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Estratégias para reduzir o envelhecimento ovariano em modelos animais

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“Everything you want's a dream away
Under this pressure, under this weight
We are diamonds taking shape.”
Adventure of a Lifetime, Coldplay, 2015

Resumo

SACCON, Tatiana Dandolini. **Estratégias para reduzir o envelhecimento ovariano em modelos animais.** 2020. 110f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

O envelhecimento ovariano pode ser definido como um esgotamento da reserva folicular ovariana associada a uma diminuição progressiva da fertilidade conforme as fêmeas envelhecem. A reserva ovariana é constituída, principalmente do conjunto de folículos primordiais que permanecem quiescentes até serem recrutados. O eixo somatotrópico, constituído pelo hormônio do crescimento (GH) e fator de crescimento semelhante a insulina (IGF-I), é essencial para o crescimento somático e determinação do tamanho corporal adulto e desempenham funções reguladoras na reprodução. Além disso, o eixo GH/IGF-I aumenta a sensibilidade de ovários para gonadotropinas e, assim, aumenta a estimulação e o desenvolvimento folicular. Quebras de fitas duplas de DNA são um dos principais tipos de danos de DNA que ocorre em células. Oócitos de mamíferos permanecem presos na prófase I da meiose por um período prolongado, até várias décadas em algumas espécies, incluindo os seres humanos, o qual favorece o acúmulo de danos de DNA, e com isso reduzindo a qualidade oocitária. Em adição ao dano de DNA, inflamação também aumenta com a idade e pode afetar negativamente a fertilidade. Por estes motivos, o objetivo do estudo foi avaliar reserva ovariana e qualidade de oócitos em camundongos *Ames dwarf* (df/df) deficientes em GH, camundongos bGH que superexpressam GH e macacos Rhesus submetidos a restrição calórica (RC) na vida adulta. Ovários de camundongos df/df de 6 meses e 18 meses, bGH e macacos Rhesus submetidos a RC foram utilizados, sendo que df/df de 18 meses foram submetidos ao tratamento de GH exógeno. Número de folículos foi avaliado em camundongos df/df de 6 meses. Dano de DNA (anti- γH2AX e contagem de macrófagos foi realizada por imunofluorescência. Número de células da granulosa de folículos primordiais e primários foram estimados. Camundongos df/df com 6 meses apresentaram uma maior reserva ovariana, indicado pela maior quantidade de folículos primordiais. Camundongos df/df de 6 meses e 18 meses, e de macacos submetidos a RC apresentaram oócitos e células da granulosa mais saudáveis em folículos primordiais e primários, indicado pela menor quantidade de quebras duplas no DNA (DSBs). Apresentaram um menor número de macrófagos no ovário de camundongos df/df, sendo que, o tratamento com GH exógeno em df/df de 18 meses foi capaz de aumentar as DSBs e a infiltração de macrófagos. Por outro lado, camundongos bGH apresentaram uma maior quantidade de DSBs em oócitos e células da granulosa de folículos primordiais e primários, além de uma maior infiltração de macrófagos. Em conclusão, podemos observar que o eixo GH/IGF-I está associado ao envelhecimento ovariano, demostrado pela presença de danos de DNA e infiltração de macrófagos. Além disso, a restrição calórica em macacos Rhesus, previne danos no DNA de oócitos e de células da granulosa em folículos primordiais e primários, demostrando o benefício da restrição calórica na qualidade oocitária durante o envelhecimento.

Palavras-chave: Envelhecimento ovariano, hormônio do crescimento, dano de DNA, inflamação.

Abstract

SACCON, Tatiana Dandolini. **Strategies to reduce ovarian aging in animal models.** 2020. 110f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Ovarian aging can be defined as a depletion of the ovarian follicular reserve associated with a decline in fertility. The ovarian reserve consists mainly of the number of primordial follicles that remain quiescent until they are recruited. Hormones of the somatotropic axis, growth hormone (GH) and insulin-like growth factor I (IGF-I), are essential for somatic growth and determination of adult body size and play regulatory functions in reproduction. In addition, the GH/IGF-I axis increases the sensitivity of ovaries to gonadotropins and thus increases stimulation and follicular development. DNA double strand breaks (DSBs) are one of the main types of DNA damage that occurs in cells. Mammalian oocytes remain trapped in meiosis prophase I for a long period, up to several decades in some species, including humans, which can increase the accumulation of DNA damage, thereby reducing oocyte quality. In addition to DNA damage, inflammation also increases with age and can negatively affect fertility. For these reasons, the objective of the study was to evaluate ovarian reserve and oocyte quality in GH deficient Ames dwarf (df/df) mice, bGH mice that overexpress GH and Rhesus monkeys subjected to caloric restriction (CR) in adulthood. Ovaries from df/df mice of 6-months and 18-months-old, bGH and Rhesus monkeys submitted to CR were used, and df/df of 18-months-olf were submitted to the treatment of exogenous GH. Follicles counting was evaluated in 6-months-old. DNA damage (anti- γH2AX) and macrophage counting (anti-CD68) were evaluated by immunofluorescence. Number of granulosa cells of primordial and primary follicles were estimated. 6-month-old df/df mice had a larger ovarian reserve, indicated by the increased number of primordial follicles. 6-month and 18-month df/df mice and monkeys submitted to CR showed healthier oocytes and granulosa cells in primordial and primary follicles, indicated by the lower number of DSBs, in addition, a reduced number of macrophages in the ovary of df/df mice, and treatment with exogenous GH in df/df of 18 months was able to increase DSBs and macrophage infiltration. Inversely, bGH mice had higher quantity of DSBs in both oocytes and granulosa cells of primordial and primary follicles, had ovarian tissue with more macrophage infiltration compared to normal mice. In conclusion, the present study indicates that the GH/IGF-I axis is associated with ovarian aging, demonstrated by the presence of DNA damage and macrophage infiltration. In addition, caloric restriction in Rhesus monkeys prevents damage to the DNA of oocytes and granulosa cells in primordial and primary follicles, demonstrating the benefit of caloric restriction in oocyte quality during aging.

Keywords: Ovarian reserve, growth hormone, DNA damage, inflammation.

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1 INTRODUÇÃO GERAL

O envelhecimento ovariano está associado ao esgotamento da reserva folicular ovariana, que é definida durante a vida fetal em mulheres (te Velde et al., 1998) e logo após o nascimento em camundongos (Peters, 1969). A reserva ovariana constituída principalmente de folículos primordiais contendo o oócito e uma camada de células da granulosa (Li et al., 2015, Peters, 1969). Os folículos são recrutados da reserva a cada ciclo, desenvolvem-se e podem sofrer ovulação ou tornarem-se atrésicos, processo que é irreversível (Baker, 1963). O recrutamento e a ativação dos folículos primordiais levam a depleção da reserva ovariana, culminando com o esgotamento dessa reserva e início da menopausa nas mulheres (Liu and Lebrun, 2006).

O eixo hormônio do crescimento (GH) / fator de crescimento semelhante à insulina (IGF-I) é crucial para determinar a longevidade em camundongos. Camundongos Ames Dwarf (df/df) carreiam uma mutação no gene profeta da pituitária 1 (Prop-1) que impede o desenvolvimento da glândula pituitária anterior, resultando em deficiência na secreção de GH (Sornson et al., 1996). Estes animais tem níveis circulantes muito baixos de fator de crescimento semelhante à insulina tipo 1 (IGF-I) e vivem em torno de 35-75% mais do que animais normais (Ahmed and Farquharson, 2010, Zhou et al., 2015, Brown-Borg et al., 1996). Em contrapartida, animais transgênicos que superexpressam GH (bGH), possuem níveis plasmáticos elevados de GH, o que resulta no aumento de IGF-I no plasma sanguíneo, e vivem cerca de 50% menos que animais normais (Bartke et al., 1988, Bartke, 2003). As mesmas características fenotípicas de camundongos df/df podem ser observadas em camundongos submetidos a restrição calórica (RC) (Nikolai et al., 2015). A RC pode ser definida como uma redução da ingestão calórica abaixo do *ad libitum* (à vontade) (Genaro Pde et al., 2009, Li et al., 2015), geralmente entre 20 e 40% de restrição, sem comprometer a ingestão de nutrientes essenciais (Canto and Auwerx, 2009). Assim, tanto redução do eixo GH/IGF-I como de calorias resulta em aumento da expectativa de vida.

Hormônios do eixo somatotrópico desempenham funções reguladoras na reprodução. O eixo GH / IGF-I aumenta a sensibilidade de ovários para gonadotropinas e, assim, aumenta a estimulação e o desenvolvimento folicular (Mahran et al., 2015). Já é bem estabelecido em estudos de biologia reprodutiva que o eixo GH/IGF-I é importante para a função normal do ovário (Chandrashekar et al., 2004). Um declínio progressivo e esgotamento da reserva de folículos ovarianos é o principal determinante da idade ao início da menopausa (Fortune et al., 2013). Além disso, concomitante com o número reduzido de folículos, a qualidade dos oócitos restantes nas fêmeas geralmente diminui com o avançar da idade (Richardson et al., 2014). Neste sentido, o eixo funcional GH/IGF-I é importante para a normal função do ovário (Mahran et al., 2015). O tempo de vida reprodutiva dos camundongos df/df é prolongada, indicado pela presença de atividade ovariana em uma idade avançada, aos 12 meses (Schneider et al., 2017) e aos 18 meses (Saccon et al., 2017). Esta condição parece ocorrer devido à redução da progressão de folículos do estágio primordial para o estágio primário em camundongos df/df (Schneider et al., 2014). Já camundongos bGH tem puberdade adiantada e a taxa de ovulação aumentada (Naar et al., 1991), e possui reserva ovariana diminuída, comparada a normais controle (Saccon et al., 2017). Da mesma maneira, estudos indicam que a RC auxilia na preservação da reserva ovariana, mostram que roedores submetidos a RC apresentam uma maior reserva ovariana além de apresentarem atividade ovariana em idade avançada (Xiang et al., 2012, Shi et al., 2013, Garcia et al., 2019a).

A quantidade decrescente de folículos é acompanhada da diminuição da qualidade do oócito (Broekmans et al., 2009) devido ao acúmulo de danos, em parte pelo aumento do estresse oxidativo, à medida que a mulher envelhece e está associada com maior frequência de abortos e o aumento de anormalidades cromossômicas (te Velde et al., 1998). Este longo período de dormência ao qual oócitos são submetidos durante a vida aumenta as chances de acumulo de danos no DNA, como as quebras de fita dupla (DSBs, do inglês, *double strand breaks*) (Titus et al., 2013). O efeito dos danos causados no DNA em folículos primordiais tem um grande impacto para a qualidade e capacidade de fertilidade oocitária (Adriaens et al., 2009, Kirk and Lyon, 1982). Estudo mostra que oócitos de folículos primordiais e

primários de primatas mais velhos perdem a capacidade de combater radicais livres, apresentando menores quantidades de enzimas antioxidantes, como a glutationa peroxidase e redutase (Wang et al., 2020), podendo ser um fator de aumento de danos de DNA. Os dados indicam que o reparo de DSBs seja um dos mecanismos de envelhecimento de oócitos em mamíferos (Titus et al., 2013).

Vários relatos apoiam a ideia de que macrófagos agem diretamente nos folículos saudáveis e atréticos, bem como nos corpos lúteos, participando da apoptose e angiogênese (Wu et al., 2004, Turner et al., 2011). No entanto, poucos estudos têm sugerido que uma maior infiltração de macrófagos no ovário pode estar envolvida na maturação de folículos em mamíferos (Nteeba et al., 2013, Baravalle et al., 2015). Considerando que a glicoproteína CD68 modula a atividade fagocitária dos macrófagos no ovário, é possível sugerir que o aumento no número de macrófagos no ovário poderia estar relacionado a uma maior ativação folicular e danos plausíveis dos folículos ovarianos (Nio-Kobayashi et al., 2015), visto o papel de um estado pró-inflamatório da depleção da reserva ovariana (Bromfield and Sheldon, 2013, Uri-Belapolsky et al., 2014).

2 REVISAO BIBLIOGRAFICA

2.1 Modelos animais com distúrbios no eixo somatotrópico

O eixo somatotrópico, constituído pelo hormônio do crescimento (GH) e fator de crescimento semelhante a insulina (IGF-I), é essencial para o crescimento somático e determinação do tamanho corporal adulto. O hormônio do crescimento (GH) é um hormônio anabólico com efeitos pleiotrópicos sobre o crescimento, a diferenciação e o metabolismo das células. O GH é liberado pela hipófise sob controle hipotalâmico e embora os receptores de GH sejam encontrados em todo o corpo, a maioria dos efeitos de GH são mediadas pelo fator de crescimento semelhante à insulina tipo 1 (IGF-I), um hormônio produzido, principalmente no fígado, mas também em vários outros órgãos que são alvos do GH (Nicholls and Holt, 2016).

A ausência de ação de GH, quer através da falta de sua secreção ou pela ausência de receptores para ele, resulta em um fenótipo anão com longevidade prolongada em roedores. Uma linhagem de camundongos muito estudada e que são conhecidos por apresentar alterações no eixo somatotrópico é o Ames dwarf (*df/df*)(Bartke and Brown-Borg, 2004). Camundongos *df/df* foram inicialmente descritos na literatura por Schaible e Gowen em 1961 (Schaible and Gowen, 1961). Sornson e colaboradores em 1996 mostraram que o fenótipo destes animais é devido a uma mutação recessiva espontânea no gene profeta da pituitária 1 (Prop-1) (Sornson et al., 1996). Camundongos homozigotos com esta mutação desenvolvem anormalidades em sua glândula pituitária, que carece de três tipos de células: somatotrópicas, lactotróficas e tireotróficas (Bartke et al., 2001). Consequentemente, estes animais df/df possuem níveis plasmáticos indetectáveis destes hormônio que seriam produzidos por essas células, isto é, GH, prolactina e hormônio estimulador da tireóide (TSH), respectivamente (Zhou et al., 2015). Esses camundongos apresentam características fenotípicas consistentes com a deficiência primária de GH incluindo redução do crescimento pós natal e diminuição do tamanho corporal adulto (Bartke et al., 2001). Além disso, possuem níveis plasmáticos reduzidos de prolactina, GH, TSH, IGF-I, insulina, glicose, diminuição do dano oxidativo (em proteínas, lipídios e DNA mitocondrial), diminuição da produção de espécies reativas de oxigênio (ROS),

melhora na sensibilidade à insulina e nas defesas antioxidantes (Brown-Borg et al., 2001), secreção aumentada de adiponectina (Al-Regaiey et al., 2005), aumento da resistência ao estresse, diminuição da temperatura corporal (Hunter et al., 1999), esterilidade devido à falha luteal (Bartke, 2006) e puberdade tardia. A característica mais marcante desses camundongos, por possuírem esta deficiência de secreção do GH, IGF-I e insulina muito baixos, e que vivem em torno de 35-75% mais do que animais normais (Brown-Borg et al., 1996).

Similarmente aos camundongos *df/df*, os camundongos *knockout* para o gene do receptor do GH (GHRKO) e camundongos normais submetidos a uma restrição calórica (RC) moderada, também tem concentrações séricas de IGF-1 reduzidas e apresentam uma extensão do tempo de vida similar (Coschigano et al., 2003, Masoro, 2005). Em contraste, camundongos transgênicos superexpressando GH são caracterizados por uma redução significativa do tempo de vida comparado com controles normais (Ge et al., 2013). A terapia de reposição com GH em camundongos *df/df* quando jovens por um período de 6 semanas diminui significativamente o tempo de vida, similares ao tempo de vida de camundongos normais (Panici et al., 2010). Estas evidências indicam que o eixo GH/IGF-1 e seus mediadores intracelulares tem um papel essencial no processo de envelhecimento e longevidade (Barbieri et al., 2003). Dessa forma, muitos estudos têm buscado elucidar as vias metabólicas ativas nestes animais e que são essenciais para o controle do envelhecimento.

2.2 Restrição calórica

A RC pode ser definida como uma redução da ingestão calórica (Genaro Pde et al., 2009, Li et al., 2011), geralmente entre 20 e 40% a menos quando comparado a ingestão *ad libitum*, sem comprometer a ingestão de nutrientes essenciais (Canto and Auwerx, 2009). Desde 1935, quando Clive McCay et al. (McCay et al., 1989) observaram que a RC com teor adequado de proteínas, vitaminas e minerais em ratos aumentou a longevidade dos mesmos em comparação com ratos alimentados *ad libitum*, muitos estudos têm demonstrado o efeito benéfico da RC na longevidade de diferentes espécies como leveduras, *Caenorhabditis elegans* (verme nematoide) e *Drosophila melanogaster* (mosca da fruta) e mamíferos como ratos, camundongos

(Kenyon, 2010) e macacos rhesus (Colman et al., 2014). A RC tem sido praticada como um método para aumentar a longevidade em até 50% sem comprometer a qualidade de vida (Bartke, 2006). Efeitos benéficos em macacos Rhesus sugerem que a RC também tem efeitos similares em primatas (Colman and Anderson, 2011, Kemnitz, 2011), o que aproximaria estes resultados na espécie humana. Na Figura 1 podemos observar, resumidamente, os alvos sugeridos cuja restrição calórica pode atuar no organismo já documentados em estudos anteriores.

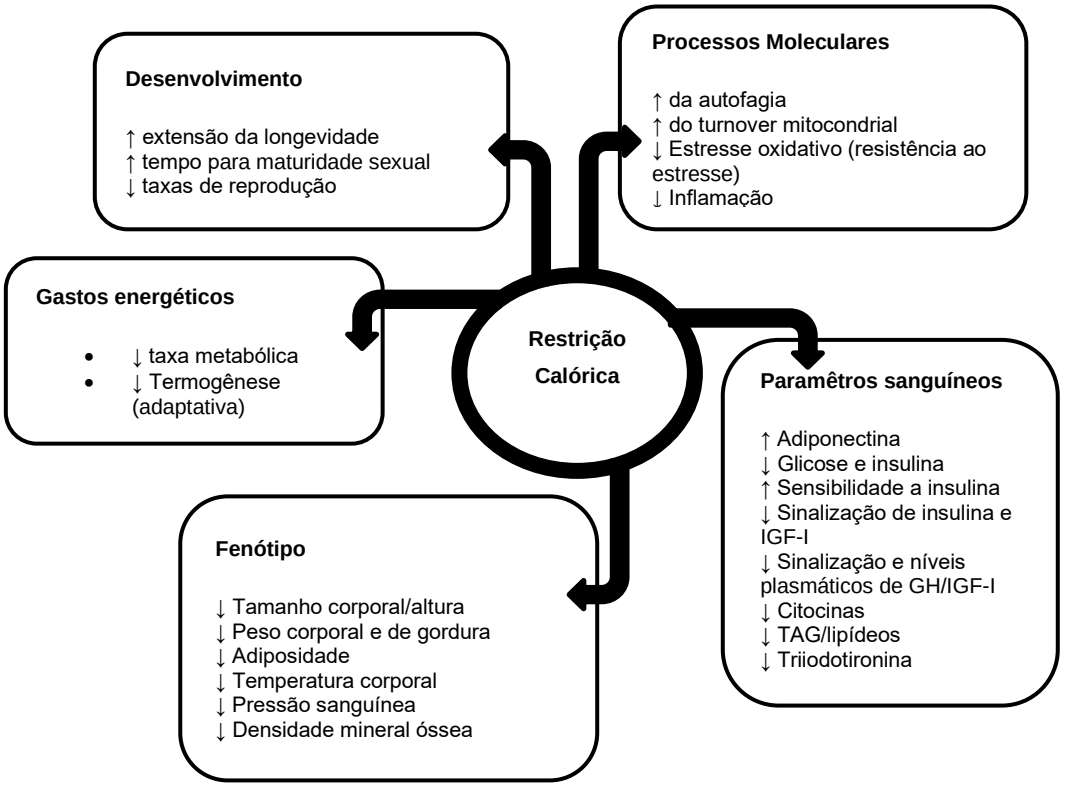

Figura 1. Visão geral esquemática dos alvos sugeridos cuja restrição calórica pode atuar no organismo já documentados em estudos anteriores. Alterada de Nikolai et al., 2015 (Nikolai et al., 2015). IGF-I (fator de crescimento semelhante à insulina 1), GH (hormônio do crescimento) e TAG (triacilglicerideos).

