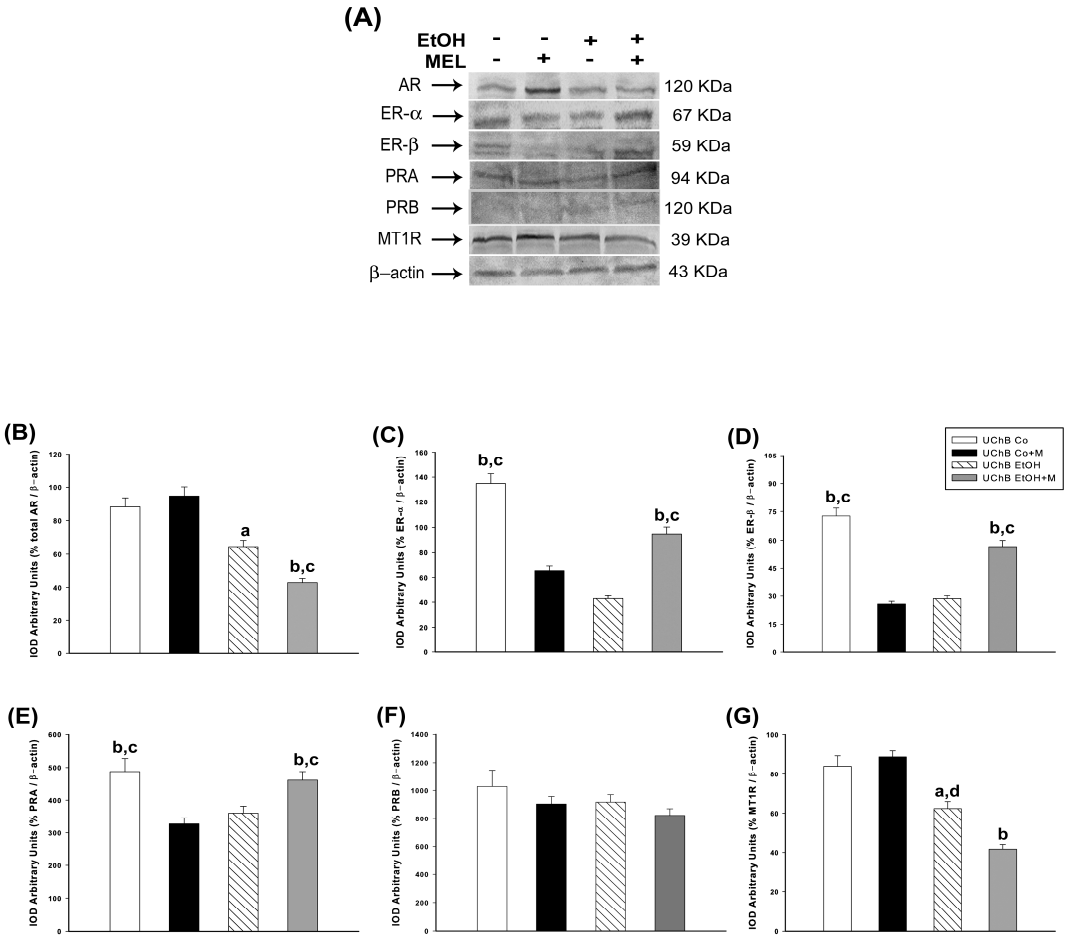

(A)
EtOH - - + +
MEL - + - +
AR → 120 KDa
ER-α → 67 KDa
ER-β → 59 KDa
PRA → 94 KDa
PRB → 120 KDa
MT1R → 39 KDa
β–actin → 43 KDa
(B)
IOD Arbitrary Units (% total AR/ β–actin)
(C)
IOD Arbitrary Units (% ER-α / β–actin)
a
a,d
(D)
IOD Arbitrary Units (% ER-β / β–actin)
b,c
UChB Co
UChB Co+M
UChB EtOH
UChB EtOH+M
(E)
IOD Arbitrary Units (% PRA / β–actin)
a
b
(F)
IOD Arbitrary Units (% PRB / β–actin)
a
b
(G)
IOD Arbitrary Units (% MT1R / β–actin)
a
d

Figure 4


(A)
EtOH - - + +
MEL - + - +
AR → 120 KDa
ER-α → 67 KDa
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PRA → 94 KDa
PRB → 120 KDa
MT1R → 39 KDa
β–actin → 43 KDa
(B)
IOD Arbitrary Units (% total AR / β–actin)
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b,c
(C)
IOD Arbitrary Units (% ER-α / β–actin)
b,c
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(D)
UChB Co
UChB Co+M
UChB EtOH
UChB EtOH+M
IOD Arbitrary Units (% ER-β / β–actin)
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(E)
IOD Arbitrary Units (% PRA / β–actin)
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IOD Arbitrary Units (% PRB / β–actin)
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IOD Arbitrary Units (% MT1R / β–actin)
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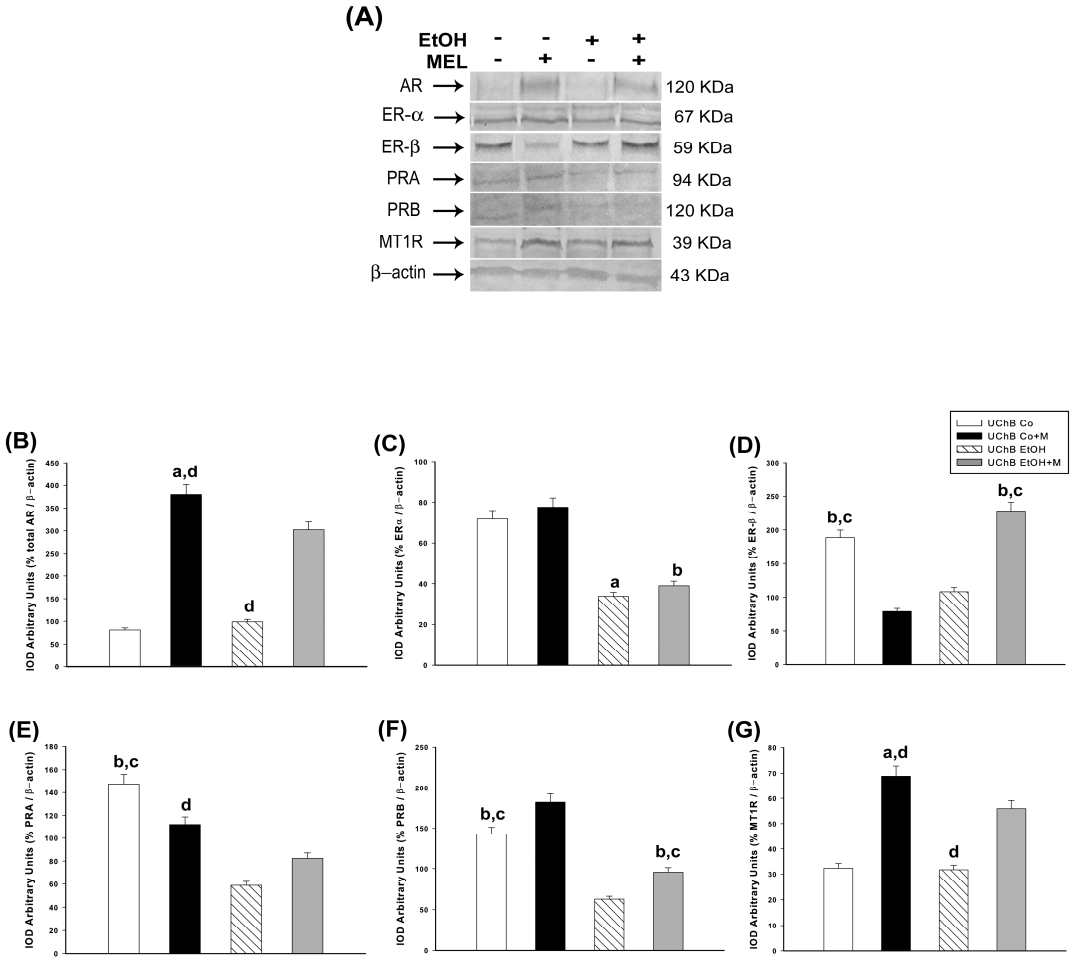
Figure 5


(A)
EtOH - - + +
MEL - + - +
AR
ER-α
ER-β
PRA
PRB
MT1R
β–actin
120 KDa
67 KDa
59 KDa
94 KDa
120 KDa
39 KDa
43 KDa
(B)
IOD Arbitrary Units (% total AR / β–actin)
a,d
d
(C)
IOD Arbitrary Units (% ERα / β–actin)
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(D)
UChB Co
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Capítulo 2

ALCOHOLISM: CLINICAL AND EXPERIMENTAL RESEARCH

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Long-Term Exogenous Melatonin Treatment Modulates Overall Feed Efficiency and Protects Ovarian Tissue Against Injuries Caused by Ethanol-Induced Oxidative Stress in Adult UChB Rats

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Background: Chronic ethanol intake leads to reproductive damage including reactive oxygen species formation, which accelerates the oxidative process. Melatonin is known to regulate the reproductive cycle, food/liquid intake, and it may also act as a potent antioxidant indoleamine. The aim of this study was to verify the effects of alcoholism and melatonin treatment on overall feed efficiency and to analyze its protective role against the oxidative stress in the ovarian tissue of UChB rats (submitted to 10% [v/v] voluntary ethanol consumption).

Methods: Forty adult female rats (n = 10/group) were finally selected for this study: UChB Co: drinking water only; and UChB EtOH: drinking ethanol at 2 to 6 ml/100 g/d + water, both receiving 0.9% NaCl + 95% ethanol 0.04 ml as vehicle. Concomitantly, UChB Co + M and UChB EtOH + M groups were infused with vehicle + melatonin (100 μg/100 g body weight/d) intraperitoneally over 60 days. All animals were euthanized by decapitation during the morning estrus (4 AM).

Results: Body weight gain was reduced with ethanol plus melatonin after 40 days of treatment. In both melatonin-treated groups, it was observed a reduction in food-derived calories and liquid intake toward the end of treatment. The amount of consumed ethanol dropped during the treatment. Estrous cycle was longer in rats that received both ethanol and melatonin, with prolonged diestrus. Following to oxidative status, lipid hydroperoxide levels were higher in the ovaries of ethanol-preferring rats and decreased after melatonin treatment. Additionally, antioxidant activities of superoxide dismutase, glutathione peroxidase activity, and glutathione reductase activity were increased in melatonin-treated groups.

Conclusions: We suggest that melatonin is able to affect feed efficiency and, conversely, it protects the ovaries against the oxidative stress arising from ethanol consumption.

Key Words: Melatonin, Feed Efficiency, Ovary, Ethanol, Lipid Peroxidation.

A CUTE AND CHRONIC ethanol intake is associated with several dysfunctions of female reproduction, including amenorrhea, blockade of ovulation, early menopause, spontaneous miscarriage, and infertility (Carrara et al., 1993; Henderson et al., 2007). Ethanol drinker strains (UChB) are derived from original Wistar rats and have been selectively bred at the University of Chile for almost 60 years (Mardones and Segovia-Riquelme, 1983). These ethanol-preferring rats are considered a special model for the understanding of the basis of alcoholism-linked characteristics such as those found in alcohol-related human diseases.

In mammals, the photoperiodic hormone melatonin (*N*-acetyl-5-methoxytryptamine) is secreted by the pineal gland and it has been documented as an important modulator of reproductive function because of its stimulation of ovarian activity, promotion of estrous cycles, gonadal atrophy (Horton and Yellon, 2001; Ocal-Irez, 1989), and regulation of folliculogenesis and ovulation (Zhao et al., 2000). More directly, ethanol intake has been proposed to induce diurnal melatonin production (Fonzi et al., 1994), and as recently described, chronic ethanol consumption plays a defective role in the synchronization of circadian rhythms (Rosenwasser et al., 2005), resulting in sleep disorders and alcohol-induced mental

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dysfunctions (Clark et al., 2007). Melatonin may be useful as a hormonal therapeutic replacement as it minimizes sleep disorders and stress agents arising from chronic alcoholism.

There are some experimental support involving actions of melatonin on nutritional parameters, such as feed efficiency, body mass gain, adiposity index, and both energy intake and expenditure (Korkmaz et al., 2009; Mustonen et al., 2002). These controversial effects are over-linked to specific diet regimen, age, and gender, but none has evaluated the effects associated with chronic ethanol consumption.

It is noteworthy that melatonin presents antioxidant properties both in vivo and in vitro. Melatonin can stimulate the activities and expressions of antioxidant enzymes that contribute to the protection against the damages caused by oxidative stress (Rodriguez et al., 2004). Several studies have demonstrated that melatonin detoxifies a variety of free radicals and reactive oxygen molecules, including hydroxyl radical (OH), superoxide, peroxynitrite, singlet oxygen, and others. Melatonin acting as a free radical scavenger of oxidative products prevents lipid peroxidation in reproductive tissues (Armagan et al., 2006; El-Sokkary et al., 1999; Peyrot and Ducrocq, 2008) and can be an useful tool against the oxidative damage arising from chronic alcoholism (Hu et al., 2009). It is known that the metabolism of ethanol induces reactive oxygen species (ROS) formation, mainly through the P450 CYP2E1 microsomal system evidenced by high rate of oxidized NADPH, where is produced large quantities of superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2) both in humans and ethanol-fed rodents (Gouillon et al., 2000; Ronis et al., 2004). Oxidative stress may be a cause of poor oocyte quality, because it produces severe cell damage, including deterioration of membrane lipids, apoptosis, and inhibition of fertilization (Agarwal et al., 2005; Noda et al., 1991). Melatonin also increases the synthesis of enzymes such as superoxide dismutase (SOD), glutathione (GSH), peroxidase (GSH-Px, GSH), reductase (GSH-Rd), and catalase (Armagan et al., 2006; Subramanian et al., 2007; Tan et al., 2007). Furthermore, recent approaches involving chronic ethanol consumption revealed a depletion of mtGSH content (Fernandez-Checa and Kaplowitz, 2005) and a decline in the enzymatic activity of liver Cu–Zn SOD, catalase, and GSH-Px (Polavarapu et al., 1998). The role of melatonin on the female reproductive tract in ethanol-fed rats is still poorly understood.

