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INJEÇÃO INTRACITOPLASMÁTICA DE ESPERMATOZOIDE: MÉTODOS DE ATIVAÇÃO OOCITÁRIA E DESESTABILIZAÇÃO DA MEMBRANA ESPERMÁTICA

Santa Maria, RS, Brasil
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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Sanidade e Reprodução Animal, da Universidade Federal de Santa Maria (UFSM-RS), como requisito parcial para obtenção do grau de **Mestre em Medicina Veterinária**.

Orientador: Prof. Paulo Bayard Dias Gonçalves
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elaborada por
João Ricardo Malheiros de Souza

Como requisito parcial para obtenção do grau de
Mestre em Medicina Veterinária

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RESUMO

INJEÇÃO INTRACITOPLASMÁTICA DE ESPERMATOZOIDE: MÉTODOS DE ATIVAÇÃO OOCITÁRIA E DESESTABILIZAÇÃO DA MEMBRANA ESPERMÁTICA

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O desenvolvimento de embriões bovinos produzidos por injeção intracitoplasmática de espermatozóides (ICSI) é baixo em relação aos embriões fertilizados. Deficiência na ativação de oócitos, capacitação inadequada de espermatozóides e falta de descondensação de espermatozóides são os principais transtornos que afetam o sucesso de ICSI em bovinos. No presente trabalho, foram testados métodos de ativação com o intuito de estabelecer um protocolo efetivo para a ativação de oócitos bovinos após a ICSI. Para isso, um inibidor específico de CDK1 (RO-3306) e um ativador específico de PKC (OAG) foram utilizados após a incubação com Ionomicina (ION) para avaliar a retomada da meiose em oócitos bovinos. Além disso, a incubação em meio Fert durante 6 h e a eletroporação (El) de espermatozoides previamente a ICSI foram testadas para verificar o efeito sobre a descondensação do espermatozoide e formação do pró-núcleo (PN) masculino. Inicialmente oócitos bovinos foram incubados, com diferentes concentrações e períodos de exposição aos tratamentos. Foram avaliados conforme a taxa de ativação oocitária (formação de PN), clivagem, desenvolvimento a blastocisto e número de células nos embriões que se desenvolveram a blastocisto. As taxas de PN foram maiores ($P \leq 0,01$) nos grupos ativados com ION+RO (48,5 %) e ION+RO+OAG (65,6 %) comparado com ION (12,3 %) e ION+OAG (9,2 %). Não houve efeito significativo entre as concentrações 5,0, 7,5 e 10,0 μM de RO sobre a taxa de ativação. A taxa de ativação foi significativamente maior ($P \leq 0,01$) em oócitos tratados com RO por 240 min (84,6 %) comparado a 60 (53,6 %) e 120 min (60,0%). No entanto, não houve diferença significativa na taxa de ativação quando o tratamento com RO foi iniciado a 0, 30 ou 60 minutos após a incubação com ION. A taxa de clivagem foi inferior nos grupos ION (11,8 %) e ION+OAG (22,8 %) comparada aos grupos ION+RO (70,2 %) e ION+RO+OAG (62,4 %). A taxa de blastocistos também foi maior no grupo ION+RO+OAG (24,1 %), mas não houve diferença estatística entre os grupos ION+RO (19,7 %) e ION+OAG (9,5 %). Não houve desenvolvimento a blastocisto quando os oócitos foram tratados somente com ION. Não foi detectada diferença estatística entre os tratamentos sobre o número médio de células por blastocistos. No segundo experimento, espermatozoides não tratados (ICSI-Cont) ou tratados por electroporação (ICSI-El) foram microinjetados em oócitos, os quais foram ativados com ION+RO por 240 min e fixados cerca de 15 h após a injeção para determinar a taxa de formação de PNs masculino e feminino (2PN). A maioria dos oócitos apresentaram o PN feminino bem desenvolvido (66,4 %). A formação de 2PN (masculino e feminino) foi maior no grupo ICSI-El (33,3 %) comparado ao grupo ICSI-Cont (9,4 %). Em conclusão, esse estudo demonstrou que a inibição específica da CDK1 após o tratamento com ION promove a ativação de oócitos bovinos. O tratamento de espermatozoides com eletroporação melhora a formação do PN masculino após ICSI, mas a taxa é inferior a formação do PN feminino. Esses resultados indicam que a deficiente descondensação do espermatozoide é o principal limitante para estabelecer um protocolo eficaz para ICSI em bovinos.

Palavras Chave: Ativação de oócitos, CDK1, PKC, ICSI, bovino.

ABSTRACT

INTRACYTOPLASMIC SPERM INJECTION: METHODS OF OOCYTE ACTIVATION AND SPERM MEMBRANE DESTABILIZING

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Development of bovine embryos produced by intracytoplasmic sperm injection (ICSI) is low compared to fertilized embryos. Deficient oocyte activation, inappropriate sperm capacitation, and lack of sperm decondensation are thought to be the main constrains affecting ICSI success in cattle. In the present study, activation compounds were tested to establish an effective protocol for activation of bovine oocytes, and then used for oocyte activation after ICSI. The activation approach consisted of exposing in vitro matured oocytes to Ionomycin (ION) following by a specific CDK1 inhibitor (RO-3306), a specific PKC activator (OAG) or both RO+OAG. In the first experiment, the rate of activation (pronuclear (PN) formation), cleavage, development to the blastocyst stage, and number of cells per blastocyst were evaluated after oocyte treatment. The PN rates were higher ($P \leq 0.01$) in the groups activated with ION+RO (48.5 %) and ION+RO+OAG (65.6 %) compared to ION (12.3 %) and ION+OAG (9.2 %). There was no significant effect between the RO concentrations tested (5, 7.5 and 10 μM) on oocyte activation. The PN rate was significantly higher ($P \leq 0.01$) when oocytes were exposed to RO for 240 min (84.6 %) compared to 60 (53.6 %) and 120 min (60.0 %). However, there was no difference between groups when treatment with RO started at 0, 30 or 60 min after on ION exposure. Cleavage rate was higher in ION+RO (70.2 %) and ION+RO+OAG (62.4 %) groups compared to ION (11.8 %) and ION+OAG (22.8 %). Blastocyst rate was also higher in the ION+RO+OAG (24.1 %) group, but not statistically different between ION+RO (19.7 %) and ION+OAG (9.5 %) groups. There was no development to the blastocyst stage after treatment with ION alone. The average cell number in blastocysts was not statistically different among treatments. In the second experiment, the effect of activation with ION+RO (10 μM for 240 min) was tested after ICSI using control (ICSI-Cont) or treated by electroporation (ICSI-El) sperm. Most oocytes presented a well-developed female PN (66.4%). Male PN formation was higher ($P \leq 0.05$) in the ICSI-El (33.3%) compared to the ICSI-Cont (9.4%) group. In conclusion, this study revealed that the specific inhibition of CDK1 after ION treatment is an effective approach to activate bovine oocytes. Male pronuclear formation after ICSI is increased by sperm electroporation, but is lower than female pronuclear formation. This indicates that deficient sperm decondensation and male PN formation rather than deficient oocyte activation is likely the main problem to develop an effective protocol for bovine ICSI.

