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CARACTERIZAÇÃO MOLECULAR DOS COMPONENTES DO SISTEMA ANGIOTENSINA-(1-7) DURANTE A DIVERGÊNCIA FOLICULAR E EXPRESSÃO DE GENES DE REPARO DA FITA DUPLA DE DNA EM EMBRIÕES BOVINOS

TESE DE DOUTORADO

Marcos Henrique Barreta

Santa Maria, RS, Brasil

2012

CARACTERIZAÇÃO MOLECULAR DOS COMPONENTES DO SISTEMA ANGIOTENSINA-(1-7) DURANTE A DIVERGÊNCIA FOLICULAR E EXPRESSÃO DE GENES DE REPARO DA FITA DUPLA DE DNA EM EMBRIÕES BOVINOS

Marcos Henrique Barreta

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Orientador: Prof. Paulo Bayard Dias Gonçalves

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elaborada por
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como requisito parcial para obtenção do grau de
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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

CARACTERIZAÇÃO MOLECULAR DOS COMPONENTES DO SISTEMA ANGIOTENSINA-(1-7) DURANTE A DIVERGÊNCIA FOLICULAR E EXPRESSÃO DE GENES DE REPARO DA FITA DUPLA DE DNA EM EMBRIÕES BOVINOS

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ORIENTADOR: PAULO BAYARD DIAS GONÇALVES
Data e Local da Defesa: Santa Maria, 24 de Fevereiro de 2012.

O primeiro estudo caracterizou a expressão do receptor MAS e de enzimas responsáveis pela produção de Ang-(1-7), tais como, enzima conversora de angiotensina 2 (ACE_2), endopeptidase neutra (NEP) e prolil endopeptidase (PEP) durante o desenvolvimento folicular. Além disso, a regulação local do sistema Ang-(1-7) foi avaliada após a injeção intrafolicular de fulvestrant (inibidor do receptor de estradiol) no folículo dominante. As vacas foram ovariectomizadas quando o tamanho entre o maior (F1) e o segundo maior folículo (F2) não era estatisticamente diferente (D2), ligeiramente (D3) ou marcadamente diferente (D4). A expressão de RNAm do receptor MAS, ACE_2, NEP e PEP foi avaliada nas células foliculares do F1 e F2. O receptor MAS foi mais expresso nas células da granulosa do F2 após o estabelecimento da divergência folicular (D4), enquanto a expressão de PEP aumentou durante (D3) e após (D4) o processo de divergência. Entretanto, a expressão de ACE_2 foi maior nas células da granulosa do F1 durante e após a divergência. A expressão de PEP não foi regulada no F1 e F2. O receptor MAS foi imunolocalizado nas células da teca e granulosa do F1 e F2 durante a divergência folicular. A expressão de RNAm do receptor MAS aumentou quando o F1 foi tratado com fulvestrant *in vivo*. Em conclusão, o perfil de expressão do receptor MAS, ACE_2, NEP e PEP nos folículos dominante e subordinado indicam que a Ang-(1-7) apresenta uma função na regulação da dominância folicular em bovinos. Em um segundo estudo investigamos a expressão de genes que controlam o reparo do DNA através das vias de recombinação homóloga (HR; 53BP1, ATM, RAD50, RAD51, RAD52, BRCA1, BRCA2, NBS1) e união terminal não homóloga (NHEJ; KU70, KU80, DNAPK) em embriões bovinos com alta, média ou baixa competência de desenvolvimento. Foi também avaliado se embriões bovinos podem responder a quebra na fita dupla de DNA (DSBs), induzida por irradiação UV, através da regulação de genes envolvidos nas vias de reparo HR e NHEJ. Embriões com alta, média ou baixa competência de desenvolvimento foram selecionados pelo tempo de clivagem após a fertilização in vitro e foram removidos do cultivo antes (36 h), durante (72 h) ou após (96 h) o momento esperado para a ativação do genoma embrionário (AGE). Todos os genes foram expressos antes, durante e após a AGE independentemente da competência de desenvolvimento dos embriões. A expressão de 53BP1 e RAD52 foi maior antes da AGE em embriões com baixa competência de desenvolvimento. A expressão de 53BP1, RAD51 e KU70 foi mais baixa as 72 h e maior as 168 h pós fertilização em embriões com DSBs induzida por irradiação UV. Em conclusão, genes importantes para o controle das vias de reparo HR e NHEJ são expressos em embriões bovinos independentemente do tempo de cultivo ou da competência de desenvolvimento. A menor competência de desenvolvimento embrionário parece estar associada com maior expressão de 53BP1 e RAD52. Os embriões bovinos respondem a DSBs após a AGE mas as vias HR e NHEJ são reguladas principalmente no estágio de blastocisto.

Palavras chave: MAS. ECA_2. PEP. Recombinação homóloga. NHEJ.

ABSTRACT

Tese de Doutorado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

MOLECULAR CHARACTERIZATION OF THE ANGIOTENSIN-(1-7) SYSTEM COMPONENTS DURING FOLLICULAR DEVIATION AND EXPRESSION OF DNA DOUBLE-STRANDED REPAIR GENES IN BOVINE EMBRYOS

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ADVISOR: PAULO BAYARD DIAS GONÇALVES
Date and Place of Defense: Santa Maria, February 24th, 2012.

The first study characterized the expression of MAS receptor and key enzymes for Ang-(1-7) production, such as, ACE_2, NEP and PEP during follicular development. Furthermore, the regulation of local Ang1-7 system was evaluated after the intrafollicular injection of fulvestrant (an estradiol-receptor inhibitor) in the dominant follicle. Cows were ovariectomized when the size between the largest (F1) and the second largest follicle (F2) was not statistically different (Day 2), slightly different (Day 3), or markedly different (Day 4). The mRNA abundance of genes encoding MAS receptor, ACE_2, NEP and PEP was evaluated in the follicular cells from F1 and F2. The mRNA expression of MAS receptor was upregulated in the granulosa cells of F2 after the establishment of follicular deviation (Day 4), while PEP mRNA increased during (Day 3) and after (Day 4) the deviation process. However, the mRNA expression of ACE_2 was upregulated in the granulosa cells of F1 during and after the deviation process. The mRNA expression of NEP was not regulated in F1 and F2. The MAS receptor was immunolocated in the granulosa and theca cells of F1 and F2 during follicular deviation. Moreover, MAS receptor gene expression increased when the F1 was treated with the estrogen receptor-antagonist *in vivo*. In conclusion, the expression profile of MAS receptor, ACE_2, NEP and PEP in dominant and subordinate follicles indicated that Ang-(1-7) play a role in the regulation of the follicular dominance in cattle. A second study was performed to investigate the expression of genes that control homologous recombination (HR; 53BP1, ATM, RAD50, RAD51, RAD52, BRCA1, BRCA2 and NBS1), and non-homologous end-joining (NHEJ; KU70, KU80 and DNAPK), DNA-repair pathways in bovine embryos with high, intermediate or low developmental competence. We also evaluated whether bovine embryos can respond to DNA double-stranded breaks (DSBs) induced by ultraviolet (UV) irradiation by regulating the expression of genes involved in the HR and NHEJ repair pathways. Embryos with high, intermediate or low developmental competence were selected based on the cleavage time after in vitro fertilization and were removed from in vitro culture before (36 h), during (72 h) and after (96 h) the expected period of embryonic genome activation (EGA). All studied genes were expressed before, during and after the EGA period regardless the developmental competence of the embryos. Higher mRNA expression of 53BP1 and RAD52 was found before EGA in embryos with low developmental competence. Expression of 53BP1, RAD51 and KU70 was downregulated at 72 h and upregulated at 168 h post-fertilization in bovine embryos with DSBs induced by UV irradiation. In conclusion, important genes controlling HR and NHEJ repair pathways are expressed in bovine embryos before, during or after EGA. Lower developmental competence seems to be associated with a higher mRNA expression of 53BP1 and RAD52. Bovine embryos can response to UV-induced DSBs after the EGA but HR and NHEJ repair pathways seem to be particularly regulated at the blastocyst stage.

Key words: MAS. ECA_2. PEP. Homologous recombination. NHEJ.

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LISTA DE ABREVIATURAS E SIGLAS

53BP1 – p53 binding protein 1

ACE_2 – Angiotensin-converting enzyme II

Ang-(1-7) – Angiotensin-(1-7)

ATM – Ataxia telangiectasia mutated

BRCA1 – Breast cancer 1

BRCA2 – Breast cancer 2

DNAPK – DNA-dependent protein kinase

DSBs – DNA double-stranded breaks

EGA – Embryionic genome activation

F1 – Largest follicle

F2 – Second largest follicle

hpf – Hours post-fertilization

HR – Homologous recombination

KU70 – 70 kDa subunit of Ku antigen

KU80 – 80 kDa subunit of Ku antigen

MAS – Receptor MAS

NBS1 – Nijmegen breakage syndrome 1

NEP – Neutral endopeptidase

NHEJ – Non-homologous end-joining

PEP – Prolyl endopeptidase

RAD50 – DNA repair protein RAD50

RAD51 – DNA repair protein RAD51

RAD52 – DNA repair protein RAD52

UV – Ultraviolet irradiation

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1. INTRODUÇÃO

Está bem estabelecido que o crescimento folicular em espécies monovulatórias ocorre em ondas sendo primariamente orquestrado por fatores endócrinos, principalmente gonadotrofinas (FSH e LH), seus receptores (FSHr e LHr) e esteroides ovarianos. A fase de divergência folicular é caracterizada por uma diminuição dos níveis plasmáticos de FSH e diminuição da capacidade esteroidogênica dos folículos subordinados com consequente início do processo de atresia (GINTHER et al., 1996). Durante a fase em que os níveis de FSH atingem o seu nível mais baixo, vários genes são diferencialmente expressos no microambiente folicular para permitir a sobrevivência das células da teca e da granulosa permitindo que o folículo dominante se torne "independente de FSH" e continue seu crescimento (MIHM et al., 2006). Recentemente, o foco sobre o microambiente folicular tem aumentado e estudos demonstram que alterações ocorridas nesse ambiente são responsáveis pelo ajuste fino do desenvolvimento folicular (FORTUNE et al., 2004; KNIGHT & GLISTER, 2006; MIHM et al., 2008). Entretanto, o controle autócrino e parácrino do desenvolvimento folicular ainda é pouco compreendido em espécies monovulatórias.

Participando da busca pelo conhecimento dos fatores locais que atuam no microambiente folicular, nosso grupo iniciou uma série de estudos investigando o papel do sistema renina-angiotensina (RAS) durante o crescimento folicular, ovulação e maturação oocitária em bovinos. Esses estudos demonstram que angiotensina II (Ang II) apresenta um papel indispensável para o crescimento folicular (PORTELA et al., 2008; FERREIRA et al., 2011a; FERREIRA 2011b; para revisão GONÇALVES et al., 2012), ovulação (FERREIRA et al., 2007; PORTELA et al., 2011) e maturação nuclear de oócitos bovinos (GIOMETTI et al., 2005; BARRETA et al., 2008). No entanto, alguns trabalhos têm sugerido que a Ang II não é o único peptídeo ativo do RAS capaz de controlar eventos fisiológicos em diversos microambientes locais. Vários estudos têm demonstrado que a angiotensina-(1-7) (Ang-(1-7)), ao contrário do que se pensava inicialmente, possui uma atividade biológica. Esse heptapeptídeo atua sobre os sistemas nervoso central, renal e cardiovascular, além de funcionar como um regulador de desenvolvimento dos tecidos vasculares (para revisão SANTOS et al., 2000). Recentemente, foi demonstrado que camundongos *knockout* para o receptor MAS apresentam uma marcada redução na produção espermática diária (LEAL et al., 2009). No ovário, a Ang-(1-7) induziu um aumento na concentração de estradiol e

progesterona em ovários de ratas (COSTA et al., 2003). Também foi demonstrado que a expressão de RNAm do receptor MAS e da enzima conversora de Ang II (ACE_2) aumentou em ovários de ratas tratadas com eCG (PEREIRA et al., 2009), sugerindo uma possível regulação mediada por gonadotrofinas. Entretanto, a função da Ang-(1-7) no ovário é pouco conhecida. Portanto, o primeiro trabalho apresentado neste manuscrito foi desenvolvido com o objetivo de caracterizar a expressão do receptor MAS e das principais enzimas responsáveis pela produção de Ang-(1-7), tais como ACE_2, endopeptidase neutra (NEP) e prolil endopeptidase (PEP) durante o desenvolvimento folicular de bovinos.

O segundo artigo apresentado nesta tese foi dedicado ao estudo de genes envolvidos no reparo da fita dupla de DNA em embriões bovinos produzidos *in vitro* (PIV). Ao longo das últimas décadas, a PIV de embriões tem sido utilizada como um modelo básico de pesquisa em fisiologia, biologia celular e para aplicações comerciais. Entretanto, os resultados de pesquisa não têm demonstrado um incremento significativo na eficácia da PIV de embriões bovinos ao longo dos anos, uma vez que, apenas 30 a 40% dos oócitos maturados *in vitro* atingem o estágio de blastocisto (XU et al., 1992; RIZOS et al., 2002; GOOVAERTS et al., 2009; FIELDS et al., 2011). Na tentativa de melhorar a eficiência dos sistemas de PIV de embriões, uma série de alterações nos meios ou condições de cultivo *in vitro* foram realizadas ao longo dos anos. Porém, o sucesso desse tipo de abordagem tem sido limitado, embora os resultados tenham gerado benefícios no que tange a simplificação dos sistemas de PIV de embriões.

Um estudo recente demonstrou que aproximadamente 30% dos blastocistos bovinos PIV tem lesões de DNA (STURMEY et al., 2009). Se levarmos em consideração que a maioria dos oócitos maturados *in vitro* não passam do estágio de 4 a 8 células (MEIRELLES et al., 2004; LEIDENFROST et al., 2011), pode-se suspeitar então que o número de estruturas com danos de DNA pode ser superior a apontada por Sturmey et al. (2009) se zigotos de 2 a 8 células fossem avaliados. Alguns pesquisadores acreditam que o bloqueio do desenvolvimento embrionário durante as primeiras clivagens possa ser devido a incapacidade do zigoto reparar danos de DNA durante o cultivo *in vitro* (BETTS & KING, 2001; MEIRELLES et al., 2004).

O DNA está sujeito à uma série de injurias e a quebra fita dupla está entre os mais perigosos indutores de danos genotóxico e de morte celular via apoptose (RICH et al., 2000). A presença de uma única quebra na fita dupla de DNA já é suficiente para bloquear a divisão celular e desencadear a morte da célula (DOHERTY & JACKSON, 2001). As quebras na fita

dupla de DNA podem ser reparadas através de duas vias: 1) recombinação homóloga (HR), a qual é livre de erros; 2) União terminal não homóloga (NHEJ), a qual está sujeita a erros. Essas duas vias de reparo são controladas por um grupo bem orquestrados de genes que parecem estar presentes em todos os organismos (BRENDEL et al., 1997).

A expressão de RNAm dos principais genes responsáveis pela ativação e controle da via de reparo HR, tais como, ATM, 53BP1, RAD50, RAD51, RAD52, BRCA1, BRCA2 e NBS1 foi detectada em oócitos e blastocistos de humanos (JAROUDI et al., 2009). Esse mesmo grupo também demonstrou a expressão de RNAm de KU70, KU80 e XRCC4 os quais, são os principais genes responsáveis pela ativação e controle da via de reparo NHEJ. Recentemente, foi descoberto que o tratamento com hRAD51 recombinante antes da irradiação com krypton-78 ou UV-B diminui marcadamente a fragmentação citoplasmática e os danos de DNA em oócitos bovinos (KUJJO et al., 2011). Esses resultados indicam que as vias de reparo HR e NHEJ podem ser uma importante forma de proteção e reparo do genoma de oócitos e embriões bovinos. Entretanto, pouco é conhecido sobre o perfil de expressão dos genes responsáveis por ativar e controlar essas duas vias de reparo ao longo do desenvolvimento embrionário. Portanto, o objetivo do segundo estudo apresentado neste manuscrito foi caracterizar o perfil de expressão dos principais genes responsáveis pelo controle e ativação das vias de reparo HR (53BP1, ATM, RAD50, RAD51, RAD52, BRCA1, BRCA2 e NBS1) e NHEJ (KU70, KU80 e DNAPK) em embriões bovinos com alta, média ou baixa competência de desenvolvimento embrionário. Além disso, nós também avaliamos se os embriões bovinos são capazes de responder a quebras na fita dupla de DNA e em que momento após a fertilização *in vitro* as vias de reparo HR e NHEJ podem ser efetivamente reguladas.

2. REVISÃO BIBLIOGRÁFICA

2.1. Desenvolvimento Folicular Antral

A função ovariana é regulada primariamente pelo controle endócrino, principalmente pelas gonadotrofinas hipofisiárias, hormônio folículo estimulante (FSH) e hormônio luteinizante (LH), seus receptores (FSHr e LHr) e esteróides ovarianos. Mais recentemente, tornou-se evidente que fatores produzidos localmente atuam em um controle autócrino/parácrino da foliculogênese, desempenhando um papel essencial na modulação do desenvolvimento folicular (DE LA SOTA et al., 1996; BERISHA & SCHAMS, 2005).

O FSH é um dos principais hormônios que regula as ondas foliculares em bovinos. Isso é demonstrado pelo aumento da sua concentração circulante simultâneo ao início de uma onda, quando são recrutados até 24 folículos com diâmetro aproximado de 5mm, fenômeno denominado emergência folicular (WILTBANK et al., 2000; MIHM et al., 2002). Os níveis de FSH começam a diminuir nos dias 2 e 3 após a emergência e então alguns folículos da onda cessam o desenvolvimento até que apenas um (futuro folículo dominante) é selecionado, enquanto os restantes da mesma onda (folículos subordinados) entram em atresia. Essa mudança no ritmo de desenvolvimento folicular é denominada divergência ou desvio folicular e inicia com uma redução nas taxas de desenvolvimento dos folículos subordinados em contraste com a manutenção (BEG et al., 2002) ou aumento (FORTUNE et al., 2001a, 2001b) da taxa de desenvolvimento do futuro folículo dominante. A divergência folicular ocorre quando o maior folículo se encontra com um diâmetro em torno de 8,5mm em raças taurinas (BEG & GINTHER, 2006) e 6,0mm em zebuínas (GIMENES et al., 2008).

Existem evidências de que a capacidade do folículo dominante crescer e se diferenciar mesmo em baixos níveis de FSH é devida a diversos fatores como a aquisição gradativa de receptores para LH nas células da granulosa, a maior disponibilidade de IGF (fator de crescimento semelhante a insulina) livre (FORTUNE et al., 2004; WEBB et al., 2004) e a fatores de crescimento da família FGF (fatores de crescimento fibroblástico, BERISHA et al., 2004; BURATINI et al., 2007). No entanto, acredita-se que esses não sejam os únicos fatores locais responsáveis pelo desenvolvimento folicular durante o período de divergência, havendo outros diversos fatores produzidos pelo oócito, células da granulosa e teca (para revisão, KNIGHT & GLISTER, 2006). Esses fatores determinam que folículos dominantes tenham

níveis mais elevados de RNAm para receptores de gonadotrofinas e enzimas envolvidas na síntese de andrógenos e progestágenos (17α-OH, P450scc, 3β-HSD, e StAR) do que os subordinados (FORTUNE, 2001a, 2001b).