In view of the above-mentioned findings, this study was designed to investigate the effects of ethanol, associated or not with melatonin treatment, on overall feed efficiency and oxidative stress in the ovaries of UChB ethanol-preferring rats (10% [v/v] ethanol voluntary drinkers).

MATERIALS AND METHODS

Animals and Experimental Design

Forty adult female rats (*Rattus norvegicus albinus*), 60 days old (225 to 240 grams at baseline), were obtained from the Department of Anatomy, Bioscience Institute/Campus of Botucatu (IBB), UNESP—Univ Estadual Paulista. The animals were randomly divided into 4 groups (n = 10/group). UChB EtOH group: rats fed 10% (v/v) ethanol ad libitum (free choice for water or ethanol) drinking from 2.0 to 6.0 ml/100 g body weight (BW)/d and receiving vehicle solution; UChB Co group: Ethanol-naïve rats without access to ethanol, used as a control group, receiving vehicle solution; UChB EtOH + M group: rats fed 10% (v/v) ethanol (free choice for water or ethanol) drinking from 2.0 to 6.0 ml/100 g BW/d and receiving vehicle + melatonin; and UChB Co + M group: without access to ethanol and receiving vehicle + melatonin. When the UChB EtOH rats reached 60 days of age, they were given during 20 days, a choice between 2 bottles containing either water ad libitum (1) or 10% (v/v) ethanol (2). After this period, 10 animals per group displaying ethanol consumption higher than 2.0 ml of ethanol/100 g BW/d were finally selected according to Mardones and Segovia-Riquelme (1983). To predict ethanol intake, blood acetaldehyde levels serve as good marker and they are remarkably high in UChB rats (Tampier et al., 2008). For this study, the preference ratio associated with ethanol-seeking behavior was about 65%. Besides that, to ensure more efficiency and maintenance of constant consumption throughout the experiment, the animals were kept under observation for 10 days when they started with melatonin treatment. Thus, after 90 days old, females received ethanol and melatonin during 60 consecutive days (Fig. 1*A*). After the melatonin treatment, rats in the morning estrus at 4 AM (or Zeitgeber Time 22:00 ZT 22) monitored by vaginal swabs in a dark room using red light were anesthetized and euthanized by decapitation for further analysis. All animals were individually housed in polypropylene cages (43 cm × 30 cm × 15 cm) with laboratory-grade pine shavings as bedding and also maintained under controlled room temperature (23 ± 1°C) and lighting conditions (12L, 12D photoperiod, lights switched on at 6 AM). The rats received a standard rodent chow (3074 SIF; Purina Ltd, Campinas, Brazil) and filtered tap water ad libitum. Experimental protocols followed the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and were approved by Ethics Committee on Animal Experimentation (Protocol no. 85/07).

Procedures of Melatonin Administration

For the animals designated to receive exogenous melatonin treatment, successive doses of melatonin (100 μg/100 g BW) (M-5250; Sigma Chemical, St. Louis, MO) were dissolved in 95% ethanol 0.04 ml, using 0.9% saline solution as a vehicle (Chuffa et al., 2011;

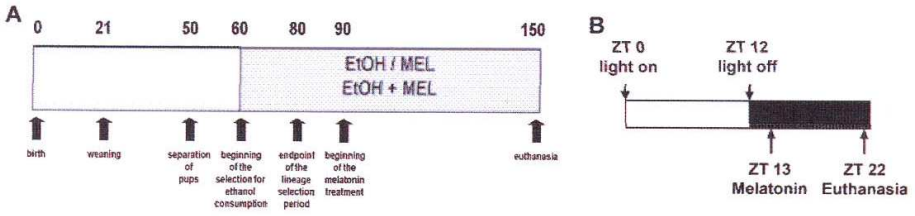

Fig. 1. (**A**) Chronological scheme for overall chronic treatment. (**B**) Schematic protocol used for melatonin (MEL) treatment based on Zeitgeber Time (ZT) corresponding to environmental circadian time. EtOH, ethanol.

Kim and Lee, 2000; Vázquez et al., 2007). The intraperitoneal infusions (only vehicle or vehicle + melatonin) were daily administered between 18:30 and 19:00 PM (ZT 13) (Fig. 1*B*).

Food and Liquid Intake

Feeding content was prepared in lots of 5 days, always at the same time of day (15:00 hours) using a marked test tube and analytical balance (Ohaus Traveler™; Ohaus Corporation, México, D.F, MÉXICO, MX). The profile of liquid ingestion (caloric value of water + ethanol = 7.1 kcal/g ethanol) and food (caloric value of standard chow = 2,930 kcal/kg) were assigned according to the standards of necessary care. Total energy intake (kcal/d) and feed efficiency (weight gain/consumed calories × 100) were evaluated as metabolic parameters. BW was also measured, and melatonin dosages were individually adjusted for each weight. At the end of treatment, the reproductive organs (uterine horn, ovaries, and oviducts) were dissected and weighed. The determination of BW and organ weight was carried out using an analytical balance (OwaLabor, Oschatz, Germany).

Glycemia Measurements

The glycemic index was measured with a blood glucose sensor (One Touch Ultra System kit; Lifescan, Milano, Italy), using blood samples from the caudal vein of animals. To avoid variation, all samples were assessed with an equal volume collected from each animal after 12 hours of fasting.

Assessment of Estrous Cyclicity

During the second half of the experiment, animals exhibiting estrous cycles were accompanied by colpocytological examination (vaginal swabs). Cells detaching from the vaginal epithelium were removed with a pipette (Lab Mate 0.5 to 10 μl; International Labmate Ltd, St. Albans, UK). The filter tips containing 10 μl of 0.9% saline solution (Marcondes et al., 2002) were discarded after the vaginal secretion had been transferred to clean slides. Colpocytological examination time was fixed at 9 AM. Each slide was analyzed under a Zeiss Axiophot II microscope (Carl Zeiss, Oberköchen, Germany) at 10× and 25× magnification and digitally photographed.

Determination of Lipid Hydroperoxide and Antioxidant Systems

After 60 days of melatonin treatment (100 μg/100 g BW/d), the ovaries were rapidly removed. Each right ovary was weighed, and tissue samples of 40 mg were immediately frozen in liquid nitrogen and stored at −80°C. The ovary samples were homogenized using a motor-driven Teflon Potter Elvehjem tissue grinder (Omni International, Kennesaw, GA) in 1.25 ml of cold 0.1 M phosphate buffer (pH 7.4) with the addition of 1 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 10,000×*g* for 15 minutes. The supernatant fraction was removed for the determination of total proteins (Seiva et al., 2010), lipid hydroperoxide LHP (Jiang et al., 1991), and antioxidant substances (Seiva et al., 2008). LHP was measured by Fe^{2+} to Fe^{3+} oxidation in the presence of xylenol orange at 560 nm. Total antioxidant substances (TAS) were assessed by the inhibition of LHP formation. Spectrophotometric assays were carried out using a spectrophotometer with a temperature-controlled cuvette chamber (UV/visible Ultraspec with Swift II software; Pharmacia Biotech, Cambridge, UK).

The enzymatic antioxidant system was investigated using the extracted supernatant. Afterward, GSH-Px activity (E.C. 1.11.1.9.) was analyzed using GSH oxidation reacted with H_2O_2 and cumene hydroperoxide (Nakamura et al., 1974). GSH-Rd activity was evaluated by monitoring NADPH oxidation (reduced nicotinamide adenine dinucleotide phosphate) at 340 nm (Miller and Blakely, 1992). The reactive mixture included 1 mM Tris buffer, pH 8.0, 5 mM EDTA, 33 mM GSSG, and 2 mM NADPH. The activity of SOD (E.C. 1.15.1.1), observable in a reduction of nitroblue tetrazolium by superoxide radicals, was verified by mixing NADH and phenazine methosulfate at a physiological pH (Ewing and Janero, 1995). Catalase (E.C. 1.11.1.6.) activity was assayed during the decomposition of H_2O_2 to $H_2O + O_2$ (Aebi, 1974). The assays of antioxidant activities were performed at 25°C using a μQuant microplate spectrophotometer (MQX 200 with KCjunior software; Bio-Tek Instruments, Winooski, VT).

Statistical Analysis

Statistical comparisons were performed by 2-way analysis of variance (ANOVA) with post hoc Tukey's test. Nonparametric Kruskal–Wallis test complemented by Dunn were applied according to the chosen parameter. All results are given as means (SEM) or median (min; max) values. Significance was set at $p < 0.01$ and $p < 0.05$. The statistical software used was Sigma Plot version 11.0 (Systat Software, Inc., Chicago, IL) and GraphPad Instat version 4 for graphic design (GraphPad Software, Inc., San Diego, CA).

RESULTS

After 60 days of treatment, there were significant differences on BW among the groups. The interaction between ethanol and melatonin promoted a constant decline in BW gain after the second half of treatment, when compared with animals receiving only ethanol (Fig. 2*A*). In the final days of treatment, the UChB Co + M group had higher BW gain compared with animals that were given both melatonin and ethanol (Fig. 2*A*). Initially, based on the time points examined, UChB EtOH and UChB EtOH + M rats exhibited an identical drop in food consumption, differently from the controls (Fig. 2*B*). Only the UChB Co + M rats had increased food intake at several time points, indicating the effects of melatonin, especially during the second half of treatment. After 40 days of treatment, the interaction between melatonin and ethanol caused a reduction in food consumption average than those receiving only ethanol (Fig. 2*B*). Furthermore, after 50 days of treatment, animals receiving ethanol in combination with melatonin showed a marked loss of appetite with a fall in food intake. Feed efficiency was reduced in UChB EtOH rats and became even more pronounced when melatonin was given. The group that received ethanol and melatonin had the lowest amount of stored calories, half of that found in UChB Co + M rats (Fig. 3*A*). Glucose levels were reduced after melatonin treatment, except in the animals receiving ethanol, despite these animals already demonstrated a considerable reduction in glucose levels (Fig. 3*B*). There was a negative correlation between ethanol intake and exogenous melatonin administration throughout the 60 days of treatment ($r = -0.79$; $p < 0.001$), evidenced by a mild reduction in total ethanol consumption observed prior to starting and during the experiment (Fig. 3*C*).

In melatonin-treated rats, energy and food intake declined toward the end of treatment. As shown in Table 1, during overall 60-day treatment, calories derived from ethanol and food, in the presence of melatonin, were poorly taken by the

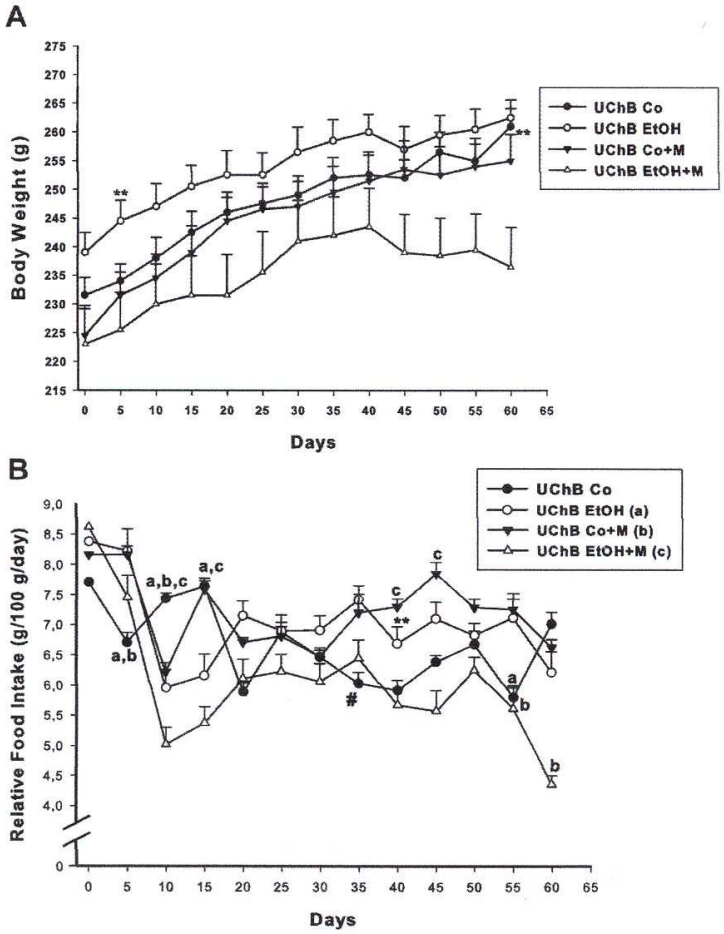

Fig. 2. (**A**) Ethanol (EtOH) and melatonin (M) effects (100 μg/100 g/d) on body weight gain in all experimental groups during 60 days of treatment. $^{**}p < 0.01$ versus UChB EtOH + M group at 5, 20, 45 to 60 days. At the end of treatment, UChB EtOH + M showed significant difference from UChB Co + M group. (**B**) Influence of melatonin and ethanol administration on relative food intake over 60 days in rats receiving standard chow. $^{**}p < 0.01$ versus UChB EtOH + M group on days 40 to 60. $^{\#}p < 0.01$ versus UChB Co + M group on days 35 to 55. $^{a-c}$Significant difference from UChB EtOH, UChB Co + M and UChB EtOH + M groups, respectively. Values are expressed as mean ± SEM (n = 10/group). Two-way ANOVA with Tukey's post hoc test. Day 0: food consumption before beginning of the treatment.

UChB EtOH rats. Following the experiment, the interaction between ethanol and melatonin contributed to the decrease in food and total energy intake, but not the energy provided by ethanol itself. In ethanol-preferring rats, the relative daily water intake was significantly decreased over the experiment, regardless of melatonin. It was also proved that after melatonin administration, total ethanol intake was reduced by 14.4% whereas untreated animals increased their consumption by about 11.3% (Table 1).

