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Faculdade de Veterinária
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Tese

17α-Estradiol e restrição calórica no envelhecimento reprodutivo de machos e fêmeas

José Victor Vieira Isola

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“Biology is the study of complicated things that have the appearance of having been designed with a purpose.”

Richard Dawkins

Resumo

ISOLA, José Victor Vieira. **Efeitos do 17α-Estradiol na reprodução de camundongos.** 2022. 81f. Tese (Doutorado em Ciências) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2022.

O 17α-estradiol (17α-E2) é uma intervenção pró-longevidade bem estabelecida na literatura, capaz de beneficiar a saúde de camundongos machos. No entanto, nenhum estudo avaliando os efeitos do 17α-E2 na fertilidade de camundongos havia sido relatado até o momento, portanto, nesta tese, objetivamos avaliar os efeitos do 17α-E2 na fertilidade de camundongos de ambos os sexos e também no envelhecimento ovariano. Sabe-se que o 17α-E2 leva a uma diminuição na ingestão de alimentos de maneira semelhante à restrição calórica (RC) branda. Sendo assim, também avaliamos os efeitos da RC branda (10%) no envelhecimento ovariano e na fertilidade de camundongos fêmeas em contraste com 17α-E2 e um RC moderada (30%) por um período de 24 semanas. Para tanto, utilizamos camundongos fêmeas C57BL/6 (n=9/grupo), e avaliamos a reserva ovariana, produção de embriões, taxa de prenhez e tamanho da ninhada. Tanto a RC de 10% quanto a de 30% provocaram efeitos positivos na preservação da reserva ovariana e na fertilidade, conforme evidenciado por maiores taxas de prenhez após a realimentação. O 17α-E2 no entanto, não afetou a preservação ovariana ou fertilidade. Para a avaliação da fertilidade de machos, duas coortes de camundongos C57BL/6 machos foram tratados com 17α-E2 (n=10 e 30) de três a sete ou oito meses de idade. Esses camundongos foram acasalados com fêmeas não tratadas e com estro sincronizado para coleta de embriões ou contagem de locais de implantação uterina. Além do desempenho reprodutivo dos machos quando acasalados com as fêmeas, também foram avaliados os níveis hormonais, a qualidade do sêmen e morfologia testicular. Apesar dos machos recebendo 17α-E2 ganharem menos peso ao longo do estudo, não foram encontradas diferenças para a totalidade das variáveis reprodutivas, mostrando que 17α-E2 não afeta a fertilidade de camundongos machos adultos. Coletivamente, nossos dados indicam 17α-E2 em uma dose de 14,4 ppm na dieta, que é estabelecida como eficaz para induzir efeitos pró-longevidade em camundongos machos, não afeta os parâmetros reprodutivos em camundongos de ambos os sexos e também não afeta o envelhecimento ovariano em camundongos fêmeas.

Palavras-chave: envelhecimento ovariano; fertilidade; intervenções anti-envelhecimento; restrição calórica.

Abstract

ISOLA, José Victor Vieira. **Effects of 17α-Estradiol on mice reproduction.** 2022. 81f. Thesis (Doctor degree in Sciences) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2022.

17α-estradiol (17α-E2) is a well-established pro-longevity intervention with benefits to male mice healthspan. However, no studies assessing 17α-E2 effects on male fertility had been reported so far, hence we aimed to evaluate the effects of 17α-E2 on fertility of mice from both sexes and also ovarian aging. 17α-E2 has been shown to decrease feed intake in a similar fashion that a mild calorie restriction (CR). However, the effects of mild CR (10%) on ovarian aging had not yet been addressed. Hence, we also assessed effects of mild calorie restriction on female ovarian aging and fertility in contrast to 17α-E2 and a standard – moderate (30%) – CR. For this purpose, we subjected C57BL/6 female mice (n=9/group) to treatment with 17α-E2 or two levels of CR over a 24-week period. In these mice we evaluated ovarian reserve, embryo production, pregnancy rate and litter size. Both 10% and 30% CR elicited positive effects on the preservation of the ovarian reserve, whereas 17α-E2 did not affect parameters associated with ovarian function. Following refeeding, both 10% and 30% increased fertility as evidenced by greater pregnancy rates. 17α-E2 however, failed to improve ovarian reserve preservation or fertility. For the male fertility assessment, two cohorts of male C57BL/6 mice were supplemented with 17α-E2 (n=10 and 30) from three to seven or eight months of age. These mice were mated with non-treated previously synchronized three-month-old females for embryo collection or counting of uterine implantation sites. Plasma hormonal levels, sperm quality, testes morphology were also evaluated. Despite males treated with 17α-E2 having lower body weight gain, no differences were found for the totality of the reproductive variables, showing that 17α-E2 does not affect the fertility of adult male mice. Collectively, our data indicate that 14.4 ppm of 17α-E2, which is established as effective to improve healthspan effects in male mice, does not affect reproductive parameters in mice of both sexes, and also fails to affect ovarian aging in female mice.

Keywords: calorie restriction; fertility; healthspan interventions; ovarian aging.

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Lista de Abreviaturas e Siglas

17α-E2	17 alpha-Estradiol
17β-E2	*17 beta-Estradiol*
Actb	*Actin Beta Gene*
AgRP	*Aguti-Related Peptide*
ALH	*Amplitude of Lateral Head Displacement*
Amh	*Anti-Mullerian Hormone Gene*
ANCOVA	*Analysis of Covariance*
ANOVA	*Analysis of Variance*
B2m	*Beta-2-Microglobulin Gene*
BCF	*Beat Cross Frequency*
BSA	*Bovine Serum Albumin*
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CASA	*Computer Assisted Sperm Analysis*
CHO	*Carbohydrate Content*
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CR	*Calorie Restriction*
CR10	*10% Calorie Restriction Group*
CR30	*30% Calorie Restriction Group*
CT	*Cycle Threshold*
CTL/COM	*Control Group*
DHT	*Dihydrotestosterone*
eCG	*Equine Chorionic Gonadotropin*
ELISA	*Enzyme-Linked Immunosorbent Assay*
ERα	Receptor de Estradiol Alfa
ERαKO	Knockout do Receptor de Estradiol Alfa
ERβ	Receptor de Estradiol Beta

Esr1	*Estrogen Receptor Alpha*
Esr2	*Estrogen Receptor Beta*
FAPERGS	Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul
FAT	*Fat Content*
FITC	*Fluorescein Isothiocyanate*
Foxo3	*Forkhead Box O3 Gene*
FSH	*Follicle-Stimulating Hormone*
GH	*Growth Hormone*
GnRH	*Gonadotropin Releasing Hormone*
hCG	*Human Chorionic Gonadotropin*
HOS	*Hyposmotic Swelling Test*
HPG	*Hypothalamic-Pituitary-Gonadal Axis*
Igf1	*Insulin-Like Growth Factor 1 Gene*
IP	*Intraperitoneal Injection*
IU	*International Unity*
KISS	*Kisspeptin*
Kit	*Proto-OncoGene Receptor Tyrosine Kinase Gene*
Kitl	*Kit ligand Gene*
LH	*Luteinizing Hormone*
Mtor	*Mechanistic Target Rapamycin Kinase Gene*
mTOR	*Mammalian Target of Rapamycin*
NPY	*Neuropeptide Y*
PMO	*Progressive Motility*
POMC	*Proopiomelanocortin*
Ppia	*Peptidylprolyl Isomerase A Gene*
PRO	*Protein Content*
RC	Restrição Calórica
ROS	*Reactive Oxygen Species*
TMO	*Total Motility*
TRIS	*Tris(hydroxymethyl)aminomethane Buffer*
VAP	*Velocity Average Path*
VCL	*Velocity Curved Line*
VSL	*Velocity Straight Line*

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1 Introdução

A expectativa de vida tem aumentado na população humana nas últimas décadas (CHRISTENSEN *et al.*, 2009). No entanto, com o aumento da idade ocorre um declínio das funções metabólicas, aumentando a ocorrência de doenças crônicas como obesidade e diabetes (LOPEZ-OTIN *et al.*, 2013). Existem diversas intervenções capazes de modular as vias intracelulares de detecção de nutrientes que podem retardar o envelhecimento fisiológico. Intervenções relacionadas à restrição no consumo de calorias estão bem estabelecidas como capazes de aliviar o declínio na saúde decorrente do avançar da idade em diversos modelos animais (FONTANA e PARTRIDGE, 2015). Em humanos, no entanto, efeitos colaterais e a baixa aceitabilidade dificultam o emprego de tais intervenções (LEE *et al.*, 2021; TREPANOWSKI *et al.*, 2017). Sendo assim faz-se necessária a busca de alternativas farmacológicas que possam proporcionar resultados semelhantes.

Uma das intervenções farmacológicas que vem sendo estudada é o 17α estradiol (17αE2). O 17α-E2 é um diastereoisômero natural do 17β-Estradiol (17β-E2) que foi predominantemente estudado como um hormônio neuro-protetor em modelos de isquemia, doença de Alzheimer e Parkinson (PEREZ *et al.*, 2005). Recentemente, os efeitos do 17α-E2 no envelhecimento sistêmico, longevidade e condições que promovem o envelhecimento, como a obesidade, vem sendo avaliados e o composto se demonstra capaz de modular beneficamente tais parâmetros (HARRISON *et al.*, 2014; STEYN *et al.*, 2018; STOUT *et al.*, 2017; STRONG *et al.*, 2016). O 17αE2, quando fornecido a camundongos, reverte várias condições associadas com o avanço da idade, incluindo adiposidade visceral, acúmulo de lipídeos ectópicos, intolerância à glicose, resistência à insulina, inflamação crônica de baixo grau e danos ao DNA de hepatócitos (HARRISON *et al.*, 2014; STOUT *et al.*, 2017; STRONG *et al.*, 2016). Além disto, o 17α-E2 também apresenta efeitos no cérebro, causando redução na ingestão de calorias através da modulação de vias anorexígenas hipotalâmicas (STEYN *et al.*, 2018). Estes efeitos anorexígenos podem ser observados também em camundongos com obesidade induzida e geneticamente propensos à hiperfagia (MANN *et al.*, 2020b).

Os mecanismos de ação do 17α-E2 ainda não foram completamente elucidados. Até recentemente, existia controvérsia a respeito de qual receptor de estradiol era responsável pela resposta ao 17α-E2. A afinidade do 17α-E2 com o receptor de estradiol β (ERβ) é baixa (ANSTEAD; CARLSON e KATZENELLENBOGEN, 1997), e acredita-se que por este motivo o 17α-E2 não apresenta efeitos feminilizantes (STOUT *et al.*, 2017). No entanto, a importância do receptor de estradiol α (ERα) não havia sido demonstrada até recentemente. Utilizando-se camundongos transgênicos com knockout do gene do ERα (ERαKO), pode-se observar que na ausência de um receptor ERα funcional, o 17α-E2 não é capaz de desempenhar seus efeitos, o que representa uma forte evidência quanto aos efeitos do 17α-E2 serem desencadeados via ERα (MANN *et al.*, 2020a). Estes resultados são um grande avanço para o melhor entendimento dos mecanismos de ação do 17α-E2 e abrem novas possibilidades para seu entendimento na função reprodutiva.

Além disto, resultados recentes nos sugerem que grande parte dos efeitos benéficos do 17α-E2 são mediados pelo fígado, ainda que o hipotálamo também expresse ERα e esteja envolvido na ativação de vias anorexígenas que causam a redução no consumo de alimentos em resposta ao 17α-E2 (STEYN *et al.*, 2018). No entanto, as gônadas também parecem estar envolvidas no mecanismo de ação do 17α-E2. Garratt et al ao relatarem vários benefícios de 17α-E2 em camundongos machos, incluindo melhor tolerância à glicose e sensibilidade à insulina, aumento da sinalização hepática de mTORC2 (GARRATT *et al.*, 2017), aumento do ciclo da ureia hepática (GARRATT *et al.*, 2018) e preservação prolongada do músculo esquelético (GARRATT *et al.*, 2019), observaram uma grande diminuição de tais efeitos em machos castrados, sugerindo que o metabolismo dos andrógenos pode estar envolvido nos efeitos mediados por 17αE2. Além disto, fêmeas com ovários normais não obtiveram benefício do tratamento com 17αE2, enquanto fêmeas ovariectomizadas apresentaram modulação da atividade de mTORC1 no fígado quando tratadas (GARRATT *et al.*, 2017). Estas observações corroboram dados recentes, que demonstram que o 17α-E2 previne obesidade e perda óssea somente em fêmeas ovariectomizadas (MANN *et al.*, 2020b). Desta maneira, parece que os tecidos reprodutivos têm uma influência direta sobre os efeitos benéficos do 17α-E2. Existe a possibilidade de que exista uma competição entre 17α-E2 e 17β-E2 por receptores estrogênicos. Neste caso a produção endógena de altos níveis de 17β-E2

na fêmeas poderia ser capaz de suprimir a ligação do 17α-E2 ao Erα, uma vez que o 17β-E2 possui afinidade maior com ambos os receptores (Erα e Erβ) (ANSTEAD; CARLSON e KATZENELLENBOGEN, 1997), explicando a baixa responsividade das fêmeas intactas.