Key words: Oocyte activation, CDK1, PKC, ICSI, bovine.

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μg – micrograma;
μL – microlitro;
μM – micromolar;
AI – anáfase da meiose I;
AP – ativação partenogenética;
APC – complexo promotor de anáfase;
ATP – trifosfato de adenosina;
ATII – anáfase II ou telófase II
BSA – albumina sérica bovina;
cAMP – monofosfato de adenosina cíclica;
Ca^{2+} – cálcio;
Caf – cafeina;
CaMKII – proteína cinase 2 dependente da calmodulina;
CB – citocalasina B;
CC – células do *cumulus*;
CCOs – complexos *cumulus-oócito*;
CDK – cinase dependente de ciclina;
CDKi – inibidor de cinase dependente de ciclina;
cGMP – guanosina-mono-fosfato;
CHX – *cycloheximide*;
CIV – cultivo *in vitro*;
CO_2 – dióxido de carbono;
CP – corpúsculo polar;
CSF – fator citostático*;*
CyB –ciclina B;
D – dia de cultivo;
DAPI – 4',6-diamidino-2-phenylindole;
DAG – diacylglycerol;
DMSO – dimetilsulfóxido;
DNA – ácido desoxirribonucleico;
DTT – dithiothreitol;
EGF – fator de crescimento epidermal;
El – eletroporação ou eletropermeabilizarão;
ER – reticulo endoplasmico;
FIV – fecundação *in vitro*;
FSH – hormônio folículo estimulante;
Gs – proteína G;
GPR3 – receptor acoplado a Proteína G;
GSH – glutationa;
GV – vesícula germinativa;
GVBD – quebra da vesícula germinativa;
IA – inseminação artificial;
ION – *Ionomycin*;
IP3 – inositol trifosfato;
ICSI – Injeção intracitoplasmática;
IVF – *in vitro fertilization*;
LH – hormônio luteinizante;

LL – *lysolecithin*;
LOPU – *Laparoscopic Ovum Pick-Up;*
MAPK – proteína cinase ativada por mitógenos;
MAP2K, MAP3K, MAP4K – cinases ativadoras de cinases ativadas por mitógenos;
MEK – cinase regulada por sinal extracelular (MAP2K);
MI – metáfase da meiose I;
MII – metáfase da meiose II;
MIV – maturação *in vitro*;
mL – mililitro;
mg – miligrama;
mM – milimolar;
MOET – múltipla ovulação seguida de transferência embrionária;
MPF – fator promotor da maturação;
Myt1, Wee1 e CDC25 – proteínas cinases envolvidas na atividade do MPF;
OAG – *1-Oleoyl-2-acetyl-sn-glycerol*;
OPU – *Ovum Pick-Up;*
PBS – solução tamponada de fosfato;
PDE3A – fosfodiasterase 3º;
PI – prófase I;
PIVE – produção *in vitro*;
PN – Pró-núcleo;
PKA – proteína cinase A;
PKC – proteína cinase C;
PKC_a – ativador de proteína cinase C;
PLCz – *phospholipase C zeta;*
PVA – álcool polivinílico;
PVP – polivinilpirrolidona;
RE – retículo endoplasmático;
RNAm – ácido ribonucléico mensageiro;
RO-3306 – *5-(6-Quinolinylmethylene)-2-[(2-thienylmethyl)amino]-4(5H)-thiazolone;*
SFB – soro fetal bovino;
TCM-199 – meio de cultura de tecidos 199;
TE – transferência de embrião;
TI – telófase da meiose I;
TX – triton X-100;
UI – unidades internacionais;
2°CP – segundo Corpúsculo Polar;
6-DMAP – *6-dimethylaminopurine*.

SUMÁRIO

1. INTRODUÇÃO

A produção *in vitro* de embriões é uma tecnologia que consiste na manipulação dos gametas masculino e feminino fora do trato reprodutivo. Considerada a terceira geração de biotecnologia da reprodução, após a inseminação artificial e a transferência de embriões (THIBIER, 1990), a PIVE proporciona, entre suas vantagens, a possibilidade de aumentar o número de indivíduos geneticamente superiores, diminuir o intervalo entre gerações e ampliar o número de descendentes (VAN WAGTENDONK-DE LEEUW et al., 2000).

Embora a PIVE seja utilizada em diversas espécies de interesse econômico, como: suínos, equinos, caprinos e ovinos, é na espécie bovina que é realizada em maior representatividade. No Brasil é considerada uma ferramenta de reprodução assistida em ascensão, sendo que no ano de 2014 o país foi responsável por mais da metade da produção *in vitro* mundial na espécie bovina, ultrapassando a marca dos 345.000 de embriões transferidos, representando quatro vezes mais que o número de embriões coletados e transferidos *in vivo* (PERRY, 2015).

Responsável pela expansão da tecnologia em larga escala no país, a FIV convencional é uma biotecnologia que permite que ocorra a fecundação do oócito pelo espermatozoide de forma a desencadear o processo de desenvolvimento embrionário de forma fisiológica. Embora o sucesso da realização da FIV seja dependente de condições de cultivo (LONERGAN & FAIR, 2008), ela apresenta resultados considerados satisfatórios em comparação a outras espécies domésticas.

Quando ocorre o insucesso da FIV convencional, pode-se utilizar a Injeção Intracitoplasmática de Espermatozoides. Por ultrapassar as barreiras do oócito e da membrana plasmática, a ICSI também auxilia animais com problemas seminais ou que possuem alterações morfofisiológicas decorrentes do envelhecimento. Recentemente, tem sido amplamente estudada em bovinos na tentativa de complementar tecnologias já existentes, como por exemplo, favorecer a produção de embriões a partir de sêmen sexado (JO et al., 2014) e possibilitar a reprodução de machos e fêmeas pré-púberes.

Porém o sucesso da ICSI em bovinos está aquém do esperado. A ausência de ativação fisiológica nos oócitos é considerada a principal responsável pelos resultados insatisfatórios (FLAHERTY et al., 1998). Além disso, a capacitação ineficiente do espermatozoide de touros antes da técnica (STRICKER, 1999), e a descondensação inadequada do espermatozóide após técnica (GALLI et al., 2003) também são observadas.