During second half of daily melatonin treatment, from the 4th until the 8th week of intervention, the vaginal smears revealed estrous cycle irregularities, namely, more extensive cycles in UChB EtOH + M rats than in other groups, with an elevated frequency of prolonged diestrous phase when compared with those receiving only ethanol. After 4 weeks of cytological examination, the increase in metaestrous stage was even more evident in UChB Co + M group (Table 2). Despite these irregularities, there were no anovulatory cycles.

The relative and total ovarian weights were significantly reduced in animals receiving the ethanol plus melatonin in relation to those receiving either ethanol or melatonin (Table 3). Also, the combination of ethanol and melatonin negatively affected the uterine horns weights. On the other hand, melatonin had no significant effect on oviduct weight (Table 3).

With respect to ovarian tissues, total protein concentration was high in the UChB EtOH and UChB Co + M groups. The interaction between ethanol and melatonin showed a significant decrease in TAS concentrations when compared with

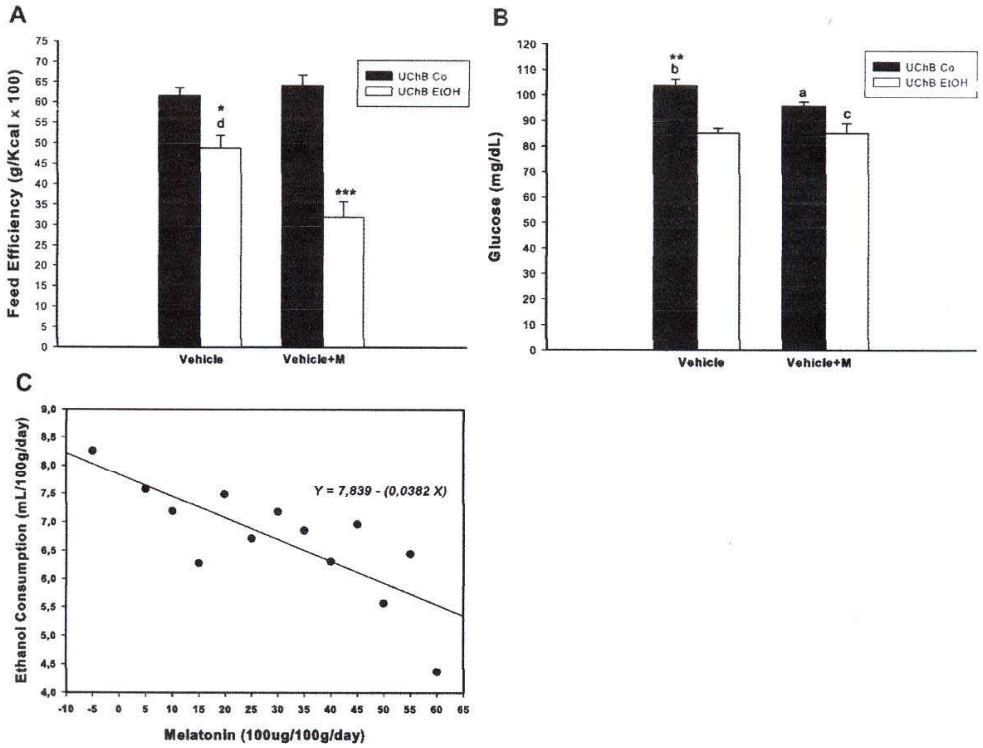

Fig. 3. (**A**) Feed efficiency (%) over the 60 days of melatonin administration (100 μg/100 g/d) and ethanol (EtOH) intake. $^{d}p < 0.05$ versus UChB EtOH + M group; $^{*}p < 0.05$, $^{***}p < 0.001$ versus UChB Co + M group. (**B**) Effects of melatonin administration alone or in combination with ethanol intake on plasma glucose levels of nonfasted rats. $^{b}p < 0.01$ versus UChB EtOH group; $^{a,c}p < 0.05$ versus UChB Co and UChB EtOH + M groups, respectively; $^{**}p < 0.01$. Values are expressed as mean ± SEM (n = 10/group). Two-way ANOVA complemented by Tukey's test. (**C**) Scattergram showing the correlations between melatonin administration and ethanol consumption throughout the 60 days ($r < -0.79$; $p < 0.001$). Day 0: beginning of the melatonin treatment.

Table 1. Status of Food Consumption (g/d), Total Energy Intake (kcal/d), Relative Water/Ethanol Intakes (ml/100 g/d), and Ethanol-Derived Calories (kcal/d) in Rats Receiving Only Vehicle and/or Melatonin.

	Vehicle		Melatonin	
Parameters	UChB Co	UChB EtOH	UChB Co	UChB EtOH
Food consumption	16.32 ± 0.40	15.90 ± 0.38	17.03 ± 0.51	14.49 ± 0.57[a]
Energy intake (food + ethanol)	47.83 ± 1.17	49.59 ± 1.26	49.90 ± 1.50	46.36 ± 1.90[a,b]
Relative water intake	13.47 ± 0.15	9.89 ± 0.30[c]	12.58 ± 0.44	7.48 ± 0.58[a]
Ethanol intake (PT)	0.00	4.86 ± 0.67	0.00	6.58 ± 0.62
Ethanol intake (AT)	0.00	5.48 ± 0.57	0.00	5.63 ± 0.57[*]
Ethanol calories	0.00	2.99 ± 0.14	0.00	3.41 ± 0.21

Values are expressed as mean ± SEM. N = 10/group.
Means followed by lowercase letters indicate statistical differences among the groups ($p < 0.05$). Two-way ANOVA with post hoc Tukey's test.
PT, prior treatment; AT, after treatment; EtOH, ethanol.
$^{a,b}p < 0.05$ versus UChB EtOH and UChB Co + M groups, respectively.
$^{c}p < 0.01$ versus UChB Co group.
$^{*}p < 0.05$ versus UChB EtOH + M (PT).

animals receiving only melatonin (Table 4). The LHP/TAS ratio was enhanced after ethanol intake, because LHP levels increase with chronic alcoholism; however, no changes were found after melatonin treatment (Table 4 and Fig. 4).

LHP levels were significantly reduced after melatonin treatment. In UChB EtOH group, the levels of LHP were higher than those found in control group in which the ethanol-treated rats evidenced the highest levels of LHP formation (note that melatonin improved LHP levels at 18.5% in UChB EtOH group) (Fig. 4). SOD and GSH-Px activities were increased with the interaction of ethanol on melatonin, compared with both groups receiving either

Table 2. Effects of Ethanol and Melatonin on Estrous Cyclicity (Days) and Frequency (%) of Lengthiness in Each Phase Among the Experimental Groups ($N = 10$/Group).

Groups	Cycle duration (days)	Estrus persistent/cycle (%)	Metaestrus persistent/cycle (%)	Diestrus persistent/cycle (%)
UChB Co	7.45 ± 0.50	6.67 (0; 26.67)	13.33 (0; 40.00)[a]	6.66 (0; 10.00)
UChB Co + M	6.55 ± 0.34	13.33 (0; 30.00)	18.33 (6.67; 40.00)	15.00 (6.67; 26.67)
UChB EtOH	6.45 ± 0.44	16.67 (0; 26.67)	16.67 (0; 43.33)	10.00 (3.33; 23.33)
UChB EtOH + M	10.35 ± 1.10**	6.67 (0; 30.00)	16.66 (0; 36.67)	21.66 (3.33; 46.67)[b]

Values are expressed as mean ± SEM and median (minimum–maximum).
EtOH, ethanol.
[a] $p < 0.05$ versus UChB Co + M group.
[b] $p < 0.05$ versus UChB EtOH group. Kruskal–Wallis test complemented by Dunn.
** $p < 0.01$ versus UChB Co + M and UChB EtOH groups, respectively. Two-way ANOVA with post hoc Tukey's test.

Table 3. Data of Total (grams) and Relative (g/100 g Body Weight) Reproductive Organs Weights in Female Rats Receiving Vehicle or Melatonin at the End of the Treatment.

	Vehicle		Melatonin	
Parameters	UChB Co	UChB EtOH	UChB Co	UChB EtOH
Ovary weight	0.098 ± 0.01	0.098 ± 0.01	0.098 ± 0.02	0.069 ± 0.02[a,b]
Ovary relative weight	0.037 ± 0.00	0.037 ± 0.00	0.038 ± 0.01	0.029 ± 0.01[a,b]
Oviduct weight	0.034 ± 0.01	0.034 ± 0.00	0.033 ± 0.01	0.030 ± 0.01
Oviduct relative weight	0.013 ± 0.01	0.013 ± 0.00	0.013 ± 0.00	0.013 ± 0.01
Uterine horn weight	0.52 ± 0.13	0.51 ± 0.13	0.50 ± 0.06	0.40 ± 0.11[a]
Uterine horn relative weight	0.19 ± 0.04	0.19 ± 0.06	0.20 ± 0.02	0.17 ± 0.04

Values are expressed as mean ± SEM. $N = 10$/group.
Two-way ANOVA complemented by the Tukey's test.
EtOH, ethanol.
[a] $p < 0.01$ versus UChB EtOH group.
[b] $p < 0.001$ versus UChB Co + M group.

Table 4. Determinations of Total Protein (%) mg Protein/mg Tissue, Total Antioxidant Substances (% TAS) and Lipid Hydroperoxide (LHP)/TAS Ratio (g/Tissue) in Ovarian Tissue of 10% (v/v) Ethanol (EtOH)-Preferring Rats Receiving Melatonin at Doses of 100 μg/100 g BW/d for 60 Days.

	Vehicle		Melatonin	
Parameters	UChB Co	UChB EtOH	UChB Co	UChB EtOH
Protein	38.80 ± 3.33[a,b]	50.10 ± 6.26	48.60 ± 4.66	42.89 ± 5.53
TAS	64.84 ± 1.67	63.22 ± 2.25	67.02 ± 1.49	61.70 ± 1.98[b]
LHP/TAS	9.54 ± 0.27[a]	13.22 ± 0.23	9.84 ± 0.15	11.69 ± 0.32

Values are expressed as mean ± SEM. $N = 7$ animals/group.
Letters indicate statistical differences among the groups ($p < 0.05$). Two-way ANOVA complemented by Tukey's test.
[a] $p < 0.001$ versus UChB EtOH.
[b] $p < 0.05$ versus UChB Co + M.

ethanol or melatonin (Fig. 5*A* and 5*B*). Catalase activity did not differ between ethanol-treated rats, and conversely, when melatonin alone was given, the catalase activity was completely restored (Fig. 5*C*). All melatonin-treated animals showed an increase in GSH-Rd activity compared with rats that received or not ethanol (Fig. 5*D*).

DISCUSSION

Appetite regulation and energy intake are fundamental for the maintenance of caloric balance and BW. Ethanol and melatonin are thought to interact directly on the maintenance of BW, mainly during the second half of treatment. Previous studies involving several doses and routes of melatonin administration in rats found an associated BW reduction in those that had been castrated (Puchalski et al., 2003), in adult rats (Bojková et al., 2008; Rasmussen et al., 2001) and in those consuming a high fat diet (Prunet-Marcassus et al., 2003). On the other hand, when melatonin is used in nocturnal progressive doses ranging from 3,000 to 15,000 pg/ml, it promotes food intake and BW gain in both male and female rats (Angers et al., 2003). Ethanol per se produces weak satiating signals and in humans, depending on metabolism and nutritional status, appears to increase food consumption and appetite (Yeomans, 2004; Yeomans et al., 1999). These differences may rely on factors such as age, gender, or body composition of the experimental animals. Taken together, melatonin seems to be related with the periodicity of food

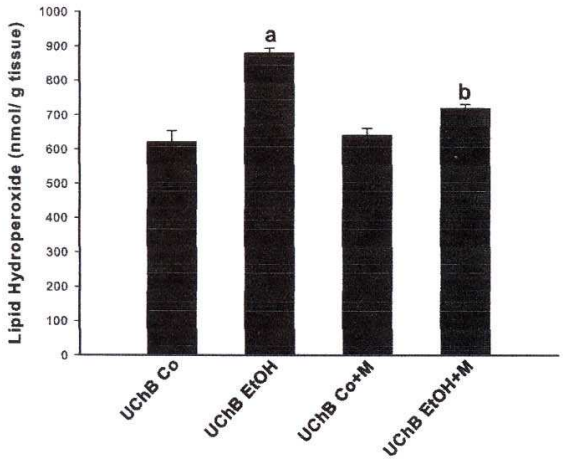

Fig. 4. Lipid hydroperoxide concentration (nmol/g tissue) in ovarian tissue of control and ethanol (EtOH)-treated rats, receiving or not melatonin (M) (100 μg/100 g/d) after 60 days of treatment. $^{a}p < 0.05$ versus UChB Co group; $^{b}p < 0.001$ versus UChB EtOH group. Values are means ± SEM (n = 10/group). Two-way ANOVA complemented by Tukey's test.

intake by influencing the gastrointestinal tract (Bubenik, 2002). Taking into account that NADH, produced by ethanol-acetaldehyde conversion, plays key roles in cellular bioenergetics and can modulate fatty acid synthesis as well as suppress β-oxidation (Lieber, 2004), these mechanisms could explain, in part, the differences in BW associated with food consumption by the animals receiving either ethanol or melatonin and both ethanol and melatonin. In addition, the caloric value of ethanol (empty calories) did not contribute to BW gain, and furthermore, lipid synthesis is blocked by the inhibition of glucose-6-phosphate dehydrogenase (Ayene et al., 2002), which is a key enzyme in the pentose shunt for generating NADPH and essential for lipogenesis. Similar approaches have been obtained with rats submitted to various diet regimens and exposed to chronic ethanol intake for 20 weeks (Smith et al., 2008) and 8 consecutive weeks (Monteiro et al., 2009).

