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Avaliação da função do óxido nítrico na capacitação do espermatozoide equino criopreservado

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RESUMO

FRANCO-SILVA, D. **Avaliação da função do óxido nítrico na capacitação do espermatozoide equino criopreservado.** [Evaluation of the role of nitric oxide in capacitation of cryopreserved equine spermatozoa by flow cytometry and computerized sperm motility (CASA) analyses]. 2013. 116f. Dissertação (Mestrado em Ciências) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, 2013

A capacitação é um pré-requisito fisiológico importante para que a célula espermática fertilize o oócito. O óxido nítrico (NO) é sintetizado *in vivo* durante a conversão da L-arginina em L-citrulina por reações oxidativas catalisadas pela enzima óxido nítrico sintase (NOS) desempenhando um papel importante na regulação da motilidade e na capacitação dos espermatozoides. Estudos indicam que o NO é capaz de regular a concentração da AMP cíclico e, por conseguinte, através da atividade da adenil ciclase, estimular a capacitação espermática em várias espécies. O objetivo deste estudo foi avaliar a função do NO na capacitação de espermatozoides equinos criopreservados. Três ejaculados foram colhidos de três garanhões (n=9). O sêmen foi diluído em meio Botu-Crio® na concentração final de 200×10^6 células/mL, envasado em palhetas de 0,5 mL e criopreservado usando um sistema automatizado. Para cada análise, foram descongeladas quatro palhetas da mesma partida e do mesmo garanhão em banho-maria a 37°C/30 s. e em seguida, o sêmen foi submetido à centrifugação em meio FIV. Posteriormente, o sêmen foi incubado neste mesmo meio na presença de L-arginina, com ou sem inibidor da enzima óxido nítrico sintase o (L-NAME), e com ou sem o removedor de NO (azul de metileno) nos tratamentos: 1) C= (FIV); 2) A= L-arginina (10 mM); 3) L = L-NAME (1 mM); 4) M = azul de metileno (100 mM); 5) AL = L-arginina (10 mM) + L-NAME (1 mM); 6) AM = L-arginina (10 mM) + azul de metileno (100 mM). As amostras foram incubadas a 38°C e 5 % de CO_2. Após a incubação realizou-se a análise computadorizada da motilidade do espermatozoide e as análises por citometria de fluxo. Para a análise computadorizada da motilidade espermática foram avaliados os tempos de incubação de 0, 60, 120 e 300 min. e para as análises por citometria de fluxo os tempos de 60, 120 e 300 min. Para avaliar a integridade das membranas plasmática e acrossomal usou-se a associação FITC-PSA e IP. Para a detecção da fosforilação do aminoácido tirosina, usou-se o anticorpo antifosfotirosina conjugado a uma fluoresceína (DAF-2). A fim de dosar a quantidade de NO produzido

pelo espermatozoide equino criopreservado foi utilizada a sonda DAF e para avaliar a peroxidação lipídica da membrana espermática utilizou a sonda C11-BODIPY. A sonda H33342 foi usada com a finalidade de evitar que partículas do mesmo tamanho e granulosidade da célula espermática fossem incluídas na contagem das análises por citometria de fluxo. Os dados foram analisados por meio da ANOVA e a comparação das médias, dentro de cada tempo, pelo teste de Tukey, com o nível de significância de 5 %, usando o software SAS. A remoção do NO do meio de cultura inibiu a motilidade das células espermáticas em todos os tempos de incubação. A motilidade total e motilidade progressiva foram reduzidas nos grupos M e AM. Os espermatozoides incubados com o removedor do NO apresentaram maior porcentagem de células com membrana plasmática e acrossomal íntegras nos 60 e 120 minutos de incubação ($p<0,05$). A reação acrossomal foi induzida nos tratamentos que receberam L-arginina (A; AL). Dentro de cada tratamento, a quantidade de NO produzido pelo espermatozoide, a fosforilação do aminoácido tirosina e a peroxidação lipídica não apresentaram diferenças entre os tempos ($p>0,05$). Foi verificada uma redução destas variáveis nos grupos M e AM ($p<0,05$). Contudo, a dose de 1 mM de L-NAME, não foi suficiente para inibir a NOS em espermatozoides criopreservados de equinos. A remoção do NO mantém a integridade das membranas plasmática e acrossomal, entretanto inibe totalmente a motilidade espermática, sugerindo um papel benéfico do NO endógeno na manutenção da motilidade dos espermatozoides equinos criopreservados.