Na tentativa de melhorar as taxas de ativação oocitária, vários métodos de ativação têm sido utilizados. Estímulos elétricos (HWANG et al., 2000) além de estímulos químicos, como o etanol (HAMANO et al., 1999; ABDALLA et al., 2009) e a ionomicina (RHO et al., 1998; JO et al., 2014) são empregados para iniciar o influxo de cálcio intra-oocitário. Ainda que seja possível obter a retomada da meiose unicamente com eles, raros são os embriões produzidos quando utilizados sozinhos. Por isso, são associados aos tratamentos com cycloheximide (SUTTNER et al., 2000; ABDALLA et al., 2009) ou 6-dimethylaminopurine (CHUNG et al., 2000; ABDALLA et al., 2009; JO et al., 2014), os quais são responsáveis por manter baixo o fator promotor da maturação, passo fundamental para o término da meiose. No entanto, diversos trabalhos comprovam a intercorrência de alterações cromossômicas devido aos seus focos de atuação.

Um dos desafios da ciência têm sido o aumento de oócitos com a presença de pró-núcleos masculinos após a ICSI em bovinos. GOTO et al. (1990) avaliaram a reação acrossômica em espermatozoides tratados com a cafeína, enquanto WEI and FUKUI (1999) testaram cálcio ionóforo e TOMKINS and HOUGHTON (1988) a eletropermeabilização. Detergentes capazes de romper as ligações presentes na membrana da cabeça de espermatozoides bovino, como dithiothreitol (GALLI et al., 2003; LEE et al., 2015), lysolecithin (LEE et al., 2015; ZAMBRANO et al., 2016) e Triton X-100 (ZAMBRANO et al., 2016) também têm sido estudados. Contudo, WATANABE et al. (2010) comprovaram que tais procedimentos podem interferir no material genético contido no esperma de camundongos, produzindo embriões com aberrações cromossômicas.

Nesse contexto, estudar métodos capazes de induzir a retomada da meiose torna-se fundamental para produzir um protocolo que resulte em embriões viáveis após a ICSI. Entre os mecanismos fisiológicos que ocorrem após a fecundação do oócito pelo

espermatozóide e que podem ser melhor explorados estão a ativação de Proteína Cinase C, a qual foi responsável por reiniciar a meiose em camundongos (SUN et al., 1997) e suínos (NAKAI et al., 2016), e a inibição específica de Ciclinas Dependentes de Cinase 1 ligadas ao Fator Promotor da Maturação, que tem sido empregada com sucesso na sincronização do ciclo celular (VASSILEV, 2006).

A hipótese investigada é que ativadores específicos de PKC e inibidores específicos de CDK1 são capazes de ativar o oócitos, auxiliando assim a retomada da meiose e favorecendo a formação do pró-núcleo feminino. Além disso, que a taxa de pró-núcleos masculinos pode ser aumentada através da pré-incubação e eletropermeabilização de espermatozoides utilizados na realização da ICSI.

Neste trabalho foram estudados métodos de ativação oocitária capazes retomar a meiose e iniciar o desenvolvimento de embriões produzidos por Injeção Intracitoplasmática de Espermatozóides previamente tratados. Os quatro primeiros experimentos foram realizados com o objetivo de validar o protocolo de ativação a partir da incubação de oócitos em diferentes tratamentos e da avaliação das taxas de retomada da meiose. O quinto experimento desafiou o melhor tratamento testado anteriormente sobre a taxa de desenvolvimento e de qualidade embrionária. O sexto experimento aferiu a retomada da meiose de embriões produzidos com espermatozóides pré-incubados e eletropermeabilizados antes da ICSI.

2. REVISÃO BIBLIOGRÁFICA

2.1. FECUNDAÇÃO *IN VITRO*

Conforme reafirmado por KOO et al. (2005), FIV é a união ou cocultivo de espermatozoides capacitados e oócitos maturados fora do trato feminino. Ocorre pela forma convencional, quando ocorre o contato direto do oócito maturado com os espermatozoides ou através da ICSI, técnica que consiste na injeção de um único espermatozoide, previamente selecionado, no citoplasma de oócitos em MII.

2.1.1. FECUNDAÇÃO *IN VITRO* CONVENCIONAL

Quando é realizada a fecundação in vitro convencional, acontece a fusão do espermatozoide com o oócito em MII, causando a ativação fisiológica do oócito, o qual estimula a liberação maciça de Ca^{2+}, a exocitose dos grânulos corticais, impermeabilização da zona pelúcida, queda no MPF e a retomada da meiose com extrusão do segundo corpúsculo polar e a formação do pró-núcleo feminino (CROZET, 1991). Além disso, o núcleo espermático se descondensa, transformando-se no pró-núcleo masculino (HAFEZ & HAFEZ, 2004). Os pró-núcleos masculino e feminino migram para o centro do oócito, o envelope nuclear se desintegra e ocorre a associação dos cromossomos para a primeira divisão mitótica, a clivagem, iniciando o desenvolvimento embrionário (GONÇALVES et al., 2008).

2.1.2. INJEÇÃO INTRACITOPLASMÁTICA DE ESPERMATOZOIDE

A ICSI foi descrita no ano de 1992, e consiste na microinjeção de um único espermatozoide no interior do citoplasma do oócito. Essa técnica pode ser utilizada quando se trata de graves alterações seminais (PALERMO et al., 1992) ou em espécies que ocorre um insucesso da técnica convencional (por exemplo em suínos e equinos),

uma vez que ultrapassa as barreiras do oócito e da membrana plasmática. Para sua realização, é fundamental posicionar o oócito com a pipeta de manutenção de forma que o primeiro corpúsculo polar fique na posição “6 ou 12 horas”. Assim, evita-se que a pipeta cause lesão à placa metafásica, uma vez que esta encontra-se na maioria das vezes na região subjacente ao CP, o que causaria um distúrbio na extrusão do 2ºCP (PAYNE, 1995).

Para a penetração do citoplasma pela ICSI convencional, é realizada a aspiração da membrana plasmática pela pipeta de injeção, visando rompê-la e só então a pipeta é introduzida mais profundamente para a liberação do espermatozoide. Entretanto, frequentemente podem ocorrer erros nesse procedimento, causando lesões ao oócito ou uma deposição incorreta do espermatozoide no espaço perivitelínico, pela falta de ruptura da membrana.

Um equipamento denominado de pipeta piezo, utilizado por KIMURA and YANAGIMACHI (1995) para a realização de ICSI, permitiu ampliar a taxa de sobrevivência de oócitos de 16 % para 80 % em camundongos, abrindo caminho para a ampliação da taxa de blastocistos produzido por ICSI em suínos (MARTIN, 2000), equinos (HINRICHS et al., 2006) e bovinos (DEVITO et al., 2010). A ação desse equipamento está baseada no efeito piezo-elétrico, que faz com que a pipeta avance curtas distâncias a uma alta velocidade. Esses pulsos transmitidos à pipeta de injeção promovem um movimento pontual, perfurando a zona pelúcida e a membrana plasmática com pouca distorção da célula (KIMURA & YANAGIMACHI, 1995). Posteriormente, esse equipamento foi empregado na ICSI em humanos, resultando em altas taxas de sobrevivência e formação de pró-núcleos, sem necessidade da aspiração do citoplasma para a deposição do espermatozoide no interior do oócito (HUANG et al., 1996).

