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MARIA VALÉRIA DE OLIVEIRA SANTOS

ATIVIDADE ANTIOXIDANTE DO ÓLEO ESSENCIAL DE *Syzygium aromaticum* L. SOBRE GAMETAS BOVINOS

MOSSORÓ–RN
2018

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Dissertação apresentada ao Programa de Pós-Graduação em Ciência Animal da Universidade Federal Rural do Semi-Árido como requisito para obtenção do título de Mestre em Ciência Animal.

Linha de Pesquisa: Morfofisiologia e Biotecnologia Animal.

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MARIA VALÉRIA DE OLIVEIRA SANTOS – Nascida em Russas, CE, no dia 07.07.1995, filha de Maria de Fátima de Oliveira Santos e Francisco Valdenir dos Santos. Graduou-se em Biotecnologia pela Universidade Federal Rural do Semi-Árido (UFERSA, 2012–2016). Durante esse período, estagiou no Laboratório de Biotecnologia Animal da UFERSA (Programa Institucional Voluntário de Iniciação Científica, 2013–2014, Programa Institucional de Bolsas de Iniciação em Desenvolvimento Tecnológico e Inovação, 2014–2015, e Programa Institucional de Bolsas de Iniciação Científica, 2015–2016). Além disso, foi monitora voluntária da disciplina de Cultura Celular Básica do Curso de Biotecnologia (UFERSA) durante o período de 2014–2015. Em julho de 2016, após ser aprovada no processo seletivo para o Mestrado acadêmico em Ciência Animal (PPGCA/UFERSA), iniciou suas atividades de mestrado no Laboratório de Biotecnologia Animal com bolsa de auxílio financeiro pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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“Que os vossos esforços desafiem as impossibilidades, lembrai-vos de que as grandes coisas do homem foram conquistadas do que parecia impossível.”

(Charles Chaplin)

ATIVIDADE ANTIOXIDANTE DO ÓLEO ESSENCIAL DE *Syzygium aromaticum* L. SOBRE GAMETAS BOVINOS

SANTOS, Maria Valéria de Oliveira. ATIVIDADE ANTIOXIDANTE DO ÓLEO ESSENCIAL DE *Syzygium aromaticum* L. SOBRE GAMETAS BOVINOS. 2018. Dissertação (Mestrado em Ciência Animal: Morfofisiologia e Biotecnologia Animal) – Universidade Federal Rural do Semi-Árido (UFERSA), Mossoró, RN, 2018.

RESUMO: O uso de antioxidantes naturais em meios de cultivo pode ser uma alternativa para minimizar os efeitos negativos do estresse oxidativo produzido pelas condições *in vitro*. Nesse sentido, o óleo essencial de *Syzygium aromaticum* (OESA) possui propriedades terapêuticas, incluindo atividade antioxidante, e poderia ser utilizado durante a maturação *in vitro* (MIV) de oócitos e incubação de espermatozoides bovinos. Portanto, o objetivo foi avaliar a atividade antioxidante do OESA sobre os gametas bovinos, especificamente sua adição (i) durante a MIV e influência sobre a maturação nuclear, maturação citoplasmática, viabilidade das células do *cumulus*, níveis de espécies reativas de oxigênio (EROs), potencial de membrana mitocondrial ($\Delta\Psi m$) e desenvolvimento embrionário partenogenético; e (ii) durante a incubação por 1 e 6 h de espermatozoides epididimários e sua influência sobre a qualidade espermática e níveis de EROs. Para tanto, gametas foram cultivados em meios contendo as seguintes suplementações: OESA0 (sem antioxidante), OESA10 (10 µg/mL de OESA), OESA15 (15 µg/mL de OESA), OESA20 (20 µg/mL de OESA), CIS (100 µM de cisteamina, somente para os oócitos) e Controle (somente para espermatozoides frescos, imediatamente avaliados). Assim, quanto à influência do OESA durante a MIV, nenhuma diferença foi observada para as taxas de maturação. Contudo, OESA15, OESA20 e CIS melhoraram a viabilidade das células do *cumulus* após a MIV, sendo somente o OESA20 maior que o OESA0 ($P < 0,05$). Além disso, embora nenhuma diferença tenha sido observada para os níveis de EROs ($P > 0,05$), oócitos derivados dos grupos OESA15, OESA20 e CIS mostraram $\Delta\Psi m$ menor que os oócitos do grupo OESA0 ($P < 0,05$). Adicionalmente, embora nenhuma diferença tenha sido observada para as taxas de clivagem de oócitos ativados partenogeneticamente, OESA20 melhorou as taxas de blastocistos, tanto quando calculada pelo número total de oócitos cultivados, quando pelo número total de oócitos clivados. Ainda, essas taxas de blastocistos foram diferentes do OESA0 e similares ao grupo CIS. Quanto à qualidade dos embriões produzidos, os grupos OESA15 e OESA20 mostraram um maior número de blastômeros quando comparados ao OESA0 ($P < 0,05$). Já quanto à influência do OESA durante a incubação dos espermatozoides, nenhuma diferença foi observada para a morfologia espermática ($P > 0,05$). Contudo, espermatozoides incubados na presença de OESA preservaram os parâmetros ao longo do tempo, especialmente para integridade estrutural e funcional da membrana plasmática, atividade mitocondrial, velocidade curvilínea e atividade metabólica ($P < 0,05$). Além disso, espermatozoides derivados do grupo OESA15 mantiveram resultados similares ao grupo controle para velocidade média após 6 h de incubação, integridade funcional da membrana e percentual de membrana danificada e mitocôndria inativa ($P < 0,05$). Ainda, nenhuma diferença foi observada para os níveis de EROs após a incubação. Em conclusão, OESA a 20 µg/mL e 15 µg/mL adicionado aos meios durante a MIV e a incubação espermática, respectivamente, pode ser uma interessante alternativa de antioxidante para manutenção da qualidade de gametas bovinos *in vitro*.

Palavras-chave: produção *in vitro* de embriões, estresse oxidativo, antioxidante natural, cravo.

ANTIOXIDANT ACTIVITY OF ESSENTIAL OIL OF *Sygygium aromaticum* L. ON BOVINE GAMETES

SANTOS, Maria Valéria de Oliveira. ANTIOXIDANT ACTIVITY OF ESSENTIAL OIL OF *Syygygium aromaticum* L. ON BOVINE GAMETES. 2018. Dissertação (Mestrado em Ciência Animal: Morfofisiologia e Biotecnologia Animal) – Universidade Federal Rural do Semi-Árido (UFERSA), Mossoró, RN, 2018.