2.3 Envelhecimento ovariano e ativação folicular

O termo “reserva ovariana” refere-se ao potencial funcional do ovário e reflete o número e qualidade dos oócitos (Richardson et al., 2014). A reserva ovariana é constituída por folículos primordiais, que são folículos em estado de dormência, juntamente com folículos já recrutados, que estão nas fases de desenvolvimento pré-antrais (primários e secundários) e antrais (terciários) (Zou et al., 2009). A reserva de folículos ovarianos primordiais é estabelecida durante o desenvolvimento embrionário na maioria dos mamíferos, inclusive nos seres humanos (te Velde et al., 1998), ou logo após o nascimento, que é o caso dos roedores (Peters, 1969). Esta reserva finita de folículos primordiais constitui a totalidade de oócitos que está disponível para uma fêmea durante sua vida reprodutiva. Portanto, o tempo de vida reprodutiva de uma fêmea depende do tamanho inicial da reserva de folículos ovarianos primordiais e a taxa de esgotamento presente no ovário (Fortune et al., 2013). Com a diminuição gradual do número de folículos com a idade, uma sequência de eventos reprodutivos ocorre, começando com baixa fecundidade e esterilidade natural e progredindo através de irregularidades no ciclo menstrual até uma cessação completa da menstruação na menopausa (Richardson et al., 2014). Sabe-se que, em relação a mulheres em pré-menopausa, mulheres no periodo pós-menopausa tem risco aumentado de desenvolver diabetes tipo II, doença cardiovascular e diversos tipos de câncer (Broekmans et al., 2009).

Os oócitos dos folículos primordiais estão dormentes na prófase meiótica I (Gleicher et al., 2011) e a sua ativação leva ao seu crescimento culminando na ovulação ou atresia em algum momento do ciclo de crescimento. Essa ativação é irreversível, ou seja, uma vez ativado o folículo não retorna mais a reserva (Baker, 1963). Um folículo primordial quando ativado, se torna um folículo primário, que é aquele que contém um oócito rodeado por uma única camada de células da granulosa cuboides. Um folículo é determinado como sendo um folículo secundário se for rodeado por mais do que uma camada de células da granulosa cuboides, sem antro visível. Um folículo é determinado como sendo um folículo antral ou terciário se possuir um espaço antral claramente definido e preenchido por liquido folicular e uma camada

de células da granulosa de cumulus em torno do oócito (Li et al., 2015). A Figura 2 ilustra as características de cada folículo.

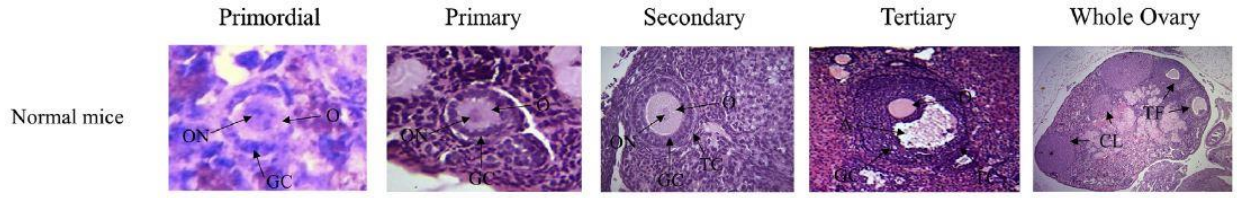

Figura 2. Representação histológica de folículos ovarianos. Imagens histológicas detalhadas de cada estágio do folículo (primordial, primário, secundário e terciário) e seções ovarianas inteiras para camundongos normais. O - Oócito, ON - Núcleos de oócitos, GC - Células da granulosa, TC - Células da teca, A - Antro, CL – Corpo lúteo, TF - Folículo terciário. Fonte:(Schneider et al., 2017).

É importante salientar que a ativação de folículos primordiais é um processo estritamente regulado coordenado por vários fatores parácrinos e endócrinos(Fortune, 2002). Hormônios do eixo somatotrópico desempenham funções reguladoras na reprodução. Além disso, o eixo GH / IGF-I aumenta a sensibilidade de ovários para gonadotropinas e, assim, aumenta a estimulação e o desenvolvimento folicular (Mahran et al., 2015). Já é bem estabelecido em estudos de biologia reprodutiva que o eixo GH/IGF-1 é importante para a função normal do ovário (Chandrashekar et al., 2004). Camundongos fêmeas *df/df* apresentam ciclos estrais normais e conseguem ovular (Bartke et al., 2001), e a gestação normal pode ser estabelecida através do tratamento com prolactina após a ovulação (Bartke, 1966). Camundongos GHRKO, apresentam atividade ovariana em uma idade avançada, quando camundongos normais já esgotaram as reservas foliculares do ovário (Sluczanowska-Glabowska et al., 2012). Esta condição parece ocorrer devido à redução da progressão de folículos do estágio primordial para o estágio primário em camundongos GHRKO (Slot et al., 2006), que também é observada em camundongos sob restrição de calorias (Li et al.,

2011, Garcia et al., 2019b). Estudos de nosso grupo indicam que os camundongos *df/df* também apresentam reserva ovariana diminuída (Saccon et al., 2017, Schneider et al., 2017). No entanto, mais estudos são necessários para confirmar esta hipótese e o papel do GH neste cenário. O prolongamento da vida reprodutiva é interessante e pode ter muitas implicações na saúde feminina, pois o atraso no início da menopausa pode conferir às mulheres um tempo fértil mais prolongado, que se tornou uma demanda social dadas as mudanças impostas pela vida moderna e a necessidade de ajuste do planejamento familiar. Além disso, mulheres pós-menopausa aumentam drasticamente o risco de desenvolver diabetes tipo II, doença cardiovascular e diversos tipos de câncer (Broekmans et al., 2009).

A via de sinalização da fosfotidilinositol 3 quinase (Pi3k)/proteína quinase B (Akt1) é importante na regulação da proliferação e sobrevivência celular e tem sido implicada na ativação de folículos primordiais (Brown et al., 2010, Reddy et al., 2008). É sabido que a ativação da via Pi3k/Akt1 e do fator de transcrição forkhead box O3a (FOXO3a) no oócito é um regulador chave do processo de ativação de folículos primordiais (Castrillon et al., 2003). A hiperativação do Akt1 resulta em hiperfosforilação do FOXO3a culminando com a ativação global de folículos primordiais e falha ovariana prematura (Kalich-Philosoph et al., 2013). A via do *mammalian target of rapamycin* (mTOR) também tem uma relação direta com a via do Pi3k/Akt1 (Caron et al., 2010), sendo que sua ativação acelera o recrutamento de folículos primordiais em sinergismo com a via Pi3k/Akt1 (Adhikari et al., 2010). A FOXO3a tem sido referida como um potencial determinante do início da menopausa, portanto um potencial alvo para intervenção com agonistas visando reduzir o declínio da fertilidade em fêmeas com idade avançada (Pelosi et al., 2013).

A via substrato de insulina-Pi3k-Akt1 é estimulada tanto pela insulina quanto pelo IGF-1 e sua ativação reduzida parece estar envolvida com a maior longevidade nos modelos de camundongos anões ou submetidos à restrição calórica (Bartke, 2008). Portanto, é possível que o aumento da vida reprodutiva nestes camundongos também esteja relacionado com a ativação desta via intracelular. Resultados de nosso grupo indicam que camundongos df/df aos 12 meses tem menor fosforilação da FOXO3a nos folículos primordiais, comparado a camundongos N/df , o que pode estar

associado ao prolongamento da janela fértil neste modelo (Schneider et al., 2014). Além disso, camundongos df/df 18 meses apresentaram maiores niveis da proteína FOXO3a não fosforilada em oócitos de folículos primordiais, sendo que o tratamento com GH diminuiu os níveis desta proteína enquanto aumentava os níveis da forma fosforilada (Saccon et al., 2017). Além de nossos achados, FOXO3a tem papel central como fator de transcrição que media vários processos fisiológicos e patológicos, incluindo genes que estão envolvidos na apoptose (Uri-Belapolsky et al., 2014), proliferação celular (Bromfield and Sheldon, 2013), progressão do ciclo celular (Broekmans et al., 2009), sobrevivência (Reddy et al., 2008) e dano de DNA (Fluteau et al., 2015). FOXO3a também responde a vários estímulos como radiação UV e estresse oxidativo (Wang et al., 2012). Além disso, FOXO3a tem sido fortemente associado com a longevidade humana (Willcox et al., 2008). Portanto, as evidências anteriores nos mostram que a via de ativação de FOXO3a dependente de GH/IGF-I pode ser um dos principais mecanismos envolvidos na longevidade reprodutiva em camundongos deficientes em GH.

Outros resultados de nossos estudos também indicam que o eixo GH/IGF-1 e suas vias de sinalização tem um papel importante na vida reprodutiva feminina. Camundongos *df/df* apresentam uma reserva ovariana maior aos 12 meses caracterizada pela redução da ativação de folículos primordiais e não transição ao estágio secundário (Schneider et al., 2017). Os animais df/df com 18 meses de idade apresentaram uma quantidade de folículos primordiais maior do que animais normais de mesma idade, mostrando que esses folículos estão retidos nessa fase e que a ausência de GH está envolvida nesta ativação, já que o tratamento com GH exógeno por seis semanas foi capaz de reverter este processo em animais df/df. Similarmente observamos que a reserva de folículos primordiais é muito maior em camundongos GHRKO (Isola et al., 2020). Em contrapartida observamos que animais transgênicos que superexpressam GH, aos 12 meses de idade, possuem uma quantidade de folículos primordiais menor que os normais de mesma idade, mostrando que o aumento de GH leva a uma maior ativação da reserva ovariana (Saccon et al., 2017), reafirmando o papel do eixo GH/IGF-1 na vida reprodutiva.

Em camundongos submetidos à RC o número de folículos primordiais maior em relação ao grupo controle, e estes camundongos permaneceram férteis durante um período de tempo muito mais longo comparado aos controle (Gosden, 1985). Resultados encontrados por Li et al., em 2011, mostraram que ratos do grupo RC apresentaram maior porcentagem de folículos primordiais e menores percentuais de folículos primários, secundários e atrésicos, indicando uma diminuição da transição, ativação dos folículos primordiais para os estágios posteriores. Assim sendo, fatores que inibem a transição de um folículo primordial para um folículo primário e reduzem a atresia do folículo podem aumentar a reserva de oócitos e retardar a depleção do folículo, atrasando o envelhecimento ovariano (Li et al., 2011). Xiang et al. (2012), relacionou os efeitos da RC na preservação ovariana observando que camundongos submetidos à RC apresentaram 47% mais folículos primordiais que camundongos submetidos a uma dieta controle (Xiang et al., 2012). Resultados de nosso grupo também confirmam estas observações de que RC de 30% por um período de 3 meses é capaz inibir a ativação de folículos primordiais (Garcia et al., 2019a). Resumindo, os mecanismos pela qual a RC atua ainda não estão totalmente elucidados, porém sabe-se que a RC tem efeito benéfico sobre o envelhecimento ovariano, assim como na longevidade aumentada. A RC diminui a transição dos folículos primordiais para folículos primários, diminuindo a depleção ovariana e prolongando a vida reprodutiva em camundongos fêmeas e ratas.

A Figura 3, desenvolvida pelo nosso grupo de pesquisa, ilustra uma possível via de sinalização envolvida na preservação dos folículos primordiais em camundongos Ames Dwarf e submetidas à RC.

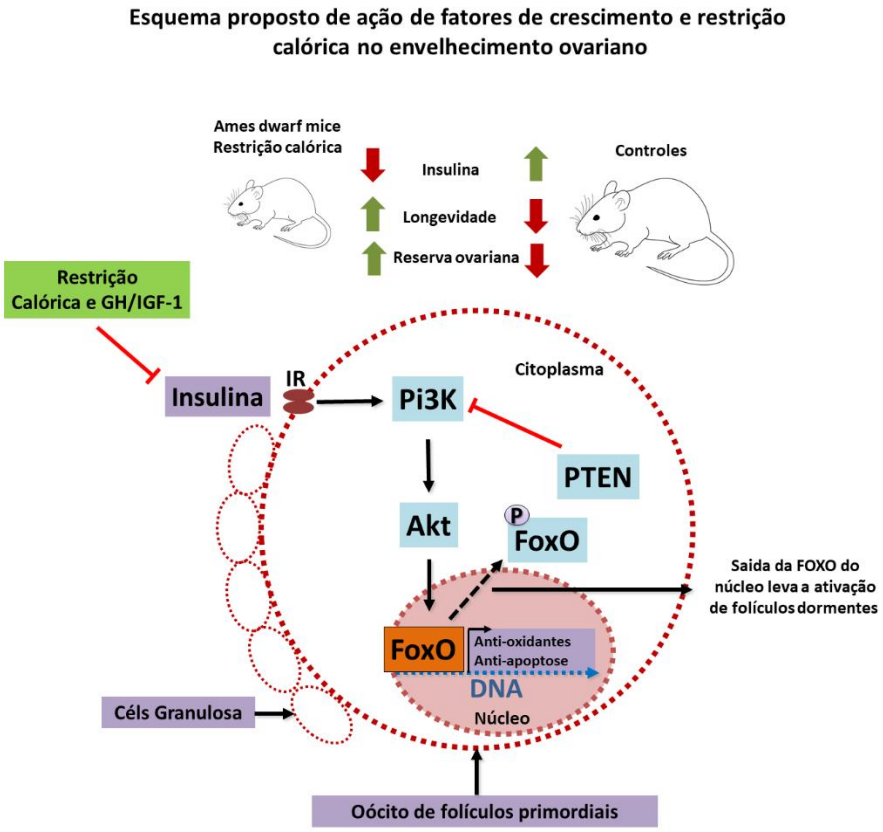

Figura 3. Via de sinalização proposta na preservação dos folículos primordiais em estado dormente em oócitos de camundongos Ames Dwarf e submetidos à restrição calórica. A ligação da insulina ao seu receptor no óocito leva à ativação de fosfoinositideo-3-quinase (PI3k), que ativa proteína B quinase (Akt) que leva a fosforilação do fator de transcrição forkhead box (FOXO), mais especificamente a FOXO3a fator de transcrição presente no núcleo do óocito, uma vez fosforilado FOXO3a sai do núcleo para o citoplasma da célula, o que leva a ativação de folículos dormentes. Em camundongos normais essa via seria a responsável pela depleção ovariana que ocorre durante a vida reprodutiva das fêmeas, por conta do aumento nos níveis de insulina, porém em camundongos submetidos Ames Dwarf e submetidos à restrição calórica (RC), que possuem uma diminuição dos níveis de insulina supõem-se que esta via esteja reduzida ocasionando um atraso da ativação de folículos primordiais, mantendo assim a reserva ovariana e atrasando o envelhecimento ovariano. O hormônio do crescimento (GH) e fator de crescimento semelhante a insulina 1 (IGF-I) também pode se ligar ao receptor da insulina desencadeando a ativação da via.

2.4 Danos de DNA em folículos ovarianos e inflamação

Vários fatores podem influenciar a qualidade do oocito durante o envelhecimento. Os efeitos adversos de danos de DNA causados pelos fatores ambientais em células somáticas tem sido extensivamente reportadas (Saini and Gordenin, 2018). Oócitos de mamíferos permanecem presos na prófase I (na fase do diplóteno) da meiose por um período prolongado, até várias décadas em algumas espécies, incluindo os seres humanos (Mehlmann, 2005, Chiang et al., 2012). Esta longa dormência fornece a oportunidade para o acúmulo de danos de DNA. Durante um longo período de tempo, é provável que os oócitos são influenciados por vários tipos de fatores que causam quebras de cadeia dupla de DNA (DSB - *double-strand breaks*), o que levaria a fertilidade reduzida (Zhang et al., 2014b). Uma resposta eficaz a danos no DNA é crucial para todas as células, incluindo oócitos (Sancar et al., 2004). Os danos extensivos que ocorrem ao longo da meiose podem ter consequências graves se uma resposta adequada não é tomada e pode resultar em infertilidade ou desenvolvimento de embriões com defeito incapazes de levar a gestação a termo (Kirk and Lyon, 1982, Adriaens et al., 2009). Em resposta aos DBSs, quinases são conhecidas por fosforilar a histona 2Ax (H2Ax) na serina 139 (Bakkenist and Kastan, 2003, Burma et al., 2001). Tal modificação pós-transcricional no local da lesão, proporciona uma plataforma para Ataxia-telangiectasia mutada (ATM) que através da via que sinaliza danos de DNA regula a reparação de DSBs no DNA (Collins and Jones, 2016). Os oócitos são, portanto, propensos a danos, pois a vida de uma única célula é muito longa e podem ocorrer danos significativos, o que pode explicar o declínio da fertilidade à medida que os animais envelhecem. Uma célula pode responder de muitas formas aos danos no DNA, incluindo a indução de uma parada no ciclo celular ou a iniciação de apoptose se o dano é grave (Roos and Kaina, 2006, Roos and Kaina, 2013, Sancar et al., 2004). Na verdade, é sabido que oócitos primordiais com DNA danificado sofrem apoptose facilmente (Suh et al., 2006, Livera et al., 2008, Kerr et al., 2012). A maioria dos folículos submetidos a atresia ao longo da vida pós-natal, são submetidos principalmente por meio de apoptose (Kim and Tilly, 2004). Tem sido demonstrado que a fragmentação de DNA, que é uma característica de células que sofrem apoptose, ocorre em folículos atrésicos (Tilly, 1996), juntamente

com a produção de DSB, que desencadeia a via apoptótica mitocondrial (Roos and Kaina, 2006).

Células germinativas de mamíferos desenvolveram um mecanismo complexo para identificar danos no DNA e ativam uma resposta necessária para manter a integridade do genoma. Estes mecanismos incluem a detecção de danos no DNA, reparo, parada do ciclo celular e apoptose, os quais operam juntos para proteger a concepção de danos de DNA contra danos no DNA originados nos gametas ou nas células somáticas do embrião (Jaroudi and SenGupta, 2007). Danos graves que ocorrem na meiose podem ter sérias consequências se uma resposta celular adequada não for ativada, podendo resultar em infertilidade ou desenvolvimento de embriões defeituosos, que podem evoluir para falha na formação de blastocisto, fragmentação nuclear em corpos apoptóticos e falhas na formação do fuso mitótico, os quais podem resultar na incapacidade de desenvolvimento de uma gravidez a termo (Adriaens et al., 2009, Kirk and Lyon, 1982). Portanto, sugere-se que a manutenção de altos níveis de fatores de proteção ao DNA possa ser um elemento-chave para preservar a reserva ovariana por um período mais longo.