Palavras chave: Espermatozoide. Capacitação. Óxido Nitrico. L-arginina. L-NAME. Azul de metileno.

ABSTRACT

FRANCO-SILVA, D. **Evaluation of the role of nitric oxide in capacitation of cryopreserved equine spermatozoa** [Avaliação da função do óxido nítrico na capacitação do espermatozoide equino criopreservado]. 2013. 116f. Dissertação (Mestrado em Ciências) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, 2013

Capacitation is an essential physiological prerequisite in order to sperm cell fertilize the oocyte. Nitric oxide (NO) is synthesized in vivo during the conversion of L-arginine in L-citruline by oxidative reactions catalyzed by nitric oxide synthase enzyme (NOS) and plays an important role in regulation of motility and in sperm capacitation. Studies indicated that NO is capable of regulating cAMP concentration and, therefore, by adenylyl cyclase, stimulate sperm capacitation in several species. The aim of this study was to evaluate the function of nitric oxide in cryopreserved equine sperm capacitation.Three ejaculates from three stallions were collected (n=9). Semen samples were diluted with Botu-Crio® extender to a final concentration of 200×10^6 sperms/mL, and then packaged in 0.5mL straws and cryopreserved using an automated freezing system. For each analysis, four straws from the same batch and the same stallion were thawed in a water bath at 37°C/30 s. washed by centrifugation in FIV medium. Thereafter, samples were incubated in FIV medium in the presence of L-arginine, with or without the inhibitor of nitric oxide sinthase (L-NAME), and with or without the scavenger of NO (Methylene blue) in the following treatments: 1) C = Control (FIV); 2) A = L-arginine 10 mM; 3) L = L-NAME 1mM; 4) M = Methylene blue 100 mM; 5) AL = L-arginine (10 mM) + L-NAME (1 mM); 6) AM = L-argine (10 mM) + Methylene blue (100 mM). The treatments were incubated at 38°C and CO_2 at 5 %. After incubation, the computer-assisted sperm motility (CASA) and flow cytometry analyses were performed. For CASA analysis, the incubation times of 0, 60, 120 e 300 min. were evaluated and for flow cytometry analyses times 60, 120 e 300 min. were evaluated. Plasma and acrosomal membranes integrity were evaluated by FITC-PSA and PI association. In order to detect amino acid tyrosine phosphorylation, we used the anti-phosphotyrosine antibody conjugated to a fluorescein (DAF-2). In order to quantify the amount of nitric oxide produced by cryopreserved equine sperm, the fluorescent probe DAF was used, and to evaluate the lipid peroxidation of sperm membrane we

used the probe BODIPY-C11. The probe H33342 was used in order to prevent that particles of the same size and granularity of sperm cell were included in the counting of flow cytometry analyses. Data were analyzed by ANOVA and comparison of means within each time by the Tukey test, at a significance level of 5%, using SAS software. Removing NO from the culture medium inhibited the motility of sperm cells at all incubation times. Total and progressive motilities were reduced in both groups, M and AM. Sperms incubated with the scavenger of NO had the highest percentage of cells with intact plasma and acrosomal membranes at 60 and 120 minutes of incubation ($p < 0.05$). Acrosomal reaction was induced in treatments with L-arginine (A, AL). Within each treatment, the amount of NO produced by sperms, the level of amino acid tyrosine phosphorylation and lipid peroxidation had no differences between the times used ($p > 0.05$). A reduction of these variables in groups M and AM ($p < 0.05$) was observed. However, a dose of 1 mM L-NAME was not sufficient to inhibit NOS in cryopreserved equine sperm. Removal of NO maintains plasma and acrosomal membranes integrity, however completely inhibits sperm motility, suggesting a beneficial role of endogenous NO in the maintenance of motility of cryopreserved equine spermatozoa.

Keywords: Sperm. Capacitation. Nitric Oxide. L-arginine. L-NAME. Methylene blue.