ABSTRACT: The use of natural antioxidants in culture media may be an alternative to minimize the negative effects of oxidative stress produced by *in vitro* conditions. In this sense, the essential oil of *Syzygium aromaticum* (EOSA), has therapeutic properties, including antioxidant activity, and could be used during *in vitro* maturation (IVM) of bovine oocytes and incubation of bovine spermatozoa. Therefore, the aim was to evaluate the antioxidant activity of EOSA on bovine gametes, specifically its addition (i) during IVM and influence on nuclear maturation, cytoplasmic maturation, viability of *cumulus* cells, reactive oxygen species (ROS) levels, mitochondrial membrane potential ($\Delta\Psi m$), and parthenogenetic embryonic development; and (ii) during incubation for 1 and 6 h of epididymal spermatozoa and its influence on sperm quality and ROS levels. Thus, gametes were cultured in media containing the following supplements: EOSA (without antioxidants), EOSA 10 (10 μg/mL of EOSA), EOSA15 (15 μg/mL of EOSA), EOSA20 (20 μg/mL of EOSA) (100 μM of cysteamine, only for oocytes) and Control (only for fresh spermatozoa, immediately evaluated). Thus, regarding the influence of EOSA during IVM, no difference was observed for maturation rates. Nevertheless, EOSA15, EOSA 20 and CYS improved the viability of *cumulus* cells after IVM, being only the EOSA20 group greater than EOSA0 ($P < 0.05$). Moreover, although no difference was observed for ROS levels ($P > 0.05$), oocytes derived from the EOSA15, EOSA20 and CYS groups showed $\Delta\Psi m$ lower than the EOSA0 ($P < 0.05$) oocytes. Additionally, although no differences was observed for cleavage rates of the parthenogenetically activated oocytes, EOSA20 improved blastocyst rates, both when calculated by the total number of cultured oocytes, and by the total number of cleaved oocytes. Also, these blastocyst rates were different from EOSA0 group and similar to CYS group. As to the quality of the embryos produced, OESA15 and OESA20 groups showed a higher number of blastomeres when compared to OESA0 ($P < 0.05$). Regarding the influence of EOSA during sperm incubation, no difference was observed for sperm morphology ($P > 0.05$). Nevertheless, spermatozoa incubated in the presence of EOSA preserved the parameters over time, especially for structural and functional integrity of the plasma membrane, mitochondrial activity, curvilinear velocity and metabolic activity ($P < 0.05$). Moreover, spermatozoa derived from the EOSA15 group maintained similar results to the control group for medium velocity after 6 h of incubation, functional integrity of the membrane and percentage of damaged membrane and inactive mitochondria ($P < 0.05$). Also, no difference was observed for ROS levels after incubation. In conclusion, OESA at 20 μg/mL and 15 μg/mL added to media during IVM and sperm incubation, respectively, may be an interesting antioxidant alternative for maintaining the quality of bovine gametes *in vitro*.

Keywords: *in vitro* embryo production, oxidative stress, natural antioxidant, clove.

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LISTA DE SÍMBOLOS E SIGLAS

%	Percentual
<	Menor
>	Maior
±	Mais ou menos
ΔΨm	Potencial de membrana mitocondrial
®	Marca registrada
°C	Graus Celsius
µL	Microlitro
µM	Micromolar
1PB	First polar body
6-DMAP	6-dimethylaminopurine
ALH	Amplitude of lateral head displacement
AP	Ativação partenogenética (*PA: parthenogenetic activation)*
B	Blastocyst
BCF	Beat/cross-frequency
BSA	Bovine serum albumin
CASA	Computer assisted sperm assessment
CCOs	Complexos *cumulus*-oócito *(COCs: cumulus-oocyte complexes)*
CEUA	Comitê de Ética de Uso de Animais
CYS	Cysteamine
CMXRos	Mito Tracker Red®
CO_2	Dióxido de carbono
DIV	Desenvolvimento *in vitro (IVD: in vitro development)*
eB	Expanded blastocyst
EROs	Espécies reativas de oxigênio (*ROS: reactive oxygen species*)
et al.	E outros
FBS	Fetal bovine serum
FIV	Fecundação *in vitro (IVF: in vitro fertilization)*
FSH	Follicle stimulating hormone
g	Grama
G	Gauge
GSH	Glutathione

h	Hora
H_2DCFDA	2′, 7′-dichlorodihydrofluorescein diacetate
H_2O_2	Hydrogen peroxide
hB	Blastocyst hatching/hatched
i.e.,	Isto é
iB	Initial blastocyst
ICSI	Intracytoplasmic sperm injection
kg	Quilo
KI	Kovat Index
LBA	Laboratório de Biotecnologia Animal
LH	Luteinizing hormone
LIN	Linearity
Mg	Miligrama
MII	Metaphase plate
Min	Minuto
MIV	Maturação *in vitro* (IVM: *in vitro* maturation)
mL	Mililitro
mm	Milímetro
mOsm/L	Miliosmol por litro
MTT	3-(4,5-dimetylthiazol-2-yl) -2,5-diphenyltetrazolium bromide
n	Número
NaCl	Cloreto de sódio
OESA	Óleo essencial de *Syzygium aromaticum (EOSA: Essential oil from Syzygium aromaticum)*
ORM	Oocyte recovery medium
P	Valor-P
PBS	Solução tampão fosfato
PI	Propidium iodide
PIVE	Produção *in vitro* de embriões *(IVEP: In vitro embryo production)*
SCNT	Somatic cell nuclear transfer
SOF	Synthetic Oviductal Fluid
STR	Straightness
TALP	Tyrode's albumin lactate pyruvate
TCM199	Tissue culture medium 199

USDA	United States Department of Agriculture
VAP	Average pathway velocity
VCL	Curvilinear velocity
Vs.	*Versus*
VSL	Straightline velocity
x	Vezes

SUMÁRIO

CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

1. INTRODUÇÃO

A produção *in vitro* de embriões (PIVE) pode ser dividida basicamente em dois sistemas: a fecundação *in vitro* (FIV) e a ativação partenogenética (AP), os quais podem ser empregados em inúmeras proposições, como pesquisas básicas em biologia do desenvolvimento (CAMARGO et al., 2016; CARROCERA et al., 2016) e aplicações comerciais (NIVET et al., 2012; VIANA et al., 2017).

Assim, para a obtenção de embriões por PIVE, normalmente são realizadas as seguintes etapas: colheita de oócitos imaturos, maturação *in vitro* (MIV) desses oócitos, FIV com espermatozoides previamente capacitados ou AP artificial e desenvolvimento *in vitro* (DIV) dos presumíveis embriões (MACHATY et al., 2012). No caso da AP, não há participação do gameta masculino sendo necessário induzir a ativação oocitária com agentes químicos e, por essa razão, os embriões não tem capacidade de se desenvolver a termo (CAMARGO et al., 2016). Por outro lado, esses embriões podem ser considerados uma alternativa de menor custo para avaliação da eficiência de meios de cultivo sem interferência do efeito touro (BARCELÓ-FIMBRES et al., 2011).

Desde o nascimento do primeiro bovino produzido por técnicas *in vitro* (BRACKETT et al., 1982), vários estudos têm sido desenvolvidos visando aumentar a eficiência dos sistemas de PIVE (MESALAM et al., 2017; KHAN et al., 2018). Em geral, todas as etapas dessa técnica apresentam peculiaridades e limitações que influenciam diretamente no sucesso final. Essas limitações podem ser relacionadas às condições de cultivo *in vitro* que devem favorecer o desenvolvimento de gametas e embriões, minimizando os efeitos negativos externos, como o estresse oxidativo (SANTOS et al., 2018). Tal estresse durante a PIVE tem sido relacionado à tensão de oxigênio, interferência de luz e calor, presença de espermatozoides, constituintes dos meios e ausência da proteção antioxidante materna (CROCOMO et al., 2012). Esses fatores podem causar desequilíbrio na produção de espécies reativas de oxigênio (EROs), promovendo uma série de danos celulares devido a elevada instabilidade e reatividade dessas moléculas (ROCHA-FRIGONI et al., 2013). Tais características fazem com que as EROs se liguem a qualquer molécula celular promovendo a oxidação das mesmas (AGARWAL et al., 2005). Dessa forma, o processo oxidativo prejudica drasticamente a atividade dos gametas, além do desenvolvimento embrionário *in vitro* (ALI et al., 2003).