Vários relatos apoiam a ideia de que macrófagos agem diretamente nos folículos saudáveis e atréticos, bem como nos corpos lúteos, participando da apoptose e angiogênese (Wu et al., 2004, Turner et al., 2011). No entanto, poucos estudos têm sugerido que uma maior infiltração de macrófagos no ovário pode estar envolvida na maturação de folículos em mamíferos (Nteeba et al., 2013, Baravalle et al., 2015). Considerando que a glicoproteína CD68 modula a atividade fagocitária dos macrófagos no ovário, é possível sugerir que o aumento no número de macrófagos no ovário poderia estar relacionado a danos plausíveis dos folículos ovarianos (Nio-Kobayashi et al., 2015). Alguns estudos recentes mostram que uma dieta hipercalorica está associada com maior quantidade de macrófagos no ovários e estes podem estar mediando o aumento da inflamação e aumento do dano aos folículos com consequente redução da fertilidade (Rodriguez-Castelan et al., 2017). Camundongos deficientes de interleucina 1 possuem uma maior reserva ovariana indicado pelo maior numero de folículos primordiais, alem de possuirem uma fertilidade aumentada, comparado a fêmeas controle (Uri-Belapolsky et al., 2014). Um de nossos estudos

anteriores identificou por sequenciamento de RNA que camundongos df/df velhos possuem mais de 150 processos relacionados com a inflamação e resposta imune regulados quando comparados com camundongo N/df de mesma idade (Schneider et al., 2017), apontando para o papel da inflamação na senescência reprodutiva. Interessantemente, o transplante de ovários jovens em camundongos mais velhos, está associado com uma redução de inflamação e aumento da longevidade (Habermehl et al., 2019). Além disso, sabe-se que camundongos df/df possuem menores níveis de citocinas pro-inflamatórias, interleucina-6 e fator de necrose tumoral (TNF-α) no sangue e em tecido adiposo (Berryman et al., 2004, Wang et al., 2006). Acredita-se que esta redução de inflamação seja uma das principais razoes pela qual camundongos df/df tenham um aumento na longevidade (Masternak and Bartke, 2012). Neste contexto, a presença de células inflamatórias no ovário talvez seja um importante parâmetro, os macrófagos são as células inflamatórias mais abundantes no ovário (Turner et al., 2011). Uma dieta rica em gordura resulta em um aumento da depleção de folículos primordiais e no aumento da infiltração de macrófagos em camundongos (Skaznik-Wikiel et al., 2016). A inflamação resulta no estresse oxidativo, levando a produção elevada de radicais livres que reagem com os ácidos graxos e proteínas da membrana, diminuindo sua função (Durackova, 2010). Além disso, o excesso de radicais livres leva a uma maior produção de citocinas pro-inflamatórias e aumenta os danos de DNA, um fator predisponente para várias doenças relacionadas com o envelhecimento (Khansari et al., 2009).

O microambiente folicular também pode ser influenciado pela deficiência de GH/IGF-I e restrição calórica. Células da granulosa de folículos pre-antrais, como primordiais e primários, são intimamente conectadas com oócitos e ambos desempenham um papel importante no crescimento e desenvolvimento folicular (Eppig et al., 1997, Su et al., 2009). O crescimento oocitario é acompanhado pelo acúmulo de RNA mensageiros, proteínas e lipídios, e por modificações na configuração da cromatina e metilação no DNA, processos os quais requerem energia (Munakata et al., 2016). A proliferação e multiplicação das células da granulosa em folículos primordiais está associado com a ativação e desenvolvimento folicular (Munakata et al., 2016). Um conjunto de mediadores moleculares que interagem entre

células da granulosa e oócito, controlando seu desenvolvimento, foi descrito. Um modelo proposto por Zhang et al. (2014) tem 2 principais passos. O primeiro passo está relacionado com a sinalização da *mTOR complexo 1 (*mTORC1), o qual é sinalizado nas células da granulosa que age como uma importante chave no processo de ativação folicular (Zhang et al., 2014a). O segundo passo envolve o canal de comunicação entre as células da granulosa e oócitos pela via da KITL-KIT, a qual desencadeia a ativação do oócito através da fosforilação da FOXO3a (Zhang et al., 2014a). É proposto que esses processos mantem a ativação progressiva em um número limitado de folículos primordiais durante a vida reprodutiva. Então, é possível que a ativação folicular seja iniciada por moléculas e mudanças celulares nas células da granulosa, gradualmente seguido da ativação dos oócitos quiescentes.

Além disso, o crescimento oocitario está associado com um aumento da atividade da expressão gênica (Moore and Lintern-Moore, 1974), indicando que o oócito está acordando todos os processos necessários para o crescimento e diferenciação. Observamos em um estudo anterior que oócitos de folículos primordiais e primários de camundongos bGH tinham um aumento no diâmetro nuclear e oocitario (Saccon et al., 2017), sugerindo que o tamanho está associado com a ativação folicular. Portanto, todos estes dados podem nos indicar que a deficiência de GH está associado com a diminuição da proliferação das celulas da granulosa, a qual pode reduzir a ativação folicular.

3 OBJETIVOS

3.1 Objetivo Geral

Estudar estratégias para retardar o envelhecimento ovariano, avaliando o efeito de hormônios e dietas sobre o tamanho da reserva de folículos primordiais, presença de dano no DNA e presença de macrófagos em camundongos e em macacos Rhesus.

3.2 Objetivos Específicos

- Caracterizar o número de folículos primordiais, primários, secundários e terciários em camundongos N/df e df/df com 6 meses para comparação com os dados já obtidos de animais com 12 e 22 meses;

- Caracterizar o dano de DNA em oócitos e em células da granulosa de camundongos N/df e *df/df* tratados com GH ou solução salina aos 18 meses e em camundongos N/df e df/df aos 6 meses pela medida na forforilacao de γH2AX;

-Realizar avaliação de tamanho oócitário e contagem de células da granulosa de folículos primordiais e primários de camundongos N/df e *df/df* tratados com GH ou solução salina aos 18 meses e em camundongos N/df e df/df aos 6 meses;

- Avaliar a infiltração de macrófagos em camundongos N/df e *df/df* tratados com GH ou solução salina aos 18 meses e em camundongos N/df e df/df aos 6 meses;

- Caracterizar o número de folículos primordiais, primários, secundários e terciários em macacos Rhesus submetidos à restrição calórica;

- Caracterizar o dano de DNA em oócitos e em células da granulosa de macacos Rhesus submetidos à restrição calórica;

- Realizar avaliação de tamanho nuclear, oócitário e folicular e contagem de células da granulosa de folículos primordiais e primários em macacos Rhesus submetidos à restrição calórica.

4 CAPÍTULO 1

Manuscrito 1 – Primordial follicle reserve, DNA damage and macrophage infiltration in the ovaries of the long-living Ames dwarf mice (Conforme normas do periódico Experimental Gerontology).

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Primordial follicle reserve, DNA damage and macrophage infiltration in the ovaries of the long-living Ames dwarf mice

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Abstract

The aim of this study was to evaluate the effect of growth hormone (GH) deficiency in primordial follicle reserve, DNA damage and macrophage infiltration in the ovaries of young mice. Ovaries from six-month-old GH-deficient Ames Dwarf (df/df) and Normal (N/df) mice were used. The number of primordial follicles was higher in df/df mice ($p = 0.0026$). Also, df/df mice had a lower number of primary ($p = 0.023$), secondary ($p = 0.0052$) and tertiary ($p = 0.019$) follicles. These findings indicate a slower rate of primordial follicle activation in df/df mice. Female df/df mice had decreased γH2AX foci intensity in oocytes of primordial ($p = 0.015$) and primary ($p = 0.0004$) follicles compared to N/df mice. Also, df/df mice had reduced γH2AX intensity in granulosa cells of primordial ($p = 0.0002$) and primary ($p < 0.0001$) follicles. Overall, this indicate to us that df/df mice accumulate less DNA damage in the ovarian reserve compared to N/df mice. Additionally, macrophage infiltration was also reduced in ovaries of df/df mice compared to N/df mice ($p = 0.033$). Interestingly, df/df mice had a reduced number of granulosa cells around primordial ($p = 0.0024$) and primary ($p = 0.007$) follicles compared to N/df mice. Also, df/df mice had a small diameter of primordial follicle nuclei ($p = 0.0093$), secondary follicle oocyte ($p = 0.046$) and tertiary follicle ($p = 0.012$). This points to the role of granulosa cell proliferation and oocyte growth for primordial follicle activation. The current study points to the role of the GH/IGF-1 axis in extending lifespan of reproductive health, along with maintenance of oocyte DNA integrity and reduced ovarian inflammation.

Keywords: Growth hormone; Insulin-like growth factor-1; ovarian reserve; DNA damage; macrophages.

1. Introduction

A progressive decline and consequent depletion of the ovarian primordial follicle reserve is the main determinant of the onset of menopause in women (Faddy et al. 1992). Along with the reduction in follicle number, the quality of the remaining oocytes also decreases with age (Faddy et al. 1992; Hirshfield 1994). It is well known that the age-related decline in women's

fertility is paralleled by a decrease in the ovarian reserve and an increase in chromosomally abnormal conceptions (McGowan and McCoy 2013). The underlying cause of the increased risk to pregnancy outcome with age is commonly attributed to the exponential rise in oocyte chromosome mis-segregations, leading to karyotypic imbalances and aneuploidies in the offspring (Woods et al. 2017). Many of these karyotypic abnormalities result in spontaneous abortion in the first trimester and thus contribute to the high frequency of pregnancy loss during this time window (Ashaat and Husseiny 2012). However, advanced maternal age also poses an increased risk of serious complications that manifest in later pregnancy, these include miscarriage, late fetal and perinatal death, stillbirth, preterm and extreme preterm birth, low birth weight, placenta praevia and pre-eclampsia (Abel et al. 2002; Jacobsson et al. 2004).

In mammalian ovaries, the majority of oocytes are meiotically arrested and surrounded by a layer of flattened somatic granulosa cells, a structure known as primordial follicle (Hirshfield 1991; Adhikari and Liu 2009). In order to ensure the proper reproductive lifespan, most of the oocytes are maintained in this quiescent state within primordial follicles (Zhang et al. 2014). Granulosa cells play a fundamental role in initiating the growth of primordial follicles (Zhang et al. 2014). Several molecular networks mediate the interaction between somatic cells and germ cells in controlling the development of dormant mammalian oocytes (Laplante and Sabatini 2012; Zhang et al. 2014). Mammalian target of rapamycin (mTOR), for example, is stimulated by nutrition, oxygen, energy and several growth factors (Laplante and Sabatini 2012). Granulosa cells produced mTOR stimulates Kit which in the oocyte stimulates the phosphoinositide 3-kinase (Pi3k)/protein kinase B (Akt1) pathway and the transcription factor Forkhead Box O3a (FoxO3a) phosphorylation resulting in primordial follicle activation (John et al. 2008; Reddy et al. 2008; Zhang et al. 2014). In mice this pathway is already active after birth and has a role in promoting activation of primordial follicles as early as post-natal day 10 (Zhang et al. 2014). Mathematical models and in vivo evaluation of the primordial reserve suggest a severe reduction in the number of primordial follicles in the reserve from birth to puberty that can play a key role in reproductive longevity of mice (Bristol-Gould et al. 2006).

Ames Dwarf mice (df/df) have a defective Prophet of Pit1 (Prop1) gene, which impairs anterior pituitary gland development, resulting in deficient growth hormone (GH) secretion (Sornson et al. 1996). As a result, df/df mice have very low levels of circulating insulin-like growth factor I (IGF-1), are significantly smaller, have delayed puberty and live around 30 to

65% longer than normal littermates (N/df) (Chandrashekar and Bartke 1993; Brown-Borg et al. 1996). The GH/IGF-1 axis also has a central role in ovarian function (Bachelot et al. 2002; Zaczek et al. 2002; Chandrashekar et al. 2004). We have shown that older GH-deficient df/df mice have a larger ovarian primordial follicle reserve than N/df mice and that treatment with exogenous GH activated the primordial follicle reserve in both df/df and N/df mice (Saccon et al. 2017). This indicates that increased longevity is correlated with increased reproductive lifespan. Furthermore, mice overexpressing bovine GH (bGH) had a smaller ovarian reserve than normal mice (Saccon et al. 2017), showing that the GH/IGF-1 axis has a central role in ovarian function. Despite the evidences that df/df mice have increase ovarian reserve little is known about the quality of these oocytes.

Several factors can influence oocyte quality during aging. The adverse effects of DNA damage caused by environmental factors on somatic cells have been extensively reported (Saini and Gordenin 2018). Mammalian oocytes enclosed in primordial follicles remain arrested in prophase I of meiosis up to several decades in some species, including humans (Mehlmann 2005; Chiang et al. 2012). This long dormancy period increases the chances of accumulating DNA damage, as shown in female mice and human, both of which, accumulate double strand breaks (DSBs) in primordial follicle oocytes with aging (Titus et al. 2013). Extensive damage occurring throughout meiosis can have serious consequences if an adequate cellular response is not activated, and may result in infertility or development of defective embryos that are unable to result in full-term pregnancy (Kirk and Lyon 1982; Adriaens et al. 2009). In response to DSBs, kinases are known to phosphorylate histone 2Ax (γH2AX) on serine 139 (Burma et al. 2001; Bakkenist and Kastan 2003). Such post-transcriptional modification at the lesion site provides a platform for the ataxia-telangiectasia mutated (ATM)–mediated DNA damage signaling pathway to regulates the repair of DNA DSBs (Collins and Jones 2016). Oocytes are therefore prone to such damage as their lifetime for a single cell is very long and significant damage can accumulate, which may explain the fertility decline as animals become older.

Along with DNA damage, inflammation also increases with age and can negatively affect fertility (Bektas et al. 2018). Interleukin 1 deficient female mice have more primordial follicles and increased fertility than control females (Chen et al. 2018). We identified by RNASeq that old df/df mice have more than 150 down-regulated gene ontology terms related to the inflammatory/immune response compared to N/df mice (Schneider et al. 2017), pointing to a

role of inflammation in reproductive senescence. Interestingly, transplant of young ovaries to older recipients in mice was associated to a reduced inflammation and increased longevity (Habermehl et al. 2019). Also, it is well known that adipose tissue and blood levels of pro-inflammatory cytokines, interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) are reduced in df/df mice (Berryman et al. 2004; Wang et al. 2006). This reduced inflammation is believed to be one of the main reasons for the increased longevity of df/df mice (Masternak and Bartke 2012). In this context, the presence of inflammatory cells within the ovaries is an important parameter, as macrophages are the most abundant immune cells within the ovary (Turner et al. 2011). A high-fat diet results in increased primordial follicle depletion and increased ovarian macrophage infiltration in mice (Skaznik-Wikiel et al. 2016). Inflammation results in oxidative stress, reducing cellular antioxidant capacity, leading to overproduction of free radicals that react with cell membrane fatty acids and proteins impairing their function permanently (Durackova 2010). In addition, excess free radicals increase DNA damage, a predisposing factor for several age-related disorders (Khansari et al. 2009).

Based on this evidence, the aim of this study was to evaluate the primordial follicle reserve, DNA damage and macrophage infiltration in the ovaries of young df/df and N/df mice.

2. Materials and methods:

2.1. Animals

For these experiments, six-month-old female Ames dwarf (df/df, n = 6) and normal littermate control (N/df, n = 6) mice were used. Mice were maintained under temperature (22 ± 2°C) and humidity (40-60%) controlled conditions. All experiments were approved by the Institutional Animal Care and Use Committee from Southern Illinois University School of Medicine, IL, USA.

1. Tissue collection and processing

The mice were anesthetized after fasting for 12 h and the pair of ovaries was collected, dissected from surrounding adipose tissue and placed in 10% formalin buffered solution. After that, the ovaries were removed from the formalin solution, dehydrated in alcohol, cleared in xylene and embedded individually in Paraplast Plus (Sigma Chemical Company®, St. Louis, MO, USA). One ovary of the pair was then serially sectioned at 5µm using a semi-automated

rotary microtome (RM2245, Leica Biosystems, San Diego, CA, USA). The sections started at the beginning of the visible area of the ovarian surface until the end of the structure, and every sixth section was selected and placed on a standard histological slide for staining and counting. Intermediate sections were randomly selected for immunofluorescence analysis using slides impregnated with 3% organosilane (Sigma Chemical Company®, St. Louis, MO, USA) in ethanol.

2. Follicular classification, counting and measurement

The slides were dried at 56^{o} C for 24 h, stained with hematoxylin-eosin, and mounted with coverslips and synthetic resin (Sigma Chemical Company®, St. Louis, MO, USA). Images of the ovarian sections were captured with a digital camera coupled to a microscope (Nikon Eclipse E200, Nikon Corporation, Japan) using the 10 and 40 objectives, assisted by the software Moticam 5.0 (Motic, Hong Kong, China). Nucleus, oocytes and follicles diameters were measured using Motic Images Plus 2.0® (Motic, Hong Kong, China). For each mouse 3 random follicles/category were evaluated for diameter measurement (n=18/group/category). Only follicles containing an oocyte with clearly visible nucleus were counted in each slide. Follicle classification was based on Myers et al, 2004. Follicles were classified as primordial (oocyte surrounded by a single layer of flattened granulosa cells, Figure 1A), primary (oocyte surrounded by a single layer of cuboidal granulosa cells, Figure 1B), secondary (oocyte surrounded by two or more layers of cuboid granulosa cells without a visible antrum, Figure 1C) and tertiary (follicles with a clearly defined antral space and multiple layers of granulosa cells around the oocyte, Figure 1D) (Myers et al. 2004). To estimate actual follicle quantity the number of follicles in each category was multiplied by six to account for the section sampling and by two to account for the fact that only one ovary of the pair was used for sampling (Saccon et al. 2017).

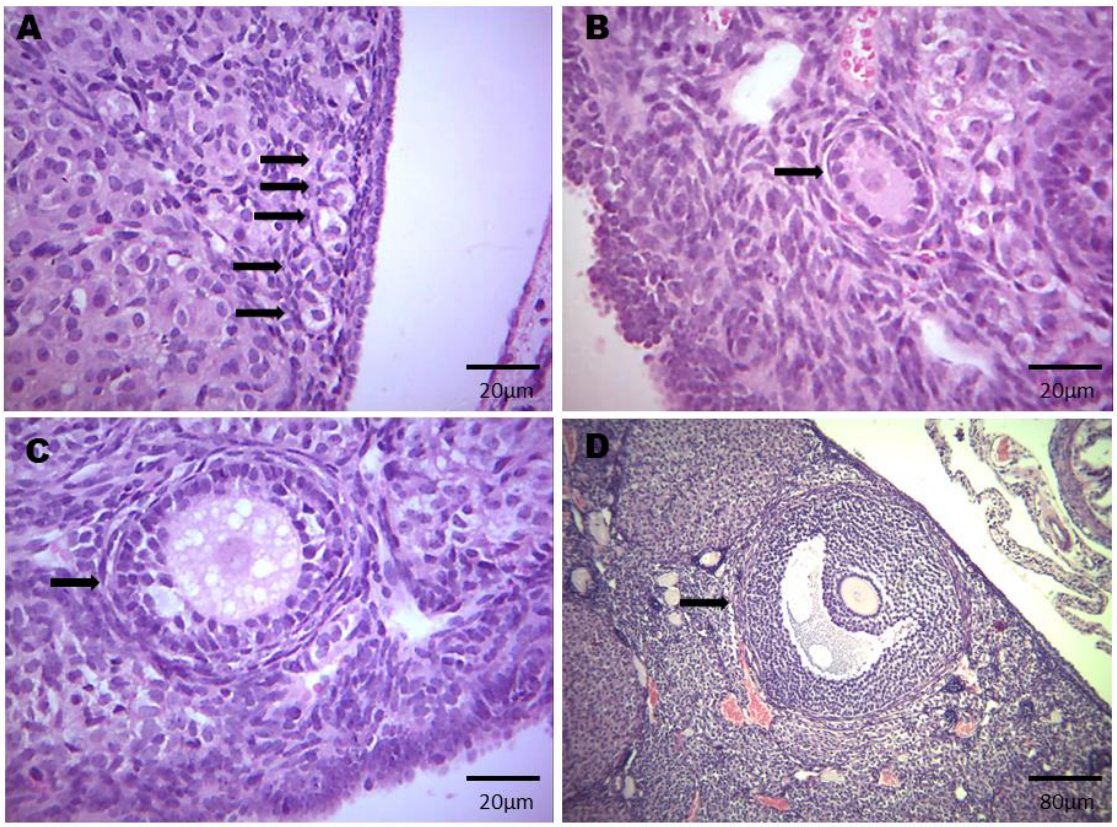

Figure 1. Morphological classification of follicles. They were classified according to (Myers et al. 2004) as (A) primordial (oocyte surrounded by a single layer of flattened granulosa cells), (B) primary (oocyte surrounded by a single layer of cuboidal granulosa cells), (C) secondary (oocyte surrounded by two or more layers of cuboid granulosa cells without a visible antrum) and (D) tertiary (follicles with a clearly defined antral space and multiple layers of granulosa cells around the oocyte). Black arrows indicate the follicle.