O papel da aquisição de receptores de LH pelas células da granulosa no estabelecimento da dominância folicular é controverso. Beg et al. (2001) afirmam que a expressão de RNAm para LHr nas células da granulosa do futuro dominante está aumentada 8 horas antes da divergência e que esta expressão não sofre alteração no segundo maior folículo, indicando que este é um mecanismo de seleção do folículo dominante. Contudo, isso contradiz o relato de níveis indetectáveis de LHr, pela técnica de hibridização *in situ* de células da granulosa, durante a seleção folicular (EVANS & FORTUNE, 1997). Nogueira et al. (2007) utilizando folículos de fêmeas Nelore, identificaram as isoformas de RNAm do LHr em células da granulosa de folículos com 8mm, e em apenas um dos seis folículos de 7mm analisados. Considerando-se que a divergência folicular ocorre aos 6mm de diâmetro nesta raça, assume-se que a expressão do gene que codifica o LHr nas células da granulosa foi detectada após a seleção do folículo dominante. Evans & Fortune (1997) também descreveram que a seleção do folículo dominante ocorre na ausência da expressão de receptores de LH nas células da granulosa. O mesmo grupo demonstrou a participação de fatores locais que suportam o desenvolvimento folicular durante essa fase de baixos níveis séricos de FSH (FORTUNE et al., 2001a; RIVERA et al., 2001; FORTUNE et al., 2004). Dentre esses fatores, a participação do IGF no desenvolvimento folicular de bovinos, assim como suas proteínas de ligação (IGFBPs) e proteases específicas, está bem estabelecida (FORTUNE et al., 2004; SPICER & AAD, 2007).

2.2. Sistema Renina-Angiotensina

Classicamente, o sistema renina-angiotensina (RAS) é definido como um sistema hormonal circulante que está envolvido no controle da pressão osmótica, equilíbrio de sais e manutenção da homeostase dos fluidos. De acordo com essa visão sistêmica, o precursor do RAS, o angiotensinogênio, é expresso no fígado e é clivado pela enzima renina, secretada pelos rins para a circulação, formando o decapeptídeo angiotensina I (Ang I). A Ang I é então clivada na ligação Phe^{8}-His^{9} pela enzima conversora de angiotensina (ACE), que está presente no endotélio vascular (PEACH, 1977). A clivagem da Ang I dá origem à Ang II, componente

bioativo responsável por mediar a maioria das funções do RAS. No entanto, a identificação de componentes do RAS em diversos tecidos tem levado ao novo conceito de sistema local de formação de Ang II e outros peptídeos bioativos do mesmo sistema. Além disso, a regulação dos sistemas locais parece ser independente do controle sistêmico do RAS. Esses sistemas locais de angiotensina parecem atuar de forma autócrina/paracrina em alguns tecidos, tendo funções descritas no coração, vasos sanguíneos, rins, cérebro e glândulas endócrinas (FERRARIO et al., 1998; PHILLIPS & SUMNERS, 1998; KIM & IWAO, 2000).

2.3. Angiotensina II no desenvolvimento folicular, ovulação e maturação de oócito

Os receptores para Ang II foram descritos nas células da teca e granulosa de ratas (HUSAIN et al., 1987), coelhas (YOSHIMURA et al., 1996a) e vacas (BERISHA et al., 2002; PORTELA et al., 2008); e em macacas predominantemente nas células da teca (AGUILERA et al., 1989). Com base nas diferentes propriedades farmacológicas e bioquímicas, os receptores para Ang II foram classificados em dois subtipos (DEGASPARO et al., 1995). O receptor tipo 1 (AT1 ou AGTR1) tem sido demonstrado mediando um número de funções bem conhecidas da Ang II como contração de musculatura lisa, síntese e secreção de aldosterona e angiogênese (para revisão DINH et al., 2001). Já o receptor tipo 2 (AT2 ou AGTR2) é responsável por efeitos opostos ao receptor AT1 e por mediar funções reprodutivas como esteroidogênese, maturação de oócitos e ovulação em algumas espécies (YOSHIMURA et al., 1996b; FERREIRA et al., 2007; BENETTI et al., 2008; para revisão GONÇALVES et al., 2012).

Em bovinos, a presença de receptores de Ang II nas células foliculares (BERISHA et al., 2002; PORTELA et al., 2008) e o aumento nas concentrações de Ang II após o pico de LH (ACOSTA et al., 2000) sugerem uma atividade biológica desse peptídeo no desenvolvimento folicular. Nosso grupo tem demonstrado a participação da Ang II na ovulação (FERREIRA et al., 2007; PORTELA et al., 2011), maturação oocitária (GIOMETTI et al., 2005; STEFANELLO et al., 2006; BARRETA et al., 2008) e, mais recentemente, demonstramos que a aplicação intrafolicular de saralasina (antagonista de Ang II) inibe o desenvolvimento folicular antral, sendo esta ação revertida pela administração sistêmica de FSH (FERREIRA et al., 2011a). Ainda, é importante frisar que os níveis de Ang II aumentam no fluído folicular durante o momento esperado para a divergência folicular e após o

estabelecimento da dominância folicular (FERREIRA et al., 2011b). Esses dados demonstram que a Ang II possui uma ação local indispensável para o desenvolvimento folicular e sugerem que esta ação ocorre somente no período em que as concentrações de FSH estão reduzidas, ou seja, durante a fase de transição de folículo FSH-dependente para LH-dependente. Em outro ensaio *in vivo*, foi demonstrado que a Ang II participa do desenvolvimento folicular regulando enzimas esteroidogênicas assim como marcadores de diferenciação das células da granulosa (aromatese, 3ßHSD, LHr e SerpinE2; FERREIRA et al., 2011a). Cabe salientar que o processo de diferenciação das células da granulosa e aquisição de receptores de LH nessas células é um passo indispensável para aquisição da dominância folicular e parecem ser regulados pela Ang II. Recentemente, foi demonstrado um aumento de Ang II no fluído folicular do folículo dominante nos dias 3 e 4 após o início da onda de crescimento folicular (durante e após o momento esperado para a divergência folicular; FERREIRA et al., 2011b). Nesse mesmo estudo, foi demonstrado um aumento da expressão de RNAm para ACE nas células da granulosa do folículo após o estabelecimento da dominância folicular (dia 4 após o início da onda). Os recentes resultados demonstram uma regulação dos componentes do sistema Ang II (PORTELA et al., 2008) e a participação dos mesmos durante o desenvolvimento folicular de bovinos (FERREIRA et al., 2011a; FERREIRA et al., 2011b). No entanto, alguns trabalhos têm sugerido que a Ang II não é o único peptídeo ativo do RAS. Outros fatores como Ang III (Ang-(2-8); ZINI et al., 1996), Ang IV (Ang-(3-8); BRASZKO et al., 1988; WRIGHT et al., 1993) e Ang-(1-7) (SCHIAVONE et al., 1988; BARNES et al., 1990; FELIX et al., 1991) também podem mediar as ações do RAS em diferentes sistemas, principalmente a Ang-(1-7) (COSTA et al., 2003; SAMPAIO et al., 2007; LEAL et al., 2009).

2.4. Angiotensina-(1-7)

A Ang-(1-7) é um componente ativo do sistema renina-angiotensina que pode ser formada por uma rota independente da ACE. Esse heptapeptídeo pode ser gerado a partir da Ang I pela endopeptidase neutra (NEP) e prolyl endopeptidase (PEP), ou a partir da Ang II pela PEP e prolyl carboxipeptidases (PCP; SANTOS et al., 1992). Recentemente, uma enzima formadora de Ang-(1-7) homóloga a enzima conversora da angiotensina I (ACE) foi descoberta e nomeada de ACE_2. A ACE_2 é uma carboxiaminopeptidase não responsiva a ação do captopril (inibidor seletivo da ACE) e é capaz de gerar Ang-(1-7) diretamente da Ang II ou indiretamente da Ang I, convertendo-a em Ang-(1-9), a qual, por ação da ACE forma a Ang-

(1-7) (DONOGHUE et al., 2000). A ACE_2 exibe uma alta eficiência catalítica para a conversão de Ang II a Ang-(1-7), quase 500 vezes maior que para a conversão de Ang I a Ang-(1-9). A partir de um ensaio com mais de 120 peptídeos, somente dois (dinofirna A e apelina 13) foram hidrolisados pela ACE_2 com cinética comparável à conversão de Ang II a Ang-(1-7). Assim, a ACE_2 converte o vasoconstritor e promotor de crescimento Ang II em Ang-(1-7), um peptídeo com propriedades vasodilatadoras e anti-proliferativas; provendo um aparente mecanismo para balancear diretamente os níveis de Ang II e Ang-(1-7), modulando os eixos pressor/mitogênico e depressor/anti-proliferativo do RAS (GALLAGHER et al., 2006).

Uma vez formada, a Ang-(1-7) pode ser hidrolisada por amino-peptitases, dando origem a Ang-(2-7) e a Ang-(3-7); ou pela ACE dando origem a Ang-(1-5). A atividade da ACE parece ser um importante mecanismo de inativação da Ang-(1-7) sistêmica e tecidual (YAMADA et al., 1998; CHAPPELL et al., 2000). As concentrações séricas de Ang-(1-7) são similares àquelas observadas para Ang II; no entanto, as concentrações de Ang-(1-7) aumentam após a inibição da ACE (CAMPBELL et al., 1991).

Nos últimos anos, foram descobertos vários componentes do sistema renina-angiotensina (RAS) em diversos tecidos, e novos conceitos de regulação do RAS estão sendo propostos. A descoberta da ACE_2 e do receptor MAS (receptor para Ang-(1-7)) levou a uma reavaliação da cascata original do RAS e o surgimento de um novo seguimento do RAS: o eixo ACE_2/Ang-(1-7)/MAS (para revisão XU et al., 2010). Nessa nova visão de regulação local do RAS, Lin et. al, (2010) demonstraram que a Ang II estimula a expressão de ACE_2 em fibroblastos isolados do coração de humanos. Um aumento na expressão de RNAm para ACE_2, mediado pela Ang II, também foi observado na aorta de ratos hipertensos o que corrobora com a premissa de que a Ang II exerce uma ação regulatória positiva sobre a ACE_2 (IGASE et. al, 2005, 2008). A maior expressão de ACE_2 induzida pela Ang II pode ser um mecanismo de autocontrole que tem como função impedir um aumento exagerado nas concentrações de Ang II. A atividade da ACE_2 parece ser regulada pelos níveis de estrógeno circulantes, pois ratas suplementadas com estrógeno apresentaram uma maior atividade renal de ACE_2 (JI et al., 2008).

2.5. Receptor MAS

O protooncongene MAS foi detectado originalmente em uma linhagem de células de carcinoma epidermóide humano (YOUNG et al., 1986). O MAS codifica uma proteína da classe das proteínas G, e discute-se seu envolvimento nas ações da Ang II. Essa proteína possui sete domínios transmembrana hidrofóbicos, sendo que o N- e C-terminal são hidrofílicos, e pertence portanto, à classe de receptores celulares de superfície acoplados à proteína G (JACKSON et al., 1988; VON BOHLEN UND HALBACH et al., 2000). O primeiro estudo que demonstrou a função do MAS foi realizado por Jackson et al. (1988), os quais demonstraram que células de mamíferos transfectadas com a sequência de DNA do receptor MAS apresentavam mobilização intracelular de Ca2+ em resposta a Ang II e Ang III. Dessa forma, a proteína MAS foi descrita inicialmente como um receptor funcional para angiotensinas.

Santos et al. (2003) demonstraram que o MAS é responsável pela ligação e mediação dos efeitos da Ang-(1-7). Nesse trabalho, foi demonstrando que a deleção do receptor MAS anula a ligação da Ang-(1-7) em rins de camundongo e inibe a ação antidiurética promovida pela Ang-(1-7) após sobrecarga hídrica. Ainda, esse estudo demonstrou que a resposta vasodilatadora promovida pela Ang-(1-7) é abolida na aorta de camundongos *knockout* para o receptor MAS. A expressão de RNAm para o receptor MAS foi verificada em vários tecidos, sendo abundantemente expresso em testículos de ratos e humanos (METZGER et al., 1995; REIS et al., 2010). Recentemente, têm sido demonstrado que as ações da Ang-(1-7) podem ser especificamente inibidas quando o receptor MAS é bloqueado com d-Ala7-Ang-(1–7), também conhecido como A-779 (CANGUSSU et al., 2009; LEAL et al., 2009; DILAURO & BURNS, 2009). Estudos utilizando o antagonista A-779 (bloqueador seletivo para o receptor MAS) e antagonistas de Ang II sugerem que as ações da Ang-(1-7) não são mediadas pelos receptores clássicos de Ang II (AT1 e AT2; FREEMAN et al., 1996; FONTES et al., 1997; IYER et al., 1998).

Sabe-se que a Ang-(1-7) ativa as MAPK (*mitogen-activated protein kinases*), através da ligação ao receptor MAS em células mesangiais humanas e que essa ativação está associada com a estimulação da fosfolipase A2 e liberação do ácido araquidônico (ZIMPELMANN & BURNS, 2009). Já em células endoteliais humanas, foi demonstrado que a Ang-(1-7) atua na via fosfatidilinositol 3-quinase (PI3K) / proteína quinase B / Akt, na

liberação de óxido nítrico (SAMPAIO et al., 2007), sendo que essa rota também está envolvida na ativação das MAPK.

2.6. Funções da Ang-(1-7) no ovário

No que diz respeito à função da Ang-(1-7), esta pode realizar efeitos similares, opostos ou distintos da Ang II. Vários estudos têm demonstrado que a Ang-(1-7), ao contrário do que se pensava inicialmente, possui uma atividade biológica. Esse heptapeptídeo atua sobre os sistemas nervoso central, renal e cardiovascular, além de funcionar como um regulador de desenvolvimento dos tecidos vasculares (para revisão SANTOS et al., 2000). Recentemente, foi demonstrado que camundongos *knockout* para o receptor MAS apresentam uma marcada redução na produção espermática diária (LEAL et al., 2009). No entanto, pouco se sabe sobre a função ovariana da Ang-(1-7). Em ratas, os níveis ovarianos de Ang-(1-7) estão mais elevados no proestro e estro quando comparado aos níveis do metaestro e diestro (COSTA et al., 2003). Os mesmos autores demonstraram que Ang-(1-7) induz um aumento nas concentrações de estradiol e progesterona e esse efeito foi inibido por um bloqueador específico de Ang-(1-7) (A-779). Esse mesmo grupo de pesquisa imunolocalizou Ang-(1-7) e o receptor MAS nas células intersticiais e tecais de folículos antrais e pré-ovulatórios de ratas tratadas com eCG. Foi também demonstrado que a expressão de RNAm para MAS e ACE_2, em homogenatos de ovário, aumenta em ratas tratadas com eCG (PEREIRA et al., 2009), o que sugere uma possível regulação mediada por gonadotrofinas. Utilizando o bovino como modelo experimental, nosso grupo demonstrou recentemente que a concentração de Ang-(1-7) aumenta no fluído folicular de folículos pré-ovulatórios, 24h após a administração intramuscular de um análogo sintético do GnRH (SANTOS et al., 2011). Além disso, foi demonstrado que existe regulação da expressão de RNAm de ACE_2, NEP e PEP nas células da granulosa de folículos pré-ovulatórios após o desafio com GnRH.

Uma vez que a função da Ang II em ratos (SPETH et al., 1999) parece ser diferente das demais espécies (YOSHIMURA et al., 1996c; FERREIRA et al., 2011a), no que tange ao desenvolvimento folicular, a indução da esteroidogênese causada pela Ang-(1-7) pode ser devida a uma ação direta, ou simplesmente uma inibição da ação apoptótica da Ang II nas células da granulosa. Entretanto, o papel do sistema Ang-(1-7) na função ovariana e desenvolvimento folicular permanece ainda desconhecido.

2.7. Detecção e reparo de danos de DNA no embrião

A expressão de genes responsáveis pelo reparo do DNA são de suma importância para a manutenção da integridade do genoma e consequentemente para o adequado desenvolvimento embrionário em mamíferos. Estudos com camundongos *knockout* têm demonstrado que a supressão da expressão de genes responsáveis pelo controle das principais vias de reparo de DNA podem causar sérios problemas de desenvolvimento ou serem incompatíveis com a vida (FRIEDBERG & MEIRA, 2006). Grande parte das perdas embrionárias ocorrem durante a fase de implantação o que sugere que o embrião necessita de um adequado sistema de reparo durante essa fase de rápida proliferação e diferenciação celular (VINSON & HALES, 2002). Em bovinos, o destino do embrião depende do resultado das primeiras quatro divisões de clivagem, as quais, geralmente têm uma elevada taxa de insucesso (LEIDENFROST et al., 2011). A morte embrionária antes do período de implantação é considerada como um dos maiores determinantes de casos de infertilidade em bovinos (BETTS & KING, 2001). Um recente estudo demonstrou que cerca de 30% dos blastocistos produzidos *in vitro* apresentam danos de DNA (STURMEY et al., 2009). É provável que a percentagem de embriões com danos fosse ainda maior se a avaliação feita por Sturmey et al. (2009) tivesse sido realizada antes da ativação do genoma embrionário. Essa afirmação pode ser suportada pelo fato de que a maioria dos oócitos que atingem o estádio de metáfase (MII) e completam a primeira clivagem *in vitro*, não são capazes de se desenvolver além dos estádios de 4 a 8 células (TELFORD et al., 1990). Além disso, alguns pesquisadores acreditam que a incapacidade de reparar danos de DNA é uma das principais causas de bloqueio do desenvolvimento antes da ativação do genoma de embriões cultivados *in vitro* (BETTS & KING, 2001; MEIRELLES et al., 2004).

Várias proteínas atuam conjuntamente em uma via celular complexa que tem como função detectar e reparar os diferentes tipos de danos de DNA. As enzimas de reparo de DNA parecem estar presentes em todos os organismos (BRENDEL et al., 1997) e sua conservação evolutiva confirma que são necessárias para o funcionamento normal e reprodução dos organismos vivos (ARAVIND et al., 1999). Até o ano de 2005 mais de 150 genes de reparo de DNA foram clonados e sequenciados em humanos (WOOD et al., 2005).

As principais vias de reparo de DNA ativas nas células de mamíferos são: *base excision repair* (BER), *double strand break repair* (DSBR), *mismatch repair* (MMR), *nucleotide excision repair* (NER) e *post-replication repair* (PRR) (VINSON & HALES,

2002; WOOD et al., 2005). Os diferentes tipos de lesões de DNA são reconhecidos por diversas proteínas de reparo, as quais, identificam a lesão de acordo com seu substrato e dessa forma, permitem que uma via específica de reparo possa ser ativada. Os danos de DNA podem ser amplamente classificados em dois tipos: 1) lesões e 2) quebras da fita de DNA. As lesões são caracterizadas por alterações na estrutura de bases e resulta em alterações químicas e/ou físicas da estrutura do DNA, as quais, podem produzir mutações pontuais ou distorções físicas do DNA que podem impedir a replicação e a transcrição. Os principais mecanismos responsáveis pelo reparo das lesões de DNA são: NER, BER e MMR. Essa três vias utilizam a excisão de bases como forma de reparo e seu funcionamento pode ser resumido em quatro passos comuns: 1) detecção da área lesada; 2) uma DNA endonuclease (BER and NER) ou exonuclease (MMR) corta um fragmento da fita de DNA que contém a lesão; 3) uma DNA polimerase sintetiza uma nova cópia, baseada na sequência da fita complementar, para preencher o espaço criado durante o corte e 4) uma DNA ligase liga o fragmento sintetizado completando dessa forma o reparo da fita lesada (HAKEM, 2008).