Nesse contexto, estudos vêm sendo realizados visando avaliar a suplementação de meios com antioxidantes em diferentes etapas da PIVE (SALZANO et al., 2014; KHAN et al., 2018). Esses antioxidantes podem ser de origem natural ou sintética, e estão envolvidos em reações que permitem reduzir a formação ou ação de EROs, contribuindo para aumentar o desenvolvimento de embriões (BANSAL; BILASPURI, 2011; KHAN et al., 2018).

Atualmente, antioxidantes naturais têm sido amplamente estudados durante o cultivo *in vitro* de gametas (SANTOS et al., 2018). Nesse contexto, algumas plantas se destacam em virtude da presença de substâncias com essa propriedade, tornando-as promissoras para uso em meios a fim de minimizar o estresse oxidativo (MALEKI et al., 2014). Nesse cenário, encontra-se a *Syzygium aromaticum* L., conhecida pelo seu botão de flor, chamado de cravo, o qual apresenta atividade antioxidante eficaz, especialmente devido à presença de compostos fenólicos em sua composição (BAGHSHAHI et al., 2014). O eugenol é o componente majoritário do cravo e exibe propriedades antioxidantes evidenciadas em diversos estudos (MAHAPATRA et al., 2009; OU et al., 2006).

Trabalhos anteriores avaliaram o extrato aquoso e etanólico do *S. aromaticum* e observaram forte atividade sequestradora de radicais livres em ensaios bioquímicos (VIUDA-MARTOS et al., 2010; EL-MAATI et al., 2016). Já em estudos aplicados à reprodução, o extrato hexânico de *S. aromaticum* administrado oralmente em camundongos (15 mg por kg de peso corporal por dia) aumentou a motilidade espermática e atividade secretória do epidídimo e vesículas seminais (MISHRA; SINGH, 2013). Além disso, Baghshahi et al. (2014) avaliaram o extrato etanólico de *S. aromaticum* (35 e 75 µg/mL) em diluente de sêmen ovino durante o resfriamento e criopreservação e observaram um efeito benéfico do extrato sobre a motilidade, integridade da membrana e viabilidade espermática.

Quimicamente, a obtenção de um óleo essencial envolve procedimentos mais complexos em relação aos extratos; contudo, o óleo essencial permite uma combinação mais pura de bioativos sem a interferência que solventes utilizados na obtenção de extratos podem proporcionar (EL-MAATI et al., 2016). Portanto, devido às propriedades antioxidantes e efeitos positivos observados na reprodução, bem como as grandes quantidades de eugenol, o óleo essencial de *S. aromaticum* (OESA) pode ser uma alternativa para a suplementação antioxidante nos meios de cultivo *in vitro* de gametas bovinos.

Assim, o objetivo foi avaliar a atividade antioxidante do OESA sobre a qualidade e o estresse oxidativo de gametas bovinos, especificamente durante a MIV e incubação de espermatozoides epididimários.

2. FUNDAMENTAÇÃO TEÓRICA

2.1. ASPECTOS BOTÂNICOS E COMERCIAIS DA *S. aromaticum* L.

A *S. aromaticum* L., cujo sinômino é *Eugeia caryophyllata* Trunb., é comumente conhecida como cravo ou craveiro, da família Mirtaceae, nativa das ilhas Maluku, no leste da Indonésia (DUKE, 2002). Essa espécie é frequentemente cultivada em áreas costeiras com altitudes máximas de 200 m acima do nível do mar (CORTÉS-ROJAS et al., 2014). No Brasil, o cravo é cultivado na região nordeste, especialmente na Bahia, onde podem ser encontrados aproximadamente 8000 hectares com produção de 2500 toneladas por ano (OLIVEIRA et al., 2007; CORTÉS-ROJAS et al., 2014).

O craveiro (**Figura 1A**) é caracterizado como uma árvore com copa alongada que pode atingir em média 8–12 m de altura (CORTÉS-ROJAS et al., 2014). Suas folhas possuem características ovais, aromáticas e tem de 7–11 cm de comprimento. Já as suas flores são pequenas, dispostas em corimbos terminais e os frutos são de drupa elipsoide com coloração avermelhada (LORENZI; MATOS, 2002). O botão de flor (**Figura 1B e 1C**), também conhecido como cravo, possui odor fortemente aromático, sabor ardente e marcante (SILVESTRI et al., 2010).

Os principais produtos comercializados dessa planta são os botões florais secos (**Figura 1C**), os quais têm sido amplamente empregados na culinária como ingrediente em diferentes cardápios e para a produção de essências aromáticas (AFFONSO et al., 2012). Além disso, na forma de chá ou óleo essencial, o cravo tem sido utilizado na medicina popular e tradicional, como anestésico odontológico e tratamento para alívio de dores estomacais, intestinais, reumáticas e musculares (BHOWMIK et al., 2012).

2.2. EXTRATOS E ÓLEOS ESSENCIAIS DERIVADOS DE *S. aromaticum*

Os estudos voltados para as propriedades terapêuticas de *S. aromaticum* envolvem tanto o emprego de extrato (BAGHSHAHI et al., 2014), quanto de óleo essencial (SHEWEITA et al., 2016) produzidos a partir de botões florais secos. Quimicamente, extratos vegetais são definidos como uma combinação de vários tipos de compostos fitoquímicos com diferentes polaridades (SASIDHARAN et al., 2011). Desta maneira, o processo de extração permite a obtenção de porções ativas da planta usando solventes seletivos com o objetivo de separar os metabólitos vegetais solúveis, descartando o resíduo celular. Em geral, as técnicas mais

utilizadas para a obtenção de extrato são a maceração, decocção, infusão e percolação (AZWANIDA, 2015).