3. Immunofluorescence

For immunofluorescence analysis, the ovarian samples were deparaffinized with xylene and rehydrated with graded alcohols. The primary monoclonal antibodies were obtained from Abcam (Abcam Plc, Cambrigde, UK) and diluted in 1.5% BSA solution. The anti-gamma H2AX (γH2AX) phospo S139 antibody (ab11174), to indicate DNA damage (Titus et al. 2013) and anti-CD68 antibody (ab955), to indicate the presence of macrophage (Skaznik-Wikiel et al. 2016) were used at a final dilution of 1:500. The blockage of the endogenous peroxidase activity was achieved with hydrogen peroxide blocking solution, while the antigen recovery was

performed in humid heat, during 3 min after the boiling point, in citrate solution at pH 6.0. Non-specific background staining was reduced by covering the tissue sections that received protein block with BSA and goat serum. Thereafter, slides were incubated overnight with the primary antibody in a humid chamber at 4°C. The slides with anti- γH2AX and anti-CD68 antibodies were incubated for 1 hour with secondary Alexa Fluor® 488 (ab150113) and Hoechst (ab228550) for nuclei staining during 15 min. The slides were mounted with a drop of mounting medium under coverslips. The images of the follicles were captured by confocal microscope (Olympus FluoView™ 1000). Fluorescence intensity quantification for γH2AX, measured as pixel intensity in the nuclei area (Anderson et al. 2013), and macrophage counting, measured as number of identified cells/slide, was performed by image analysis software Image J® (Schneider et al. 2012). γH2AX was measured in 3 follicles for each category (primordial, primary, secondary) per mice (n=18 follicles/category/group). Macrophage counting was performed as a total number of macrophages per sections of ovary. Each mouse had a total of 4 random sections from the center of the ovary counted for macrophage infiltration. The number of granulosa cells was also counted in 3 follicles/ category/mouse, using the Hoechst (ab228550) for nuclei staining of surrounding granulosa cells (Braw-Tal and Yossefi 1997).

4. Statistical analyzes

All statistical analyzes were performed using GraphPad Prism 6 (GraphPad Inc., La Jolla, CA, USA). *T*-test was performed for comparing the number and size of follicles and immunostaining between df/df and N/df mice. A *p*-value lower than 0.05 was considered as significant.

3. Results

The number of primordial follicles was higher in df/df mice ($p = 0.0026$, Figure 2A). Also, df/df mice had a lower number of primary ($p = 0.023$, Figure 2B), secondary ($p = 0.0052$, Figure 2C) and tertiary ($p = 0.019$, Figure 2D) follicles, suggesting a reduced activation rate of primordial follicles in 6-month-old df/df mice. The number of total follicles was not different between both groups ($p > 0.05$).

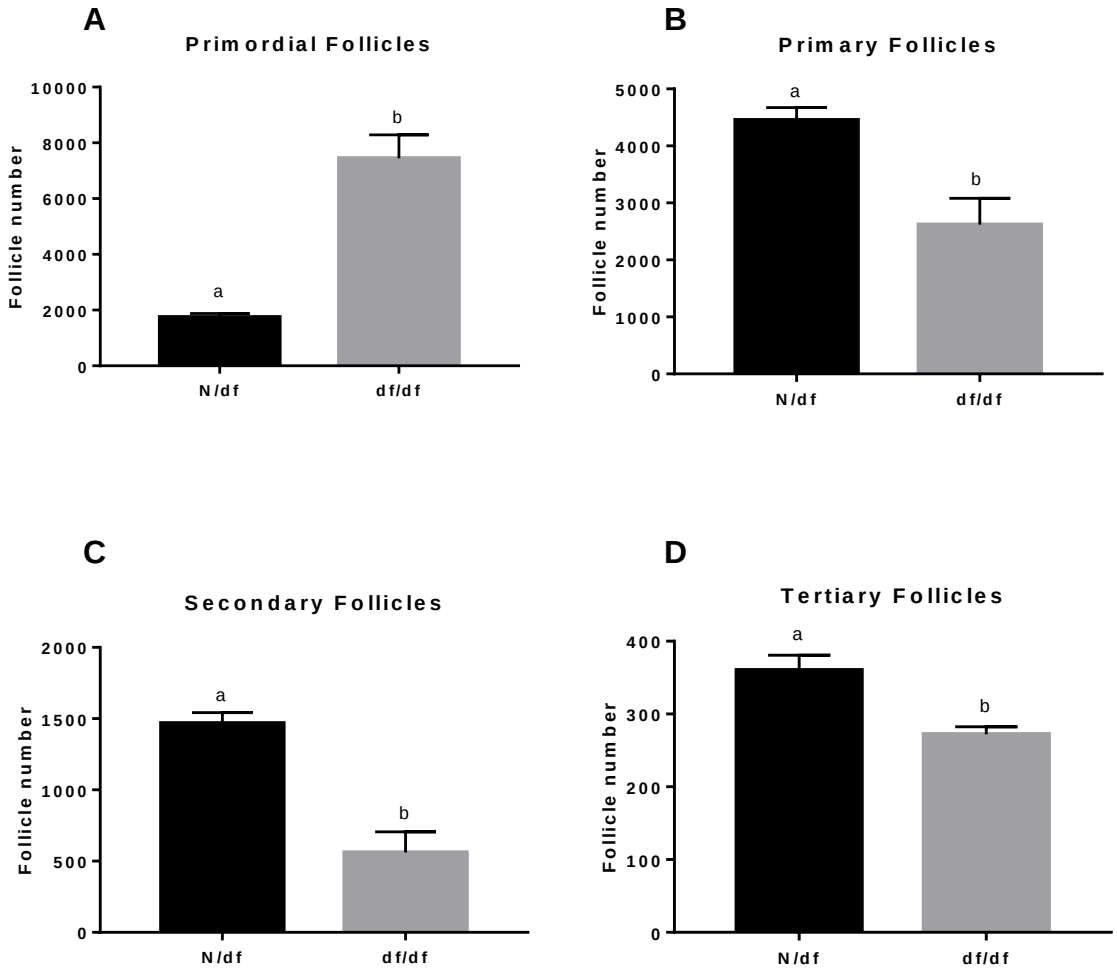

Figure 2. Comparison between the number of (A) primordial, (B) primary, (C) secondary and (D) tertiary follicles in N/df (n = 6) and df/df (n = 6) mice. Different letters indicate significant differences ($p < 0.05$). Data presented as media ± SEM.

Female df/df mice had healthier primordial and primary follicles, as indicated by the lower quantity of DSBs, measured by lower fluorescence intensity for γH2AX foci in oocytes from primordial ($p = 0.015$, Figure 3A) and primary ($p = 0.0004$, Figure 3B) follicles compared to N/df mice. Also, df/df mice had reduced γH2AX intensity in granulosa cells of primordial ($p = 0.0002$, Figure 3C) and primary ($p < 0.0001$, Figure 3D) follicles. Representative images of

immunofluorescence of anti-γH2AX are shown in Figure 4. Df/df mice also had ovarian tissue with reduced macrophage infiltration compared to N/df mice (p = 0.033, Figure 5C).

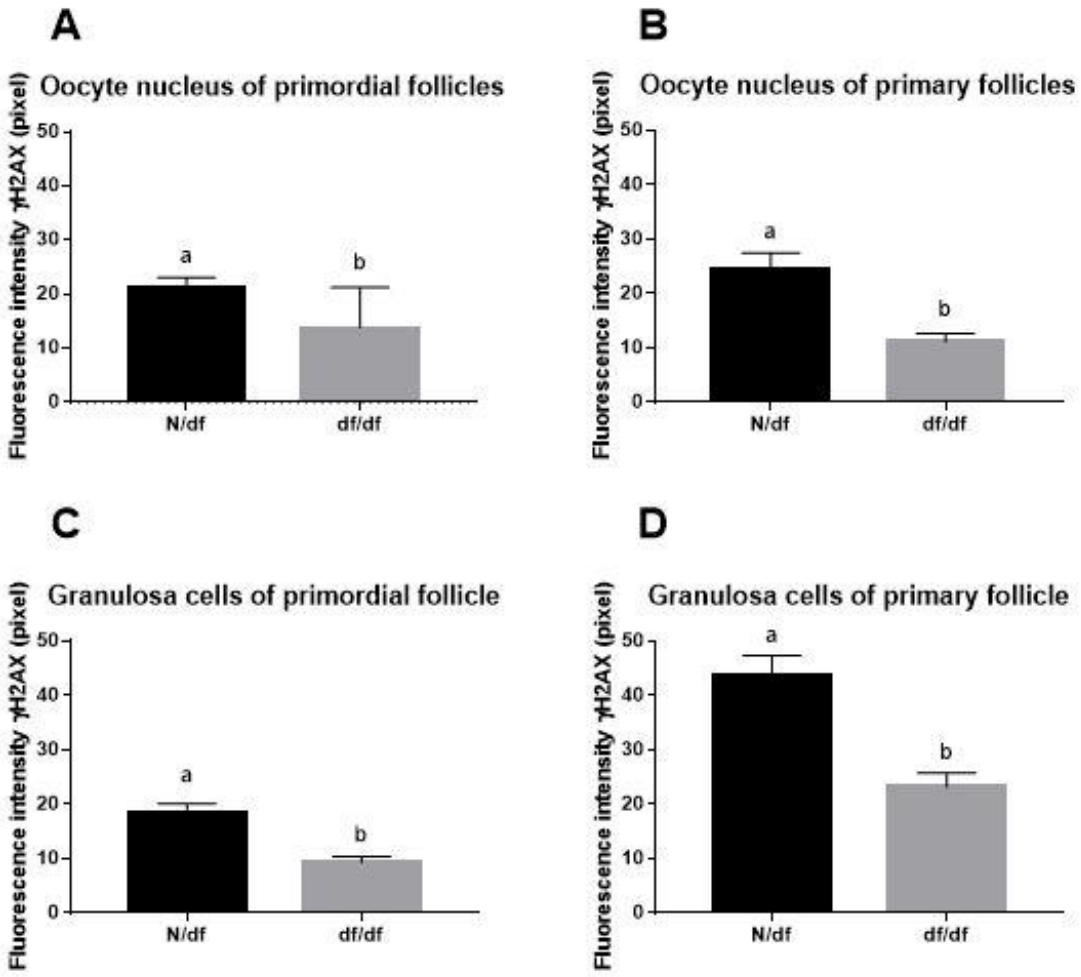

Figure 3. Fluorescence intensity in pixels of γH2AX immunostaining in oocytes nucleus of primordial follicle (n = 18, A), oocyte nucleus of primary follicle (n = 18, B) and granulosa cell nuclei from primordial (n = 18, C) and primary follicles (n = 18, D) of N/df and df/df mice. Different letters indicate significant differences (p < 0.05). Data presented as media ± SEM.

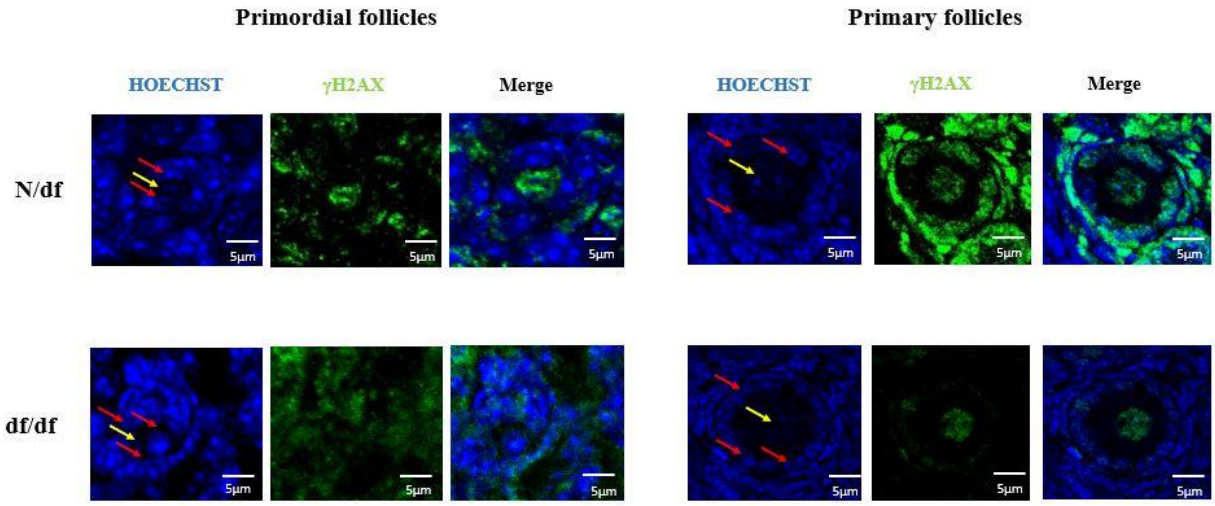

Figure 4. Representative images of immunofluorescence of anti-γH2AX in primordial and primary follicles of N/df and df/df mice. Blue images represent Hoechst (staining genetic material) and greens represents anti-γH2AX staining γH2AX protein. Merged images are showed as both Hoechst and anti-γH2AX staining combination. Red arrows represent some granulosa cells and yellow arrow represents oocyte nucleus.

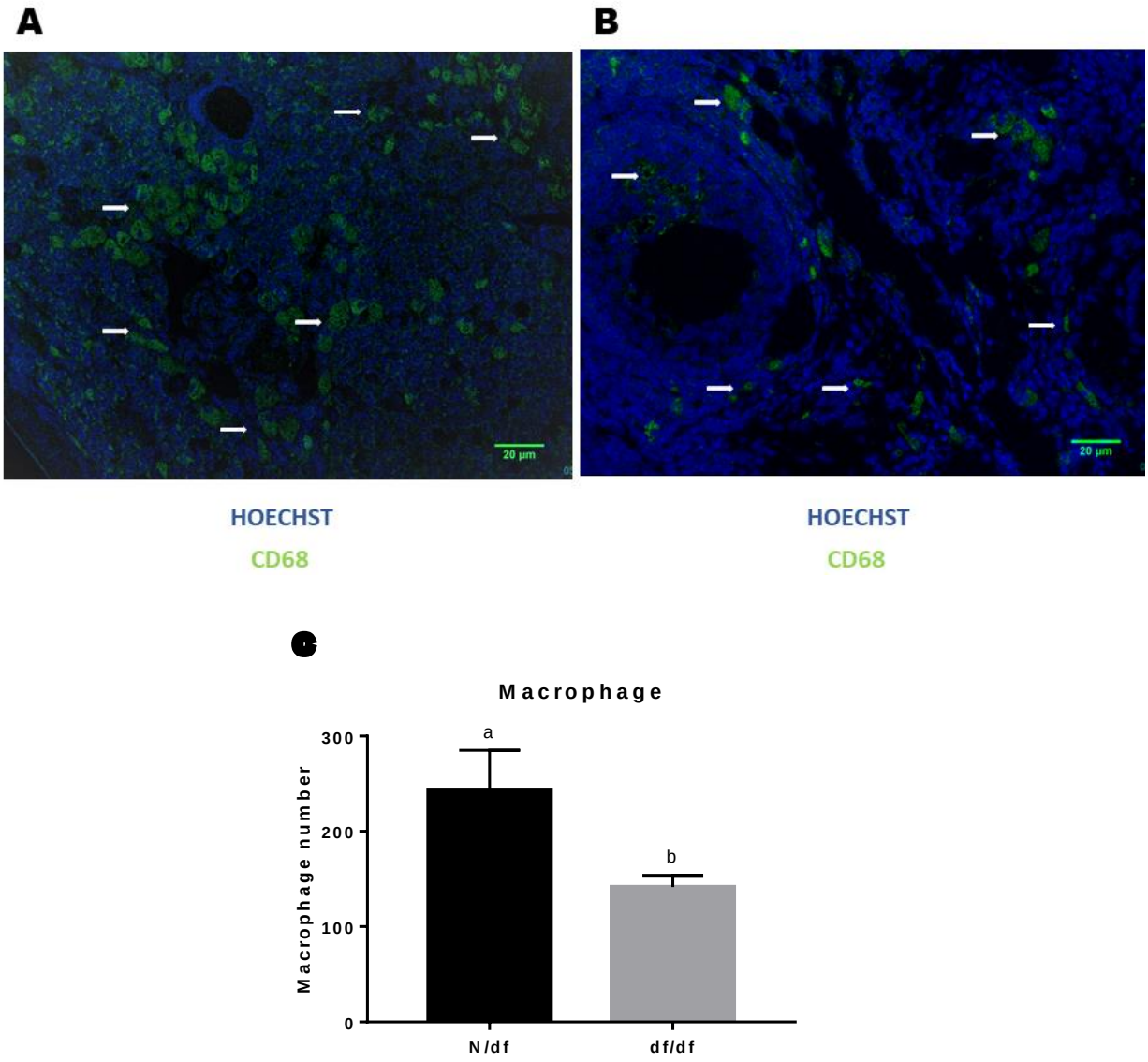

Figure 5. Representative immunofluorescence of anti-CD68 marker of N/df (A) and df/df (B) mice and macrophage number (C) on ovarian section of N/df (n = 18) and df/df (n = 18) mice. White arrows indicate macrophages staining. Different letters indicate significant differences ($p < 0.05$).

We also evaluated morphological follicle parameters. Female df/df mice had a lower number of granulosa cells in primordial ($p = 0.0028$, Table 1) and primary ($p = 0.021$, Table 1) follicles compared to N/df mice. Also, df/df mice had a smaller diameter of primordial follicle

nuclei (p = 770.0028, Table 1), secondary follicle oocyte (p = 0.009, Table 1) and tertiary follicle (p = 0.046, Table 1). No difference was found for other parameters such as nuclei diameter of primary, secondary and tertiary follicles, oocyte diameter of primordial, primary and tertiary follicles and follicle diameter of primordial, primary and secondary follicles (p > 0.05).

Table 1. Number of granulosa cells and diameters of nucleus, oocyte and follicle of primordial, primary, secondary and tertiary follicles of N/df and df/df mice.