A introdução de determinadas substâncias químicas, juntamente com o espermatozoide no momento da ICSI, é outro ponto importante a ser discutido. A PVP é uma substância comumente utilizada nos trabalhos de ICSI para o preparo da suspensão de espermatozoides a serem microinjetados. Utilizada para promover um aumento da viscosidade do meio, causando uma redução da motilidade dos espermatozoides, para que eles possam ser aspirados ou imobilizados pela pipeta. Além disso, a PVP reduz a

aderência dos espermatozoides nas paredes da pipeta, facilitando o procedimento de aspiração e deposição do espermatozoide no oócito.

Acredita-se que a PVP não causa alterações celulares ou danos aos cromossomos. Entretanto, foi comprovado que a PVP pode causar uma estabilização da membrana do espermatozoide, o que afetaria a taxa de formação de pró-núcleo masculino ou ainda, quando injetada em maior quantidade, forma uma gota ao redor do espermatozoide, isolando-o no interior do oócito e impedindo o seu contato com substâncias do citoplasma do oócito, responsáveis pela sua descondensação (DOZORTSEV et al., 1995).

2.2.1. ATIVAÇÃO OOCITÁRIA

A ativação oocitária, obtida pela fecundação ou pela partenogênese, é uma cascata de eventos desencadeada para aumentar os níveis de Ca^{2+} intracelular (SHEN et al., 2008). A cascata é iniciada com o aparecimento de uma molécula pequena e hidrossolúvel, chamada de IP3, que sai da membrana citoplasmática do oócito e se difunde rapidamente no citosol (FISSORE et al., 2002).

Quando o IP3 alcança o RE, se liga aos canais dependentes de Ca^{2+} de IP3, que estão presentes na membrana do RE e consequentemente consegue abri-los. A função principal do IP3 é estimular a liberação de Ca^{2+} armazenado no RE, aumentando sua concentração citosólica (LEE et al., 2006). O significado das oscilações de Ca^{2+} nos oócitos ainda não está totalmente estabelecido, mas é sabido que o Ca^{2+} está envolvido nos eventos da exocitose dos grânulos corticais e da progressão do ciclo celular (DUCIBELLA et al., 2002).

Alguns tratamentos associando dois estímulos de ativação são frequentemente utilizados, garantindo melhores resultados no desenvolvimento embrionário de embriões obtidos pela ICSI e transferência nuclear (KEEFER et al., 2001). A associação é feita com agentes ativadores de Ca^{2+} e com inibidores da síntese proteica ou da fosforilação, para garantir a manutenção do MPF em níveis basais até as primeiras divisões mitóticas (ALBERIO et al., 2001).

2.2.2. INIBIDOR DE CDK1

Um grande número de proteínas são fosforiladas no momento de início da divisão celular, muitas mediadas por CDK. ADHIKARI et al. (2012) demonstraram que a deleção específica de CDK1 resultou no bloqueio da fase GV em oócitos de camundongo, resultando em incapacidade para fosforilar e suprimir queda de PP1, com subsequente falha de fosforilar a lamin A/C, que é necessário para a desmontagem do envelope nuclear. Enquanto que a deleção de CDK2 não foi suficiente para bloquear a meiose ou impedir a fecundação.

Diversos inibidores da proteína cinase (6-DMAP) (LE BEUX et al. (2003) e de síntese de proteínas (CHX) (MOTLIK et al. (1998) têm sido utilizados na tentativa de manipular o MPF, porém podem estar associados ao aumento do risco de desenvolvimento de anormalidades em fases de desenvolvimento (LONERGAN et al., 1998).

Embora a utilização de inibidores específicos de CDK1 ainda seja desconhecida em oócitos bovinos, o bloqueio de oócitos suínos em GV foi obtido com utilização do inibidor RO-3306 (JANG et al., 2014). O qual também foi responsável por manter células humanas em fase G2/M do ciclo celular, permitindo o isolamento das células mitóticas sem o uso de drogas de micro túbulos (VASSILEV, 2006).

2.2.3. ATIVADOR DE PKC

Durante o processo de fecundação fisiológica, a fusão do espermatozoide à membrana oocitária desencadeia oscilações de cálcio (Ca^{2+}) e a produção de diacilglicerol (DAG), os quais são ativadores de Proteina Cinase C (PKC). Esta ativação está associada com os componentes do citoesqueleto (INAGAKI et al., 1988), micro túbulos e microfilamentos de actina (CAPCO, 2001). Os filamentos de actina desempenham um importante papel durante a fecundação. São responsáveis pela incorporação do espermatozoide, pela exocitose dos grânulos corticais, pela

movimentação do fuso e pela extrusão do corpúsculo polar (CAPCO et al., 1992). A produção de embriões a partir de ativadores específicos de PKC já foi registrada em camundongos (SUN et al., 1997) e suínos NAKAI et al. (2015), mas ainda não foi testada em bovinos.

3. CAPÍTULO 1

TRABALHO A SER ENVIADO PARA PUBLICAÇÃO:

CDK1 INHIBITOR INDUCES BOVINE OOCYTE ACTIVATION AFTER ICSI

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CDK1 inhibitor induces bovine oocyte activation after ICSI

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Abstract

Development of bovine embryos produced by intracytoplasmic sperm injection (ICSI) is low compared to fertilized embryos. Deficient oocyte activation, inappropriate sperm capacitation, and lack of sperm decondensation are thought to be the main constraints affecting ICSI success in cattle. The main objectives in this study were to establish a protocol for bovine oocyte activation based on the modulation of the CDK1 and PKC activity, and to test if this protocol would improve the sperm decondensation after ICSI. For the first objective, oocytes were matured in vitro for 24 h, stripped from cumulus cells, and then parthenogenetically activated with one the following treatments: (1) 5 µM Ionomycin for 5 min (ION); (2) Ion followed by 4 h in the presence of 10 µM of the CDK1 inhibitor RO-3306 (ION+RO); (3) Ion followed by 4 h in the presence of 100 µM the PKC activator 1-Oleoyl-2-acetyl-sn-glycero (ION+OAG); and (4) Ion followed by 4 h in the presence of RO and OAG (ION+RO+OAG). Oocyte activation rates were higher ($P<0.01$) for treatments ION+RO (48.5%) and ION+RO+OAG (65.6%) compared to ION (12.3%) and ION+OAG (9.2%). The effect of three concentrations of RO (5, 7.5 or 10 µM) were then tested and revealed no significant differences on oocyte activation. Next, the effect of three exposure times (60, 120, or 240 min) to 10 µM RO was tested. Activation rate was significantly higher ($P\leq0.01$) in oocytes treated for 240 min (84.6 %) compared to 60 (53.6 %) and 120 (60.0 %) min. We then evaluated if the interval from ION treatment to the beginning of RO exposure affect oocyte activation and observed similar activation rates between groups exposed to RO for 240 min starting at 0, 30 or 60 min after ION. To evaluate embryo development and quality, oocyte were activated, cultured for 4 h in the presence of 7.5 µg/mL cytochalasin B to prevent second polar body extrusion, and then thoroughly rinsed and