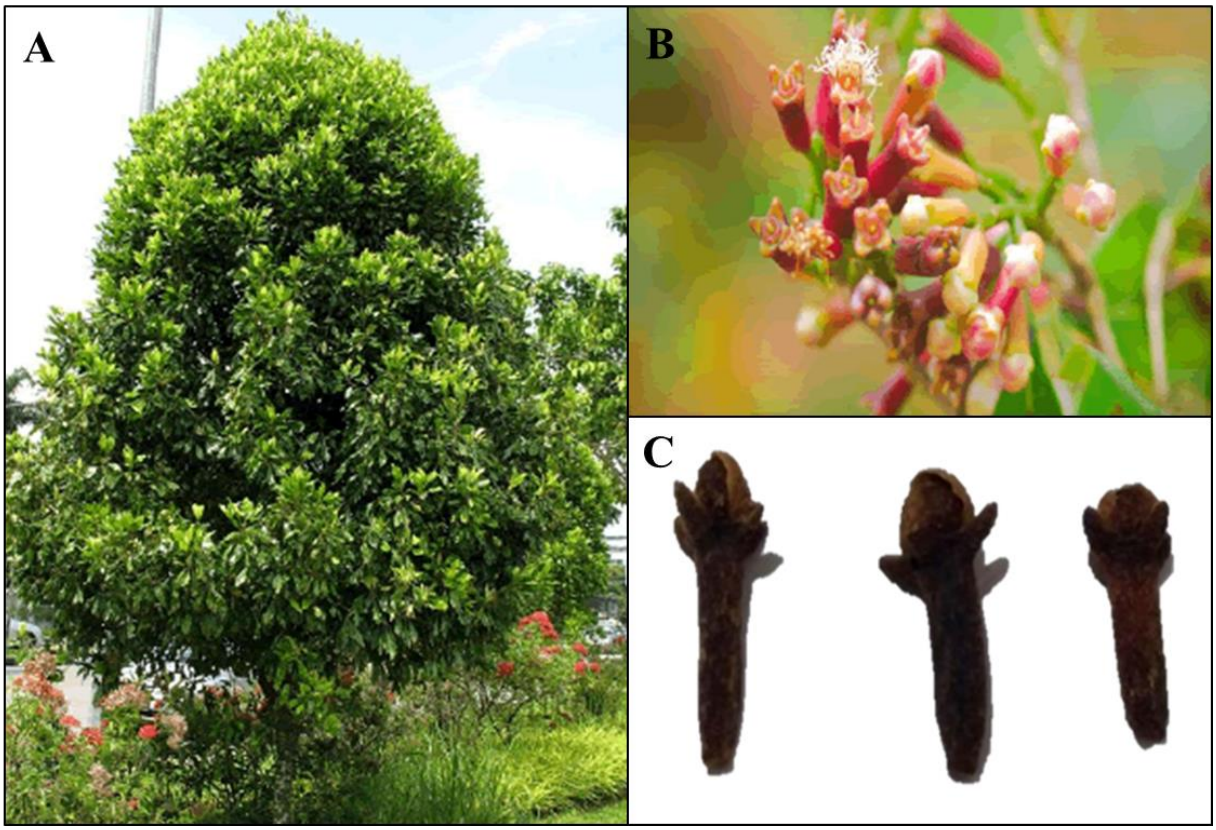

Figura 1. Características botânicas de *S. aromaticum*. (**A)** Exemplar de *S. aromaticum* com porte arbóreo e copa alongada, Fonte: Ranasinghe et al. (2016). (**B)** Botões florais, Fonte: Ranasinghe et al. (2016). (**C)** Botões florais secos, Fonte: LBA.

Já os óleos essenciais consistem em um líquido hidrofóbico concentrado contendo compostos aromáticos voláteis da planta, principalmente álcoois, fenóis, aldeídos, hidrocarbonetos, ésteres e cetonas (RASSEM et al., 2016). A obtenção destes óleos é realizada por diferentes metodologias, como a hidrodestilação, arraste com vapor d'água, extração por solvente e CO_2 supercrítico (WENQIANG et al., 2007). Contudo, o método convencional para a obtenção de óleos essenciais consiste na hidrodestilação, na qual uma mistura de água com os materiais vegetais é aquecida gerando o vapor com os constituintes voláteis. Esses vapores são liquefeitos em um condensador e coletados em recipiente apropriado (RASSEM et al., 2016; **Figura 2**).

Para o *S. aromaticum*, tanto o extrato quanto o óleo essencial apresentam componentes majoritários similares (NASSAR et al., 2007; XU et al., 2016). Contudo, o emprego de

solventes nos procedimentos de obtenção de extratos influencia criticamente na composição e concentração dos constituintes (AZWANIDA, 2015; EL-MAATI et al., 2016). Já os óleos essenciais permitem uma combinação mais pura de bioativos sem a interferência que diferentes solventes podem fornecer, mantendo maior similaridade com suas fontes vegetais correspondentes (EL-MAATI et al., 2016; RASSEM et al., 2016). Além disso, as concentrações dos constituintes, tanto de extratos quanto de óleos essenciais, podem ser alteradas pelos métodos de obtenção e por fatores inerentes da planta, como a parte utilizada, local de origem e época do ano (AFFONSO et al., 2012). Apesar disso, estudos relacionados ao estabelecimento de bioativos naturais envolvem inicialmente pesquisas com misturas químicas e seus efeitos, os quais podem ser resultado ou não do sinergismo entre componentes majoritários e minoritários (AZWANIDA et al., 2015).

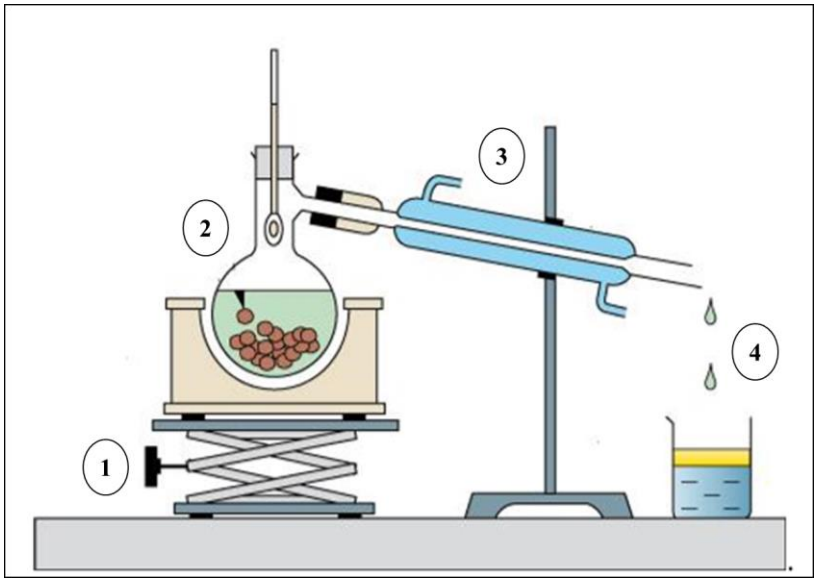

Figura 2. Obtenção de óleo essencial usando sistema de hidrodestilação. (1) Fonte de calor. (2) Mistura de água e material vegetal. (3) Condensador que liquefaz os vapores gerados. (4) Recipiente para colheita. Fonte: Rassem et al. (2016).

Em geral, o componente majoritário do cravo é o eugenol ou 2-metoxi-4-prop-2-enilfenol (**Figura 3A**), o qual representa cerca de 60–90% da composição (CRAVEIRO et al., 1976). O eugenol, um fenilpropanóide, é um ácido fraco solúvel em solventes orgânicos, tem aroma picante e peso molecular de 164,2 g/mol (MOHAMMADI et al., 2017). Já os outros constituintes têm sido principalmente o β-cariofileno e o acetato de eugenila, variando entre o segundo e terceiro lugar na quantidade presente no óleo (CHAIEB et al., 2007; XU et al., 2016). Assim, o β-cariofileno [(1R, 4E, 9S) -4,11,11-trimetil-8-metilidenobiciclo [7.2.0]

undec-4-eno] (**Figura 3B**) é um sesquiterpeno bicíclico natural, com peso molecular de 204,3 g/mol, pouco solúvel em água, conhecido por ter uma estrutura química considerada raridade na natureza e pelo sabor doce característico encontrado em vários alimentos (LIU et al., 2013). Finalmente, o acetato de eugenila ou (2-metoxi-4-prop-2-enilfenil) acetato, (**Figura 3C**) é um éster derivado do eugenol, pouco solúvel em água, com peso molecular de 206,2 g/mol (LOSS et al., 2016).