1 INTRODUÇÃO

A criopreservação é uma biotécnica que causa danos aos espermatozoides (WATSON, 2000). A congelação e a descongelação são passos importantes na criopreservação espermática, uma vez que provoca efeitos sobre a estrutura e função celular (HAMMERSTEDT; GRAHAM; NOLAN, 1990), reduz a motilidade e capacidade fertilizante (HAMMERSTEDT, 1993), induz a capacitação prematura e a descondensação nuclear (CORMIER; SIRARD; BEILEY, 1997). Portanto, mesmo levando em consideração o uso de crioprotetores como o glicerol, a gema de ovo e o leite, alterações significativas são evidenciadas no acrossoma (WATSON, 1995), nas mitocôndrias e na membrana plasmática (FORERO-GONZALEZ et al., 2012). Por este motivo, é importante enfatizar que, a maior parte da população de células espermáticas sobreviventes exibe algum grau de danos subletais, tais como a peroxidação lipídica, o envelhecimento precoce das células (ação das espécies reativas de oxigênio (EROS) e a criocapacitação) e as transições de fase de membrana (WATSON, 2000), que diminuem seu tempo de vida dentro do trato reprodutor da fêmea, sugerindo uma situação problemática, principalmente para as espécies que apresentam longos períodos de estro, onde exames frequentes são necessários para executar inseminações artificiais próximas o suficiente da ovulação (FERRUSOLA et al., 2009a).

Um dos grandes problemas da técnica de criopreservação espermática na espécie equina, está relacionada a grande variabilidade inter-individual na sobrevivência de espermatozóides durante os procedimentos de congelação e descongelação. Tal variabilidade é muitas vezes atribuída ao fato de que a maioria dos garanhões foram selecionados pelo seu desempenho e fenótipo, e não pela qualidade espermática. No entanto, as razões fisiológicas e bioquímicas por trás dessa variabilidade permanecem sem explicação (FERRUSOLA et al., 2009b)

Watson, (2000) descreveu que a criopreservação induz a formação de um excesso de espécies reativas de oxigênio (EROS) tais como, o peróxido de hidrogênio, o anion superóxido e os radicais hidroxila, (BAUMBER et al., 2000; AGARWALL; SAID, 2005) reduzindo a qualidade espermática em várias espécies. Os espermatozoides de equinos são capazes de gerar (EROS) (BAUMBER et al., 2000) por um mecanismo que se acredita envolver uma oxidase nicotinamida adenina dinucleótido

fosfato (NADPH) semelhante ao relatado em espermatozoides de humanos (AITKEN; BUCKINGHAM; WEST, 1992). A criopreservação nas células espermáticas de equinos pode induzir a produção de óxido nítrico (NO) mesmo em espermatozoides de garanhão com boa qualidade pós-descongelação (FERRUSOLA et al., 2009a).

A capacitação é um termo descrito como uma série de eventos que ocorrem no espermatozoide para que ele fertilize o oócito. Embora a capacitação seja conseguida de forma sinérgica e eficientemente no trato reprodutivo da fêmea, ela também pode ser realizada *in vitro* em meios bem definidos em várias espécies de mamíferos (O'FLAHERTY; RODRIGUEZ; SRIVASTAVA, 2004). Os eventos da capacitação não são bem caracterizados e o seu impacto sobre a fertilidade do garanhão ainda é pouco conhecida.

A capacitação pode ser conseguida *in vitro* em solução salina equilibrada contendo concentrações apropriadas de eletrólitos, fontes de energia metabólica, e albumina sérica bovina (BSA). Em humanos, um método para medir a capacitação é através da eficiência dos espermatozoides em apresentarar reação acrossômica (ICKOWICZ; FINKELSTEIN; BREITBART, 2012). Salienta-se que nos espermatozoides de equinos uns dos eventos mais importantes para determinar a capacitação espermática é a presença da fosforilação dos resíduos do aminoácido tirosina (MCPARTLIN et al., 2008). Diferentes estímulos, como a progesterona, o fluido folicular, zona pelúcida, e o cálcio (Ca^{2+}) podem desencadear a reação acrossômica. Durante a capacitação, as mudanças no espermatozoide como alteração das concentrações de íons intracelulares, a fluidez da membrana plasmática e motilidade são eventos fundamentais para este processo (YANAGIMACHI, 1994). Além disso, o aumento da concentração da adenosina monofosfato cíclica (AMP cíclico) (KOPF; VISCONTI; GALANTINO-HOMER, 1999) e a fosforilação dos resíduos do aminoácido tirosina também estão envolvidos (VISCONTI et al., 1995). Estudos recentes indicam que o anion superóxido ($O_2^{\bullet -}$) (de LAMIRANDE, GAGNON, 1992; GRIVEAU; RENARD; LE LANNOU, 1995) o peróxido de hidrogênio, (H_2O_2) (GRIVEAU; RENARD; LE LANNOU, 1994) e óxido nítrico (NO) (VEDANTAM; ATREJA; GARG, 2012) participam da capacitação espermática. No entanto, embora o número de estudos sobre esses eventos estejam crescendo, as interações entre as diferentes vias de sinalização envolvidas na capacitação espermática ainda permanecem pouco elucidadas.

