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AVALIAÇÃO DO EFEITO DA RESTRIÇÃO CALÓRICA MATERNA GESTACIONAL SOBRE ASPECTOS REPRODUTIVOS DA PROLE E DA MÃE

Porto Alegre
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RESUMO

A restrição calórica (RC) é considerada um fator de intervenção ideal para a promoção da saúde, conhecida por aumentar a expectativa de vida, especialmente através da modulação redox. Interferências no ambiente materno são conhecidas por reprogramar a resposta metabólica da prole, tendo impacto na vida pós-natal. Entretanto, pouco se sabe sobre os efeitos da RC gestacional sobre parâmetros redox e mecanismos moleculares em ovários e testículos da prole na sua vida adulta. Nosso objetivo, neste trabalho, foi verificar os efeitos de uma RC moderada (20%) nos níveis de espécies reativas, nas defesas antioxidantes enzimáticas e não enzimáticas, nos parâmetros de dano oxidativo, na expressão molecular das enzimas SIRT1 e SIRT3, bem como avaliar os parâmetros morfológicos em ovários e testículos de ratos submetidos à RC gestacional. Ratas Wistar fêmeas adultas foram submetidas à RC moderada durante o período gestacional, recebendo suplementação de vitaminas e minerais a fim de equilibrar o consumo de micronutrientes em relação ao grupo controle, que recebeu ração *ad libitum*. Os filhotes foram eutanasiados no dia pós-natal (PND) 60, quando ovários e testículos foram removidos para posterior análise. Massa e potencial de membrana mitocondrial, níveis de superóxido mitocondrial, conteúdo de espécies reativas, conteúdo de óxido nítrico e um índice de lipoperoxidação foram analisados por citometria de fluxo; a atividade antioxidante enzimática e os conteúdos de carbonilas e sulfidrilas foram analisados espectrofotometricamente; o conteúdo de glutationa reduzida (GSH) foi analisado fluorimetricamente; o imunoconteúdo de SIRT1 e SIRT3 foi analisado por Western Blotting; as análises morfológicas foram feitas com coloração por hematoxilina-eosina; os úteros maternos foram corados com solução de NaOH 2%; e os dados obtidos foram analisados por teste *t* e considerados significativos quando $p<0,05$. O protocolo utilizado foi analisado e aprovado pela Comissão de Ética no Uso de Animais (CEUA) da UFRGS, sob o n° 34056. Filhotes fêmeas demonstraram altos níveis de oxidantes e aumento do dano oxidativo a lipídeos, embora tenha ocorrido aumento da atividade da enzima superóxido-dismutase em ovários. Em filhotes machos, foi observado diminuição das defesas antioxidantes enzimáticas e não enzimáticas por diminuição da atividade da enzima glioxalase I e diminuição do conteúdo de glutationa reduzida nos testículos. O dano oxidativo a proteínas não foi afetado em nenhum dos órgãos. Ovários e testículos demonstraram aumento da expressão de SIRT3, uma desacetilase relacionada à bioenergética celular e ao metabolismo da RC. Nossos dados sugerem que, embora a RC gestacional promova melhora de parâmetros moleculares na SIRT, tal intervenção nutricional afetou negativamente o estado redox em órgãos reprodutivos de ratos jovens adultos.

Palavras-chave: ovários; testículos; estresse oxidativo; mitocôndria; desenvolvimento inicial; ambiente; nutrição; restrição calórica; gestação.

ABSTRACT

Caloric Restriction (CR) is a well-known lifespan extensor, remarkably known for its redox modulation, which contributes to diminished cellular aggression. Interferences on maternal environment are known to reprogram the offspring metabolic response, impacting its postnatal life. However, little is known about gestational CR effects on adult offspring's ovaries and testis regarding antioxidant parameters and molecular mechanisms. We aimed to assess the effects of a moderate (20%) CR on reactive species levels, enzymatic and non-enzymatic antioxidant defenses, oxidative damage parameters, molecular expression of SIRT1 and SIRT3 deacetylase enzymes and morphological parameters on ovaries and testis of adult rats which underwent gestational CR. Female Wistar rats underwent moderate CR during pregnancy, supplemented with vitamins and minerals to balance consumption to control group, that was fed *ad libitum*. The offspring was euthanized in postnatal day (PND) 60, and ovaries and testis were dissected for analysis. Mitochondrial mass and membrane potential, mitochondrial superoxide levels, oxidants content, nitric oxide content, and lipid peroxidation where measured by flow cytometry. Enzymatic antioxidant activity, carbonyl and sulfhydryl levels were analyzed by spectrophotometry. Reduced glutathione content was analyzed by fluorimetry. SIRT1 and SIRT3 immunocontents were analyzed by Western blotting. Morphological analyzes were made by hematoxylin and eosin staining. The mother's uterus were stained with 2% NaOH. The data was analyzed by multiple *t* tests and significant when $p<0.05$. The protocol was approved by the local Ethic's Comission under protocol n° 34056. Female pups demonstrated high levels of oxidants and increased oxidative damage to lipids, although superoxide dismutase activity was increased in ovaries. In male pups we found decreased enzymatic enzymatic and non-enzymatic defenses by diminished glyoxalase I activity and decreased glutathione content in testis. Oxidative damage to proteins was not affected in neither structure. The expression of SIRT3, a deacetylase enzyme with mechanisms related to cellular bioenergetics and to CR metabolism, was increased in both reproductive organs. Our data suggest that, although improving molecular mechanisms related to SIRT3, intrauterine CR negatively affected the redox status of reproductive organs from young adult rats.

Keywords: ovary; testis; oxidative stress; mitochondria; early development; environment; nutrition; caloric restriction; pregnancy.

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1 INTRODUÇÃO COMPREENSIVA

Infertilidade é um termo que se refere a "um problema do sistema reprodutivo caracterizado por não gravidez após 12 meses ou mais de tentativas falhas" (ZEGERS-HOCHSCHILD *et al.,* 2009). De acordo com a Organização Mundial da Saúde (OMS), existem dois tipos de infertilidade: infertilidade primária, em que a mulher não é capaz de gestar uma criança, sendo ineficaz de conceber e/ou manter a gestação a termo; e infertilidade secundária, caracterizada por mulheres que, mesmo com gestações anteriores, não conseguem manter uma gestação a termo por uma outra vez (MASCARENHAS *et al.*, 2012). Com relação aos índices mundiais, é difícil averiguar e definir com exatidão a taxa de infertilidade. No mesmo estudo em que se baseia a OMS (MASCARENHAS *et al*., 2012), é demonstrado que as taxas de infertilidade primária variam muito mundialmente, inclicando a se manter abaixo de 1% em países como Polônia e Bolívia; entre 1 e 2% em países como Canadá e Brasil e podendo chegar a mais de 3% em países como Marrocos e Ucrânia. Com relação à infertilidade secundária, os índices tendem a ser mais elevados: até 9% em países como Marrocos, Brasil e México e até 13% em países como Rússia e Polônia. Por ser um índice extremamente variado, acredita-se que fatores genéticos e ambientais devem estar associados à infertilidade.

Com relação à infertilidade feminina, muito se deve a problemas ovulatórios, que caracterizam 25% dos casos de infertilidade; obstrução e adesão de tecido às tubas uterinas caracterizam 30% dos problemas de concepção, além de patologias como síndrome do ovário policístico, que pode levar à falta de ovulação (anovulação) (BARRETT, 2006; GARDNER *et al.*, 2007). Fatores masculinos relacionados à infertilidade tendem a ser de ordem gamética, como baixa contagem espermática e anormalidades morfológicas e de motilidade do espermatozoide (BARRETT, 2006), bem como endocrinopatias, problemas genéticos, infecções, uso de anabolizantes ou idiopáticas (AZAMBUJA, 2017). De acordo com a Sociedade Americana de Medicina Reprodutiva, fatores de risco para infertilidade incluem, além de problemas genéticos, idade avançada (materna e paterna), obesidade ou anorexia, falta de exercício, consumo de drogas, doenças sexualmente transmissíveis e má-nutrição.

1.1 ESTADO REDOX

Um importante fator relacionado à fisiopatologia da infertilidade é o estresse oxidativo. Definido como o desequilíbrio entre produção de espécies reativas de oxigênio (EROs) e Espécies Reativas de Nitrogênio (ERNs) versus a proteção pelo sistema de defesa antioxidante (PERSSON *et al.,* 2014), o estresse oxidativo surge em um contexto de produção aumentada de tais espécies danosas e/ou de diminuição da capacidade antioxidante fisiológica do organismo em lidar com esses ataques oxidativos a biomoléculas. Já foi demonstrada a correlação entre estresse oxidativo e mais de 100 patologias, tanto como causa ou resultado patológico (LIN e BEAL, 2006; RANI *et al.*, 2016).

Exemplificando, cabe ressaltar que mulheres que sofrem de endometriose ou de infertilidade idiopática são mais propensas a terem níveis aumentados de EROs no fluido peritoneal (WANG *et al.*, 1997), enquanto que no fluido folicular, que circunda as células germinativas, mulheres grávidas tendem a ter níveis elevados de EROs, sendo que esse pode ser um potencial marcador de sucesso para a fertilização *in vitro* (ATTARAN *et al.,* 2000). Com relação ao homem, sabe-se que existe uma correlação negativa entre qualidade espermática de homens inférteis e geração de EROs (GOMEZ *et al.*, 1998), sendo a mitocôndria a maior geradora de EROs em espermatozoides desses homens (PLANTE *et al.,* 1994).

O estresse oxidativo foi primariamente caracterizado por Sies (1991) como “um distúrbio do pró-oxidante para o antioxidante em favor das espécies oxidantes levando a dano potencial”, ou seja, uma produção excessiva de EROs, resultado de um desequilíbrio entre a produção e a remoção das mesmas. Corroborando, Jones (2006) redefiniu o termo, defendendo a hipótese de que o aumento do estresse oxidativo é consequência do desequilíbrio na relação glutationa reduzida/glutationa oxidada (GSH/GSSG). O termo foi atualizado por Sies, que hoje caracteriza o estresse oxidativo como um desafio oxidante excessivo que causa dano às biomoléculas, entretanto a manutenção de um nível fisiológico desse mesmo desafio oxidante (denominado "*oxidative eustress*") é essencial para processos vitais por meio da sinalização redox (SIEST *et al.*, 2017).

As espécies reativas incluem os radicais livres e outras moléculas capazes de gerar espécies radicalares. Radicais livres representam espécies químicas reativas devido a um elétron sem pareamento na sua camada orbital mais externa (GUTTERIDGE e HALLIWELL, 2000). EROs de importância biológica incluem o peróxido de hidrogênio (H2O2), o ânion superóxido (O2˙), o radical hidroxil (˙OH) e o oxigênio singlet (1/2 O2˙). Algumas ERNs,

como óxido nítrico (NO˙), além de espécies reativas de ferro, cobre e enxofre, também são encontradas nesse contexto (HALLIWELL *et al.,* 1992; RILEY, 1994). A mitocôndria é responsável pela maioria das fontes endógenas de espécies reativas identificadas (JONES, 2006; MAILLOUX, 2015). Além da mitocôndria, espécies reativas também podem ser advindas da reação de Fenton, que produz radical hidroxil (reação entre peróxido de hidrogênio com ferro), complexo enzimático citocromo P450, beta-oxidação no peroxissomo e ativação de células fagocitárias (GUTTERIDGE e HALLIWELL, 1993; GILCA *et al*., 2007; POLJSAK *et al.,* 2013). Devido à produção endógena, muitos substratos celulares são alvos dessas. Lipídeos são considerados os mais suscetíveis à oxidação; as proteínas podem ter a sua cadeia lateral e esqueleto oxidados, permitindo a interação com aminoácidos e a formação de radicais carbonila; ácidos nucleicos podem ser danificados, podendo levar a oxidação, quebra de fita ou formação de adutos no DNA (GANDHI e ABRAMOV, 2012).

As defesas antioxidantes são diversas, incluindo formas endógenas (enzimáticas e não enzimáticas) e formas exógenas (provenientes da dieta). O conceito de antioxidante biológico se refere a "qualquer composto que, quando presente em baixas concentrações, comparado a um substrato oxidável, é capaz de atrasar ou prevenir a oxidação do substrato" (HALLIWELL e GUTTERIDGE, 2007; GODIC *et al.,* 2014; PISOSCHI e POP, 2015). O sistema antioxidante não enzimático contém fontes endógenas e exógenas. De fontes endógenas, ressalta-se a glutationa reduzida (GSH), sintetizada no citoplasma celular (HALLIWELL e GUTTERIDGE, 2007), sendo considerada o principal tampão redox da célula (AOYAMA e NAKAKI, 2013; AOYAMA e NAKAKI, 2015) em virtude da sua interação com defesas antioxidantes enzimáticas e da reação direta com EROs e ERNs (HALLIWELL e GUTTERIDGE, 2007). Após sua oxidação e utilização, a GSH oxidada é reciclada por ação enzimática, utilizando NADPH em uma reação catalisada pela glutationa redutase (HALLIWELL e GUTTERIDGE, 2007). De fontes exógenas, ressaltam-se as vitaminas do complexo B, substratos essenciais para a síntese de coenzimas do ciclo do ácido cítrico e da cadeia transportadora de elétrons mitocondrial, em que sua deficiência é relacionada ao aumento de estresse oxidativo (DEPEINT *et al.,* 2006). A vitamina E compreende um grupo de lipídeos naturais (tocoferóis e tocotrienois) que, graças a sua característica lipossolúvel, é amplamente distribuída no organismo e capaz de inibir a peroxidação lipídica (HALLIWELL e GUTTERIDGE, 2007), agindo diretamente sobre espécies reativas (MACHLIN e BENDICH, 1987), podendo ser regenerada pela GSH ou pela vitamina C após a oxidação (HALLIWELL e GUTTERIDGE, 2007). A vitamina C reage com superóxido e com outras espécies reativas (HALLIWELL, 1999; MAY, 2000). No entanto, essa vitamina se torna pró-

oxidante após sua redução, podendo catalisar a geração de hidroxil, uma ERO (HALLIWELL e GUTTERIDGE, 2007).

Enzimaticamente, existem apenas antioxidantes endógenos. A enzima superóxido dismutase (SOD) (EC 1.15.1.1) tem três isoformas celulares: MnSOD, dependente de manganês e encontrada na mitocôndria; CuZnSOD, dependente de cobre e de zinco e encontrada no citoplasma, nos lisossomos, nos peroxissomos, no núcleo e extracelularmente; e EcSOD, também dependente de cobre e de zinco. As três isoformas desempenham a mesma função de dismutação do O2•-, formando O2 e H2O2 (MCCORD e FRIDOVICH, 1969; HALLIWELL e GUTTERIDGE, 2007). Entretanto, a exposição prolongada ao H2O2 pode levar à inibição da forma citosólica sem afetar sua forma mitocondrial (HALLIWELL e GUTTERIDGE, 2007). Com relação ao H2O2, mesmo pouco danoso, esse pode atravessar membranas e interagir com lipídeos, levando a formação do radical hidroxil (OH•) (HALLIWELL e GUTTERIDGE, 2007; BRINKMANN e BRIXIUS, 2013). A principal enzima responsável pela sua detoxificação é a catalase (CAT) (EC 1.11.1.6), encontrada nos peroxissomos, dependente de ferro e que utiliza H2O2 como substrato, produzindo água e moléculas de O2 (HALLIWELL e GUTTERIDGE, 2007; SIES, 2014). Além da CAT, a enzima glutationa peroxidase (GPx) (EC 1.11.1.9) também realiza a eliminação de H2O2, além de ter o potencial de reagir com hidroperóxidos orgânicos e com peroxinitrito (HALLIWELL e GUTTERIDGE, 2007; BRIGELIUS-FLOHÉ e MAIORINO, 2013). A GPx existe em oito isoformas, distribuídas entre frações citosólicas, mitocondriais, extracelulares e plasmáticas (MATÉS *et al.*, 1999; BELA *et al.*, 2015), sendo o principal mecanismo de remoção de H2O2 no citosol e na mitocôndria. Entretanto, a enzima necessita de duas moléculas de GSH reduzida para a catálise enzimática de redução de H2O2 em água (HALLIWELL e GUTTERIDGE, 2007), em que essa se torna glutationa oxidada (GSSG), sendo reciclada enzimaticamente pela enzima glutationa redutase. A GSH também atua na via glicolítica e de lipídeos e proteínas, catalisando o tóxico metilglioxal em um subproduto menos danoso. O metilglioxal é reduzido em hemiacetal pela GSH (ALLAMAN *et al.*, 2015). Entretanto, hemiacetal ainda é uma forma danosa. A fim de eliminá-lo, esse subproduto é catalisado pela enzima Glioxalase I (GLO I), que o reduz em S-d-lactoilglutationa, sendo finalmente liberado em forma de ácido lático (ALLAMAN *et al.*, 2015).

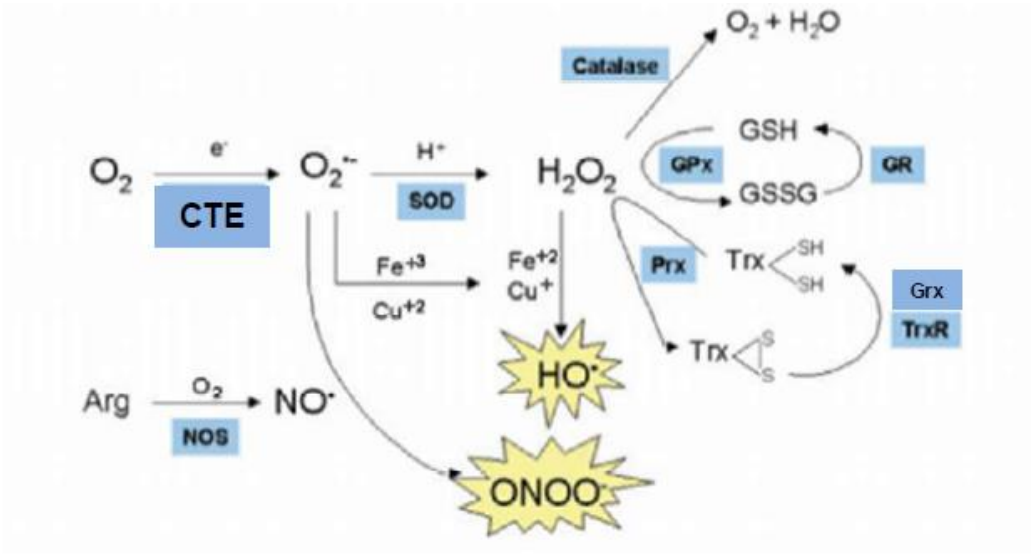

Figura 1. Esquema das reações e respectivas enzimas envolvidas na produção de espécies reativas de oxigênio e nitrogênio.[1] NOS: óxido-nítrico-sintase; SOD: superóxido dismutase; GPx: glutationa peroxidase; Prx: peroxirredoxinas; GR: glutationa redutase; Grx: glutarredoxina; Trx: tiorredoxina; GSH: glutationa reduzida; GSSG: glutationa oxidada.