Scalera and colleagues (2008) also reported reduction in food consumption and increased leptin levels in melatonin-treated rats. In rabbits, treatment with melatonin was followed by 4 weeks of reduced food consumption, BW, and glucose levels (Hussein et al., 2007). Also supporting our findings, Strbák and colleagues (1998) demonstrated that when ethanol consumption rises, food consumption is often reduced, depending on age and particular lineage. In female rats, ethanol preference has also been linked to a reduction in water consumption (Bell et al., 2004). Considering our rat model (UChB), the low water intake occurred because of increased ethanol/ethanol + water ratio. As observed in the UChB Co + M group, ethanol deprivation may lead to withdrawal-induced food intake increases (Kampov-Polevoy et al., 2004; Krahn et al., 2006). In this condition, the melatonin would interact synergistically.

Feed efficiency was reduced concurrently with ethanol consumption and became more pronounced when combined with melatonin, resulting in a low calorie-derived BW gain. This association suggests changes in energetic metabolism and in the ability to store energy as fat mass. Additionally, glucose blood levels were lower in the presence of ethanol or melatonin. In fact, it is well documented that ethanol inhibits gluconeogenesis and melatonin plays a role in carbohydrate metabolism related to insulin sensitivity (Muhlbauer et al., 2009; She et al., 2009). As expected, the effects of ethanol in combination with melatonin promoted a greater reduction in glucose levels. Similar studies have demonstrated that both melatonin (Prunet-Marcassus et al., 2003) and chronic ethanol consumption (Choi et al., 2006) induced low feed efficiency in rats beyond the effects that ethanol alone has on malnutrition (DiCecco and Francisco-Ziller, 2006).

Ethanol consumption decreased after the beginning of melatonin treatment. Accordingly, the ethanol intake dropped when melatonin (25 μg/animal/d) was administered over 11 weeks (Rudden and Symmes, 1981). Noticeably, it has been reported the prominent actions of melatonin on the opioidergic system, producing the analgesic effect in a dose-dependent manner that is similar to naloxone (opioid receptor antagonist) (Yu et al., 2000). Furthermore, whether melatonin is able to stabilize the reward system (Tahsili-Fahadan et al., 2005), it could be avoiding the ethanol reinforcement. Nevertheless, the effects of melatonin on ethanol dependence syndrome remain to be investigated (Arnedt et al., 2007; Fonzi et al., 1994).

Following chronic ethanol consumption, the main time-dependent effects over 60-day treatment have already been confirmed by our previous study in which ovarian tissue was structurally compromised (Chuffa et al., 2009). The ethanol-induced alterations include higher incidence of degenerating granulosa cells and follicular atresia, fluctuations in follicular fluid composition, ultrastructural changes such as autophagy, excessive lipid droplets formation, as well as disruption of follicular basement membrane. In this context, melatonin administration may act as a protective factor by preventing ethanol-induced ovarian failures.

In this study, rats receiving only ethanol or melatonin presented persistent estrus, while the positive interaction between ethanol and melatonin promoted the longest estrous cycles, which featured an extended diestrous phase. It is known that melatonin dissolved in drinking water can increase the frequencies of diestrous or estrous phases in rodents (Kachi et al., 2006) by modulation of GnRH hormones. Furthermore, rats receiving melatonin (200 μg/100 g BW/d) showed longer estrous periods, reduction in polycystic ovary syndrome, and ovary weight (Prata Lima et al., 2004). As seen before, there may also be a positive interaction between ethanol and melatonin for decreasing ovarian weights, because they act on reproduction-linked hormones. It was clearly demonstrated that high melatonin levels is directly related to functional hypogonadotropic hypogonadism (Bergiannaki et al., 1995; de Roux

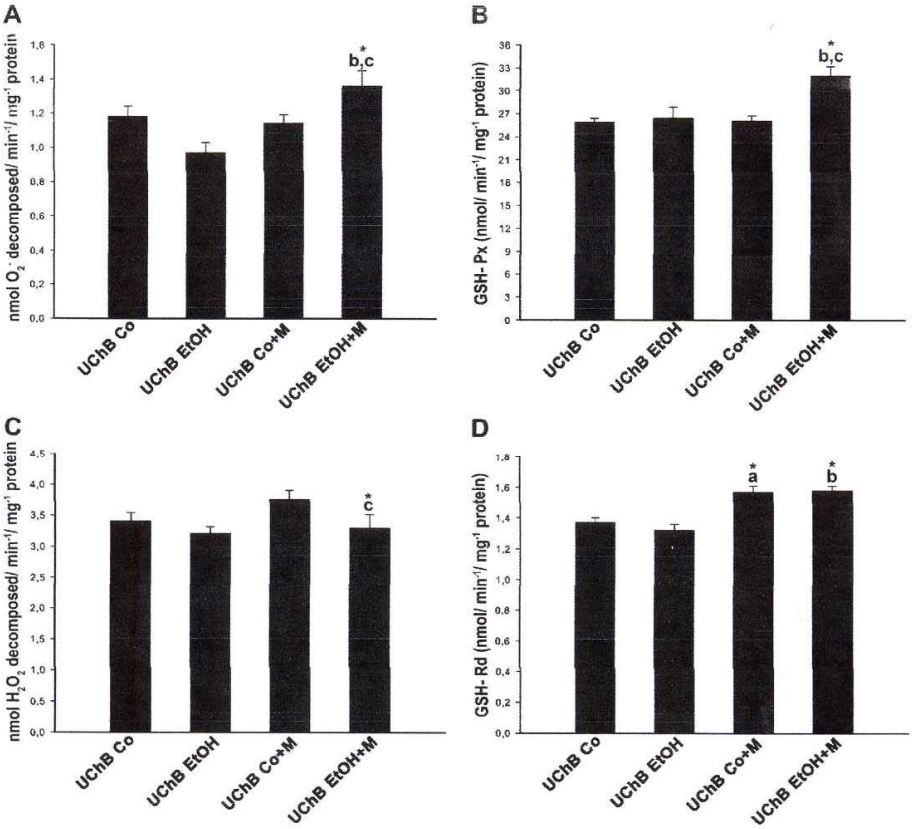

Fig. 5. Antioxidant activity (nmol/mg protein) in ovarian tissue of UChB rats receiving or not melatonin (M) (100 μg/100 g/d) after 60 days of treatment. (**A**) Activity of superoxide dismutase. (**B**) Activity of glutathione peroxidase (GSH-Px). (**C**) Activity of catalase. (**D**) Activity of glutathione reductase (GSH-Rd). $^{a-c}$Significant differences from UChB Co, UChB EtOH, UChB Co + M groups, respectively; $^*p < 0.05$. Values are expressed as means ± SEM (n = 10/group). Two-way ANOVA complemented by Tukey's test. EtOH, ethanol.

et al., 2003). Moreover, administration of exogenous melatonin induces a decrease in luteinizing hormone (LH) secretion, which in turn blocks the ovulation, leading to an extensive luteal phase (Voordouw et al., 1992), thus providing endocrine disruption and gonadal atrophy as it was also noted. Consistently, our previous study has found a reduction in relative ovary weight and an extensive estrous phase in UChB strain and it was associated with fall in LH levels (Chuffa et al., 2009). In this context, the addition of melatonin may suppress further release of GnRH, resulting in prolonged diestrous. On the other hand, Dardes and colleagues (2000) pointed out that low serum levels of melatonin tend to increase the duration of estrus, while treatment with melatonin was effective in regulating the estrous cycle.

The total protein concentration in ovaries of UChB EtOH and UChB Co + M rats was enhanced, suggesting increased enzymatic activity because of cellular metabolism. Particularly, in UChB EtOH rats, melatonin did not influence TAS concentration. However, the LHP/TAS ratio was increased when only ethanol was given. Indeed, to validate these findings, it is necessary to analyze the individual enzymatic and nonenzymatic antioxidant status.

There seems to be a little doubt that ethanol-induced oxidative stress is linked to the metabolism of ethanol. As a result of ethanol and subsequent acetaldehyde oxidation, there is a significant increase in the hepatic NADH/NAD^+ redox ratio (Das et al., 2005). Under a variety of pathophysiological conditions, including acute and chronic ethanol consumption, the increased levels of NADPH-oxidase generates O_2^- and H_2O_2, which in the presence of Fe^{3+}, produces powerful oxidants such as the OH (Kessova and Cederbaum, 2003), enhancing the lipid peroxidation (Le Lan et al., 2004). Ethanol is well reported to deplete GSH levels via its own pro-oxidative character or by inhibiting the mitochondrial GSH transporter (Kannan et al., 2004; Wheeler et al., 2003). Interestingly, the LHP levels were higher in animals consuming ethanol, similar to those described by other authors (Ashakumary and Vijayammal, 1996), and underwent a remarkable reduction after the administration of melatonin, confirming a positive interaction.

Undoubtedly, melatonin has an important role in attenuating ROS deleterious effects by promoting mRNA synthesis for antioxidant enzymes (Rodriguez et al., 2004). Considering these functions, the protective effect observed in the ovaries is believed to be attributed to both properties of melatonin as ROS scavenger and increasing the antioxidant status (Chuffa et al., 2011). Similar results elucidating the protective effect of melatonin and other antioxidants have been found in the testis of ethanol-treated rats (Oner-Iyidogan et al., 2001). According to Amanvermez and colleagues (2005), the chronic ethanol consumption leads to lipid and protein oxidation in rat ovaries and also reduces the GSH content in testes. Replacement therapy using melatonin and 17β-oestradiol in ovariectomized rats caused reduction in lipid peroxidation and increased GSH content and SOD activity (Feng and Zhang, 2005).

As previously described, ROS such as OH, peroxyl radicals (ROO^-), and H_2O_2 are known to be detrimental to the oocyte (Tamura et al., 2008), and in this study, doses of melatonin increased SOD and GSH-Px activities in ethanol-preferring rats. Similar evidence has been documented after treatment with vitamin E in adult rat ovaries (Rao et al., 2009). Moreover, supporting these findings, daily injection of melatonin (10 mg/kg) in rat tissues increased SOD activity after 7 days (Ozturk-Urek et al., 2001), while Liu and Ng (2000) found higher SOD activity after a single injection of melatonin (5 mg/kg). In accordance with our results, Tomas-Zapico and Coto-Montes (2005) and Rodriguez and colleagues (2004) emphasized that administration of melatonin promotes increased activity of SOD, GSH-Px, and GSH-Rd. Melatonin did not affect catalase activity in the ovaries. It is likely that this enzyme is being used for peroxisome metabolism during chronic ethanol consumption.

In conclusion, we demonstrated that there are interactions between melatonin and ethanol on feed efficiency, with melatonin acting as a hormone regulating weight gain, stored calories, and ethanol intake. Moreover, the administration of melatonin, although has caused estrous cycle disruption, was able to protect the ovaries of UChB rats against oxidative stress, preventing attack by free radicals, and promoting antioxidant defenses.

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REPRODUCTIVE BIOLOGY AND ENDOCRINOLOGY

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Melatonin reduces LH, 17 beta-estradiol and induces differential regulation of sex steroid receptors in reproductive tissues during rat ovulation

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Abstract

Background: Melatonin is associated with direct or indirect actions upon female reproductive function. However, its effects on sex hormones and steroid receptors during ovulation are not clearly defined. This study aimed to verify whether exposure to long-term melatonin is able to cause reproductive hormonal disturbances as well as their role on sex steroid receptors in the rat ovary, oviduct and uterus during ovulation.

Methods: Twenty-four adult Wistar rats, 60 days old (+/- 250 g) were randomly divided into two groups. Control group (Co): received 0.9% NaCl 0.3 mL + 95% ethanol 0.04 mL as vehicle; Melatonin-treated group (MEL): received vehicle + melatonin [100 μg/100 g BW/day] both intraperitoneally during 60 days. All animals were euthanized by decapitation during the morning estrus at 4 a.m.

Results: Melatonin significantly reduced the plasma levels of LH and 17 beta-estradiol, while urinary 6-sulfatoximelatonin (STM) was increased at the morning estrus. In addition, melatonin promoted differential regulation of the estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR) and melatonin receptor (MTR) along the reproductive tissues. In ovary, melatonin induced a down-regulation of ER-alpha and PRB levels. Conversely, it was observed that PRA and MT1R were up-regulated. In oviduct, AR and ER-alpha levels were down-regulated, in contrast to high expression of both PRA and PRB. Finally, the ER-beta and PRB levels were down-regulated in uterus tissue and only MT1R was up-regulated.

Conclusions: We suggest that melatonin partially suppress the hypothalamus-pituitary-ovarian axis, in addition, it induces differential regulation of sex steroid receptors in the ovary, oviduct and uterus during ovulation.

Background

Melatonin (*N*-acetyl-5-methoxytryptamine) also known as "chemical expression of darkness" is an indolamine produced by pineal gland and secreted in a circadian manner during the night [1]. It is indisputable that melatonin has been potentially implicated as a therapeutic agent in several conditions. In mammals, melatonin can affect the reproductive function through activation of receptor sites within the hypothalamic-pituitary-gonadal axis [2]. Previous evidence has suggested that changes consistent with inhibition of GnRH release occur after melatonin implants [3]. Melatonin is found inside ovarian follicles [4], thus proving its direct action in ovarian function. It has also been proposed that pre-ovulatory follicles contain high amount of melatonin which were indirectly linked to the 17 β-estradiol (E2) and progesterone (P4) synthesis [5]. In melatonin-deprived rats, an increased estrous frequency was inversely related to the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels [6]. According to Soares et al. [7],

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the low melatonin levels lead to a reduction of P4 and its receptors while increasing E2 levels. Moreover, it was reported that melatonin might decrease E2 levels during the premenopausal period [8]. Most studies investigating the mechanism(s) by which melatonin modulates the reproduction have focused mainly in the pituitary and hypothalamus or in evaluating the effects of pinealectomy, with little attention devoted to the relationship between exogenous melatonin treatment and female reproductive tissues during ovulation. Furthermore, these reproductive actions promoted by long-term melatonin administration in a non-seasonal breeder (e.g. rat) are yet poorly understood.