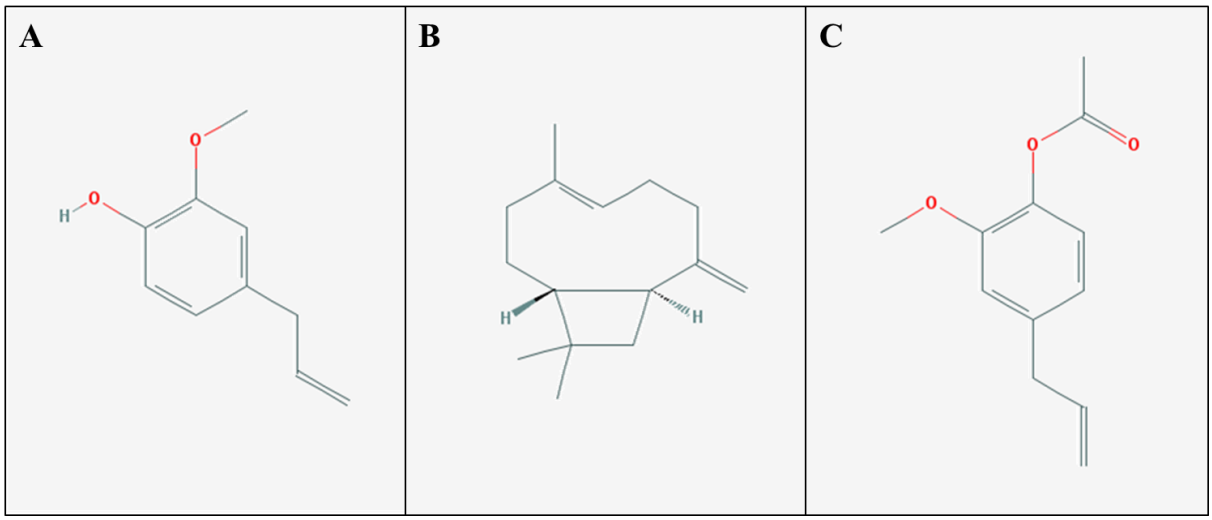

Figura 3. Estrutura química do **(A)** eugenol, **(B)** β-cariofileno e **(C)** acetato de eugenila, Fonte: NCBI (2018a, b, c).

Assim, em virtude de sua composição, o *S. aromaticum* apresenta várias propriedades terapêuticas (EL-MAATI et al., 2016). Inicialmente, o óleo essencial ou produtos derivados do cravo podem ser empregados como anestésico em procedimentos odontológicos (AFFONSO et al., 2012) e em tratamentos de queimaduras e ferimentos pela redução do tempo de cicatrização (BHOWMIK et al., 2012). Além disso, atividades antibacteriana (CHAIEB et al., 2007), antiviral (HUSSEIN et al., 2000), antifúngica (PINTO et al., 2009), anticarcinogênica (VENUGOPAL et al., 2017), antidiabetes (SHUKRI et al., 2010) e antioxidante (BAGHSHAHI et al., 2014) já foram identificadas para o cravo.

2.3. PROPRIEDADE ANTIOXIDANTE DE *S. aromaticum*

O *S. aromaticum* é amplamente conhecido como uma planta com eficaz atividade antioxidante em virtude da presença de componentes fenólicos em sua composição (AFFONSO et al., 2012). Em estudos realizados com óleo essencial, extrato aquoso e

etanólico obtidos de seus botões florais, foi observada uma marcante ação antioxidante usando diferentes ensaios bioquímicos, os quais quantificaram a capacidade de sequestro de radicais livres, doação de hidrogênio, ação quelante de íons metálicos e ação contra a peroxidação lipídica (VIUDA-MARTOS et al., 2010; EL-MAATI et al., 2016). Além disso, observou-se que em alguns ensaios bioquímicos os extratos ou óleos essenciais do cravo podem apresentar atividade antioxidante mais elevada em relação aos seus principais constituintes isolados (DORMAN et al., 2000; LEE et al., 2001).

Já em pesquisas relacionadas à reprodução *in vivo*, foi investigado o efeito da exposição oral ao extrato hexânico (15 mg, 30 mg e 60 mg/kg peso corporal ao dia) de botões florais de *S. aromaticum* sobre um ciclo espermatogênico (35 dias) de camundongos (MISHRA; SINGH, 2008). Nesse estudo, o tratamento não induziu a toxicidade, sendo a dose de 15 mg/kg capaz de aumentar a produção de testosterona e as doses de 30 mg/kg e 60 mg/kg responsáveis pela redução de testosterona e indução de alterações degenerativas nos túbulos seminíferos associados à diminuição na produção de espermatozoides diários. Posteriormente, Mishra e Singh (2013), novamente utilizando o mesmo extrato, observaram que a dose de 15 mg/kg/dia aumentou a motilidade espermática e estimulou as atividades secretoras do epidídimo e vesículas seminais. Por outro lado, doses de 30 e 60 mg/kg/dia resultaram em uma redução significativa na motilidade, no número de espermatozoides na cauda epididimária e atividade secretora do epidídimo e vesículas seminais. Ambos os estudos demostraram a importância do uso de doses adequadas de substâncias naturais para obtenção de efeitos positivos sobre a reprodução.

Já em um estudo *in vitro* relacionado à conservação de sêmen ovino, Baghshahi et al. (2014) observaram que o extrato etanólico de cravo adicionado ao diluidor de sêmen para o resfriamento (4°C por 120 min) em concentrações de 35 e 75 µg/mL, promoveu um aumento significativo nos valores de motilidade total (60,5% e 62,7% *vs*. diluidor na ausência de extrato: 53,5%), motilidade progressiva (56,5% e 58,6% *vs*. 49,1%) e viabilidade espermática (65,7% e 76,3% *vs*. 57,1%). No mesmo estudo, 75 µg/mL de extrato em comparação ao diluidor na ausência de extrato durante a criopreservação de sêmen aumentou a motilidade total (40,0% *vs*. 27,9%), motilidade progressiva (34,4% *vs*. 22,8%), viabilidade espermática (43,0% *vs*. 29,9%) e integridade da membrana plasmática (39,0% *vs*. 25,6%). Tais efeitos positivos foram atribuídos a propriedade antioxidante do *S. aromaticum*, comprovada por ensaio bioquímico de sequestro de EROs (BAGHSHAHI et al., 2014).

2.3.1. Propriedade antioxidante do eugenol

Inicialmente, o eugenol (1, 5, 10, 15 e 20 µg/mL) foi avaliado quanto ao seu efeito antioxidante sobre macrófagos murinos tratados com nicotina (MAHAPATRA et al., 2009). Nesse estudo, os autores observaram um efeito protetor dose-dependente mais evidenciado na concentração de 15 µg/mL, que promoveu diminuição na geração de ânion superóxido e peroxidação lipídica, e aumentou a atividade de superóxido dismutase. Além disso, o eugenol também atenuou a toxicidade da nicotina através da diminuição da geração de EROs e danos a lipídios, proteínas, DNA e status antioxidante endógeno. Já Ou et al. (2006) observaram um efeito protetor antioxidante em células endoteliais da veia umbilical humana sob estresse induzido, onde o eugenol (12,5–100 µM) inibiu a geração de EROs, o esgotamento do potencial de membrana mitocondrial e a ativação de caspase-3.