Em contrapartida, a fita dupla de DNA pode sofrer quebras físicas, as quais, podem estar associadas a uma ou as duas fitas de DNA. Qualquer tipo de quebra física da fita de DNA é suficiente para bloquear a divisão e desencadear a morte celular (DOHERTY & JACKSON, 2001). Geralmente, esse tipo de dano ocorre devido a exposição do DNA a radiação ionizante, estresse ambiental (VAN ATTIKUM & GASSER, 2005), radiação UV (KUJJO et al., 2011) e em casos severos de exposição a espécies reativas ao oxigênio (BILSLAND & DOWNS, 2005). A quebra da fita dupla de DNA está entre os mais perigosos indutores de danos genotóxicos e de morte celular por apoptose (RICH et al., 2000). Por serem o objeto alvo deste estudo e devido a sua importância, as formas de reparo da fita dupla de DNA serão discutidas em um tópico separado.

Logo após a detecção do dano, as vias de reparo são ativadas e o ciclo celular é bloqueado transitoriamente para que o reparo do DNA possa ser efetuado (BRANZEI & FOIANI, 2008). Entretanto, esses mesmos autores citam que quando a lesão não é reparada, o ciclo celular pode ser permanentemente suspenso ou as vias apoptóticas podem ser ativadas para que a célula seja eliminada. Existem limitadas informações sobre os mecanismos de reparo de DNA durante as primeiras fases do desenvolvimento embrionário, principalmente em modelos animais não roedores. Experimentos com zigotos de camundongos têm indicado que lesões na cromatina paterna podem ser reconhecidas e reparadas durante a fertilização (DERIJCK et al., 2008). Desde então, tem sido considerado que a capacidade de reparo de

DNA observada nas fases iniciais do desenvolvimento embrionário é uma característica materna. Baseado nessa hipótese, foi postulado que o oócito pode acumular RNAm para enzimas de reparo, as quais, serão importantes durante a fertilização e nas fases iniciais do desenvolvimento embrionário. Recentemente, foi observado que oócitos no estádio de metáfase II (MII) e blastocistos de humanos expressam RNAm de enzimas fundamentais para a ativação e controle das principais vias de reparo de DNA descritas em mamíferos (JAROUDI et al., 2009). Esse estudo demonstrou que 15 dos 25 genes envolvidos no reparo da fita dupla de DNA são expressos em oócitos MII e blastocistos. Esse resultados indicam que a expressão de genes responsáveis pelo reparo de DNA pode ter um grande impacto durante as primeiras fases do desenvolvimento embrionário. Entretanto, apesar de a expressão de genes de reparo ter sido detectada em embriões de mamíferos (para revisão JAROUDI & SENGUPTA, 2007; JAROUDI et al., 2009), o perfil de expressão gênica ao longo do desenvolvimento embrionário e a habilidade de reparo frente a quebra da fita dupla de DNA não tem sido investigados.

2.8. Reparo de quebras na fita dupla de DNA

As duas vias envolvidas no reparo de quebras na fita dupla de DNA são: recombinação homóloga (HR) e a união terminal não homóloga (NHEJ). A via HR é livre de erros e está ativa durante as fases S e na transição G2-M do ciclo celular onde uma cromátide irmã atua como molde para o reparo (JOHNSON & JASIN, 2000). Na via NHEJ as porções finais da fita dupla de DNA quebrada são ligadas independentemente de pertencerem ao mesmo cromossomo e, por isso, essa via é sujeita a erros que podem ocasionar mutações ou perda de funções em partes específicas do DNA (BRANZEI & FOIANI, 2008). Nas células de mamíferos, a via NHEJ está predominantemente ativa durante a transição das fases G0 para G1(JOHNSON & JASIN, 2000; LIEBER, 2010). Oócitos e blastocistos de humanos expressam 12 dos 19 genes pertencentes a via HR e 3 dos 6 genes pertencentes a via NHEJ (JAROUDI et al., 2009). Entretanto, o reparo da fita dupla pela via HR parece ser mais ativo em oócitos e embriões. Esse resultado de certa forma era esperado uma vez que, a via HR é livre de erros e mantém a integridade do genoma.

Várias investigações ainda tentam elucidar como a célula determina se o reparo ocorrerá via HR ou NHJE. Essa determinação pode ser exclusivamente operacional uma vez que, se uma sequencia homóloga não estiver próximo a fita dupla danificada durante a fase

S/G2 a via de reparo HR não pode ser ativada. Durante a fase S, se uma cromátide irmã está fisicamente próxima a fita danificada a célula pode dar preferência para a via de reparo HR. Entretanto, fora das fases S e G2 a NHEJ acaba sendo escolhida como a via de preferência para o reparo. Existe ainda a hipótese de que a via de reparo escolhida pela célula depende de uma competição entre proteínas da família KU ou RAD. Nesse modelo se as proteínas KU70 e KU80 forem as primeiras a se ligar na região danificada do DNA a reparação ocorreria pela via NHEJ. Em contrapartida, se proteínas pertencentes a família RAD (principalmente RAD51 e 52) forem as primeiras a se ligar ao DNA a reparação aconteceria pela via HR. Todas essas vias citadas foram recentemente revisadas por Libier (2010) e ainda não existe um consenso sobre o mecanismo que a célula utiliza para determinar se o reparo ocorrerá pela via HR ou NHEJ.

2.9. Reparo da fita dupla de DNA por recombinação homóloga (HR)

Durante o reparo pela via HR, a fita de DNA danificada se aproxima de uma cromátide irmã íntegra, a qual, tem uma sequência homologa que será utilizada como molde para o reparo (JOHNSON & JASIN, 2000; BRANZEI & FOIANI, 2008). A via HR é iniciada pela ligação do complexo MRE-RAD50-NBS1 ao fragmento de DNA danificado. Essa ligação promove a ressecção de alguns nucleotídeos na direção 5'- 3' e expõe a porção terminal da fita de DNA. Imediatamente após esse evento, as proteínas RAD51 e proteína de replicação A (RPA) unem-se a RAD52 e aos fragmentos da fita de DNA para formar um filamento de nucleoproteína (KAGAWA et al., 2001). A enzima BRCA1, a qual é ativada pela proteína ATM, auxilia na ligação da RAD51 e da BRCA2 ao DNA o que acaba atraindo a proteína RAD52 (VALERIE & POVIRK, 2003). A ligação da RAD52 a esse complexo de proteínas evita a digestão exonucleolítica da fita de DNA (VALERIE & POVIRK, 2003). A proteína 53BP1 é essencial para fosforilação da proteína ATM, uma vez que a supressão da 53BP1 com siRNA causa falhas na ativação da ATM (DITULLIO et al., 2002). O filamento de nucleoproteína formado pela RAD51, RPA e RAD52 localiza uma região homóloga na cromátide irmã, que irá servir como molde, e invade a fita dupla dessa cromátide formando uma *Holliday junction* (VAN DYCK et al., 2001). A DNA polimerase utilizará a cromátide irmã como molde e fará então a extensão da região terminal 3' da molécula de DNA danificada. Após esse processo a região complementar produzida pela DNA polimerase será ligada a fita de DNA por uma DNA ligase do tipo I. Finalmente, as *Holliday junctions* são

clivadas e ligadas para então produzir duas moléculas intactas de DNA. Um resumo de todo esse processo está exposto na Figura 1.

O RNAm das principais enzimas responsáveis pela ativação e controle da via HR são expressos em oócitos e embriões de humano (JAROUDI et al., 2009). Esse estudo demonstrou a expressão de RNAm para BRCA1, BRCA2, NBS1, RAD50, RAD51, RAD52, ATM e 53BP1 tanto em oócitos quanto em blastocistos. Recentemente, foi demostrado que a microinjeção de hRAD51 recombinante em oócitos de bovino previne a fragmentação citoplasmática induzida pela caspase 3 e reduz o dano ao DNA induzido pela irradiação UV-B ou com krypton-78 (KUJJO et al., 2011). Esses resultados indicam que a HR pode ser uma importante via de proteção e reparo do genoma em oócitos e embriões de bovinos. Entretanto, o conhecimento sobre essa via de reparo tanto em oócitos quanto em embriões da referida espécie é escasso e mais estudos são necessários para entendermos como essa via se comporta ao longo do desenvolvimento embrionário.

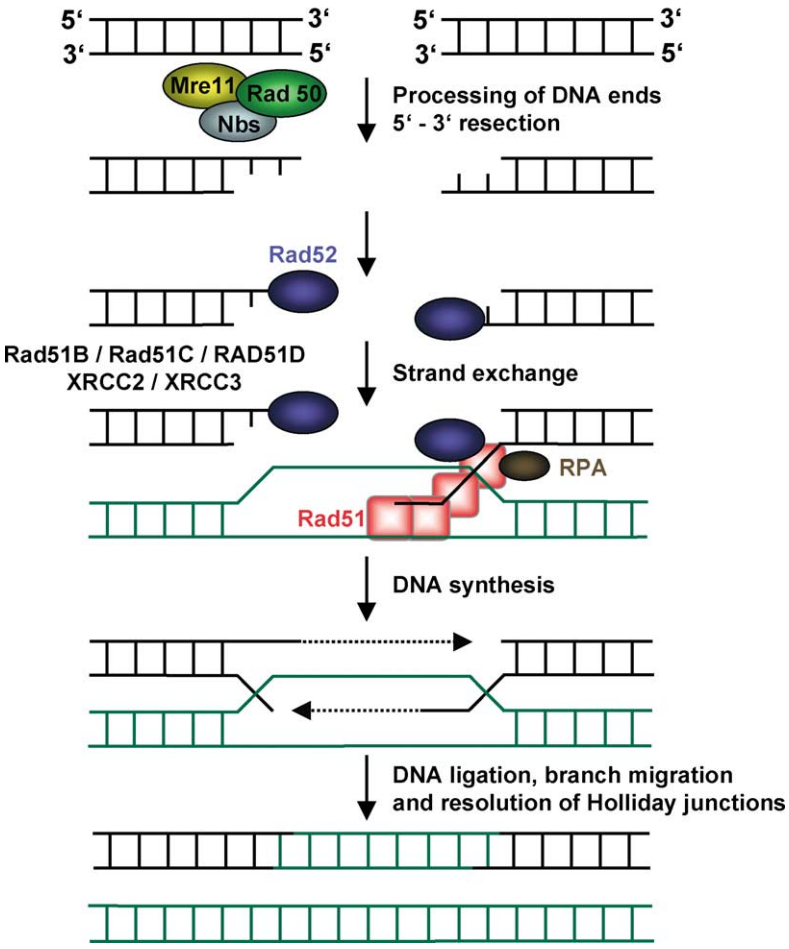

Figura 1 - Mecanismo de reparo por recombinação homóloga (HR). A HR inicia pela ressecção de nucleotídeos na dupla fita de DNA quebrada na direção 5’-3’ pelo complexo

MRE11–Rad50–NBS1 permitindo dessa forma a ligação da RAD52. A RAD52 inicia a formação do filamento de nucleoproteína pela interação com a uma série de proteínas tais como: RAD51, RPA, BRCA1, BRCA2, ATM e 53BP1. O filamento de nucleoproteína invade a cromátide irmã permitindo a cópia de um segmento homólogo pela DNA polimerase. Após a síntese, as *Holliday junctions* são clivadas e o DNA é ligado para então produzir duas moléculas intactas de DNA. Adaptado de Christmann et al. (2003).

2.10. Reparo da fita dupla de DNA pela união terminal não homóloga (NHEJ)

A via de reparo NHEJ inicia pela união das proteínas KU70 e KU80 ao DNA danificado (JEGGO et al., 1992). Essa ligação protege a fita dupla de DNA impedindo sua degradação por exonucleases. O complexo KU associa-se então à proteína quinase dependente de DNA (DNAPK) promovendo a sua ativação (SIPLEY et al., 1995). O alvo da DNAPK ativa é a proteína XRCC4 (X-ray repair complementing defective repair in Chinese hamster cells 4) que forma um estável complexo com a proteína LIG4 (DNA ligase 4; LEBER et al., 1998). A ligação do complexo XRCC4-LIG4 a DNAPK permite que as extremidades do DNA quebrado sejam ligadas de maneira complementar. A maioria das quebras de fita dupla precisam de um processamento da fita de DNA com o objetivo de tornar as extremidades 3'-5' complementares antes da ligação do complexo XRCC4-LIG4 (LEE et al., 2003). Esse processamento é realizado pelo complexo MRN o qual, remove o excesso de DNA da região 3' (MASER et al., 1997), ou pela FEN1 (flap endonuclease 1), a qual remove o excesso de DNA da região 5'. A deficiência do complexo FEN1 leva a uma severa redução do uso da via NHJE para o reparo da fita dupla de DNA (WU et al., 1999). Um resumo de todo esse processo está exposto na Figura 2.

Assim como já citado para a via HR, as principais proteínas responsáveis pela ativação e controle da via NHEJ também são expressas em oócitos e blastocistos de humanos (JAROUDI et al., 2009). A expressão de RNAm para KU70, KU80, XRCC4 e FEN1 foi detectada tanto no oócito quanto no embrião de humanos. Entretanto, o RNAm para a proteína LIG4 não foi detectado tanto no embrião quanto no oócito. Pelo perfil de expressão de RNAm encontrado, Jaroudi et al. (2009) sugerem que a via NHEJ seja mais ativa em blastocistos que em oócitos. Entretanto, os trabalhos realizados em mamíferos não têm abordado a expressão via NHEJ ao longo do desenvolvimento embrionário e tão pouco em

que fase do desenvolvimento o embrião pode utilizar essa via para o reparo da fita dupla de DNA.

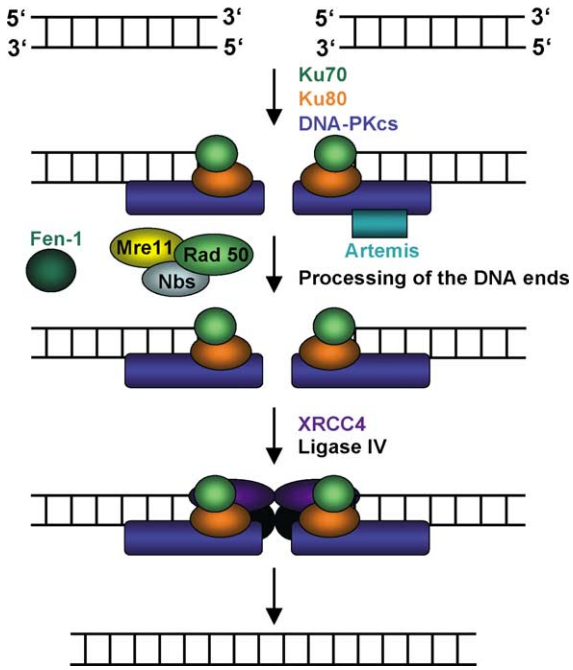

Figura 2 - Mecanismo de reparo pela união terminal não homóloga (NHEJ). O complexo KU70-KU80 reconhece a quebra na fita dupla de DNA e liga-se a ela. Logo após, o complexo KU liga-se à proteína DNAPK. A DNAPK ativa o complexo XRCC4–ligase IV, o qual se liga à fita dupla de DNA quebrada. Antes da re-ligação do DNA pelo complexo XRCC4–ligase IV, as extremidades 3' e 5' são processadas pelo complexo MRN e pela proteína FEN1. Adaptado de: Christmann et al. (2003).

3. ARTIGO 1

TRABALHO SUBMETIDO PARA PUBLICAÇÃO:

THE COMPONENTS OF ANGIOTENSIN-(1-7) SYSTEM ARE DIFFERENTIALLY EXPRESSED DURING FOLLICULAR WAVE IN CATTLE.

Marcos Henrique Barreta, Bernardo Garziera Gasperin, Rogério Ferreira, Monique Rovani, Gabriel Ribas Pereira, Rodrigo C. Bohrer, João Francisco de Oliveira, Paulo Bayard Dias Gonçalves.

REPRODUCTION, 2012.

The components of Angiotensin-(1-7) system are differentially expressed during follicular wave in cattle[1].

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Footnotes

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Abstract

The objective of this study was to characterize the expression of Ang-(1-7) system components, such as MAS receptor, angiotensin-converting enzyme 2 (ACE_2), neutral endopeptidase (NEP) and prolyl endopeptidase (PEP) during follicular development. Furthermore, the regulation of local Ang-(1-7) system during health/atresia transition was tested by inducing follicular atresia with intrafollicular injection of an estradiol-receptor inhibitor. Cows were ovariectomized when the size between the largest (F1) and the second largest follicle (F2) was not statistically different (Day 2), slightly different (Day 3), or markedly different (Day 4). The mRNA abundance of genes encoding MAS receptor, ACE_2, NEP and PEP was evaluated in the follicular cells from F1 and F2. The mRNA expression of MAS receptor was upregulated in the granulosa cells of F2 after the establishment of follicular deviation (Day 4), while PEP mRNA increased during (Day 3) and after (Day 4) the deviation process. However, the mRNA expression of ACE_2 was upregulated in the granulosa cells of F1 during and after the deviation process. The mRNA expression of NEP was not regulated in F1 and F2. The MAS receptor was immunolocated in the granulosa and theca cells of F1 and F2 during follicular deviation. Moreover, MAS receptor gene expression increased when the F1 was treated with the estrogen receptor-antagonist *in vivo*. In conclusion, mRNA for MAS receptor, ACE_2, NEP and PEP is expressed in follicular cells throughout follicular development. The MAS receptor was upregulated in granulosa cells of subordinate follicles and in dominant follicles treated with an estradiol-receptor inhibitor.

Keywords: MAS receptor, ACE_2, PEP, NEP, follicular deviation, bovine.

Introduction

It is well established that the follicular growth in single-ovulating species occurs in waves being primary orchestrated by endocrine factors, mainly gonadotrophins (FSH and LH), their receptors (FSHr and LHCGr) and ovarian steroids. The transient increase in the FSH levels stimulates the growth of a cohort of follicles until one follicle is selected to continue growing, while the other follicles regresses due to the low levels of FSH in the follicular deviation stage (Ginther *et al.* 1996). During nadir of FSH secretion, several genes are differentially expressed in microenvironment of follicles to allow the survival of granulosa and theca cells allowing the dominant follicle to become "FSH independent" and to continue its growth (Mihm *et al.* 2006). More recently, focus has shifted to the microenvironment of follicles and an emerging appreciation that changes in that environment provide a critical "fine-tuning" of follicular regulation. The deviation phase is one of development stages that depend on changes in the follicular microenvironment. Previous studies have characterized some important local factors, such as members of IGF and TGFβ family, in follicular development (Fortune *et al.* 2004, Knight & Glister 2006, Mihm *et al.* 2008). However, the autocrine and paracrine control of folliculogenisis is poorly understood in single-ovulating species.