Além da questão da baixa responsividade de fêmeas ao tratamento com 17αE2, outras lacunas ainda precisam ser elucidadas. Entender os possíveis efeitos do 17α-E2 na reprodução de camundongos é uma delas. Não temos conhecimento a respeito de nenhuma publicação reportando dados reprodutivos de camundongos machos e fêmeas tratados com 17α-E2. Dados os efeitos benéficos antienvelhecimento desta molécula, analisar seus efeitos tanto na fertilidade de camundongos de ambos os sexos, quanto na reserva folicular e envelhecimento ovariano de camundongos fêmeas é de suma importância para que se possa melhor compreender seus mecanismos de ação e para que estudos em humanos possam avançar com segurança.

A reserva ovariana compreende o número de folículos existentes nos ovários de uma fêmea. Esta reserva é finita, e uma vez ativados os folículos primordiais seguirão se desenvolvendo até a ovulação ou sofrerão atresia em alguma fase do processo. Portanto, quanto maior a taxa de ativação destes folículos mais cedo se dará o esgotamento da reserva (BROEKMANS; SOULES e FAUSER, 2009). Uma vez esgotada esta reserva, as mulheres iniciam a menopausa, uma fase da vida associado ao maior risco de desenvolvimento de diabetes e doenças cardíacas (WELLONS *et al.*, 2012). Menopausa precoce está inclusive associada à redução na expectativa de vida (OSSEWAARDE *et al.*, 2005), evidenciando esta relação entre envelhecimento ovariano e longevidade. A maioria das intervenções antienvelhecimento também apresenta efeitos positivos na manutenção da reserva ovariana, através da diminuição da taxa de ativação de folículos primordiais (SCHNEIDER *et al.*, 2020). Em um estudo prévio, fizemos o primeiro relato a respeito da reserva ovariana de camundongos tratados com 17α-E2 (ISOLA *et al.*, 2020). Neste estudo, surpreendentemente, não observamos nenhum efeito do 17α-E2 na preservação da reserva ovariana de camundongos do tipo selvagem, diferente do que ocorre para a RC, ou para o tratamento com rapamicina e metformina (GARCIA *et al.*, 2019; QIN *et al.*, 2019). Ainda que os efeitos do 17α-E2 sejam mais brandos em fêmeas, mais estudos avaliando sua relação com a fisiologia ovariana e fertilidade de camundongos fêmeas precisam ser realizados.

A diminuição acentuada da fertilidade com o aumentar da idade, que observamos em fêmeas não é tão óbvia em machos, uma vez que nas fêmeas o envelhecimento reprodutivo está diretamente relacionado com o declínio no número de folículos ovarianos e maior taxa de abortos (BROEKMANS; SOULES e FAUSER, 2009). No entanto, sabe-se que homens idosos possuem níveis mais baixos de testosterona (FELDMAN *et al.*, 2002) e espermatozoides menos viáveis (NG *et al.*, 2004). Em roedores com idade avançada, a literatura reporta qualidade espermática inferior (PARKENING; COLLINS e AU, 1988), capacidade de fertilização diminuída (PARKENING, 1989), espermatogênese e histologia testicular anormal (HUMPHREYS, 1977; WANG; LEUNG e SINHA-HIKIM, 1993). O número de filhotes também pode ser reduzido quando gerados por machos de idade avançada (FRANKS e PAYNE, 1970). Estudos que abordam intervenções pró-longevidade na fertilidade masculina, seja visando uma manutenção da longevidade reprodutiva, ou mesmo apenas visando proteção contra o envelhecimento sem prejudicar a fertilidade, são escassos. Os dados sobre os efeitos da RC na fertilidade masculina permanecem controversos. Alguns autores reportam danos reprodutivos aos machos durante o período de RC (COMPAGNUCCI *et al.*, 2002; MARTINS *et al.*, 2020; RIZZOTO *et al.*, 2019), enquanto outros não observaram diferença (ROCHA *et al.*, 2007). Estas controvérsias devem-se provavelmente a diferenças na intensidade da RC induzida e na idade dos animais. No que diz respeito a outras intervenções, algumas, como a rapamicina, podem ser prejudiciais à fertilidade (KIRSANOV *et al.*, 2020; LIU *et al.*, 2015), e outras, como a quercetina, podem ser prejudiciais (RANAWAT *et al.*, 2013) ou até mesmo benéficas (KHAKI *et al.*, 2010), dependendo da dose. Embora nenhum efeito feminilizante do 17α-E2 tenha sido relatado, existe a preocupação de que ele possa ter efeitos negativos na fertilidade masculina, não apenas através da perda de peso, mas também através dos receptores de estrogênio. Por outro lado, sabe-se que o ERα está envolvido na reprodução de ambos os sexos. Na ausência de um ERα funcional em machos, os túbulos seminíferos são alterados e a produção de sêmen e a função espermática são prejudicadas (EDDY *et al.*, 1996; SINKEVICIUS *et al.*, 2009). O ERα também possui impacto na reprodução feminina. Camundongos fêmeas com knockout do ERα são inférteis (DUPONT *et al.*, 2000; LUBAHN *et al.*, 1993), e embora estas fêmeas apresentem progressão normal pelos estágios de folículo primordial, primário e antrais, a maturação dos folículos pré-ovulatórios é comprometida (SCHOMBERG *et al.*, 1999). Em zebrafish, no entanto, o knockout do

ERα resulta na hiperativação dos folículos primordiais (CHEN *et al.*, 2018). Da mesma forma, em humanos, defeitos no gene que codifica ERα, resultam em depleção prematura de folículos ovarianos ou foliculogênese interrompida (GOSWAMI e CONWAY, 2005).

Sendo assim, nossos objetivos foram verificar os efeitos do 17α-E2 na fertilidade de camundongos machos e fêmeas e na reserva ovariana de camundongos fêmeas. Para tanto, produzimos dois artigos. O Artigo 1 aborda os efeitos do 17α-E2 na reserva ovariana e fertilidade de camundongos fêmeas, em contraste com a restrição calórica moderada (30%) e branda (10%). O Artigo 2, por sua vez, aborda os efeitos do 17α-E2 sobre a fertilidade de camundongos machos, avaliando parâmetros como qualidade espermática, morfologia testicular, níveis hormonais e produção de embriões.

2 Objetivos

De forma geral, nesta tese, objetivamos avaliar os efeitos do 17α-E2 na reprodução de camundongos. Mais especificamente, buscamos avaliar se a fertilidade de camundongos machos e fêmeas era afetada pelo tratamento crônico com 14.4 ppm de 17α-E2. Também buscamos avaliar possíveis efeitos do 17α-E2 na manutenção da reserva ovariana de camundongos fêmeas. Além disto, em camundongos fêmeas objetivamos contrastar os efeitos do 17α-E2 com efeitos da restrição calórica de 30% e uma restrição mais branda (10%) similar à promovida pelos efeitos anorexígenos do composto.

3 Artigos

3.1 Artigo 1

Mild calorie restriction, but not 17α-estradiol, extends ovarian reserve and fertility in female mice

José V.V. Isola, Bianka M. Zanini, Jessica D. Hense, Joao A. Alvarado-Rincón, Driele N. Garcia, Giulia C. Pereira, Arnaldo D. Vieira, Thais L. Oliveira, Tiago Collares, Bernardo G. Gasperin, Michael B. Stout, Augusto Schneider

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Mild calorie restriction, but not 17α-estradiol, extends ovarian reserve and fertility in female mice

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Highlights

- Mild calorie restriction (10%) protects against age-related declines in ovarian reserve
- Pregnancy rates are increased during refeeding following periods of mild (10%) and moderate (30%) calorie restriction
- 17α-estradiol administration fails to alter ovarian reserve or fertility

Keywords: Follicular progression, menopause, ovarian aging, pro-longevity interventions

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Competing Interests
The authors declare no conflicts or competing interests.

Abstract

Calorie restriction (CR) (25-40%) is the most commonly studied strategy for curtailing age-related disease and has also been found to extend reproductive lifespan in female mice. However, the effects of mild CR (10%), which is sustainable, on ovarian aging has not yet been addressed. 17α-estradiol (17α-E2) is another intervention shown to positively modulate healthspan and lifespan in mice but its effects on female reproduction remain unclear. We evaluated the effects of mild CR (10%) and 17α-E2 treatment on ovarian reserve and female fertility over a 24-week period, and compared these effects with the more commonly employed 30% CR regimen. Both 10% and 30% CR elicited positive effects on the preservation of ovarian reserve, whereas 17α-E2 did not alter parameters associated with ovarian function. Following refeeding, both 10% and 30% increased fertility as evidenced by greater pregnancy rates. In aligned with the ovarian reserve data, 17α-E2 also failed to improve fertility. Collectively, these data indicate that 10% CR is effective in preserving ovarian function and fertility, while 17α-E2 does not appear to have therapeutic potential for delaying ovarian aging.

1. Introduction

Human life expectancy has increased significantly over the past several decades, primarily due to public health initiatives and improvements in western medicine capabilities (Christensen et al., 2009). An unanticipated consequence of extending human lifespan was the concomitant increase in chronic disease burden which has more recently been linked to multimorbidity and the onset of frailty syndrome (Lopez-Otin et al., 2013). In particular, postmenopausal females display an accelerated aging phenotype (Levine et al., 2016) and greater disease burden as compared to premenopausal females. Furthermore, an early onset of menopause has been linked to a reduction in life expectancy (Ossewaarde et al., 2005). These observations suggest a connection between gonadal and systemic aging processes, which has led to the search for interventional strategies that not only extend lifespan and compress the period of morbidity in mid-to-late life, but also curtail ovarian aging.

The most commonly studied strategy for extending lifespan and curtailing age-related disease is calorie restriction (CR). Preclinical studies employing CR have been found to extend lifespan and delay disease onset in a variety of species ranging from invertebrates to mammals (Fontana and Partridge, 2015, Masoro, 2005). The mechanisms underlying these observations are multifactorial, but have been linked to declines in growth signaling & pro-inflammatory stress and improvements in insulin sensitivity & nutrient-sensing plasticity. In addition to extending lifespan and delaying disease onset, CR has also been found to slow the ovarian aging process, thereby extending reproductive longevity (Schneider et al., 2021).

Female reproductive longevity is dictated by the ovarian reserve, which is determined by the number of quiescent primordial follicles in the ovary (te Velde et al., 1998). Ovarian reserve is finite, and once activated, primordial follicles enter an irreversible growth phase that results in either ovulation or atresia (Richardson et al., 2014). Therefore, a greater activation rate of primordial follicles will more rapidly result in the exhaustion of ovarian reserve which results in the onset of menopause (Broekmans et al., 2009). Commonly employed CR regimens (30-40%) have been shown to reduce primordial follicle activation and extend ovarian reserve in mice (Garcia et al., 2019, Xiang et al., 2012). The primary mechanism underlying these effects is believed to be the suppression of mammalian target of rapamycin (mTOR) activity (Li et al., 2015). CR has also been shown to decrease ROS production,

increase SIRT3 activity, improve oocyte-specific meiotic spindle assembly & maintenance, while enhancing chromosome stability (Selesniemi et al., 2011).

Despite the fact that chronic CR improves ovarian reserve, ovulation and fertility are actually reduced during an active bout of CR due to declines in GnRH activity, which adversely affects cyclicity (Sun et al., 2021). However, the aforementioned improvements in primordial follicle number and oocyte quality promote greater fertility, fecundity, and offspring survival when refeeding periods follow bouts of chronic CR (Selesniemi et al., 2008). Although the benefits of CR on ovarian reserve and fertility outcomes while refeeding are well-established, human compliance when employing a robust CR regimen (25-30%) is poor (Flanagan et al., 2020), and tends to decrease over time. For example, in the first 6 months of the CALERIE 2 trial, in which the goal was 25% CR, the percentage of CR achieved dropped to only 9% after the first six months (Ravussin et al., 2015). Intriguingly, even this level of CR was capable of eliciting health benefits including weight loss and declines in metabolic risk factors. In rats, mild CR (10-20%) has also been shown to elicit benefits that are comparable to more stringent levels of CR (Richardson et al., 2016), despite there being a scarcity of data addressing the idea. However, no studies to date have evaluated the effects of mild CR (10%) on ovarian aging, which could represent a more viable intervention option for individuals attempting to delay ovarian failure and extend reproductive lifespan.

In addition to exploring the effects of mild CR on reproductive outcomes in these studies, we also evaluated the effects of 17α-estradiol (17α-E2); a natural-occurring diastereomer of 17β-estradiol (17β-E2) that has been shown to extend lifespan (Harrison et al., 2014, Strong et al., 2016) and elicit beneficial effects on metabolic and aging-related parameters in a manner similar to CR (Miller et al., 2020, Stout et al., 2017). We have also established that 17α-E2 suppresses calorie intake by modulating hypothalamic anorexigenic pathways in male mice (Steyn et al., 2018), which at least partially underlies the improvements in metabolic outcomes following 17α-E2 administration. We more recently reported that 17α-E2 reverses ovariectomy-mediated changes in adiposity and bone density in female mice (Mann et al., 2020b), indicating that specific populations of females may also benefit from 17α-E2 treatment. What remains unclear is if 17α-E2 can beneficially alter ovarian reserve or fertility in a manner similar to CR. We have previously shown that 17α-E2 treatment appears to have negligible effects on ovarian reserve in WT mice, although we did not evaluate

fertility in that study (Isola et al., 2020). Based on the aforementioned results, the aim of this study was to evaluate the effects of a mild CR (10%) and 17α-E2 treatment on ovarian reserve and female fertility, while also comparing these interventions to a more standard CR regimen (30%).