cultured in SOF medium for 7 days. Cleavage rate was higher in ION+RO (70.2%) and ION+RO+OAG (62.4%) groups compared to ION (11.8%) and ION+OAG (22.8%). blastocyst rate was also higher in the ION+RO+OAG (24.1%) group, but not statistically different between ION+RO (19.7%) and ION+OAG (9.5%) groups. There was no development to the blastocyst stage after treatment with ION alone. No statistical difference was detected between the average cell number in blastocysts among ION+RO (78.4±6.3), ION+OAG (90.0±0.0) and ION+RO+OAG (101.6±15.2). For the second objective, oocytes were activated with ION+RO (10μM for 240 min) after ICSI. The effect of incubating sperm for 6h in Fert medium alone (ICSI-Cont) or 6 h in Fert medium followed by an electroporation pulse (ICSI-El) on the rate of male pronuclear formation was evaluated. At 15 h after ICSI, most oocytes presented a well-developed female PN (66.4%). Male PN formation was higher ($P \leq 0.05$) in the ICSI-El (33.3%) compared to the ICSI-Cont (9.4%) group. In conclusion, this study revealed that the specific inhibition of CDK1 after ION treatment is an effective approach to activate bovine oocytes. Male pronuclear formation after ICSI is increased by sperm electroporation, but is lower than female pronuclear formation. This indicates that deficient sperm decondensation and male PN formation rather than deficient oocyte activation is likely the main problem to develop an effective protocol for bovine ICSI.

Key words: Oocyte activation, CDK1, PKC, ICSI, bovine.

1. Introduction

Intracytoplasmic sperm injection (ICSI) has been widely applied in humans and domestic animal species. This technique is an alternative for cases where in vitro

fertilization (IVF) does not provide enough embryos, due to either spermatozoa related defects or immature oocyte. By applying ICSI techniques in humans [1], mice [2], rabbits [3], and horses [4], the injection procedure itself is sufficient to activate the oocyte, to decondense the sperm and to start embryonic development [5]. In contrast to other species, the protamine disulfide bond in bull sperm is more stable, which makes sperm decondensation more difficult and decreases pronuclear formation. Deficient oocyte activation is considered the main cause of fertilization failure by conventional ICSI [6]. Other potential causes include inefficient sperm capacitation before ICSI [7], and unsuitable sperm head decondensation after ICSI [5].

Oocyte activation consists of a series of events capable of releasing the oocyte from metaphase-II (MII) stage. In most species, a process triggered by the fusion of oocyte-sperm membranes regulates oocyte activation. During this process, the phospholipase C zeta (PLCz) is released into the ooplasm promoting the production of diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP3) [8]. The presence of these messengers inside the cytoplasm causes intracellular calcium oscillations (Ca^{2+}) that activate cellular events. DAG activates protein kinase C (PKC), which in turn leads to exocytosis of cortical granules, promoting the hardening of the zona pellucida (zona reaction), deactivates mitogen-activated protein kinase (MAPK) allowing the pronuclei formation, and activates calmodulin-dependent protein kinase II (CaMKII). The IP3 binds to its receptors in the endoplasmic reticulum (ER) causing the release of Ca^{2+} [9] and activation of CaMKII, which results in the inhibition of cytostatic factor (CSF) and release of anaphase-promoting complex (APC). The reduction of maturation-promoting factor (MPF) activity, which comprises CDK1 and Cyclin B1, triggers the exit from meiotic arrest [10], extrusion of the second polar body, and formation of the female pronucleus.

Pig oocytes exposed to a PKC activator similar to 1-Oleoyl-2-acetyl-sn-glycerol (OAG) were activated and formed pronuclei [11, 12]. The selective inhibitor CDK1 (RO-3306) was also used in pigs to arrest them at the germinal vesicle (GV) stage [13]. However, there are no studies that evaluated the effects of PKC activators and CDK1 inhibitors either alone or combined in the activation of bovine oocytes.

Different strategies have been used to improve activation of bovine oocytes after ICSI. Among the tested approaches are electric pulses [14], ethanol [15, 16], ionomycin (ION) [17, 18], cycloheximide (CHX) [16, 19], and 6-dimethylaminopurine (6-DMAP) [16, 18, 20]. Other treatments have focused on the sperm and include induction of acrosome reaction with caffeine [21], calcium ionophore (A23187) [22], electropermeabilization [23], or plasma membrane-disrupting agents such as dithiothreitol (DTT) [5, 24], lysolecithin (LL) [24, 25], and Triton X-100 (TX) [25].

The working hypothesis in this study is that PKC activation and CDK1 inhibition would effectively activate bovine oocytes and along with treatment that destabilize the sperm membrane would improve male pronuclear formation after ICSI. Therefore, our objectives were to: i) establish a suitable oocyte activation method for bovine by inducing PKC activation and inhibiting CDK1; and ii) test the activation protocol after ICSI performed with control or electroporated sperm.

2. Material and methods

All chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO), except otherwise stated.

2.1. Oocyte collection and in vitro maturation

Cow ovaries were obtained from a commercial abattoir (Frigorífico Silva/Best Beef, Santa Maria, Brazil), and transported to the laboratory at 30ºC in saline solution (NaCl, 0.9 %) supplemented with penicillin (100 IU/mL) and streptomycin sulfate (50 µg/mL). Cumulus-oocyte complexes (COCs) were collected from follicles of 2-8 mm in diameter using a vacuum pump (vacuum rate of 20 mL water/min) into 15 mL centrifuge tubes, allowed to sediment for 10 min, and then COCs were recovered under a stereomicroscope. Quality I and II COCs were selected and matured in groups of 20-40 in 400 µl of maturation medium in four-well dishes (Nunc; Thermo Fisher Scientific Inc., Roskilde, Denmark) for 24 hours. 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 sodium bicarbonate, 5.0 µg/mL LH (Bioniche, Belleville, ON, Canada), 0.5 µg/mL FSH (Bioniche, Belleville, ON, Canada), 10 % fetal bovine serum (FBS) (Gibco Labs, Grand Island, NY, USA), 100 IU/mL penicillin, and 50 µg/mL streptomycin sulphate. The oocytes were maintained under a temperature of 38.5 ºC, saturated humidity and an atmosphere of 5 % CO_2 in air. After maturation, cumulus cells were removed by pipetting in the presence of 0.1 % hyaluronidase, and the oocytes were selected by the presence of the first polar body.