More recently, it was noted that administration of melatonin at night induces prolonged diestrous phase in normal rats [9,10]. There seem to be little doubt that exogenous melatonin restores the basal gonadotropin concentrations (FSH and LH) in aged rats as similar to young rats [11], also having a stimulatory effect on E2 levels and pituitary responsiveness to LHRH [12]. Nevertheless, the effects of melatonin on reproductively active rats, at the timing of ovulation, remain a matter of debate.

In reproductive system, melatonin may interact with sex steroids [13-15]. It is well-known that sex steroid receptors might regulate a variety of physiological responses in the ovary, oviduct and uterus tissue when they are activated [13,16,17]. Estrogen receptor (ER), a member of the nuclear receptor superfamily, has two functional isoforms designated as ER-α and ER-β [18]. In ovaries, the granulosa cells express higher levels of ER-β than ER-α, while ER-β is reportedly expressed at lower levels in uterus [19]. Importantly, a repetitive loss of ER-β expression or a decrease in ER-β/ER-α ratio is linked to ovarian epithelial tumorigenesis [20]. Other study showed a decreased number of uterine estrogen receptor with concomitant increase of PR after 15-day melatonin treatment [13]. However, none have evaluated the role of melatonin considering different steroid receptors isoforms. Despite of considerable effort, the effects of long-term melatonin focused on reproductive hormones and its specific receptors involving the ovaries, oviducts and uterus are not well discussed.

Progesterone receptors (PRs), one of the well-characterized estrogen-regulated genes, are expressed as PR-A and PR-B isoforms [21]. PRA has a transactivation role in some cells whereas it functions as a repressor of PRB (heterodimer form) and androgen receptor [16]. Although it has long been emphasized that E2 up-regulates PR, little is known as to whether PRA and B expression is modulated by either E2 or melatonin. Furthermore, E2 seems to alter expression from PRB to PRA dominancy in oviduct and uterus [22,23]. Not surprisingly, PRA, but not PRB expression, is necessary and sufficient for ovulation process [17]. More recently, melatonin significantly increased P4 as well as the number of total PR in ovarian tissue at proestrus [15]. Otherwise, Soares Jr. et al. [7] found a diminution of 6-sulfatoximelatonin (STM) metabolite and PR levels after pinealectomy surgery. To date, the melatonin effects on selective PRA and PRB have not been demonstrated.

Melatonin signals through at least two G protein-coupled receptors, the MTR1 and MTR2 membrane receptors, or via putative cytoplasmatic/nuclear sites mediating the physiological responses [24,25]. Among other actions, MTR1-binding melatonin is thought to cause down-regulation of both ER-α protein and ER-α mRNA [26] and, alternatively, it may inhibit the ligation of E2-ER complex to the estrogen response elements (ERE) on DNA [14,26], thus dampening the E2-mediated effects. Since melatonin is a potential agent controlling the reproduction, its long-term effects related to reproductive tissues, at estrous phase, have never been identified through MT1R receptors.

Therefore, the present study was undertaken to verify whether exposure to long-term melatonin is able to cause reproductive hormonal disturbances as well as their role upon sex steroid receptors in the rat ovary, oviduct and uterus during ovulation process.

Methods

Animals and experimental design

Twenty-four adult female rats (*Rattus norvegicus albinus*), 60 days old (± 250 g) were obtained from the Department of Anatomy, Bioscience Institute, UNESP - Univ Estadual Paulista, Campus of Botucatu. All animals were housed in polypropylene cages (43 cm × 30 cm × 15 cm) with laboratory-grade pine shavings as bedding and also maintained under controlled room temperature (23 ± 1°C) and lighting conditions (12 L, 12 D photoperiod, lights switched on at 6 a.m). Initially, the animals were randomly divided into two experimental groups (n = 12/group). Control group: rats fed standard chow and tap water *ad libitum* and receiving 95% ethanol 0.04 mL + 0.9% NaCl 0.3 mL (1:7 v/v) as vehicle; Melatonin-treated group: rats fed standard chow and tap water *ad libitum* receiving vehicle + melatonin. At 90 days old, females started to receive successive doses of melatonin over 60 consecutive days. After melatonin treatment period, all rats were monitored by vaginal swabs in a dark room using a red dim illumination, and during the early morning of estrus (timing of ovulation) at 4 a.m (or Zeitgeber Time, (ZT) 22, corresponding to the environmental circadian time) they were anesthetized and euthanized by decapitation for further analysis. Experimental protocols were previously accepted by Ethical Committee of the Institute of Bioscience/UNESP, Campus of Botucatu, SP, Brazil (Protocol n° 85/07).

Procedures of melatonin administration

Successive doses of melatonin [100 μg/100 g BW] (M-5250, purchased from Sigma Chemical, St Louis, MO) were dissolved in 95% ethanol 0.04 mL, using 0.9% NaCl solution as a vehicle [9]. The intraperitoneal infusions (only vehicle or vehicle + melatonin) were daily administered between 18:30 - 19:00 p.m (ZT 13).

Urine and reproductive organs collection

In the evening before they were killed, all animals received the last injection of melatonin and they were kept inside metabolic cages (Techniplast, Exton, PA, USA) by 10 h in order to collect individual urine samples. Thereafter, all samples were centrifuged at 10,000 × g for 20 min at 4°C and stored at - 20°C. On the next day and after sacrifice, all reproductive organs (ovaries, oviducts and uterine horns) were entirely dissected and weighed for further assays.

Sex hormones assay

Blood samples were collected from the trunk of decapitated rats into heparinized tubes. Afterwards, plasma was obtained by centrifugation at 1,200 × g for 15 min at 4°C and stored at - 20°C until assayed by radioimmunoassay (RIA). Plasma samples were assayed for FSH and LH by double-antibody RIA with specific kits provided by the "National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases" (NIADDK, Baltimore, MD, USA). The FSH primary antibody was anti-rat FSH-S11, and the standard FSH-RP2. The antiserum for LH was LH-S10 using RP3 as reference. The lower limit of detection for FSH and LH was 0.2 ng/mL and the intra-assay coefficient of variation was 3% and 4%, respectively. Plasma concentrations of E2 and P4 were determined using Estradiol and Progesterone Maia kits (Biochem Immunosystems, Serotec, Italy). The lower detection limit and the intra-assay coefficient of variation were respectively 7.5 pg/ml and 2.5% for E2 and 4.1 ng/ml and 3.7% for P4. All samples were measured in duplicate and at different dilutions, if necessary. In order to prevent interassay variation, all samples were assayed in the same RIA.

Determination of plasma melatonin and urinary 6-sulfatoximelatonin (STM)

Melatonin was initially extracted from plasma (n = 12 samples/group) using methanol HPLC grade followed by separation into columns Sep-Pak Vac C-18, reverse phase, 12.5 nm (Water Corporation, Milford, Massachusetts, USA). Thereafter, 50 μL of reconstituted samples were assayed with coat-a-count melatonin ELISA kits and measured photometrically at a wavelength of 405 nm. The intra-assay coefficient of variation was 3%. Urinary 6-STM (a metabolite of melatonin) was assayed with solid-phase melatonin sulfate ELISA kits and, finally, read at 450 nm. The intra-assay coefficient of variation was 5.2%. Samples were double assayed at the time to avoid interassay variations. All reagents and microtiter plate were provided by IBL (IBL International, Hamburg, Germany).

Western blotting analysis and protein quantification

After 60 days of melatonin treatment (100 μg/100 g BW/day), the ovaries, oviducts and uterine horns were rapidly removed and tissue samples of 50 mg were immediately frozen in liquid nitrogen and stored at -80° C. All tissues were homogenized with RIPA lysis buffer (Pierce Biotechnology, Rockford, IL, USA), 10X (0.5 M Tris-HCl, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA, pH 7.4) and protease inhibitor cocktail (Sigma Chemical Co.) using a homogenizer (IKA® T10 basic Ultra, Staufen, Germany). Aliquots containing 1:10 (v/v) of Triton X-100 were added to homogenates and samples were placed on dry ice under agitation by 2 h in order to improving extraction. These suspensions were centrifuged at 21,912 × g for 20 min at 4°C and the pellet discarded. The protein concentrations were measured by the Bradford micro-method for colorimetric determination. Total proteins were dissolved in 1.5 × sample buffer previously described by Laemmli and used for SDS-PAGE (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein (70 μg) of each sample were loaded per well onto preformed gradient gels, 4-12% acrylamide (Amersham Biosciences, Uppsala, Sweden) with a Tris-glycine running buffer system for electrophoresis (60 mA fixed during 2 h). After electrophoresis, total proteins were electro-transferred (200 mA fixed by 1 h 30 min) onto 0.2 μm nitrocellulose membranes in a Tris-glycine-methanol buffer. Kaleidoscope Prestained Standards (Bio-Rad) were used as molecular weight markers. Thereafter, the membranes were blocked with TBS-T solution containing 3% BSA at room temperature (RT) for 60 min and then incubated at 4°C overnight with rabbit primary antibody AR-N20 anti-androgen receptor (AR); rabbit clone E115 anti-ERα; rabbit clone 68-4 anti-ERβ; mouse monoclonal [C262] anti-PRA and PRB and rabbit polyclonal anti-MT1R (dilutions of 1:1000; 1:250; 1:500; 1:350; 1:500; 1:500 were carried out at 1% BSA, respectively). This was followed by washing 3 × 5 min in TBS-T solution and then incubated for 2 h at RT with rabbit or mouse HRP-conjugated secondary antibodies (diluted 1:1000 in 1% BSA; Sigma, St. Louis, MO, USA). After sequential washing with TBS-T, signals were enhanced and peroxidase activity was finally detected by mixing 10 mL PBS, 8 μl H_2O_2 and 0.02 g diaminobenzidine (DAB) chromogen (Sigma Chemical Co.). Immunoreactive bands of each protein (arbitrary units) were

obtained from separate blots of six rats/group using image analysis software (NIS-Elements, Advanced Research, Nikon). β-actin was used as an endogenous control and all results were expressed as mean ± SEM. Immunoblotting concentrations (%) were represented as optical densitometry values (band intensity/β-actin ratio).

Statistical analysis

Data of plasma FSH, LH, E2, P4, melatonin, urinary 6-SMT and western blotting analysis were performed by Student's t test with independent samples. Statistical significance was set at $P < 0.05$ and significant results are expressed as mean ± SEM. The statistical software used was *GraphPad Instat version 4 and Sigma Plot version 11.0* for graphic design.

Results

Plasma sex hormones, melatonin and urinary 6-STM levels

After eight weeks of treatment, total LH and E2 levels were reduced in melatonin-treated rats ($p < 0.05$). Conversely, FSH and P4 levels had not been influenced by melatonin at the estrus phase ($p > 0.05$). These data confirmed our previous reports in which long-term melatonin administration leads to a reduced ovarian mass and prolonged metaestrus and diestrus duration, without blocking ovulation (recently published data). Additionally, there was no evidence for increased plasma melatonin levels in animals receiving the treatment, but the urinary 6-STM levels were significantly higher at the morning of estrus ($p < 0.01$; Figure 1A-F).

Analysis of ovarian AR, ER-α, ER-β, PRA, PRB and MT1R levels after treatment

Sex steroid receptors in reproductive female tract were differentially expressed at the end of melatonin treatment. In the ovarian tissue, despite of AR and ER-β levels were not affected along the treatment, melatonin significantly reduced ER-α and PRB levels ($p < 0.05$; Figure 2A, B), beyond the ER-α/ER-β ratio (melatonin 1.17 ± 0.2 *vs* control 1.27 ± 0.3). Moreover, it was observed that melatonin induced significant overexpression of PRA subunit and of its own receptor MT1R ($p < 0.01$; Figure 2A, B). There was also an increase of the PRA/PRB ratio after melatonin treatment (melatonin 1.34 ± 0.6 *vs* control 0.73 ± 0.5).

Analysis of oviduct AR, ER-α, ER-β, PRA, PRB and MT1R levels after treatment

Regarding to the oviduct tissue, expressions of AR and ER-α, in addition to ER-α/ER-β ratio (melatonin 1.45 ± 0.8 *vs* control 1.73 ± 0.6) were significantly lower in melatonin-treated group ($p < 0.05$), while both PRA and PRB subunits had a remarkable increase after melatonin treatment ($p < 0.01$; Figure 3A, B). No significant PRA/PRB ratio was seen between the groups (melatonin 0.87 ± 0.2 *vs* control 0.85 ± 0.4). Furthermore, the oviduct ER-β and MT1R levels kept unchanged in the presence of melatonin (Figure 3A, B).

Analysis of uterine AR, ER-α, ER-β, PRA, PRB and MT1R levels after treatment

Following to uterus tissue, there were no differences for AR, ER-α and PRA levels ($p > 0.05$; Figure 4A, B). Although melatonin had significantly reduced the ER-β and PRB subunits in the uterine tissues, its selective receptor MT1R was clearly overexpressed ($p < 0.05$; Figure 4A, B). Moreover, in contrast to ovary and oviduct tissue, melatonin significantly increased the uterine ER-α/ER-β ratio (melatonin 0.90 ± 0.2 *vs* control 0.34 ± 0.4) but not the PRA/PRB ratio (melatonin 0.63 ± 0.6 *vs* control 0.59 ± 0.5).

Discussion

The present study found that melatonin is able to reduce LH and E2, but not FSH and P4 levels at estrous. Although melatonin may act as a synchronizer of the reproductive function, the cellular and molecular characteristics of melatonin binding sites are so far unknown. It seems obvious that melatonin does not act directly on GnRH neurons [27,28] but, instead, exert indirect actions on *Kiss 1*/GPR54 system responsible for controlling reproduction via neural-axis by inducing low circulating gonadotropins and sex steroids levels [27,29]. In this context, the long-term melatonin treatment may be linked to the phenotype of hypogonadotropic hypogonadism, evidenced by loss of ovarian mass, as previously demonstrated by our group [9]. It has been proposed that preovulatory LH surge, until the onset of estrus, depends on the lowest melatonin levels [30,31] where increased E2 could suppress its production. It is also known that human preovulatory follicles contain amounts of melatonin in a concentration higher than those in the circulating serum, where it strongly regulates the steroid synthesis by the gonads [32]. Ultimately, melatonin may drastically influence the success of ovulation.