Parameter	**N/df**	**df/df**	***p*-Value**
Granulosa cells number (SEM)	**(n=18)**	**(n=18)**	
Primordial Follicles	8.0 (±0.4)	5.3 (±0.5)	0.0028
Primary Follicles	17.9 (±2.7)	12.3 (±0.7)	0.021
Diameters (µm) (SEM)			
Primordial Follicle			
Nuclei diameter (µm)	3.1 (±0.2)	2.6 (±0.1)	0.009
Oocyte diameter (µm)	6.3 (±0.4)	5.6 (±0.3)	0.31
Follicle diameter (µm)	9.5 (±1.0)	8.0 (±0.3)	0.17
Primary Follicle			
Nuclei diameter (µm)	3.5 (±0.2)	3.3 (±0.2)	0.61
Oocyte diameter (µm)	9.3 (±0.9)	9.5 (±0.8)	0.88
Follicle diameter (µm)	16.3 (±1.4)	14.9 (±1.2)	0.47
Secondary Follicle			
Nuclei diameter (µm)	6.6 (±0.6)	5.9 (±0.3)	0.29
Oocyte diameter (µm)	25.2 (±1.3)	21.8 (±1.0)	0.046
Follicle diameter (µm)	53.5 (±3.5)	54.0 (±5.0)	0.93
Tertiary Follicle			
Nuclei diameter (µm)	5.56 (±0.4)	5.2 (±0.8)	0.65
Oocyte diameter (µm)	30.6 (±1.0)	29.8 (±1.3)	0.43
Follicle diameter (µm)	180.6 (±8.0)	147.0 (±9.7)	0.012

4. Discussion

The current study points to the role of the GH/IGF-1 axis in keeping the primordial follicle reserve in the ovary, along with maintenance of oocyte DNA integrity and reduced ovarian macrophage infiltration. GH-deficient df/df mice are known for being smaller-sized and living longer and healthier life than normal littermate controls (Chandrashekar and Bartke 1993;

Brown-Borg et al. 1996). Our present study confirms that these mice have a larger ovarian primordial follicle reserve than N/df already at six months of age. Dwarf mice had almost four times more primordial follicles than N/df mice. This increased primordial ovarian reserve can increase the ovarian lifespan. A previous study from our group demonstrated that df/df mice had a larger ovarian reserve than N/df mice at 18-months-old, with twice as many primordial follicles (Saccon et al. 2017). Also, evaluating 12-month-old df/df mice, we observed three times more primordial follicles (Schneider et al. 2017). This suggests that the difference in the ovarian primordial reserve between df/df and N counterparts decreased with aging, as the reserve becomes smaller. Additionally, we observed that df/df mice had a lower number of primary, secondary and tertiary follicles, which further supports the notion that df/df mice are activating a lower number of follicles, explaining the origins of its prolonged reserve. This means in the absence of GH less primordial follicles are activated to primary follicles and are kept in the reserve for longer periods, therefore, extending the ovarian lifespan. Young female GH receptor gene knockout (GHRKO) mice, which also have lower serum IGF-1 and increased longevity (Bartke 1966), also have an increased number of primordial follicles (Slot et al. 2006), and still have ovarian activity at older ages, when age matched normal mice have completely exhausted the ovarian follicular reserve (Sluczanowska-Glabowska et al. 2012). These GHRKO females were able to successfully deliver pups when normal littermates already exhausted reproductive function (Selesniemi et al. 2008; Sluczanowska-Glabowska et al. 2012). Increasing the number of primordial follicles in the ovary can improve the reproductive lifespan. This was demonstrated in calorie restricted mice, which had a increased primordial follicle reserve and delivered pups much later in life when control mice stopped reproducing (Selesniemi et al. 2008).

Activation of the Pi3k/Akt1 signaling pathway and the phosphorylation of downstream effector FoxO3a is an essential step for the activation of the primordial follicle and its irreversible growth (Castrillon et al. 2003; John et al. 2007). Hyperphosphorylation of FoxO3a results in its nuclear exclusion, culminating in the global activation of primordial follicles and premature ovarian failure (Castrillon et al. 2003). Therefore, the presence of FoxO3a in its non-phosphorylated form is crucial to maintaining the primordial follicles in their quiescent state (John et al. 2008). Primordial follicles begin to grow irreversibly when oocyte FoxO3a is phosphorylated by stimulus from the surrounding granulosa cells (John et al. 2007; John et al.

2008; Zhang et al. 2014). We previously demonstrated a higher level of non-phosphorylated FoxO3a in primordial follicles oocytes of 18-month-old df/df mice (Saccon et al. 2017). In addition, GH treatment reduced the FoxO3a protein level, while it increased its phosphorylated form in primordial follicles oocytes (Saccon et al. 2017). Another study observed that pFoxO3a protein levels in primordial/primary follicles were lower in 12-month-old df/df compared to N/df mice, despite no difference in total FoxO3a protein levels (Schneider et al. 2014), which suggests an age-dependent FoxO3a regulation in the ovary. Besides our findings, FOXO3a is a central transcription factor that mediates multiple physiological and pathological processes by inducing transcription of target genes involved in apoptosis (Chen et al. 2018), proliferation (McClelland Descalzo et al. 2016), cell cycle progression (McGowan and McCoy 2013), survival (Joseph et al. 2016) and DNA damage (Fluteau et al. 2015). It also respond to several cellular stresses such as UV irradiation (Wang et al. 2012) and oxidative stress (Lim et al. 2017; Wang et al. 2017). Besides, FOXO3a is strongly associated with human longevity (Willcox et al. 2008). Therefore, this previous evidence shows us that GH/IGF-1-dependent activation of the FoxO3a pathways could be one of the main mechanisms involved in increased reproductive longevity in df/df mice and it is effects can be in 6-month-old df/df mice.

DNA damage in oocytes and granulosa cell can be caused by various endogenous or exogenous stresses, including oxidative stress, telomere erosion, oncogenic mutations, genotoxic stress, and metabolic stress (Lopez-Otin et al. 2013). DNA damage is a challenge that all somatic and germline cells are exposed during their lifetime (Jackson and Bartek 2009). Cells interpret DSBs as a serious threat to their integrity, activating DNA damage checkpoints (DDC) and reacting with a DNA damage response resulting in cell cycle arrest as a downstream effect, allowing activation of repair mechanisms (Bartek and Lukas 2007). DSBs trigger a DDC response by activating two major kinases, i.e. ATM and ataxia telangiectasia and Rad3-related (ATR) (Reinhardt and Yaffe 2009; Smith et al. 2010). Taking advantage of the cell cycle arrest, the cell can then repair the damaged DNA. At the DNA damage site, ATM gives rise to the phosphorylated form of the H2AX histone, which acts as a catalyst for the recruitment of the necessary checkpoint and repair factors (Burgoyne et al. 2007). Snell dwarf mice, endocrinologically identical to df/df mice, have increased cellular DNA repair capacity and upregulation of several DNA repair-related genes compared to normal littermates (Podlutsky et al. 2017). Our data, therefore, prompts several considerations on the relevance of DNA damage

in df/df mice oocytes. Our study showed that df/df mice have a higher number of primordial follicles; therefore, more oocytes are kept longer in the ovary, considering also that these mice live 30-70% longer than N/df mice. Additionally, we observed that oocytes from primordial and primary follicles of df/df mice had fewer DSBs than those of N/df mice, indicating that df/df primordial and primary oocytes have a better quality. This is interesting, as it demonstrates that GH-deficiency not only preserves the ovarian primordial reserve for a longer period, but also oocytes accumulate less DNA damage. The impairment of DNA DSBs repair is associated with accelerated loss of ovarian follicular reserve and accumulation of DSBs in human and mouse oocytes (Titus et al. 2013). Other have also observed in monkeys that DSBs increase with age in granulosa cells (Zhang et al. 2015), also suggesting this damage is not confined to the oocyte as we observed. This suggests, that besides FoxO3a activation, reduced DSBs in primordial follicle oocytes can be a mechanism of increased ovarian reserve in df/df mice. Moreover, the expression of Breast Cancer 1 protein (BRCA1) and other key genes in the ATM-pathway decline with age in human oocytes, associated with an increase in γH2AX foci (Titus et al. 2013), suggesting a relationship between DNA DSB repair and ovarian aging. The same decline in repair mechanisms was observed for monkey granulosa cells (Zhang et al. 2015). We previously observed a three-fold reduction in *BRCA1* gene expression with aging in N mice, although its expression did not change with aging in df/df mice (Schneider et al. 2017). Therefore, it is suggested that maintenance of high levels of DNA protecting factors could be a key element for preserving the ovarian reserve for a longer period. The agreement between mouse and human data pinpoints impaired DNA DSBs repair as a universal mechanism of oocyte aging in mammals. However, further studies on DSB repair in oocytes of df/df mice are needed to establish a mechanistic link.

GH-deficient df/df mice exhibited reduced ovarian macrophage infiltration associated with reduced ovarian aging. The long-living df/df, GHRKO and calorie restricted mice have all been extensively characterized as having a reduced pro-inflammatory profile, which may represent one of the major mechanisms promoting increased insulin sensitivity and extended longevity in these mice (Masternak and Bartke 2012). Macrophages located in the ovaries, by secreting growth factors and/or cytokines, may play a synergistic role in stimulating cellular proliferation and follicle growth (Fukumatsu et al. 1992). Indeed, co-culture of rat granulosa cells with peritoneal macrophages results in proliferation of the granulosa cells (Fukumatsu et

al. 1992). Some of the macrophage-derived factors that are known to impact follicular growth are hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), tumor necrosis factor (TNF) α and β, and IGF-1 (Stirling et al. 1991; Spaczynski et al. 1999; Nakayama et al. 2003). Macrophages are present in atretic and well as healthy follicles (Turner et al. 2011). Ablation of ovarian macrophages disrupts vascular integrity indicating its pivotal role in ovarian function (Turner et al. 2011). On the other hand, prolonged exposure (both short-term and long-term) to a high-fat diet in young adult female mice reduced primordial follicle numbers, compromised fertility, produced higher systemic proinflammatory cytokine levels, and increased ovarian macrophage infiltration in the stroma, independent of obesity (Skaznik-Wikiel et al. 2016). Pro-inflammatory Interleukin 1 deficient female mice have more primordial follicles and increased fertility than control females (Chen et al. 2018). Also, LPS exposure in mice reduced the primordial follicle pool mediated by TLR4 (McClelland Descalzo et al. 2016), further indicating the role of inflammation on ovarian reserve depletion. Our ovarian transcriptome study showed that the top 150 down-regulated terms between old df/df and old N mice were related to the inflammatory/immune response (Schneider et al. 2017). It was observed that macrophage chemotaxis, macrophage activation and macrophage differentiation were also among the top down-regulated biological processes in old df/df compared to old N mice. Therefore, these findings point to an association between the depletion of the ovarian reserve and an increased ovarian pro-inflammatory status, which were both attenuated in the ovaries of aging df/df mice. Osteopetrotic mice have severely reduced numbers of mature macrophages due to a natural mutation in the CSF-1 gene (Cohen et al. 2002). These mice have reduced numbers of ovarian macrophages, which may be either a cause or effect of the decreased follicle growth. Caloric restriction also reduced the numbers of macrophages surrounding preovulatory follicles in rats (Duggal et al. 2002). Taken together, this evidence provides an interesting link between ovarian longevity and macrophage population, although more functional studies are necessary.

In addition to reduced activation of primordial follicle numbers in df/df mice, the microenvironment within follicles and ovaries also could be influenced by GH/IGF-1 deficiency. Particularly, pre-antral follicles granulosa cells are intimately connected with oocytes, and both play critical roles in oocyte growth and follicular development (Eppig et al. 1997; Su et al. 2009). Oocyte growth is accompanied by the active accumulation of mRNA,

proteins, and lipids, and by modifications of chromatin configuration and DNA methylation status, and these molecular reactions require enough energy (Munakata et al. 2016). The number of granulosa cells in primordial follicles is associated with the energy sufficiency of oocytes (Munakata et al. 2016), which can affect oocyte and follicle growth. A complete set of molecular networks that mediate the interaction between granulosa cells and oocytes in controlling the development of dormant mammalian oocytes had been described. The model proposed by Zhang et al. (2014) has two key steps. The first step is the mTOR complex 1 (mTORC1) signaling in granulosa cells that acts as the key decision-making process regarding whether or not a primordial follicle will be activated (Zhang et al. 2014). The second step involves the tightly regulated communication channel from the granulosa cells to the oocytes via KITL-KIT to trigger the awakening of the oocyte through FoxO3a phosphorylation (Zhang et al. 2014). It is proposed that these processes ensure the progressive activation of a limited number of primordial follicles throughout the reproductive lifespan. Thus, it is likely that follicular activation is initiated by molecular and cellular changes in the granulosa cells that are followed by awakening of the dormant oocytes. Our study shows that df/df mice had fewer granulosa cells surrounding oocytes than N/df mice in both primordial and primary follicles, supporting the reduced activation of primordial follicle observed for df/df mice. Also, df/df mice had a smaller oocyte nuclei diameter for primordial follicles and oocyte diameter for secondary follicles. Growth of oocyte nuclei is associated with increased gene expression activity (Moore and Lintern-Moore 1974), indicating that the oocyte is awakening the machinery necessary for its growth and differentiation. We previously observed that oocytes from primordial and primary follicles of bGH mice had increased nuclei and oocyte diameter (Saccon et al. 2017), suggesting the opposite effect associated to increased primordial follicle activation. Overall this may be an indication that GH deficiency is associated with decreased granulosa cell proliferation, which leads to reduced primordial oocyte activation, resulting in a smaller nucleus.

5. Conclusion

In conclusion, the present study indicates a reduced primordial reserve in young long-living df/df mice in comparison to N/df mice. Oocytes from the primordial and primary follicles of df/df mice presented fewer DSBs than N/df mice, indicating that GH-deficiency not only

increases the primordial reserve, but also the oocytes accumulate less DNA damage. Additionally, df/df mouse ovaries presented less macrophage infiltration. It is suggested that reduced DSBs along with a reduced number of granulosa cells surrounding the primordial oocyte are involved in the preservation of the ovarian reserve. Overall, these important observations confirm, that delayed aging phenotype in these unique long-living df/df mice are associated to beneficial characteristics of longevity in the female reproductive organs.

6. Acknowledgments

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5 CAPITULO 2

Manuscrito 2 - Growth hormone increases DNA damage in ovarian follicles and macrophage infiltration in the ovaries

(Conforme normas do periódico Growth hormone and IGF research)

Growth hormone increases DNA damage in ovarian follicles and macrophage infiltration in the ovaries

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Abstract

Objective: The aim of the study was to evaluate the role of growth hormone (GH) in DNA damage, macrophage infiltration and the granulosa cells number of primordial and primary follicles.
Methods: For these experiments six groups of female mice were used. Four groups consisted of Ames dwarf mice (Prop-1^{df}, df/df, n=12) and their normal littermates (N/df, n=12), between sixteen and eighteen month-old, receiving GH (n=6 for df/df, and n=6 for N/df mice) or saline injections (n=6 for df/df, and n=6 for N/df mice). The other two groups consisted of ten to twelve-month-old transgenic mice overexpressing bovine GH (bGH) (n=6) and normal mice (N, n=6). DNA damage (anti- γH2AX) and macrophage counting (anti-CD68) were evaluated by immunofluorescence. Number of granulosa cells of primordial and primary follicles were estimated.
Results: Female df/df mice had lower γH2AX foci intensity in both oocytes and granulosa cells of primordial and primary follicles ($p<0.05$), indicating less DNA double strand breaks (DSBs). GH treatment increased DSBs in both df/df and N/df mice. Inversely, bGH mice had higher quantity of DSBs in both oocytes and granulosa cells of primordial and primary follicles ($p<0.05$). Df/df mice showed ovarian tissue with less macrophage infiltration than N/df mice ($p<0.05$) and GH treatment increased macrophage infiltration ($p<0.05$). In contrast, bGH mice had ovarian tissue with more macrophage infiltration compared to normal mice ($p<0.05$). Df/df mice had fewer granulosa cells in primordial and primary follicles than N/df mice ($p<0.05$). GH treatment did not affect the granulosa cells number ($p>0.05$). However, bGH mice had an increased number of granulosa cells on primordial and primary follicles compared to normal mice ($p<0.05$).
Conclusion: The current study shows that GH increases DNA DSBs in oocyte and granulosa cells and also raises macrophage infiltration in the ovaries, pointing to the role of the GH/IGF-I axis in maintenance of oocyte DNA integrity and macrophage ovarian infiltration in mice.

Keywords: Growth hormone; Insulin-like growth factor-1; DNA damage; macrophages; granulosa cells.

Introduction

Ames dwarf mice (df/df) have a defective Prophet of Pit1 (Prop1) gene, which impairs anterior pituitary gland development, resulting in deficient growth hormone (GH), prolactin and TSH production [1]. As a result, df/df mice have very low levels of circulating insulin-like growth factor 1 (IGF-1), are significantly smaller and live between 30 to 65% longer than normal littermates (N/df) [2]. In contrast, transgenic mice overexpressing bovine GH (bGH) have elevated plasma levels of GH, resulting in increased circulating IGF-I levels and adult body size [3, 4]. The lifespan of bGH mice is approximately 50% shorter than for normal (N) littermates [4]. Collectively, this evidence points to an important role of the GH/IGF-I axis in the aging process and longevity.

A progressive decline with aging and consequent depletion of the ovarian primordial follicle reserve is the main cause of the onset of menopause in women [5]. Along with the reduction in follicle numbers, the quality of the remaining oocytes enclosed in these follicles also decreases with age [5, 6]. It is well established that the age-related decline in fertility is paralleled by a decrease in the ovarian reserve and the increase in chromosomally abnormal conceptions [7]. One of the primary causes of the increased risk to miscarriages with age is frequently attributed to the exponential rise of the oocyte chromosome mis-segregations, leading to karyotypic imbalances and aneuploidies in the offspring [8]. Many of these karyotypic abnormalities result in spontaneous abortion in the first trimester and thus contribute to the high frequency of pregnancy loss during this time window [9]. However, advanced maternal age also poses an increased risk of serious complications that manifest in later pregnancy. These include miscarriage, late fetal and perinatal death, stillbirth, preterm and extreme preterm birth, low birth weight, placenta praevia and pre-eclampsia [10, 11].

The GH/IGF-I axis has a central role in ovarian function [12-14]. Previous studies of our group have shown that middle-aged and old GH-deficient df/df mice have a larger ovarian primordial follicle reserve than N/df mice [15, 16]. This indicates that increased longevity is correlated with increased reproductive lifespan. In contrast, bGH mice had a smaller ovarian reserve and early decline in fertility than N mice [17, 18], showing that the GH/IGF-I axis has a central role in the reproductive lifespan. Despite the evidences for the role of GH/IGF-I in the

preservation of the ovarian reserve, little is known about its effects on the other ovarian parameters as the mice age.

Several factors can influence fertility during aging. The adverse effects of DNA damage caused by environmental factors on somatic cells have been extensively reported [19]. Mammalian oocytes enclosed in primordial follicles remain arrested in prophase I of meiosis up to several decades in some species, including humans [20, 21]. During this long period of dormancy which oocyte are submitted to increase the chances of accumulating DNA damage, as shown in female mice and human, both of which, accumulate double strand breaks (DSBs) in primordial follicle oocytes with aging [22]. Substantial damage occurring throughout meiosis can have serious consequences if an adequate cellular response is not activated, and may result in infertility, abortions or development of defective embryos that are unable to result in full-term pregnancy [23, 24]. As a reaction to DSBs, kinases are known to phosphorylate histone 2Ax (γH2AX) on serine 139 [25, 26]. This post-transcriptional modification at the lesion site provides a platform for the ataxia-telangiectasia mutated (ATM)–mediated DNA damage signaling pathway to regulates the repair of DNA DSBs [27].