O NO é um radical livre produzindo a partir de uma série de reações moduladas pela enzima oxido nítrico sintase (NOS) (MONCADA; PALMER; HIGGS, 1991). Até o momento, três isoformas de NOS foram purificadas, sequenciadas e caracterizadas: duas delas são NOS neuronal (nNOS) e NOS endotelial (eNOS) que têm sido referidas como constitutivas (cNOS), ativadas por cálcio e calmodulina, e são seletivamente inibidas pela N^G-nitro-L-arginina metil éster (L-NAME) e N^G-nitro-L-arginina (L-NA). Existe também uma terceira forma induzível (iNOS), que é cálcio independente (MONCADA; PALMER; HIGGS, 1991). O NO é um importante modulador das funções celulares. É um vasodilatador potente que tem sido identificado em numerosos processos fisiológicos, farmacológicos e patológicos (MONCADA; PALMER; HIGGS, 1991). Também é uma molécula mensageira muito importante, envolvida em vários aspectos da reprodução, tais como, o desenvolvimento folicular (JABLONKA-SHARIFF; OLSON, 2000) a ovulação, (OLSON et al., 1999) a esteroidogenese (JABLONKA-SHARIFF; OLSON, 1998) e a maturação de oócitos (SCHWARZ et al., 2008). Em espermatozoides, o NO parece desempenhar um papel importante na regulação da motilidade e na capacitação (HELLSTROM et al., 1994; LEWIS et al., 1996). Herrero et al. (2000) relataram que o NO é capaz de regular a concentração de AMP cíclico e, por conseguinte, através da atividade da adenil ciclase (AC) estimular a capacitação espermática em várias espécies (MURAD, 1994; HELLSTROM et al., 1994; LEWIS et al., 1996). Estudos indicam que baixas concentrações de NO melhoram a motilidade e promovem um aumento significativo da capacitação espermática e reação acrossômica em humanos e ratos (ZINI; de LAMIRANDE; GAGNON, 1995; HERRERO; de LAMIRANDE; GAGNON, 1999; REVELLI et al., 2001). No entanto, pouco se sabe sobre o efeito do NO na capacitação de espermatozoides de equinos.

O NO está presente nos espermatozoides equinos (FERRUSOLA et al., 2009a). No entanto, são escassos na literatura científica, trabalhos que elucidem a importância deste radical livre na motilidade e na capacitação do espermatozoide equino criopreservado. O presente experimento foi realizado com o objetivo de verificar o efeito da inibição, da indução e da remoção do NO, no meio de capacitação, sobre os padrões de motilidade, hiperativação, integridade de membrana plasmática e acrossomal, fosforilação do aminoácido tirosina, quantidade de NO produzido e peroxidação lipídica de espermatozoides equinos criopreservados.

9 CONCLUSÃO

1. Na concentração de L-arginina utilizada (10 mM) não foi possível detectar um aumento da produção de NO em espermatozoides equinos criopreservados aos 60 minutos de incubação em meio de capacitação.
2. O L-NAME na concentração utilizada (1 mM) não é um eficiente inibidor da NOS do espermatozoides equinos, uma vez que a sua adição não inibiu a ação da L-arginina e nem a produção de NO em espermatozoides equinos criopreservados.
3. O azul de metileno é um potente removedor e inibidor da produção, e dos efeitos do NO em espermatozoides equinos incubadoss em meio de capacitação.
4. A incubação de espermatozoides equinos pós-descongelação com L-arginina no meio de incubação causou uma redução da motilidade total e progressiva, da VAP, VSL, LIN e RAP no início da incubação (0 min), porém não foi efetiva em induzir a fosforilação dos resíduos do aminoácido tirosina e nem sobre a peroxidação lipídica da membrana plasmática. No entanto, ela foi eficaz em elevar numéricamente as células com reação acrossômica.
5. O L-NAME foi efetivo apenas quando associado com a L-arginina, causando a alteração de alguns padrões de motilidade, como redução da VSL (aos 300 min) e da VCL, aumento da BCF (aos 120 min) e a redução da hiperativação.
6. Ao longo do tempo houve uma tendência das células espermáticas equinas a se comportarem com características de capacitação.

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