The Renin-Angiotensin System (RAS) has emerged as an important local system in the regulation of reproductive events (Yoshimura *et al.* 1992, Yoshimura *et al.* 1996, Giometti *et al.* 2005, Ferreira *et al.* 2007, Barreta *et al.* 2008, Portela *et al.* 2008, Pereira *et al.* 2009, Ferreira *et al.* 2011a, Portela *et al.* 2011). Among the peptides of the RAS, angiotensin II (Ang II) is one of the major bioactive compounds. Ang II receptors are present in theca and granulosa cells in cattle (Portela *et al.* 2008) and the levels of Ang II increase in dominant follicle during the follicular deviation (Ferreira *et al.* 2011b). Also, we have previously

67 demonstrated that RAS components are differentially regulated during development of
68 dominant and subordinate follicles (Ferreira *et al.* 2011b), that saralasin (a competitive
69 inhibitor of Ang II) inhibits the follicular growth and that Ang II play a key role in the
70 follicular microenvironment during nadir of FSH secretion in cattle (Ferreira *et al.* 2011a).
71 Taken together, these results strongly suggest that Ang II has an important biological activity
72 during the follicular development.

73 Angiotensin-(1-7) (Ang-(1-7)) has been shown to be another important active
74 compound of the RAS. This peptide results either from the cleavage of angiotensin I (Ang I)
75 by neutral endopeptidase (NEP) and prolyl endopeptidase (PEP) or from Ang II by
76 angiotensin-converting enzyme II (ACE_2) and PEP (Santos *et al.* 2008). Ang-(1-7) acts
77 through the G protein-coupled receptor MAS (Santos *et al.* 2003) and can be specifically
78 inhibited with d-Ala7-Ang-(1-7), also known as A-779 (MAS receptor antagonist; Dilauro &
79 Burns 2009). Serum concentrations of Ang-(1-7) are similar to those observed for Ang II
80 (Campbell *et al.* 1991). However, the effect of Ang II and Ang-(1-7) are similar in some
81 tissues (i.e., brain; Santos *et al.* 2000) and different in others (i.e., kidney and heart; Santos *et*
82 *al.* 2008).

83 In the ovarian tissue, Ang-(1-7) induced an increase in estradiol and progesterone
84 levels in perfused rat ovary, which was inhibited by A-779 (Costa *et al.* 2003). It was also
85 shown that the expression of mRNA for MAS receptor and ACE_2 increased in rat ovaries
86 treated with eCG (Pereira *et al.* 2009), suggesting a possible regulation mediated by
87 gonadotropins. However, the function of Ang-(1-7) is little known in the ovary and far less is
88 known in single-ovulating species. Cattle, a single-ovulating specie, provide an excellent
89 model for studying the role of local factors in the control of follicle development, as the
90 follicular wave can be accurately monitored on a day-to-day basis by ultrasonography *in vivo*
91 (Savio *et al.* 1988, Ginther *et al.* 1989, Evans & Fortune 1997) and the follicular environment

can be easily modified by an ultrasound-guided intrafollicular injection (Kot *et al.* 1995, Ferreira *et al.* 2007). The present study characterized the mRNA expression of MAS receptor and key enzymes for Ang-(1-7) production, such as, ACE_2, NEP and PEP during follicular development. Furthermore, follicular atresia was induced by intrafollicular injection of an estradiol-receptor inhibitor to evaluate the regulation of local Ang1-7 system during health/atresia transition.

Results

Gene expression of MAS receptor, ACE_2, NEP and PEP in theca and granulosa cells before, during and after the expected moment to follicular deviation

This experiment was performed to evaluate whether MAS receptor, ACE_2, NEP and PEP are differentially expressed in the granulosa and theca cells of the largest and second largest follicle during follicular wave development. The experimental design allowed the recovery of follicles when the follicular size of the largest and the second largest was not statistically different (Day 2), slightly different (Day 3), or markedly different (Day 4) as previously described by our group (Ferreira *et al.* 2011b). With this in vivo experimental model, we observed that the mRNA expression of MAS receptor was upregulated in the granulosa cells of second largest follicle after the establishment of follicular deviation (Fig. 1A), while PEP mRNA increased during and after the deviation process (Fig. 1C). However, the mRNA expression of ACE_2 was upregulated in the granulosa cells of the largest follicle at the expected moment and after the establishment of follicular deviation (Fig. 1B). The mRNA expression of MAS, PEP and ACE_2 was detected in the theca cells of largest and second largest follicle, but was not regulated during follicular wave development (data not showed). The mRNA expression of NEP enzyme was weakly detected in the granulosa and theca cells, but was not regulated throughout development of largest and second largest follicle (data not

showed). The MAS receptor protein was detected by immunofluorescence in the granulosa and theca cells of largest and second largest follicles (Fig. 2).

Gene expression of MAS receptor, ACE_2 and PEP in granulosa cells of dominant follicles induced to atresia

This experiment was conduced in an in vivo model where the dominant follicle was induced to atresia by intrafollicular treatment with fulvestrant (a selective estrogen receptor antagonist; 100 uM). Intrafollicular treatment with fulvestrant decreased CYP19A1 gene expression and induced the atresia of dominant follicle 12 h after treatment (data previously confirmed by authors). The mRNA expression of ACE_2 and PEP enzymes did not differ between fulvestran- and saline-treated follicles at 12 and 24 h after intrafollicultar treatment. However, the mRNA expression of MAS receptor was upregulated in fulvestrant-treated follicles at 12 and 24 h after intrafollicular treatment (Fig. 3).

Discussion

Our significant findings are: 1) the mRNA expression for RAS components to produce intracellular Ang-(1-7) were demonstrated during follicular deviation using a bovine in vivo model; 2) differential mRNA expression of MAS receptor, ACE_2 and PEP enzymes were observed in granulosa but not in theca cells during follicular deviation; 3) MAS receptor was immunolocated in granulosa and theca cells of largest, second largest and third largest follicles during the expected follicular deviation moment; 3) NEP enzyme was expressed, but not regulated, in granulosa and theca cells during follicular deviation; and 4) mRNA expression of MAS receptor was upregulated in granulosa cells of dominant follicles induced to atresia.

A well-established experimental in vivo model proposed by Rivera *et al.* (2001) was used in this study to found that the mRNA expression to MAS receptor is upregulated in

granulosa cells of subordinate follicle after establishment of follicular deviation. Moreover, the induction of dominant follicle atresia after intrafollicular treatment with fulvestrant upregulated the mRNA expression of MAS receptor 12 and 24 h after intrafollicular treatment. This is the first time that the expression of MAS receptor was correlated with follicular atresia. The activation of MAS receptor by Ang-(1-7) is able to inhibit the cell growth in several local systems. The antimitogenic effects of Ang-(1-7) were initially shown in vitro and in vivo in vascular smooth muscle cells (Freeman *et al.* 1996) and cardiac myocytes (Loot *et al.* 2002, Tallant *et al.* 2005) and this effect was mediated by decreased mitogen-stimulated protein synthesis (Tallant *et al.* 2005). Previous studies have demonstrated that Ang-(1-7) inhibits the growth of human lung cancer cells in vitro (Gallagher & Tallant 2004) and tumor angiogenesis in vivo through activation of the MAS receptor (Soto-Pantoja *et al.* 2009). This effect appears to be mediated, at least in part, by reducing the expression and activity of MMP-2 and MMP-9 in the pulmonary tissue (Ni *et al.* 2012). Together, these results suggest that a higher mRNA expression to MAS receptor in granulosa can be a required mechanism to mediate the Ang-(1-7) effect on follicular atresia. However, these findings contradict the results obtained in multiovulatory species, where the MAS receptor was upregulated in healthy and estrogen active follicles (Pereira *et al.* 2009). This difference between species is similar for the AT_2 receptor, which is selectively expressed in atretic follicles in the rat (Daud *et al.* 1988, Pucell *et al.* 1991) while it is upregulated in granulosa cells of healthy follicles in cattle (Portela *et al.* 2008).

The mRNA expression of ACE_2 was upregulated in dominant follicle during and after the establishment to follicular deviation (Day 3 and 4 after the beginning of follicular wave). These results were expected since, using the same experimental model in vivo, our group demonstrated that the mRNA expression of CYP19 and the levels of estrogen and Ang II increase in dominant follicle during days 3 and 4 after the beginning of the follicular wave

(Ferreira *et al.* 2011b). Lin *et al.* (2010) demonstrated that Ang II stimulates the ACE_2 expression in fibroblasts isolated from human heart. An increase in the mRNA expression of ACE_2, mediated by Ang II, was also observed in the aorta of hypertensive rats, which confirms the assumption that Ang II exerts a positive regulatory action on ACE_2 (Igase *et al.* 2005, Igase *et al.* 2008). Together, these results denote that increased ACE_2 expression, induced by Ang II, may be a mechanism of self-control that is designed to prevent an excessive increase in the local concentrations of Ang II. Furthermore, the ACE_2 activity also appears to be regulated by circulating levels of estrogen; therefore, rats supplemented with estrogen had an increased renal ACE_2 activity (Ji *et al.* 2008). Thus, we can infer that estradiol and Ang II levels in follicular fluid can regulate the ACE_2 expression in the dominant follicle during and after the establishment of follicular divergence, and this event can be an important mechanism for controlling intrafollicular levels of Ang II. However, more studies are needed to understand how the ACE_2 expression is regulated in granulosa cells and which are its effects on follicular development.

The mRNA expression of PEP enzyme was upregulated in granulosa cells of subordinate follicle during and after the establishment of follicular deviation. Our result contradicts the findings of Pereira *et al.* (2009), which demonstrated that the PEP mRNA expression increases in ovarian homogenates of eCG-treated rats. However, Pereira *et al.* (2009) evaluated the PEP mRNA expression in ovarian homogenates from immature female (control) compared with eCG-treated rats and thus the effect observed by these authors on PEP and MAS receptor expression may be due to an increase in ovarian activity produced by eCG treatment. As the ACE_2 expression is lower in subordinate follicles, the conversion of Ang II into Ang-(1-7) or Ang I into Ang-(1-9) does not seem to be the main pathways to Ang-(1-7) production in subordinate follicles. Thus, high PEP mRNA expression during and after the establishment of follicular deviation suggests that the conversion of Ang I into Ang-(1-7)

190 may be the choice pathway for Ang-(1-7) production in subordinate follicles. However,
191 further studies are needed to confirm these statements and to understand how this regulation
192 works during follicular deviation. We did not find regulation in NEP mRNA expression
193 during follicular development, raising the possibility of an absence of action in the ovary or
194 the regulatory involvement on other ovarian peptides (Pinto *et al.* 1999, Pereira *et al.* 2009).

195 In conclusion, mRNA for MAS receptor, ACE_2, NEP and PEP is expressed in theca
196 and granulosa cells of dominant and subordinate follicles before, during and after follicular
197 deviation phase. The differential mRNA expression of these enzymes during follicular
198 deviation suggest the involvement of Ang-(1-7) system in the regulatory process of follicular
199 deviation in cattle. The upregulation of MAS receptor mRNA expression in granulosa cells of
200 subordinate follicle after follicular deviation and in dominant follicles treated with an
201 estradiol-receptor inhibitor, suggest an involvement of this receptor with the follicular atresia
202 and with the establishment of follicular dominance.

203 **Materials and Methods**

204 ***Experiment 1: MAS receptor, ACE_2, PEP and NEP mRNA expression during bovine***
205 ***follicular wave***

206 This experiment was performed using an experimental model to investigate the mRNA
207 expression of MAS receptor, ACE_2, PEP and NEP before, during, and after follicular
208 divergence. Thirty-six weaned beef cows (predominantly Hereford and Aberdeen-Angus),
209 with an average body condition score of 3 (1–5, emaciated to fat) were treated with two doses
210 of a PGF2a analogue (cloprostenol®, 125 μg; Schering-Plough Animal Health, Brazil)
211 intramuscularly (im), 12 h apart. The estrus signs were observed within 3–5 days after PGF2a,
212 and the experiment was performed during the first follicular wave of the estrous cycle.
213 Ovaries were then examined once a day by transrectal ultrasonography, using an 8-MHz

linear-array transducer (Aquila Vet scanner®, Pie Medical, Netherlands), and all follicles larger than 5 mm were drafted using 3 to 5 virtual slices of the ovary allowing a three-dimensional localization of follicles and monitoring individual follicles during follicular wave (Jaiswal *et al.* 2004). The day of the follicular emergence was designated as Day 0 of the wave and was retrospectively identified as the last day on which the dominant follicle was 4 or 5 mm in diameter (Rivera & Fortune 2001). The cows were randomly assigned to be ovariectomized by colpotomy at days 2, 3, and 4 of the follicular wave (4 cows for each day) to recover the largest and the second largest follicle from each cow.

Experiment 2: MAS receptor, ACE_2, PEP mRNA expression during initial atresia

This experiment was performed using an experimental model to investigate whether mRNA expression of MAS receptor, ACE_2 and PEP is regulated during initial atresia of a dominant follicle. Twenty *Bos taurus taurus* adult cyclic cows (as previously described) were synchronized with a progesterone-releasing intravaginal device (1 g progesterone, DIB® – Intervet Schering Plough), an injection of 2 mg estradiol benzoate im (Genix, Anápolis, Brazil), and two injections of 250 μg sodium cloprostenol im (12 h apart; Ciosin® - Intervet Schering Plough). All treatments performed at the same time on Day 0. Four days later, the progesterone devices were removed and the ovaries were monitored daily until the largest follicle of the growing cohort reached a diameter of 7-8 mm. At this moment, an intrafollicular injection of fulvestrant (a selective estrogen receptor antagonist) in a final concentration of 100 μM (based on a previous dose-response experiment; data not shown) or saline was given. The cows were ovariectomized 12 (n=3/group) or 24 h (n=3/group) after the intrafollicular injection. The injections were given as previously described (Ferreira *et al.* 2007). All experimental procedures using cattle were reviewed and approved by the Federal University of Santa Maria Animal Care and Use Committee (ACUC).

Processing of ovarian follicles

The ovariectomy was realized by colpotomy and follicular fluid, granulosa, and theca cells were recovered from F1 and F2 (experiment 1) and from fulvestrant- or saline-treated follicles (experiment 2) and stored at -80ºC. Follicular fluid estradiol levels from all follicles were determined by ELISA following the manufacturer's instructions (Cayman Biochemical). Cross-contamination of the theca and the granulosa cells was tested by qRT-PCR to detect cytochrome P450 aromatase (CYP19A1) and 17a-hydroxylase (CYP17A1) mRNA. The granulosa cells that expressed CYP17A1 and the theca cells that expressed CYP19A1 were discarded (Buratini *et al.* 2005).

The largest and second largest follicles of the first follicular wave of the estrous cycle were isolated from the ovaries of one cow (experiment 1) during the expected moment to follicular deviation (Day 3 relative to beginning of follicular wave). The isolated follicles were fixed to immunolocation of MAS receptor in granulosa and theca cells.

Nucleic Acid Extraction and Real-Time qRT-PCR

Total RNA was extracted using Trizol (theca cells) or a silica-based protocol (granulosa cells; Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions and was quantified by absorbance at 260 nm. Total RNA (1 µg) was firstly treated with 0.2 U DNase (Invitrogen) at 37°C for 5 min. to digest any contaminating DNA, followed by heating to 65°C for 3 minutes. The RNA was reverse transcribed (RT) in the presence of 1 µM oligo(dT) primer, 4 U Omniscript RTase (Omniscript RT Kit; Qiagen, Mississauga, ON, Canada), 0.5 µM dideoxynucleotide triphosphate (dNTP) mix, and 10 U RNase Inhibitor (Invitrogen) in a volume of 20 µL at 37°C for 1 hour. The reaction was terminated by incubation at 93°C for 5 minutes.

Real-time polymerase chain reaction (qPCR) was conducted in a Step One Plus instrument (Applied Biosystems, Foster City, CA) with Platinum SYBR Green qPCR SuperMix (Invitrogen) and bovine-specific primers (Table 1). Common thermal cycling parameters (3 minutes at 95°C, 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C) were used to amplify each transcript. Melting-curve analyses were performed to verify product identity. The samples were run in duplicate and were expressed relative to GAPDH as the housekeeping gene. The relative quantification of gene expression across treatments was evaluated using the ddCT method (Livak & Schmittgen 2001). Briefly, the dCT was calculated as the difference between the CT of the investigated gene and that of GAPDH in each sample. The ddCT of each investigated gene was calculated as the difference between the dCT in each treated sample and the dCT of the sample with lower gene expression (higher dCT). The fold change in relative mRNA concentrations was calculated using the formula 2^{-ddCT}. Bovine-specific primers (Table 1) were taken from literature and synthesized by Invitrogen.

Immunofluorescence assessment

Ovaries from cows were collected by ovariectomy as described in experiment 1. The largest and second largest follicles of the first follicular wave of the estrous cycle were isolated from the ovaries (Richard & Sirard 1996). The isolated follicles were fixed in 4% paraformaldehyde for 6 h and paraffin embedded. Histological sections with 5 μm in thickness and slides preparations were made to perform immunofluorescence analysis. Slides were deparaffinized using Xylene for 15 min., rehydrated through a graded alcohol series (one times for 5 min. in each 100%, 90%, 80%, 70% and 50% dilution), and rinsed for 15 min. in ddH_2O. Endogenous peroxidase activity was then blocked for 20 min in 0.3% H_2O_2 and washed three times in PBS1X for 5 min. After washing, the slides were carefully blotted using a PAP pen (Vector Laboratory, Burlingame, CA) around the tissue. A blocking solution

(PBS1X with 3% of Bovine Serum Albumin and 0.2% Twen-20) was used to block non-specific sites during 2 h at room temperature in a humidify chamber. After washed three times in PBS1X during 5 min., the same blocking solution was used to incubate the primary MAS receptor antibody (sc-135063; 1:50; Santa Cruz Biotechnology) in a humidified chamber overnight at 5^{o}C. After this incubation, samples were washed three times in a PBS1X containing 0.2% Tween-20 for 5 min. before being incubated for 1 h at room temperature to a goat anti-rabbit IgG antibody conjugated with AlexaFluor 488 (1:500; Invitrogen). Then, slides were washed in three times in a PBS1X containing 0.2% Tween-20 for 5 min. Finally, to enable nuclear staining visualization, samples were incubated with 300 nM of 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) in PBS1X for 5 min. at room temperature. Then, slides were mounted with a space between the coverslip, filled with 50 µl drop of Aqueous Mounting Medium (Fluoromount; Sigma) and sealed with nail polish. Laser-scanning confocal microscopy was performed using a Confocal Microscope Espectral FV1000 (Olympus). Laser scanning microscope was equipped with two lasers for the simultaneous excitation of Alexa Fluor 488 fluorescent for MAS receptor and DAPI for DNA. Image software FV-Viewer (Olympus) was used to obtain sample images.