1.2 ESTRESSE OXIDATIVO E PROGRAMAÇÃO METABÓLICA

O termo programação metabólica compreende uma situação em que um insulto ou estímulo durante o desenvolvimento intrauterino leva a mudanças fisiológicas em órgãos e tecidos, que podem perdurar por longo prazo (LUCAS, 1991). Com base nessa observação, foi desenvolvida uma hipótese para explicar como a má-nutrição durante o desenvolvimento fetal leva a adaptações fisiológicas e metabólicas para a sobrevivência caso a condição de desnutrição se mantenha na vida pós-natal (HALES e BARKER, 1992), o que inclui preservar o desenvolvimento de órgãos vitais, como, por exemplo, o cérebro, em detrimento de outros tecidos e órgãos. Nesse âmbito, recentemente expandiu-se tal termo para Origens Desenvolvimentistas da Saúde e da Doença (DOHaD; do inglês, "Developmental Origins of Health and Disease"), que fornece a base patofisiológica que explica a influência ambiental que ocorre durante o desenvolvimento inicial e que pode influenciar o risco de doenças crônicas não transmissíveis na vida adulta (HEINDEL *et al*., 2016).

Com relação ao estado de estresse oxidativo, sabe-se que o embrião se desenvolve

[1] Adaptado de Lívea Fujita Barbosa, Marisa H.G. de Medeiros e Ohara Augusto. Quim. Nova, Vol. 29, No. 6, 1352-1360, 2006.

com um baixo nível de oxigênio (THOMPSON e AL-HASAN, 2012), tendo baixa capacidade antioxidante e, portanto, sendo altamente sensível ao dano oxidativo a biomoléculas (HITCHLER e DOMANN, 2007; DENNERY, 2010). A placenta permite troca de oxigênio entre mãe e feto e, portanto, leva ao aumento da geração intracelular de EROs (BURTON, 2009), o que promove a diferenciação celular pela indução da transcrição de diversos genes importantes para a mesma e pela proliferação celular (CASTAGNE *et al.*, 1999; SCHAFER e BUETTNER, 2001; BURTON, 2009). Considerando defesas antioxidantes, estudos demonstram que a suplementação materna com micronutrientes, durante o período de desenvolvimento intrauterino, induz o aumento de óxido nítrico e a diminuição dos níveis de ânion superóxido nos filhotes (THAKOR *et al*., 2010).

Sendo o estresse oxidativo um fator de risco para infertilidade, é importante adotar estratégias nutricionais que diminuam o estresse oxidativo sem promoção de risco para a saúde. Ao se considerar o estado nutricional e o estresse oxidativo, cabe ressaltar sua influência durante a gestação. Segundo a hipótese de Barker, a gestação é um período crítico do desenvolvimento. A má nutrição pré-natal e após o nascimento leva à programação do desenvolvimento de fatores de risco (BARKER, 1992; GODFREY e BARKER, 2001), ou seja, em que o estresse gerado durante o período de desenvolvimento intrauterino pode levar a adaptações, que repercutem na vida pós-natal.

1.3 RESTRIÇÃO CALÓRICA

Na sociedade ocidental, a maior parte da população vive em um regime sedentário, com alimentação de baixa qualidade, ingerida em grandes quantidades (USDA, 2010; DIGITAL, 2017). Quando associados, superalimentação e estilo de vida sedentário, podem levar a diferentes complicações, como infarto do miocárdio, obesidade, problemas hormonais e até mesmo morte precoce (EYRE *et al.*, 2004).

Com relação à extensão da expectativa de vida e à dieta, existem muitos estudos que demonstram um efeito benéfico quando há diminuição da ingestão diária sem promover má-nutrição, uma intervenção denominada RC (COLMAN *et al*., 2014; LEE e LONGO, 2016). A RC é conhecida como o padrão ouro com relação ao aumento da expectativa de vida, desde leveduras até primatas (DANG, 2014). Segundo Sinclair (2005), o efeito da RC em modular o tempo de vida provém de uma resposta em longo prazo ao estresse, que leva a adaptação do organismo e, portanto, aumenta as chances desse sobreviver a adversidades. Já foi

demonstrado o potencial da RC na modulação da homeostase redox e em defesas antioxidantes (DUBEY *et al*., 1996; LASS *et al.*, 1998), especialmente se comparado às dietas *ad libitum* (SOHAL e FORSTER, 2014).

Desde 1917, (LOEB e NORTHROP, 1917; OSBORNE *et al.*, 1917), bem como demonstrado em 1935 (MCCAY *et al*., 1935), existe e é aceita a ideia de que a quantidade de alimentos ingeridos pode afetar o envelhecimento. Com o advento de técnicas mais modernas e de estudos mais aprofundados, pode-se perceber que o envelhecimento tem grande relação com prejuízos à mitocôndria, levando ao estresse oxidativo por aumento de EROs/ERNs e pela diminuição das defesas antioxidantes (MERRY, 2002; CHISTIAKOV *et al*., 2014). Além de efeitos mitocondriais, López-Lluch e Navas (2016) afirmam que uma diminuição da ingestão de alimentos leva ao desequilíbrio metabólico, em que diferentes mecanismos regulatórios interagem para que haja retorno ao equilíbrio. Dentre esses, cabe ressaltar a família das Sirtuínas (SIRT). Sendo um conjunto de enzimas desacetiladoras dependentes de NAD+, as SIRT são controladas pelo balanço celular de NAD+/NADH (YANG *et al*., 2007; HOUTKOOPER *et al.*, 2010). Tais enzimas são extremamente conservadas entre os organismos (IMAI *et al*., 2000; MICHAN e SINCLAIR, 2007) e, dentre as 7 isoformas presente em mamíferos, as isoformas 1 (SIRT1) e 3 (SIRT3) são as de maior interesse em um contexto de estratégia nutricional. As SIRT1 e 3 têm localizações subcelulares diferentes e extremamente estudadas. A SIRT1 depende do tipo celular, do estresse sofrido e de interações moleculares, sendo que já se observou interação com proteínas citosólicas e nucleares, podendo estar presente nestes dois compartimentos celulares (COHEN *et al.*, 2004; MICHISHITA *et al*., 2005; TANNO et al., 2007). Com relação a SIRT3, essa foi a primeira a ser localizada na matriz mitocondrial (SCHWER *et al*., 2002). Ambas enzimas têm papeis essenciais em relação à percepção do estado redox celular (YANG *et al.*, 2007; HE *et al*., 2010), podendo levar a efeitos positivos quando em situação de estresse oxidativo, como expressão de genes relacionados à bioenergética celular (ZHONG e MOSTOSLAVSKY, 2011).

Considerando estratégias nutricionais, a expressão de tais isoformas tende a promover os mesmos efeitos da RC em diferentes tecidos (BORDONE *et al*., 2007; PALACIOS et al., 2009). Em relação à SIRT1, estudos em roedores demonstram que há aumento da expressão dessa em tecidos com altos índices metabólicos, como cérebro e rins, por exemplo (COHEN, *et al*., 2004), bem como há diminuição de colesterol sérico, adiposidade e insulinemia (BORDONE *et al*., 2007; BANKS *et al.*, 2008). De maneira oposta, estudos utilizando camundongos com deficiência para o gene da SIRT1 demonstraram um menor tempo de vida,

algo contrário ao que ocorre durante a RC, podendo, desta forma, demonstrar os efeitos similares entre essa enzima e a intervenção nutricional (CHEN *et al.*, 2005; LI *et al*., 2008; NOGUEIRAS *et al*., 2012). Em relação à SIRT3, sabe-se que está aumentada na RC (PALACIOS *et al*., 2009; SUNDARESAN *et al.*, 2009), Provou-se também que a ingestão calórica está inversamente relacionada com a ativação da SIRT3 quando, em estudos com roedores obesos, observou-se uma diminuição de sua expressão (SHI *et al*., 2005; NOGUEIRAS *et al*., 2012).

1.4 RESTRIÇÃO CALÓRICA, SIRTUÍNAS E REPRODUÇÃO

Estudos demonstram que, em modelos animais, níveis moderados de RC na idade adulta podem garantir a função reprodutiva feminina até idades avançadas (SELESNIEMI *et al*., 2008), inibindo o desenvolvimento folicular ovariano e, portanto, diminuir a perda de folículos (LIU *et al*., 2015), Em relação à função reprodutiva masculina, poucos dados demonstram o papel da RC nesse processo.

Com relação aos ovários, existem alguns estudos sobre a relação entre esse tecido e a RC. Bernal e colaboradores (2010) estudaram o efeito de uma restrição alimentar (RA) de 50% sem aporte de micronutrientes (também considerada uma subnutrição) (KOWALTOWSKI, 2011). Foi o primeiro artigo a estudar o efeito da RA severa durante o período pré e pós-natal (durante amamentação) em roedores, bem como a função em alguns parâmetros oxidativos. Tal estudo demonstrou diminuição de folículos ovarianos, correspondente a diferentes insultos nutricionais durante a gestação. Por exemplo, animais cujas mães foram submetidas à RC durante o período gestacional ou durante o período de lactação tinham apenas diminuição de folículos antrais, enquanto filhotes cujas mães foram submetidas à RC durante a gestação e a lactação tinham diminuição de folículos primordiais, secundários e antrais. Com relação ao estresse oxidativo, o mesmo estudo verificou que, nas fêmeas filhotes de mães submetidas à RC durante a gestação e durante a gestação aliada a lactação, houve aumento do conteúdo de carbonilas, indicando dano oxidativo a proteínas.

Entretanto, os dados relacionados à RA e ao ovário são contraditórios. Um estudo feito por Li e colaboradores (2015) demonstrou que a RA de 55% promoveu a reserva de folículos ovarianos primordiais e não a depleção, como demonstrado por Bernal. Entretanto, cabe ressaltar que o estudo conduzido por Li e colaboradores demonstrou o efeito da RA no animal submetido a mesma e não na prole, como é o caso de Bernal. Tais efeitos visualizados por Li

e colaboradores devem-se à modulação da sinalização da rapamicina. Essa é um alvo comum da SIRT1, em que a inibição da ativação da rapamicina também demonstrou aumento da reserva folicular em um estudo conduzido por Liu e colaboradores (2015). Um estudo entre múltiplas gerações, conduzido por Harrath e colaboradores (2017), demonstrou que a RC materna pode ter efeitos na primeira e na segunda geração de sua prole, em que se levanta a hipótese de que tal insulto durante o desenvolvimento leva a um fenótipo melhorado para enfrentar a escassez de alimentos.

Com relação ao estresse oxidativo, sabe-se que EROs tem papel crucial durante a gravidez e o parto natural (FAINARU *et al*., 2002; MYATT e CUI, 2004). Entretanto, tem-se pouca evidência do efeito dessas espécies reativas sobre parâmetros ovarianos, como maturação folicular, ovulação, entre outros (ISHIKAWA, 1993; SUZUKI *et al*., 1999). Além disso, ainda não se sabe exatamente qual o papel do estresse oxidativo sobre o ovário e sobre o útero, em que esse pode estar relacionado a patologias como pré-eclâmpsia e abortos, por exemplo (ŁAGÓD *et al*., 2001; TRANQUILLI *et al*., 2004). Em relação à RC gestacional e ao estresse oxidativo, não existem resultados disponíveis na literatura, até o momento, sobre o efeito dessa intervenção sobre o estado oxidativo dos ovários, bem como há poucas evidências sobre moduladores do metabolismo, como é o caso das enzimas SIRT1 e SIRT3.

Não existem muitos estudos disponíveis ao se falar sobre reprodução masculina em um contexto de RC. Dos dados disponíveis considerando RC, tende-se a avaliar o efeito da RA severa (50%) em um contexto de restrição de crescimento intrauterino (DAI *et al*., 2012). Com relação à RC e ao estresse oxidativo, não foi encontrada literatura disponível que dispusesse de um panorama geral sobre o estado oxidativo testicular dos animais. Com relação aos moduladores do metabolismo, um estudo conduzido por Coussens e colaboradores (2008) menciona que a deficiência do gene para SIRT1 é responsável pela diminuição da espermatogênese e da função celular germinativa. Corroborando, um estudo posterior desenvolvido por Bell e colaboradores (2014) menciona a necessidade do gene para SIRT1 para diferenciação das células germinativas masculinas. Da mesma forma que nos ovários, quando se correlaciona RC durante gestação, estresse oxidativo e parâmetros testiculares, a literatura é completamente inexistente. Considerando fatores de modulação metabólica, há poucos estudos, e os mesmos focam na expressão de SIRT1 e em sua atividade, enquanto que a SIRT3 não aparece muito na literatura a partir do ponto de vista reprodutivo.

2 JUSTIFICATIVA

Diante do aumento da infertilidade na atualidade, cabe estudar estratégias que visem aumentar o potencial reprodutor e a vida útil dos órgãos reprodutivos femininos e masculinos. É visível o potencial efeito da RC como estratégia nutricional no prolongamento de vida útil de diferentes órgãos. Além disso, são poucos os estudos que demonstram qual o efeito de uma RC gestacional nos órgãos da prole, sendo escassos os estudos que avaliam órgãos reprodutivos e parâmetros de estresse oxidativo. Assim, ressalta-se a necessidade de estudar o possível papel de tal intervenção nutricional durante a gestação, tentando compreender os efeitos que uma intervenção nutricional durante o desenvolvimento pode ter sobre os parâmetros metabólicos e reprodutivos da prole.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Investigar os efeitos da RC materna moderada (20%) durante a gestação sobre o imunoconteúdo de sirtuínas, parâmetros bioquímicos e morfológicos em ovários e em testículos da prole em idade reprodutiva, bem como avaliar as implantações embrionárias no útero materno.

3.2 OBJETIVOS ESPECÍFICOS

São objetivos do trabalho: avaliar possíveis alterações do estado redox nos ovários e nos testículos da prole adulta de ratas submetidas à restrição calórica moderada durante a gestação através de:

a) avaliação do imunoconteúdo de SIRT1 e SIRT3 por meio de *Western blotting*;

b) avaliação dos níveis de espécies reativas, como o superóxido mitocondrial, o óxido nítrico e a oxidação da diclorofluoresceína utilizando citometria de fluxo;

c) avaliação da massa e do potencial de membrana mitocondrial por citometria de fluxo;

d) avaliação da atividade das enzimas antioxidantes SOD, CAT, GPx e GLO1;

e) avaliação do conteúdo GSH;

f) avaliação morfológica tecidual em lâmina histopatológica corada com hematoxilina-eosina (HE).

Também foi realizada a avaliação, após o período gestacional, do número de implantações uterinas fetais nas ratas mães por coloração uterina, a fim de verificar o índice de viabilidade fetal.

4 ARTIGO CIENTÍFICO

DEVELOPMENTAL PROGRAMMING: INTRAUTERINE CALORIC RESTRICTION PROMOTES UPREGULATION OF MITOCHONDRIAL SIRTUIN WITH MILD IMPACT ON OXIDATIVE PARAMETERS IN OFFSPRING'S OVARY AND TESTIS

Running Title: Molecular and antioxidant effect of Gestational CR

Summary-sentence: Intrauterine caloric restriction has low impact on ovaries and testis antioxidant defense function and oxidative stress status of young adult pups, even though SIRT3 expression was increased.

Key-Words: ovary; testis; oxidative stress; mitochondria; early development; environment; nutrition; caloric restriction; pregnancy.

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ABSTRACT

Interferences on maternal environment are known to reprogram the offspring metabolic response, impacting its postnatal life, in accord to Developmental Origins of Health and Disease (DOHaD) concept. Caloric Restriction is a well-known lifespan extensor, remarkably known for its redox modulation which contributes to diminished cellular aggression. However, little is known about caloric restriction effects on offspring's ovaries and testis regarding antioxidant parameters and molecular mechanisms of action. We aimed to assess the effects of a moderate (20%) caloric restriction on redox status parameters, molecular expression of SIRT1/3 deacetylase enzymes and histopathological markers in ovaries and testis of adult rats which underwent gestational caloric restriction. Female pups demonstrated high levels of oxidants and some oxidative damage, although superoxide dismutase activity was increased. Male pups displayed little effects, although gestational caloric restriction decreased enzymatic antioxidant defenses, evidenced by diminished glyoxalase I activity and reduced glutathione content. Ovaries and testis presented increased SIRT3 expression, a deacetylase enzyme with mechanisms related to cellular bioenergetics. Histopathological evaluation did not show any difference in the offspring's ovaries and testis. In concern to ovaries, different data suggest that diminished antioxidant metabolism can lead to premature failure. Unfortunately, there is few evidence available in the literature assessing testis redox profile. Our article is the first assessing the redox network in both organs, suggesting that, although improving molecular mechanisms, intrauterine caloric restriction has a negative impact on antioxidant network and redox status on reproductive organs of young adult rats.

INTRODUCTION

Metabolic programming during intrauterine development could impact the offspring's health in a long-term way. Over recent years, the field of Developmental Origins of Health and Disease (DOHaD) became a growing issue. Studies on DOHaD are focused in the effects and importance of nutritional interventions during early development, such as modulation in the intrauterine environment, modulating the susceptibility of non-communicable diseases later on life [1]. Maternal environment can influence on early embryo and fetal development, modulating offspring's life health and even conferring resistance to diseases [2].

In the occidental society, most of the population live in a sedentary regimen, with low quality and high volume of food ingestion [3, 4]. When associated, hyperalimentation and sedentary lifestyle lead to different complications, like myocardial infarction, obesity, hormonal issues and even earlier death [5]. In such context, there is an rising set of studies demonstrating the benefic role of caloric restriction (CR) on health, leading to increased health and life span [6, 7]. CR consists in diminishing the daily calorie intake, without promoting malnutrition [8, 9]. It is known as a gold-standard intervention to extend lifespan in different organisms, from yeast to primates [10]. One of such benefits is the redox modulation promoted by CR [11] which contributes to diminished cellular aggression, maintaining cellular efficiency for a longer period in life, leading to tissue longevity [12, 13].

Regarding redox modulation and lifespan extension, oxidative stress is a major cause of the aging process. The free radical theory of aging postulates that aging is a result of accumulated deleterious effects caused by free radicals and the organism's ability to manage oxidant induced cellular damage have an important role in determining lifespan [14]. Oxidative stress is caused by an unbalance between oxidants production and antioxidant defenses, and influences different biochemical processes, cellular stability and viability [15]. The oxidants, formed mainly by the mitochondrial electron transport system (METS), play a crucial role in cell signaling, like immune regulation processes [16]. Although oxidants in low levels are physiological and not harmful, high concentrations may not be removed properly by the enzymatic and non-enzymatic defense systems, leading to damage to cellular components, such as lipids, proteins or DNA [14]. The antioxidant defense system is composed by complementary enzymatic and non-enzymatic antioxidants, which act by scavenging reactive species [15].

There is a concern about reproduction and its lifespan [17, 18]. Throughout life, women and men have different reproductive lifespans. While both start in puberty, women have timed reproductive life while men's is conserved until elder ages. For women, the reproductive tissue is under different influences that can lead to reproductive problems, even in earlier ages [19, 20]. Healthy physiological processes are mandatory for correct ovarian function [21]. Nevertheless, a healthy and intact ovarian follicle reserve features functions that prevents metabolic disturbances, like osteoporosis [22, 23]. The follicle reserve tends to diminish if the organism faces potentially harmful situations, like oxidative stress [24]. For ovarian tissue, a theory links aging and oxidative stress, suggesting an ability to modulate fertility during aging [25], influencing reproductive lifespan and even menopause [26]. Other authors suggest that higher oxidant concentrations lead to follicular atresia and aging,

diminished endocrine function and follicular dysfunction, which can contribute to genetic problems if fertilization occurs [27]. Regarding pregnancy, redox homeostasis and oxidative stress had been associated to disturbances like pre-eclampsia and bortion [28]. In concern to men, the reproductive capacity does not suffer an age-related decline. Chirurgical procedures, e.g. vasectomy or hormonal and idiopathic infertility issues are able to affect reproductive capacity [29]. Nonetheless, oxidative stress can also affect men in a similar way that in women, by attacking germ cells [30].