2. Methodology

2.1 Animal Diets: TestDiet, a division of Purina Mills (Richmond, IN), prepared the diets for these studies. We used TestDiet 58YP (66.4% CHO, 20.5% PRO, 13.1% FAT) ± 17α-E2 (14.4 ppm; Steraloids, Newport, RI).

2.2 Animals and Experimental Design: Ten- to eleven-week old female C57BL/6 mice (N=72) were obtained from UFPel Central Vivarium and housed three per cage at 24 ± 0.5°C on a 12:12-hour light-dark cycle with *ad libitum* access to water throughout the study. Following a two-week facility acclimation, mice were randomly assigned into one of four groups: Control (CTL; n=18) receiving *ad libitum* access to TestDiet 58YP; 17α-E2 (n=18) receiving *ad libitum* access to TestDiet 58YP + 17α-E2; 10% calorie restriction (CR10; n=18) receiving TestDiet 58YP; or 30% calorie restriction (CR30; n=18) receiving TestDiet 58YP. The number of calories that the 10% and 30% CR groups received was determined by assessing calorie consumption in the CTL group, with 10% or 30% fewer calories being provided per day during the following week. Daily food rations were provided at noon each day for the calorie restricted groups. The experimental period was 24 weeks. Body mass was evaluated at baseline, week four, and every two weeks thereafter for the first 18 weeks of treatment. After 18 weeks of treatment, half of the females were prepared for mating. These females began *ad libitum* feeding at this point in preparation for mating, therefore body mass assessments were halted. All procedures were approved by the Animal Experimentation Ethics Committee from the Universidade Federal de Pelotas.

2.3 Embryo and Ovary Collections: Following 24 weeks of treatment, half of the mice in each group (n=9) were IP injected with equine chorionic gonadotrophin (eCG; 5 IU) five days prior to euthanasia and human chorionic gonadotrophin (hCG; 5 IU) three days prior to euthanasia for synchronized superovulation. Immediately following the hCG injections, all females were paired with three-month old males and allowed to mate. Mice were fasted for 12 hours and euthanized. After euthanasia, embryos were

then collected by washing the uterine tubes with saline solution. Any female found to have at least one embryonic structure were considered responsive to the synchronization protocol. From those females that were fertilized the total number of embryos collected was recorded. Ovaries were also collected from these mice for the evaluation of ovarian reserve and gene expression.

2.4 Histological Assessment of Ovarian Reserve: Ovaries were collected and processed as previously described (Isola et al., 2020). Nine sections (three from the distal end, three from the middle, three from the proximal end) from each ovary (n=5/group) were evaluated for follicular density. Only follicles with clearly visible oocyte nuclei were counted. Follicles were classified as done previously (Isola et al., 2020) The total number of follicles from each classification were then divided by the total section area (mm^2), thereby providing follicular density (follicles/mm^2) (Ansere et al., 2021).

2.5 DNA Damage by Immunofluorescence: Immunofluorescence was performed to assess DNA damage in ovarian follicles as previously described (Saccon et al., 2020)

2.6 Ovarian Gene Expression: Total ovarian RNA was extracted and processed as previously described (Stout et al., 2011). *B2m* (Beta-2 microglobulin), *Actb* (Actin beta) and *Ppia* (Peptidylprolyl isomerase A) were evaluated as candidate housekeeper genes using the geNorm software (Vandesonpele, 2002). *B2m* was found to be the most stably expressed gene and used as the internal control. The target genes evaluated were *Mtor* (Mechanistic target of rapamycin kinase), *Kit* (Proto-oncogene receptor tyrosine kinase), *Kitl* (Kit ligand), *Foxo3* (Forkhead box O3), *Amh* (Anti-Mullerian hormone) *Igf1* (Insulin-like growth factor 1), *Esr1* (Estrogen receptor alpha) and *Esr2* (Estrogen receptor beta). Relative gene expression was calculated using the comparative CT method (Livak and Schmittgen, 2001). Primer sequences used in these analyses are shown in Table 1.

2.7 Fertility Assessment: Following 18 weeks of treatment, the other half of the females, those females not randomly selected to be subjected to superovulation (n=9/group), were provided *ad libitum* access to their respective diets for 10 days in order to restore normal ovulatory patterns (Selesniemi et al., 2008). The group

receiving 17α-E2 was kept under treatment for the whole period, including pregnancy. After the refeeding period, the females were paired with three-month old males and allowed to mate for a period of 14 days. One breeding cage in the 30% CR group had to be excluded due to death of the male. Visibly pregnant females were separated into individual cages. After birth, all pups were counted and weighed. The number of females that delivered pups were used to calculate the pregnancy rate.

2.9 Statistical Analysis: Results are presented as mean ± SEM with *p* values less than 0.05 considered to be significant unless otherwise specified. Normal distribution of data was evaluated using the Shapiro-Wilk test. Differences between groups in body mass were evaluated using the mixed procedure repeated measures test in SAS (Cary, NC, USA). Follicular density was also compared using the mixed procedure test in SAS. Of note, different ovarian histological sections were accounted for in the model as within subject replicates. The remaining statistical analyses were performed using GraphPad Prism 8 software (La Jolla, CA, USA). Variables expressed as a percentage, including pregnancy rate and percent of females responsive to ovarian synchronization, were compared using the Chi square test. The other continuous variables were compared using one-way ANOVA followed by the Tukey post-hoc test.

Table 1: Primers used in the study.

Gene	NCBI	Forward Primer Sequence	Reverse Primer Sequence	Amplicon Size (bp)
Actb	NM_007393.5	GAGACCTTCAACACCCCAGC	ATGTCACGCACGATTTCCC	263
B2m	NM_009735.3	AAGTATACTCACGCCACCCA	CAGGCGTATGTATCAGTCTC	217
Ppia	NM_008907	GAGCTGTTTGCAGACAAAGTTC	CCCTGGCACATGAATCCTGG	125
Mtor	NM_020009.2	CGGCAACTTGACCATCCTCT	TGCTGGAAGGCGTCAATCTT	101
Kitl	NM_013598.	TTCGCACAGTGGCTGGTAAC	TTCACAGCGAAGCACTCTGC	163
Kit	NM_001122733.1	CTCCCCCAACAGTGTATTCAC	TAGCCCGAAATCGCAAATCTT	90
Foxo3a	NM_001376967.1	CGGCTCACTTTGTCCCAGAT	GCCGGATGGAGTTCTTCCA	106
Amh	NM_007445.3	TCCTACATCTGGCTGAAGTGATATG	CAGGTGGAGGCTCTTGGAACT	66
Igf1	NM_010512.5	CTGAGCTGGTGGATGCTCTT	CACTCATCCACAATGCCT	118
Erα	NM_001302531.1	CCTCCCGCCTTCTACAGGT	CACACGGCACAGTAGCGAG	128
Erβ	NM_010157.3	CTGTGATGAACTACAGTGTTCCC	CACATTTGGGCTTGCAGTCTG	80

3. Results

3.1 CR prevents gains in body mass more robustly than 17α-E2 in lean female mice

As expected, both regimens of CR (10% & 30%) reduced gains in body mass over the treatment period as compared to *ad libitum* fed CTL mice (Figure 1A). 30% CR immediately affected body mass, whereas 10% CR only elicited effects after 6 weeks, and 17α-E2, interestingly did not cause body mass differences from the CTL until 16 weeks of treatment (Figure 1A). As shown in Figure 1B, the CTL mice increased their body mass by over 20% during the course of the study, whereas 17α-E2 and 10% CR groups gained approximately 13% and 2% respectively, and 30% CR females lost 6% of body mass during this period.

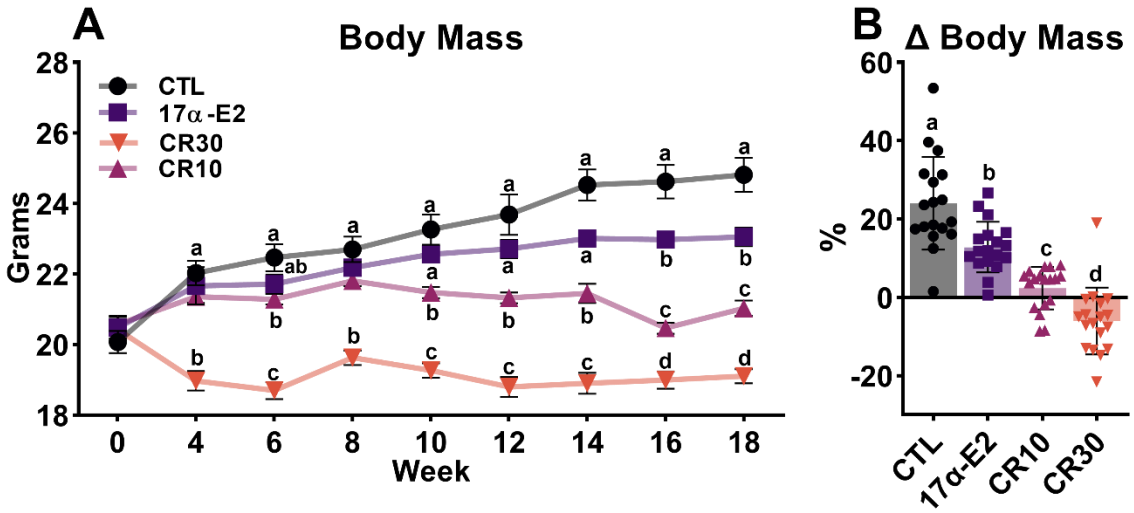

Figure 1: Calorie restriction (CR) and 17α-E2 differentially modulate body mass in female mice. (A) Body mass and (B) % change in body mass in female control (CTL) mice and those treated with 17α-E2 or CR regimens (10% or 30%) for 18 weeks starting at 12 weeks of age. All data are presented as mean ± SEM and were analyzed by repeated measures ANOVA or one-way ANOVA where appropriate. Different letters indicate statistical differences ($p<0.05$) between groups (n=18/group).

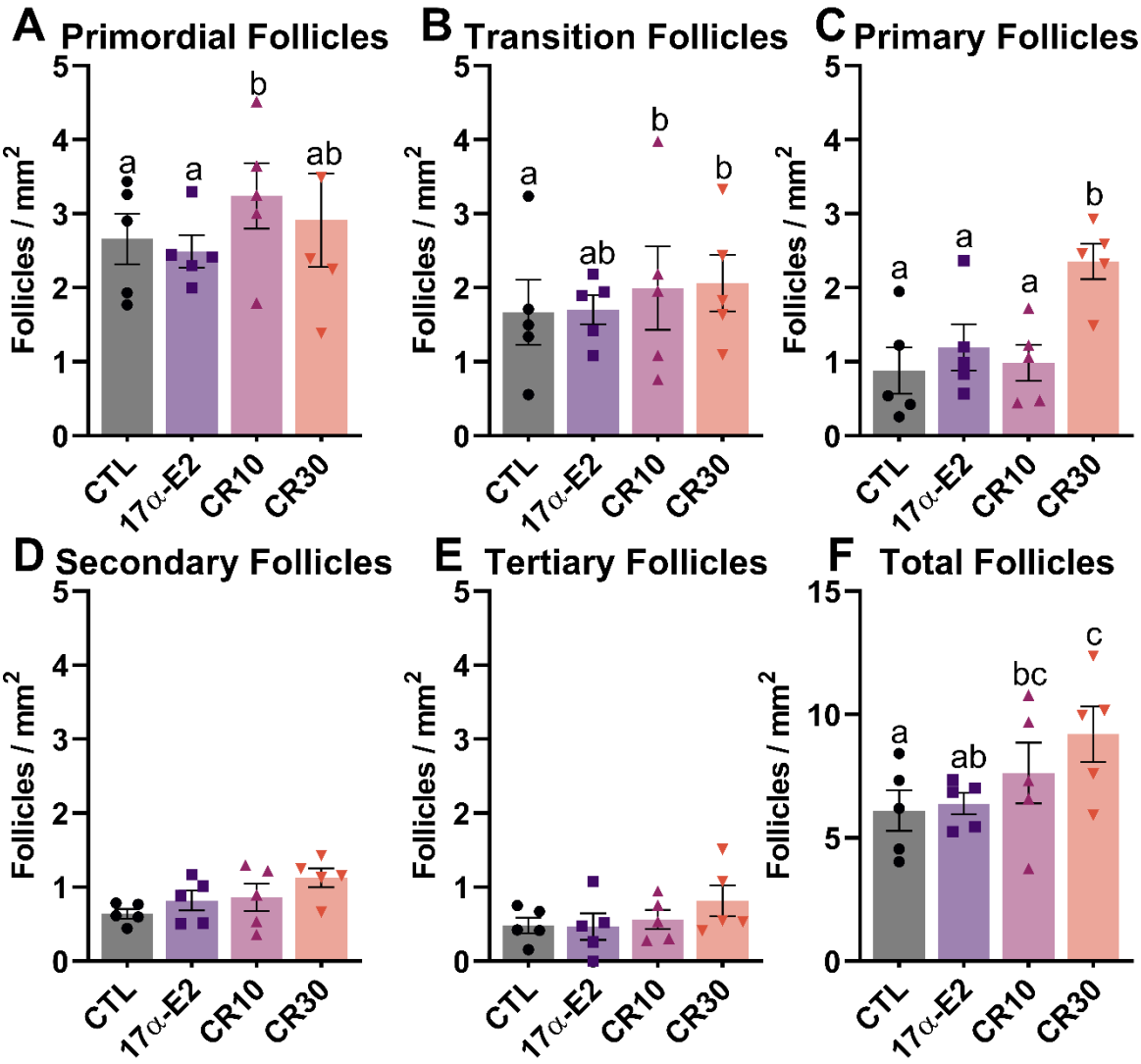

Figure 2: Both mild (10%) and moderate (30%) calorie restriction (CR) regimens positively affect ovarian reserve. Number of primordial (A), transition (B), primary (C), secondary (D), tertiary (E) and total follicles (F) per section area (mm^2) from female control (CTL) mice and those treated with 17α-E2 or CR regimens (10% or 30%) for 24 weeks starting at 12 weeks of age (n=9 sections/mouse; 5 mice/group). All data are presented as mean ± SEM and were analyzed using the mixed procedure test in SAS. Different letters indicate statistical differences ($p<0.05$) between groups.