Statistical analysis

The regulation of mRNA-encoding MAS receptor, ACE_2, PEP and NEP proteins was analyzed by ANOVA and a multicomparison between days or groups was performed by least square means. Data were tested for normal distribution using Shapiro-Wilk test and normalized when necessary. All analyses were performed using JMP software (SAS Institute Inc., Cary, NC) and a $P<0.05$ was considered statistically significant. Data are presented as means ± sem.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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458 **Legend figures:**

459

460 **Figure 1** Relative mRNA expression of MAS receptor (MAS; A), angiotensin converting
461 enzyme 2 (ACE_2; B) and prolyl endopeptidase (PEP; C) in granulosa cells during follicular
462 development. Granulosa cells were recovered from the largest (black bar) and the second
463 largest (open bar) follicle (mean±SEM) collected at days 2 (n=4), 3 (n=4), and 4 (n=4) of the
464 first follicular wave of a cycle. Asterisk (*) indicates statistical difference in the relative
465 mRNA expression between the largest and the second largest follicle assessed by a paired
466 Student's T test using the cow as subject ($p \leq 0.05$).

467

468 **Figure 2** MAS receptor localization in the granulosa and theca cells as detected by confocal
469 immunofluorescence microscopy. The largest and second largest follicles were recovered
470 from ovaries collected at days 2, 3 and 4 of the first follicular wave of a cycle. Scale bars = 70
471 μm. Magnification = 200x.

472

473 **Figure 3** Relative mRNA expression of MAS receptor, angiotensin converting enzyme 2
474 (ACE_2) and prolyl endopeptidase 2 (PEP) in granulosa cells 12 or 24 h after intrafollicular
475 selective estrogen receptor antagonist (fulvestrant) treatment. Granulosa cells were recovered
476 from saline (open bar)- and fulvestrant (black bar)- treated follicles 12 (n=3/group) and 24h
477 (n=3/group) after intrafollicular injection (mean±SEM). Asterisk (*) indicates statistical
478 difference in the relative mRNA expression between groups ($p \leq 0.05$).

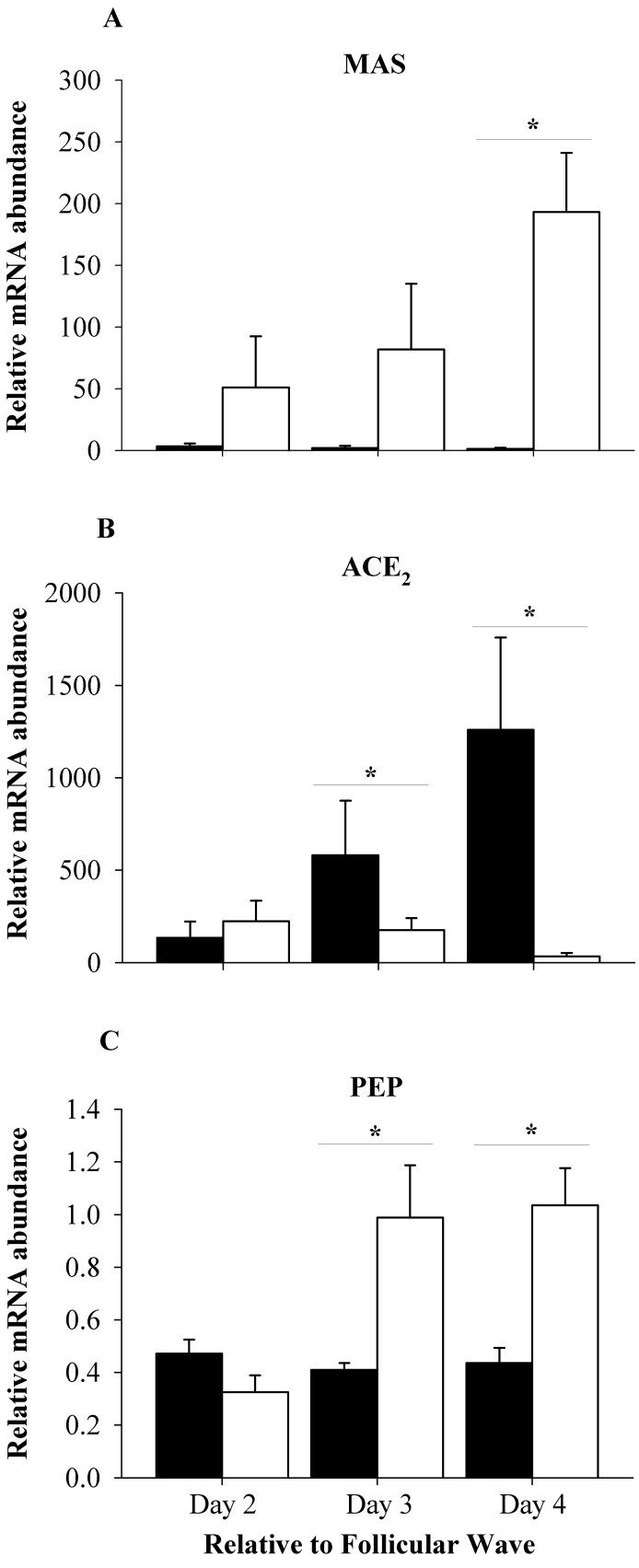
479

480 **Table 1.** Primers used in the expression analysis of candidate genes. Primer sequences and
481 concentrations used to amplify each product are described.

Gene		Sequence	Conc. (nM)	Reference
ACE_2	F	TGACTACAGCCGTGACCAGTTG	200	(Santos *et al.* 2011)
	R	CAACTTTGCCCTCACATAAGCA	200	
GAPDH	F	GATTGTCAGCAATGCCTCCT	200	(Ferreira *et al.*
	R	GGTCATAAGTCCCTCCACGA	200	2011b)
MAS	F	CGTGATCATCTTCATAGCCATTCT	200	(Santos *et al.* 2011)
	R	CCACGAGTTCTTCCGGATCTT	200	
PEP	F	GTTCCTCGACCCCAACACACT	200	(Santos *et al.* 2011)
	R	GGCACTCAGACCATAGGCAAA	200	
NEP	F	CCTGCTGCTCACCATCATTG	200	(Santos *et al.* 2011)
	R	TCGAGCGGCTGATTTTATGC	200	

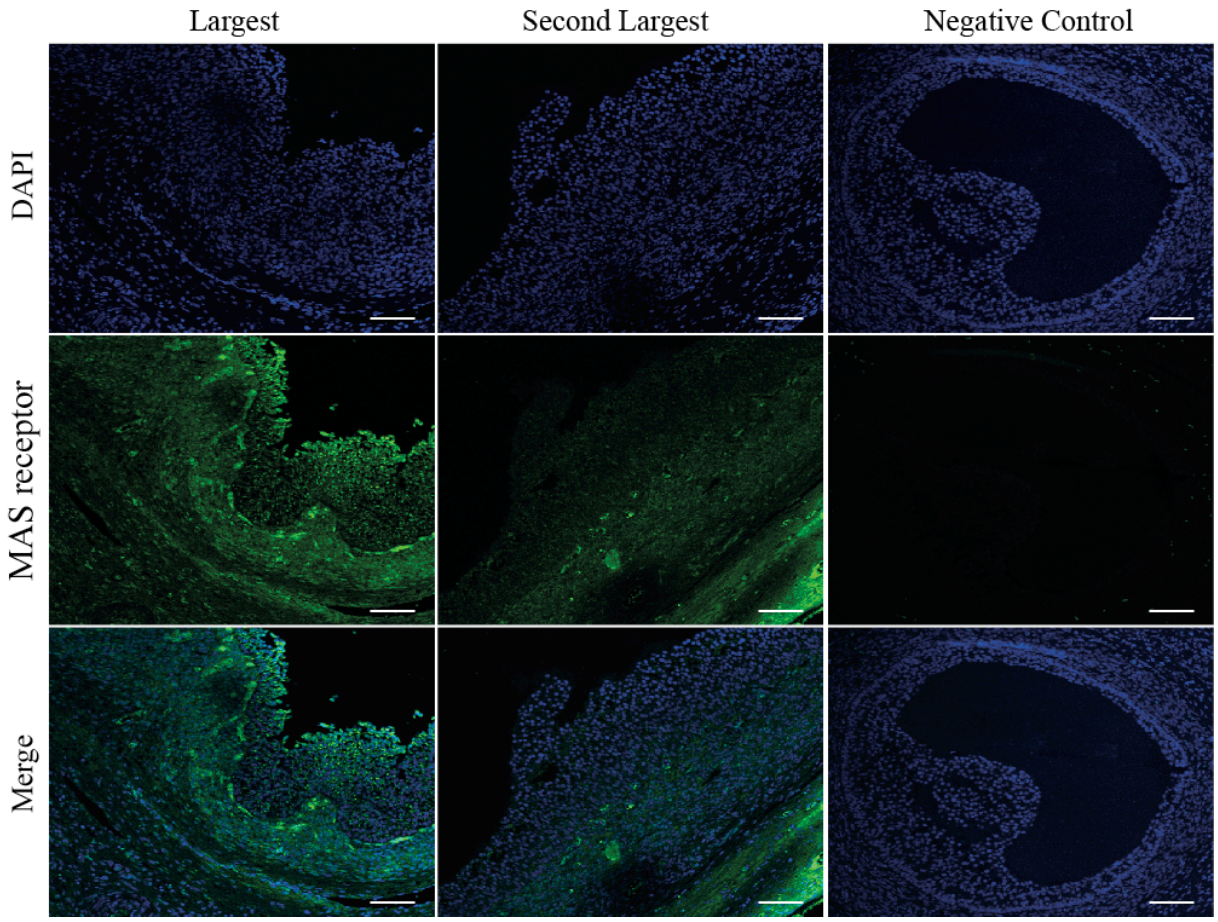
482 F, Forward primer; R, Reverse primer; Conc., primer concentration used for gene
483 amplification.

Figure 1


A
MAS
Relative mRNA abundance
300
250
200
150
100
50
0
*
B
ACE2
Relative mRNA abundance
2000
1500
1000
500
0
*
*
C
PEP
Relative mRNA abundance
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Day 2
Day 3
Day 4
Relative to Follicular Wave

484

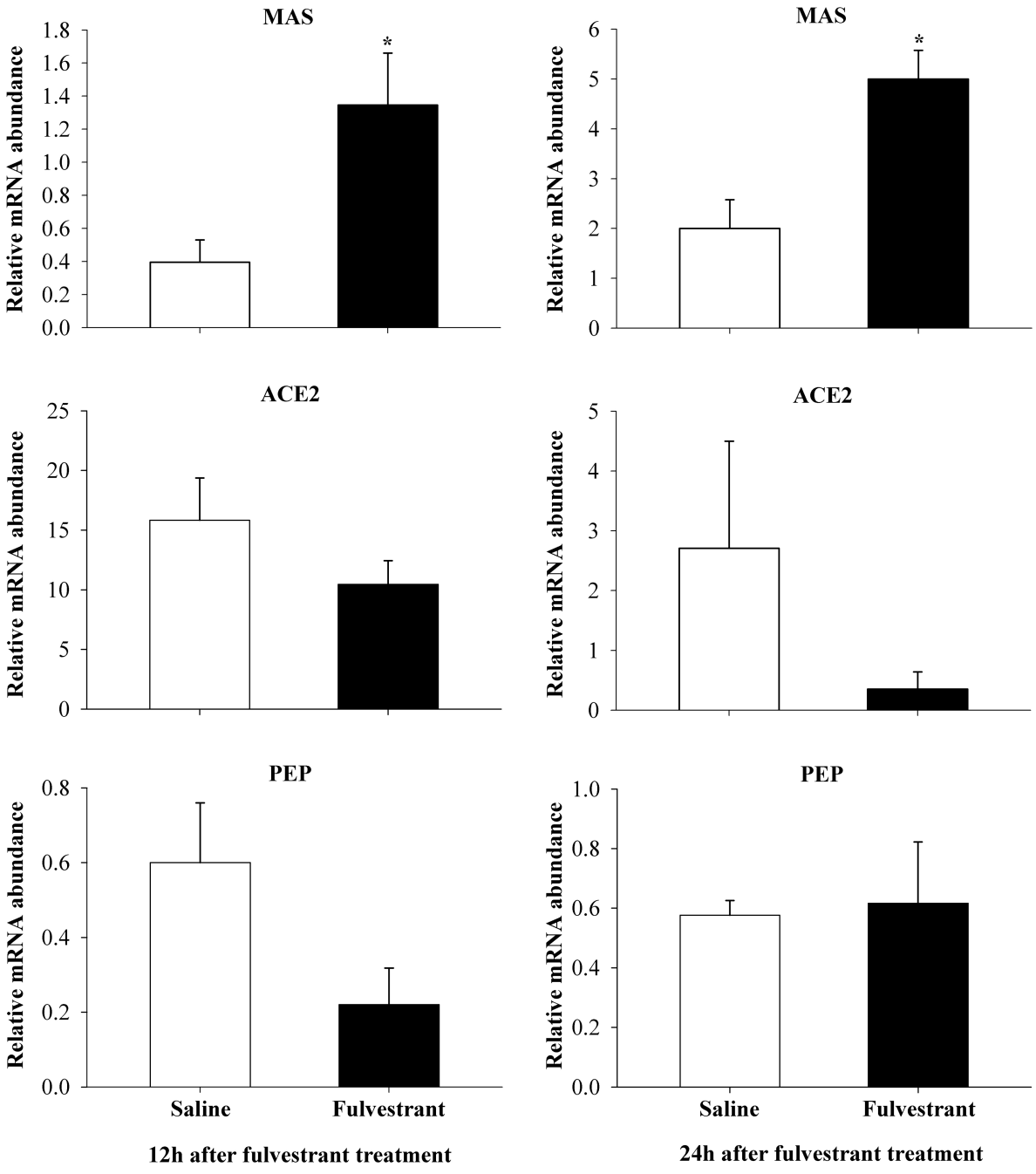
Figure 2



485

486

Figure 3



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488

4. ARTIGO 2

TRABALHO SUBMETIDO PARA PUBLICAÇÃO:

HOMOLOGOUS RECOMBINATION AND NON-HOMOLOGOUS END-JOINING REPAIR PATHWAYS IN BOVINE EMBRYOS WITH DIFFERENT DEVELOPMENTAL COMPETENCE.

Marcos Henrique Barreta, Bernardo Garziera Gasperin, Vitor Braga Rissi, Matheus Pedrotti de Cesaro, Rogério Ferreira, João Francisco de Oliveira, Paulo Bayard Dias Gonçalves, Vilceu Bordignon.

EXPERIMENTAL CELL RESEARCH, 2012.

Homologous recombination and non-homologous end-joining repair pathways in bovine embryos with different developmental competence[A]

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Short Title: DNA double-straded repair in bovine embryos.

Footnotes

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Abstract

The objective of this study was to investigate the expression of genes that control homologous recombination (HR; 53BP1, ATM, RAD50, RAD51, RAD52, BRCA1, BRCA2, NBS1), and non-homologous end-joining (NHEJ; KU70, KU80, DNAPK), DNA-repair pathways in bovine embryos with high, intermediate or low developmental competence. We also evaluated whether bovine embryos can respond to DNA double-stranded breaks (DSBs) induced with ultraviolet (UV) irradiation by regulating the expression of genes involved in the HR and NHEJ repair pathways. Embryos with high, intermediate or low developmental competence were selected based on the cleavage time after in vitro fertilization and were removed from in vitro culture before (36 h), during (72 h) and after (96 h) the expected period of embryonic genome activation (EGA). All studied genes were expressed before, during and after the EGA period regardless the developmental competence of the embryos. Higher mRNA expression of 53BP1 and RAD52 was found before EGA in embryos with low developmental competence. Expression of 53BP1, RAD51 and KU70 was downregulated at 72 h and upregulated at 168 h post-fertilization in bovine embryos with DSBs induced by UV irradiation. In conclusion, important genes controlling HR and NHEJ repair pathways are expressed in bovine embryos before, during or after EGA. Lower developmental competence seems to be associated with a higher mRNA expression of 53BP1 and RAD52. Bovine embryos can respond to UV-induced DSBs after the EGA but HR and NHEJ repair pathways seem to be particularly regulated at the blastocyst stage.

Adittional keywords: DNA repair, double-strand break, bovine, embryo.

Introduction

Since the last few decades, in vitro production of embryos (IVP) from different mammalian species has been used as a model for research in different fields, e.g. cell biology, development, physiology, cryobiology, toxicology, as well as for commercial applications. However, the overall efficiency of a conventional IVP system remains very low when compared with in vivo embryo development, because only 30 to 40% of the oocytes reach the blastocyst stage (Xu *et al.* 1992; Rizos *et al.* 2002; Goovaerts *et al.* 2009; Fields *et al.* 2011).

Studies conducted with different species of animals have shown that in vitro-produced embryos differ in many aspects including gene expression profile (Enright *et al.* 2000; Corcoran *et al.* 2006; McHughes *et al.* 2009), morphology and ultrastructure (Holm *et al.* 2002; Bhojwani *et al.* 2007), metabolic activity (Lopes *et al.* 2007), and kinetics of development (Van Soom *et al.* 1992; Holm *et al.* 2002). Moreover, a recent study demonstrated that about 30% of in vitro produced bovine blastocysts have DNA damage (Sturmey *et al.* 2009). It is known that about 80-90% of fertilized oocytes are able to cleave but only 35-50% of the cleaved embryos reach the blastocyst stage (Rizos *et al.* 2002; Gilchrist and Thompson 2007). Indeed, most oocytes that reach metaphase II (MII) stage and complete the first cleavage in vitro do not develop further than 4- to 8-cells stage (Telford *et al.* 1990; Barnes and First 1991). It was proposed that this developmental block could be a consequence of the inability of early-stage embryos to repair DNA injuries triggered by in vitro environment (Betts and King 2001; Meirelles *et al.* 2004). Thus, the results presented by Sturmey *et al.* (2009) could be even more striking if DNA damage would have been evaluated in early-stage embryos.

DNA damage can have severe cellular consequences in addition to block cell development if not properly repaired. DNA damage is broadly grouped into lesions and strand breaks. Lesions are alterations in base structures and result in changes in the chemical and/or physical structure of the DNA, which can produce point mutations or physical distortions and can prevent transcription and replication. The main mechanisms responsible to repair DNA lesions in mammalian embryos are: nucleotide excision repair (NER), base excision repair (BER) and mismatch repair (MMR). In contrast, strand breaks are physical breaks in the DNA strand (for review Jaroudi and SenGupta 2007; Ozturk and Demir 2011). They can be single strand breaks or more severe double strand breaks. The presence of a single break is sufficient to block the cellular division and trigger cell death (Doherty and Jackson 2001). Generally, this type of damage results from ionizing radiation, ultraviolet (UV) radiation, environmental stresses, stalling of the DNA replication fork (Van Attikum and Gasser 2005), and reactive oxygen species (Bilsland and Downs 2005).