Um dos mecanismos propostos para atuação positiva do eugenol na proteção celular, diz respeito a sua capacidade de se incorporar na membrana devido à natureza hidrofóbica (NAGABABU; LAKSHMAIAH, 1992) e inibir o ataque de EROs, diminuindo a peroxidação lipídica (KUMARAVELU et al., 1996). Além disso, em estudos com macrófagos peritoneais murinos *in vitro*, o eugenol demonstrou a capacidade de aumentar significativamente a atividade e os níveis de glutationa, que é um importante antioxidante endógeno celular (MAHAPATRA et al., 2009).

2.3.2. Propriedade antioxidante do β-cariofileno e acetato de eugenila

O efeito protetor e antioxidante do β-cariofileno (2,5 µM) foi estudado em células de neuroblastoma humano expostas a um agente neurotóxico, onde foi observada uma melhora significativa na viabilidade celular, atividade mitocondrial e níveis de glutationa, bem como diminuição nos níveis de EROs e da taxa de apoptose devido a menor expressão de genes apoptóticos (WANG et al., 2018). Já com células de glioma de rato com citotoxicidade induzida, também foi obtido diminuição dos níveis de EROs, melhoria da viabilidade celular, aumento dos níveis de glutationa e reestabelecimento da atividade mitocondrial, com o tratamento usando 0,5 e 1 µM de β-cariofileno (ASSIS et al., 2014).

Quanto ao acetato de eugenila também foi relatado efeito antioxidante na concentração de 160 µg/mL em ensaio bioquímico de quantificação da peroxidação lipídica (LEE et al., 2001). Adicionalmente, Vanin et al. (2014) observaram uma capacidade sequestradora de EROs com a concentração de 500 µg/mL de acetato de eugenila. Contudo, por não apresentar

um grupo fenólico, sua atividade antioxidante é possivelmente devido ao grupo éster (LEE et al., 2001).

Portanto, todos os constituintes majoritários do OESA apresentam propriedade antioxidante e podem atuar de forma sinérgica para o efeito total do óleo a fim de evitar e minimizar os efeitos negativos causados pelo estresse oxidativo.

3. JUSTIFICATIVA

A produção *in vitro* de embriões bovinos proporciona uma série de vantagens aos programas de produção animal, aumentando a produtividade e a qualidade de rebanhos. Desde sempre, a espécie bovina tem se destacado em virtude de sua grande importância científica e econômica, sendo o Brasil o detentor de 22,64% de bovinos do mundo, perdendo apenas para a Índia, segundo os dados do Departamento de Agricultura dos Estados Unidos (USDA, 2017). Além disso, o Brasil é, atualmente, o maior produtor de embriões bovinos usando a técnica de fecundação *in vitro* em oócitos imaturos aspirados *in vivo* (VIANA et al., 2017).

Apesar disso, algumas limitações podem diminuir o sucesso da produção de embriões, como o estresse oxidativo causado por fatores inerentes das condições de cultivo *in vitro*. Nesse sentido, visando minimizar os danos gerados pelas EROs, meios suplementados com substâncias antioxidantes têm sido empregados, visando manter ou aumentar a qualidade de gametas e embriões. Embora agentes antioxidantes sintéticos possam ser empregados, como a cisteamina usada no meio de maturação oocitária, resultados promissores com agentes naturais isolados ou não de plantas têm sido observados.

Nesse contexto, na visão de uso de antioxidantes naturais de plantas de fácil obtenção, tem-se a *S. aromaticum*, a qual apresenta grande potencial antioxidante para aplicação em sistemas de cultivo de gametas, em virtude dos compostos fenólicos presentes em sua composição, bem como sua eficiência demonstrada em estudos bioquímicos e celulares de diferentes espécies.

Portanto, estabelecer uma suplementação antioxidante natural como o óleo essencial de *S. aromaticum* para oócitos e espermatozoides poderia reduzir o estresse oxidativo, aumentando o quantitativo de embriões bovinos produzidos *in vitro*.

4. HIPÓTESES CIENTÍFICAS

I – O OESA, nas concentrações de 10, 15 ou 20 µg/mL, usado durante a maturação *in vitro* de oócitos bovinos influencia positivamente a maturação oocitária e o desenvolvimento embrionário partenogenético;

II – O OESA, nas concentrações de 10, 15 ou 20 µg/mL, usado durante a incubação *in vitro* de espermatozoides epididimários bovinos por 6 h influencia positivamente nos parâmetros de qualidade espermática e redução nos níveis de EROs.

5. OBJETIVOS

5.1. OBJETIVO GERAL

Avaliar a atividade antioxidante do OESA sobre a maturação *in vitro* de oócitos e incubação *in vitro* de espermatozoides epididimários bovinos.

5.2. OBJETIVOS ESPECÍFICOS

- Avaliar a adição do OESA (10, 15 e 20 µg/mL) durante a MIV sobre a viabilidade das células do *cumulus*, maturação nuclear e maturação citoplasmática de oócitos bovinos, em comparação ao meio sem antioxidante ou com antioxidante sintético (cisteamina);

- Comparar o níveis de EROs e potencial de membrana mitocondrial ($\Delta\Psi m$) de oócitos bovinos maturados na ausência ou presença de diferentes concentrações de OESA (10, 15 e 20 µg/mL) ou cisteamina;

- Analisar o efeito do OESA (10, 15 e 20 µg/mL) em relação ao meio sem antioxidante ou com cisteamina durante a MIV de oócitos bovinos sobre o desenvolvimento embrionário partenogenético;

- Avaliar a adição de OESA (10, 15 e 20 µg/mL) durante a incubação por 1 e 6 h de espermatozoides epididimários bovinos e sua influência sobre os parâmetros de qualidade espermática, em comparação ao controle fresco ou sem antioxidante;

- Verificar o efeito do OESA (10, 15 e 20 µg/mL) sobre a produção de EROs em espermatozoides epididimários bovinos durante a incubação por 1 e 6 h, comparando ao controle fresco ou sem antioxidante.

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CAPÍTULO 2 – USO DE ANTIOXIDANTES NATURAIS NA PRODUÇÃO *IN VITRO* DE EMBRIÕES DE MAMÍFEROS

Artigo de revisão: Use of natural antioxidants in *in vitro* mammalian embryo production

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Use of natural antioxidants in *in vitro* mammalian embryo production

Uso de antioxidantes naturais na produção *in vitro* de embriões de mamíferos

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Abstract

In vitro embryo production (IVEP) contributes to the quantitative and qualitative aspects of animal reproduction. Nevertheless, inherent technical factors such as oxidative stress can negatively influence the result and this can impair cell metabolism, thus decreasing the rates of in vitro development, and necessitating the supplementation of culture medium with antioxidants. In this context, compounds of natural origin with this property have been highlighted because of the positive results obtained at different stages of IVEP. Thus, this review aims to present the results obtained by using natural antioxidants to minimize the effects of oxidative stress on gametes and embryos. A variety of natural isolated substances and mixtures (essential oils and extracts) have been studied for supplementation of IVEP media, at stages of in vitro maturation, sperm capacitation, in vitro fertilization, and in vitro development of embryos in different mammalian species. Generally, beneficial effects are observed according to the concentration used, thus demonstrating the potential of several natural antioxidants. Therefore, the main challenges in using these compounds as antioxidants during IVEP include proving their efficiency against free radicals and determining the best concentration at each stage. In addition, understanding the mechanisms of action of such antioxidants is crucial to establishing their use in IVEP biotechnology.