Osborne et al. showed that CR from 1.5 to 6 months-of-age restored fertility at elder ages and extended lifespan of female rats [31]. However, there are few studies concerning gestational CR and oxidative stress [32] and none of them regards ovarian and testis function associated to its molecular basis. In the context of evaluating CR's effect in ovaries and testis tissue, different signaling pathways are onsidered, pathways related to the Sirtuins (SIRT) family are relevant. As a family of enzymatic NAD+- dependent deacetylases, controlled by the cellular NAD+/NADH ratio [33, 34], SIRT are found in different organisms [35, 36]. Among the seven SIRT isoforms expressed in mammals, SIRT1 and SIRT3 play major roles in sensing and perceiving cellular redox state [34, 37], that can lead to benefic effects when in oxidative stress situation, like cellular bioenergetics gene expression [38]. Concerning nutritional strategies, it is reported that SIRT1 and SIRT3 overexpression tend to promote the same effects as CR nutritional intervention in different tissues [39, 40]. Regarding probably protective functions in oxidative stress situation and positive regulation when in CR [41], studies correlate those SIRT expressions to benefits for ovarian content [42]. For testicular content, however, the literature mentions two opposite situations: while SIRT1 deficiency can benefit germ cells [43], it also postulates that overexpression increase acrosome biogenesis during spermatogenesis [44].

While most animal studies evaluate CR effects on adult animals, there is sparse evidence about intrauterine CR effects in the reproductive organs of pups. Thus, the aim of this study was to assess the effects of 20% gestational CR on ovarian and testicular redox profile and SIRT-related signaling pathways, as well as reproductive organs morphology.

METHODS

ANIMALS AND REAGENTS

Thirty-five adult female Wistar rats (120 days-of-age, nulliparous) were mated with 18 males, in proportion of 2 females:1 male. All animals were obtained from the Central Animal House of Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12h-12h light/dark cycle in constant temperature of 22 ± 1 oC. The conception was diagnosed by the presence of sperm in vaginal smears in the day after mating. After, animals were housed in groups of three female rats per cage, and randomly divided into two groups: control and caloric restriction. The procedures were approved by local Ethics Comission (Comissão de Ética no Uso de Animais/Universidade Federal do Rio Grande do Sul) under the number 34056, and followed the national animal rights regulation (Law 11.794/2008), the National Council of Control on Animal Experimentation (CONCEA/Brazil) on the guidelines about euthanasia, the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication No 80-23, revised in 1996) and the guidelines of the Canadian Council on Animal Care (CCAC). We attest that all the efforts were made to minimize the suffering of the animals used.

CHEMICALS

The chemicals were obtained from SIGMA® Chemical Co. (St. Louis, MO, USA), Invitrogen (Carlsbad, CA, EUA), Abcam (Cambridge, UK), Cellsignaling® (Danvers, MA, USA), and Thermo Fischer Scientific (Waltham, MA, USA).

CALORIC RESTRICTION PROTOCOL

Pregnant Wistar rats were divided into control and CR groups. Control group received food and chow ad libitum. CR group received 20% less commercial chow than control. To ensure similar micronutrients consumption between groups, CR dams were supplemented daily with a multivitamin and mineral mix via gavage. To abolish the gavage stressor effect, control group received vehicle (sucralose 0.1% and methylparaben 0.1%). All animals were weighted daily throughout pregnancy, and the diet was adjusted accordingly to the body weight, using the control consumption as standard for CR group's chow consumption. Such protocol was carried throughout the 21 days of pregnancy.

After delivery, CR pups were cross-fostered: CR pups were housed with control dams and control pups with another control dam to abolish the CR effect during lactation. To ensure equal food offer, the litter was adjusted to 8 pups, which were kept with the dam up to weaning on postnatal day (PND) 21. At weaning, the animals were relocated in new home-cages by gender, in number of 4 animals per cage until PND 60. On PND 60, female and male littermates were euthanized by decapitation and the ovaries and testis were dissected on Petri dish on ice, and the samples were stored in -80 °C for further analyses.

For flow cytometry assay, ovaries and testis were used freshly and prepared as described below.

MORPHOLOGICAL ANALYZES

Delivery Index

Uteruses of control and CR pregnant dams were dissected and immersed on 2% sodium hydroxide alkaline solution for one hour [45]. Rats which presented spermatozoids in vaginal smears and were not pregnant also had the uterus dissected to act as negative control for the uterine staining method. The post- implantation ratio was calculated based on the number of implantation sites/number of pups delivered x 100 [46].

Histopathological evaluation

Pups'ovaries and testis were embedded in 10% buffered formalin. Tissues were paraffinized, sectioned (5µm), and stained with hematoxylin and eosin for general histomorphological evaluation.

FLOW CYTOMETRY

The ovarian tissue samples (100 mg) were dissociated with 1.5mL of PBS pH 7.4 containing 1mg% of collagenase IV and the testis tissue sample was dissociated in 1 mL of PBS pH 7.4 containing 1mg% of collagenase IV, both filtered and incubated with probe.

Mitochondrial superoxide was measured using the probe MitoSOX® red. Nitric oxide was measured using the probe 4-amino-5-methylamino-20,70- difluorescein (DAF-FM®). Mitochondrial membrane mass and potential were analyzed by MitoTracker® Green FM and MitoTracker® Red CM-H2XRos respectively. Oxidants content was measured by dichlorofluoroscein oxidation (DCFH). Lipid oxidation was measured using the probe BODIPY 581/591® in a FACScalibur flow cytometer (BD BiosciencesVR , San Jose, CA). Sixty microliters of ovarian sample and twenty microliters of testis samples were incubated at 37 °C during 30 min in the presence of MitoSox® red in a final concentration of 1 µM and DCFH probe. Sixty microliters of ovarian sample and twenty microliters of testis samples were incubated at 37 °C during 1 hour in the presence of DAF-FM® in the final concentration of 10 µM. Sixty microliters of ovarian sample and twenty microliters of testis samples were incubated at 37 °C during 1 hour in the presence of MitoTracker® Green FM and MitoTracker® Red CM-H2XRos [46]. Sixty microliters of ovarian sample and twenty microliters of testis samples were incubated at 37 °C during 30 minutes in the presence of BODIPY 581/591. After that, 30,000 cells were evaluated per sample in the flow cytometer. Data were analyzed using the software FlowJo® (Ashland, OR).

SAMPLE PROCESSING FOR REDOX STATUS ASSAYS

The tissue was homogenized in 10 volumes (1:10, w/v) of phosphate buffered saline (PBS) pH 7.4, added 1mM ethyleneglycoltetraacetic acid (EGTA) and 1mM phenylmethanesulfonyl fluoride (PMSF). Homogenates were centrifuged at 1000 × g for 10 min at 4 °C, to discard nuclei and cell debris. The pellet was discarded, and the supernatant was taken to biochemical assays.

ANTIOXIDANT ANALYZES

Superoxide Dismutase

SOD enzyme (EC 1.15.1.1) activity was assayed according to Misra and Fridovich [47]. It was measured the total SOD activity by quantifying the inhibition of superoxide-dependent epinephrine autoxidation at 480 nm in a SpectraMax M5 microplate reader

(Molecular Devices, Sunnyvale, CA, USA). SOD activity is expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, which is equal to 1 unit. Data are expressed as Units/mg protein.

Catalase

Catalase (CAT, EC 1.11.1.6) activity was assayed according to Aebi [48]. The decrease in the absorbance at 240 nm was measured in a reaction medium containing 20 mM H2O2, 0.1% Triton X-100 and 10 mM potassium phosphate buffer, pH 7.0, using the SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The CAT unit is defined as 1 µmol of H2O2 consumed per minute. Data of specific activity are expressed as Units/mg protein.

Glutathione Peroxidase

GPx enzyme (EC 1.11.1.9) activity was assayed according to Wendel [49]. NADPH disappearance was monitored at 340 nm using the SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The reaction medium contained 100 mM potassium phosphate buffer, pH 7.7, containing 1mM EDTA, 2 mM GSH, 0.15 U/mL glutathione reductase (EC 1.8.1.7), 0.4 mM azide, 0.1 mM NADPH, and 0.5 mM tert-butyl hydroperoxide as enzyme substrate. The GPx unit is defined as 1 µmol of NADPH consumed per minute and the specific activity is represented as Units/mg protein.

Glyoxalase I

Glyoxalase I (GLO I; EC 4.4.1.5) activity was measured using GSH and methylglyoxal (MG) [50]. S-D-lactoylglutathione formation was monitored at 240 nm by SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The reaction medium contained 4 mM GSH and 4 mM MG in a 60 mM potassium phosphate buffer, pH 6.6. One GLO I unit is defined as the enzymatic quantity that catalyzes formation of 1 µmol of S-D-lactoylglutathione per minute.

NON-ENZYMATIC ANTIOXIDANTS

Reduced Glutathione Concentration

Reduced glutathione (GSH) concentration was measured according to Browne and Armstrong [51], where GSH reacts with the fluorophore o-phthalaldehyde. The proteins in supernatant were initially precipitated with meta-phosphoric acid (1:1, v:v), centrifuged at 5,000 × g, for 10 min, at 25 ºC. Forty-two µL of supernatant was incubated with 154,2 µL 120 mM sodium phosphate buffer pH 8.0, containing 5 mM ethylenediaminetetraacetic acid (EDTA) at room temperature for 15 minutes. A blank was parallelly performed. To measure fluorescence, the wavelengths were at 350 nm excitation and 420 nm emission. Calibration curve was prepared with standard GSH (0.01-1 mM) and the concentration were presented as nmol/mg protein.

BIOMOLECULE OXIDATIVE PARAMETERS

Total Sulfhydryl Content

The assay is based on the 50 -dithiobis-(2-nitrobenzoic acid) (DTNB) reduction by sulfhydryl groups, which are oxidized (disulfide), generating a yellow derivative (TNB), that can be measured spectrophotometrically at 412 nm [52]. Briefly, 50 µL of homogenate were added to 1 mL of phosphate saline buffer (PBS pH 7.4) containing 1 mM EDTA. Subsequent addition of 30 mL of 10 mM DTNB, prepared in a 0.2 M potassium phosphate solution pH 8.0 was performed. Incubation at room temperature was performed in a dark room for 30 min. Absorbance was measured at 412 nm. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nmol TNB/mg protein.

Carbonyl Levels

As a marker of protein oxidative damage, protein carbonyl content was assayed based on the reaction of such proteins with dinitrophenylhydrazine (DNPH) forming a yellow

compound, measured by absorbance at 370 nm [53]. The assay consisted of 1 mg sample protein treatment with 20% trichloroacetic acid, centrifuged at 4,000 × g for 5 minutes at 4 °C. Briefly, the pellet was dissolved in 0.2 M NaOH, then added 10 mM dinitrophenylhydrazine, prepared in HCl 2 M. The samples were kept in the dark during an hour, vortexed each 15 min. Then, centrifuged at 20,000 × g, for 5 min at 4 °C. The supernatant was discarded, and the pellet washed three times with ethanol:ethyl acetate (1:1, v/v), being centrifuged between washes at 20,000 g, for 5 min at 4 °C. The final supernatant was discarded and the pellet washed in 8 M urea pH 2.3 at 60 °C. After that, the samples were centrifuged at 20.000 × g for 5 min and absorbance was measured at 370 nm. Protein carbonyl content was expressed as nmol/mg protein.

WESTERN BLOT

Ovaries and testis from pups on PND60 were homogenized in ice-cold lysis buffer containing 4% sodium dodecyl sulfate (SDS), 2 mM EDTA, 50 mM Tris, and 1% protease inhibitor cocktail. The homogenates were denatured at 100 °C for 5 min, and then centrifuged at 10,000 × g for 30 min. After this, the supernatant containing the cytosolic fraction was collected, β-mercaptoethanol was added to a final concentration of 5%, and then, the samples were stored at -80 °C until use. Equal concentration of protein (50 μg) was loaded and immunodetected as previously described [54]. Membranes were incubated for 60min at 4°C in blocking solution (Tris-buffered saline containing 5% non-fat milk and 0.1% Tween-20, pH 7.4) prior the incubation with the primary antibody. Membranes were incubated overnight at 4 °C in blocking solution containing one of the following primary antibodies: anti-SIRT1 (1:500, Santa Cruz Technologies, catalog number #sc-15404), anti-SIRT3 (1:500, Abcam, catalog number #ab189860), and rabbit monoclonal anti-β-actin (1:2000, Cell Signaling, catalog number #4967). After washing, the membranes were incubated with secondary antibody containing peroxidase-conjugated anti-rabbit IgG (1:2000, GE Healthcare Life Sciences, catalog number #NA934V) for 1 h. The chemiluminescence was detected using a digital imaging system (Image Quant LAS 4000, GE Healthcare Life Sciences) and analyzed using the Image J Software. The average optical density for the control group was designated as 100%.

PROTEIN CONCENTRATION ASSAY

Protein concentration was measured by Lowry's method [55], using bovine serum albumin as standard.

STATISTICAL ANALYSIS

Data were analyzed by Student's t test using GraphPad Prism 6.0. Data were considered statistically significant when $p<0.05$.

RESULTS

CR EFFECTS IN DELIVERY INDEX

Delivery index parameters were evaluated on maternal uteruses. Figure 1 shows the difference between uterus of non-pregnant and pregnant rats. Results showed that there was no statistical difference [$p=0.641$, $t(24)=0.472$] between delivery and fetus implantation sites between control and CR dams.

CR EFFECTS IN OVARIAN AND TESTIS HISTOLOGY

Ovarian and testis histology were evaluated on PND60 male and female pups submitted to gestational CR. Results showed no cellular differences between ovaries and testis from control and CR pups (Figure 2).

CR EFFECTS ON OVARIAN AND TESTICULAR MITOCHONDRIAL FUNCTION AND REACTIVE SPECIES CONTENT

Mitochondrial mass and membrane potential, mitochondrial superoxide levels, oxidant levels and nitric oxide content were evaluated on PND60 male and female pups submitted to

gestational CR. Results show that, in ovaries, mitochondrial mass [t(16)=0.58;p=0.564] and membrane potential [t(15)=1.42;p=0.174] were no altered, mitochondrial superoxide levels [t(12)=3.02;p=0.01] and oxidant levels [t(14)=2.14;p=0.049] were increased and nitric oxide content [t(12)=2.13;p=0.054] was no altered by gestational CR (Figure 3). In testis, mitochondrial mass [t(14)=0.29;p=0.774] and membrane potential [t(13)=2.03;p=0.062], mitochondrial superoxide levels [t(15)=0.80;p=0.431], oxidant levels [t(16)=1.60;p=0.127], and nitric oxide content [t(14)=0.07;p=0.940] were no altered by gestational CR (Figure 3).

CR EFFECTS ON OVARIAN ENZYMATIC ANTIOXIDANT DEFENSES

SOD, CAT, GLO I, and GPx ovarian activities were evaluated on PND60 female pups submitted to gestational CR (Figure 4). Results showed that gestational CR promoted increased SOD [t(11)=2.89;p=0.014] and decreased GLO I activity [t(10)=2.38;p=0.038], while CAT [t(9)=1.66;p=0.129] and GPx [t(11)=1.20;p=0.253] were not altered by gestational CR.

CR EFFECTS ON TESTIS ENZYMATIC ANTIOXIDANT DEFENSES

SOD, CAT, GLO I, and GPx activities were evaluated on PND60 male pups submitted to gestational CR. (Figure 4). Results showed that gestational CR promoted diminished GLO I activity [t(13)=2.42; 250 p=0.030], while SOD [t(10)=1.93;p=0.082], CAT [t(11)=1.35;p=0.203], and GPx [t(11)=1.06;p=0.336] were not altered by gestational CR.

CR EFFECTS ON OVARIAN AND TESTIS NON-ENZYMATIC ANTIOXIDANT DEFENSES

GSH content was assessed in ovaries and testis of adult females and male rats exposed to CR during pregnancy (Figure 4). While the diet showed no statistical significant effect on the GSH ovarian content [t(9)=1.93;p=0.085], it was able to reduces the non-enzymatic antioxidant defense in the rat's testis [t(10)=3.18;p=0.009].

CR EFFECTS ON OVARIAN AND TESTIS OXIDATIVE DAMAGE PARAMETERS

Carbonyl and sulfhydryl content, as well as lipid peroxidation levels were assessed as indexes of oxidative damage on PND60 male pups submitted to gestational CR. Carbonyl and sulfhydryl content showed no alteration in both structures [ovaries: carbonyl: $t(18)=0.90;p=0.376$; sulfhydryl: $t(13)=7.03;p>0.999$; testis: carbonyl: $t(11)=2.081;p=0.061$; sulfhydryl: $t(11)=0.133;p=0.896$]. On the other hand, lipid peroxidation was increased in ovaries [$t(16)=2.39;p=0.029$], but showed no alteration in testis [$t(12)=0.83;p=0.419$] (Figure 5).

CR EFFECTS ON OVARIAN AND TESTIS SIRT1 AND SIRT3 EXPRESSION

SIRT1 and SIRT3 expression were evaluated on PND60 male and female pups submitted to gestational CR. Results showed increased SIRT3 expression in ovaries [$t(10)=2.36;p=0.039$] and testis [$t(14)=2.22;p=0.043$], while SIRT1 in ovaries [$t(10)=1.27;p=0.237$] and testis [$t(10)=2.01;p=0.071$] were not altered by gestational CR (Figure 6).

DISCUSSION

Metabolic programming comprehends the consequence of an intervention during development, leading to physiological long-term changes in organs and tissues [56]. Recently expanded to the Developmental Origins of Health and Disease (DOHaD) concept, metabolic programming demonstrates the pathophysiological base explaining the environmental influence during development leading to chronical diseases or health promotion in adulthood [1].

In this sense, gestational CR had multiple effects on ovaries and testis of adult rats. It is already known that CR can upregulate different enzymes, protecting different organs and tissues, although for ovaries, the studies focus on molecular explanations rather than on the enzymatic and non- enzymatic antioxidant defenses [24, 57]. We observed that gestational CR increased SOD activity in the ovaries of pups. The majority of the works focus on SOD activity, only demonstrating that a decrease in such enzyme activity is related to

unsuccessful in vitro fertilization [58]. Concerning superoxide related parameters, mitochondrial superoxide content was also increased in the ovaries and may be involved in SOD activation. Cooperatively, such variation might be explained by the measurement of total SOD cellular activity, rather than only mitochondrial isoform. While the increase of SOD activity is an effect of gestational CR, the up-rise in superoxide content can also be explained by the ovarian cells hormonal shift during each estrus phase, mainly by the fact that ovulation, a hormonal induced process, needs higher levels of superoxide to occur [59]. Albeit SOD activity peaks during the pro-estrus phase [60, 61] the correlation cannot be assured resultantly from the non-assessment of estrous cycle. Gestational CR promoted an increase in mitochondrial superoxide levels, without eliciting any alteration in mitochondrial biogenesis markers in pup's ovaries. Concerning mitochondrial related parameters, SIRT3, a deacetylase enzyme capable of perceive cellular redox state, leading to benefic effects due to expression of genes related to cellular bioenergetics [33], was increased in the ovaries of pups submitted to intrauterine CR. Studies mention the CR ability to modulate SIRT3 [40, 62]. In the same context, Qiu et al. [63] observed that in caloric restricted mice, SIRT3 was able to activate mitochondrial SOD (SOD2) in white adipose tissue, modulating oxidants detoxification, something that was not observed in mice fed ad libitum in the same study. In addition, while SIRT1 expression was not altered in the ovaries of pups from a gestational CR model, it is known that CR is able to modulate SIRT1 expression and possibly modulate SOD activity in adult animal models [64-66].