3.2 Mild CR positively affects ovarian reserve

30% CR positively affected the ovarian reserve, as seen by the overall greater follicle density in this group. However, no statistical difference was observed for primordial follicles, as would be expected. This result can be due to the methodology used, which compares the number of follicles/mm^2 and do not estimate the number of follicles in the ovary, which makes the discrepancy less obvious. Moreover, 10% CR had an effect on the number of both primordial and total follicles (Figure 2). The overall number of follicles in the 17α-E2 group, however, was similar to the control group,

suggesting that 17α-E2 does not affect the ovarian reserve. We also performed anti-γH2AX immunostaining, which is a marker of DNA damage and observed no difference between groups for both primordial and primary follicles (Figure 3). We also assessed ovarian gene expression, and found no differences for genes involved on the pathway of follicle activation (*Igf1, Mtor*, *Kit*, *Kitl*, *Foxo3*), a fertility marker (*Amh*) or the estrogen receptors (*Esr1, Esr2*) (Figure 4).

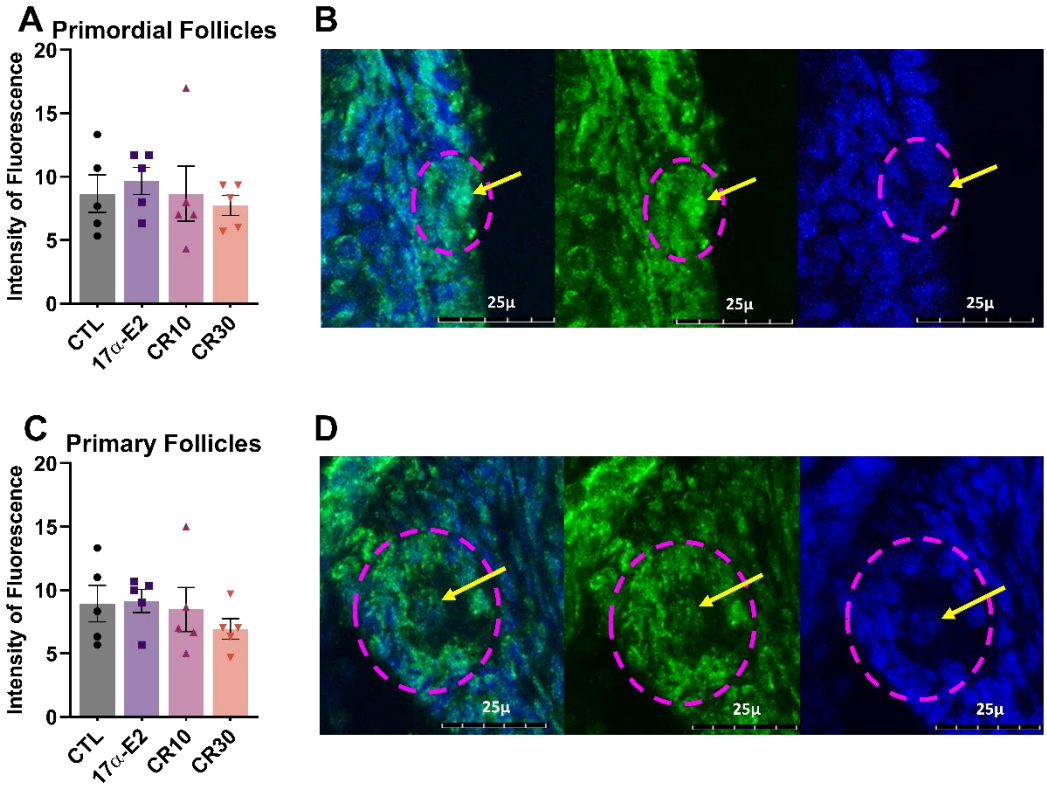

Figure 3: Calorie restriction (CR) and 17α-E2 fail to reduce DNA damage in the oocyte of primordial and primary follicles. Average fluorescence intensity of γH2AX immunostaining in oocytes from (A) primordial and (C) primary follicles from female control (CTL) mice and those treated with 17α-E2 or CR regimens (10% or 30%) for 24 weeks starting at 12 weeks of age. Representative images demonstrate γH2AX protein (green) and blue represent DAPI staining of genetic material in (B) primordial and (D) primary follicles. The dashed pink line represents the follicles being evaluated, whereas the line arrows point out the nuclei being evaluated. The images are stacked slices in the maximum projection mode, at a 400x magnification in a TCS SP8 CLSM Leica microscope. Values presented are averages of 3 follicles nuclei for each mouse (n = 5 mice/group). All data are presented as mean ± SEM and were analyzed by one-way ANOVA.

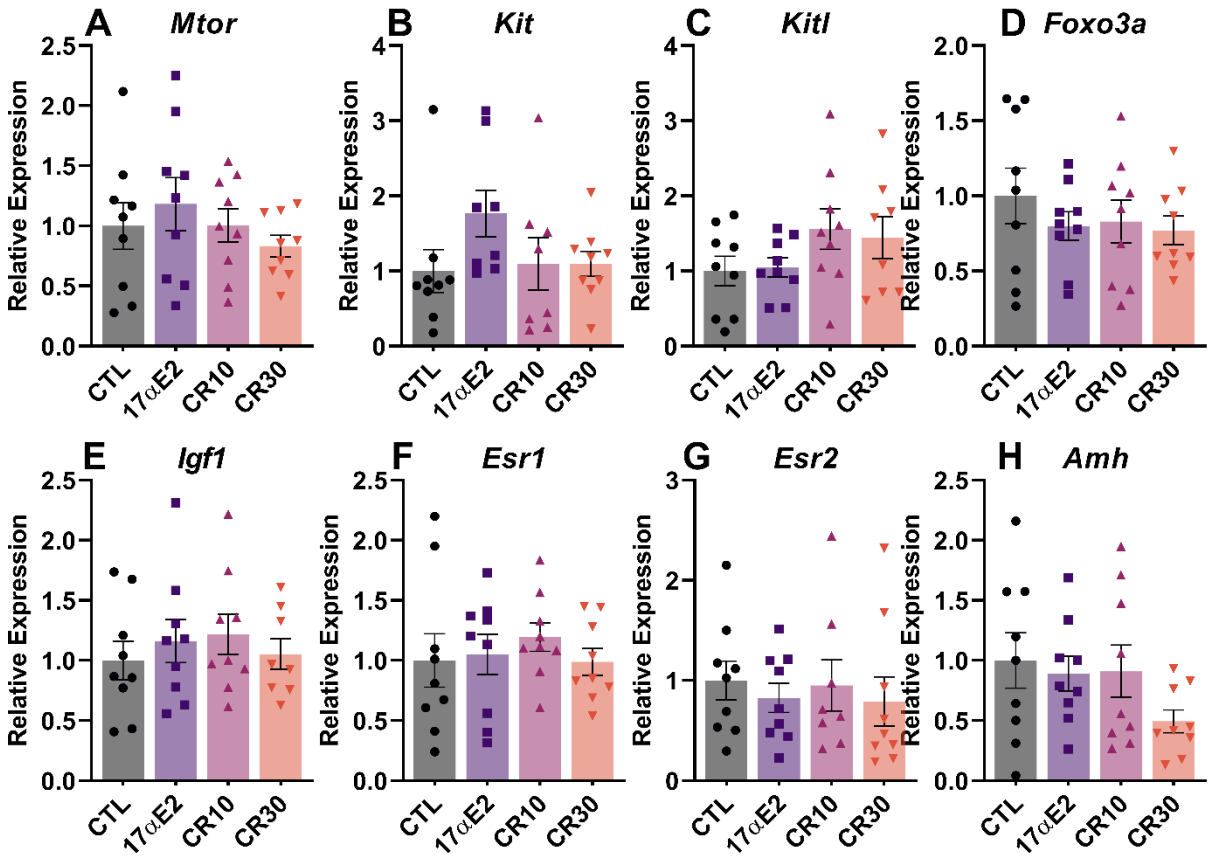

Figure 4: Calorie restriction (CR) and 17α-E2 did not alter the expression of genes related to fertility and follicle activation. Relative expression of (A) *Mtor* (Mechanistic target of rapamycin kinase), (B) *Kit* (Proto-oncogene receptor tyrosine kinase), *Kitl* (Kit ligand), *Foxo3* (Forkhead box O3), *Amh* (Anti-Mullerian hormone), *Igf1* (Insulin-like growth factor 1), *Esr1* (Estrogen receptor alpha) and *Esr2* (Estrogen receptor beta) in ovaries of female control (CTL) mice and those treated with 17α-E2 or CR regimens (10% or 30%) for 24 weeks starting at 12 weeks of age (n=9/group). All data are presented as mean ± SEM and were analyzed by one-way ANOVA.

3.3 In the absence of refeeding, CR decreases responsiveness to ovulatory synchronization

The 30% CR group had a poorer response to the superovulation protocol than other groups (33.33%), whereas the 10% CR group had an intermediate response (55.55%) and the CTL and 17α-E2 groups had a higher and similar response (77.77% and 66.66%, respectively) (Figure 5A). Regarding the number of embryos recovered, no difference was observed among groups ($p = 0.985$) (Figure 5).

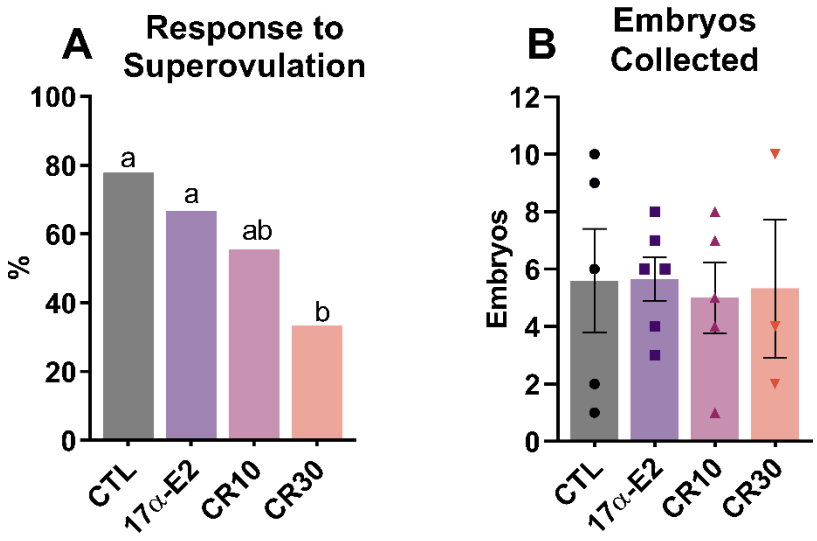

Figure 5: Calorie restriction (CR) decreases % of females responsive to superovulation treatment, but does not affect the number of structures (oocytes/embryos) produced by those that respond. (A) Percentage of females that had at least one embryo or oocyte recovered by uterine flushing after ovulation induction and mating (n=9/group) and (B) number of embryos produced by those that responded to the protocol and were fertilized (n = 5 CTL; 6 17α-E2; 5 CR10; 3 CR30) amongst female control (CTL) mice and those treated with 17α-E2 or CR regimens (10% or 30%) for 24 weeks, starting at 12 weeks of age. (A) data are presented in % and were analyzed by Chi-square (7/9 CTL; 7/9 17α-E2; 5/3 CR10; 3/9 CR30), (B) data are presented as mean ± SEM and were analyzed by one-way ANOVA. Different letters indicate statistical differences ($p<0.05$) between groups.

3.4 Both mild and moderate CR regimens increase pregnancy rates after refeeding

After 10 days of refeeding for the CR groups, we observed a pregnancy rate of 100% in females submitted to 30% CR, while groups CTL and 17α-E2, presented a lower rate (44.44%) and the group 10% CR presented an intermediate rate (77.77%) (Figure 6). Litter size was also similar among groups. The weight of neonates in the 17α-E2 group, interestingly, was lower than that of the CTL group, although it was not different from the CR groups (Figure 6).