Indeed, exogenous melatonin induces a decrease in LH surge, blocking ovulation and luteal phase with increase in P4 levels, without affecting FSH or E2 levels [33]. Melatonin was also seen to inhibit steroidogenesis by altering cAMP levels through a direct action on theca or granulosa cells of the follicles [32,34]. This dual effect of melatonin allowed us to believe that low E2 levels can be associated with direct inhibition of pathway for E2 biosynthesis since FSH levels was unchanged. Furthermore, a positive feedback on LH secretion does not occur when E2 levels are low, thus explaining, in

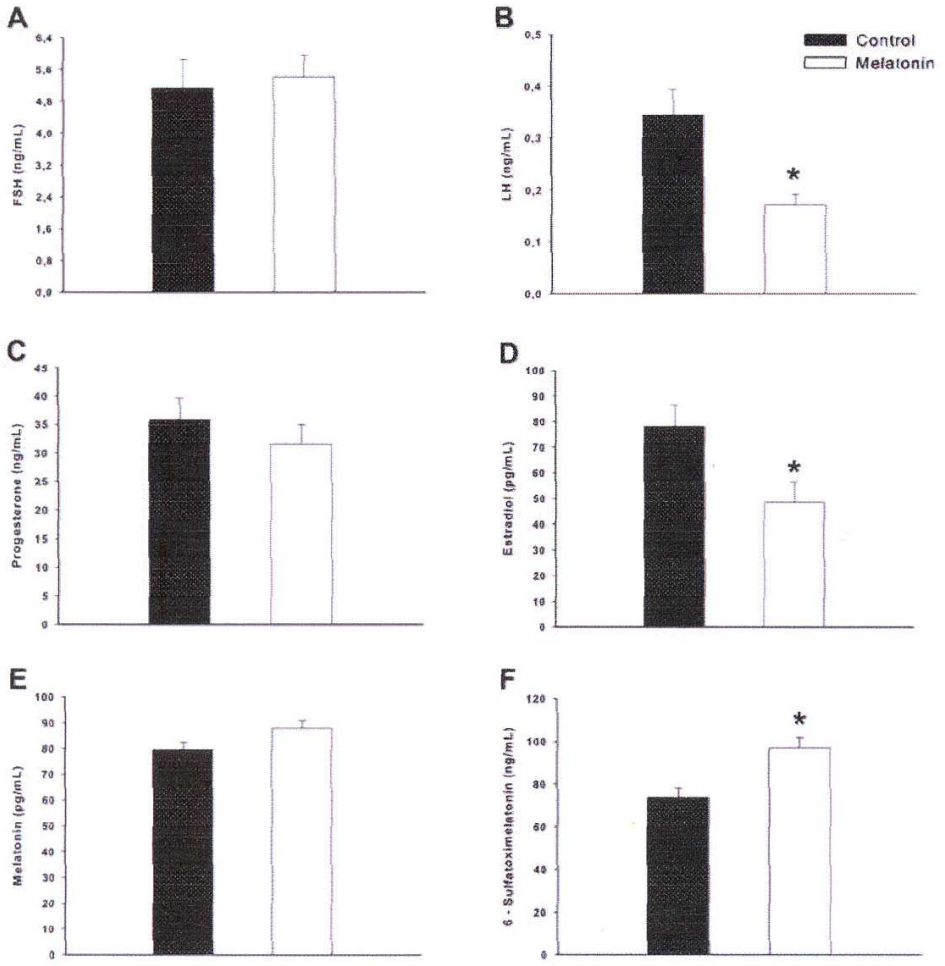

Figure 1 Hormonal profile after 60-day melatonin treatment at morning estrus. **(A)** Plasma FSH levels (ng/mL), **(B)** Plasma LH levels (ng/mL), **(C)** Plasma P4 levels (ng/mL), **(D)** Plasma E2 levels (pg/mL), **(E)** Plasma melatonin levels (pg/mL), **(F)** urinary 6-STM levels (ng/mL). Values are expressed as mean ± SEM (N= 12 animals/group). * $p < 0.05$ vs. control group.

part, the hormonal disturbances in female reproduction caused by melatonin. Melatonin also regulates the expression and activity of aromatase [35], acting as a selective estrogen enzyme modulator, and further contributing to a decrease in E2 levels. Following the treatment, although the urinary 6-STM levels were raised at morning estrus, plasma melatonin levels were unchanged. This is due to the short half-life of melatonin, where it is rapidly converted into 6-STM prior to elimination. In accordance to Graham et al. [36], the increased 6-STM level is a good biomarker to predict the effectiveness of treatment.

The activity of melatonin directly influencing the ovary function and estrous cycle was first described by Wurtman et al. [37] and as expected, similar findings were previously confirmed by our group [9,38]. Recently, Adriaens et al. [39] demonstrated that melatonin increased P4 and androgen production in mouse preantral follicles. These contradictory results are partially due to different melatonin concentration, time and route of administration and period of estrus stage evaluation. Brzezinski et al. [40] reported that melatonin itself has no effect on basal P4 productions, but when combined with LH analogues, melatonin potentiated the

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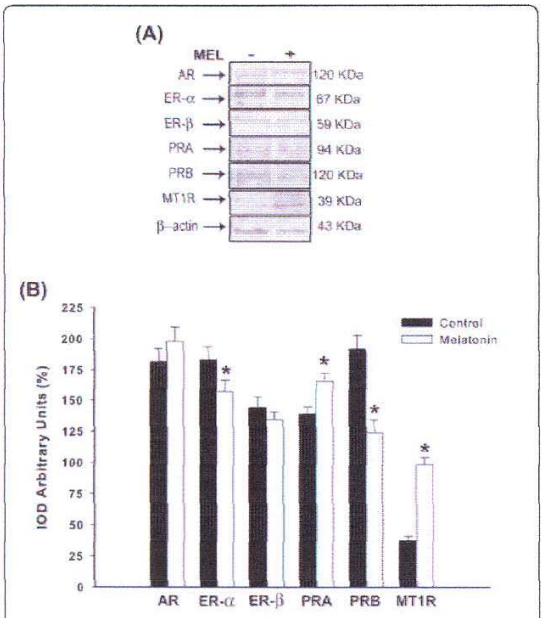

Figure 2 Analysis of ovarian receptors. (A) Representative Western blotting analysis of androgen receptor (AR), estrogen receptor (ER-α and ER-β), progesterone receptor (PRA and PRB) and melatonin receptor (MT1R) in ovarian tissue of rats receiving melatonin [100 μg/100 g B.W]. Indicated concentrations of each total protein (70 μg extracted from a pool of 6 organs/group) were used to detect specific protein expression levels in the blots (upper panel). **(B)** Densitometry values for AR, ER-α, ER-β, PRA, PRB and MT1R levels were studied following normalization to the house-keeping gene (β-actin). All results are expressed as mean ± SEM (N= 6 animals/group). * $p < 0.05$ vs. control group.

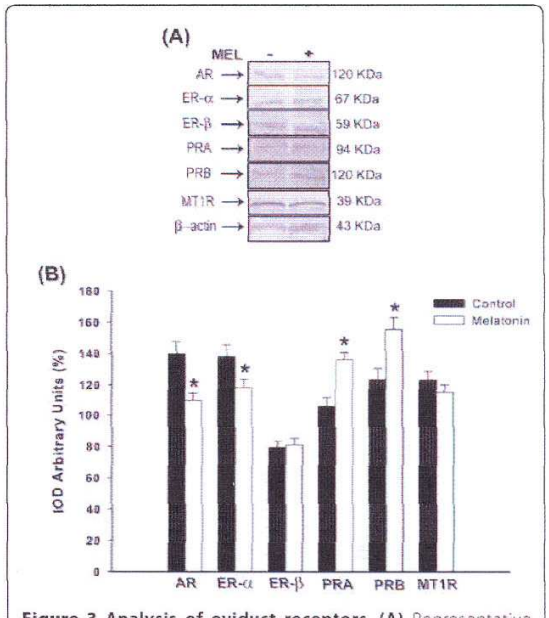

Figure 3 Analysis of oviduct receptors. (A) Representative Western blotting analysis of androgen receptor (AR), estrogen receptor (ER-α and ER-β), progesterone receptor (PRA and PRB) and melatonin receptor (MT1R) in oviduct tissue of rats receiving melatonin [100 μg/100 g B.W]. Indicated concentrations of each total protein (70 μg extracted from a pool of 6 organs/group) were used to detect specific protein expression levels in the blots (upper panel). **(B)** Densitometry values for AR, ER-α, ER-β, PRA, PRB and MT1R levels were studied following normalization to the house-keeping gene (β-actin). All results are expressed as mean ± SEM (N= 6 animals/group). * $p < 0.05$ vs. control group.

stimulatory effect on intraovarian P4 production. Our data corroborate those findings, in which P4 was unaltered by melatonin treatment and even LH levels were insufficient to produce activity on P4 secretion. The present study showed that long-term melatonin is able to reduce the ER-α and PRB ovarian levels while increasing PRA and its receptor MT1R at morning estrus. Indeed, ER-α seems to be activated when intracellular cAMP is elevated after non-transcriptional mechanisms mediated by estrogens [41]. Alternatively, melatonin acting through membrane-bound G protein-coupled MT1 receptor can inhibits adenylate cyclase activity, thus decreasing cAMP levels [42]. This reduction may be a direct effect by which melatonin decreases E2-induced ER-α transcriptional activity. As a favorable condition, the reduction in ER-α/ER-β ratio represents a protective action of melatonin against estrogen-dependent tumor. Both PRA and PRB have been shown to function as ligand-dependent repressors of ER-mediated transcriptional activity [43]. Furthermore, PRA may act as a transdominant inhibitor of PRB and AR gene expression [44]. In this context, melatonin treatment might be accentuating PRA activity, thereby providing a negative regulation of ER-α and PRB expression. Since PRA isoform is essential for ovulation to occur [17], the long-term melatonin treatment could delay but not abolish the ovulation, as we had already been noted. Curiously, melatonin-deprived rats had lower expression of P4 and PR than controls [7,15], thus proving that melatonin is a key factor in PR regulation. Previous study has indicated that melatonin binding receptor is high during estrus, proestrus and diestrus, in contrast to low levels in metaestrus when E2 and P4 are reduced [45,46]. Thus, it allows us to conclude that both E2 and P4 regulate MT1 receptor binding activity.

We demonstrated for the first time that total expression of oviduct PRA and PRB was enhanced while AR and ER-α decreased after melatonin treatment. Generally, PRB is transcriptionally more active than PRA [47], and it is well documented that PRA acts as a

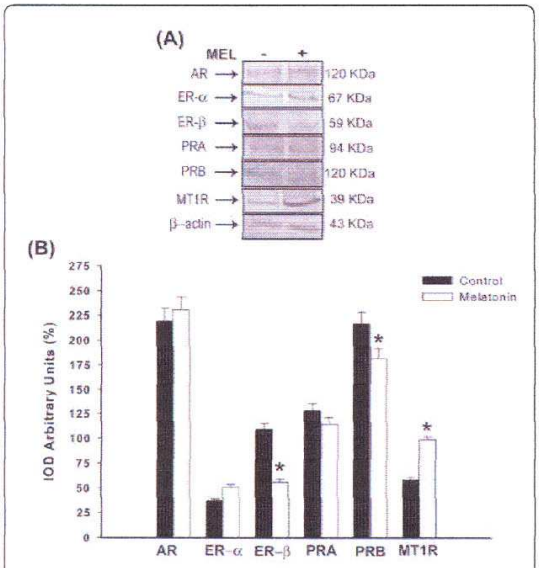

Figure 4 Analysis of uterine receptors. (A) Representative Western blotting analysis of androgen receptor (AR), estrogen receptor (ER-α and ER-β), progesterone receptor (PRA and PRB) and melatonin receptor (MT1R) in uterus tissue of rats receiving melatonin [100 μg/100 g B.W]. Indicated concentrations of each total protein (70 μg extracted from a pool of 6 organs/group) were used to detect specific protein expression levels in the blots (upper panel). **(B)** Densitometry values for AR, ER-α, ER-β, PRA, PRB and MT1R levels were studied following normalization to the housekeeping gene (β-actin). All results are expressed as mean ± SEM (N= 6 animals/group). * $p < 0.05$ vs. control group.

repressor of PRB-dependent activation genes and, likewise, it inhibits the transactivation of AR [16]. Surprisingly, oviduct PRB has been up-regulated after melatonin exposure. It is likely that melatonin promoted a differential effect upon its regulatory mechanism(s) independently of either P4 or PRA functions. Moreover, the distinct transactivation properties, including presence or absence of the PRB-specific AF-3 domain, are probably due to the broad repertoire of physiological responses to P4 [48]. Nevertheless, the regulation of sex steroid receptors in oviduct is not yet fully clarified. Similarly to the ovary, melatonin led to a downregulation of oviduct ER-α through its direct effect or indirectly by the fall in E2 levels. Hence, an inverse ER-α/ER-β ratio also brings up a positive action of melatonin to the oviduct. The oviduct MT1R was not affected over the treatment, showing that, in fact, the ovary and uterus are more responsive to the effects of melatonin mediated by MT1R. Besides that, it seems plausible that melatonin-induced changes occur through different signaling pathway. It is well emphasized that melatonin may exert its physiological function by binding to melatonin receptors or even through nuclear signaling involving RZR/ROR receptors [49]. However, additional studies are needed for a better understanding of melatonin binding sites.