2.2 Oocyte activation

Oocytes were activated by incubation with 5 mM ION in TCM-199 medium supplemented with 2 mg BSA for 5 min. Oocytes were then incubated with 100 µM of OAG or RO at a final concentration of 10 µM for 240 min (except otherwise stated on

experimental design) at 38.5°C, with a saturated humidity atmosphere of 95 % air and 5 % CO_2. The culture medium consisted of synthetic oviductal fluid (SOF medium) supplemented with 5 % FBS, 0.36 mM sodium pyruvate, 1 % antibiotics (100 IU/mL penicillin and 50 µg/mL streptomycin sulphate). To evaluate the effect of the activation treatment on embryo development oocytes were incubated in the presence of 7.5 µg/mL cytochalasin B for 4h to prevent the extrusion of the second polar body.

2.3 Sperm preparation and pretreatment

Semen of a bull with proven fertility was thawed in a water bath (37ºC, 30 sec) and motile spermatozoa were obtained by centrifugation on a Percoll® discontinuous density gradient for 30 min at 750 X g. Viable spermatozoa were washed in Fert medium containing 10 mg/mL heparin, 30 mg/mL penicilinamine,15 mM hypotaurineand and 1 mM epinephrine. Sperm were incubated in Fert medium for 6 h at 38.5 ºC, and then electroporated (ICSI-El) or not (ICSI-Cont) before ICSI. Electroporation treatment consisted of a single pulse of 500 V for 30 µs using an Eppendorf multiporator system (Eppendorf AG, Hamburg, Germany). Immediately before ICSI, sperm were resuspended in TCM-199 HEPES buffered medium containing 10 % polyvinyl pyrrolidone (PVP).

2.4 Intracytoplasmic Sperm Injection (ICSI)

ICSI was performed using an inverted microscope (Nikon Eclipse 80i microscope,Nikon, Tokyo, Japan) and hydraulic micromanipulators (Narishige International, New York, NY, USA) at x 200 magnification. The oocytes were

manipulated in 40 µl droplets of TCM-199 HEPES buffered medium supplemented with 10 % FBS under mineral oil in a tissue culture dish. A single sperm was aspirated into the injection pipette (9-11 µm internal diameter). One oocyte, with its polar body located either at the 6 or 12 o'clock position, was held with the holding pipette. After breaking the plasma membrane by aspiration, the spermatozoa was injected into the oocyte at the 3 o'clock position. After ICSI, oocytes were washed three times in maturation medium and then activated.

2.5 Pronuclear formation

Oocytes were fixed in 4 % paraformaldehyde for 10-15 min and then stored at 4 °C in PBS containing 0.3 % BSA and 0.1 % Triton X-100. Samples were exposed to 10 µM Hoechst 33342 for 5 min and washed three times in PBS containing 0.3 % BSA and 0.1 % Triton X-100. Oocytes were then mounted into slides using Mowiol and the pronuclear formation was evaluated under the epifluorescence microscope (Leica, DMI 4000B).

2.6 Embryo culture and evaluation

Embryos were culture in SOF medium supplemented with 5 % FBS, 0.36 mM sodium pyruvate, 1 % antibiotics (100 IU/mL penicillin and 50 µg/mL streptomycin sulphate. Cleavage rate was recorded on Day 2 and blastocyst rate at Day 7 of culture. Embryos that developed to the blastocyst stage were rinsed in PBS containing 0.1 % polyvinyl alcohol (PBS-PVA) and fixed in 4 % paraformaldehyde for 10-15 min. Fixed embryos were rinsed in PBS-PVA and then stored at 4 °C in PBS containing 0.3 % BSA

and 0.1 % Triton X-100. DNA was stained by exposing the embryos to 10 μM Hoechst 33342 for 5 min. Embryos were then mounted on slides using Mowiol and the number of cell nuclei per embryo was counted under epifluorescence microscope (Leica, DMI 4000B).

2.7 Statistical analysis

The percentage data were arcsine-transformed. The activation rates and development data were subjected to ANOVA using the Standard Least Squares procedure and analyzed by LSMeans Student t test with JMP software (SAS institute Inc., Cary, NC). Differences between means were considered statistically significant when $P \leq 0.05$.

2.8 Experimental design

Experiment 1. We compared the ability of pronuclear formation after the following treatments: (1) ION (control group), matured oocytes were exposed to 5 μM ION for 5 min; (2) ION+RO, matured oocytes were exposed to ION for 5 min and then incubated in the presence of 10 μM RO for 240 min; (3) ION+OAG, matured oocytes were exposed to ION for 5 min and then incubated in the presence of 100 μM OAG for 240 min; and (4) ION+RO+OAG, matured oocytes oocytes were exposed to ION for 5 min and then incubated in the presence of 10 μM RO and 100 μM OAG for 240 min.

Experiment 2. The effect of three concentrations of RO on oocyte activation was tested in this experiment. Matured oocytes were exposed to 5 μM ION for 5 min and

then divided in three groups and incubated in the presence of 5, 7.5 or 10 μM RO for 240 min.

Experiment 3. The effect of the time of exposure to RO on the activation rate was evaluated in this experiment. Matured oocytes were exposed to 5 μM ION for 5 min and then divided in three groups and incubated in the presence of 10 μM RO for 60, 120 or 240 min.

Experiment 4. The effect of the interval between ION and RO treatment on the activation rate was evaluated in this experiment. Matured oocytes were exposed to 5 μM ION for 5 min and then divided in 4 groups, which were culture without RO (Control) or in the presence of 10 μM RO for 60 min starting immediately (T0), 30 min (T30), or 60 min (T60) after ION treatment. Between ION treatment and RO incubation, the oocytes were maintained in SOF medium.

Experiment 5. Embryo cleavage and development after activation were evaluated in this experiment. Oocytes were activated as described in the experiment 1 but also incubated in the presence of 7.5 μg/mL cytochalasin B for 240 min after ION treatment, and then cultured in SOF medium for 7 days.

Experiment 6. The effect of activation and pronuclear formation after ICSI was evaluated in this experiment. ICSI was performed using either control (ICSI-Cont) or electroporated (ICSI-El) sperm and oocytes were activated by exposure to 5 μM ION for 5 min followed by incubation in the presence of 10 μM RO for 240 min.

3 Results

3.1. Experiment 1: Effect of oocyte activation with ION, ION+RO, ION+OAG or ION+RO+OAG on pronuclear formation

The effect of oocyte activation on activation and pronuclear formation is shown in table 1. We found that treatment with RO in the absence (48.5 %) or presence (65.6 %) of OAG significantly increased pronuclear formation compared with ION alone (12.3 %) or OAG (9.2 %) alone. The average rate of the normal activated oocytes, i.e., those having 1PN and 2 polar bodies, was lower ($P \leq 0.01$) in the ION (9.8 %) and OAG (5.9 %) groups than RO (29.2 %) and RO+OAG (27.2 %) groups.

3.2. Experiment 2: Effect of RO concentration on oocyte activation rate

The activation rates were not statistically different when oocytes were exposed to 5, 7.5 or 10 µM RO for 240 min after ION treatment. The average of activated oocyte were 71.8 %, 82.4 %, 85.3 % for the 5, 7.5 and 10 µM RO concentrations, respectively ($P \leq 0.01$).