Gestational CR did not elicit any effect on CAT and GPx activities, conversely, increased GLO I activity in ovaries. Since there is a sparse enzymatic antioxidant activity in ovaries, hormonal function may be playing a significant role. Among the hormonal effect on enzymatic defenses, CAT activity fluctuate throughout the estrous cycle, depending on different gonadotropic hormones concentration [67]. Along with hormonal variation, CAT activity is also modulated by SIRT1 [64]. The basal state of CAT in the ovarian content can be related to SIRT1 basal levels found in this work. Regarding non-enzymatic antioxidant defenses, the diet did not alter the GSH content. In concern to GLO I diminished activity, Lurderer et al. assessed the variation of GSH content, correlated enzymes and hormonal shift [68]. However, GSH modulation related to hormonal mechanisms are not elucidated yet. One approach to solve the question can be the future estrous cycle assessment, carefully studying animals within the same hormonal window.

Moreover, intrauterine CR increased total oxidant content, measured by DCFH oxidation, without any change in nitric oxide content in pup's ovaries. The main oxidants that

interact with DCFH are hydrogen peroxide and hydroxyl radical [69]. Regarding protein and lipid damage, gestational CR elicited lipid peroxidation without promoting changes in carbonyl or sulfhydryl contents in ovarian tissue. The increase in oxidants may be related to increased lipid peroxidation, also promoted by gestational CR, and a well-known effect of oxidative stress and a pro-aging agent [14]. The higher lipid peroxidation can form glyoxal along with monosaccharides and glycated proteins degradation [58]. In such scenario, diminished GLO I antioxidant activity and augmented oxidant levels could lead to early ovarian aging [25, 27, 28].

In testis, CR was also able to induce SIRT3 overexpression and did not elicit any effect on oxidants content, SOD, CAT, and GPx activities or oxidative damage parameters. Moreover, SIRT1 was not altered in the testis as well. Gestational CR and the effects on testicular tissue have to be more explored in the literature, there is very few data concerning this intervention. Concerning different dietary interventions in rodents, rats submitted to protein restriction (10%) during intrauterine development present decreased SOD activity at 110 days of age [70]. In the same study, Rodríguez-González et al. showed that the pups display increased DCFH oxidation and lipid peroxidation. It is mandatory to observe that SIRT3 is a mitochondrial enzyme and, despite up-regulation by gestational CR, mitochondrial parameters, such as biogenesis, are not affected. In addition, we observed decreased GSH content and GLO I activity in the testis of young animals. However, it is stablished that lipid and protein metabolism, along with glycolysis pathway, can lead to MG formation and, thus, need for GSH and GLO I detoxification [71]. It is well described that aging decreases antioxidant defenses in several tissues [72], however, intrauterine CR was able to jeopardize antioxidant defenses even in young adult animals. It is essential to mention that although there are no signs of oxidative stress in the testis, studies report the fact that controlled and low concentrations of oxidants are important to different physiological process in the organ, such as sperm capacitation, acrosome reaction, and signaling that ensures fertilization [73]. In relation to hormonal variation, studies demonstrate that moderate CR (30%) does not impact testosterone biosynthesis gene expression or related cells maintenance in young or older Rhesus monkeys [74]. Sitzmann et al. also demonstrated minor effects of CR in testis morphology of old animals, meaning that long-term CR can affect cell's architecture, however the relation to fertility remains unclear. Another study by Gedik et al. [75] describes the impact of 30% dietary restriction for 17 months on rats' testis. In comparison to one-month old rats, the dietary restricted animals displayed diminished glutathione reductase and GPx activities, without alterations in SOD and

CAT activities. Rebrin et al. [76] demonstrated that 40% CR on adult mice impacted on oxidized glutathione (GSSG) and GSH contents only in testis of 26 months-old animals, when compared to 4 months old peers. However, when it was compared mice fed ad libitum and CR animals, the GSH:GSSG ratio was affected in 22 months-old mice testis, observed in the same study. Combined, such data demonstrate that, although CR has impacted on testis parameters, the effects are only present in elder ages, rather than on early adulthood.

Additionaly, intrauterine CR did not elicit any effect on the cell's architecture of neither tissue evaluated, as depicted by morphological analyses. Nevertheless, CR does not alter the delivery index of pregnant dams, demonstrated by no significant difference in the uterine staining of control and CR dams.

In summary, we showed for the first time that gestational CR exert long-lasting effects on the offspring's ovaries and testis metabolism in early adult age by inducing adaptive changes during development. Our findings reveal that gestational CR has long-term influence on offspring metabolism, being able to modify the molecular expression of SIRT3, an enzyme known for modulating cellular bioenergetics. In addition, our findings demonstrate a first look on different redox status parameters of ovaries and testis of young adult offspring that underwent gestational CR, demonstrating that such programming effects were still evident later-on on life, suggesting a negative impact on reproduction.

REFERENCES

1. Heindel JJ, Balbus J, Birnbaum L, Brune-Drisse MN, Grandjean P, Gray K, Landrigan PJ, Sly PD, Suk W, Slechta DC. Developmental origins of health and disease: integrating environmental influences. Endocrinology 2016; 2016:17-22.
2. Hanson Ma, Gluckman P. Early developmental conditioning of later health and disease: physiology or pathophysiology? Physiological reviews 2014; 94:1027-1076.
3. Digital N. Statistics on obesity, physical activity and diet, England 2017. 2017.
4. USDA U. Dietary guidelines for Americans, 2010. US Department of Agriculture, US Department of Health and Human Services, Washington, DC 2010.
5. Eyre H, Kahn R, Robertson RM, Committee AAACW. Preventing cancer, cardiovascular disease, and diabetes: a common agenda for theAmerican Cancer Society, the American Diabetes Association, and the American Heart Association. CA Cancer J Clin 2004; 54:190-207.
6. Lee C, Longo V. Dietary restriction with and without caloric restriction for healthy aging. F1000Res 2016; 5.
7. Colman RJ, Beasley TM, Kemnitz JW, Johnson SC, Weindruch R, Anderson RM. Caloric restriction reduces age-related and all-cause mortality in rhesus monkeys. Nat Commun 2014; 5:3557.

8. McCay CM, Crowell MF, Maynard LA. The effect of retarded growth upon the length of life span and upon the ultimate body size. 1935. Nutrition 1989; 5:155-171; discussion 172.
9. Cerqueira FM, Cunha FM, Laurindo FR, Kowaltowski AJ. Calorie restriction increases cerebral mitochondrial respiratory capacity in a NO*-mediated mechanism: impact on neuronal survival. Free Radic Biol Med 2012; 52:1236-1241.
10. Dang W. The controversial world of sirtuins. Drug Discov Today Technol 2014; 12:e9-e17.
11. Kim HJ, Jung KJ, Yu BP, Cho CG, Choi JS, Chung HY. Modulation of redox-sensitive transcription factors by calorie restriction during aging. Mechanisms of ageing and development 2002; 123:1589-1595.
12. Yu BP. Aging and oxidative stress: modulation by dietary restriction. Free radical biology and medicine 1996; 21:651-668.
13. Sohal RS, Weindruch R. Oxidative stress, caloric restriction, and aging. Science 1996; 273:59-63.
14. Harman D. Aging: a theory based on free radical and radiation chemistry. J Gerontol 1956; 11:298-300.
15. Sies H. Oxidative stress. Elsevier; 2013.
16. Fleury C, Mignotte B, Vayssiere JL. Mitochondrial reactive oxygen species in cell death signaling. Biochimie 2002; 84:131-141.
17. Shadyab AH, Macera CA, Shaffer RA, Jain S, Gallo LC, Gass M, Waring ME, Stefanick ML, LaCroix AZ. Ages at menarche and menopause and reproductive lifespan as predictors of exceptional longevity in women: the Women's Health Initiative. Menopause (New York, NY) 2017; 24:35-44.
18. Skakkebaek NE, Rajpert-De Meyts E, Buck Louis GM, Toppari J, Andersson A-M, Eisenberg ML, Jensen TK, Jørgensen N, Swan SH, Sapra KJ. Male reproductive disorders and fertility trends: influences of environment and genetic susceptibility. Physiological reviews 2015; 96:55- 97.
19. Johansson HKL, Svingen T, Fowler PA, Vinggaard AM, Boberg J. Environmental influences on ovarian dysgenesis - developmental windows sensitive to chemical exposures. Nat Rev Endocrinol 2017; 13:400-414.
20. Crain DA, Janssen SJ, Edwards TM, Heindel J, Ho SM, Hunt P, Iguchi T, Juul A, McLachlan JA, Schwartz J, Skakkebaek N, Soto AM, et al. Female reproductive disorders: the roles of endocrine- disrupting compounds and developmental timing. Fertil Steril 2008; 90:911-940.
21. Burger HG, Dudley EC, Robertson DM, Dennerstein L. Hormonal changes in the menopause transition. Recent Prog Horm Res 2002; 57:257-275.
22. Ettinger B, Pressman A, Sklarin P, Bauer DC, Cauley JA, Cummings SR. Associations between low levels of serum estradiol, bone density, and fractures among elderly women: the study of osteoporotic fractures. J Clin Endocrinol Metab 1998; 83:2239-2243.
23. Meema S, Meema HE. Menopausal bone loss and estrogen replacement. Isr J Med Sci 1976; 12:601-606.
24. Behrman HR, Kodaman PH, Preston SL, Gao S. Oxidative stress and the ovary. J Soc Gynecol Investig 2001; 8:S40-42.
25. Buffenstein R, Edrey YH, Yang T, Mele J. The oxidative stress theory of aging: embattled or invincible? Insights from non-traditional model organisms. Age (Dordr) 2008; 30:99-109.
26. De Bruin J, Dorland M, Spek E, Posthuma G, Van Haaften M, Looman C, Te Velde E. Ultrastructure of the resting ovarian follicle pool in healthy young women. Biology of reproduction 2002; 66:1151-1160.
27. Lim J, Luderer U. Oxidative damage increases and antioxidant gene expression

decreases with aging in the mouse ovary. Biology of reproduction 2011; 84:775-782.
28. Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. Reprod Biol Endocrinol 2005; 3:28.
29. Mann T, Lutwak-Mann C. Male reproductive function and semen: themes and trends in physiology, biochemistry and investigative andrology. Springer Science & Business Media; 2012.
30. Aitken RJ, Gibb Z, Baker MA, Drevet J, Gharagozloo P. Causes and consequences of oxidative stress in spermatozoa. Reprod Fertil Dev 2016; 28:1-10.
31. Osborne TB, Mendel LB, Ferry EL. The effect of retardation of growth upon the breeding period and duration of life of rats. Science 1917; 45:294-295.
32. Agale S, Kulkarni A, Ranjekar P, Joshi S. Maternal caloric restriction spares fetal brain polyunsaturated fatty acids in Wistar rats. Brain and Development 2010; 32:123-129.
33. Houtkooper RH, Canto C, Wanders RJ, Auwerx J. The secret life of NAD+: an old metabolite controlling new metabolic signaling pathways. Endocr Rev 2010; 31:194-223.
34. Yang H, Yang T, Baur JA, Perez E, Matsui T, Carmona JJ, Lamming DW, Souza-Pinto NC, Bohr VA, Rosenzweig A, de Cabo R, Sauve AA, et al. Nutrient-sensitive mitochondrial NAD+ levels dictate cell survival. Cell 2007; 130:1095-1107.
35. Imai S-I, Armstrong CM, Kaeberlein M, Guarente L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature 2000; 403:795.
36. Michan S, Sinclair D. Sirtuins in mammals: insights into their biological function. Biochemical Journal 2007; 404:1-13.
37. He W, Wang Y, Zhang MZ, You L, Davis LS, Fan H, Yang HC, Fogo AB, Zent R, Harris RC, Breyer MD, Hao CM. Sirt1 activation protects the mouse renal medulla from oxidative injury. J Clin Invest 2010; 120:1056-1068.
38. Zhong L, Mostoslavsky R. Fine tuning our cellular factories: sirtuins in mitochondrial biology. Cell metabolism 2011; 13:621-626.
39. Bordone L, Cohen D, Robinson A, Motta MC, van Veen E, Czopik A, Steele AD, Crowe H, Marmor S, Luo J, Gu W, Guarente L. SIRT1 transgenic mice show phenotypes resembling calorie restriction. Aging Cell 2007; 6:759-767.
40. Palacios OM, Carmona JJ, Michan S, Chen KY, Manabe Y, Ward Iii JL, Goodyear LJ, Tong Q. Diet and exercise signals regulate SIRT3 and activate AMPK and PGC-1α in skeletal muscle. Aging (Albany NY) 2009; 1:771.
41. Zhang J, Fang L, Lu Z, Xiong J, Wu M, Shi L, Luo A, Wang S. Are sirtuins markers of ovarian aging? Gene 2016; 575:680-686.
42. Tatone C, Di Emidio G, Barbonetti A, Carta G, Luciano AM, Falone S, Amicarelli F. Sirtuins in gamete biology and reproductive physiology: emerging roles and therapeutic potential in female and male infertility. Human reproduction update 2018; 24:267-289.
43. Coussens M, Maresh JG, Yanagimachi R, Maeda G, Allsopp R. Sirt1 deficiency attenuates spermatogenesis and germ cell function. PLoS One 2008; 3:e1571.
44. Liu C, Song Z, Wang L, Yu H, Liu W, Shang Y, Xu Z, Zhao H, Gao F, Wen J, Zhao L, Gui Y, et al. Sirt1 regulates acrosome biogenesis by modulating autophagic flux during spermiogenesis in mice. Development 2017; 144:441-451.
45. YAMADA T, OHSAWA K, OHNO H. The usefulness of alkaline solutions for clearing the uterus and staining implantation sites in rats. Experimental Animals 1988; 37:325-331.
46. Marcelino TB, Longoni A, Kudo KY, Stone V, Reck A, de Assis A, Scherer EB, da Cunha MJ, Wyse AT, Pettenuzzo LF, Leipnitz G, Matte C. Evidences that Maternal Swimming Exercise Improves Antioxidant Defenses and Induces Mitochondrial Biogenesis in Brain of Young Wistar Rats. Neuroscience 2013.
47. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine

and a simple assay for superoxide dismutase. J Biol Chem 1972; 247:3170-3175.
48. Aebi H. Catalase in vitro. Methods Enzymol 1984; 105:121-126.
49. Wendel A. Glutathione peroxidase. Methods Enzymol 1981; 77:325-333.
50. Thornalley P, Tisdale M. Inhibition of proliferation of human promyelocytic leukaemia HL60 cells by SD-lactoylglutathione in vitro. Leukemia research 1988; 12:897-904.
51. Browne RW, Armstrong D. Reduced glutathione and glutathione disulfide. Methods Mol Biol 1998; 108:347-352.
52. Aksenov MY, Markesbery WR. Changes in thiol content and expression of glutathione redox system genes in the hippocampus and cerebellum in Alzheimer's disease. Neurosci Lett 2001; 302:141-145.
53. Reznick AZ, Packer L. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods Enzymol 1994; 233:357-363.
54. Hoppe JB, Coradini K, Frozza RL, Oliveira CM, Meneghetti AB, Bernardi A, Pires ES, Beck RC, Salbego CG. Free and nanoencapsulated curcumin suppress β-amyloid-induced cognitive impairments in rats: involvement of BDNF and Akt/GSK-3β signaling pathway. Neurobiology of learning and memory 2013; 106:134-144.
55. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193:265-275.
56. Lucas A. Programming by early nutrition in man. The childhood environment and adult disease 1991; 1991:38-55.
57. Selesniemi K, Lee H-J, Muhlhauser A, Tilly JL. Prevention of maternal aging-associated oocyte aneuploidy and meiotic spindle defects in mice by dietary and genetic strategies. Proceedings of the National Academy of Sciences 2011; 108:12319-12324.
58. Tatone C, Amicarelli F. The aging ovary—the poor granulosa cells. Fertility and sterility 2013; 99:12-17.
59. Kaneko T, Iuchi Y, Kawachiya S, Fujii T, Saito H, Kurachi H, Fujii J. Alteration of glutathione reductase expression in the female reproductive organs during the estrous cycle. Biology of reproduction 2001; 65:1410-1416.
60. Laloraya M, Laloraya MM. Changes in the levels of superoxide anion radical and superoxide dismutase during the estrous cycle of Rattusnorvegicus and induction of superoxide dismutase in rat ovary by lutropin. Biochemical and biophysical research communications 1988; 157:146-153.
61. Laloraya M, Kumar G, Laloraya M. Histochemical study of superoxide dismutase in the ovary of the rat during the oestrous cycle. Journal of reproduction and fertility 1989; 86:583-587.
62. Hirschey MD, Shimazu T, Goetzman E, Jing E, Schwer B, Lombard DB, Grueter CA, Harris C, Biddinger S, Ilkayeva OR. SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation. Nature 2010; 464:121.
63. Qiu X, Brown K, Hirschey MD, Verdin E, Chen D. Calorie restriction reduces oxidative stress by SIRT3-mediated SOD2 activation. Cell metabolism 2010; 12:662-667.
64. Tatone C, Di Emidio G, Vitti M, Di Carlo M, Santini S, D'Alessandro AM, Falone S, Amicarelli
F. Sirtuin functions in female fertility: possible role in oxidative stress and aging. Oxidative medicine and cellular longevity 2015; 2015.
65. Kanfi Y, Peshti V, Gozlan YM, Rathaus M, Gil R, Cohen HY. Regulation of SIRT1 protein levels by nutrient availability. FEBS letters 2008; 582:2417-2423.
66. Allard JS, Perez E, Zou S, De Cabo R. Dietary activators of Sirt1. Molecular and cellular endocrinology 2009; 299:58-63.
67. Singh D, Pandey R. Changes in catalase activity and hydrogen peroxide level in rat

ovary during estrous cycle and induction of catalase in rat ovary by estradiol-17 beta. Indian journal of experimental biology 1998; 36:421-423.

68. Luderer U, Kavanagh TJ, White CC, Faustman EM. Gonadotropin regulation of glutathione synthesis in the rat ovary☆. Reproductive Toxicology 2001; 15:495-504.

69. LeBel CP, Ischiropoulos H, Bondy SC. Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol 1992; 5:227-231.

70. Rodríguez-González GL, Reyes-Castro LA, Vega CC, Boeck L, Ibáñez C, Nathanielsz PW, Larrea F, Zambrano E. Accelerated aging of reproductive capacity in male rat offspring of protein-restricted mothers is associated with increased testicular and sperm oxidative stress. AGE 2014; 36:9721.

71. Allaman I, Bélanger M, Magistretti PJ. Methylglyoxal, the dark side of glycolysis. Frontiers in neuroscience 2015; 9:23.

72. Currais A, Maher P. Functional consequences of age-dependent changes in glutathione status in the brain. Antioxidants & redox signaling 2013; 19:813-822.

73. Bansal AK, Bilaspuri G. Impacts of oxidative stress and antioxidants on semen functions. Veterinary Medicine International 2011; 2011.

74. Sitzmann BD, Brown DI, Garyfallou VT, Kohama SG, Mattison JA, Ingram DK, Roth GS, Ottinger MA, Urbanski HF. Impact of moderate calorie restriction on testicular morphology and endocrine function in adult rhesus macaques (Macaca mulatta). Age 2014; 36:183-197.