In addition to DNA damage, inflammation also increases with age and can negatively affect fertility [28]. Interleukin 1 deficient female mice have more primordial follicles and increased fertility compared to control females [29]. One of our previous RNASeq studies identified that old df/df mice have more than 150 down-regulated gene ontology terms related to the inflammatory/immune response compared to N/df mice [30], pointing to a role of inflammation in ovarian aging. It was shown that adipose tissue and blood levels of pro-inflammatory cytokines, interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) are reduced in df/df mice [31, 32]. This reduced inflammatory response is believed to be one of the main reasons for the increased longevity of df/df mice [33]. Given this background, the presence of inflammatory cells within the ovaries is an important parameter, as macrophages are the most numerous immune cells within the ovary [34]. Inflammation can result in oxidative stress and vice-versa, reducing cellular antioxidant capacity, leading to overproduction of free radicals that react with cell membrane fatty acids and proteins impairing their function permanently [35]. Furthermore, excess free radicals can increase DNA damage, a predisposing factor for several age-related disorders [36].

In mammalian ovaries, the majority of oocytes are meiotically arrested and surrounded by a layer of flattened somatic granulosa cells, a structure known as primordial follicle [37, 38]. To ensure the proper reproductive lifespan, most of the oocytes are maintained in this quiescent state within primordial follicles [39]. Granulosa cells play a fundamental role in initiating the growth of primordial follicles [39]. The number of granulosa cells can be a useful marker of follicular activation and development [40]. Several molecular networks mediate the interaction between somatic cells and germ cells in controlling the development of dormant mammalian oocytes [39, 41]. For example, granulosa produced mammalian target of rapamycin (mTOR) stimulates Kit which in the oocyte stimulates the phosphoinositide 3-kinase (Pi3k)/protein kinase B (Akt1) pathway and the phosphorylation of transcription factor Forkhead Box O3a (FoxO3a) results in primordial follicle activation [39, 42, 43]. We have previously shown that GH has a central role in primordial follicle activation through activation of FoxO3a [16], however, to date little is known about its effects on granulosa cells number in primordial and primary follicles.

Based on this evidence, the aim of the present study was to evaluate the DNA damage, macrophage infiltration and the granulosa cells number of primordial and primary follicles in the ovaries of aged df/df and N/df mice with or without treatment with exogenous porcine GH and in short-living bGH transgenic mice.

Materials and methods:

Animals and treatment

Experiment 1 was ran with 4 groups of Ames dwarf mice (Prop-1^{df}, df/df, n=12) and their normal littermates (N/df, n=12), between sixteen and eighteen month-old, receiving GH (n=6 for df/df, and n=6 for N/df mice) or saline injections (n=6 for df/df, and n=6 for N/df mice). Experiment 2 consisted in 2 groups of ten to twelve-month-old bGH (n=6) and normal mice (N, n=6) (Figure 1). Mice were maintained under temperature (22 ± 2°C) and humidity (40–60%) controlled conditions. Mice treated with GH received recombinant porcine GH (pGH) subcutaneous injections (4 µg/g of body weight; Alpharma, Inc., Victoria, Australia) twice daily, beginning at fourteen months of age for six weeks. Control mice received saline injections in the same way as GH treated mice. After six weeks of treatment, mice were kept two more

weeks in the same conditions until euthanasia. The GH treatment used in the current study was proven effective for increasing serum IGF-I concentrations and body weight gain in previous studies using the same dose and treatment length in young (three weeks-old) [44, 45], middle age (five month-old) [46] and old mice (sixteen month-old)[44]. Body weights were measured before the first GH injection and at the end of the six-week treatment to confirm effectiveness of the treatment [16]. All experiments were approved by the Ethics Committee for Animal Experimentation from the University of Southern Illinois, IL, USA.

Tissue collection and processing

The mice were anesthetized and euthanized after fasting for 12 h and the pair of ovaries was collected, dissected from surrounding adipose tissue and placed in 10% formalin buffered solution. After that, the ovaries were removed from the formalin solution, dehydrated in alcohol, cleared in xylene and embedded individually in Paraplast Plus (Sigma Chemical Company®, St. Louis, MO, USA). One ovary of the pair was then serially sectioned at 5µm using a semi-automated rotary microtome (RM2245, Leica Biosystems, San Diego, CA, USA). Sections were randomly selected for immunohistochemistry analysis using slides impregnated with 3% organosilane (Sigma Chemical Company®, St. Louis, MO, USA) in ethanol.

Immunofluorescence

For immunofluorescence analysis, the ovarian samples were deparaffinized with xylene and rehydrated with graded alcohols. The primary monoclonal antibodies were obtained from Abcam (Abcam Plc, Cambrigde, UK) and diluted in 1.5% BSA solution. The anti-gamma H2AX (γH2AX) phospo S139 antibody (ab11174), to indicate DNA damage [22] and anti-CD68 antibody (ab955), to indicate the presence of macrophage [47] were used at a final dilution of 1:500. The blockage of the endogenous peroxidase activity was achieved with hydrogen peroxide blocking solution, while the antigen recovery was performed in humid heat, during 3 min after the boiling point, in citrate solution at pH 6.0. Non-specific background staining was reduced by covering the tissue sections that received protein block with BSA and goat serum. Thereafter, slides were incubated overnight with the primary antibody in a humid chamber at 4°C. The slides with anti- γH2AX and anti-CD68 antibodies were incubated for 1 hour with secondary Alexa Fluor® 488 (ab150113) and Hoechst (ab228550) for nuclei stanning

during 15 min. The slides were mounted with a drop of mounting medium under coverslips. The images of the follicles were captured by confocal microscope (Olympus FluoView™ 1000)**.** Fluorescence intensity quantification for γH2AX and macrophage counting was performed by image analysis software Image J® [48]. γH2AX and granulosa cells number were evaluated in 3 follicles/class/mouse. Macrophage counting was performed as a total number of macrophages per sections. Each mouse had a total of 4 random sections from the center of the ovary counted. The granulosa cells were counted as visible nuclei using the Hoechst (ab228550) for nuclei staining.

Statistical analyzes

All statistical analyzes were performed using Graphpad Prism 6 (Graphpad Inc., La Jolla, CA, USA). Two-way ANOVA was used for comparing the fluorescence intensity, number of granulosa cells and macrophage number of df/df and N/df mice (effect of the genotype, treatment with GH and the interaction). T-test was performed for comparing fluorescence intensity, number of granulosa cells and macrophage number between bGH and normal mice. A P value lower than 0.05 was considered as statistically significant.

Results

Experiment 1 – df/df mice

As shown in Figure 2, female df/df mice had fewer DNA DSBs, as indicated by lower fluorescence for γH2AX in oocytes from primordial (p <0.0001) and primary (p <0.0001) follicles, when compared to N/df mice. Also, the granulosa cells of primordial (p = 0.0299) and primary (p = 0.0002) follicles of df/df mice had less DNA DSBs. GH treatment increased (p < 0.0001) DNA DSBs in the oocytes and granulosa cells of primordial and primary follicles of both genotypes, Representative images of anti-γH2AX intensity in oocyte and granulosa cells of primordial and primary follicles are shown in Figure 4. Similar behavior was observed on macrophage infiltration on the ovary. df/df mice presented reduced macrophage infiltration (p <0.0001) and the GH treatment increased macrophage infiltration (p = 0.0007) in both genotypes (Figure 5A).

Df/df mice had less granulosa cells on primordial (p = 0.0002) and primary (p <0.0001) follicles than N/df mice, and GH treatment did not affect this parameter in any follicular types (Table 1; p >0.05).

Experiment 2 – bGH mice

Following a similar pattern to the described in experiment 1, animals with higher GH levels (bGH) presented higher (p = 0.0001) DNA DSBs in the oocyte from primordial and primary follicles (Figure 3). The granulosa cells from primordial (p <0.0001) and primary (p = 0.0466) had also more DNA DSBs in bGH mice than in N mice (Figure 3 C and D). Representative images of anti-γH2AX intensity in oocyte and granulosa cells of primordial and primary follicles are shown in Figure 4. Higher GH levels (bGH) also increased macrophage infiltration (p <0.0001) in the ovary (Figure 5 B and D) and the number of granulosa cells in primordial (p <0.0001) and primary (p <0.0001) follicles, when compared to normal mice (Table 1).

Discussion

The key novel results of the study indicates that GH/IGF-I, which has a central role in somatic and gonadal aging, can also prevent accumulation of DNA damage, further contributing to the younger phenotype observed in GH-deficient mice, pointing to a role of the GH/IGF-I axis in the regulation of oocyte DNA integrity and ovarian macrophage infiltration. GH-deficient df/df mice are known for being smaller-sized and living longer and healthier life than normal littermate controls [2, 49], while bGH mice are known for a bigger-sized body and short lifespan [4]. Our previous study [16], had shown that df/df mice have more ovarian primordial follicles than normal littermates, and that GH treatment increased primordial follicle activation and reduced the ovarian reserve. Additionally, we had shown that bGH mice have decreased primordial follicle reserve. Therefore, our previous results indicated the increased ovarian reserve depletion promoted by GH, and our current findings, showing a role of GH in increasing oocyte and granulosa DNA DSBs and inflammation. Taken together, these results suggest that beyond preserving the follicular reserve for longer periods, GH/IGF-1 axis is involved in

activation or operation of DNA damage repair mechanisms, whose deficiency reduces oocyte DNA damage accumulation, impacting fertility.

DNA DSBs in oocytes and granulosa cells from primordial and primary follicles were reduced in old GH-deficient df/df mice, while GH exogenous treatment increased accumulation of DNA DSBs. In contrast, bGH mice had increased accumulation of DNA DSBs in oocytes and granulosa cells from primordial and primary follicles. DNA damage is a challenge that all somatic and germline cells are exposed to during their lifetime [50]. Cells respond to DSBs as a serious threat to their integrity, activating DNA damage checkpoints (DDC) and reacting with a DNA damage response resulting in cell cycle arrest as a downstream effect, allowing activation of repair mechanisms [51]. DSBs trigger a DDC response by activating two major kinases, i.e. ATM and ataxia telangiectasia and Rad3-related (ATR) [52, 53]. Taking advantage of the cell cycle arrest, the cell can then repair the damaged DNA. At the DNA damage site, ATM gives rise to the phosphorylated form of the H2AX histone, which acts as a catalyst for the recruitment of the necessary checkpoint and repair factors [54]. Snell dwarf mice, which have a mutation at Pituitary Factor1 gene and are endocrinologically identical to df/df mice, have increased cellular DNA repair capacity and upregulation of several DNA repair-related genes compared to normal littermates [55]. Our data, therefore, points to a role for the GH/IGF-I axis in DNA damage in mice oocytes and surrounding granulosa cells from the ovarian follicles. Mouse and human oocytes accumulate DNA DSBs with age which contributes to reproductive aging [22]. Additionally, granulosa cells are essential for oocyte growth and activation, and others have shown increased DNA DSBs in monkey granulosa cells with age [56]. The decrease of DNA DSBs repair is also associated with accumulation of DSBs in human and mouse oocytes [22]. The expression of Breast Cancer 1 protein (BRCA1) and other key genes in the ATM-pathway decline with age in human oocytes, leading to accumulation of DNA DSBs [22]. We previously observed a three-fold reduction in *BRCA1* gene expression with aging in N mice. Its expression did not change with aging in df/df mice [30], further contributing to DSB accumulation coordinated by GH/IGF-I axis. Overall, this paper shows that GH/IGF-I deficiency beyond preserving the ovarian reserve for longer periods, can also contribute to maintain oocyte DNA integrity, increasing the chances of successful pregnancy in older ages.

Old df/df mice exhibited reduced ovarian macrophage infiltration, and the treatment with GH in adult life increased the number of macrophages in the ovary. Moreover, bGH mice had increased macrophage infiltration. This evidence suggests the GH/IGF-I axis as central to regulating ovarian macrophage infiltration. The long-living df/df, growth hormone receptor knockout (GHRKO) mice and calorie restricted mice have all been extensively characterized as having a reduced pro-inflammatory profile, which may represent one of the major mechanisms promoting increased insulin sensitivity and extended longevity in these mice [33]. Macrophages located in the ovaries, by secreting growth factors and/or cytokines, may play a synergistic role in stimulating cellular proliferation and follicle growth [57]. Some of the macrophage-derived factors that are known to impact follicular growth are hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), tumor necrosis factor (TNF) α and β, and IGF-I [58-60]. On the other hand, prolonged exposure (both short-term and long-term) to a high-fat diet in young adult female mice reduced primordial follicle numbers, compromised fertility, produced higher systemic proinflammatory cytokine levels, and increased ovarian macrophage infiltration in the stroma, independent of obesity [47]. Pro-inflammatory Interleukin 1 deficient female mice have more primordial follicles and increased fertility than control females [29]. Also, lipopolysaccharide exposure reduced the primordial follicle pool mediated by toll-like receptor 4 in mice [61], indicating the role of inflammation on ovarian reserve depletion. Our ovarian transcriptome study showed that the top 150 down-regulated terms between old df/df and old N mice were related to the inflammatory/immune response [30]. Macrophage chemotaxis, macrophage activation and macrophage differentiation were also among the top down-regulated biological processes in old df/df compared to old N mice. These findings point to an association between the GH/IGF-1 axis and the inflammationinn ovaries of old df/df mice. For the bGH mice have, there are no data on ovarian inflammation, however, these transgenic mice have an increase pro-inflammatory profile in kidney [62] and age related increased pro-inflammatory markers in blood [63]. Another study with osteopetrotic mice showed reduced numbers of mature macrophages due to a natural mutation in the CSF-1 gene [64]. These osteopetrotic mice have reduced numbers of ovarian macrophages, whereby is not still clear if this is a cause or an effect of the decreased follicle growth on these mice. Our evidence provides an interesting connection between ovarian

longevity and macrophage population coordinated by the GH/IGF-I axis, however, more studies are necessary to understand the role of macrophages in ovarian aging.

The microenvironment within follicles and ovaries also could be influenced by GH/IGF-I deficiency. Our study shows that df/df mice had fewer granulosa cells surrounding oocytes than N/df mice in both primordial and primary follicles, supporting the late activation of primordial follicle observed for df/df mice in a previous study. Moreover, bGH mice had a larger number of granulosa cells compared to normal mice, suggesting that granulosa cell number is an indication of successful follicle activation and development, since bGH mice presented fewer primordial follicles than normal mice. Particularly, granulosa cells and cumulus cells are intimately connected with oocytes, and both play critical roles in oocyte growth and follicular development and could also influence oocyte quality [65, 66]. Oocyte growth is accompanied by the active accumulation of mRNA, proteins, and lipids, and by modifications of chromatin configuration and DNA methylation status, and these molecular reactions require sufficient energy [67]. The number of granulosa cells in follicles is associated with the energy supply to oocytes [67], which can affect oocyte and follicle growth. A complete set of molecular networks that mediate the interaction between somatic cells and germ cells in controlling the development of dormant mammalian oocytes had been described. The model proposed by Zhang et al. (2014) has two key steps. The first step is the mTOR complex 1 (mTORC1) signaling in granulosa cells that acts as the key decision-making process regarding whether or not a primordial follicle will be activated [39]. The second step involves the tightly regulated communication channel from the granulosa cells to the oocytes via KITL-KIT to trigger the awakening of the oocyte through FoxO3a phosphorylation [39]. It is proposed that these processes ensure the progressive activation of a limited number of primordial follicles throughout the reproductive lifespan. It is likely that follicular activation is initiated by molecular and cellular changes in the granulosa cells that are followed by awakening of the dormant oocytes. The regulation of granulosa cell number by GH/IGF-I is in line with our previous work showing that FoxO3a activation is regulated by the GH/IGF-I axis in mice primordial and primary oocytes. Therefore, regulation of granulosa cell number by GH/IGF-I can be a critical point in determining ovarian lifespan.

Conclusion

In conclusion, the present study indicates that GH/IGF-I is one of major modulators of oocyte and granulosa cell DNA damage and ovarian macrophage infiltration. Adding to our previous results, the current study demonstrates that beyond preserving the ovarian primordial reserve, GH/IGF-I deficiency in long-living df/df mice reduces accumulation of DNA damage and inflammation, both factors significantly associated with fertility in older ages. Overall, these observations confirm that the role of GH/IGF-I in the regulation of health span and life span e in these mice is also reflected in ovarian aging.

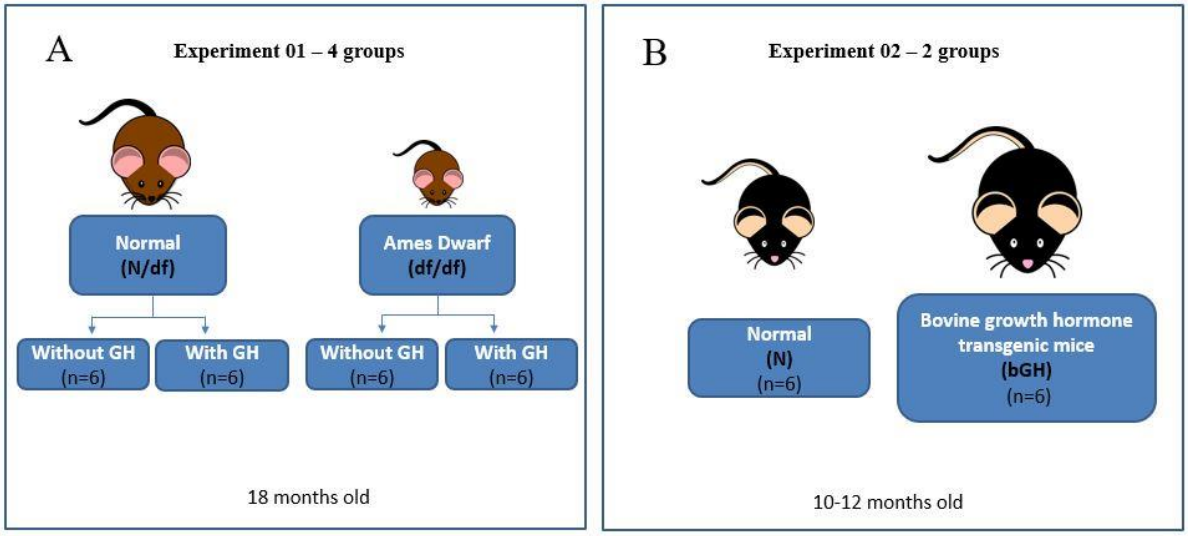

Figure 1. Animal groups used in experiment 01 (A) of N/df and df/df mice treated with growth hormone (GH) and experiment 02 (B) of bGH mice.