3.3. Experiment 3: Effect of exposure time to RO-3306 on bovine oocyte activation rates

This experiment revealed that the period of exposure to RO after ION treatment affects the activation rate (Fig 1). Significantly more oocytes were activated and formed pronuclei ($P \leq 0.01$) when exposed to 10 µM RO for 240 min (84.6 %) compared to those

265 treated for 120 min (60.0 %), 60 min (53.6%), or not treated with RO (47.5 %) after
266 ION.
267
268 *3.4. Experiment 4: Effect of the RO incubation time after ION treatment on oocyte*
269 *activation rates*
270
271 There was no difference in the total activation rate among groups that were
272 exposed to RO starting a T0 (95.5 %), T30 (95.8 %) and T60 (94.4 %) after ION. All
273 treatments produced significantly higher rates of activation ($P \leq 0.01$) compared to the
274 ION group (53.0 %) not exposed to RO (Fig 2).
275
276 *3.5. Experiment 5: Effect of oocyte activation on embryo development and quality*
277
278 Cleavage rates were lower when oocytes were activated with ION (11.8 %) or
279 ION+OAG (22.8 %) compared to ION+RO (70.2 %) and ION+RO+OAG (62.4 %)
280 treatments ($P \leq 0.01$; Table 2). Blastocyst rates at Day 7 were lower for the ION+OAG
281 treatment (9.5 %) compared to RO (19.7 %) and ION+RO+OAG (24.1 %) treatments
282 ($P \leq 0.01$). The average number of cells per blastocyst were not statistically different
283 between treatments (Table 2).
284
285 *3.6. Experiment 6: Effect of sperm pretreatment with subsequent oocyte activation on*
286 *pronuclear formation*
287
288 The rate of female pronuclear formation after ICSI and activation was 66.4 %,
289 which was higher than the rate of male pronuclear formation. Sperm electroporation

before ICSI significantly increased male pronuclear formation (33.3 %) compared to control injected sperm (9.4 %) ($P \leq 0.05$; Table 3).

4 Discussion

In this study, cattle oocytes were used to investigate activation methods for in vitro production of embryos after injection of pre-treated spermatozoa. We observed that: meiosis resumption rate was higher with CDK1 inhibitor combined with PKC activator compared to negative control; the rates of activation, cleavage and blastocyst no differ among CDK1 inhibitor treatment alone or with PKC activator in combination; the time of exposure to CDK1 inhibitor was 240 min; the use of electroporation increase numerically of male formation rate.

Our primary objective in this study was that specific PKC activator and specific CDK1 inhibitor are capable of releasing the oocyte from MII. The MPF activity in bovine oocytes is decreased in the germinal vesicle stage, subsequently reaching its peak in the MI stage, suffering a decrease in the stages of anaphase I and telophase I and increasing in MII [26]. Although PKC is able to alter the kinetics of oocyte maturation in the same periods in which increase of MPF concentrations [27], the PKC activator only when associated with CDK1inhibitor was able to resumption of meiosis. Moreover, CDK1inhibitor only has been sufficient to drive resumption of meiosis. This suggests that specific CDK1 inhibitor are capable of releasing the oocyte from MII, form female pronuclear, assisting cleavage and blastocyst stage. Studies with swine oocytes have established that RO is efficient to blocking meiotic progression [13]. On the other hands, has been proposed also for cell cycle synchronization in the G2/M

phase [28]. The success in the results is due to the specificity of RO, that has a 10–100-fold stronger inhibitory effect on CDK1 than on CDK2 or CDK4 [28].

In order to explore the potential mechanism associated with the releasing from MII in oocytes treated with CDK1inhibitor, we evaluated the exposure time for treatment in bovine oocyte activation. Increased oocyte activation rate was demonstrated at 240 min after CDK1inhibitor treatment when compared other treatments, but does not appear necessary at first 60 min after ION incubation. These results support the hypothesis that is important to maintain low levels of maturation promoting factor (MPF), independent of period the intracellular Ca^{2+} oscillations. The reduction of the MPF complex, through the inhibition of CDK1, is necessary for trigger resumption of meiosis [10], elimination of half of the sister chromatids (second polar body extrusion), and then female pronuclear formation. We are suggest, that this is the factor responsible for the formation of a higher rate of normal pronuclear formation, important aspect to ensure efficient protocol of activation.

Our second objective in this study was that acrossome reaction of bull spermatozoa before injection are capable of increased pronuclear formation rates of cattle embryos produced in ICSI procedure. In this study, we have confirmed that treatmente with ION+RO after ICSI promoted high rates of female pronuclear formation but low rates of both male and female pronuclear formation. The rates of two pronuclear formation (male and female) were improved when sperm pretreated in Fert medium incubation for 6h and electroporated. Membrane modifications occurs in response to eletric pulse, improving the decondensation of sperm and pronuclei formation. Head decondensation is lower in various species after ICSI, improvement has been reported after sperm treatment with DTT in pigs [29] and cows [5]. On other hand, its effects on the chromosomal disorders produces chromosomal damage in

identified in mice [30]. Recent studies demonstrated that treatment of spermatozoa with Triton was sufficient to support the formation of the male pronuclei in sheep [31] and cattle [25], however they used of the piezo-ICSI method. This technique produce high frequency of vibration on micro-pipette excited by the piezo-electric that necessary to oocyte pierce, and may has induced sperm membrane damage producing male pronuclei.

5 Conclusions

Findings from this study indicate that the specific inhibition of CDK1 after ION treatment can effectively promote the activation of bovine oocytes. Activation of bovine oocytes after ICSI with ION and RO result in higher rates of female pronuclear formation lower rates of both male and female pronuclei. Electroporation treatment of sperm before ICSI results in higher rates of sperm decondesation and pronuclear formation compared to control sperm. Collectively, our findings suggest that difficulties to implement an effective protocol for ICSI is bovine are mainly associated to defective sperm decondensation rather than insufficient oocyte activation.

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Conflict of interest

None of the authors have any conflict of interest to declare.

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458

Figure legends

Figure 1. Effect of 60 min, 120 min and 240 min exposure to RO on activation of bovine oocytes. Data are presented as average ± standard error of the mean (SEM). Different letters indicate significant differences between groups (P≤ 0.01).

Figure 2. Effect of the interval between ION and RO treatment on activation of bovine oocytes. Data are presented as average ± standard error of the mean (SEM). Different letters indicate significant differences between groups (P≤ 0.01). ION represents the control group (not exposed to RO); and T0, T30, and T60 represent groups treated at 0, 30 and 60 min after ION, respectively.

470 **Table 1.** Effect of treatment with ION, ION+RO, ION+OAG or ION+RO+OAG on the
471 activation of bovine oocytes.