In this study, the uterine ER-β and PRB was down-regulated whereas MT1R was up-regulated. It is established that E2 and P4 acts on the uterus by an interdependent regulation of ER and PR [50,51]. Noticeably, it has been suggested that E2 decreases the expression of uterine ER but not PR, while P4 reduces the levels of both receptors [52]. Taking into account that E2 levels, which are responsible for increasing PR levels, were suppressed by the treatment, our results could be explained, in part, by the down-regulation of uterine PRB expression. In this context, the regulation of PRB appears to be more sensitive than PRA, considering the fall in E2. It has already been proposed that uterine ER-β, but not ER-α, is detected under low amounts at the cellular level [53]. On the other hand, our data pointed to an increase in ER-α/ER-β ratio. These effects may be due to differential ER expression associated with variations into estrus period. Uterine MT1R was up-regulated after melatonin treatment during ovulation, thereby supporting a direct regulation by melatonin itself. However, it cannot be assumed that melatonin-bound uterine MT1R is involved in down-regulation of ER-β. In contrast, MT1R was found to be depleted after E2 has raised [54], thus demonstrating a negative correlation. Finally, the uterine AR levels seem to be not affected by melatonin during ovulation.

Conclusions

In summary, we reported that long-term melatonin is able to partially suppress the neuroendocrine reproductive axis during ovulation, indirectly causing disturbances to ovary, oviduct and uterus.

Moreover, melatonin promoted differential regulation of the sex steroid receptors on the reproductive tissues, mostly acting "in situ" through its MT1R receptor (especially in ovarian and uterine tissue) or by altering the dynamics and responsiveness of sex steroid receptor isoforms after binding to E2 or P4. These data represent therefore an important benchmark for furthering the understanding of melatonin-reproduction interface during ovulation process.

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Authors' contributions
LGAC, FEM: collected and analyzed the data and drafted the manuscript, beyond conceiving the main idea of the study. FRFS, WJF, GRT, FKD, and AAHF: performed the ELISA assays and Western Blotting analysis given substantial interpretation of data. JPAA, LOM, BAF, MM and PFFP: participated in the acquisition of data, in the design of the study and in the intellectual conception. JAAF: participated in all RIA dosages and during interpretation of these data. The authors helped to perform the statistical analysis. All authors read and approved the final version of the manuscript.

Competing of interests
The authors declare that they have no competing interest.

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Capítulo 4

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Long-term melatonin treatment reduces ovarian mass and enhances tissue antioxidant defenses during ovulation in the rat

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Abstract

Melatonin regulates the reproductive cycle, energy metabolism and may also act as a potential antioxidant indoleamine. The present study was undertaken to investigate whether long-term melatonin treatment can induce reproductive alterations and if it can protect ovarian tissue against lipid peroxidation during ovulation. Twenty-four adult female Wistar rats, 60 days old (± 250-260 g), were randomly divided into two equal groups. The control group received 0.3 mL 0.9% NaCl + 0.04 mL 95% ethanol as vehicle, and the melatonin-treated group received vehicle + melatonin (100 µg·100 g body weight^{-1}·day^{-1}) both intraperitoneally daily for 60 days. All animals were killed by decapitation during the morning estrus at 4:00 am. Body weight gain and body mass index were reduced by melatonin after 10 days of treatment ($P < 0.05$). Also, a marked loss of appetite was observed with a fall in food intake, energy intake (melatonin 51.41 ± 1.28 *vs* control 57.35 ± 1.34 kcal/day) and glucose levels (melatonin 80.3 ± 4.49 *vs* control 103.5 ± 5.47 mg/dL) towards the end of treatment. Melatonin itself and changes in energy balance promoted reductions in ovarian mass (20.2%) and estrous cycle remained extensive (26.7%), arresting at diestrus. Regarding the oxidative profile, lipid hydroperoxide levels decreased after melatonin treatment (6.9%) and total antioxidant substances were enhanced within the ovaries (23.9%). Additionally, melatonin increased superoxide dismutase (21.3%), catalase (23.6%) and glutathione-reductase (14.8%) activities and the reducing power (10.2% GSH/GSSG ratio). We suggest that melatonin alters ovarian mass and estrous cyclicity and protects the ovaries by increasing superoxide dismutase, catalase and glutathione-reductase activities.

Key words: Melatonin; Ovary mass; Lipid peroxidation; Antioxidant defenses; Superoxide dismutase

Introduction

The rhythm of melatonin (N-acetyl-5-methoxytryptamine) secretion is important for the synchronization of the reproductive response with appropriate environmental conditions in photoperiodic animals (1). Melatonin is known to modulate some physiological functions such as seasonal reproduction, energy metabolism and thermoregulation in mammals (2). Experimental studies have indicated the action of melatonin in nutrition and feed efficiency affecting body mass and adiposity index and both energy intake and expenditure, with melatonin preferentially acting by reducing fat deposits, thus preventing obesity (3,4). However, these controversial effects are not yet fully understood.

It has been shown that melatonin plays key roles in reproductive function due to its stimulation of ovarian activity and the promotion of estrous cyclicity and gonadal atrophy depending on photoperiod length (5). Moreover, melatonin also regulates folliculogenesis and ovulation (6) since acute suppression of luteinizing hormone levels is notable after melatonin treatment (7). Also, it has been emphasized that melatonin affects the axis by directly binding to granulosa cells in the ovary (8).

During ovulation, the mechanisms causing follicular rupture result in exposure of the ovarian surface to deleterious agents such as free radicals (9,10). Thus, repeated

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ovulation and its complex activities are linked to inflammation, reactive oxygen species (ROS) formation, and special cytokine expression, and may also be involved in the etiopathogenesis of ovarian cancer (10,11). Some reports have described the presence of ROS in the female reproductive tract, including the ovaries (12). Under normal conditions, ROS appear to be directly involved in the reproductive physiological functions such as steroidogenesis, oocyte development and fertility (12,13). Interestingly, the major sources of ROS, including ·OH, O_2^- and H_2O_2, are derived from macrophages and neutrophils, as they are present in the ovaries during ovulation (14). The preovulatory follicle has a powerful antioxidant defense, which is depleted by the intense peroxidation. As an example, the activity of glutathione peroxidase (GSH-Px) may also maintain low levels of hydroperoxides inside the ovarian follicles.

The use of melatonin has been investigated as a therapeutic strategy to improve oocyte quality in patients failing to get pregnant in earlier *in vitro* fertilization (15). In addition, melatonin has an important role as a ROS scavenger, acting directly and/or indirectly as a hormone preventing attack by free radicals (16). A previous study showed that elevated melatonin levels in preovulatory follicles are able to protect granulosa cells and the oocyte from the free radicals induced by ovulation (17). Melatonin also positively increases both antioxidant enzyme activity and gene expression of superoxide dismutase (SOD), catalase and GSH-Px, which are fundamental to detoxify most of the ROS produced (18).

Although melatonin treatment has been widely used as an effective and useful adjuvant for several therapies, including to maintain oocyte quality (15), its adverse time-dependent effects are still obscure and require further study. The study of long-term exposure to melatonin may provide new insights into the understanding of the physiological and cellular mechanisms underlying metabolic changes, appetite signaling and protective factors in female reproduction. Additionally, it should be remembered that possible changes in nutritional status could improve or compromise the reproductive viability. Melatonin can contribute to decreasing fat pad storages (4), providing a better lifespan, and may reduce lipid oxidation, which would be of benefit for reproduction, in addition to having a known detoxifying role. However, the role of melatonin in such events remains unclear and conflicting results have been observed.

The present study was undertaken to determine if long-term melatonin administration is able to induce reproductive changes and how melatonin exerts its protective action related to lipid oxidation and antioxidant activities in the rat ovary during ovulation.

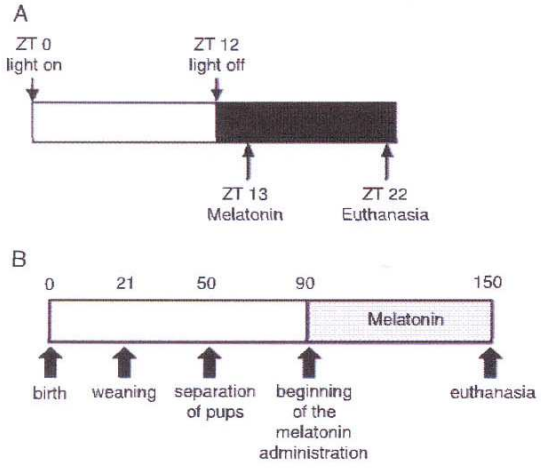

Figure 1. *A*, Protocol for melatonin treatment based on Zeitgeber time (ZT) corresponding to the environmental circadian time. *B*, Rats received 100 µg melatonin·100 g body weight^{-1}·day^{-1}, *ip*, for 60 days.

Material and Methods

Animals and experimental design

Twenty-four adult female rats (*Rattus norvegicus albinus*), 60 days old (± 250-260 g), were obtained from the Department of Anatomy, Bioscience Institute, Botucatu Campus (IBB, UNESP). The rats were divided into two groups of 12 animals each: control, rats receiving standard chow and tap water *ad libitum* and 0.04 mL 95% ethanol + 0.3 mL 0.9% NaCl (1:7, v/v) as vehicle; melatonin-treated group, rats receiving standard chow and tap water *ad libitum* and vehicle + melatonin. All animals were housed in polypropylene cages (43 cm x 30 cm x 15 cm) with laboratory-grade pine shavings as bedding under conditions of controlled room temperature (23 ± 1°C) and lighting (12-h light/12-h dark photoperiod, lights switched on at 6:00 am). At 90 days of age, females received melatonin daily for 60 consecutive days (Figure 1A and B). After the period of melatonin treatment, rats cycling in the morning of estrus (period of ovulation) at 4:00 am (or Zeitgeber time 22:00, ZT 22, corresponding to the environmental circadian time set) and monitored by vaginal swabs in a dark room using a red light, were anesthetized and killed by decapitation for further analysis. The experimental protocols were approved by the Ethics Committee of the Institute of Bioscience, UNESP, Botucatu, SP, Brazil (protocol #85/07).

Melatonin administration

For the animals designated to receive exogenous melatonin treatment, successive doses of melatonin (100 µg/100 g body weight; M-5250, purchased from Sigma Chemical, USA) were dissolved in 0.04 mL 95% ethanol, using 0.3 mL 0.9% NaCl (1:7, v/v) as vehicle (19). The injections (only vehicle or vehicle + melatonin) were administered daily *ip* between 6:30 and 7:00 pm (ZT 13; Figure 1A).

Food and liquid intake

The diet of the animals was prepared in lots of 5 days, always at the same time of day (3:00 pm) using a marked

test tube and an analytical scale (Ohaus Traveler™, Analytica S.A, Colombia). Liquid and food consumption (caloric value of standard chow = 2930 kcal/kg) was determined. Total energy intake (kcal/day) and feed efficiency (weight gain/consumed calories x 100) were evaluated as metabolic parameters. Body weight was also measured. At the end of treatment, the reproductive organs (uterine horn, ovaries and oviducts) were dissected and weighed. Body weight and organ weight were measured using an analytical balance (OwaLabor, Germany).

Glycemia measurements

The glycemic index was measured with a blood glucose sensor (One Touch Ultra System Kit, Lifescan, Italy) using blood samples from the caudal vein of the animals. To avoid variation, all samples were assessed in an equal volume collected from each animal after 12 h of fasting.

Assessment of estrous cyclicity

During the second part of the experiment, animals exhibiting estrous cycles were monitored by colpocytological examination (vaginal smears) (20). Cells detaching from the vaginal epithelium were removed with a pipette (Lab Mate 0.5-10 µL, UK). The filter tips containing 10 µL 0.9% saline were discarded after the vaginal secretion had been transferred to clean slides. Colpocytological examination time was at 9:00 am. Each slide was analyzed under a Zeiss Axiophot II microscope (Carl Zeiss, Germany) at 10X and 25X magnification and digitally photographed.

Determination of lipid hydroperoxide and antioxidant systems

After 60 days of melatonin treatment (100 µg·100 g body weight^{-1}·day^{-1}), the ovaries were removed rapidly. Each ovary was weighed and tissue samples of 40 mg were frozen immediately in liquid nitrogen and stored at -80°C. The ovary fragments were homogenized using a motor-driven Teflon Potter Elvehjem (Scientific Ltd., England) tissue homogenizer in 1.25 mL cold 0.1 M phosphate buffer, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 10,000 *g* for 15 min. The supernatant fraction was removed for the determination of total proteins, lipids, hydroperoxide, serum antioxidant capacity, and total antioxidant substances (test Kit Randox Laboratories Ltd., Crumlin, Co., UK).

Lipid hydroperoxide (LHP) was measured by Fe^{2+} to Fe^{3+} oxidation, using a 100-mL sample and a 900-mL reaction mixture containing 250 mM $FeSO_4$, 25 mM H_2SO_4, 100 mM xylenol orange, and 4 mM butyl hydroxytoluene in 90% (v/v) methanol. Absorbance was measured at 560 nm (21). Total antioxidant substances (TAS) were measured by the inhibition of LHP formation and measured at 500 nm (22). Spectrophotometric assays were carried out using a spectrophotometer with a temperature-controlled cuvette chamber (Ultraspec with Swift II software, Pharmacia Biotech, UK).

The enzymatic antioxidant system, reduced glutathione (GSH) and oxidized glutathione (GSSG), was investigated using the extracted supernatant (22). GSH-Px activity (E.C. 1.11.1.9) was then analyzed using glutathione oxidation reacted with hydrogen peroxide and cumene hydroperoxide. GSH reductase (GSH-Rd) activity was determined by monitoring NADPH oxidation at 340 nm. The mixture contained 1 mM Tris buffer, pH 8.0, 5 mM EDTA, 33 mM GSSG and 2 mM NADPH. SOD activity (E.C. 1.15.1.1) was determined with NADH and phenazine methosulfate (PMS) and reduction of nitroblue tetrazolium (NBT) by superoxides at physiological pH. The complete reaction system consisted of 50 mM phosphate buffer, pH 7.4, 0.1 mM EDTA, 50 mM NBT, 78 mM NADH, and 3.3 mM PMS. Catalase (E.C. 1.11.1.6) activity was assayed during the decomposition of H_2O_2 to H_2O + O_2. The assays of antioxidant activities were performed at 25°C using a µQuant microplate spectrophotometer (MQX 200 with KCjunior software, Bio-Tek Instruments, USA). All chemicals were purchased from Sigma.