DNA double-strand breaks are among the most dangerous inducers of genotoxic damage and cell death via apoptosis (Rich *et al.* 2000). Two pathways are involved in the repair of double-strand breaks: 1) homologous recombination (HR), which is error-free and active in late S and G2-M stages of the cell cycle where a sister chromatid acts as a template (Johnson and Jasin 2000), and 2) non-homologous end-joining (NHEJ), which is error-prone and the predominant pathway in mammalian cells at G0/G1 stages of the cell cycle (Haber

2000; Johnson and Jasin 2000). A well-orchestred group of genes control the HR and NHEJ repair pathways. Most of these genes are expressed in mammalian oocytes and embryos. For instance, mRNA expression of BRCA1, BRCA2, NBS1, RAD50, RAD51, RAD52, ATM and 53BP1 genes was detected in human oocytes and blastocyst (Jaroudi *et al.* 2009). These are the main genes responsible for triggering and controlling the HR repair pathway in mammalian gametes and embryos (for review Jaroudi and SenGupta 2007; Ozturk and Demir 2011). Jaroudi *et al.* (2009) showed that KU70, KU80 and XRCC4, the main genes responsible for triggering and controlling the NHEJ repair pathway, are also expressed in human oocytes and blastocyst.

Recently, it has been demonstrated that microinjection of recombinant hRAD51 prior to krypton-78 or UV-B irradiation markedly decreased both cytoplasmic fragmentation and DNA damage in bovine oocytes (Kujjo *et al.* 2011). These results indicate that the HR pathway may be an important form of DNA-damage protection and repair in bovine oocytes and embryos. Although several studies have shown the presence of both HR and NHEJ repair factors in mammalian oocytes and embryos, little is still known about the expression profile of the different components involved in these two double-stranded DNA repair pathways during early embryo development. Thus, the present study was designed to: a) assess the expression profile of important genes participating in the HR and NHEJ DNA-repair pathways in bovine embryos of high, intermediate and low developmental competence; and b) evaluate whether bovine embryos at different stages after in vitro fertilization are able to respond to UV-induced double-stranded DNA breaks by regulating the expression of genes participating in HR and NHEJ DNA-repair pathways.

Material and Methods

All chemicals used were purchased from Sigma Chemicals Company, St. Louis, MO, USA, unless otherwise indicated in the text.

In vitro embryo production

Cow ovaries were obtained from a local abattoir and transported to the laboratory in saline solution (0.9% NaCl; 30°C) containing 100 IU mL^{-1} penicillin and 50 μg mL^{-1} streptomycin sulphate. Cumulus oocyte complexes (COCs) from 3 to 8 mm diameter follicles were aspirated with a vacuum pump (vacuum rate of 20 mL of water/$minute^{-1}$). The COCs

were recovered and selected according to Leibfried and First (1979) under a stereomicroscope. Grade 1 and 2 COCs were randomly distributed into 400 µL of maturation medium and cultured in an incubator at 39°C in a saturated humidity atmosphere containing 5% CO_2 and 95% air, for 24 h. The maturation medium used was TCM199 containing Earle's salts and L-glutamine (Gibco Labs, Grand Island, NY, USA) supplemented with 25 mM HEPES, 0.2 mM pyruvic acid, 2.2 mg mL^{-1} sodium bicarbonate, 5.0 mg mL^{-1} LH (Bioniche, Belleville, ON, Canada), 0.5 mg mL^{-1} FSH (Bioniche, Belleville, ON, Canada), 10% (v/v) bovine calf serum (Gibco Labs, Grand Island, NY, USA), 100 IU mL^{-1} penicillin and 50 µg mL^{-1} streptomycin sulphate.

After in vitro maturation, oocytes were fertilized with tested frozen semen that was thawed and fractionated on discontinuous Percoll (Amersham Biosciences AB, Uppsala, Sweden) gradients, as described by Parrish *et al.* (1986). The sperm were diluted to a final concentration of $2x10^6$ sperm mL^{-1} in Fert-TALP medium containing 10 µg mL^{-1} heparin, 30 µg mL^{-1} penicilinamine, 15 µM hypotaurine and 1 µM epinephrine (Parrish *et al.* 1988). In vitro fertilization was carried out by co-culture of sperm and oocytes for 18 h in four-well plates in the same atmospheric conditions as the ones used for maturation. After gamete co-incubation period, the cumulus cells were removed by 2 min of vortex. Presumptive zygotes were cultured at 39°C in an incubator culture chamber (CBS Scientific, Del Mar, CA, USA) with saturated humidity atmosphere containing 5% CO_2, 5% O_2 and 90% N_2 in 400 µL synthetic oviduct fluid (SOF) medium in four-well plates (Nunc, Roskilde, Denmark).

Estimation of the total cell number after embryo culture

After 7 days in culture, embryos were rinsed in PBS containing 0.1% PVA and fixed during 15-20 minutes in 4% paraformaldehyde. Fixed embryos were rinsed in PBS containing 0.1% PVA and stored at 4°C in PBS supplemented with 0.3% BSA and 0.1% Triton X-100 for no more than 1 week. Two to three embryos were placed onto each slide using a 10-µL glycerol drop containing 10 µM Hoechst 33342 and mounted under a coverslip. Slides were stored for 10-15 minutes before evaluation under an epifluorescent inverted microscope (Leica, DMI 4000B) to count the total number of cells per individual blastocyst.

RNA extraction and qRT-PCR

Total RNA was extracted using Trizol according to the manufacturer's instructions and was quantified by absorbance at 260 nm. Total RNA (50 ng) was first treated with 0.2 U

DNase (Invitrogen) at 37°C for 5 minutes to digest any contaminating DNA, followed by heating to 65°C for 3 minutes. The RNA was reverse transcribed (RT) in the presence of 1 µM oligo(dT) primer, 4 U Sensiscript RTase (Sensiscript RT Kit; Qiagen, Mississauga, ON, Canada), 0.5 µM dideoxynucleotide triphosphate (dNTP) mix, and 10 U RNase Inhibitor (Invitrogen) in a volume of 20 µL at 37°C for 1 hour. The reaction was terminated by incubation at 93°C for 5 minutes.

Real-time polymerase chain reaction (PCR) was conducted in a Step One Plus instrument (Applied Biosystems, Foster City, CA) with Platinum SYBR Green qPCR Master Mix (Applied Biosystems) and specific primers (Table 1). Common thermal cycling parameters (3 minutes at 95°C, 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C) were used to amplify each transcript. Melting-curve analyses were performed to verify product identity. The samples were run in duplicate and were expressed relative to Histone H2A as the housekeeping gene. The relative quantification of gene expression across treatments was evaluated using the ddCT method (Livak and Schmittgen 2001). Briefly, the dCT was calculated as the difference between the CT of the investigated gene and that of Histone H2A in each sample. The ddCT of each investigated gene is calculated as the difference between the dCT in each treated sample and the dCT of the sample with lower gene expression (higher dCT). The fold change in relative mRNA concentrations was calculated using the formula 2^{-ddCT}. Specific primers (Table 1) were designed in Primer Express Software v 3.3 (Applied Biosystems) and synthesized by Invitrogen for quantitative analysis of the mRNA abundance.

Ultraviolet irradiation

Ultraviolet (UV) irradiation was achieved by placing embryos in 400 µL of maintenance medium in an open, four-well plate. The four-well plates containing the embryos were placed on a thermal plate at 30°C into a laminar flow and submitted to UV irradiation for 0 (control group), 1, 2.5, 5 or 10 minutes. Control group was submitted to the same manipulation used to treated groups. However the control group was not exposed to UV irradiation. The maintenance medium consisted of TCM199 containing Earle's salts and L-glutamine supplemented with 25 mM HEPES, 0.2 mM pyruvic acid, 10% (v/v) bovine calf serum, 100 IU mL^{-1} penicillin and 50 µg mL^{-1} streptomycin sulphate. After exposure to UV irradiation, embryos were cultured at 39°C in an incubator culture chamber with saturated

183 humidity atmosphere containing 5% CO_2, 5% O_2 and 90% N_2 in 400 µL SOF medium in four-
184 well plates. The culture time after exposure to UV irradiation varied according to the
185 experiment.

186

187 *Immunofluorescence detection of phosphorylated histone H2AX*

188 Embryos were rinsed in PBS containing 0.1% PVA and fixed during 15-20 minutes in
189 4% paraformaldehyde. Fixed embryos were rinsed in PBS containing 0.1% PVA and stored at
190 4°C in PBS supplemented with 0.3% BSA and 0.1% Triton X-100 for no more than 1 week.
191 Fixed embryos were incubated for 1 h at room temperature in blocking solution (3% BSA and
192 0.2% Tween-20 in PBS), and then maintained overnight in the presence of the primary
193 antibody diluted in blocking solution.

194 Mouse monoclonal anti-phospho-H2A.X (Serine 139) (Upstate Cell Signaling
195 Solutions, NY) was used as primary antibody in a dilution rate of 1:500. Negative controls
196 were incubated in the absence of primary antibody. Embryos were then washed three times
197 for 20 min each in blocking solution and incubated for 1 h at room temperature (24-26°C) in
198 the presence of 1:500 diluted anti-mouse IgG Cy3-conjugated secondary antibody (Jackson
199 ImmunoResearch Lab. Inc. PA). Samples were washed three times (20 min each) in blocking
200 solution and mounted on a microscope slides using a drop of Mowiol containing 10 mg mL^{-1}
201 of Hoechst 33342 for chromatin visualization. Slides were stored in a dark box at 4°C and
202 examined within 48 h after preparation. Specimens were examined by epifluorescence using a
203 Nikon eclipse 80i microscope (Nikon, Tokyo, Japan). Images were recorded using a Retiga
204 2000R monochromo digital camera (Qimaging, BC, Canada) and the SimplePCI Imaging
205 Software (Compix, Inc., Sewickley, PA). Nuclei were individually evaluated for the
206 immunostaining signal and classified as either positive or negative.

207

208 *Experiment 1*

209 This experiment was performed to evaluate whether the cleavage time affects the
210 competence of embryonic development of in vitro matured and fertilized bovine oocytes. To
211 conduct this experiment, 2510 bovine COCs obtained in abattoir were in vitro matured and
212 fertilized. Cleavage was evaluated at 28, 32 and 36 h post-fertilization (hpf) and the cleaved
213 embryos in each of these times were placed in a new culture well separated by cleavage time.
214 At 72 hpf, the cell number was assessed again to determine the number of embryos that
215 remain at the 2-cells stage or have progressed beyond this stage. Embryo development was

evaluated 7 days after in vitro fertilization to determine the blastocyst rate per cleaved zygote. In each replication, four embryos per group were fixed in paraformaldehyde 4% for evaluating the total number of blastomeres. The total cleavage rate (at 48 hpf) and the general blastocyst rate per oocyte were evaluated to determine the efficiency of in vitro culture system. This experiment was performed in quadruplicate.

Experiment 2

The experimental design used here was similar to the experiment 1 with small modifications. Bovine embryos with high, intermediate or low competence of embryonic development were selected based on the cleavage time (28, 32 and 36 hpf, respectively) and removed from in vitro culture before, during or after the expected moment of embryonic genome activation (36, 72 or 96 hpf, respectively). Immediately after the removal from in vitro culture, embryos were washed with PBS, placed in 100 ul of Trizol and stored at -80^{o}C until RNA extraction. This approach allowed us to evaluate mRNA expression of eleven genes involved in double-stranded DNA repair and two genes involved in cell apoptosis. The studied genes are listed in Table 1. Four replications were performed per group totalizing twelve replications (4 replicas x 3 groups: 36, 72 and 96 hpf). A total of 400-600 COCs were used in each group/replicate, totalizing approximately 6000 COCs for the twelve replications.

Experiment 3

In this experiment the scriptaid (a histone deacetylase inhibitor that has a general property of transcriptional facilitation) was used during different phases of in vitro production of embryos in an attempt to advance the embryos cleavage and thereby improve embryonic development. Scriptaid (500 mM) was used during the final 12 h of in vitro maturation (n=263), during the initial 12 h of in vitro fertilization (n=266) or during in vitro culture (36 to 48 hpf; n=229). As controls, 267 untreated COCs were submitted to conventional in vitro production system. The COCs and sperm were co-incubated for 12 h in all groups. The cleavage kinetic was evaluated at 28, 32, 36 and 48 hpf and the blastocyst rate was evaluated at 168 hpf. Twelve embryos per group were fixed in 4% paraformaldehyde to estimate total cell number. This experiment was performed in triplicate.

Experiment 4

The goal of this experiment was to indentify an UV-irradiation exposure time enough to induce DNA lesions without completely impairing embryo cleavage. COCs obtained from

abattoir ovaries were submitted to conventional system of in vitro maturation and fertilization. Eighteen hours after in vitro fertilization embryos were exposed to UV irradiation for 0 (n=160), 1 (n=158), 2.5 (n=158), 5 (n=159) or 10 minutes (n=159). After exposure, embryos returned to the culture system and the cleavage rates were evaluated at 28, 32, 36 and 48 hpf. Around forty embryos per group were submitted to UV irradiation in each replication. This experiment was performed in quadruplicate.

Experiment 5

This experiment was conducted to evaluate at what point of development the embryo can respond to UV-induced DNA damage and by assessing the expression of genes involved in HR and NHEJ repair pathways. Double-stranded DNA damages were induced by exposure of embryos to UV irradiation for 2.5 minutes at 18 (n=118), 36 (n=102), 72 (n=119), 96 (n=130) or 168 hpf (n=79). Only embryos with at least two cells at 36 h, eight cells at 72 h, more than eight cells at 96 h and blastocyst at 168 hpf were used in this experiment. The control group consisted of embryos at the same time-points and were submitted to the same manipulation used to treated groups, however they were not exposed to UV irradiation (n=118 at 18 h; n=90 at 36 h; n=130 at 72 h; n=128 at 96 h and n=77 at 168 hpf). Control and irradiated embryos were cultured for 6 h after each time-point (equivalent to 6 h after UV exposure in irradiated group) and then four embryos per group were fixed in each replication to detection of phosphorylated H2A.X (γH2A.X). The remaining embryos from each group were washed with PBS, placed into 100 µl of Trizol and stored at -80^{o}C until RNA extraction to evaluate the expression of genes involved in double-stranded DNA repair. This experiment was performed in triplicate and 53BP1, RAD51, RAD52 and KU70 genes were evaluated.

Statistical analysis

Data from cleavage, two cells block and blastocyst rates were analyzed by logistic regression, using the PROC GENMOD (generalized linear model). The total number of blastomeres and the regulation of mRNA expression were analyzed by ANOVA and a multicomparison between groups was performed by least square means. Data were tested for normal distribution using Shapiro-Wilk test and normalized when necessary. In all analyses a $p<0.05$ was considered statistically significant. Non-categorical data are presented as means ± sem.

Results

Early cleavage is associated with high developmental competence.

This experiment was performed to evaluate whether the time to the first cleavage after fertilization is associated to developmental competence. The approach used in this experiment allowed us to assess the development ability of early, intermediate and late-cleaved embryos (28, 32 and 36 hpf, respectively). Most oocytes (41,3%) submitted to in vitro maturation and fertilization cleaved early (28 hpf), whereas a small proportion (10,9%) of oocytes cleaved late (36 hpf; Fig. 1A). Approximately 50% of embryos that cleaved later remain at the 2-cells stage at 72 hpf, while only a small percentage (10,6%) of early and intermediate-cleaved embryos were blocked in two-cells stage at this moment (Fig. 1B). The timing of cleavage post-fertilization was decisive to embryonic development since, the early-cleaved embryos showed a higher competence of embryonic development than intermediate or late-cleaved embryos (Fig. 1C and D). The in vitro system used in this experiment allowed a final cleavage rate of 86.6% (48 hpf) and 42.5% of total oocytes developed to blastocyst stage. Approximately 80% of produced embryos in this system were obtained from embryos that cleaved until 32 hpf. The cleavage moment (28, 32 or 36 hpf) did not affect the total number of blastomeres (103.4 ± 9.5, 114.9 ± 7.2 and 96.5 ± 17.5, respectively; $p>0.05$).

Genes involved in double-stranded DNA repair are differentially regulated in embryos with different developmental competence.

Using a similar experimental model as described in the first experiment, we characterized the expression of genes responsible to trigger and control both HR and NHEJ repair pathways in bovine embryos with high, intermediate or low developmental competence before, during and after the expected moment of embryonic genome activation. All evaluated genes were detected before, during or after the expected moment for embryonic genome activation in embryos with high, intermediate or low competence for development (Table 2). However, only two genes were regulated before the expected moment for embryonic genome activation (36 hpf). The mRNA expression of 53BP1 was upregulated in embryos with lower competence of embryonic development (Fig. 2A), while the mRNA expression of RAD52 was upregulated in embryos with intermediate or low developmental competence (Fig 2B). We did not observe significant gene expression regulation in high, intermediate or low developmental competence embryos removed from in vitro culture during or after the expected moment of embryonic genome activation (72 and 96 hpf, respectively; Table 2).

Effect of Scriptaid treatment on cleavage kinetics and embryonic development

In this experiment, the hypothesis that scriptaid could advance the cleavage kinetics and improve the embryonic development rate was rejected. Scriptaid treatment during in vitro maturation, fertilization or culture did not affect the cleavage kinetics or the final cleavage rate at 48 hpf (Fig. 3A). Moreover, when oocytes were treated with scriptaid during the final 12 h of in vitro maturation, embryo development rate was impaired but the total number of blastomeres increased. Scriptaid treatment during in vitro fertilization or culture did not affect embryonic development rate neither the total number of blastomeres (Fig. 3B and C).

The ultraviolet irradiation impairs embryo cleavage in a time-dependent manner

The purpose of this experiment was to find an optimal exposure time to UV irradiation, which impairs, but not completely blocks the embryo cleavage. UV irradiation negatively affected the embryo cleavage in a time-dependent manner. Approximately 40% of embryos exposed to UV for 2.5 minutes were able to cleave while the exposure to UV for 5 or 10 minutes inhibited the cleavage of nearly all treated embryos. However, the exposure to UV irradiation for 1 minute delayed cleavage kinetics (Fig. 4A) but did not affect the final cleavage rate at 48 hpf (Fig. 4B).

Effect of ultraviolet irradiation on expression of genes involved in double-stranded DNA repair

This experiment was conducted to evaluate whether bovine embryos are able to respond to UV-induced DNA damage by regulating the expression of genes involved in the double-stranded DNA repair. The exposure of embryos to UV irradiation for 2.5 minutes induced double-stranded DNA break at several points, which were detected by immunolocation of phosphorylated H2A.X (data not shown). It was observed that 6 h after UV-induced DNA damage mRNA levels of 53BP1, RAD51 and KU70 were reduced at 72 h but increased at 168 hpf (Fig. 5A, B and D). However, RAD52 mRNA was not regulated after by UV exposure. These results indicated that bovine embryos are able to respond to UV-induced double-stranded DNA damage. However, both HR and NHEJ repair pathways seem to be effectively regulated only at 168 hpf.

Discussion

The significant findings of this study are: 1) Most bovine oocytes cleaved until 32 h after in vitro fertilization; 2) Early-cleaved embryos have higher developmental competence than intermediate and late-cleaved; 3) A large number of late-cleaved embryos was blocked at the two-cell stage at the expected time of embryonic genome activation; 4) mRNA expression of 53BP1 and RAD52 was upregulated only before embryonic genome activation in embryos with intermediate or low developmental competence; 5) Bovine embryos are able to respond to UV-induced double-stranded DNA damage just after the embryonic genome activation.