75. Gedik CM, Grant G, Morrice PC, Wood SG, Collins AR. Effects of age and dietary restriction on oxidative DNA damage, antioxidant protection and DNA repair in rats. European journal of nutrition 2005; 44:263-272.

76. Rebrin I, Kamzalov S, Sohal RS. Effects of age and caloric restriction on glutathione redox state in mice. Free Radic Biol Med 2003; 35:626-635.

FIGURE LEGENDS

Figure 1: Uterine staining for non-gravid uterus (A), birth day uterus (B) of dams. Black arrow indicates implantation site. n = 11-15.

Figure 2: Effect of intrauterine caloric restriction (CR) on ovarian and testis morphology, regarding: control ovaries (A), CR ovaries (B), control testis (C), and CR testis (D). n = 10.

Figure 3: Effect of intrauterine caloric restriction (CR) on ovarian and testis parameters, such as: dichlorofluorescein oxidation content (A), mitochondrial superoxide formation (B), nitric oxide (C), mitochondrial mass (D) and membrane potential (E). Results are expressed as mean ± S.E.M. for n = 9. *p<0.05, **p<0.001 (Student's test).

Figure 4: Effect of intrauterine caloric restriction (CR) on ovarian and testis enzymatic and non- enzymatic antioxidant parameters, such as: superoxide dismutase (SOD) (A), catalase (CAT) (B), glyoxalase 1 (GLO I) (C), glutathione peroxidase (GPx) (D) activities and GSH content (E). Results are expressed as mean ± S.E.M. for n = 6-9. *p<0.05 (Student's test).

Figure 5: Effect of intrauterine caloric restriction (CR) on ovarian and testis oxidative damage parameters such as: carbonyl (A) and sulfhydryl (B) content and lipid peroxidation (C). Results are expressed as mean ± S.E.M. for n = 6-10. *p<0.05, **p<0.001 (Student's test).

Figure 6: Effect of intrauterine caloric restriction (CR) on SIRT1 expression (A) and SIRT3 expression (B). Results are expressed as mean ± S.E.M. for n = 6-8. *p<0.05, **p<0.001 (Student's test).

FIGURES

Figure 1

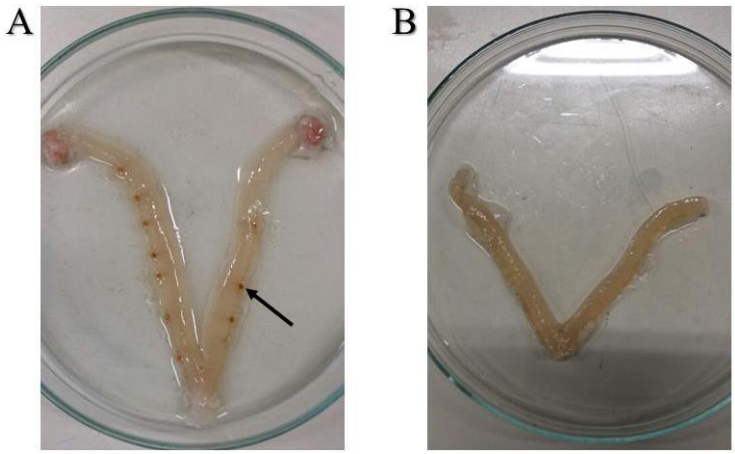


Figure 2

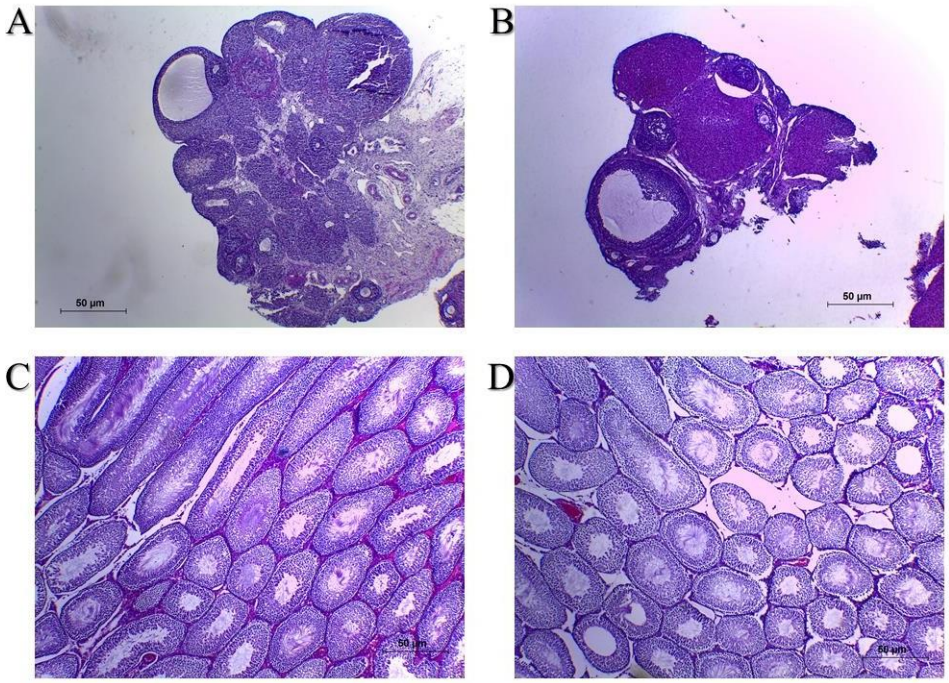


Figure 3

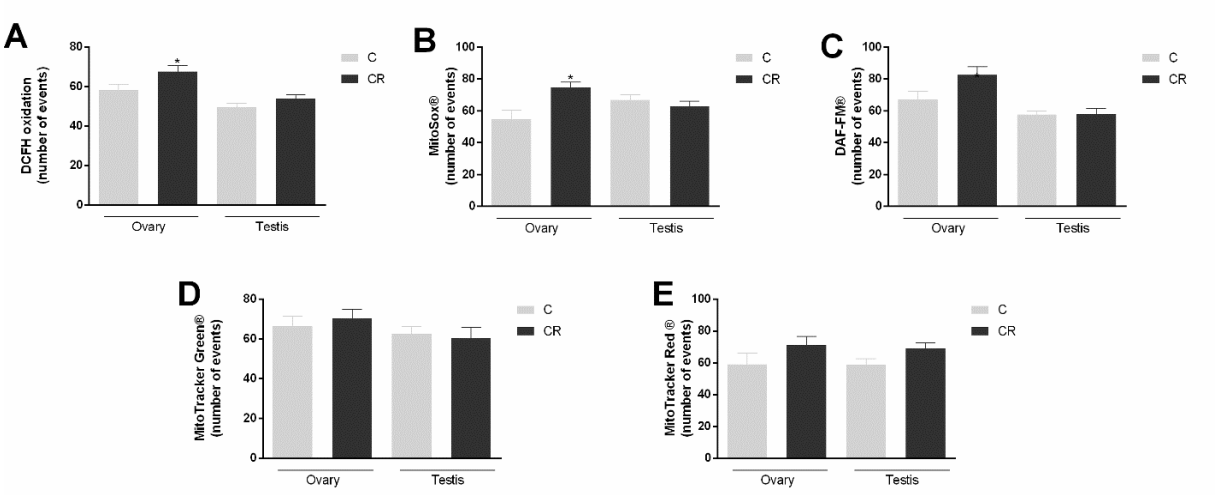

A
DCFH oxidation (number of events)
80
60
40
20
0
*
C
CR
Ovary
Testis
B
MitoSox® (number of events)
100
80
60
40
20
0
*
C
CR
Ovary
Testis
C
DAF-FM® (number of events)
100
80
60
40
20
0
C
CR
Ovary
Testis
D
MitoTracker Green® (number of events)
80
60
40
20
0
C
CR
Ovary
Testis
E
MitoTracker Red ® (number of events)
100
80
60
40
20
0
C
CR
Ovary
Testis

Figure 4

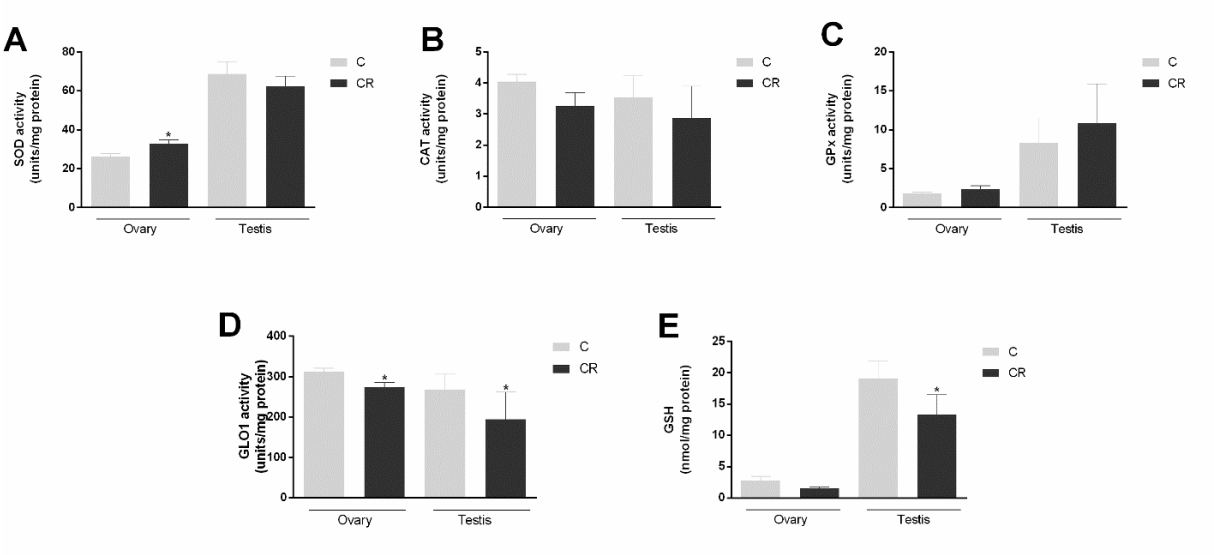

A
SOD activity (units/mg protein)
C
CR
Ovary
Testis
B
CAT activity (units/mg protein)
C
CR
Ovary
Testis
C
GPx activity (units/mg protein)
C
CR
Ovary
Testis
D
GLO1 activity (units/mg protein)
C
CR
Ovary
Testis
E
GSH (nmol/mg protein)
C
CR
Ovary
Testis

Figure 5

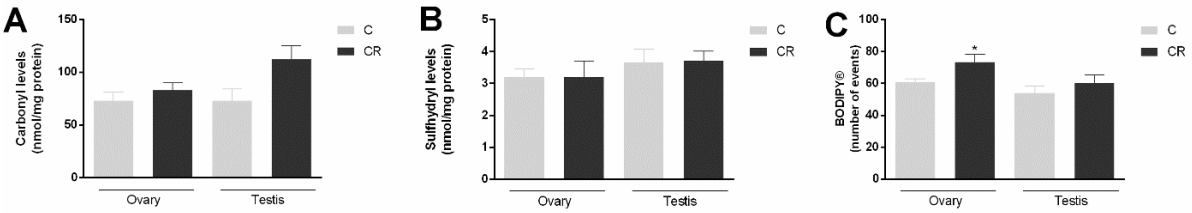
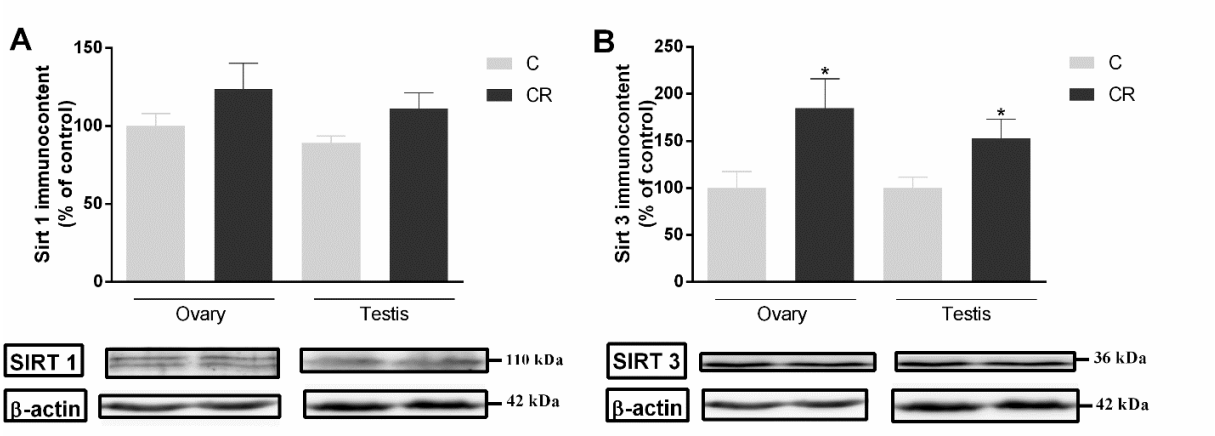

A
Carbonyl levels (nmol/mg protein)
150
100
50
0
C
CR
Ovary
Testis
B
Sulfhydryl levels (nmol/mg protein)
5
4
3
2
1
0
C
CR
Ovary
Testis
C
BODIPY® (number of events)
100
80
60
40
20
0
*
C
CR
Ovary
Testis

Figure 6


A
Sirt 1 immunocontent (% of control)
150
100
50
0
C
CR
Ovary
Testis
SIRT 1
β-actin
110 kDa
42 kDa
B
Sirt 3 immunocontent (% of control)
250
200
150
100
50
0
*
*
C
CR
Ovary
Testis
SIRT 3
β-actin
36 kDa
42 kDa

5 CONCLUSÕES E PERSPECTIVAS

O presente estudo demonstrou que a RC gestacional é capaz de promover alterações no estado redox e na função mitocondrial de ovários e testículos da prole aos 60 dias de vida. Embora os resultados obtidos sejam preliminares, eles realçam a importância de mais investigações nessa área de pesquisa.

De forma geral, pode-se avaliar que a RC durante o período gestacional tende a levar a diferentes modulações nos órgãos reprodutivos masculinos e femininos na idade adulta. A literatura sugere que a RC, na vida adulta, é um modelo que tende a levar a um aumento do tempo útil de muitos órgãos, por modulação benéfica do estado redox. Ao se estudar órgãos reprodutivos em um contexto de RC gestacional, demonstra-se que tal intervenção, durante o desenvolvimento, pode levar a modulações negativas. Em ovários, observamos um aumento no imunoconteúdo da SIRT3, entretanto o estado redox foi afetado de forma relevante. Enquanto que há aumento da atividade da SOD, há diminuição da atividade da GLO I. Além disso, a concentração de oxidantes é ampliada, levando ao aumento da oxidação lipídica. Em testículos, encontramos redução de parâmetros antioxidantes enzimáticos e não enzimáticos, entretanto sem modulação de espécies reativas ou dano oxidativo. Da mesma forma que nos ovários, há modulação de vias de sinalização metabólicas, graças a superexpressão de SIRT3, entretanto, nos dois órgãos, não foi possível explicar tal superexpressão pelos parâmetros estudados no presente trabalho.

Vale ressaltar que as modulações vistas deixam lacunas a serem preenchidas futuramente. O estudo de outras vias bioquímicas relacionadas a sirtuínas é essencial para um panorama mais completo da função exercida por essas nos órgãos reprodutivos femininos e masculinos. Além disso, é necessário fazer a avaliação de outras rotas bioquímicas relacionadas a enzimas antioxidantes, de modo a buscar um entendimento da atividade dos antioxidantes enzimáticos e não enzimáticos alterados nesse estudo. Além disso, cabe estudar diferentes idades da prole, desde o início da vida pós-natal, infância e idades mais avançadas da vida destes animais. De uma forma geral, mostramos que a RC gestacional afeta o metabolismo oxidativo em órgãos reprodutivos da prole, o que pode ter um impacto clínico relevante se os nossos dados puderem ser extrapolados para a condição humana.

REFERÊNCIAS

ALLAMAN, I.; BÉLANGER, M.; MAGISTRETTI, P. J. Methylglyoxal, the dark side of glycolysis. **Frontiers in neuroscience,** v. 9, p. 23, 2015. ISSN 1662-453X.

AOYAMA, K.; NAKAKI, T. Impaired glutathione synthesis in neurodegeneration. **International journal of molecular sciences,** v. 14, n. 10, p. 21021-21044, 2013.

AOYAMA, K.; NAKAKI, T. Glutathione in Cellular Redox Homeostasis: Association with the Excitatory Amino Acid Carrier 1 (EAAC1). **Molecules,** v. 20, n. 5, p. 8742-58, May 14 2015. ISSN 1420-3049 (Electronic). 1420-3049 (Linking). Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/26007177>.

ATTARAN, M. et al. The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. **International journal of fertility and women's medicine,** v. 45, n. 5, p. 314-320, 2000. ISSN 1534-892X.

AZAMBUJA, R. Reprodução Assistida - Técnicas de Laboratório. **AGE** v. 1, n. 1 ed, p. 320, 2017. ISSN 978-85-8343-320-0.

BANKS, A. S. et al. SirT1 gain of function increases energy efficiency and prevents diabetes in mice. **Cell metabolism,** v. 8, n. 4, p. 333-341, 2008. ISSN 1550-4131.

BARKER, D. J. P. Fetal and infant origins of adult disease. **BMJ**, 1992.

BARRETT, J. R. Fertile grounds of inquiry: environmental effects on human reproduction. **Environmental health perspectives,** v. 114, n. 11, p. A644, 2006.

BELA, K. et al. Plant glutathione peroxidases: emerging role of the antioxidant enzymes in plant development and stress responses. **Journal of plant physiology,** v. 176, p. 192-201, 2015. ISSN 0176-1617.

BELL, E. L. et al. SirT1 is required in the male germ cell for differentiation and fecundity in mice. **Development,** v. 141, n. 18, p. 3495-3504, 2014. ISSN 0950-1991.

BERNAL, A. B. et al. Maternal undernutrition significantly impacts ovarian follicle number and increases ovarian oxidative stress in adult rat offspring. **PLoS One,** v. 5, n. 12, p. e15558, Dec 13 2010. ISSN 1932-6203 (Electronic) 1932-6203 (Linking).

BORDONE, L. et al. SIRT1 transgenic mice show phenotypes resembling calorie restriction. **Aging Cell,** v. 6, n. 6, p. 759-67, Dec 2007. ISSN 1474-9726 (Electronic) 1474-9718 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17877786 >.

BRIGELIUS-FLOHÉ, R.; MAIORINO, M. Glutathione peroxidases. **Biochimica et Biophysica Acta (BBA)-General Subjects,** v. 1830, n. 5, p. 3289-3303, 2013. ISSN 0304-4165.

BRINKMANN, C.; BRIXIUS, K. Peroxiredoxins and sports: new insights on the antioxidative defense. **The Journal of Physiological Sciences,** v. 63, n. 1, p. 1-5, 2013. ISSN

1880-6546.

BURTON, G. J. Oxygen, the Janus gas; its effects on human placental development and function. **Journal of anatomy,** v. 215, n. 1, p. 27-35, 2009. ISSN 1469-7580.

CASTAGNE, V. et al. An optimal redox status for the survival of axotomized ganglion cells in the developing retina. **Neuroscience,** v. 93, n. 1, p. 313-320, 1999. ISSN 0306-4522.