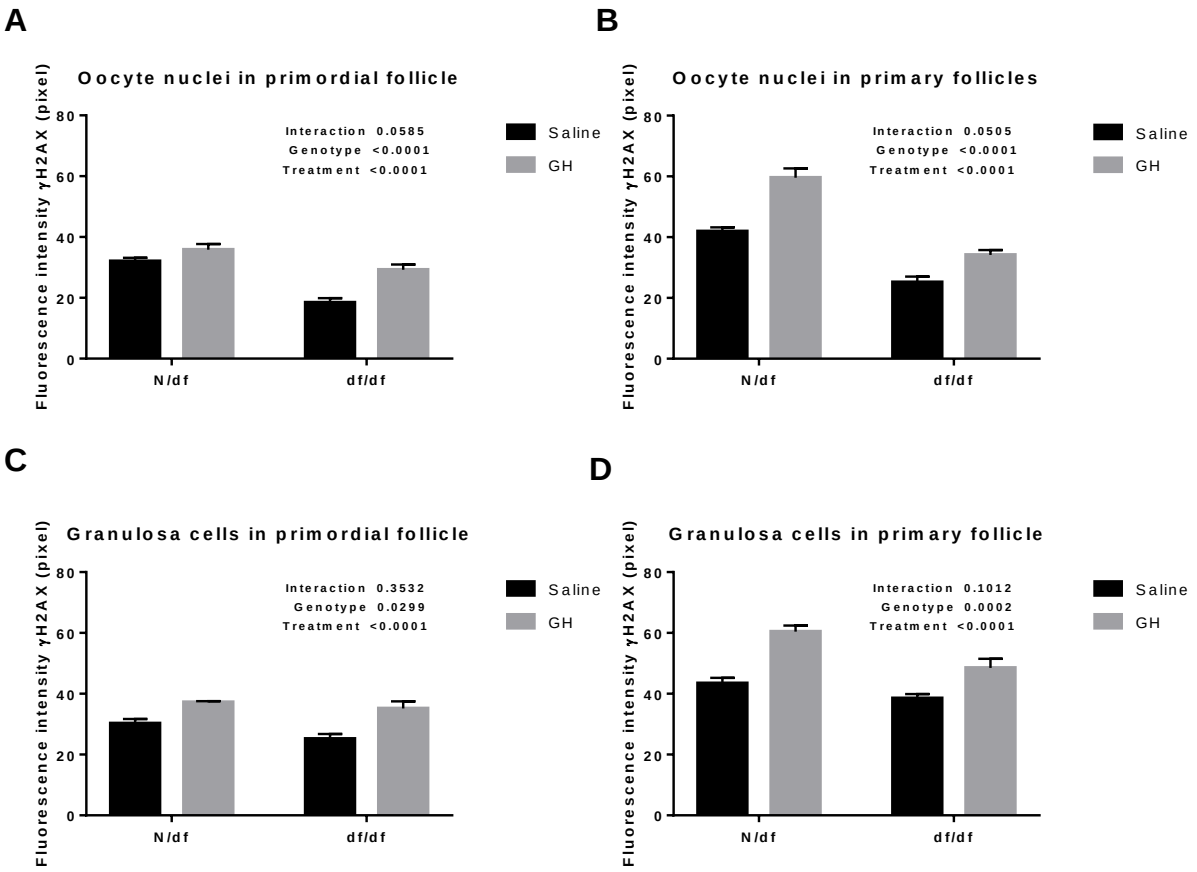

Figure 2. Fluorescence intensity of γH2AX immunostaining in nuclei of oocytes from primordial follicles (n = 18, A), primary folliclse (n = 18, B) and granulosa cell nuclei from primordial (n = 18, C) and primary follicles (n = 18, D) of N/df and df/df mice treated with exogenous GH or saline. Data presented as media ± SEM.

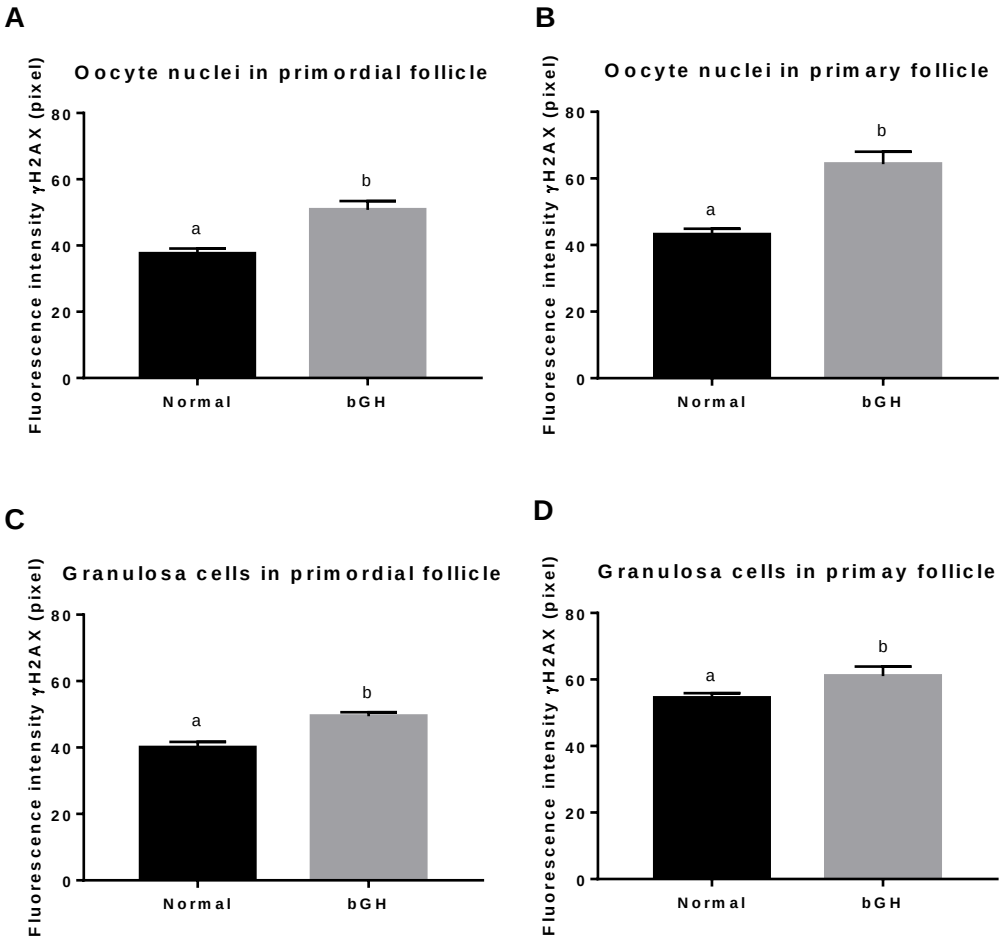

Figure 3. Fluorescence intensity of γH2AX immunostaining in nuclei of oocytes from primordial follicle (n = 18, A), primary follicle (n = 18, B) and granulosa cell nuclei from primordial (n = 18, C) and primary follicles (n = 18, D) of normal and bGH transgenic mice. Different letters indicate significant differences ($p < 0.05$). Data presented as media ± SEM.

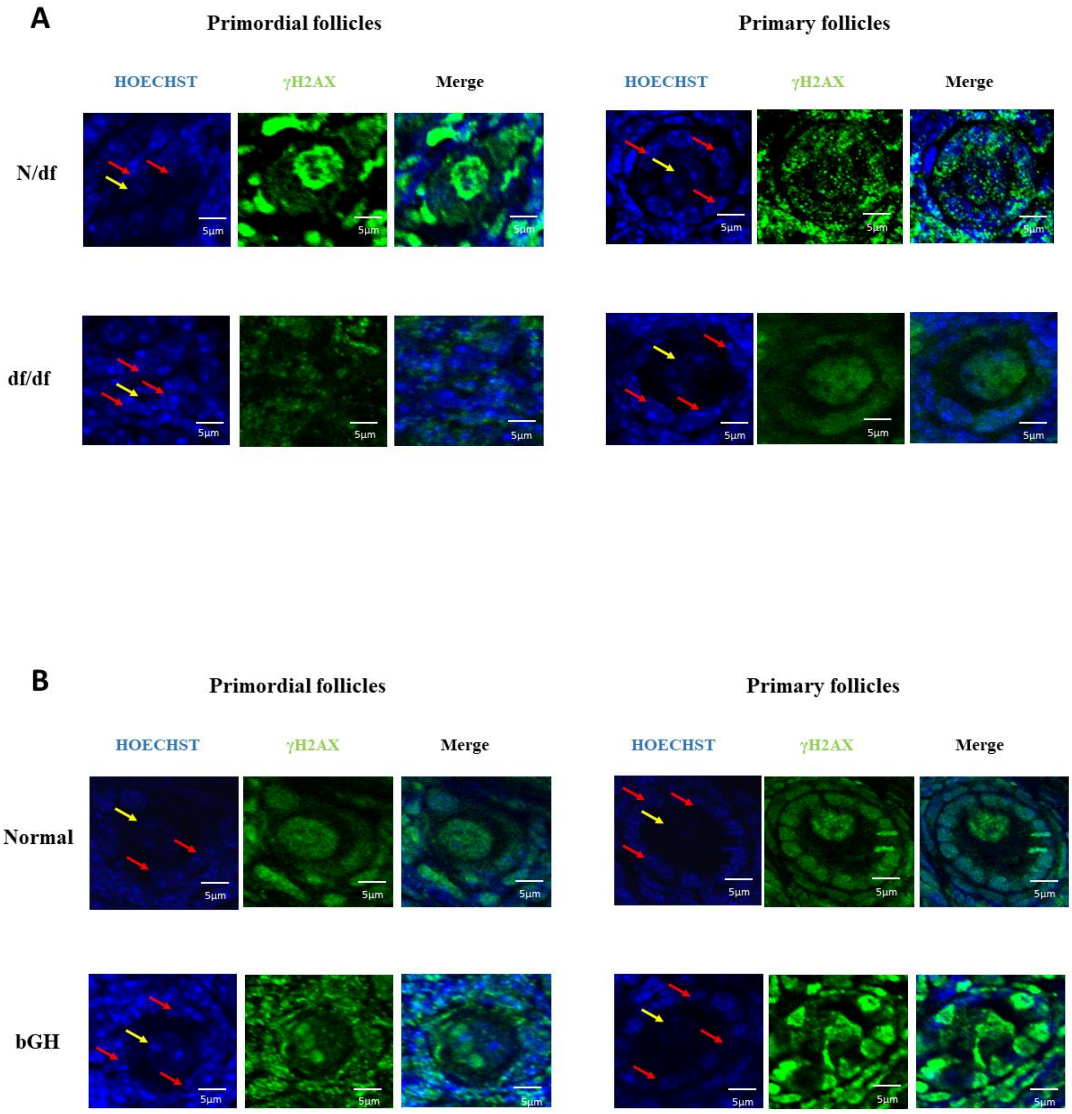

Figure 4. Representative images of immunofluorescence of anti-γH2AX in primordial and primary follicles of N/df and df/df mice – Experiment 1 (A); and normal and bGH mice – Experiment 2 (B). Blue images represent Hoechst (staining genetic material) and green represent anti-γH2AX staining γH2AX protein. Merged images are showed as both Hoechst and anti-γH2AX staining combination. Red arrows represent some granulosa cells and yellow arrow represents oocyte nucleus.

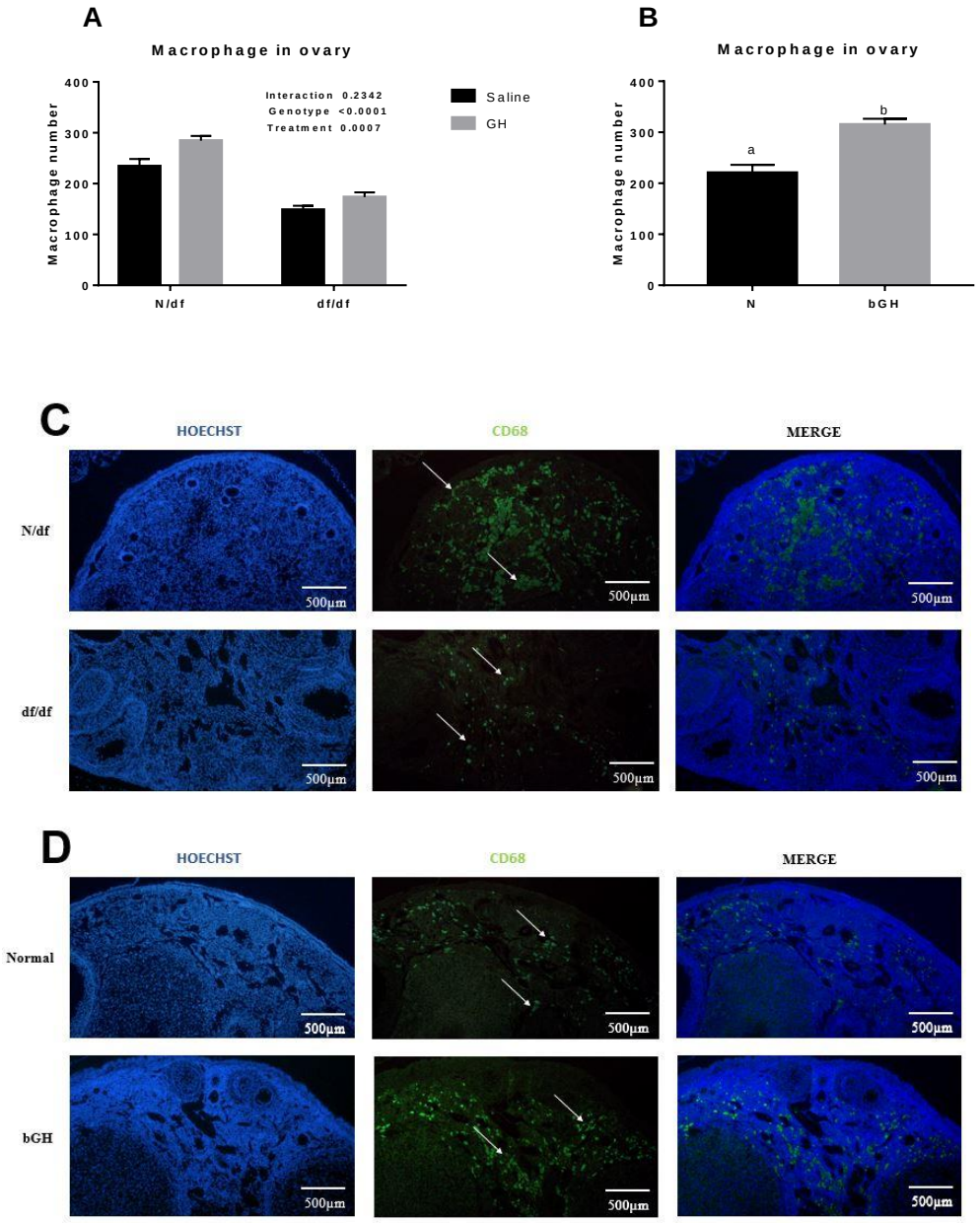

Figure 5. Macrophage number per ovarian section in N/df (n = 24) and df/df (n = 24) mice treated with exogenous GH or saline (A); and macrophage number on ovarian section of normal (n = 24) and bGH (n = 24) transgenic mice (B). Representative images of macrophage infiltration on ovarian section of N/df and df/df (C); normal and bGH transgenic mice (D). White arrow indicates macrophage staining. Different letters indicate significant differences ($p < 0.05$).

Table 1. Number of granulosa cells of df/df and N/df mice treated with exogenous GH (Experiment 1) and bGH transgenic mice (Experiment 2).

					***p*-Value[a]**		
	Granulosa cell number (n=18) (SEM)				**Genot.[1]**	**Treat.[2]**	**Genot* Treat[3]**
Follicle Class	**N/df**		**df/df**				
	Saline	**GH**	**Saline**	**GH**			
Primordial Follicles	6.0 (±0.2)	6.2(±0.2)	4.8(±0.2)	5.4(±0.2)	0.0002*	0.1389	0.5425
Primary Follicles	10.3 (±0.4)	11.4(±0.6	8.8(±0.3)	8.7(±0.3)	<0.0001*	0.2796	0.1590

			***p*-Value[b]**
	Normal	**bGH**	
Primordial Follicles	5.4 (±0.4)	9.9 (±1.1)	<0.0001*
Primary Follicles	8.2 (±0.3)	17.7 (±0.9)	<0.0001*

[a]Two-way Anova test; [b]T-test; *Significant P Value – $p<0.05$

[1]Genotype; [2]Treatment; [3]Genotype * Treatment

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6 CAPITULO 3

Manuscrito 3 - Ovarian reserve and DNA damage on Rhesus monkey submitted to caloric restriction (Conforme normas do periódico Reproductive Biology – este trabalho foi realizado em parceria com um grupo de pesquisa na University of Winsconsin Madison e fará parte de um artigo maior)

Ovarian reserve and DNA damage on Rhesus monkey submitted to caloric restriction

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Abstract

The aim of this study was to evaluate the density of follicles in the ovarian reserve, nucleus, oocyte and follicle diameters and oocyte DNA damage on Rhesus monkey submitted to caloric restriction. Rhesus monkeys were submitted to 30% calorie restriction (CR) and the ovaries collected (n=4, Control group; n=4, Caloric Restriction group). There was no difference in the follicular density of primordial, primary, tertiary follicles between control and CR groups. CR group had higher total and secondary follicular density than control group (p=0.05 and *p*=0.009). Primordial and primary oocytes and granulosa cells from the CR group had lower quantity of DSBs, measured by lower fluorescence intensity for γH2AX. No difference was found in granulosa cells number, nuclei, oocyte and follicle diameter, od primordial and primary follicles. More studies are required to elucidate the effects of caloric restriction on ovarian reserve and oocyte quality of Rhesus monkey.

1. Introduction

The marked anatomical, physiological and behavior similarities between humans and rhesus monkeys (*Macaca mulatta*) make the latter uniquely suited for providing insights into the biology of human ageing. With advancing age, many of the familiar age-related phenotypes exhibited in humans are also observed in rhesus monkey [1-4].

A level of caloric restriction (CR) between 20 and 40% without compromising the ingestion of essential nutrients [5, 6] can preserve the ovarian reserve in female mice and rats [7-9]. Rhesus monkeys, cynomolgus monkeys, and baboons play important role in CR studies because they have higher risks for obesity, insulin resistance, type 2 diabetes, immune decline, and cardiovascular diseases and impaired cognition, which are also observed during aging in humans [10-13]. CR in non-human primates initiated after adulthood was associated to a healthier life and increased insulin sensitivity, decreased hyperglycemia and incidence of type 2 diabetes, preservation of skeletal muscle function and reduced visceral fat [14].

Rhesus monkey initiates puberty at two to four years old and are physically mature at about six to ten years [15]. In the laboratory life expectancy is approximately twenty-six years, 10% survive beyond thirty-five years and maximum life expectancy is approximately forty years [15]. As a natural consequence of aging, females gradually lose the ability to reproduce.

The functional and structural changes in the IGF-I-insulin-ovarian axis that underlie menopause have been the subject of considerable research [16-19]. The reproductive aging process is thought to be dominated by a gradual decrease in both the quantity and the quality of the oocytes residing within the follicles present in the ovarian cortex [20]. The reductions in oocyte quantity and oocyte quality inherent in reproductive ageing result in clinical subfertility and increased miscarriage rates in women of advanced maternal age [21]. Several factors can influence oocyte quality during aging. The adverse effects of DNA damage caused by environmental factors on somatic cells have been extensively reported [22].

Mammalian oocytes are trapped in primordial stage and remain arrested in prophase I of meiosis up to several decades in some species, including humans [23, 24]. This long period of dormancy increases the chances of the oocyte accumulating DNA damage, as shown in mice and humans, both of which, accumulate double strand breaks (DSBs) in primordial follicle oocytes with aging [25]. As a reaction to DSBs, kinases phosphorylate histone 2Ax (γH2AX) on serine 139 [26, 27].

Based on this evidence, the aim of this study was to evaluate the density of follicles in the ovarian reserve, nucleus, oocyte and follicle diameters and DNA damage in rhesus monkey submitted to 30% CR.

2. **Methodology**

All rhesus monkeys (*Macaca mulatta*) were of Indian origin, were born at the WNPRC and were adults (7–14 years old), then introduced into the study[28]. The animals were evenly matched and randomized to control or CR diets taking into consideration baseline food intake, body weight and age. Individualized food allotments were calculated based on daily food intake data that had been collected for each animal over a 3–6-month period before the start of this study. Once animals were assigned to either control or CR group, each CR animal individually determined baseline intake was reduced by 10% per month over a 3-month period to reach the desired 30% restriction. Animals were fed a semipurified, nutritionally fortified, low-fat diet containing 15% protein and 10% fat. As part of the study design, animals were treated for presenting conditions. This study was conducted in accordance with protocols approved by the University of Wisconsin–Madison Graduate School Institutional Animal Care and Use Committee. The study design was described by Kemnitz et. Al [28]. For this study six ovaries

were used, four for the control group (16, 17, 25 and 31 years old) and four for the caloric restriction group (CR) (27, 23, 24 and 29 years old).