The first approach used in this study allowed us to find that the early-cleaved embryos have a higher potential of embryonic development than intermediate- or late-cleaved embryos. Our findings suggest that in vitro early-cleaved embryos have similar developmental potential than in vivo produced embryos (Rizos *et al.* 2002). In accordance with previous studies (Lonergan *et al.* 1999; Blondin *et al.* 2002; Bastos *et al.* 2008) it was found that the required time for the first cleavages is an important parameter to predict the developmental potential of bovine embryos. Despite the greater development potential, early-cleaved embryos had similar blastomeres number at 168 hpf in comparison to intermediate and late-cleaved. This result indicates that the mitotic ability of blastomeres was similar independently of the cleavage moment. The rate of embryos blocked at 2-cells stage during expected moment for embryonic genome activation (72 hpf) was about 5 times higher in late-cleaved than in early-cleaved embryos. The lower developmental potential of late-cleaved embryos can be explained by higher blocking rate at 72 hpf.

Previously, our group demonstrated that the proportion of positively stained nuclei for H3K14ac and HMGN2 at 50 h after parthenogenetic activation was higher in late- than in early-cleaved bovine embryos (Bastos *et al.* 2008). The H3K14ac and HMGN2 proteins have been associated with different nuclear functions including chromatin condensation, transcription, DNA replication, and DNA repair (Bustin 2001; Bianchi and Agresti 2005). Furthermore, bovine embryos with delayed development after in vitro fertilization have higher rate of positive nuclei for phosphorylated H2A.X (Bastos *et al.* unpublished data). H2A.X represents about 10-15% of total histone H2A in most mammalian cells and has been known as the DNA damage-specific “histone code” based on the observation that, the double-stranded DNA break, is often associated with phosphorylation of histone variant H2A.X (Fernandez-Capetillo *et al.* 2004). Together, these results indicate that the lower

developmental competence observed in late-cleaved embryos may be related to double-stranded DNA damage.

In the second experiment, we evaluate the mRNA expression profile of genes involved in the HR and NHEJ repair pathways in embryos with high, intermediate or low developmental competence removed from in vitro culture before, during or after the expected moment of embryonic genome activation. As shown in Table 2, all studied genes were expressed before, during and after the embryonic genome activation, regardless of developmental competence. Eight of these genes are associated with DNA HR repair, three with DNA NHEJ repair and two with apoptosis. The main genes related with HR and NHEJ repair patways were also expressed in human oocytes and blastocysts (Jaroudi *et al.* 2009). It is important to highlight that of the 13 studied genes just 53BP1 and RAD52 were upregulated. This higher expression was observed just before embryonic genome activation (at 36 hpf) in embryos with intermediate and low competence of embryonic development. The 53BP1 and RAD52 belong to the group of genes responsible for early activtion stages of HR repair pathway (Menezo *et al.* 2010; Ozturk and Demir 2011). In human embryos, the double-stranded DNA repair by HR repair pathway seems to be predominantly active before embyonic genome activation (Jaroudi *et al.* 2009) similarly to what we observed in bovine embryos. As the transcriptional activity is low in the bovine embryos until the embryonic genome activation (Barnes and First 1991; Hay-Schmidt *et al.* 2001), we suspected that the higher expression of RAD52 and 53BP1 at 36 hpf can be from maternal origin because it was only observed before genome activation in embryos with intermediate and low competence of development.

During embryonic genome activation most maternal mRNAs are degraded and the embryo starts to transcribe its own mRNAs (Wong *et al.* 2010). This can justify why the observed effect at 36 h for 53BP1 and RAD52 genes disappeared at 72 and 96 hpf. The higher mRNA expression of 53BP1 and RAD52 at 36 hpf in embryos with intermediate and low developmental competence is an intriguing fact and indicates that these mRNAs were accumulated in bovine oocyte before the beginning of in vitro maturation, *i.e.* during follicle development. Nevertheless, more studies are necessary to confirm this hypothesis and to understand if the higher mRNA expression of 53BP1 and RAD52 can be related with bovine oocyte developmental competence.

The absence of regulation in mRNA expression of all studied genes at 72 and 96 hpf led us to believe that bovine embryos may have difficulty in responding to a double-stranded DNA damage immediately after the embryonic genome activation. To evaluate this

hypothesis, we developed an experimental model that induced a double-stranded DNA damage without totally impairing the cleavage. Using this experimental model, approximately 40% of embryos exposed to UV irradiation for 2.5 minutes were able to cleave and the double-stranded DNA break was confirmed by immunolocation of phosphorylated H2A.X. Using this approach, we found that bovine embryos are able to respond to DNA damage only after the embryonic genome activation but, the ability to upregulate genes involved in the double-stranded DNA repair was only observed at 168 hpf when the embryonic genome is totally functional. The expression of double-stranded DNA repair genes was greatly increased during and after the embryonic genome activation. However, when DNA damage was induced at 72 hpf, mRNA expression of three out of four studied genes were downregulated indicating that the embryos may be translating the mRNA to protein but not being able to synthesize enough mRNA to meet the high demand induced by DNA damage. In contrast, when embryos were exposed to UV irradiation at 168 hpf a higher expression of DNA repair genes was observed indicating that the bovine embryos can have higher ability to respond to DNA damage when the embryonic genome is totally functional. The upregulation in mRNA expression of 53BP1, RAD51 and KU70 genes at 168 hpf indicates that HR and NHREJ repair pathways are present and potentially actives in bovine embryos.

Bovine oocytes submitted to UV irradiation rapidly increase the RAD51 protein synthesis while in mice this response was longer (Kujjo *et al.* 2011). The rapid increase in the RAD51 production demonstrated that bovine oocytes could be more resistant than mice oocytes to DNA damage induced by irradiation (Kujjo *et al.* 2011). This suggests that there is a significant difference between species in response to the DNA damage caused by irradiation. However, the reason why bovine oocytes respond faster than the mouse oocytes to DNA damage induced by UV irradiation is still unknown. Our results demonstrated that UV-induced increased expression of RAD51, is accompanied by upregulation of 53BP1. The 53BP1 is a nuclear protein that rapidly localizes double-stranded DNA breaks induced by ionizing radiation, etoposide, neocarzinostatin and other agents (Schultz *et al.* 2000; Rappold *et al.* 2001). In agreement with our results, Jaroudi *et al.* (2009) found that human oocytes and blastocysts have medium to high levels of 53BP1, RAD51, RAD52, KU70 and KU80 mRNA. This rapid ability to activate the HR and NHEJ repair pathways could be explained as an adaptive response to continuous exposure to solar and cosmic irradiations of bovines and humans as previously mentioned by Kujjo *et al.* (2011).

The addition of Scriptaid to in vitro maturation, fertilization and culture media did not affect the cleavage kinetics and did not improve the embryonic development rate. These

results may be due to low capacity of transcription observed in bovine embryo until the genome is completely functional. However, further studies involving different concentrations and different times of exposure to Scriptaid are required.

In conclusion, the main genes responsible to trigger and control both HR and NHEJ repair pathways are expressed before, during or after embryonic genome activation in bovine embryos with high, intermediate or low in vitro developmental competence. Bovine embryos with low in vitro developmental competence were found to have higher mRNA expression of 53BP1 and RAD52 before embryonic genome activation. Bovine embryos can respond to a double-stranded DNA damage only after the embryonic genome activation but HR and NHEJ repair pathways seem to be effectively regulated only after the embryonic genome is totally functional.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Legend Figures:

Fig. 1. Cleavage kinetics, percentage of embryos blocked at 2-cells stage at 72 h after in vitro fertilization (IVF) and development of embryos cleaved at 28, 32 and 36 h after IVF. Cleavage rates represent the proportion of oocytes that cleaved at 28, 32 and 36 h after IVF (A). The percentage of embryos blocked at the 2-cells stage was calculated based on the number of cleaved at 28, 32 and 36 h after IVF (B). Blastocyst rate was evaluated per total of cleaved at 28, 32 and 36 h after IVF (C) or per total of oocytes (D). Bars with no common letter are statistically different ($p \leq 0.05$).

Fig. 2. Relative mRNA expression (mean ± standard error of mean) of 53BP1 (A) and RAD52 (B) in bovine embryos with high, intermediate or low competence of embryonic development and removed from in vitro culture at 36 h after IVF. The time to the first cleavage was used as a parameter to predict the developmental competence. Embryos cleaved at 28, 32 and 36 h after IFV were considered to have high, intermediate and low competence for embryo development, respectively. Bars with no common letter are statistically different ($p \leq 0.05$).

Fig. 3. Cleavage kinetics (A), embryonic development (B) and total number of blastomeres (C) after treatment with 500 mM scriptaid during the final 12 h of in vitro maturation (IVM), initial 12 h of in vitro fertilization (IVF) or from 36 to 48 h after IVF (IVC). Bars with no common letter are statistically different ($p \leq 0.05$).

Fig. 4. Cleavage kinetics (A) and cleavage rate (B) of embryos exposed to ultraviolet (UV) irradiation for 0, 1, 2.5, 5 or 10 minutes at 18 h after in vitro fertilization (IVF). The cleavage rate was evaluated at 48 h after IVF. Bars with no common letter are statistically different ($p \leq 0.05$).

Fig. 5. Relative mRNA expression (mean ± standard error of mean) of 53BP1 (A) RAD51 (B), RAD52 (C) and KU70 (D) 6 h after induction of DNA damage (ultraviolet irradiation for 2.5 minutes) at 18, 36, 72, 96 and 168 h after in vitro fertilization (IVF). The asterisk (*) indicates difference between control and irradiated group at the same time ($p \leq 0.05$).

645 **Table 1.** Primers used in the expression analysis of candidate genes. Primer sequences and
646 concentrations used to amplify each gene are described.

Gene	Sequence	Conc. (nM)	Reference or accession n^{o}
Histone H2A	GAGGAGCTGAACAAGCTGTTG	200	Bettegowda *et al.* 2006
	TTGTGGTGGCTCTCAGTCTTC	200	
53BP1	ATCAGACCAACAGCAGAATTTCC	200	ENSBTAT00000028388
	CACCACGTCAAACACCCCTAA	200	
RAD52	GGCCAGGAAGGAGGCAGTA	200	ENSBTAT00000055617
	TGACCTCAGATAGTCTTTGTCCAGAA	200	
RAD51	ATGCACCGAAGAAGGAGCTAAT	200	ENSBTAT00000003788
	GATCGCCTTTGGTGGAACTC	200	
RAD50	TGTGGAACAGGGCCGTCTA	200	XM_001255278.2
	CAATTCTAGCTGTGTTGCCAGAGA	200	
KU70	AATTGACTCCTTTTGACATGAGCAT	200	XM_001789352.1
	CCATAGAACACCACTGCCAAGA	200	
KU80	TGGCATCTCCCTGCAGTTCT	200	ENSBTAT00000013758
	AGGCCCATGGTGGTCTGA	200	
BRCA1	ACAAAGCAGCAGACACAATCTCA	200	ENSBTAT00000011616
	TCATGGTCTCCCACACTGAAATA	200	
BRCA2	AAATTTCACTGCACCTGGTCAA	200	ENSBTAT00000001311
	TCATGGGTTTGCCTATAGTTATCG	200	
ATM	CTTAGGAGGAGCTTGGGCCT	200	ENSBTAT00000040104
	CCGCTGTGTGGCAAACC	200	
NBS1	GGCGTCCGATTGTAAAACCA	200	ENSBTAT00000017598
	TCAACAGGTGGGTAAAAACTTTCA	200	
DNAPK	AAAGGCAATCCGTCCTCAGA	200	ENSBTAT00000022631
	AAGGCAGGTGCTAAACTGAGATG	200	
BAX	GACATTGGACTTCCTTCGAGA	200	Mani *et al.* 2010
	AGCACTCCAGCCACAAAGAT	200	
BCL2	GTGGATGACCGAGTACCTGAAC	200	Mani *et al.* 2010
	AGACAGCCAGGAGAAATCAAAC	200	

647 F, Forward primer; R, Reverse primer; Conc., primer concentration used for gene
648 amplification.
649

Table 2. mRNA expression and regulation of genes controlling apoptosis, homologous recombination and non-homologous end-joining repair pathways in bovine embryos removed from in vitro culture at 36, 72 or 96 h post-fertilization.

Involved Pathway	Gene	36 h		72 h		96 h	
		Expressed	Regulated	Expressed	Regulated	Expressed	Regulated
HR repair	53BP1	**Yes**	**Yes**	Yes	No	Yes	No
	RAD52	**Yes**	**Yes**	Yes	No	Yes	No
	RAD51	Yes	No	Yes	No	Yes	No
	RAD50	Yes	No	Yes	No	Yes	No
	BRCA1	Yes	No	Yes	No	Yes	No
	BRCA2	Yes	No	Yes	No	Yes	No
	ATM	Yes	No	Yes	No	Yes	No
	NBS1	Yes	No	Yes	No	Yes	No
NHEJ repair	KU70	Yes	No	Yes	No	Yes	No
	KU80	Yes	No	Yes	No	Yes	No
	DNAPK	Yes	No	Yes	No	Yes	No
Apoptosis	BAX	Yes	No	Yes	No	Yes	No
	BCL2	Yes	No	Yes	No	Yes	No

HR – homologous recombination; NHEJ – non-homologous end-joining.

656

Figure 1

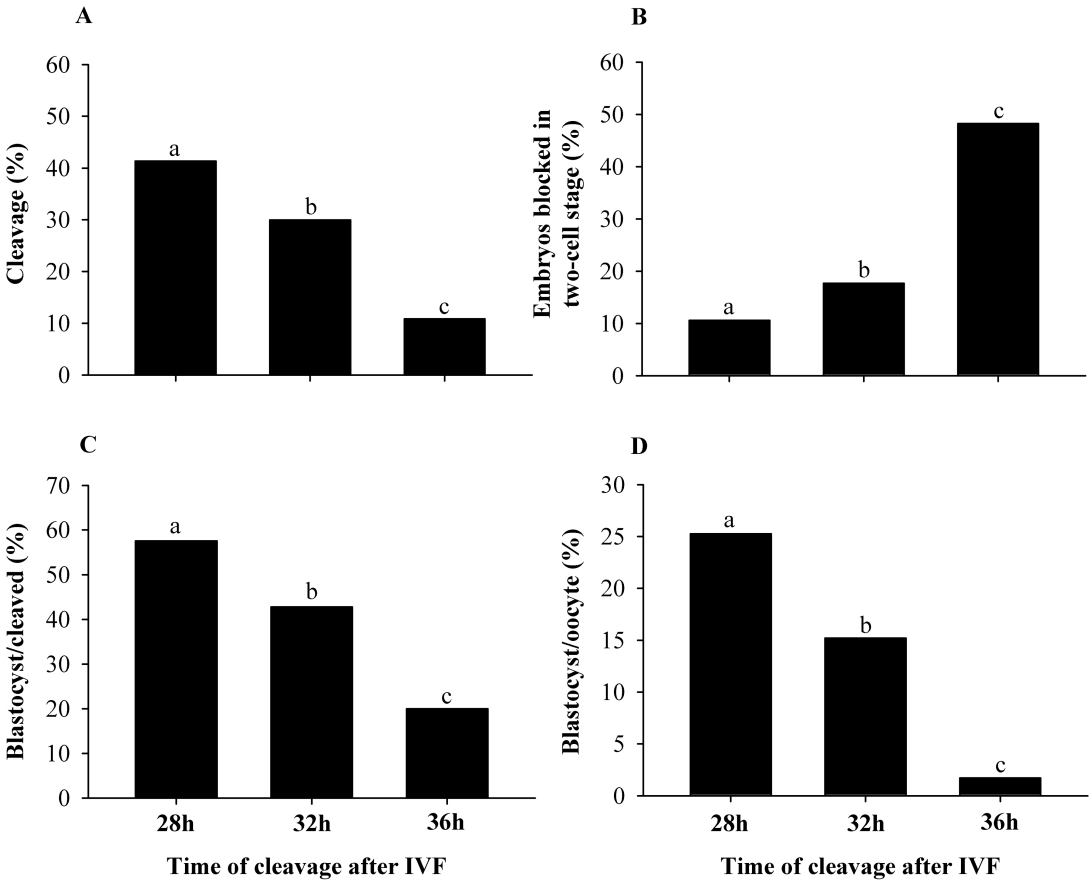

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Figure 2

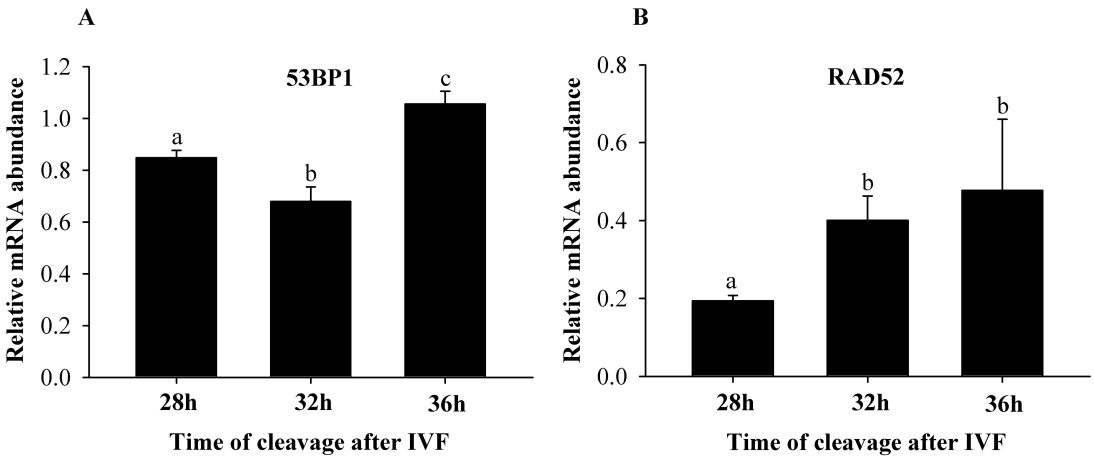

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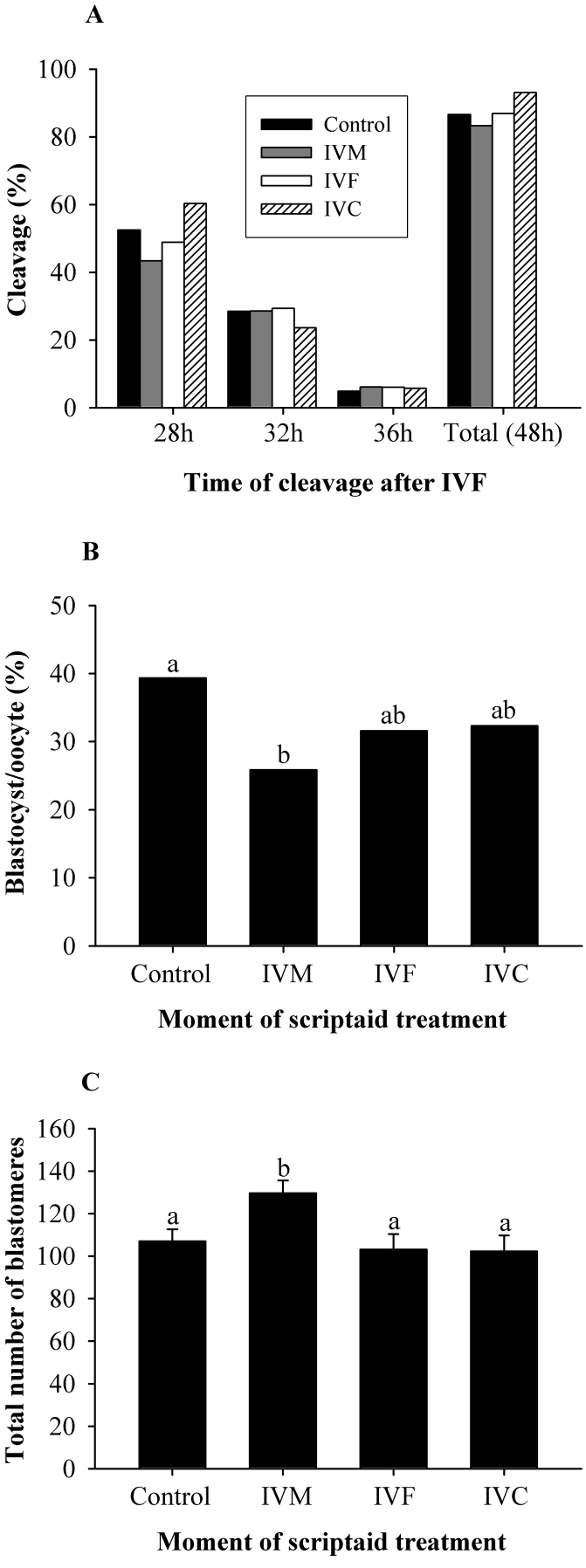
Figure 3