CHEN, D. et al. Increase in activity during calorie restriction requires Sirt1. **Science,** v. 310, n. 5754, p. 1641-1641, 2005. ISSN 0036-8075.

CHISTIAKOV, D. A. et al. Mitochondrial aging and age-related dysfunction of mitochondria. **BioMed research international,** v. 2014, 2014. ISSN 2314-6133.

COHEN, H. Y. et al. Acetylation of the C terminus of Ku70 by CBP and PCAF controls Bax-mediated apoptosis. **Molecular cell,** v. 13, n. 5, p. 627-638, 2004. ISSN 1097-2765.

COHEN, H. Y. et al. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. **science,** v. 305, n. 5682, p. 390-392, 2004. ISSN 0036-8075.

COLMAN, R. J. et al. Caloric restriction reduces age-related and all-cause mortality in rhesus monkeys. **Nat Commun,** v. 5, p. 3557, Apr 1 2014. ISSN 2041-1723 (Electronic) 2041-1723 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24691430>.

COUSSENS, M. et al. Sirt1 deficiency attenuates spermatogenesis and germ cell function. **PLoS One,** v. 3, n. 2, p. e1571, Feb 13 2008. ISSN 1932-6203 (Electronic) 1932-6203 (Linking).

DAI, Y. et al. Superimposition of postnatal calorie restriction protects the aging male intrauterine growth-restricted offspring from metabolic maladaptations. **Endocrinology,** v. 153, n. 9, p. 4216-4226, 2012. ISSN 0013-7227.

DANG, W. The controversial world of sirtuins. **Drug Discov Today Technol,** v. 12, p. e9-e17, Jun 2014. ISSN 1740-6749 (Electronic). 1740-6749 (Linking). Disponível em: http://www.ncbi.nlm.nih.gov/pubmed/25027380>.

DENNERY, P. A. Oxidative stress in development: nature or nurture? **Free Radical Biology and Medicine,** v. 49, n. 7, p. 1147-1151, 2010. ISSN 0891-5849.

DEPEINT, F. et al. Mitochondrial function and toxicity: role of the B vitamin family on mitochondrial energy metabolism. **Chemico-biological interactions,** v. 163, n. 1-2, p. 94-112, 2006. ISSN 0009-2797.

DIGITAL, N. **Statistics on obesity, physical activity and diet**. England, 2017.

DUBEY, A. et al. Effect of age and caloric intake on protein oxidation in different brain regions and on behavioral functions of the mouse. **Archives of biochemistry and biophysics,** v. 333, n. 1, p. 189-197, 1996. ISSN 0003-9861.

EYRE, H. et al. Preventing cancer, cardiovascular disease, and diabetes: a common agenda

for theAmerican Cancer Society, the American Diabetes Association, and the American Heart Association. **CA Cancer J Clin,** v. 54, n. 4, p. 190-207, Jul-Aug 2004. ISSN 0007-9235 (Print) 0007-9235 (Linking).

FAINARU, O. et al. Active labour is associated with increased oxidisibility of serum lipids ex vivo. **BJOG: An International Journal of Obstetrics & Gynaecology,** v. 109, n. 8, p. 938-941, 2002. ISSN 1471-0528.

GANDHI, S.; ABRAMOV, A. Y. Mechanism of oxidative stress in neurodegeneration. **Oxidative medicine and cellular longevity,** v. 2012, 2012. ISSN 1942-0900.

GARDNER, D. G.; SHOBACK, D.; GREENSPAN, F. S. **Greenspan's basic & clinical endocrinology**. McGraw-Hill Medical, 2007. ISBN 0071440119.

GILCA, M. et al. The oxidative hypothesis of senescence. **Journal of postgraduate medicine,** v. 53, n. 3, p. 207, 2007. ISSN 0022-3859.

GODFREY, K. M.; BARKER, D. J. Fetal programming and adult health. **Public health nutrition,** v. 4, n. 2b, p. 611-624, 2001. ISSN 1475-2727.

GODIC, A. et al. The role of antioxidants in skin cancer prevention and treatment. **Oxidative medicine and cellular longevity,** v. 2014, 2014. ISSN 1942-0900.

GOMEZ, E.; IRVINE, D.; AITKEN, R. Evaluation of a spectrophotometric assay for the measurement of malondialdehyde and 4-hydroxy-alkenals in human spermatozoa: Relationships with semen quality and sperm function. **International Journal of Andrology,** v. 21, n. 2, p. 81-94, 1998. ISSN 0105-6263.

GUTTERIDGE, J.; HALLIWELL, B. Free radicals and antioxidants in the year 2000: a historical look to the future. **Annals of the New York Academy of Sciences,** v. 899, n. 1, p. 136-147, 2000. ISSN 1749-6632.

GUTTERIDGE, J. M.; HALLIWELL, B. Invited review free radicals in disease processes: a compilation of cause and consequence. **Free radical research communications,** v. 19, n. 3, p. 141-158, 1993. ISSN 8755-0199.

HALES, C. N.; BARKER, D. J. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. **Diabetologia,** v. 35, n. 7, p. 595-601, 1992. ISSN 0012-186X.

HALLIWELL, B. Vitamin C: poison, prophylactic or panacea? **Trends in biochemical sciences,** v. 24, n. 7, p. 255-259, 1999. ISSN 0968-0004.

HALLIWELL, B.; GUTTERIDGE, J. M.; CROSS, C. E. Free radicals, antioxidants, and human disease: where are we now? **The Journal of laboratory and clinical medicine,** v. 119, n. 6, p. 598-620, 1992. ISSN 0022-2143.

HALLIWELL, B.; GUTTERIDGE, J. M. C. **Free Radicals in Biology and Medicine**. New York: Oxford University, 2007.

HARRATH, A. H. et al. Food restriction during pregnancy and female offspring fertility:

adverse effects of reprogrammed reproductive lifespan. **Journal of ovarian research,** v. 10, n. 1, p. 77, 2017. ISSN 1757-2215.

HE, W. et al. Sirt1 activation protects the mouse renal medulla from oxidative injury. **J Clin Invest,** v. 120, n. 4, p. 1056-68, Apr 2010. ISSN 1558-8238 (Electronic) 0021-9738 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/203356590>.

HEINDEL, J. J. et al. Developmental origins of health and disease: integrating environmental influences. **Endocrinology,** v. 2016, n. 1, p. 17-22, 2016. ISSN 0013-7227.

HITCHLER, M. J.; DOMANN, F. E. An epigenetic perspective on the free radical theory of development. **Free Radical Biology and Medicine,** v. 43, n. 7, p. 1023-1036, 2007. ISSN 0891-5849.

HOUTKOOPER, R. H. et al. The secret life of NAD+: an old metabolite controlling new metabolic signaling pathways. **Endocr Rev,** v. 31, n. 2, p. 194-223, Apr 2010. ISSN 1945-7189 (Electronic) 0163-769X (Linking). Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/200073260>.

IMAI, S.-I. et al. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. **Nature,** v. 403, n. 6771, p. 795, 2000. ISSN 1476-4687.

ISHIKAWA, M. Oxygen radicals-superoxide dismutase system and reproduction medicine. **Nihon Sanka Fujinka Gakkai Zasshi,** v. 45, n. 8, p. 842-848, 1993. ISSN 0300-9165.

JONES, D. P. Redefining oxidative stress. **Antioxidants & redox signaling,** v. 8, n. 9-10, p. 1865-1879, 2006. ISSN 1523-0864.

KOWALTOWSKI, A. J. Caloric restriction and redox state: does this diet increase or decrease oxidant production? **Redox Rep,** v. 16, n. 6, p. 237-41, 2011. ISSN 1743-2928 (Electronic) 1351-0002 (Linking).

ŁAGÓD, L. et al. The antioxidant-prooxidant balance in pregnancy complicated by spontaneous abortion. **Ginekologia polska,** v. 72, n. 12, p. 1073-1078, 2001. ISSN 0017-0011.

LASS, A. et al. Caloric restriction prevents age-associated accrual of oxidative damage to mouse skeletal muscle mitochondria. **Free Radical Biology and Medicine,** v. 25, n. 9, p. 1089-1097, 1998. ISSN 0891-5849.

LEE, C.; LONGO, V. Dietary restriction with and without caloric restriction for healthy aging. **F1000Research,** v. 5, 2016.

LI, L. et al. Caloric restriction promotes the reserve of follicle pool in adult female rats by inhibiting the activation of mammalian target of rapamycin signaling. **Reprod Sci,** v. 22, n. 1, p. 60-7, Jan 2015. ISSN 1933-7205 (Electronic) 1933-7191 (Linking).

LI, Y. et al. SirT1 inhibition reduces IGF-I/IRS-2/Ras/ERK1/2 signaling and protects neurons. **Cell metabolism,** v. 8, n. 1, p. 38-48, 2008. ISSN 1550-4131.

LIN, M. T.; BEAL, M. F. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. **Nature,** v. 443, n. 7113, p. 787, 2006. ISSN 1476-4687. 2006

LIU, W.-J. et al. Calorie restriction inhibits ovarian follicle development and follicle loss through activating SIRT1 signaling in mice. **European journal of medical research,** v. 20, n. 1, p. 22, 2015. ISSN 2047-783X.

LOEB, J.; NORTHROP, J. H. What determines the duration of life in metazoa? **Proceedings of the National Academy of Sciences,** v. 3, n. 5, p. 382-386, 1917. ISSN 0027-8424.

LÓPEZ-LLUCH, G.; NAVAS, P. Calorie restriction as an intervention in ageing. **The Journal of physiology,** v. 594, n. 8, p. 2043-2060, 2016. ISSN 1469-7793.

LUCAS, A. Programming by early nutrition in man. **The childhood environment and adult disease,** v. 1991, p. 38-55, 1991.

MACHLIN, L. J.; BENDICH, A. Free radical tissue damage: protective role of antioxidant nutrients. **The FASEB Journal,** v. 1, n. 6, p. 441-445, 1987. ISSN 0892-6638.

MAILLOUX, R. J. Teaching the fundamentals of electron transfer reactions in mitochondria and the production and detection of reactive oxygen species. **Redox biology,** v. 4, p. 381-398, 2015. ISSN 2213-2317.

MASCARENHAS, M. N. et al. National, regional, and global trends in infertility prevalence since 1990: a systematic analysis of 277 health surveys. **PLoS medicine,** v. 9, n. 12, p. e1001356, 2012. ISSN 1549-1676.

MATÉS, J. M.; PÉREZ-GÓMEZ, C.; DE CASTRO, I. N. Antioxidant enzymes and human diseases. **Clinical biochemistry,** v. 32, n. 8, p. 595-603, 1999. ISSN 0009-9120.

MAY, J. M. How does ascorbic acid prevent endothelial dysfunction? **Free radical biology and medicine,** v. 28, n. 9, p. 1421-1429, 2000. ISSN 0891-5849.

MCCAY, C. M.; CROWELL, M. F.; MAYNARD, L. A. The effect of retarded growth upon the length of life span and upon the ultimate body size one figure. **The journal of Nutrition,** v. 10, n. 1, p. 63-79, 1935. ISSN 0022-3166.

MCCORD, J. M.; FRIDOVICH, I. Superoxide dismutase an enzymic function for erythrocuprein (hemocuprein). **Journal of Biological chemistry,** v. 244, n. 22, p. 6049-6055, 1969. ISSN 0021-9258.

MERRY, B. Molecular mechanisms linking calorie restriction and longevity. **The international journal of biochemistry & cell biology,** v. 34, n. 11, p. 1340-1354, 2002. ISSN 1357-2725.

MICHAN, S.; SINCLAIR, D. Sirtuins in mammals: insights into their biological function. **Biochemical Journal,** v. 404, n. 1, p. 1-13, 2007. ISSN 0264-6021.

MICHISHITA, E. et al. Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. **Molecular biology of the cell,** v. 16, n. 10, p. 4623-4635,

2005. ISSN 1059-1524.

MYATT, L.; CUI, X. Oxidative stress in the placenta. **Histochemistry and cell biology,** v. 122, n. 4, p. 369-382, 2004. ISSN 0948-6143.

NOGUEIRAS, R. et al. Sirtuin 1 and sirtuin 3: physiological modulators of metabolism. **Physiological reviews,** v. 92, n. 3, p. 1479-1514, 2012. ISSN 0031-9333.

OSBORNE, T. B.; MENDEL, L. B.; FERRY, E. L. The effect of retardation of growth upon the breeding period and duration of life of rats. **Science,** v. 45, n. 1160, p. 294-295, 1917. ISSN 0036-8075.

PALACIOS, O. M. et al. Diet and exercise signals regulate SIRT3 and activate AMPK and PGC-1α in skeletal muscle. **Aging (Albany NY),** v. 1, n. 9, p. 771, 2009.

PERSSON, T.; POPESCU, B. O.; CEDAZO-MINGUEZ, A. Oxidative stress in Alzheimer's disease: why did antioxidant therapy fail? **Oxidative medicine and cellular longevity,** v. 2014, 2014. ISSN 1942-0900.

PISOSCHI, A. M.; POP, A. The role of antioxidants in the chemistry of oxidative stress: A review. **European journal of medicinal chemistry,** v. 97, p. 55-74, 2015. ISSN 0223-5234.

PLANTE, M.; DE LAMIRANDE, E.; GAGNON, C. Reactive oxygen species released by activated neutrophils, but not by deficient spermatozoa, are sufficient to affect normal sperm motility. **Fertility and sterility,** v. 62, n. 2, p. 387-393, 1994. ISSN 0015-0282.

POLJSAK, B.; ŠUPUT, D.; MILISAV, I. Achieving the balance between ROS and antioxidants: when to use the synthetic antioxidants. **Oxidative medicine and cellular longevity,** v. 2013, 2013. ISSN 1942-0900.

RANI, V. et al. Oxidative stress and metabolic disorders: Pathogenesis and therapeutic strategies. **Life sciences,** v. 148, p. 183-193, 2016. ISSN 0024-3205.

RILEY, P. Free radicals in biology: oxidative stress and the effects of ionizing radiation. **International journal of radiation biology,** v. 65, n. 1, p. 27-33, 1994. ISSN 0955-3002.

SCHAFER, F. Q.; BUETTNER, G. R. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. **Free Radical Biology and Medicine,** v. 30, n. 11, p. 1191-1212, 2001. ISSN 0891-5849.

SCHWER, B. et al. The human silent information regulator (Sir) 2 homologue hSIRT3 is a mitochondrial nicotinamide adenine dinucleotide–dependent deacetylase. **J Cell Biol,** v. 158, n. 4, p. 647-657, 2002. ISSN 0021-9525.

SELESNIEMI, K.; LEE, H. J.; TILLY, J. L. Moderate caloric restriction initiated in rodents during adulthood sustains function of the female reproductive axis into advanced chronological age. **Aging cell,** v. 7, n. 5, p. 622-629, 2008. ISSN 1474-9726.

SHI, T. et al. SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes. **Journal of Biological Chemistry,** v. 280, n. 14, p.

13560-13567, 2005. ISSN 0021-9258.

SIES, H. Oxidative stress: from basic research to clinical application. **The American journal of medicine,** v. 91, n. 3, p. S31-S38, 1991. ISSN 0002-9343.

SIES, H. Role of metabolic H2O2 generation: redox signaling and oxidative stress. **J Biol Chem,** v. 289, n. 13, p. 8735-41, Mar 28 2014. ISSN 1083-351X (Electronic) 0021-9258 (Linking).

SIES, H.; BERNDT, C.; JONES, D. P. Oxidative stress. **Annual review of biochemistry**, v. 86, p. 715-748, 2017.

SINCLAIR, D. A. Toward a unified theory of caloric restriction and longevity regulation. **Mechanisms of ageing and development,** v. 126, n. 9, p. 987-1002, 2005. ISSN 0047-6374.

SOHAL, R. S.; FORSTER, M. J. Caloric restriction and the aging process: a critique. **Free Radical Biology and Medicine,** v. 73, p. 366-382, 2014. ISSN 0891-5849.

SUNDARESAN, N. R. et al. Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. **The Journal of clinical investigation,** v. 119, n. 9, p. 2758-2771, 2009. ISSN 0021-9738.

SUZUKI, T. et al. Superoxide dismutase in normal cycling human ovaries: immunohistochemical localization and characterization. **Fertility and sterility,** v. 72, n. 4, p. 720-726, 1999. ISSN 0015-0282.

TANNO, M. et al. Nucleocytoplasmic shuttling of the NAD+-dependent histone deacetylase SIRT1. **Journal of Biological Chemistry,** v. 282, n. 9, p. 6823-6832, 2007. ISSN 0021-9258.

THAKOR, A. et al. Redox modulation of the fetal cardiovascular defence to hypoxaemia. **The Journal of physiology,** v. 588, n. 21, p. 4235-4247, 2010. ISSN 1469-7793.

THOMPSON, L. P.; AL-HASAN, Y. Impact of oxidative stress in fetal programming. **Journal of pregnancy,** v. 2012, 2012. ISSN 2090-2727.

TRANQUILLI, A. L. et al. Amniotic vascular endothelial growth factor (VEGF) and nitric oxide (NO) in women with subsequent preeclampsia. **European Journal of Obstetrics and Gynecology and Reproductive Biology,** v. 113, n. 1, p. 17-20, 2004. ISSN 0301-2115.

USDA, U. Dietary guidelines for Americans, 2010. **US Department of Agriculture, US Department of Health and Human Services, Washington, DC**, 2010.

WANG, Y. et al. Importance portance of reactive oxygen species in the peritoneal fluid of women with endometriosis or idiopathic infertility. **Fertility and sterility,** v. 68, n. 5, p. 826-830, 1997. ISSN 0015-0282.

YANG, H. et al. Nutrient-sensitive mitochondrial NAD+ levels dictate cell survival. **Cell,** v. 130, n. 6, p. 1095-107, Sep 21 2007. ISSN 0092-8674 (Print) 0092-8674 (Linking). Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/17889652>.

ZEGERS-HOCHSCHILD, F. et al. The international committee for monitoring assisted reproductive technology (ICMART) and the world health organization (WHO) revised glossary on ART terminology, 2009. **Human reproduction,** v. 24, n. 11, p. 2683-2687, 2009. ISSN 1460-2350.

ZHONG, L.; MOSTOSLAVSKY, R. Fine tuning our cellular factories: sirtuins in mitochondrial biology. **Cell metabolism,** v. 13, n. 6, p. 621-626, 2011. ISSN 1550-4131.

APÊNDICE A – PARECER DE APROVAÇÃO DA CEUA

Projeto aprovado pela Comissão de Ética no Uso de Animais da Universidade Federal do Rio Grande do Sul (CEUA/UFRGS), sob o projeto n° 34056.

Comissão De Ética No Uso De Animais aprovou o mesmo, em reunião realizada em 05/03/2018 - Sala 330 do Anexo 1 da Reitoria - Campus Centro - Porto Alegre - RS, em seus aspectos éticos e metodológicos, para a utilização de 80 ratos Wistar neonatos fêmeas, 80 ratos Wistar neonatos machos, 86 ratos Wistar fêmeas de 120 dias e 43 ratos Wistar machos de 120 dias, provenientes do CREAL, de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

CEUA/UFRGS

Carta de aprovação indisponível no Portal da UFRGS em 18 de junho de 2018.

APÊNDICE B – NORMAS DE PUBLICAÇÃO DA REVISTA BIOLOGY OF REPRODUCTION

Initial submission: For the initial submission, authors do not need to follow the BOR manuscript style and may submit all components of the manuscript for review as long as the text is double-spaced and labelled with line numbers. Please note that specific requirements for BOR submission, including style and format, must be followed for all revised manuscripts.