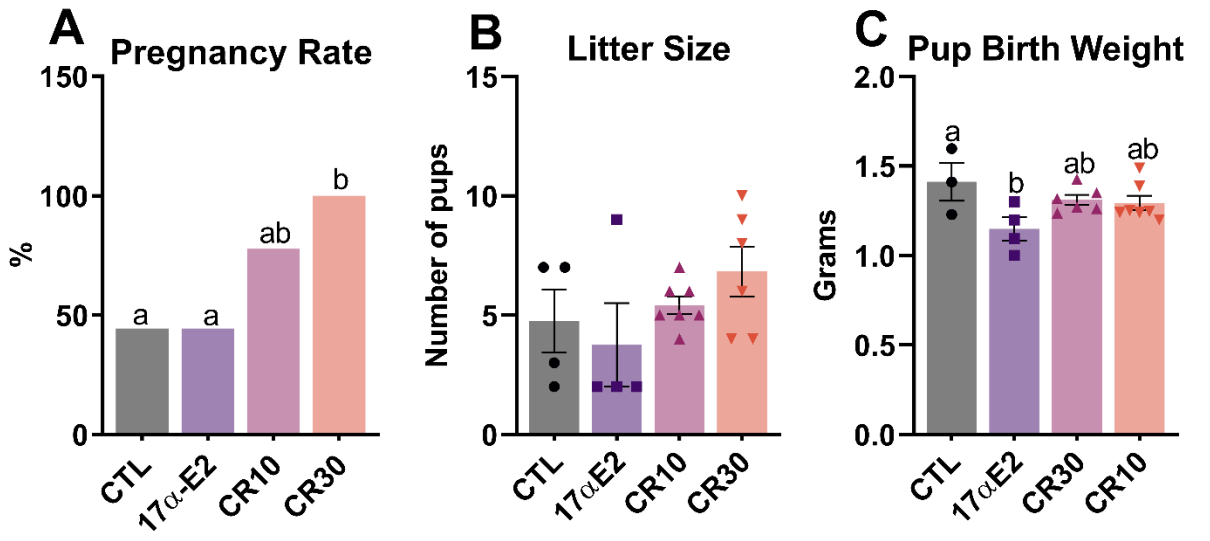

Figure 6: After refeeding, calorie restriction (CR) increases pregnancy rate in female mice. Pregnancy rate (A), litter size (B), pup birth weight (C) from female control (CTL) mice and those treated with 17α-E2 for 24 weeks or CR regimens (10% or 30%) for 18 weeks starting at 12 weeks of age and then refed for 10 days before mating. A) data are presented in % and were analyzed by Chi-square (4/9 CTL; 4/9 17α-E2; 7/9 CR10; 6/6 CR30), (B & C) data are presented as mean ± SEM and were analyzed by one-way ANOVA. Different letters indicate statistical differences ($p<0.05$) between groups.

4. Discussion

In the present study we evaluated the effects of CR (10% & 30%) and 17α-E2 treatment on ovarian reserve and female mice fertility. As a means of demonstrating tangible treatment effects, we assessed body mass for comparison purposes. As expected, 30% CR immediately prevented gains in body mass, whereas 10% CR required a longer duration of treatment before effects on body mass were observed. 17α-E2 treatment also mildly prevented gains in body mass but these effects were less pronounced when compared to the two CR regimens. Our findings support previous reports demonstrating minimal, if any, effects on body mass in wild-type females (Garratt et al., 2019, Mann et al., 2020a, Mann et al., 2020b, Sidhom et al., 2021). In fact, 17α-E2 seems to fail eliciting beneficial effects in intact females. Conversely, 17α-E2 has been found to prevent gains in body mass and adiposity, prevent bone loss, and alter the metabolome in ovariectomized mice (Garratt et al., 2018, Mann et al., 2020b), which suggests that the presence of functional ovaries and/or their endocrine factors may attenuate 17α-E2 actions in females. However, previous studies from our group have shown that life-extending strategies such as GH deficiency (Saccon et al.,

2017), 30% CR (Garcia et al., 2019) and rapamycin (Garcia et al., 2019) also result in improved ovarian reserve. Therefore, we hypothesized that 17α-E2 could have some beneficial effect in the reproductive axis or even a negative effect that would counterbalance its positive endocrine effects in females.

With regard to ovarian reserve, we observed an overall trend for a greater number of follicles in both CR treatment groups as evidenced by more primordial and transition follicles in the 10% CR group and more transition and primary follicles in the 30% CR group. In addition, the 30% CR group had slightly higher total follicle density than the 10% CR group, suggesting an incremental effect. Several previous studies have found that between 3 to 6 months of 30% CR increases the ovarian reserve (Garcia et al., 2019, Li et al., 2015, Xiang et al., 2012, Liu et al., 2015). In the current study we did not find statistical differences in the number of primordial follicles between 30% CR and the CTL group, although we did observe more primary follicles in this group. This discrepancy could be due to differences in methodological approaches for quantifying primordial follicles. Most previous reports provide an estimation of the total number of follicles in the ovaries, whereas we report the number of follicles in a given area of histological sections. Potential differences are less obvious using our methodology. However, overall, both 10 and 30% CR improved primordial/transition/primary follicle density compared to the control group, reflecting in increased total follicle density, in line with previous evidence. Of note, 17α-E2 did not alter ovarian reserve in this study, indicating its overall endocrine effect are not sufficient to affect folliculogenesis.

Interestingly, 10% CR appears to have elicited protective effects on ovarian reserve similarly to those seen in the 30% CR group, despite much milder effects on body mass. This observation is aligned with previous reports demonstrating that mild CR in preclinical studies can positively affect age-related parameters associated with disease burden (Richardson et al., 2016, Duffy et al., 2001). We perceive this to be an important observation because human compliance to more stringent CR regimens is poor (Ravussin et al., 2015). In fact, most humans only achieve a small percentage of their prescribed energy deficit (Flanagan et al., 2020), indicating that milder CR regimens may serve as an effective alternative. In addition to evaluating ovarian reserve, we also assessed fertility in this study. CR has previously been reported to modulate female fertility (Sun et al., 2021). During the period when CR in employed, cyclicity is impaired due to CR-mediated effects on the hypothalamus-pituitary-ovarian axis due to changes in gonadotrophin pulsatility (Sun et al., 2021). However, female fertility is significantly

enhanced during refeeding periods that follow bouts of CR (Selesniemi et al., 2008, Sluczanowska-Glabowska et al., 2015). In the current study, females that were used for embryo collection were not subjected to refeeding prior to initiating the superovulation protocol because it provided additional confirmation that our CR regimens were indeed modulating cyclicity. As expected, the 30% CR group displayed a significant reduction in ovulation, in alignment with other reports in the literature (Liu et al., 2015). Interestingly, 10% CR also reduced ovulation to a lesser extent, although this finding did not rise to the level of statistical significance. Nevertheless, despite fewer females responding to superovulation treatment in the CR groups, the mice that did respond produced similar numbers of structures (oocytes/embryos) as those mice in the CTL and 17α-E2 treatment groups. Response to synchronization and embryo numbers were also not affected by 17α-E2, further indicating its lack of effect in the reproductive axis.

In addition to the experiments outlined above, we also evaluated the effects of CR regimens on fertility in mice that were subjected to a ten-day *ad libitum* refeeding period. In alignment with previous reports (Selesniemi et al., 2008, Sluczanowska-Glabowska et al., 2015) we found that both regimens of CR increased pregnancy rates, although the changes in the 10% CR group did not reach statistical significance. Litter sizes and pup birth weights were not affected by either CR regimen following the refeeding period, suggesting that lower body masses during pregnancy did not adversely affect the ability of females to carry and deliver multiple pups. Females provided 17α-E2 did not display any changes in pregnancy rates, although pup birth weights were found to be lower, which has been associated with poor health outcomes (Beauchamp et al., 2015). Future studies will be needed to determine if 17α-E2 treatment during pregnancy adversely affects pup morbidity during maturation and adulthood. Despite the fact that CR clearly improved ovarian reserve and fertility in our studies, the underlying mechanism(s) promoting these benefits remain unclear. Previous studies have linked the protective effects of CR to declines in ovarian DNA damage and oxidative stress (Luo et al., 2017), which certainly contribute to improvements in follicle number and quality (Prasad et al., 2016, Selesniemi et al., 2011). However, we did not observe difference in primordial or primary oocyte DNA damage, or any transcriptional changes in genes associated with follicle activation or fertility. Future studies will need to explore alternative mechanisms by which mild CR may be modulating ovarian reserve, including its effects on mTOR activity. The

suppression of mTOR activity in the ovary decreases primordial follicle activation, thereby extending ovarian reserve (Li et al., 2015). This has been linked to declines in FOXO3a phosphorylation, which is the primary signal for the activation of dormant primordial follicles (Saccon et al., 2017, Castrillon et al., 2003). To date, it remains unclear if mild CR regimens also modulate mTOR and FOXO3a activity in the ovary.

There are a few notable limitations to our studies. First, the methodology used to assess follicle reserve consists of counting of follicles/mm^2 on histological sections, which often times leads to less obvious differences between groups as compared to estimating the overall number of follicles in the ovary. Despite not being the best methodology for comparing different treatments in age matched mice, our approach provides an exact quantification of different follicle types within the sections being evaluated. Secondly, the females being evaluated in these studies for changes in DNA damage responses were relatively young (9 months old), which may explain why we did not observe a beneficial effect of CR. Also, transcriptional analyses were done in whole ovarian extracts, therefore it prevents us from making any conclusions related to cell-type-specific mechanisms of ovarian aging. Lastly, the fertility assessments were not similar for all groups, since CR was withdrawn and 17α-E2 was maintained prior to mating and during pregnancy, explaining the higher pregnancy in CR groups. Also, we did not measure endocrine markers during estrous cycle and pregnancy. However, CR is known to impair the hypothalamus-pituitary-ovarian axis and the ovulation rate and estradiol/progesterone levels (Sun et al., 2021). However, the results suggest that 17α-E2 does not have deleterious effects on the reproductive axis, as the fertility was similar to the control group. Despite these limitations, this is the first report to our knowledge that demonstrates that mild CR can prolong ovarian reserve and increase fertility in female mice.

5. References

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3.2 Artigo 2

17α-estradiol does not adversely affect sperm parameters or fertility in male mice

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ABSTRACT

17α-estradiol (17α-E2) is referred to as a nonfeminizing estrogen that was recently found to extend healthspan and lifespan in male, but not female, mice. Despite an abundance of data indicating that 17α-E2 attenuates several hallmarks of aging in male rodents, very little is known with regard to its effects on feminization and fertility. In these studies, we evaluated the effects of 17α-E2 on several markers of male reproductive health in two independent cohorts of mice. In alignment with our previous reports, chronic 17α-E2 treatment prevented gains in body mass, but did not adversely affect testes mass or seminiferous tubule morphology. We subsequently determined that chronic 17α-E2 treatment also did not alter plasma 17β-estradiol or estrone concentrations, while mildly increasing plasma testosterone levels. We also determined that chronic 17α-E2 treatment did not alter plasma follicle-stimulating hormone or luteinizing hormone concentrations, which suggests 17α-E2 treatment does not alter gonadotropin-releasing hormone neuronal function. Sperm quantity, morphology, membrane integrity, and various motility measures were also unaffected by chronic 17α-E2 treatment in our studies. Lastly, two different approaches were used to evaluate male fertility in these studies. We found that chronic 17α-E2 treatment did not diminish the ability of male mice to impregnate female mice, or to generate successfully implanted embryos in the uterus. We conclude that chronic treatment with 17α-E2 at the dose most commonly employed in aging research does not adversely affect reproductive fitness in male mice, which suggests 17α-E2 does not extend lifespan or curtail disease parameters through tradeoff effects with reproduction

KEYWORDS: aging, androgen, estrogen, hypothalamic-pituitary-gonadal axis, reproduction

INTRODUCTION

Despite significant increases in human lifespan over the past several decades, human healthspan has failed to increase in a similar fashion. In fact, the period of morbidity in mid-to-late life, and prevalence of multimorbidity, has increased dramatically in recent decades (1). Although it is well-established that dietary interventions including chronic calorie restriction and various forms of fasting can delay and/or reverse basic mechanisms of aging, many of these strategies are poorly tolerated (2). Compliance issues remain a paramount hurdle with dietary interventions due to adverse effects on mood, thermoregulation, and/or musculoskeletal mass (3). These adverse health outcomes demonstrate the need for pharmacological approaches aimed at curtailing aging and disease.

17α-estradiol (17α-E2) is one of the more recently studied compounds to demonstrate efficacy for beneficially modulating age- and disease-related outcomes. The NIA Interventions Testing Program found that 17α-E2 administration extends median lifespan of male mice in a dose-dependent manner (4, 5). Our group has reported that 17α-E2 reduces calorie intake and adiposity while concomitantly improving several markers of metabolic homeostasis including glucose tolerance, insulin sensitivity, and ectopic lipid deposition in obese and aged male mice (6-10). Others have reported similar findings including improvements in glucose tolerance, mTORC2 signaling, hepatic amino acid composition and markers of urea cycling, markers of neuroinflammation, and sarcopenia (11-15). Although the mechanisms of action for 17α-E2 remain debated, we recently reported that the ablation of estrogen receptor α (ERα) completely attenuates all beneficial metabolic effects of 17α-E2 in male mice (6), thereby indicating that 17α-E2 signals through ERα to elicit benefits, but also that ERα could be a ‘druggable’ target for mitigating aging and disease in males.

Despite an abundance of data demonstrating that 17α-E2 improves a multitude of parameters related to metabolism and aging in males, very little has been done to determine if 17α-E2 promotes significant feminization, and more importantly, if any of these outcomes deleteriously affects male fertility.