Statistical analysis

The Student *t*-test for independent samples was used. Data are reported as means ± SEM and statistical significance was set at $P < 0.05$. The statistical software used was Sigma Plot version 11.0 and GraphPad Instat version 4 for graphic design.

Results

After 60 days of melatonin treatment, there were significant differences in body weight. Melatonin-treated rats exhibited a body weight reduction after 10 days of treatment, with a remarkable maintenance of body weight gain between days 35-60 (Figure 2). As expected, melatonin

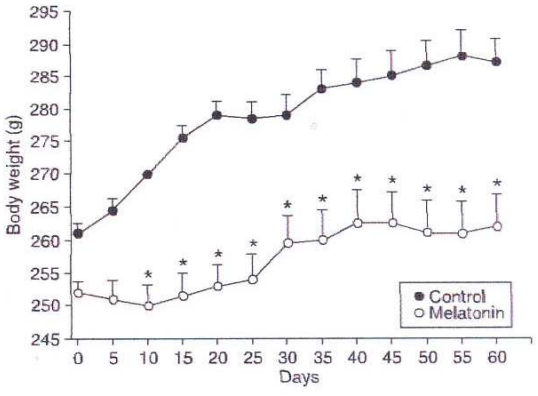

Figure 2. Effects of melatonin (100 µg·100 g body weight^{-1}·day^{-1}) on body weight gain during 60 days of treatment. Data are reported as means ± SEM for 10 animals per group. *$P < 0.01$ *vs* control group on days 10 to 60 of treatment (Student *t*-test).

treatment also reduced total weight gain and body mass index by decreasing fat mass (Table 1). Although overall food efficiency and liquid consumption were not affected by melatonin, there was a reduction in total and relative food consumption, as well as a reduced energy intake and glucose levels throughout 60 days of melatonin administration (Table 1, Figure 3).

During the second half of the daily melatonin treatment, when changes in total energy balance were detected, alterations in the reproductive cycles were observed, such as estrous irregularities, namely, more extensive cycles in melatonin-treated rats than controls, with a high frequency of continuous metaestrus and diestrus (Table 2). During this period, melatonin had no influence on estrous stage. Total ovarian mass was significantly reduced in melatonin-treated rats; however, the relative ovarian mass remained unchanged because of the reduced final body weight of the animals (Table 2).

Table 3 shows that total protein concentration remained unchanged in ovarian tissues after melatonin treatment. LHP levels increased during spontaneous ovulation and decreased when melatonin was given. Moreover, TAS were higher in melatonin-treated rats than controls, although the LHP/TAS ratio was unchanged because of the high LHP formation in control animals (Table 3). With respect to antioxidant activities, melatonin increased SOD, GSH-Rd and catalase activities in ovarian tissues during ovulation. Only GSH-Px activity was unchanged after melatonin treatment. Additionally, the reducing power (GSH/GSSG ratio) was higher in melatonin-treated rats than in controls, thus

Table 1. Nutritional parameters of female rats after 60 days of melatonin treatment.

Parameters	Control (N = 12)	Melatonin (N = 12)
Body weight gain (g)	36.5 ± 10.6	22.5 ± 11.6*
Body mass index (g/cm^2)	0.78 ± 0.05	0.66 ± 0.08*
Total food consumption (g/day)	19.57 ± 0.46	17.55 ± 0.43*
Liquid consumption (mL/day)	12.42 ± 0.23	11.06 ± 0.23
Energy intake (kcal/day)	57.35 ± 1.34	51.41 ± 1.28*
Feed efficiency (g/kcal)	63.0 ± 4.56	61.5 ± 4.76
Blood glucose (mg/dL)	103.5 ± 5.47	80.3 ± 4.49*

Data are reported as means ± SEM. *P < 0.05 *vs* control group (Student *t*-test).

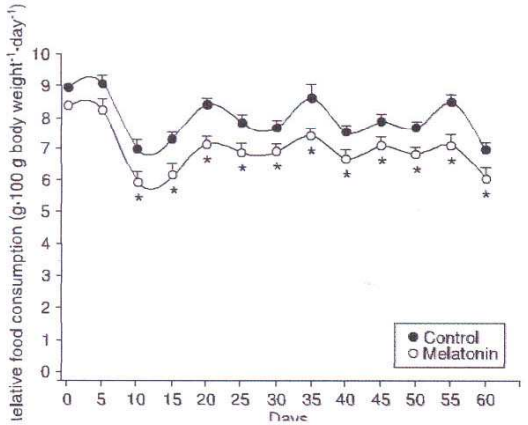

Figure 3. Influence of melatonin administration (100 µg·100 g body weight^{-1}·day^{-1}) on relative food intake for 60 days of rats receiving standard chow. Data are reported as means ± SEM for 10 animals per group. *P < 0.01 *vs* control group on days 10 to 60 of treatment (Student *t*-test). Day 0: Food consumption before the beginning of melatonin administration.

Table 2. Total and relative ovarian mass and duration of estrous cycles in female rats throughout 60 days of melatonin treatment.

Parameters	Control (N = 12)	Melatonin (N = 12)
Ovarian mass (g)	0.094 ± 0.01	0.075 ± 0.02*
Ovarian relative mass (g/100 g body weight)	0.035 ± 0.01	0.031 ± 0.01
Estrous cycle duration (days)	5.06 ± 0.20	6.90 ± 0.71*
Estrus (h/cycle)	23.92 ± 0.61	21.60 ± 0.92
Metaestrus (h/cycle)	6.72 ± 0.39	25.92 ± 0.39*
Diestrus (h/cycle)	34.56 ± 1.55	91.14 ± 1.48*

Data are reported as means ± SEM. *P < 0.05 *vs* control group (Student *t*-test).

Table 3. Oxidative and antioxidant status of rat ovaries after 60 days of melatonin administration.

Parameters	Control (N = 12)	Melatonin (N = 12)
Ovary protein (mg/100 mg tissue)	26.25 ± 2.52	35.74 ± 5.95
LHP (nmol/g tissue)	450.7 ± 15.3	419.5 ± 14.2*
TAS (%)	52.50 ± 1.47	65.06 ± 4.03*
LHP/TAS (g/tissue)	8.27 ± 0.59	7.47 ± 0.41
SOD (nmol/mg protein)	1.48 ± 0.12	1.88 ± 0.05*
GSH-Px (nmol/mg protein)	32.3 ± 1.22	30.6 ± 1.72
GSH-Rd (nmol/mg protein)	1.32 ± 0.01	1.55 ± 0.01*
CAT (nmol/mg protein)	2.85 ± 0.25	3.73 ± 0.16*
GSH/GSSG	10.5 ± 0.7	11.7 ± 0.6*

Data are reported as means ± SEM. LHP = lipid hydroperoxide; TAS = total antioxidant substances; SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; GSH-Rd = glutathione reductase; CAT = catalase; GSH = reduced glutathione; GSSG = oxidized glutathione. *P < 0.05 *vs* control group (Student *t*-test).

emphasizing the reduced glutathione formation associated with increasing GSH-Rd activities during exhaustive antioxidant renovation.

Discussion

Appetite regulation and energy sources are fundamental for the maintenance of caloric balance and body weight gain. Melatonin reduced body weight gain, body mass index, total and relative food consumption as well as energy intake and glucose levels in the present study. There are several possible explanations regarding the action of melatonin as a metabolic hormone disruptor. Melatonin seems to be directly related to the periodicity of food intake (23). Molecular evidence suggests a stronger interaction between melatonin-induced clock genes and cell metabolism, including the control of glucose homeostasis and adipogenesis (24-26), thereby reducing the lipid content. Moreover, melatonin influences the satiety process and reduces blood glucose levels (27,28). Taken together, our findings concerning the nutritional balance could be attributed to one or more of these factors. Similarly, a previous study on the effects of melatonin detected body weight reduction in animals (29). It has also been demonstrated that pinealectomized rats do not show a greater body weight and, conversely, when they are exposed to a short-day photoperiod, a marked reduction of body weight is seen (30). Furthermore, these differences may depend on factors such as gender, age, or body composition of the animals receiving appropriate treatment.

In the present study, rats receiving melatonin showed a reduction in ovarian weight and, additionally, longer estrous cycles, which featured an extended metaestrous and diestrous phases. It is known that melatonin can increase the diestrous frequencies or estrous phases in rodents (31) by modulating GnRH hormones, but the exact mechanisms involved are poorly understood. Thus, it seems true that nutritional improvements were not effectively responsible for maintaining regular cycles. Moreover, rats receiving melatonin (200 µg·100 g body weight^{-1}·day^{-1}) and exposed to continuous light showed longer estrous periods and reduction of ovarian weight (32). It is clear that melatonin does not act directly on hypothalamic GnRH neurons and its responsiveness does not change with the photoperiod (33). Disturbances involving neuroendocrine regulation of reproduction appear to be associated with hypogonadotropic hypogonadism (34) where expression of *Kiss 1* is down-regulated by melatonin (35). Thus, our findings could be partially explained if melatonin suppressed GnRH hormones.

It is noteworthy that spontaneous ovulation is a source of ROS generation due to the intensive ovarian activity (36). Conversely, there is a body of evidence suggesting that melatonin plays key roles as a ROS scavenger (15,37). Under a variety of physiological conditions, including ovulation or follicular rupture, the proteolytic cascade and vascular changes lead to ROS production (38). It seems clear that increased levels of NADPH-oxidase generate superoxide anion radicals (O_2^-) and hydrogen peroxide (H_2O_2), which in the presence of Fe^{3+} produces powerful oxidants such as hydroxyl radicals, enhancing lipid peroxidation (39). In melatonin-treated rats, LHP levels were reduced and TAS concentration was increased, although the LHP/TAS ratio was not modified by melatonin. Interestingly, lipid hydroperoxide levels were higher during spontaneous ovulation and underwent a remarkable reduction after the administration of melatonin, which quenched hydroxyl radicals within ovarian cells, confirming the protective effect of the hormone as a ROS scavenger preserving fatty acid integrity against aldehyde formation and finally improving the lipid profile (40). Also, another sub-product formed as a result of melatonin scavenging hydrogen peroxide, i.e., N(1)-acetyl-N(2)-formyl-5-methoxykynuramine, is also a potent scavenger.

Melatonin had an important effect on the ovaries, inducing higher SOD, catalase and GSH-Rd activity compared to control. It is well known that melatonin has an important role in transcriptional mRNA synthesis (i.e., gamma-glutamyl cysteine synthetase and others) for antioxidant enzymes (18), suggesting that melatonin regulation is receptor-mediated, thereby most likely implicating the MT1/MT2 receptors via second messengers such as cAMP, phospholipase C or intracellular calcium concentration, and thus, it might have improved the antioxidant profile if they are activated. The antioxidant defense system is an integrated array of enzyme and non-enzyme antioxidants. In control rats, higher ovarian LHP levels indicated that the oxidation of GSH to GSSG, reducing the GSH/GSSG ratio, led to lipoperoxidation and oxidative stress. During the reduction of hydrogen peroxide, GSH is oxidized to GSSG and GSH-Rd is activated to convert oxidized GSSG to reduced GSH. Curiously, besides increasing the GSH/GSSG ratio, melatonin also enhanced GSH-Rd activity. Our findings seem to agree with these mechanisms.

This study demonstrated that long-term melatonin treatment modulates body weight gain, calorie storage and food intake. Although melatonin reduced ovarian mass and caused estrous cycle disturbances, it was able to protect the ovaries against lipid peroxidation by reducing LHP levels during ovulation. These protective effects could be due to the properties of melatonin both as a ROS scavenger and as an agent increasing antioxidant activities.

Acknowledgments

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5. CONCLUSÕES

1. A melatonina apresenta efeito oposto ao observado após o consumo de etanol sobre o E2 e a P4 durante o período ovulatório, onde ambos atuam como desreguladores endócrinos podendo alterar o funcionamento dos ovários, ovidutos e úteros.

2. Independentemente, a melatonina e o etanol promovem regulação diferencial dos receptores esteróides sexuais AR, ER-α, ER-β, PRA e PRB nos tecidos reprodutivos, atuando principalmente através de seu receptor MT1R (especialmente no ovário e útero). A combinação da melatonina e etanol é variável sobre esses receptores, acentuando ou atenuando sua expressão, dependendo do tecido analisado;

3. Existe interação entre melatonina e etanol sobre a eficiência alimentar, onde a melatonina atua como hormônio regulador do ganho de peso, das calorias armazenadas e do consumo de etanol;

4. Embora a melatonina altere o ciclo estral, ela é capaz de proteger os ovários de ratas UChB contra o estresse oxidativo, prevenindo o ataque de radicais livres e estimulando as defesas antioxidantes.

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DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha **Tese de Doutorado** intitulada "."**AÇÃO DA MELATONINA SOBRE OS RECEPTORES ESTERÓIDES SEXUAIS NO OVÁRIO, OVIDUTO E ÚTERO E O ESTRESSE OXIDATIVO NOS OVÁRIOS DE RATAS ADULTAS UChB (CONSUMIDORAS VOLUNTÁRIAS DE ETANOL A 10%) DURANTE A OVULAÇÃO"**:

( ) não se enquadra no § 3º do Artigo 1º da Informação CCPG 01/08, referente a bioética e biossegurança.

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CERTIFICADO

Certificamos que o Protocolo nº **85/07-CEEA**, sobre *"AÇÃO DA MELATONINA SOBRE OS RECEPTORES ESTERÓIDOGÊNICOS ERA E ERB E PROGESTAGÊNICOS PRA E PRB NOS ÓRGÃOS GENITAIS FEMININOS INTERNOS E O EXTRESSE OXIDATIVO NOS OVÁRIOS DE RATAS ADULTAS UCHB (CONSUMIDORAS VOLUNTÁRIAS DE ETANOL A 10%)"*, sob a responsabilidade de **FRANCISCO EDUARDO MARTINEZ**, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado pela ***COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL*** (CEEA), em reunião de 14/12/2007.

Botucatu, 14 de dezembro de 2007.

Prof. Dr. MARCELO RAZERA BARUFFI
Presidente - CEEA

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