Treatments	n	Non activated n (%)	Total activated n (%)	Normal activated n (%)
ION	122	107 (87.7) [a]	15 (12.3) [b]	12 (9.8) [b]
ION+RO	130	67 (51.5) [b]	63 (48.5) [a]	38 (29.2) [a]
ION+OAG	119	108 (90.8) [a]	11 (9.2) [b]	7 (5.9) [b]
ION+RO+OAG	125	43 (34.4) [b]	82 (65.6) [a]	34 (27.2) [a]

472

473 Columns with different letters show statistical difference (P≤0,01). Non activated: MII,
474 ATII, and 2PB; Total activated: 1PN+2PB and 1PN+1PB; Normal activated: 1PN+2PB

475 **Table 2:** Effect of oocyte activation with ION, ION+RO, ION+OAG or

476 ION+RO+OAG on embryo development.

Treatments	n	Cleavage n (%)	Blastocyst n (%)	Embryo Cell Number (Average)
ION	93	11 (11.8)[a]	-	-
ION+RO	94	66 (70.2)[c]	13 (19.7)[ab]	78,4[a]
ION+OAG	92	21 (22.8)[b]	2 (9.5)[a]	90,0 [a]
ION+RO+OAG	93	58 (62.4)[c]	14 (24.1)[b]	101,6[a]

477 Columns with different letters show statistical difference. ($P \leq 0.01$). Cleavage and

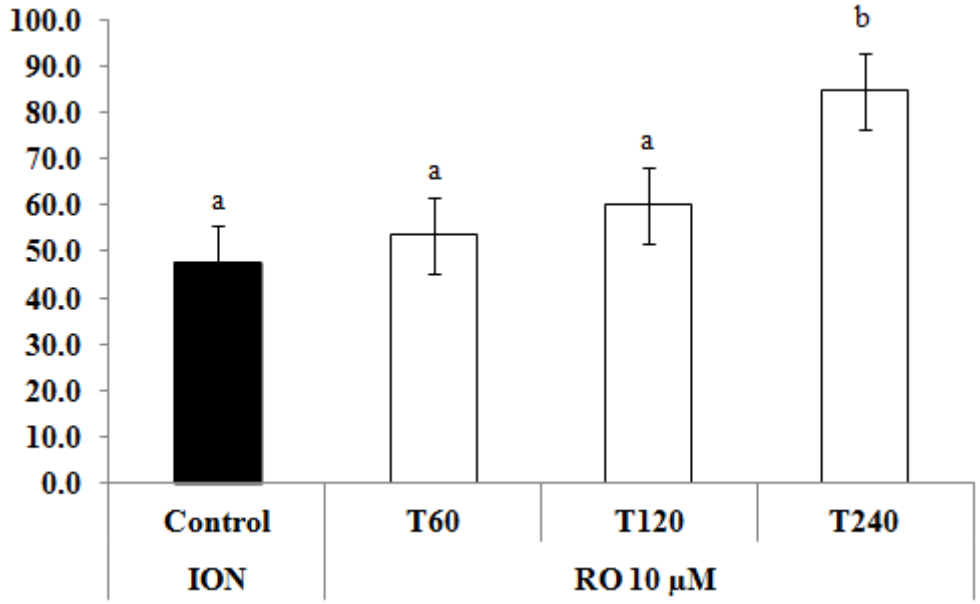
478 blastocyst rates were recorded at 48 h and on Day 7.

Table 3: Pronuclear formation after ICSI using sperm treated with different protocols and oocyte activation with ION+RO.

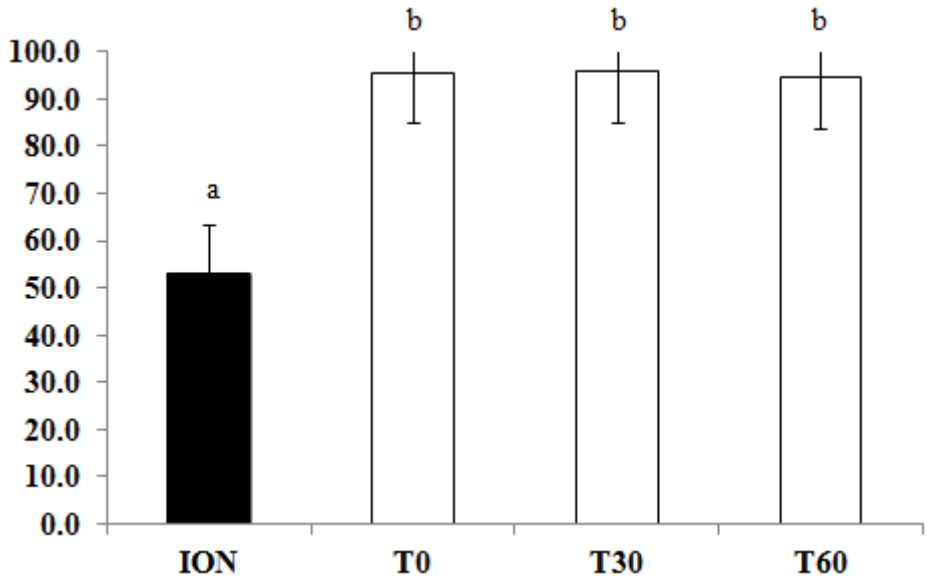
Treatments	n	MII+2PB n (%)	1PN n (%)	2PN n (%)
ICSI-Cont[1]	53	19 (35.8) [b]	29 (54.7) [b]	5 (9.4)[b]
ICSI-El[1]	60	19 (31.7) [a]	21 (35.0) [a]	20 (33.3)[a]

ICSI-Cont: Incubation in Fert medium for 6h before ICSI; ICSI-El: Incubation in Fert medium for 6h and Electroporated before ICSI. [1] Incubated with ION before RO treatment. MII+2PB: Metaphase II and 2º Polar Body extrusion; 1PN: Female Pronuclear formation; 2PN: Male and Female Pronuclear formation. Columns with different letters show statistical difference. ($P \leq 0.05$).

Figure 1


100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
a
a
a
b
Control
T60
T120
T240
ION
RO 10 µM

488 **Figure 2**



489

Highlights

• Specific inhibitor CDK1 (RO-3306) treatment is efficient to promote meiotic resumption.

• The time of exposure to Specific inhibitor CDK1 (RO-3306) influence on bovine oocyte activation and embryonic development rate.

• Specific inhibitor CDK1 (RO-3306) promote the bovine oocyte activation submitted to ICSI.

• Pretreated of sperm with Fert medium for 6h and electroporation improve rate of male pronuclear formation after ICSI.

4. CONCLUSÃO

Com base nos resultados obtidos no presente estudo, conclui-se que a combinação entre Ionomicina (ION) e o inibidor específico de CDK1 (RO-3306) pode ser utilizada como método de ativação para oócitos bovinos após a ICSI. A eletroporação de espermatozoides anteriormente a ICSI melhora a formação de pró-núcleos masculinos. Isso indica que a deficiente descondensação do espermatozoide é provavelmente o principal limitante para estabelecer um protocolo eficaz para ICSI em bovinos.

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