Interestingly, several interventions that extend lifespan can also impart some impact on reproduction. For instance, females subjected to CR, rapamycin, or metformin display a suppression of ovarian primordial follicle activation, thereby extending the reproductive window (16, 17) and improving fertility (18) once treatment has subsided. The effects of lifespan-extending interventions on male reproduction are conflicting and exceedingly unclear. CR is known to reduce libido in men (19) which could be related to a suppression of the reproductive axis (20). However, other reports indicate that CR does not alter circulating testosterone or semen quality in rhesus macaques (21, 22). In rodents, moderate CR (20-30%) has been reported to decrease testes and seminal vesicle mass (23), increase sperm defects (23, 24) and suppress serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (23). Conversely, other studies have found that CR has no effects on testes mass (25, 26), sperm production (25), serum testosterone (26) or fertility (26). The effects of rapamycin on male fertility are more consistent and clearly indicate deleterious effects related to testes mass, spermatogenesis, and testosterone production in both rodents and humans (27-29). To date, no study has thoroughly evaluated the effects of 17α-E2 on sperm parameters or fertility in male model systems.

The work outlined in this report sought to determine if chronic 17α-E2 treatment adversely affected testes morphology, sperm parameters, reproductive fitness, and/or the serum hormonal milieu in middle-aged male mice. Two independent cohorts of mice were studied and found to respond nearly identically to 17α-E2 treatment. We

determined that chronic treatment with 17α-E2 at the dose most commonly employed in aging research does not adversely affect reproductive fitness in male mice. It remains unknown if higher dosing regimens of 17α-E2 induces reproductive abnormalities in rodents, or if allometric scaling to higher-order mammals for the purpose of translational studies can be effectively undertaken.

MATERIALS AND METHODS

Control and experimental diets. TestDiet, a division of Purina Mills (Richmond, IN), prepared all the diets. LabDiet 58YP (66.4% CHO, 20.5% PRO, 13.1% FAT) was used as the control (Con) diet and LabDiet 58YP was supplemented with 17α-E2 (14.4 ppm Steraloids, Newport, RI) for the treatment diet.

Animals. All mice (C57BL/6) used in these studies were obtained from the UFPel Central Vivarium. Unless otherwise noted, all mice were group housed at 24 ± 2°C on a 12:12-hour light-dark cycle with *ad libitum* access to food and water. At three months of age mice were randomly assigned to Con or 17α-E2 treatment groups. Two cohorts of mice were evaluated independently for comparison purposes and to determine outcome repeatability. In Cohort 1, Con (n=8) and 17α-E2 (n=10) treatment groups were evaluated for five months. In Cohort 2, Con (n=30) and 17α-E2 (n=30) treatment groups were evaluated for four months. Body mass was monitored monthly. During the final week of treatment, male mice from both cohorts were housed with three-month-old female mice (1 male with 2 females) that had undergone estrus synchronizations so male fertility could be assessed. Female mice were synchronized with IP injections of equine chorionic gonadotrophin (eCG; 5UI) two days prior to exposure to males and human chorionic gonadotrophin (hCG; 5UI) when housed with males. Male mice were humanly euthanized with isofluorane one week after being paired with females and

plasma, testes, and semen were collected. Females that were bred with males from Cohort 1 were humanely euthanized with isoflurane three days after being paired with males so that embryos could be collected as previous described (30). Any female found to have at least one embryo was deemed to have been successfully fertilized by the corresponding male. Females that were bred with males from Cohort 2 were humanely euthanized with isoflurane eight days after being paired with males so that the number of implantation sites could be counted. All procedures were approved by the Ethics Committee for Animal Experimentation from the Universidade Federal de Pelotas.

Semen collection and analysis. Following euthanasia of males, the epididymis was removed and placed into a microtube containing 300 µL of preheated (36.5°C) extender solution (3.634g TRIS, 0.50g glucose, 1.99g citric acid, 6.0g BSA, 100ml of H_2O). The epididymis was then gently fractionated with scissors and agitated for 5 minutes in order to release the sperm. For sperm concentration analyses, 25 µL of the semen solution was aliquoted and diluted with 25 µL of formaldehyde-saline (8.5g NaCl, 100ml of 40% formaldehyde, 900ml of H_2O). Concentration was determined by counting sperm cells using a hemocytometer. Sperm motility was evaluated using a Zeiss Axio Scope A1 microscope (Jena, Germany) coupled to a computer-assisted semen analysis system (CASA, SpermVision, Minitube, Tiefenbach, Germany). For this assay 6 µL of semen solution was placed on a glass slide under a coverslip and observed at 200X magnification. The CASA system determined velocity average path (VAP), velocity curved line (VCL), velocity straight line (VSL), beat cross frequency (BCF), amplitude of lateral head displacement (ALH), total motility (TMO), and progressive motility (PMO). The automated system evaluated a minimum of 500 cells

for each sample with an average of 822.6 and a range of 530 to 974 cells/sample. The membrane integrity of sperm cells was assessed by a hypoosmotic swelling test (HOS) (31), which is done by determining the % of sperm tail swelling following the exposure to a hypoosmotic solution. For this analysis, 10 µL of the semen solution was aliquoted and diluted with 90 µL of HOS solution (2.7g of fructose, 1.47g of sodium citrate and 100ml of H2O) and incubated at 36.5°C for 30 min. After incubation, 200 cells were counted as non-swollen or swollen tails using an Olympus BX 51 microscope (América INC, São Paulo, SP) at 400X magnification. As a negative control, 6 µL of semen solution was smeared on a glass slide before incubation with HOS solution and 200 cells were counted for swollen tails. The percentage of membrane integrity was calculated by the number of sperm with swollen tails before and after incubation as previously described (32). For the acrosome integrity analysis, 3 µL of semen solution was aliquoted and diluted with 3 µL of a buffer containing Lectin from *Arachis hypogaea* FITC conjugate (Sigma-Aldrich, Saint-Louis, MO, USA) and incubated at room temperature in the dark for 15 min. In this analysis, by observing the emission of green fluorescence the number of intact acrosome cells was counted in a total of 100 cells. This analysis was performed on an Olympus BX 51 epifluorescence microscope at 400X magnification (América INC, São Paulo, SP, Brazil) using a WU filter (450-490 nm excitation and 516-617 emission) as previously described (33). Lastly, sperm were also evaluated for morphological defects. For this, a drop of semen was smeared on a glass slide and dried. Slides were then stained using a fast-panoptic staining method (34). One hundred cells were observed on a Nikon Eclipse E200 microscope (Tokyo, Japan) at 40X magnification and the number of normal cells or those with defects (head, midpiece, tail) were recorded (34).

Testes histology. One of the testes was fixed in formalin, dehydrated in alcohol, cleared in xylol and embedded in paraplast (Sigma-Aldrich, Saint-Louis, MO, USA). Paraplast blocks were transversally cut into 5 µm sections starting in the middle of the testicle. The sections were then dewaxed in xylene, rehydrated in descending series of alcohol, and stained with H&E. H&E stained sections were photographed by a camera coupled to a microscope using the software TC Capture (Tucsen Photomics Co.) at 40X magnification on a microscope (Nikon Eclipse E200, Nikon, Tokyo, Japan). In the images, twenty round or near round seminiferous tubules were randomly chosen for each animal. Perimeter, area and diameter of the seminiferous tubules and its lumen were measured using the Motic 2.0 software (Motic®, Hong Kong, China). For statistical comparison, the mean measurements from 20 seminiferous tubules were used.

Circulating hormone analyses. Plasma testosterone, 17β-E2, 17α-E2, and estrone were evaluated by liquid chromatography (LC/MS/MS) as previously described (10). Plasma FSH and LH levels were evaluated by ELISA (FSH: KA2330, Abnova, Taipei, Taiwan; LH: ABIN6574077, Antibodies-online, Limerick, PA, USA).

Statistical analyses. Statistical analysis was performed using the GraphPad Prism 6 or SPSS version 28. The Shapiro-Wilk test was performed to test normality. Body weight was compared by repeated measures ANOVA and percentage of fertilized females and viable embryos was compared by chi-square, whereas the other variables were compared by Student's T-test. For analysis of testes mass relative to body mass, we used a general linear model, including testes mass as the dependent variable, treatment as a fixed factor, and body weight as a continuous covariate. Non-parametric

variables from CASA were compared by Mann-Whitney test. P-values lower or equal to 0.05 were considered significant.

RESULTS

17α-E2 treatment does not affect testes mass or seminiferous tubule morphology

Similar to our previous reports (6, 9, 10), 17α-E2 treatment reduced male body mass in both cohorts we evaluated (Figure 1A, B, E, F). We did not evaluate adiposity in these studies but we have previously established that reductions in adipose accounts for the vast majority of declines in mass during 17α-E2 treatment (6, 9, 10). We next sought to determine if chronic 17α-E2 treatment altered testes mass or seminiferous tubule morphology. We found that testes mass was unaffected by 17α-E2 treatment in both cohorts of animals tested (Figure 1C, G). Evaluation of testes mass relative to body mass revealed no association between the two variables (Figure 1D, H), which is aligned with a previous report indicating that testes mass is only mildly correlated with body mass in mice (35). Subsequent analysis of seminiferous tubules within the testes revealed that 17α-E2 treatment did not adversely affect the tubule or lumen morphology (Figure 2A-B) or size (Figure 2C-H). This latter observation suggests that 17α-E2 treatment does not alter sperm parameters because changes in seminiferous tubule size, particularly lumen size, is associated with declines in sperm quality (36).

17α-E2 treatment does not alter circulating sex hormones or gonadotropins

Given that 17α-E2 is known to be a mild 5α-reductase inhibitor (37), coupled with several reports suggesting that 17α-E2 treatment elicits health benefits by

modulating androgen metabolism (11, 12, 14), we next sought to determine if the endogenous sex hormone milieu was altered in the current study. As expected, plasma 17α-E2 was robustly increased with 17α-E2 treatment (Figure 3A). Surprisingly, the plasma levels of 17α-E2 observed in the current study were several times higher than we found in much older mice receiving an identical dose in a very similar diet (10). Despite the higher level of plasma 17α-E2 in the current study, plasma 17β-E2 was essentially unchanged by 17α-E2 treatment (Figure 3B). Plasma estrone (Figure 3C) was undetectable (< 3.3 pg/ml) in most samples, and thus, nearly identical between Con and 17α-E2 treated animals. Plasma testosterone (Figure 3D) was not statistically different between treatment groups, but was found to be trending higher in mice treated with 17α-E2. This observation may result from the aforementioned 5α-reductase inhibition properties of 17α-E2, which may increase circulating testosterone levels due to a reduced capacity to convert testosterone into dihydrotestosterone (DHT) (38). Due to limited plasma volumes we were unable to directly evaluate DHT in these studies. However, we did evaluate the effects of 17α-E2 treatment on circulating gonadotropins because estrogens are known to signal in the hypothalamus and pituitary as part of a negative feedback loop to control gonadotropin production and secretion (39). We found that plasma LH (Figure 3E) and FSH (Figure 3F) were not significantly altered by 17α-E2 treatment, suggesting that exogenously administered 17α-E2 does not influence androgen-mediated hypothalamic-pituitary-gonadal (HPG) signaling in male mice.

17α-E2 treatment does not alter sperm morphology, quantity, or motility

Males with higher-than-normal plasma 17β-E2 often display low sperm counts and/or declines in sperm quality to include motility (40), which contributes to infertility

(41). Therefore, we sought to determine if chronic 17α-E2 treatment would adversely affect sperm parameters. Morphological assessment of sperm from both cohorts of mice revealed that 17α-E2 treatment did not diminish the quantity of normal sperm, or increase sperm with apparent defects, including decapitation (Figure 4). Subsequent evaluation of sperm concentrations and various measures of motility, including straight and curvilinear velocities, amplitude of lateral head displacement, and beat cross frequency, were not different between Con and 17α-E2 treatment groups in both cohorts of mice tested (Table 1). Although these variables do not directly predict fertility outcomes, they are useful indicators of sperm quality because they represent sperm kinetics (42). Both sperm cell membrane integrity and acrosomal integrity, which are important factors for determining fertilizing capacity (43), were found to be unaffected by 17α-E2 treatment in cohort 1.

17α-E2 treatment does not adversely affect male fertility

In addition to the variables outlined above, we also wanted to determine if chronic 17α-E2 treatment would adversely affect male fertility. To do this we subjected male mice from both treatment groups to female mice that had undergone estrus synchronization. We then determined the percentage of successful fertilizations in cohort 1, and the pregnancy rates and number of implanted embryos in cohort 2. We found that Con and 17α-E2 treated males were equally effective at fertilizing females in cohort 1 and at successfully fertilizing oocytes that result in implanted embryos in the cohort 2 (Table 1). These findings clearly indicate that the 17α-E2 dosing regimen used in these studies does not adversely affect the propensity for male mice to breed, or their ability to fertilize oocytes that become implanted embryos.

DISCUSSION

17α-E2 treatment was recently found to extend healthspan and lifespan in male mice (4-15). Despite several lines of evidence indicating that 17α-E2 delays aging hallmarks in male rodents, very little is known with regard to its effects on feminization, sperm quality, and fertility. We previously reported that 17α-E2 administration only mildly affects circulating testosterone levels, gonadal mass, or seminal vesicle mass in aged male mice (10). Although informative, the mice evaluated in those studies were over 18 months of age, and thus, well-beyond their reproductive window. Therefore, it remains unclear if 17α-E2 administration adversely affects reproductive fitness in breeding age males. In the current studies, we evaluated the effects of chronic 17α-E2 treatment on several parameters indicative of male reproductive health, in addition to directly assessing fecundity in young, breeding age male mice.