A
Cleavage (%)
100
80
60
40
20
0
Control
IVM
IVF
IVC
28h
32h
36h
Total (48h)
Time of cleavage after IVF
B
Blastocyst/oocyte (%)
50
40
30
20
10
0
a
b
ab
ab
Control
IVM
IVF
IVC
Moment of scriptaid treatment
C
Total number of blastomeres
160
140
120
100
80
60
40
20
0
a
b
a
a
Control
IVM
IVF
IVC
Moment of scriptaid treatment

662

Figure 4

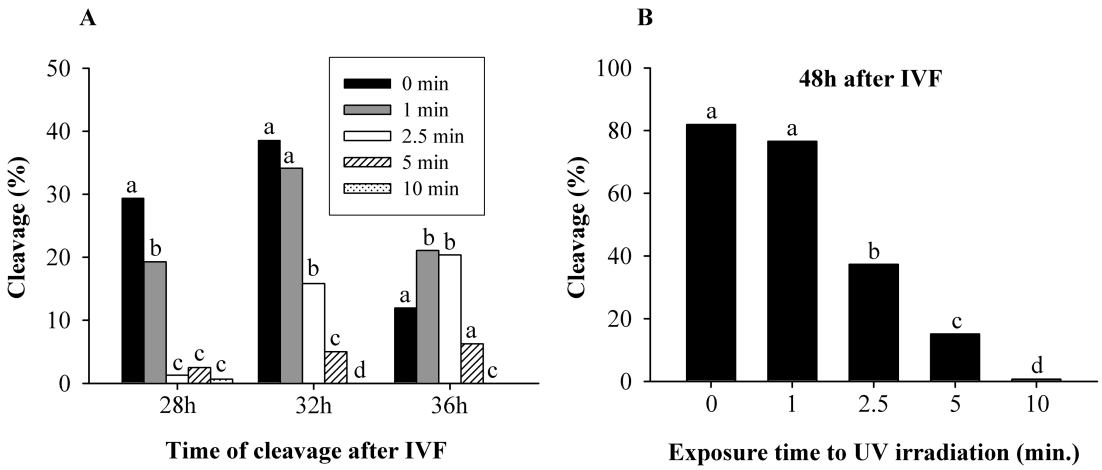


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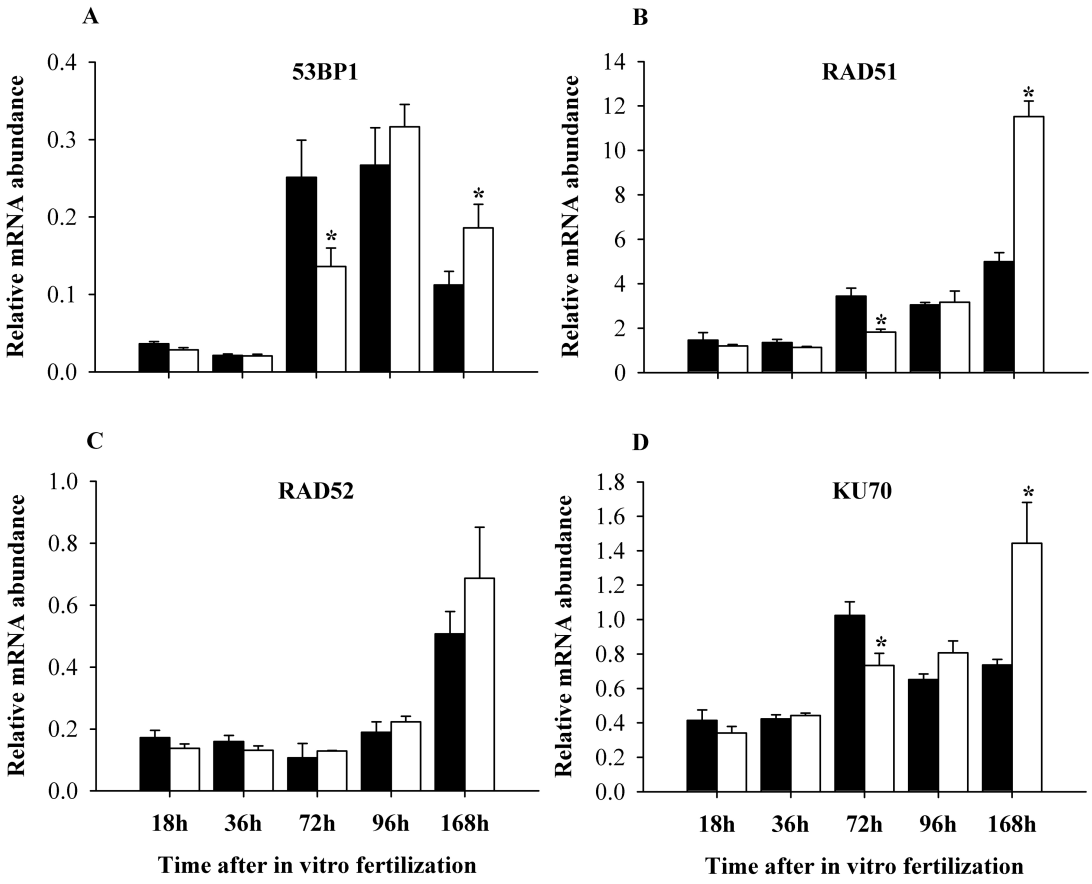
Figure 5



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5. DISCUSSÃO

O avanço nas técnicas de dosagem hormonal e o uso da ultrassonografia na medicina veterinária propiciaram, no final da década de 80 e na década de 90, um melhor entendimento sobre a dinâmica de crescimento folicular em mamíferos. Em bovinos, o desenvolvimento de folículos específicos pode ser acompanhado com precisão desde a emergência de uma onda folicular até a ovulação do folículo dominante. Além disso, o microambiente folicular pode ser facilmente manipulado em fases específicas da onda folicular utilizando técnicas de injeção intrafolicular (KOT et al., 1995; FERREIRA et al., 2007), o que permitiu o estudo de diversos fatores locais que participam do controle da divergência folicular (FERREIRA et al., 2011a; FERREIRA et al., 2011b), ovulação (FERREIRA et al., 2007; SANTOS et al., 2011); e maturação de oócitos (BARRETA et al., 2008). Por isso, a fêmea bovina pode ser considerada um bom modelo para o estudo do desenvolvimento folicular antral, ovulação e maturação de oócitos em espécies monovulatórias.

No primeiro estudo descrito neste manuscrito, foi utilizado um modelo experimental bem estabelecido (RIVERA et al., 2001) que nos permitiu acompanhar o desenvolvimento de folículos específicos e a obtenção de células da teca e granulosa do maior e do segundo maior folículo antes (Dia 2), durante (Dia 3) e após (Dia 4) o momento esperado para a divergência folicular. Com essa abordagem, foi demonstrado que o receptor MAS é mais expresso nas células da granulosa do folículo subordinado após o estabelecimento da divergência folicular (Dia 4). Utilizando o mesmo modelo experimental descrito por Ferreira et al. (2011b), foi demonstrado que a expressão do receptor MAS aumenta nas células da granulosa 12 e 24 h após a indução da atresia do folículo dominante pela injeção intrafolicular de um inibidor do receptor de estradiol. Alguns estudos têm demonstrado que a ativação do receptor MAS pela Ang-(1-7) é capaz de inibir a proliferação celular em diversos sistemas locais (FREEMAN et al., 1996; LOOT et al., 2002; GALLAGHER & TALLANT 2004; TALLANT et al., 2005; SOTO-PANTOJA et al., 2009). Esse efeito parece ser mediado, ao menos em parte, pela redução da expressão e da atividade de MMP-2 e MMP-9 no tecido pulmonar (NI et al., 2012). As metaloproteinases da matriz extracelular (MMPs), são enzimas proteolíticas que têm capacidade de degradar a matriz extracelular permitindo o remodelamento fisiológico dos tecidos (GALIS & KHATRI, 2002; SPINALE, 2002). Dessa forma, a inibição da atividade das MMPs pode ser um dos mecanismos envolvidos na ação da Ang-(1-7), via receptor MAS, sobre o desenvolvimento folicular. Pela primeira vez, foi evidenciado que a regulação da

expressão do receptor MAS nas células da granulosa pode estar relacionada com a atresia folicular.

Nós também demonstramos que as enzimas chave para produção de Ang-(1-7) são expressas nas células da teca e granulosa do maior e segundo maior folículos da onda antes, durante e após o estabelecimento da divergência. A expressão de ACE_2 foi maior no folículo dominante, enquanto que, a expressão de PEP foi maior no folículo subordinado durante e após o estabelecimento da divergência folicular. Recentemente, foi demonstrado que os níveis de Ang II, estradiol e a expressão de CYP19 aumentam no folículo dominante durante e após o estabelecimento da divergência folicular em bovinos (FERREIRA et al., 2011b). Portanto, a maior expressão de ACE_2 no folículo dominante pode estar sendo regulada pelos níveis de Ang II e estrógeno, uma vez que, ambos são responsáveis por aumentar a expressão de ACE_2 em outros sistemas locais (IGASE et al. 2005; IGASE et al. 2008; JI et al. 2008; LIN et al., 2010). Juntos, esses resultados sugerem que a expressão de ACE_2 pode ser um importante mecanismo de controle dos níveis de Ang II no fluído folicular. Entretanto, mais estudos serão necessários para entender como a expressão de ACE_2 é regulada nas células da granulosa e quais são seus efeitos sobre o desenvolvimento folicular. Como a expressão de ACE_2 é menor nos folículos subordinados, a conversão de Ang II em Ang-(1-7) ou de Ang I em Ang-(1-9) não parecem ser as principais vias de produção de Ang-(1-7) nos folículos subordinados. Portanto, a maior expressão de PEP nas células da granulosa durante e após o estabelecimento da divergência folicular sugerem que a conversão de Ang I em Ang-(1-7) pode ser a rota de escolha para a produção de Ang-(1-7) nos folículos subordinados.

Futuramente, estudos *in vivo* utilizando a técnica de injeção intrafolicular e cultivo *in vitro* de células da granulosa podem auxiliar no desenvolvimento de experimentos que visem o melhor entendimento da função e regulação do sistema Ang-(1-7) no microambiente folicular. Essas abordagens poderiam auxiliar no esclarecimento de como o sistema Ang-(1-7) participa do ajuste fino do desenvolvimento folicular e como ele interage com outros sistemas locais bem estabelecidos, tais como, Ang II, fatores de crescimento semelhante a insulina, fatores de crescimento fibroblastico, fatores de crescimento semelhante ao EGF, entre outros. Ainda, seria interessante esclarecer se os fatores endócrinos responsáveis por orquestrar o desenvolvimento folicular, tais como, FSH, LH, estradiol e progesterona podem estar envolvidos na regulação local do sistema Ang-(1-7).

No segundo estudo descrito nesta tese, nosso primeiro objetivo foi estabelecer um modelo *in vitro* que permitisse obter embriões bovinos com diferentes potenciais de desenvolvimento. Após avaliar a cinética de clivagem de aproximadamente 2500 zigotos

observamos que o tempo requerido para a primeira clivagem pós fertilização *in vitro* (FIV) é um bom parâmetro para predizer o potencial de desenvolvimento embrionário. Nesse experimento, foi demonstrado que os embriões que clivam até as 28 h pós FIV têm um maior potencial de desenvolvimento que os embriões que levam 32 ou 36 h pós FIV para realizar a primeira clivagem. Dessa forma, baseado no horário da primeira clivagem nós conseguimos desenvolver um sistema para obter embriões com alta, média ou baixa capacidade de desenvolvimento embrionário. Foi demonstrado ainda que os embriões que clivam mais tarde têm um maior percentual de bloqueio no estádio de 2 duas células durante o momento esperado para a ativação do genoma embrionário (72 h pós FIV). Alguns trabalhos publicados por nosso grupo sugerem que o menor potencial de desenvolvimento observado nos embriões que clivam tarde pode estar relacionado com danos na fita dupla de DNA, uma vez que, esses embriões apresentam uma maior quantidade de núcleos positivos para H3K14ac, HMGN2 (BASTOS et al., 2008) e H2A.X fosforilada (BASTOS et al. dados não publicados).

Após o estabelecimento do modelo *in vitro*, nós avaliamos o perfil de expressão de genes que participam do controle do reparo da fita dupla de DNA pelas vias de recombinação homóloga (HR) ou união terminal não homóloga (NHEJ) em embriões bovinos com alta, média ou baixa competência de desenvolvimento e que foram retirados do cultivo *in vitro* antes (36 h), durante (72 h) ou após (96 h pós FIV) a ativação do genoma embrionário. Foi observado que todos os genes estudados envolvidos no controle das vias HR (53BP1, ATM, RAD50, RAD51, RAD52, BRCA1, BRCA2 e NBS1) e NHEJ (KU70, KU80 e DNAPK) são expressos nos embriões bovinos independente do seu potencial de desenvolvimento ou do momento de sua retirada do cultivo. Entretanto, os genes 53BP1 e RAD52 foram mais expressos nos embriões que clivaram tarde (36 h pós FIV). Esse efeito foi observado somente quando os embriões foram retirados do sistema de cultivo antes da ativação do genoma embrionário (36 h pós FIV). O 53BP1 e RAD52 pertencem ao grupo de genes responsáveis pelos estádios iniciais de ativação da via HR (MÉNÉZO et al., 2010; OZTURK & DEMIR 2011). Após o reinício da meiose a atividade transcricional do oócito bovino torna-se muito baixa e assim perdura até a ativação do genoma embrionário (BARNES & FIRST, 1991; HAY-SCHMIDT et al., 2001). Isso nos leva a crer que a maior quantidade de RNAm de 53BP1 e RAD52 detectada as 36 h pós FIV nos embriões com baixo potencial de desenvolvimento, pode ter sido acumulada no oócito antes do início da maturação *in vitro*. Os complexos cumulus-oócitos coletados para PIV são oriundos de folículos em diferentes fases de desenvolvimento. Dessa forma, podem ser puncionados folículos saudáveis ou com diferentes graus de atresia folicular o que de certa forma pode estar afetando a integridade do

oócito. Entretanto, novos estudos serão necessários para compreender porque os embriões com baixo potencial de desenvolvimento apresentam maior quantidade de RNAm para 53BP1 e RAD52 antes da ativação do genoma embrionário e se esses genes podem servir como marcadores de competência para o desenvolvimento embrionário. Uma abordagem interessante para responder parte desses questionamentos seria comparar a expressão de 53BP1 e RAD52 em oócitos oriundo de folículos puncionados *in vivo* em fases aleatórias do desenvolvimento folicular (semelhante ao que acontece na PIV) com oócitos puncionados em diferentes momentos após a emergência de uma nova onda folicular. Além disso, abordagens comparando oócitos maturados *in vivo* e *in vitro* poderiam ser de grande valia.

Como a expressão de todos os genes estudados foi baixa as 72 e as 96 h pós FIV e não houve regulação da expressão entre os embriões com diferentes potencias de desenvolvimento, nós suspeitamos que os embriões bovinos podem ter dificuldade de responder a uma quebra na fita dupla de DNA antes do genoma embrionário estar em pleno funcionamento. Para testar essa hipótese, foi desenvolvido um modelo experimental *in vitro* que induzisse quebras na fita dupla de DNA, sem bloquear completamente o desenvolvimento dos embriões. No modelo experimental desenvolvido, podemos observar que quando os embriões eram expostos a irradiação UV por 2.5 minutos as 18 h pós FIV, 40% deles foram capazes de clivar. O dano a fita dupla de DNA foi confirmado pela imunolocalização de H2A.X fosforilada.

Utilizando essa abordagem, foi demonstrado que os embriões bovinos são capazes de responder a uma quebra na fita dupla de DNA somente após a ativação do genoma embrionário mas, uma maior expressão dos genes envolvidos no reparo da fita dupla de DNA foi observado somente as 168 h pós FIV. Esse resultado indica que os embriões bovinos podem ter plena capacidade de reparar quebras na fita dupla de DNA somente quanto o genoma embrionário estiver em pleno funcionamento. Dessa forma, torna-se compreensível porque grande parte dos embriões clivados tarde bloqueiam seu desenvolvimento antes da ativação do genoma embrionário. A maior expressão de 53BP1, RAD51 e KU70 as 168 h pós FIV indica que as vias de reparo HR e NHREJ estão presentes e potencialmente ativas nos embriões bovinos.

6. CONCLUSÃO

O presente trabalho demonstrou que o RNAm do receptor MAS e das enzimas chave para a produção de Ang-(1-7), ACE_2, NEP e PEP são expressos nas células da teca e granulosa dos folículos dominante e subordinado antes, durante e após a divergência folicular. A expressão diferencial das enzimas ACE_2, NEP e PEP durante a fase de divergência folicular sugere o envolvimento do sistema Ang-(1-7) no processo regulatório da divergência folicular em bovinos. A maior expressão do receptor MAS nas células do folículo subordinado após a divergência e em folículos induzidos a atresia, indica que esse receptor está envolvido com o processo de atresia folicular.

Com relação aos genes de reparo da fita dupla de DNA, podemos concluir que os principais genes responsáveis pela ativação e controle das vias de reparo HR e NHEJ são expressos em embriões com alta, média ou baixa competência de desenvolvimento embrionário *in vitro*. Embriões bovinos com baixa competência de desenvolvimento apresentaram maior expressão de 53BP1 e RAD52 as 36 h após a fertilização in vitro. Ainda, foi demonstrado que os embriões bovinos podem responder a quebras na fita dupla de DNA somente após a ativação do genoma embrionário mas as vias de reparo HR e NHEJ parecem ser efetivamente reguladas somente após o genoma embrionário estar totalmente funcional.

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