All manuscripts are submitted and reviewed via the journal's Editorial Manager system. New authors should create an account prior to submitting a manuscript for consideration. Questions about submitting to the journal should be sent to the editorial office at biolre.editorialoffice@oup.com.

All submissions to the journal are initially reviewed by the editors to assess appropriateness for the journal. Manuscripts viewed as potentially suitable for the journal are immediately sent for peer review, usually by two independent reviewers. However, if deemed not relevant to the purview of Biology of Reproduction or of high priority, manuscripts are returned to the author immediately without peer review; this fast rejection process means that authors are given a quick decision on papers not appropriate for the journal.

For those manuscripts subjected to peer review, a decision about suitability is made based on the review feedback and judgment of the editors. When a manuscript is promising but not acceptable in its present form, suggestions for revisions are transmitted to the author. For information on the journal's review process or a manuscript's progress, please contact the Managing Editor at biolre.editorialoffice@oup.com.

Content types

Original Research Articles: An original research article should contain no more than 8,000 words (~10 pages) in the main text (Introduction, Methods, Results and Discussion) and a maximum of 6 display items (figures and tables). Where appropriate, detailed methods may be presented as supplementary materials. Supplementary materials should be presented as a single pdf file. Large datasets should be provided separately as supplemental materials.

Reviews: A full-length review should contain no more than 10,000 words (~12 pages) and 6 display items (figures and tables).

Letters to the Editor: Letters are brief and concise reports of novel findings of general interest to the field. A Letter should start with "Dear Editor,", and contains NO abstract or

other subsections. The main text should contain <1,000 words, one figure (multiple panels allowed) and up to 10 references. If necessary, methods and materials can be included in supplemental materials, which can be presented as a single pdf file.

Research Highlights: Research Highlights aim to point the BOR readership to the latest novel findings published in high-impact journals. A Research Highlight should contain no more than 1,000 words, up to 10 references and 1 display item, and must be read and approved by the corresponding author of the paper to be highlighted before submission.

Interviews: BOR publishes interviews with prominent investigators in the field, which are usually conducted by the BOR editorial team including Editors-in-Chief, Associate Editors and members of the Board of Reviewing Editors. An interview should contain no more than 1,000 words, up to 10 references and one portrait of the interviewee.

Commentaries: Commentaries highlight original research articles published in BOR. A commentary should contain no more than 1,000 words, up to 10 references and 1 display item.

Revision

Manuscripts that receive a decision of Reconsider after Major Revisions, Acceptance if Appropriately Revised, or Conditional Acceptance may be revised and submitted only once for re-review. Revised manuscripts must be received by the Editorial Office within 90 days of the date of first decision; if a revised manuscript is received after the 90-day period, it will be treated as a new manuscript or a resubmission. If authors find that an extension is necessary, they must request an extension from the Editors-in-Chief in writing. If authors decide not to submit a revision, they are asked to send a request for their submission to be withdrawn.

When a revised manuscript is submitted, the manuscript text, all figure and supplemental files, and a "marked-up" version of the original submission (a copy of the previous submission with revision changes drafted in using font attributes such as redline, strikeout, or highlight) must be uploaded. Please upload the marked-up version as a supplemental file. A point-by-point Response to Reviewers is also required for all revisions.

Submitting Issue Cover Images

If an article is accepted for publication in BOR, the editors strongly encourage authors to submit a candidate image that may be used on the cover of an upcoming issue. Accepted authors who would like to submit an image for possible use as an issue cover should email a high resolution version of the image to the production editor at biolrepro@oup.com. They

should also include a one-sentence caption of the image.

Preparing a Manuscript for Submission

Equations. Equations must NOT be formatted using the default math editing tool in Word 2007. Instead, use the Design Science Equation Editor or MathType by clicking "Object" in the Insert ribbon and choosing either object type "Microsoft Equation 3.0" or "MathType Equation."

Figure and reference citations. Cite references, tables, figures, and supplemental data consecutively. Place reference numbers in square brackets, e.g., "as Smith [12] reported" or "as previously reported [3-5]." Authors will be charged for any alterations to References at proof stage.

File format. Preferred file formats are Microsoft Word and Corel WordPerfect.

Fonts. Only standard fonts such as Helvetica or Times New Roman should be used.

Genetic sequence deposits. Genetic sequences must be deposited to the appropriate database; this must be documented in footnote 1 on the title page.

Headers. Three levels of heads may be used. Level 1 is reserved for BIOLRE section headers (e.g.,Abstract, Introduction, Materials and Methods, etc.). Level 2 and 3 heads should consist of descriptive phrases.

Language. Choose U.S. English.

- In Word 2007 or 2010, click "Review" on the ribbon, then click "Language," and "Set Proofing Language." Select "English (U.S.)" and click "OK."
- In Word 97-2003, click "Tools" on the toolbar, then select "Language," and "Set Language." Select "English (U.S.)" and click "OK."
- In WordPerfect, click "Tools" on the toolbar, then select "Language," and "Settings." Scroll to find "English-U.S." and click "OK."
- Line numbering. Manuscripts submitted without line numbering will be returned to authors for correction.
- In Word 2007 or 2010, select the text of the Abstract through the Discussion, then go to Page Layout. Click the Line Numbers drop-down menu and choose "Line Numbering Options...." Click "Line Numbers...," then select "Add line numbering" and change the settings to start at 1, count by 5, and number continuously. Click "OK" in each window. In Word 97-2003, select the text of the Abstract through the

Discussion, then go to File > Page Setup > Layout and choose "Selected text" in the "Apply to" drop-down menu. Select "Line Numbers" and check the box to add line numbering. Start numbering at 1, count by 5, and number continuously.

- In WordPerfect, place the cursor at the start of the Abstract, then click "Format," "Line," and "Numbering," and enable the "Turn Line Numbering On" check box.

Line spacing. Double-space all text.

Manuscript length. There are currently no page or word limits for BIOLRE manuscripts; however, to contain publishing costs and reduce reader fatigue, manuscripts must be concise and avoid reiteration and redundancies.

- Do not include information in the Introduction that is provided elsewhere in the manuscript.
- Do not present discussion in the Results section.
- Cite only essential references.
- Do not include sequence information that can be accessed through a publicly available database, such as GenBank or EMBL.
- Include only essential figures and format each figure so that it occupies no more space than is necessary to convey critical information.
- Do not reiterate information in figure legends that appears in Materials and Methods orResults. Do not include lengthy descriptive information in figure legends that is more appropriately incorporated in the text of the manuscript.
- Avoid unnecessary tables; do not put data in tabular form if the information can be presented adequately in a few sentences in the text. Very large tables should be submitted as Supplemental Data.

Gene and protein nomenclature. Authors must adhere to the guidelines of the relevant species nomenclature committees.

Page numbering. Number pages at the top right.

Page setup. Choose letter-size, 8-1/2" x 11" paper. Set all margins at one inch.

Section order. Arrange research papers in the following order (for Reviews, the Introduction through Discussion sections do not apply):

- Title page
- Abstract
- Introduction
- Materials and Methods

- Results
- Discussion
- Acknowledgment (if applicable)
- References
- Figure Legends
- Supplemental Data Legends (if applicable)
- Tables (one per page)
- Figures (one per page)

Manuscript Formatting

Failure to comply with these requirements may lead to processing delays. See recent BIOLRE papers for examples of manuscript format. Please contact the Biology of Reproduction Editorial Office with any questions: biolre.editorialoffice@oup.com.

Cover Letter

All submissions must have a cover letter. The cover letter should be submitted as a separate file, saved as "Cover Letter" and submitted through Editorial Manager as a supplemental file.

The cover letter should contain: The title of the paper and a brief statement of its main point and significance.

Information about any potential conflicts of interest, including professional or financial affiliations that might be perceived as biasing the presentation.

A statement that written permission has been obtained from any author whose work is cited as a personal communication, unpublished work, or work in press, but is not an author of the manuscript. Upon acceptance, a copy of this permission will be requested.

A statement that written permission has been obtained from all publishers, individuals, or institutions that hold copyright or exclusive license for any work (figure, table, textual extract) included in the submission, whether it is the same or modified. Upon acceptance, a copy of this permission will be requested.

If Supplemental Data are uploaded, indicate whether they are to be included in the final publication or are for the reviewers only.

Videos can be published in the online article, with a still image of the video appearing in the print version. Please submit videos in MP4 format. Any supplementary videos that you do not want to be included in the article itself can be uploaded as supplementary data. All

videos should have an accompanying legend.

References can be formatted in any readable style at submission, although authors are responsible for their accuracy.

Acknowledgements and details of funding sources should be included at the end of the text. Please refer to your funding organizations to acknowledge their support. PubMed Central links will require a specific grant number to be referenced.

Please list all author contributions upon submission of the manuscript.

Please also define non-standard abbreviations at the first occurrence and number figures and tables consecutively.

Upon revision papers should be submitted in an editable file format (i.e. not PDF) and figures should be submitted as separate, high-resolution, files.

For information on Latex files, please see: http://www.oxfordjournals.org/en/authors/latex- files.html.

Title page

Title. Indicate the species studied, using italics as needed. Do not use abbreviations or acronyms. Spell out Greek characters.

Running title. A title of 50 or fewer characters, including spaces. This will appear as the running head of your published paper.

Summary sentence. A one-sentence summary of the manuscript's significance (limited to 250 characters). Do not use phrases such as "this paper demonstrates…" or "we show that…" Examples of appropriate summary statements are:

The protein greatstuff (GTSF), which is produced by the developing oocyte, is essential for preimplantation embryo development beyond the two-cell stage.

Photoperiod regulation of amino acid transport in the brain influences spawning behavior of guppies. Rat Sertoli cells promote high expression of Clutz mRNA and CLUTZ protein by spermatocytes; the phenotype of a null mutation in the Clutz gene is azoospermia.

Keywords. Title page keywords will appear on the first page of the final publication. There is no limit on the number of keywords that may be provided.

Acceptable Keywords

Acrosome	Adrenal	Aging
Acrosome reaction	Adrenal cortex	Androgens/Androgen
Activin	Adrenal medulla	receptor

Aneuploidy
Angiogenesis
Anterior pituitary
Anti-Mullerian hormone
Apoptosis
Assisted reproductive technology
Atresia
Behavior
Blastocyst
Breast cancer
Calcium
Capacitation
Carnivore reproduction
Catecholamines
Cell culture
Cell cycle
Central nervous system
Cervix
Chemotaxis
Chromatin
Circadian rhythm
Cloning
Comparative reproduction
Conceptus
Conservation
Contraception
Corpus luteum
Corticosterone
Corticotropin-releasing hormone (CRH/CRH receptor)
Cortisol
Cryobiology
Cryopreservation
Cumulus cells
Cyclic adenosine monophosphate (cAMP)
Cyclic guanosine monophosphate (cGMP)
Cytokines
Cytoskeleton
Decidua
Developmental biology
Developmental origins of health and disease
Diapause
Differentiation
DNA methylation
Domestic animal reproduction
Dopamine
Early development
Embryo
Embryo culture
Embryonic stem cells
Endocrine disruptors
Endometriosis
Endometrium
Environment
Environmental contaminants and toxicants
Epididymis
Epigenetics
Equids (horses, donkeys, zebras)
Estradiol/Estradiol receptor
Estrous cycle
Evolution
Extracellular matrix
Fallopian tubes
Female infertility
Female reproductive tract
Fertility
Fertilization
Fetal development
Fish reproduction
Follicle
Follicle-stimulating hormone (FSH/FSH receptor)
Follicular development
Follicular maturation
Follistatin
Gamete biology
Gametogenesis
Gene expression
Gene regulation
Genetics
Genomic imprinting
Genomics
Glucocorticoids/Glucocorticoid receptor
Gonadal function
Gonadal steroids
Gonadotropin-releasing hormone (GnRH/GnRH receptor)
Gonadotropins
Granulosa cells

Growth factors
Growth hormone/Growth hormone-releasing hormone
Histone
Histone modifications
Hormone
Hormone action
Hormone receptors
Humanchorionic gonadotropin (hCG/hCG receptor)
Human reproduction
Hypothalamic hormones
Hypothalamus
Hypoxia
Immunology
Implantation
Imprinted genes
In vitro fertilization (IVF)
In vitro maturation (IVM)
Induced pluripotent stem cells (IPC cells)
Inhibin
Inner cell mass
Insulin
Insulin-like growth factor (IGF/IGF receptors)
Interstitial cells
Intracytoplasmic sperm injection (ICSI)
Intrauterine growth restriction (IUGR)
Invertebrates
Ion channels Integrins
Kinases
Kisspeptin
Labor
Lactation
Leptin/Leptin receptor
Leydig cells
Luteinizing hormone (LH/LH receptor)
Litter size
Luteolysis
Macrophage
Male infertility
Male reproductive tract
Male sexual function
Mammary glands
Mechanisms of hormone action
Meiosis
Meiotic arrest
Meiotic maturation
Meiotic spindle
Melatonin Menstrual cycle
Menopause
Metabolism
MicroRNA
Mitosis
Mitochondria
Molecular biology
Morula
Müllerian ducts
Myoid cells
Myometrium
Neuroendocrinology
Neuropeptides
Neurotransmitters
Nitric oxide
Noncoding RNA
Nuclear transfer
Null mutation/knockout
Nutrition
Oocyte
Oocyte development
Oocyte-follicle interactions
Oocyte maturation
Ovary
Ovarian cancer
Oviduct
Ovine/sheep
Ovulation
Ovulatory cycle
Ovum
Ovum pick-up/transport
Oxidative stress
Oxytocin
Parturition
Penis
Pheromones
Phosphatases
Phosphodiestrases
Photoperiod
Pineal
Pituitary/Pituitary hormones
piRNA
Placenta

Placental transport
Placentation
Polypeptide receptors
Porcine/pig
Posterior pituitary
Preeclampsia
Pregnancy
Preimplantation embryo
Primates
Premature ovarian failure
Primordial germ cells
Prostaglandins
Protein kinases
Proteomics
Progesterone/Progesteron e receptor
Prolactin/Prolactin receptor
Prostate
Puberty
Relaxin
Reproductive behavior
Reproductive immunology
Reprogramming
Retinoids
Rodents (rats, mice, guinea pigs,voles)
Ruminants (cows, sheep, llama, camel)
Seasonal reproduction
Semen
Seminal plasma
Seminal vesicles
Serotonin
Sertoli cells
Sex determination
Sex differentiation
Signal transduction
Somatic cell nuclear transfer
Somatostatin
Sperm
Sperm capacitation
Sperm DNA fragmentation
Sperm hyperactivation
Sperm maturation
Sperm motility and transport
Spermatid
Spermatocyte
Spermatogenesis
Spermatogonia
Spermatogonial stem cells
Stem cells
Steroid hormones/Steroid hormone receptors
Stress Stroma
Syncytiotrophoblast
Telomeres
Teratogen
Teratology
Testicular cancer
Testis
Testosterone
Theca cells
Thyroid-stimulating hormone (TSH/TSH receptor)
Toxicology
Transcription
Transcriptional regulation
Transgenesis
Transgenic/Knockout model
Translation
Transplantation
Trophoectoderm
Trophoblast
Uterine cancer
Uterus
Vagina
Vaginal epithelium
Vas deferens
Vasopressin
Vertebrates, non-mammalian (fish, fowl, reptiles, amphibians)
Vitrification
Vitelline membrane
Wolffian duct
X chromosome
Y chromosome
Zebrafish
Zona pellucida
Zoo species (exotic species)
Zygote

Authors and affiliations. List all authors and provide the full name (including departments and/or divisions) and location (i.e., city, state, country) of each institution where work was performed. Do not use abbreviations or acronyms, and do not provide street addresses. Use superscript Arabic numerals to key the authors to the institutions.

Grant support. Indicate financial support (i.e., funding agency names and grant or contract numbers, if applicable) in footnote 1. Do not include funding information in the Acknowledgment section.

Conference presentation (if applicable). If any research in the manuscript was presented elsewhere, indicate that here (e.g., "Presented in part at the 45th Annual Meeting of the Society for the Study of Reproduction, 12-15 August 2012, State College, Pennsylvania.").

Correspondence. The corresponding author should be indicated in footnote 2; the footnote should give the complete contact information, including street address, for this person.

Additional footnotes. If two or more authors contributed equally to the work, or if an author's contact address has changed since the research was performed, this information should be provided in separate footnotes.

Abstract. In a maximum of 250 words, summarize the purpose of the work, the methods used, and the conclusions. Do not present data or cite references. Avoid abbreviations and acronyms, and spell out Greek characters.

Introduction. Provide a clear statement of the problem and cite the relevant literature on the subject. Do not include results or summary statements.

Materials and Methods

Ethics. It must be stated and documented that investigations using experimental animals or subjects were conducted in accordance with the SSR's specific guidelines and standards.

Materials

Brand names. Use generic names of chemicals, drugs, antibodies, reagents, enzymes, etc., when possible. Brand names should be used if the composition of that brand is critical to the methodology. If a brand name is given, the name of the manufacturer must also be provided. For example:

Dynabeads mRNA DIRECT kit (Invitrogen)

one-way ANOVA (PRISM software version 3.03; GraphPad) FSHR (1:1000 dilution; product no. ab65975; Abcam, Inc.)

Composition. Specify the composition of all solutions, buffers, mixtures, and culture media (including PBS) if a brand name and manufacturer are not provided.

Donated materials. Provide institutional affiliations of individuals or companies that donated supplies or reagents.

Trademark symbols. Do not use trademark or registered symbols with brand or company names.

Methods

Concisely provide readers with sufficient information to replicate the work. Unpublished work may not be cited to provide validation of methodology. Include statistical methods used for data analysis. Use references to published methods if they are identical to methods used in the current study.

Results

Present findings in appropriate detail, using the past tense. Refer to tables and figures in order, without discussion.

Nucleotide sequences should be submitted to GenBank, EMBL, or DNA Data Bank of Japan, and the accession number and date of accession noted in the text. Authors are encouraged to provide a link to the deposit rather than providing the complete sequence in the text.

Genomic and proteomic data should be deposited with the NCBI gene expression and hybridization array data repository (GEO). The GEO accession number and sequence deposit information should be referenced in footnote 1 after any funding information.

Discussion

Provide a clear and concise interpretation of the results; avoid repeating the results.

Acknowledgment

Acknowledge any non-financial assistance (e.g., statistical review, technical help, editorial assistance, animal husbandry, etc.).

References

Acceptable works. Only published articles or articles accepted for publication may be used. Articles must have appeared in peer-reviewed publications or other published works that are accessible to most scientists. Articles that have been "conditionally accepted," "submitted," or are "in process" are not acceptable. If a paper has been accepted but has not been published in final form (i.e., full citation information is not yet available), please indicate that the paper is "in press."

Abstracts. An abstract may be used as a reference only if it has been published in a regular issue of a readily available and indexed journal.

Internet/online references. All online material must be cited completely and must include the web address and date of access by the authors. Many online sources provide a suggested citation.

Unpublished data. Cite personal communications and unpublished data only if necessary. In the text, provide the name(s) of the individual(s) associated with the unpublished data. For example, "(Smith and Winter, unpublished data).

Accuracy. Authors are responsible for the accuracy of all references.

Order. All references should be cited in numerical order in the text using square brackets (e.g., "as Smith [12] reported" or "as previously reported [3-5]") and should appear in that order in the References section. If a reference citation appears only in a table or figure, number that reference last. For example, if there are 50 references in the text, four in a table, and two in a figure, the in-text references would be numbered 1-50, the references in the table would be numbered 51-54, and the references in the figure would be numbered 55-56.