In alignment with our previous reports, 17α-E2 administration prevented gains in body mass in male mice in these studies. We previously established that 17α-E2-mediated changes in body mass are due to an almost exclusive loss of adiposity, which we later linked to the modulation of hypothalamic anorexigenic signaling pathways (6, 9, 10). Given the close association between hypothalamic regulation of metabolism and reproduction (44), coupled with the close proximity of hypothalamic neurons that control satiety, metabolism, and reproduction (44), we speculated that 17α-E2 treatment would also modulate the HPG axis in a manner that would attenuate reproductive vigor. Surprisingly, we found no evidence that chronic 17α-E2 treatment altered reproductive health in male mice. Both testes mass and seminiferous tubule morphology were unchanged following several months of 17α-E2 treatment in our studies. These observations provided the first indication that 17α-E2 treatment does

not alter reproductive health in male mice, at least at the dose known to extend lifespan (4).

Our subsequent analyses provided additional evidence indicating that chronic 17α-E2 treatment fails to adversely affect reproductive health in male mice. First, 17α-E2 treatment did not alter the endogenous sex hormone milieu as evidenced by only mild changes in plasma 17β-E2, estrone, or testosterone. Interestingly, circulating testosterone trended slightly higher in the 17α-E2 treatment group, which we speculate is due to 17α-E2 being a mild 5α-reductase inhibitor (37), thus limiting the conversion of testosterone into DHT. Since we did not evaluate DHT in these studies, future experiments will be needed to determine if 17α-E2 treatment alters circulating DHT in male mice, and more importantly, if this potential change underlies benefits attributed to 17α-E2 treatment. It should be noted that Garratt et al. previously reported that responsiveness to 17α-E2 was significantly attenuated in castrated male mice (12), which could be at least partially due to a lack of endogenous DHT and its effects on adiposity (45). Regardless of the potential effects of 17α-E2 treatment on circulating DHT, our findings clearly demonstrate that chronic 17α-E2 treatment does not feminize the endogenous sex hormone milieu, which suggests 17α-E2 does not directly inhibit Leydig cell steroidogenesis as has been shown with 17β-E2 (46). Furthermore, we also determined that chronic 17α-E2 treatment does not suppress plasma gonadotropins, LH or FSH. This observation suggests that the dose of 17α-E2 employed in our studies does not interfere with gonadotropin-releasing hormone (GnRH) neuronal function, which stimulates the pituitary to produce and secrete LH and FSH (44). However, it should be noted that GnRH-mediated production of LH and FSH in the pituitary relies heavily on GnRH pulse frequency and amplitude as opposed to operating in a binary fashion (47). Since GnRH pulse frequency and amplitude have recently been linked to

male aging (47), it would be prudent for future studies to evaluate the direct effects of 17α-E2 on GnRH neuronal function and/or neuronal populations shown to modulate GnRH neuronal activity, including agouti-related peptide (AgRP), neuropeptide Y (NPY), proopiomelanocortin (POMC) and kisspeptin (KISS) (47).

Based on the lack of adverse effects of chronic 17α-E2 treatment on testes mass, seminiferous tubule morphology, and the sex hormone milieu, we surmised that sperm parameters and fertility would also be unaffected by 17α-E2 administration. Indeed, we found no evidence suggesting that chronic 17α-E2 treatment perturbed sperm quantity or quality. This suggests that our dose of 17α-E2 does not elicit ERβ responsiveness in Sertoli cells due to its role in regulating spermatogenesis (48). If true, this would support our previous work suggesting that 17α-E2 elicits the vast majority of its benefits through ERα (6). Given that sperm parameters were unchanged by 17α-E2 treatment it came as little surprise that fertility was also unaffected in animals receiving 17α-E2. Two different approaches were used to evaluate male fertility. In the first cohort we assessed the percentage of females that had fertilized oocytes in their oviducts following exposure to males from control or 17α-E2 treatment groups. We found that control and 17α-E2 treated mice successfully fertilized a similar percentage of females, with 17α-E2 treated mice actually fertilizing a greater percentage of females they were paired with. In the second cohort we evaluated the ability of control and 17α-E2 treated males to produce embryos that successfully implant in the uterus. We found that pregnancy rates and the number of implanted embryos were essentially identical in female pairs with control and 17α-E2 treated males. Since oocyte fertilization and embryo implantation represents the gold-standard for evaluating fertility, these observations strongly indicate that the dose of 17α-E2 employed in our studies does not adversely affect male fertility.

There are a few caveats to our studies that should be noted. First, we were unable to evaluate plasma DHT in our studies due to limited sample availability. Investigating how 17α-E2 potentially modulates plasma DHT could provide additional insight into how 17α-E2 beneficially alters male metabolism and aging, therefore future studies would be helpful in answering this question. We also did not measure seminal vesicle mass, an organ that is highly sensitive to androgen exposure, particularly DHT (49). A previous study reporting that plasma testosterone levels were unaffected by 17α-E2 also reported that seminal vesicle mass was slightly, but consistently, reduced following three months of treatment in old male mice (15). Given that seminal vesicles produce the majority of the proteins found in seminal plasma, which are important for promoting embryo implantation and placental development (50), it is possible that 17α-E2 treatment in males could elicit subtle effects on offspring development *in utero.* Lastly, our evaluation of circulating LH and FSH in these studies is a nice surrogate marker of central GnRH activity, but they provide only limited insight into GnRH pulse frequency and amplitude, which is reported to play a key role in male aging (47). Future studies evaluating GnRH pulsatility will provide tremendous insight into a potential mechanism by which 17α-E2 may modulate male aging.

In summary, the data presented herein are the first to show that chronic 17α-E2 treatment does not adversely affect male reproductive health, including testes mass, seminiferous tubule morphology, plasma sex hormone milieu, sperm parameters, and fertility. These observations suggest that 17α-E2 does not extend lifespan or curtail disease parameters through tradeoff effects with reproduction. Future studies are still needed to determine if 17α-E2 is mildly altering GnRH pulsatility through actions in AgRP, NPY, POMC, or KISS neurons, which may underlie its lifespan extending effects.

CONFLICTS

The authors declare no conflicts of interest.

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TABLES

Table 1: Sperm parameters and fertility in control and 17α-estradiol treated male mice

Cohort 1			
Sperm Parameters	**Con (n=8)**	**17α-E2 (n=10)**	**p value**
Epididymal concentration (x10^4 cells/uL)	2.3 ± 0.8	1.8 ± 0.9	0.28
Motility (%)	65.6 ± 3.9	62.7 ± 4.8	0.66
Progressive motility (%)	52.4 ± 4.9	50.0 ± 5.5	0.64
Average path velocity (VAP)	120.4 ± 5.6	127.6 ± 4.9	0.67
Curvilinear velocity (VCL)	229.3 ± 10.1	233.9 ± 11.0	0.76
Straight line velocity (VSL)	83.7 ± 4.5	85.9 ± 2.7	0.67
Amplitude of lateral head (ALH)	9.8 ± 0.3	10.1 ± 0.4	0.61
Beat cross frequency (BCF)	17.9 ± 0.2	17.6 ± 0.2	0.20
Hypoosmotic swelling test (Δ%)	28.3 ± 3.2	21.0 ± 2.9	0.18
Acrosomal integrity (%)	34.5 4.0	36.9 ± 3.3	0.65
Fertility Parameters	**Con (n=16)**	**17α-E2 (n=18)**	**p value**
Fertilized females (%)	75.0	94.4	0.11

Cohort 2			
Sperm Parameters	**Control (n=22)**	**17α-E2 (n=21)**	**p value**
Epididymal concentration (x10^4 cells/uL)	1.6 ± 0.6	1.6 ± 0.6	0.95
Motility (%)	57.9 ± 3.9	59.0 ± 4.4	0.85
Progressive motility (%)	46.8 ± 4.0	46.8 ± 4.6	0.60
Average path velocity (VAP)	72.2 ± 2.4	71.1 ± 2.4	0.76
Curvilinear velocity (VCL)	134.6 ± 4.9	131.3 ± 4.9	0.39
Straight line velocity (VSL)	45.5 ± 1.5	45.0 ± 1.7	0.83
Amplitude of lateral head (ALH)	4.1 ± 0.1	4.0 ± 0.1	0.33
Beat cross frequency (BCF)	30.0 ± 0.2	30.3 ± 0.2	0.46
Fertility Parameters	**Con (n=40)**	**17α-E2 (n=40)**	**p value**
Pregnancy rate (%)	20.0	22.5	0.78
Implanted embryos	13.6 ± 1.3	14.6 ± 2.7	0.77

Data presented as mean ± SEM with the exception of % fertilized females and % pregnancy rate.

FIGURES

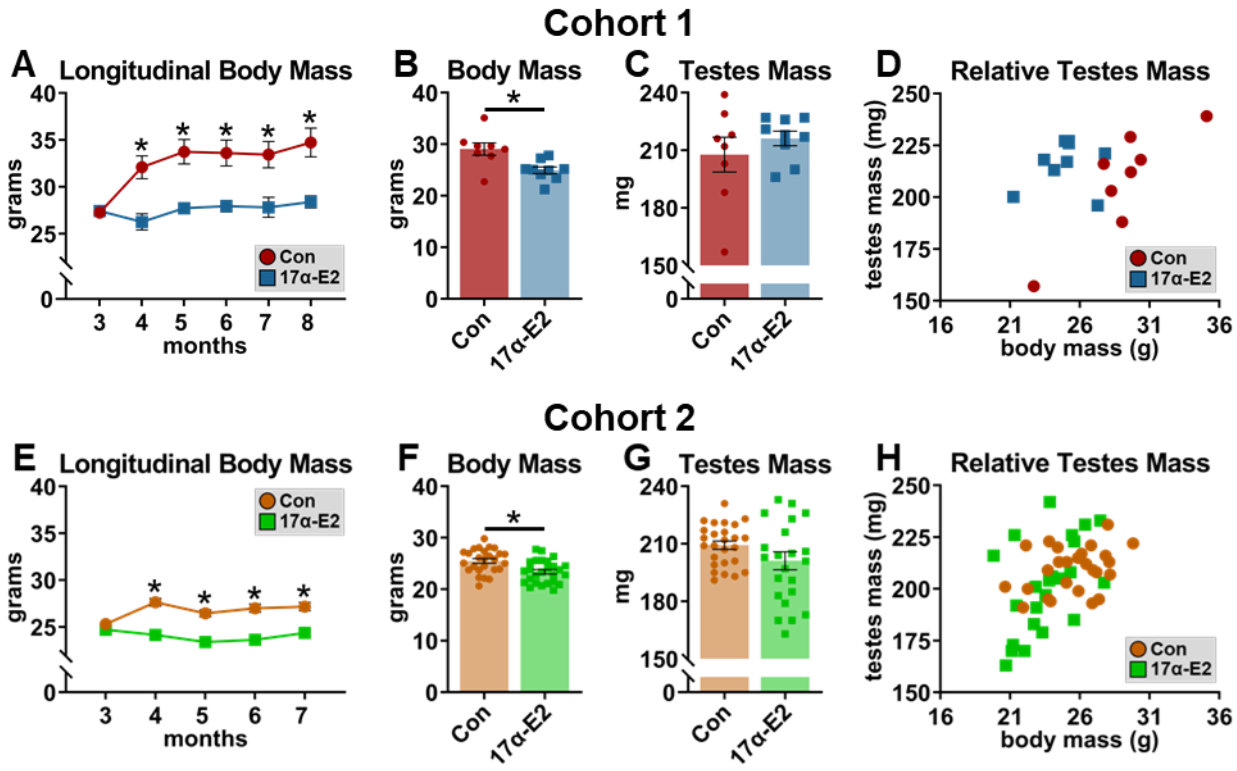

Figure 1: Chronic 17α-E2 treatment decreases body mass, but not testes mass, in male mice. (A) Longitudinal changes in body mass, (B) body mass at necropsy, (C) testes mass at necropsy, and (D) relative testes mass at necropsy in control (Con) and 17α-E2 treated male mice over a 5-month period (Cohort 1). (E) Longitudinal changes in body mass, (F) body mass at necropsy, (G) testes mass at necropsy, and (H) relative testes mass at necropsy in Con and 17α-E2 treated male mice over a 4-month period (Cohort 2). Mice received LabDiet 58YP ± 17α-E2 (14.4 ppm) throughout the intervention periods. All data are presented as mean ± SEM and were analyzed by repeated measures ANOVA, Student's t-test, or ANCOVA where appropriate. n=8-10/group for cohort 1 and 30/group for cohort 2. *p<0.05.