Key words: Culture medium. Natural bioactive. Oxidative stress. Reproduction.

Resumo

A produção *in vitro* de embriões (PIVE) contribui para os aspectos quantitativos e qualitativos da reprodução animal. Contudo, fatores inerentes da técnica, como o estresse oxidativo, podem influenciar negativamente o resultado e isso pode prejudicar o metabolismo celular, diminuindo assim as taxas de desenvolvimento in vitro, e exigindo a suplementação do meio de cultivo com antioxidantes. Neste contexto, os compostos de origem natural com essa propriedade têm se destacado devido aos resultados positivos obtidos em diferentes estágios da PIVE. Assim, esta revisão pretende apresentar os resultados obtidos usando antioxidantes naturais para minimizar os efeitos do estresse oxidativo em gametas e embriões. Uma variedade de substâncias isoladas e de misturas naturais (óleos essenciais e extratos) tem sido estudada para a suplementação de meios de PIVE, nas etapas de maturação *in vitro*, capacitação espermática, fecundação *in vitro* e desenvolvimento *in vitro* de embriões em diferentes espécies de mamíferos. Geralmente, os efeitos benéficos são observados de acordo com a concentração utilizada, demostrando assim o potencial positivo de vários antioxidantes naturais. Portanto, os principais desafios para o uso desses compostos como antioxidantes durante a PIVE incluem provar sua eficiência contra os radicais livres e determinar a melhor concentração em cada etapa. Além disso, a compreensão dos mecanismos de ação de tais antioxidantes é crucial para estabelecer o uso na biotecnologia de PIVE.

Palavras-chave: Bioativo natural. Estresse oxidativo. Meio de cultivo. Reprodução.

Introduction

In vitro embryo production (IVEP) facilitates production of viable embryos from *in vitro* procedures that mimic the natural physiological conditions of the female reproductive system. Typically, IVEP can be divided into a few main steps, starting with the recovery of immature cumulus-oocyte complexes*, in vitro* maturation (IVM), sperm capacitation, *in vitro* fertilization (IVF), *in vitro* development (IVD) of possible embryos to the blastocyst stage, and their transfer to synchronized recipients to generate offspring (PARAMIO; IZQUIERDO, 2016). In

general, at each stage of IVEP, media with different compositions and supplements are used to stimulate the different reactions required at each developmental phase.

Since the first successful studies performed in small mammals [rabbits (CHANG, 1959), mice (WHITTINGHAM, 1968), and rats (TOYODA; CHANG, 1974)], IVEP has been applied to several species of this class, mainly domestic and production mammals, with different propositions (NAGASHIMA et al., 2015; BULGARELLI et al., 2017). In this sense, over the years, IVEP has promoted numerous benefits to the productive, scientific, and technological sectors (PEREIRA et al., 2012a). Thus, we can cite genetic improvement (NIVET et al., 2012), optimization of the multiplication of animals of commercial interest, increase in offspring/female/year, use of prepubertal (MORIN-DORÉ et al., 2017), pregnant, and lactating females (TAKUMA et al., 2010), use of animals with infertility problems of noncongenital origin, obtaining knowledge about reproductive (SOUZA-FABJAN et al., 2013) and embryonic physiology (CARROCERA et al.lk, 2016), and support in conservation (AVELAR et al., 2012; ARAV, 2014), cloning (PEREIRA et al., 2014), and transgenesis (PEREIRA et al., 2013; CAMPELO et al., 2016) techniques.

Despite many applications, IVEP still presents some limitations with respect to *in vitro* culture media, which exert a great influence on system maintenance and the result, i.e., the number of viable embryos (PARAMIO; IZQUIERDO, 2016). These media must provide all the nutrients and factors necessary for the development of gametes and embryos, as well as maintain the balance of molecules that can harm the system (CROCOMO et al., 2012; SANTOS et al., 2017). In this regard, one of the main factors that can influence the success of IVEP is oxidative stress caused by the accumulation of reactive oxygen species (ROS) that impair the integrity of cells and their macromolecules thus interfering with *in vitro* development (ROCHAFRIGONI et al., 2015).

To avoid or minimize oxidative stress, antioxidants have been used as supplements in IVEP media, including enzymes, amino acids, vitamins, and compounds of natural origin such as polyphenols, carotenoids, extracts, and essential oils having this property (TAKAHASHI, 2012). These antioxidants are easily found in nature and have been studied *in vitro* mainly because of their efficiency against ROS. In addition, during IVEP these natural compounds showed promising results in protecting gametes and embryos from oxidative stress and

improving development (MALEKI et al., 2014). Thus, this review aimed to discuss the results obtained with the application of natural antioxidant compounds to minimize the effects of oxidative stress on IVEP in domestic mammals.

Oxidative stress

Some factors inherent to IVEP such as oxygen tension, interference of light and heat, presence of sperm, constituents of the medium, and absence of maternal antioxidant protection, are directly related to oxidative stress (CROCOMO et al., 2012). This stress results from an imbalance between ROS and antioxidant substances, with a predominance of the former (ROCHA-FRIGONI et al., 2015).

ROS are represented by a variety of molecules and free radicals produced from oxygen metabolism in biological systems. During cellular respiration, the oxygen molecule (O_2) must receive four electrons to be completely reduced to two H_2O molecules. Nevertheless, if O_2 is partially reduced by the receipt of only one electron, the product of this reduction will be the superoxide radical (O_2•−) (CROCOMO et al., 2012; TAKAHASHI, 2012). From this radical, other biochemical reactions can lead to the formation of hydrogen peroxide (H_2O_2) and the hydroxyl radical (•OH) (TAKAHASHI, 2012). All these oxygen-derived metabolites exhibit high instability and reactivity due to at least one unpaired electron in their outer orbit (ROCHAFRIGONI et al., 2015).

In an equilibrium situation, ROS have beneficial effects acting as signaling molecules in physiological processes such as tissue regeneration, hormonal signaling, intracellular redox regulation, and embryogenesis (ZHONG; ZHOU, 2013). However, under conditions of imbalance, ROS can oxidize any cellular molecule such as lipids, carbohydrates, amino acids, and nucleic acids, thus modifying their functions and compromising cell survival (AGARWAL; SALEH, 2002). Therefore, oxidative stress can cause negative effects on viability, gene expression, protein synthesis, development, and molecular signaling of gametes and embryos, aspects that are determinant for the success of IVEP (ZULLO et al., 2016a).

Normally, living organisms possess a wide variety of antioxidants, especially of enzymatic and food origin, that eliminate or suppress ROS formation and activity (BANSAL et al., 2011).