TIP: Search for "[" in your Word or PDF document to see a list of your in-text references. Use this list to ensure that your references are in numerical order.

Presentation. List up to 12 authors and/or editors of a publication. If there are more than 12 authors, list the only first 12 followed by "et al." Abbreviate journal names according to Serial Sources for the BIOSIS Database, Index Medicus, or PubMed's Journal Browser.Page numbers must be inclusive (e.g., 722–729, not 722–29).

NOTE: EndNotes users may download a .ENS file in BIOLRE style from the EndNotes web site (type "Biology of Reproduction" in the Publication Name field).

Abstract in Biology of Reproduction. Vo TTB, Jeung EB. Calbindin-D9k expression in GH3 cells is a biomarker of xenoestrogenic potential of parabens. In: Abstracts of the 43rd Annual Meeting of the Society for the Study of Reproduction, July 31-August 3, 2010, Milwaukee, Wisconsin. Biol Reprod 2010; 83(suppl): Abstract 275.

Kwon DK, Koo OJ, Park SJ, Kang JT, Park HJ, Kim SJ, Moon JH, Saadeldin IM, Jang G, Lee BC. Optimizing porcine oocytes electrical activation by adjusting pre- and post-activation mannitol exposure time. In: Supplement to Biology of Reproduction for the Forty-Fourth Annual Meeting of the Society for the Study of Reproduction, July 31-August 4, 2011, Portland, Oregon. Biol Reprod 2011; Suppl: Abstract 176.

Book. Sokol RR, Rohlf FJ. Biometry. New York: WH Freeman and Co; 1981:253-261.

Book chapter. Harrison RJ, Weir BJ. Structure of the mammalian ovary. In: Zuckerman S, Weit BJ (eds.), The Ovary, vol. 1, 2nd ed. New York: Academic Press; 1977:113-217.

Database. Mouse Tumor Biology Database (MTB), Mouse Genome Informatics. Bar Harbor, ME: The Jackson Laboratory; 2004. http://www.informatics.jax.org. Accessed 11 October 2012.

Internet source. Mammalian Reproductive Genetics [Internet]. Seattle, WA: University of Washington. http://mrg.genetics.washington.edu. Accessed 12 January 2012.

Journal article, 12 or fewer authors. Demas GE, Nelson RJ. Photoperiod, ambient temperature, and food availability interact to affect reproductive and immune function in adult male deer mice (Peromyscus maniculatus). J Biol Rhythms 1998; 13:253–262.

Journal article, more than 12 authors. Okasaki Y, Okazaki Y, Furuno M, Kasukawa T, Adachi J, Bono H, Kondo S, Nikaido I, Osato N, Saito R, Suzuki H, Yamanaka I, et al. Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. Nature 2002; 420:563-573.

Journal article, published ahead of print. Aitken-Palmer C, Hou R, Burrell C, Zhang Z, Wang C, Spindler R, Wildt DE, Ottinger MA, Howard J. Protracted reproductive seasonality in the male giant panda (Ailuropoda melanoleuca) reflected by patterns in androgen profiles, ejaculate characteristics, and selected behaviors. Biol Reprod 2012; (in press). Published online ahead of print 4 April 2012; DOI 10.1095/biolreprod.112.099044.

Journal article, e-journal. Yuen T, Wurmbach E, Pfeffer RL, Ebersole BJ, Sealfon SC. Accuracy and calibration of commercial oligonucleotide and custom cDNA microarrays. Nucleic Acids Res 2002; 30:e48.

Patent. Smith C, Jones K (inventors). The United States of America as represented by the Department of Health and Human Services, assignee. Adreno-medullin peptides. U.S. patent 6 320 022; 2001.

Thesis or dissertation. Wilson K. The effects of substance P, neurotensin and arginine vasopressin on reproductive function. London, UK: University of London; 1984. Thesis.

Terminology

Abbreviations. Do not use abbreviations in titles. Do spell out all abbreviations at first mention in the Abstract and in the body of the manuscript.

Beginning sentences. Do not begin sentences with very short abbreviations or acronyms, especially those that begin with a lower case letter. For example, hCG and cDNA should instead be "Human CG" and "Complementary DNA," respectively, when beginning a sentence.

Definitions. Do define all abbreviations and acronyms at first mention in the Abstract and in the body of the manuscript. Definitions are not needed for names of genes, gene products, proteins, and protein products.

Eponyms. Do not use the possessive form for an eponym. For example, do use Dulbecco Modified Eagle medium, not Dulbecco's Modified Eagle's medium; Hanks solution, not Hanks' solution; Student t-test, not Student's t-test; etc.

Joint Commission on Biochemical Nomenclature. Do not use the nomenclature for polypeptide hormones proposed by the Joint Commission on Biochemical Nomenclature. Do use follicle-stimulating hormone, not follitropin; luteinizing hormone, not lutropin; etc.

Latin terms. Do not italicize Latin terms such as et al., in situ, in vitro, or in vivo. Do use italics for gene symbols and genus-species designations.

Nomenclature. For all genes, the gene names should be spelled out in the first mention, and they should be abbreviated in acronym form in all following mentions. If there are any previous gene names, these names should be included in parentheses following the first mention of the gene. For example, "Granulosa cells produce vascular endothelial growth factor A (VEGFA; previously known as _____)."

The basic nomenclature guidelines for Biology of Reproduction are as follows:

a) Mouse and Rat

Websites for nomenclature rules:

- Mouse and rat: http://www.informatics.jax.org
- Rat only: http://rgd.mcw.edu

General nomenclature rules (mouse and rat):

- Full gene names are in roman font (not italic); e.g., insulin-like growth factor 1.

- Greek symbols are not used.

  Gene, mRNA, and cDNA symbols:

- Italic font, with only the first letter upper case; e.g., Igf1.
- Greek symbols are not used. Hyphens are rarely used.

  Protein symbols:

- Use the same symbol as the gene.
- Roman font (not italic), with all letters upper case; e.g., IGF1.

  Mutant alleles:

- Define when first mentioned; e.g., "Igf1 tm1Arge/Igf1 tm1Arge is one of several knockout alleles of Igf1."
- Italic font is used for all letters and numbers, with the allelic designation (e.g. tm1Arge) in superscript.
- After first mention, the homozygous KO can be indicated as Igf1-/-; the heterozygote can be indicated as Igf1+/-, etc.

b) Humans, nonhuman primates, chickens, domestic species, and everything that is not a mouse, rat, fish, worm, frog, or fly

Website for nomenclature rules:

- http://www.genenames.org

  General nomenclature rules:

- Full gene names are in roman font (not italic); e.g., insulin-like growth factor 1.
- Greek symbols are not used.

  Gene, MRNA, and cDNA symbols:

- Italic font, with all letters upper case; e.g., IGF1.
- Greek symbols are never used.
- Hyphens are used only in very specific cases (please refer to the nomenclature guidelines).

  Protein symbols:

- Use the same symbol as the gene.
- Roman font (not italic), with all letters upper case; e.g., IGF1.

c) Fish (applies to all fish)

Website for nomenclature rules:

- https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines

  General nomenclature rules:

- Full gene names are in italic font, with all letters lower case; e.g., cyclops.
- Greek symbols are not used.

Gene symbols:

- Italic font, with all letters lower case; e.g., cyc.
- Greek symbols are not used.

Protein symbols:

- Use the same symbol as the gene.
- Roman font (not italic), with only the first letter upper case; e.g., Cyc.

Units of measure. Standard abbreviations for units of measure and abbreviations understood by scientists outside the field of reproductive biology may be used without definition: ml, g, IU, UV, i.v., EC, cpm, dpm, P, etc.

Figures

All illustrations that are not tables (e.g., gels, blots, charts, graphs, photographs, micrographs) are considered figures. View the submission site's Help and FAQ pages for solutions to common upload problems.

Digital Image Preparation

The following is summarized from Biology of Reproduction's Guidelines for Digital Images.

General Guidelines

- Images should be minimally processed. The final image must correctly represent the original data and conform to current standards for ethical scientific imaging.
- Original, unaltered images must be provided to the Editors if requested. If the original data cannot be produced, manuscript acceptance may be revoked.
- Cases of deliberate misrepresentation of data will result in revocation of acceptance and will be reported to the corresponding author's home institution or funding agency (see Due Process).
- Please also refer to the University of Arizona's guidelines for digital imaging ethics.

Microscopy

Adjustments. Acceptable (only if uniformly applied to the whole image and equally applied to representative controls):

- Brightness
- Contrast
- Color balance

Unacceptable (unless justified to reviewers and disclosed in the figure legend):

- Threshold manipulation
- Expansion or contraction of signal ranges
- Altering of high signals
- "Pseudo-coloring"
- Nonlinear adjustments, such as gamma
- Adjustments of individual color channels (if necessary on "merged" images, must be noted in figure legend)
- Antibody use. A useful discussion is presented by Saper CB, J Comp Neurol 2005; 493:477- 478.

Immunohistochemistry and immunofluorescence. Data should include:

- Appropriate, representative controls (either in the figures or in Supplemental Data)
- Information on the full characterization of antibodies used

Materials and Methods. Information in this section should include:

Equipment:

- Make and model of microscope
- Objective lens information (e.g., numerical aperture, filter sets, wavelength cutoff, bandwidth data)
- Camera systems
- Image processing software
- Resolution at which images were acquired
- Software used for image processing; include details about operations such as: o Type of deconvolution
- 3D reconstructions
- Surface or volume rendering
- Gamma adjustments

Gels and blots

Combined gels. Vertically sliced gels that juxtapose lanes that were not contiguous in

the experiment must have a clear separation or a black line delineating the boundary between the gels.

Controls and size markers. Positive and negative controls and molecular size markers should be included on each gel and/or blot in the main figure or in an expanded supplementary figure.

Cropped gels and blots. Cropping is permissible if it improves the clarity and conciseness of the\ presentation; however, cropped gels must retain important bands, and cropped blots should retain at least six band widths above and below the band. In such cases, the cropping must be mentioned in the figure legend, and Supplemental Data for review should include full-length gels and blots wherever possible.

High-contrast gels and blots. These are discouraged, as overexposure may mask additional bands. Authors should strive for exposures with gray backgrounds. Multiple exposures should be presented in Supplemental Data if high contrast is unavoidable.

Immunoblots. If the background is faint, use a black line to indicate blot borders. For quantitative comparisons, use appropriate reagents, controls, and imaging methods with linear signal ranges.

File Types

Preferred formats:

- EPS: Encapsulated PostScript.
- AI: The native file format of Adobe Illustrator.
- PSD: The native file format of Adobe Photoshop.
- TIFF: These files must be saved using "LZW compression" to avoid a loss of image integrity during the submission conversion process.
- PDF: The native file format of Adobe Acrobat. All PDF images should be saved at the highest-quality setting.

Other formats:

- JPG: Due to the lower quality of JPG files, if this format must be used, all images should be saved at the highest-quality setting.
- DOC or PPT: Although it is often easier to label graphs and photos within Microsoft Word or PowerPoint, these file types were not created with print processes in mind, and the final print quality of such files may be compromised. If possible, it is recommended that all Word and PowerPoint files be saved as high-quality PDFs prior

to submission.

- SigmaPlot, Deltagraph, Canvas, etc.: Graphing or drawing program files should be saved as EPS or PDF files.

Image Resolution

Upon acceptance, editorial staff will check the quality of all figures and may request new files for online and print production. Edited figures will be compared against those approved by the reviewers and, if substantively different, will need be sent to an editor for approval.

The minimum resolution specifications for digital figure files at final print size are:

- 1200 dpi: Line image (black and white only; e.g., a chart).
- 600 dpi: Combination image (grayscale or color image that contains text; e.g., a photograph or blot with letter labels, arrows, or text added outside the image area).
- 300 dpi: Grayscale or color image (grayscale or color image that does not contain text; e.g., a photograph or blot with no labels, arrows, or other text added outside the image area).

Consistency. Figures should have a consistent appearance throughout the paper.

Keys. A key should be used to explain symbols and patterns on graphs.

Borders. Do not use borders in or around figures.

Lines. Pay particular attention to the quality of the lines, symbols, and patterns. Avoid using patterns in bars; use open and solid bars wherever possible. Do not use 3-dimensional graphs to show 2-dimensional data.

Numbering. Number figures consecutively with Arabic numerals (e.g., 1, 2, 3) in the order in which they are discussed in the text. This number should be included on each figure at least 0.5 cm (0.25") above or below the figure itself.

Legends

Abbreviations. Define all abbreviations that appear on the figure that have not been defined in the text.

Label consistency. Any numerical or alphabetical labels used in the figure should appear similarly in the legend.

Methods. Do not describe methods or results in figure legends.

Placement. Present figure legends in numerical order and in their own section (see

manuscript section order).

Scale/magnification. Indicate the scale used for all micrographs, if not specified in the figure itself (e.g., "Bar = 1 μm" or "Original magnification x200").

Symbols. Special symbols should not appear in the legend. Any special symbols appearing in a figure should be defined in a key in the figure and described in the legend.

Tables

Use tables only for data that are best understood in a column-and-row format.

Antibody Table

Biology of Reproduction requires authors using antibodies for immunohistochemistry, immunocytochemistry, western blots, immunoblots, immunoneutralization, or related methodology, to submit an Antibody table. This supplementary table should be numbered to indicate its position in the sequence of tables in the article (e.g. Supplementary Table 1). In the Materials and Methods section, describe appropriate positive or negative controls, antibody validation, lot number, and provide references. Authors should also determine whether the antibody has a Research Resource Identifier (RRID) by consulting the Antibody Registry and include this information, if available, in the Methods section and/or the Antibody table of the original submission. If there is not an RRID, authors are required to register the antibody and obtain one no later than the revision stage of submission. For more information, see the Resource Identification Portal.

Cell Line Authentication

Cell lines maintained in vitro represent valuable tools in biological studies. However, many cell lines are misidentified or cross-contaminated (1). Studies using misidentified cell lines may affect the reliability and accuracy of results, and thus, could have important clinically relevant implications. Given the importance of this problem, Biology of Reproduction editorial policy will require that all cell lines used and described in submitted manuscripts be authenticated. Authentication can occur using several possible techniques that are not mutually exclusive. The use of short tandem repeat profiling (STR) is an internationally recognized method of genetic profiling of cell lines (1, 2). An important advantage of STR profiling is that the data can be utilized to search major repositories to compare and confirm the maintenance of original cell line characteristics. STR profiling does not, however, give information regarding the tissue of origin. Phenotypic markers, such as the use of thyroglobulin in differentiated thyroid cancer cell lines, may help characterize the source of the thyroid cell lines. Authors should submit the date (month, year) when the

authenticity was last confirmed.

This editorial policy will concur with the ATCC® Standards Development Organization and ATCC® SDO workgroup suggestion to perform STR in the following circumstances: when a cell line is received from an outside source (repository, other investigator), for newly established cultures, If many different cell lines are employed within a given laboratory.

The identity of cell lines used in studies to be submitted for publication in the Endocrine Society journals should be confirmed and that confirmation indicated as part of the manuscript submission process. Alternative or supplemental authentication can be performed by DNA genetic analysis and/or fingerprinting, copy number variant or molecular karyotype/chromosomal analysis.

Footnote designations. Footnotes should be denoted with superscript letters or symbols, be consistent within the table, and be keyed to data in the table. Numerals may not be used as footnote designators.

Footnote length. Footnotes should be brief, descriptive statements that apply only to the data or formatting in that table. Do not duplicate text from the main body of the paper.
Graphics. Tables should be formatted as simply as possible and should be composed entirely of text characters. Large or complex tables or tables that include graphic elements should be submitted as figures. Please contact the BOR Editorial Office if it is unclear whether material should be a table or a figure.

Heads. Every column in a table should be labeled, including the first on the left.

Placement and numbering. Place each table on a separate page and number tables consecutively with Arabic numerals (i.e., 1, 2, 3) according to their order of citation in the text. Place these numbers above each table, in front of the table's title.

Rounding. Round numbers within tables to the nearest whole number or significant digit. Numbers smaller than "1" should include a zero to the left of the decimal mark.

Size. Do not create a table with only one or two rows or columns; instead, present this data in the text.

Title. The title should be one concise sentence and should appear before each table.

Digital Image Integrity

When preparing digital images, authors must adhere to the following guidelines as stated in The CSE's White Paper on Promoting Integrity in Scientific Journal Publications:

No specific feature within an image may be enhanced, obscured, moved, removed, or

introduced.

Adjustments of brightness, contrast, or color balance are acceptable if they are applied to the entire image and as long as they do not obscure, eliminate, or misrepresent any information present in the original.

The grouping of images from different parts of the same gel, or from different gels, fields, or exposures must be made explicit by the arrangement of the figure (e.g., dividing lines) and in the figure legend.

Deviations from these guidelines will be considered as potential ethical violations.

Note that this is an evolving issue, but these basic principles apply regardless of changes in the technical environment. Authors should be aware that they must provide original images when requested to do so by the Editor-in-Chief who may wish to clarify an uncertainty or concern.

[Please see paper of Rossner and Yamada (Journal of Cell Biology, 2004, 166:11–15), which was consulted in developing these policy issues, for additional discussion and a white paper on Promoting Integrity in Scientific Journal Publications, published by The Council of Science Editors, 2006.]

References

1. Korch C, Spillman MA, Jackson TA, Jacobsen BM, Murphy SK, Lessey BA, Jordan VC, Bradford AP. DNA profiling analysis of endometrial and ovarian cancer cell lines reveals misidentification, redundancy and contamination. Gynecol Oncol 127:241-8.

2. Parson W, Kirchebner R, Muhlmann R, et al. 2005 Cancer cell line identification by short tandem repeat profiling: power and limitations. FASEB J19:434–6.

Supplementary Material

Submit all material to be considered as supplementary material online at the same time as the main manuscript. Ensure that the supplementary material is referred to in the main manuscript at an appropriate point in the text. Supplementary material will be available online only and will not be copyedited, so ensure that it is clearly and succinctly presented, and that the style conforms to the rest of the paper. Also ensure that the presentation will work on any Internet browser. It is not recommended for the files to be more than 2 MB each, although exceptions can be made at the editorial office's discretion.

File formats. File size must be reduced wherever possible to ensure that all users, regardless of internet speed or computer capabilities, may access the data.

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- File size limit: 5 MB.
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Tables and other text.

- File size limit: 5 MB.
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- File size limit: 10 MB.
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- Authors must include a "still" photograph from the video in a figure in the paper.

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Legends. Provide supplemental figure legends in a Supplemental Data Legends section in the manuscript (see section order). Editorial staff will publish the legends with the supplemental data online.

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- Violation of international, federal, or state rules
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- Animals bred specifically for laboratory study are to be used whenever practical.
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- Research animals shall be properly housed and fed, and their surroundings shall be kept in sanitary condition in accordance with the National Research Council's Guide for Care and Use of Laboratory Animals (a.k.a., the NIH Guide), or the Consortium Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching.
- Research animals shall receive appropriate anesthetics, analgesics, tranquilizers, and care to minimize pain and discomfort during procedures. The choice and use of the most appropriate drug shall be made in strict accordance with the NIH Guide, and all procedures shall be those of accepted veterinary medical practice.
- If the study or the condition of the animal requires that the animal be killed, then a humane method shall be employed.
- Use of animals shall be under the direct supervision of an experienced teacher or investigator.
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