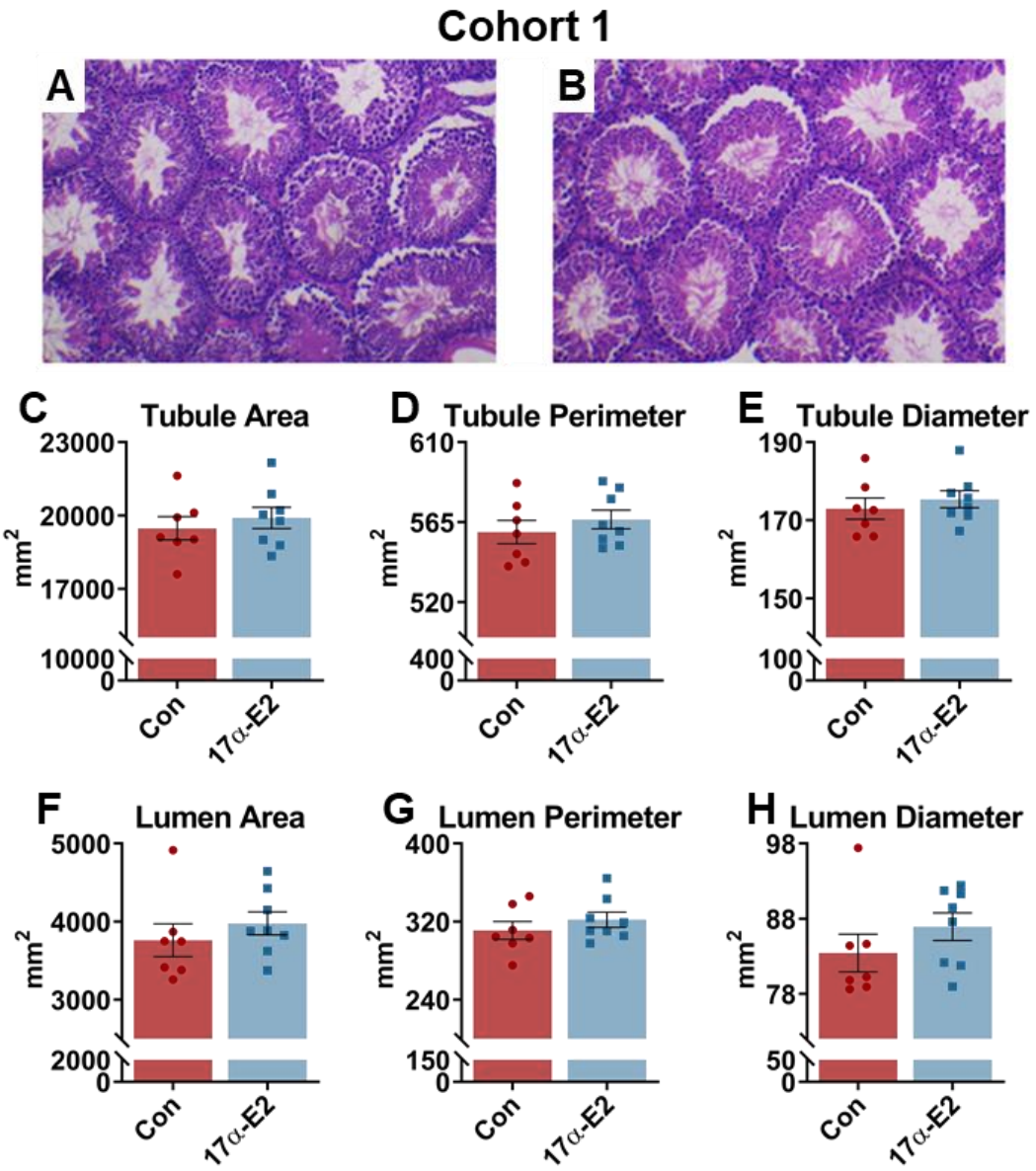

Figure 2: Chronic 17α-E2 treatment does not affect seminiferous tubule morphometry in male mice. Representative H&E stained sections of seminiferous tubules at 40X magnification from (A) control (Con) and (B) 17α-E2 treated male mice following a 5-month intervention period. Seminiferous (C) tubule area, (D) tubule perimeter, (E) tubule diameter, (F) tubule lumen area, (G) tubule lumen perimeter, and (H) tubule lumen diameter from Con and 17α-E2 treated male mice following a 5-month intervention period. Mice received LabDiet 58YP ± 17α-E2 (14.4 ppm) throughout the intervention timeframe. All data are presented as mean ± SEM and were analyzed by Student's t-test. n=7-8/group.

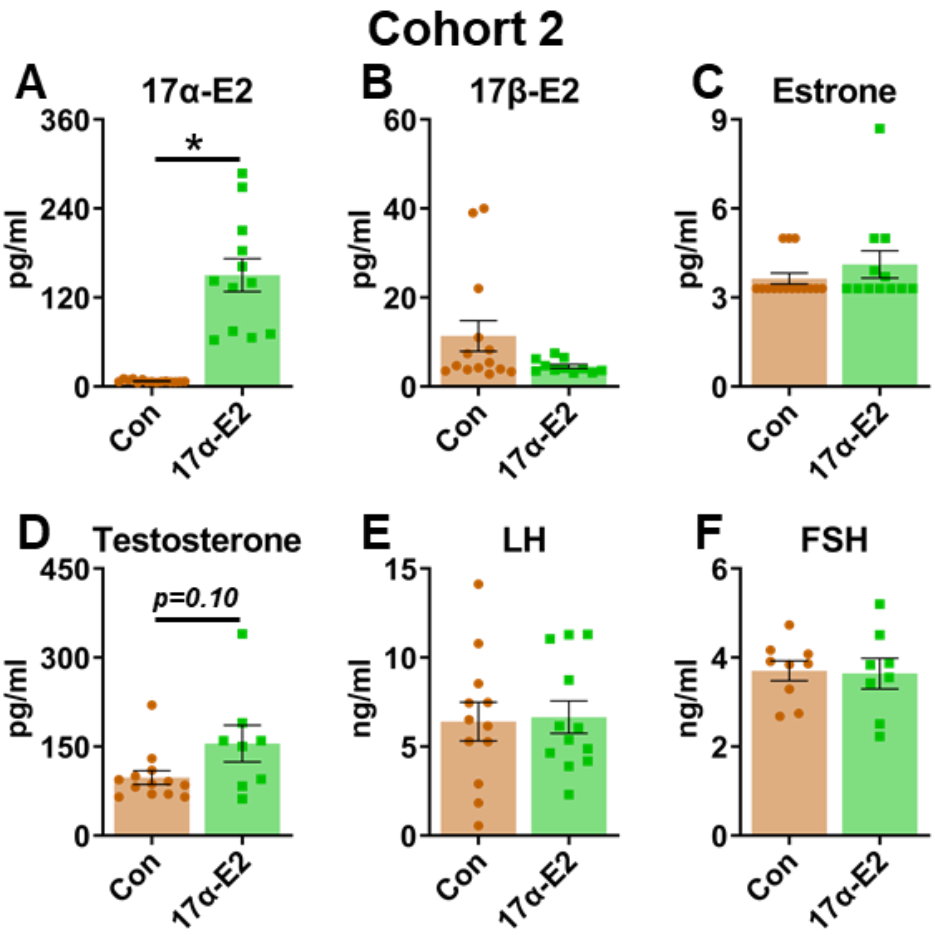

Figure 3: Chronic 17α-E2 treatment does not alter endogenous sex hormone or gonadotropin levels. Circulating (A) 17α-E2, (B) 17β-E2, (C) estrone, (D) testosterone, (E) luteinizing hormone (LH), and (F) follicle-stimulating hormone from control (Con) and 17α-E2 treated male mice following a 4-month intervention period. Mice received LabDiet 58YP ± 17α-E2 (14.4 ppm) throughout the intervention period. All data are presented as mean ± SEM and were analyzed by Student's t-test or Mann-Whitney test following the determination of normality by Shapiro-Wilk test. n=8-14/group.*$p<0.05$.

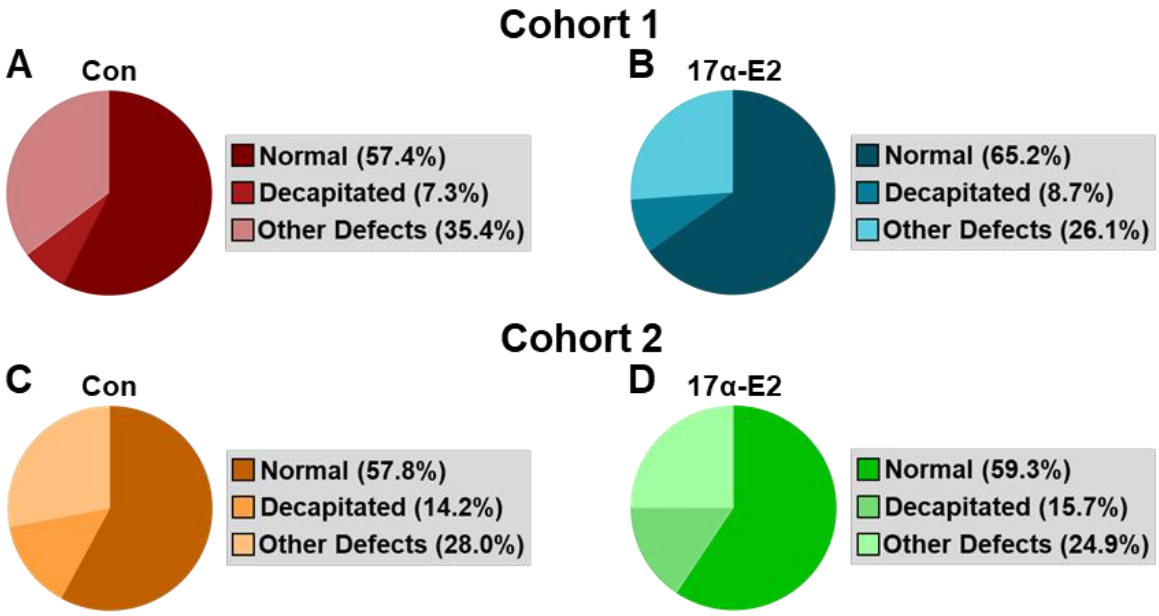

Figure 4: Chronic 17α-E2 treatment does not affect sperm morphology in male mice. Percentage of normal, decapitated, or otherwise defective sperm from (A) control (Con) and (B) 17α-E2 treated male mice following a 5-month intervention period (Cohort 1). Percentage of normal, decapitated, or otherwise defective sperm from (C) Con and (B) 17α-E2 treated male mice following a 4-month intervention period (Cohort 2). Mice received LabDiet 58YP ± 17α-E2 (14.4 ppm) throughout the intervention periods. n=8-10/group for cohort 1 and 25/group for cohort 2.

4 Considerações Finais

Em síntese, o 17α-E2, quando fornecido na dose comprovadamente eficaz como pró-longevidade para camundongos machos, não afeta a fertilidade ou reserva ovariana de camundongos, sejam estes machos ou fêmeas. Sendo assim, esta intervenção parece ser viável de ser aplicada sem a preocupação com potenciais efeitos colaterais relacionados à reprodução, o que encoraja estudos futuros.

Surpreendentemente, no entanto, a restrição calórica branda, de 10%, foi capaz de elicitar efeitos positivos na preservação reserva ovariana e taxa de prenhez após a realimentação, demonstrando a eficácia de restrições mais leves, que podem mais facilmente serem aplicadas à população humana.

Estudos adicionais ainda são necessários para melhor entender os mecanismos de ação do 17α-E2. Sabe-se que o composto age via fígado e cérebro, mas ainda se carece de maior entendimento de quais vias são mais importantes para seus efeitos benéficos. Além disto, ainda não se sabe ao certo como o 17α-E2 se compara ou se relaciona com o 17β-E2 e o porquê de seus efeitos serem muito mais brandos em fêmeas.

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Anexos

Anexos

Anexo I - Documento da Comissão de Ética e Experimentação Animal (Artigo 1)

UNIVERSIDADE FEDERAL DE PELOTAS

PARECER Nº **40/2019/CEEA/REITORIA**
PROCESSO Nº 23110.028986/2019-59

Certificado

Certificamos que a proposta intitulada **"Efeito do 17α Estradiol (17αE2) na fertilidade e envelhecimento ovariano de camundongos"**, processo nº 23110.0028986/2019-59, sob a responsabilidade de Augusto Schneider que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua execução pela Comissão de Ética em Experimentação Animal, em reunião de 16/07/2019.

Finalidade	(X) Pesquisa ( ) Ensino
Vigência da autorização	01/10/2019 a 30/09/2020
Espécie/linhagem/raça	*Mus musculus*/C57BL/6
N° de animais	117
Idade	60 dias
Sexo	45 Machos e 72 Fêmeas

Origem	Biotério Central - UFPel

Ccódigo para cadastro nº **CEEA 28986-2019**

M.V. Dra. Anelize de Oliveira Campello Felix

Presidente da CEEA

Anexo II - Documento da Comissão de Ética e Experimentação Animal (Artigo 2)

UNIVERSIDADE FEDERAL DE PELOTAS

PARECER Nº **8/2020/CEEA/REITORIA**
PROCESSO Nº 23110.050105/2019-86

Certificado

Certificamos que a proposta intitulada **"Efeito do tratamento com 17α-estradiol (17αE2) em camundongos machos na qualidade espermática e produção de embriões in vivo"**, registrada com o nº 23110.050105/2019-86, sob a responsabilidade de **Augusto Schneider** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua execução pela Comissão de Ética em Experimentação Animal, em reunião de **17 de dezembro de 2019**.

Finalidade	(x) Pesquisa ( ) Ensino
Vigência da autorização	14/01/2020 a 30/12/2021
Espécie/linhagem/raça	*Mus musculus*/C57BL/6
N° de animais	60
Idade	60 dias
Sexo	20 machos e 40 Fêmeas
Origem	Biotério Central - UFPel

Código para cadastro nº **CEEA 50105/2019-86**

M.V. Dra. Anelize de Oliveira Campello Felix

Presidente da CEEA