Ovaries were collected in 10% phosphate buffered formalin. Part of each ovary was sectioned to a thickness of 5μm with a semi-automatic microtome (RM2245, Leica Biosystems, San Diego, CA, USA). To calculate the density of follicles, a previously described methodology was used [29]. All follicles with apparent nucleus were considered every two sections. Twenty-five sections in total was considered. The total number of follicles was calculated taking in consideration the total number of sections and a correction factor, which in this case is number 2, expressing that the follicular count was performed every two sections. Follicular density was calculated based on to the number of follicles, the area and the thickness of each section.

For immunofluorescence analysis, the ovarian samples were deparaffinized with xylene and rehydrated with graded alcohols. The primary monoclonal antibody was obtained from Abcam (Abcam Plc, Cambrigde, UK) and diluted in 1.5% BSA solution. The anti-gamma H2AX (γH2AX) phospo S139 antibody (ab11174), to indicate DNA damage [25] was used at a final dilution of 1:500. The images of the follicles were captured by confocal microscope (Olympus FluoView™ 1000**).** Fluorescence intensity quantification for γH2AX, measured as pixel intensity in the nuclei area [30], and macrophage counting, measured as number of identified cells/slide, was performed by image analysis software Image J® [31]. γH2AX was measured in 3 follicles/animal for each category (primordial, primary). The number of granulosa cells was also counted in 3 follicles/ category/animal, using the Hoechst (ab228550) for nuclei staining of surrounding granulosa cells [32].

All statistical analyzes were performed using GraphPad Prism 6 (GraphPad Inc., La Jolla, CA, USA). *T*-test was performed for comparing the follicular density and size of follicles and immunostaining between CR and control group. A *p*-value lower than 0.05 was considered as significant.

3. **Results and discussion**

Rhesus monkey submitted to 30% CR had lower quantity of DSBs, measured by lower fluorescence intensity for γH2AX in oocytes nucleus from primordial ($p = 0.005$, Figure 1A) and primary ($p = 0.0031$, Figure 1B) follicles compared to the control group. Also, CR group had reduced γH2AX intensity in granulosa cells of primordial ($p = 0.0002$, Figure 1C) and

primary ($p < 0.0001$, Figure 1D) follicles. Representative images of immunofluorescence of anti-γH2AX are shown in Figure 2. DNA damage in oocytes and granulosa cell can be caused by various endogenous or exogenous stresses, including oxidative stress, telomere erosion, oncogenic mutations, genotoxic stress, and metabolic stress [33]. DNA damage is a challenge that all somatic and germline cells are exposed during their lifetime [34]. The impairment of DNA DSBs repair is associated with accelerated loss of ovarian follicular reserve and accumulation of DSBs in human and mouse oocytes [25]. Other have also observed in monkeys that DSBs increase with age in granulosa cells [35], also suggesting this damage is not confined to the oocyte as we observed. Mammalian cells have developed complex mechanisms to identify DNA damage and activate the required response to maintain genome integrity. Those mechanisms include DNA damage detection, DNA repair, cell cycle arrest and apoptosis which operate together to protect the conceptus from DNA damage originating either in parental gametes or in the embryo's somatic cells [36]. Extensive damage occurring throughout meiosis can have serious consequences if an adequate cellular response is not activated, and may result in infertility or development of defective embryos, which can developed in to a failure blastocyst formation, nuclear fragmentation into apoptotic bodies, and failures in spindle formation, that are unable to result in full-term pregnancy [37, 38]. Therefore, it is suggested that maintenance of high levels of DNA protecting factors could be a key element for preserving the ovarian reserve for a longer period.

No difference was observed in follicular density for primordial, primary and tertiary follicles ($p>0.05$). Density of secondary follicles was higher in CR group (104.7 ±21.8 follicles/mm^3) compared to control group (22.1 ±7.07 follicles/mm^3) (p=0.009). Additionally, density of total follicles was higher in CR than control monkeys ($p=0.05$). Also, no difference was found in nuclei, oocyte and follicular diameter for primordial and primary follicles ($p>0.05$). About the granulosa cells number, no difference was found in primordial and primary follicles ($p>0.05$) (Table 1). During the menopausal transition of rhesus females, there is a marked variability in levels of reproductive hormones [39, 40]. Reproductive hormone variability may be a result of follicular depletion as proposed for the human female [41]. As the female ages, there is a shift and decrease in overall follicular hormonal signaling, partly due to decreases in total numbers of follicles and possibly also due to the quality of remaining oocytes [42]. Several studies with rats and mice had shown that CR increase in the number of primordial

follicles in females [8, 43, 44], which is associated with reduced levels of circulating insulin and IGF-I [43]. GH-deficient Ames dwarf mice had almost four times more primordial follicles than N/df mice at six-months old (Saccon et al, 2020 – in press). Also, evaluating 12-month-old df/df mice, we observed three times more primordial follicles [45] and twice as many primordial follicles at 18-months-old df/df mice [46]. This suggests that the difference in the ovarian primordial reserve between df/df and N counterparts decreased with aging, as the reserve becomes smaller. It is possible that these rhesus monkey females undergo menopause, making follicular count difficult at an advanced age due the low counting of follicles number in some samples, showing no difference of primordial follicles between groups. However, CR group presented almost five times more secondary stage follicles than control group. This shows that even in this advanced age, CR group presented more ovarian activity than control group.

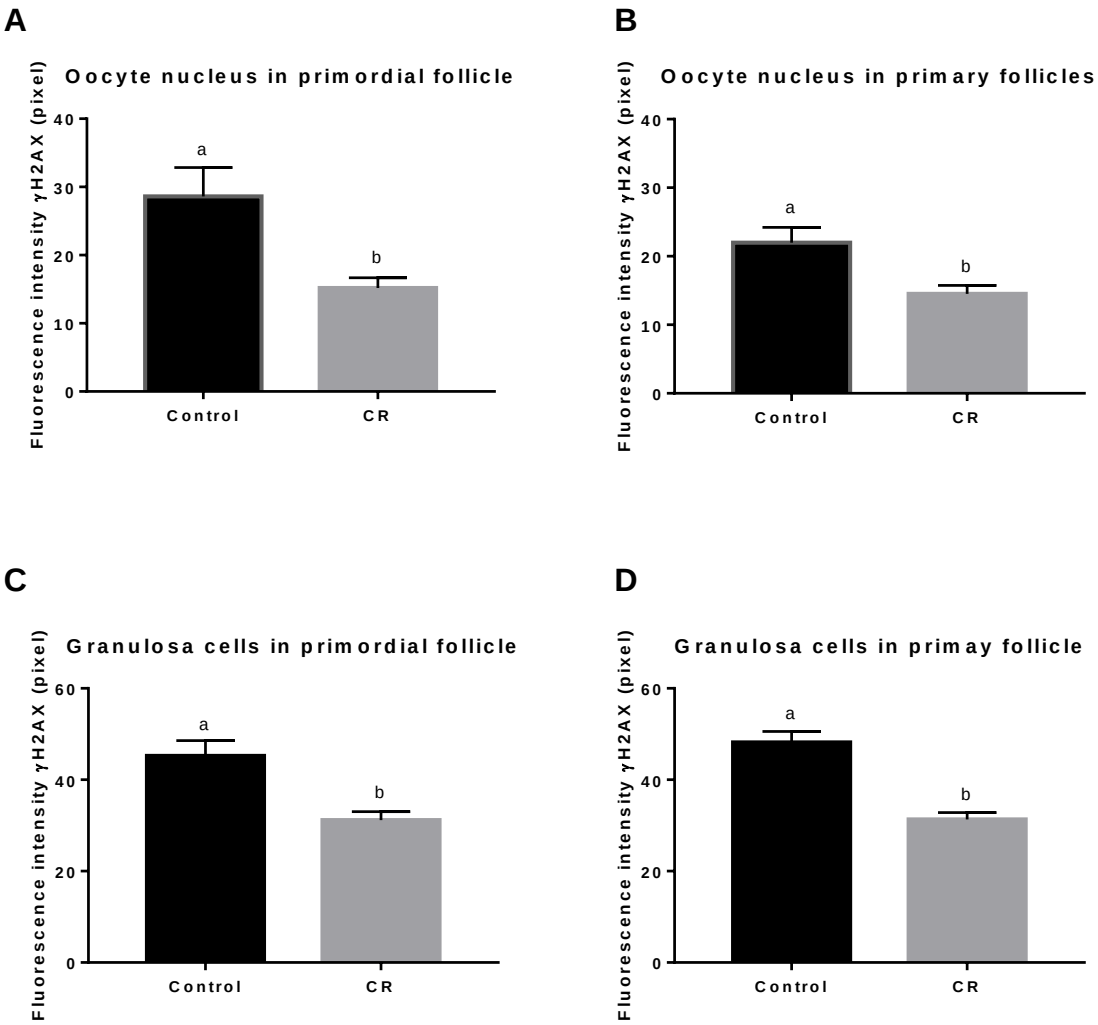

Figure 1. Fluorescence intensity in pixels of γH2AX immunostaining in oocytes nucleus of primordial follicles (n = 18, A), oocyte nucleus of primary follicle (n = 18, B) and granulosa cell nuclei from primordial (n = 18, C) and primary follicles (n = 18, D) of Rhesus monkeys, goup control and CR. Different letters indicate significant differences ($p < 0.05$). Data presented as media ± SEM.

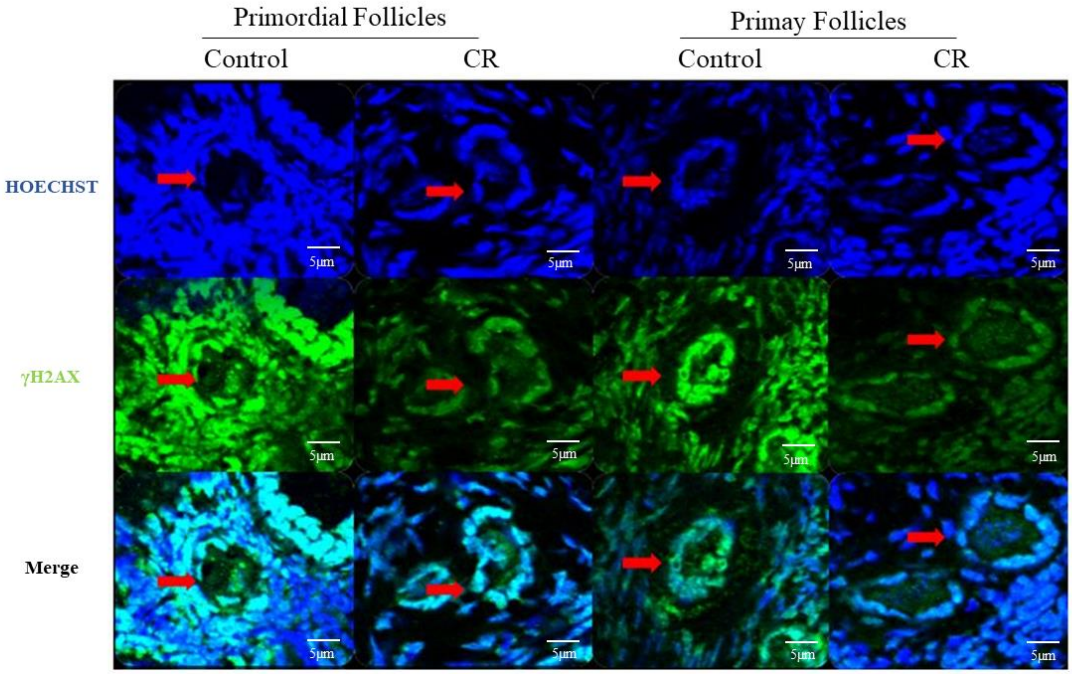

Figure 2. Representative images of immunofluorescence of anti-γH2AX in primordial and primary follicles of Rhesus monkey ovaries from control group and CR group. Blue images represent Hoechst (staining genetic material) and greens represents anti-γH2AX staining γH2AX protein. Merged images are showed as both Hoechst and anti-γH2AX staining combination. Red arrows indicate the follicles.

Table 1. Primordial and primary follicular density, diameters and granulosa cells number in ovaries of Rhesus monkey submitted to caloric restriction.

	Control	**CR**	***p*-Value**
Folicular density (follicles/mm^3)	**(n=4)**	**(n=4)**	
Primordial Follicles	35.4 (±27.6)	157 (±110)	0.44
Primary Follicles	38.8 (±10.1)	121 (±71.2)	0.26
Secondary Follicles	22.1 (±7.07)	104.7 (±21.8)	0.01
Tertiary Follicles	3.6 (±1.9)	4.7 (±1.8)	0.70
Total follicles	99.9 (±39.1)	490.3(±178.1)	0.05
Granulosa cells number (SEM)	**(n=12)**	**(n=12)**	
Primordial Follicles	9.5 (±0.5)	10.2 (±0.7)	0.30
Primary Follicles	19.2 (±1.7)	19.9 (±2.9)	0.77
Diameters (µm) (SEM)	**(n=12)**	**(n=12)**	
Primordial Follicle			
Nuclei diameter (µm)	8.9 (±04)	9.6 (±1.1)	0.53
Oocyte diameter (µm)	23.2 (±1.5)	23.4 (±1.2	0.99
Follicle diameter (µm)	37.2 (±2.6)	37.5 (±2.2)	0.92
Primary Follicle			
Nuclei diameter (µm)	15.9 (±1.2)	13.6 (±1.5)	0.3
Oocyte diameter (µm)	40.7 (±2.1)	31.8 (±6.1)	0.28
Follicle diameter (µm)	76.3 (±6.9)	64.6 (±4.1)	0.15

4. Conclusion

In conclusion, the present study indicates that oocytes from the primordial and primary follicles of Rhesus monkey submitted to CR presented fewer DSBs than control group, indicating that oocytes and granulosa cells accumulate less DNA damage. Additionally, the number of total follicles in the ovary was greater in the CR group. More studies are required to elucidate the effects of caloric restriction on ovarian reserve and oocyte quality of Rhesus monkey.

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7 CONCLUSÃO GERAL

Em conclusão, podemos observar que o eixo GH/IGF-I está associado ao envelhecimento ovariano, demostrado pela maior reserva ovariana, presença diminuída de danos de DNA e infiltração de macrófagos em camundongos df/df jovens e velhos, além do tratamento com GH exógeno reverter essas características. Além disso, a restrição calórica em macacos Rhesus, previne danos no DNA de oócitos e células da granulosa em folículos primordiais e primários, demostrando o benefício da restrição na qualidade oocitária. Portanto, estes estudos mostraram que a ação dos fatores de crescimento na reserva ovariana podem ser um importante fator regulador do envelhecimento ovariano em várias espécies mamíferas.

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9 ANEXOS

Anexo A – Cartas de aprovação do comitê de ética (University of Central Florida – Orlando, FL, US)

UNIVERSITY OF CENTRAL FLORIDA
RESEARCH & COMMERCIALIZATION

4/17/2017

Dr. Michal Masternak
Burnett School of Biomedical Sciences
Lake Nona
6900 Lake Nona Blvd
Orlando, Fl 32827

Subject: Institutional Animal Care and Use Committee (IACUC) Protocol Submission

Dear Dr. Michal Masternak:

This letter is to inform you that your following animal protocol was re-approved by the IACUC. The IACUC Animal Use Renewal Form is attached for your records.

Animal Project #:	15-26
Title:	1. MicroRNA-mediated Mechanisms of Insulin Sensitivity in the Ames Dwarf Mouse Model 2. Role of Caloric Intake During Development on Healthy Metabolism
First Approval Date:	5/20/2015

Please be advised that IACUC approvals are limited to one year maximum. Should there be any technical or administrative changes to the approved protocol, they must be submitted in writing to the IACUC for approval. Changes should not be initiated until written IACUC approval is received. Adverse events should be reported to the IACUC as they occur. Furthermore, should there be a need to extend this protocol, a renewal must be submitted for approval at least three months prior to the anniversary date of the most recent approval. If the protocol is over three years old, it must be rewritten and submitted for IACUC review.

Should you have any questions, please do not hesitate to call the office of Animal Welfare at (407) 882-1164

Please accept our best wishes for the success of your endeavors.

Best Regards,

Cristina Caamaño
Associate Director, Research Program Services

Copies: Facility Manager (when applicable.)

THE UNIVERSITY OF CENTRAL FLORIDA
INSTITUTIONAL ANIMAL CARE and USE COMMITTEE (IACUC)
Re-Approval to Use Animals

Dear Dr. Michal Masternak,

Your application for IACUC Re-Approval has been reviewed and approved by the UCF IACUC Reviewers.

Approval Date: 4/17/2017

Title: 1. MicroRNA-mediated Mechanisms of Insulin Sensitivity in the Ames Dwarf Mouse Model
2. Role of Caloric Intake During Development on Healthy Metabolism

Department: Burnett School of Biomedical Sciences

Animal Project #: 15-26

Expiration: 5/19/2018

You may purchase and use animals according to the provisions outlined in the above referenced animal project. This project will expire as indicated above. You will be notified 2-3 months prior to your expriation date regarding your need to file another renewal.

Deborah A Altomare

Deborah Altomare, Ph.D.
IACUC Chair

UNIVERSITY OF CENTRAL FLORIDA
RESEARCH & COMMERCIALIZATION

9/7/2018

Dr. Michal Masternak
Burnett School of Biomedical Sciences
Lake Nona
6900 Lake Nona Blvd
Orlando, Fl 32827

Subject: Institutional Animal Care and Use Committee (IACUC) Protocol Submission

Dear Dr. Michal Masternak:

This letter is to inform you that your following animal protocol was approved by the IACUC. The IACUC Use Approval Form is attached for your records.

Animal Project #: 18-36

Title: Role of GH/IGF1 signaling pathway on pro-longevity miRNAs

First Approval Date: 9/6/2018

Please be advised that IACUC approvals are limited to one year maximum and must be renewed annually. Should there be any technical or administrative changes to the approved protocol, they must be submitted in writing to the IACUC for approval. Changes should not be initiated until written IACUC approval is received. Adverse events should be reported to the IACUC as they occur.

If the protocol is over three years old, it must be rewritten and submitted for IACUC review and approval. A renewal must be submitted for approval at least three months prior to the anniversary date of the most recent approval.

This letter does not serve as Environmental Health and Safety (EHS) approval. EHS approval must be handled separately. EHS approval must be obtained prior to initiating animal use or procedures that need EHS approval. The contact phone number is 407-823-6300

Should you have any questions, please do not hesitate to call the office of Animal Welfare at (407) 882-1164.

Please accept our best wishes for the success of your endeavors.

Best Regards,

Cristina Caamaño
Associate Director
Office of Animal Welfare
Copies: Appropriate Facility Manager (when applicable)

THE UNIVERSITY OF CENTRAL FLORIDA
INSTITUTIONAL ANIMAL CARE and USE COMMITTEE (IACUC)
Approval to Use Animals

Dear Dr. Michal Masternak,

Your application for IACUC Approval has been reviewed and approved by the UCF IACUC Reviewers.

Approval Date:	9/6/2018
Title:	Role of GH/IGF1 signaling pathway on pro-longevity miRNAs
Department:	Burnett School of Biomedical Sciences
Animal Project #:	18-36
Expiration:	9/6/2019

You may purchase and use animals according to the provisions outlined in the above referenced animal project.

Deborah A Altomare

Deborah Altomare, Ph.D.
IACUC Chair

Contact: Cristina Caamaño, Associate Director, Office of Animal Welfare -Email:ccaamano@ucf.edu
Phone: (407) 266-2235 Fax: (407) 266-7235