Nevertheless, under *in vitro* stress conditions, endogenous antioxidants may not be sufficient to neutralize the excess free radicals. Therefore, during IVEP, is often necessary to supplement the culture medium with exogenous antioxidants that allow adequate maintenance of cellular activity (LYKKESFELDT; SVENDSEN, 2007).

Use of antioxidants in IVEP media

Antioxidants are molecules capable of donating electrons to oxidants, which eventually lose their reactivity and become harmless to cellular macromolecules (LYKKESFELDT; SVENDSEN, 2007). These compounds can be classified as enzymatic (i.e., catalase, glutathione peroxidase, and superoxide dismutase) and non-enzymatic (i.e., glutathione, cysteamine and resveratrol). Moreover, antioxidants may be naturally produced inside cells as a defense mechanism or be of exogenous origin from supplementation (BANSAL et al., 2011).

Previous studies have demonstrated that balanced presence of antioxidants and ROS in IVEP media can be beneficial for embryonic development (ALI et al., 2003; ZULLO et al., 2016a). Currently, the most used antioxidant in IVEP protocols is cysteamine, whose efficiency is mainly related to the stage of IVM. This molecule has been shown to act by stimulating embryonic development and the synthesis of glutathione (GSH), a compound naturally present in both gametes with an important role in protecting against the effects of ROS (GOTTARDI et al., 2012). Cysteine and glutathione have also been used in IVEP protocols with good results (ALI et al., 2003; SUN et al., 2015). Recently, the quercetin (2 μM), resveratrol (2 μM), vitamin C (50 μg/mL), carnitine (0.5 mg/mL), and cysteamine (100 μM) were evaluated to identify the most efficient antioxidant against the harmful effects of ROS during IVM of bovine oocytes (SOVERNIGO et al., 2017). In this study, the maturation rate with antioxidants was similar to that of the control; the blastocyst rate of all antioxidants was superior to the control; however, in the ROS and GSH evaluation, only some groups showed positive results compared to the control, and therefore, it was not possible to determine the most efficient antioxidant. Finally, in IVF, hypotaurine is widely used for its action mimicking the physiological antioxidant conditions of seminal plasma (AGARWAL; SALEH, 2002).

Despite previously observed results, much research has been conducted to establish the use of more efficient antioxidants for all stages of IVEP (GOTTARDI et al., 2012; MALEKI et al.,

2016). In this context, the use of natural *in vitro* antioxidants in culture media has received special attention, mainly due to their efficiency and low cost (WANG et al., 2013; SALZANO et al., 2014). These bioactives have been studied in the IVEP of several species (Table 1); however, positive results are only achieved with the use of appropriate concentrations. Therefore, the main challenge in the cellular antioxidant field is to establish optimal concentrations to minimize oxidative effects without impairing development. This occurs because very low concentrations of all types of antioxidants used in IVEP, natural or not, may have in significant effects whereas high concentrations may have negative effects (ALI et al., 2003; MALEKI et al., 2014; ZULLO et al., 2016a).

Methods for evaluation of antioxidants in IVEP

In general, to determinate antioxidant efficiency in gametes and embryos, specific and non-specific methods are available. As non-specific methods, morphological and developmental parameters can be evaluated. During IVEP, the maturation rate, sperm parameters, and rates of embryonic development, i.e., the number of embryos that reach the stages of cleavage, blastocyst, and hatched blastocyst, is often calculated (MALEKI et al., 2014). These numbers indicate the efficiency of culture media and the quality of oocytes, spermatozoa, and embryos (SALZANO et al., 2014).

The total number of blastocyst cells is also used as an important parameter in the evaluation of embryonic quality, since it directly influences fetal development (SALZANO et al., 2014). Additionally, this method allows differential counting of the internal cell mass and trophectoderm cells, which must be in an appropriate proportion of 20-40% (KOO et al., 2002). For this, fluorescent labels such as propidium iodide and Hoechst are usually used (SALZANO et al., 2014). Evaluation of embryonic cryotolerance has also been used as a parameter since competent embryos are more tolerant to cryopreservation procedures (CARROCERA et al., 2016). Thus, the parameters analyzed after thawing include survival and hatching rates after 24 h and 48 h of *in vitro* culture, respectively (AMBROGI et al., 2017).

Tabela 1. Natural antioxidants used in different steps of IVEP in mammals

Natural antioxidants	IVEP step	Species	Best concentration	BL (%)	Authors
Isolated substances/compounds					
Anthocyanin	IVD	Bovine	0.1 µg/Ml	28.7	Sakatani et al. (2007)
	IVM/IVD	Swine	0.1 µg/mL	51.0	You et al. (2010)
Crocetin	IVD	Bovine	1.0 µM	46.1	Zullo et al. (2016b)
Crocin	IVM	Murine	10.0 µg/mL	43.0	Maleki et al. (2014, 2016)
	Capacitation IVF	Bovine	1.0 mM	54.2	Sapanidou et al. (2015)
Green tea polyphenols	IVM	Bovine	15.0 µM	38.1	Wang et al. (2007)
	IVD		15.0 µM	N	Wang et al. (2013)
L-ergothioneine	IVM	Sheep	10.0 mM	N	Öztürkler et al. (2010)
	IVD	Bovine	0.1 mM	41,5	Zullo et al. (2016a)
Quercetin	IVM	Bovine	2.0 µM	59.5	Guemra et al. (2013)
		Swine	1.0 µg/mL	15.8	Kang et al. (2013)
	Capacitation	Human	30.0 µM	N	Moretti et al. (2012)
Resveratrol	IVM	Bovine	1 µM	30.0	Wang et al. (2014)
	IVD		0.5 µM	59.2	Salzano et al. (2014)
	IVM	Swine	2 µM	62.1	Kwak et al. (2012)
	Capacitation	Bovine	5-50 µM	N	Tvrdá et al. (2015)
		Murine	15 µg/mL	N	Mojica-Villegas et al. (2014)
		Human	0.1 mM	N	Garcez et al. (2010)
Rutin	Capacitation	Human	30.0 µM	N	Moretti et al. (2012)
Naringenin			X	N	
Epicatechin			X	N	
Verbascoside	IVM	Sheep	1.03 µM*	31.2	Dell'aquila et al. (2014)
			1 nM	37.8	Martino et al. (2016)
2-Methoxystypandrone	IVM	Bovine	1 µM	45.3	Mesalam et al. (2017)
Essential oils and extracts					
Aqueous extract of *Crocus sativus* L.	IVM	Murine	5.0 µg/mL	29.8	Tavana et al. (2012)
			40.0 mg/mL	38.0	Maleki et al. (2014)
Ethanolic extract of *Papaver rhoeas* L.	IVM	Murine	100 µg/mL	34.8	Golkar-Narenji et al. (2010)
		Sheep	50 µg/mL	N	Rajabi-Toustani et al. (2013)
Essential oil of *Lippia origanoides*	IVM	Bovine Buffaloes	2.0,5.0-10.0 µg/mL*	24.0-35.0	Sollecito et al. (2016)
Essential oil of *Thymus munbyanus*	Capacitation	Human	X	N	Chikhoune et al. (2015)
Royal jelly	IVM	Sheep	10.0 mg/mL	39.6	Eshtiyaghi et al. (2016)

BL: blastocyst rate. N: not evaluated. X: concentrations evaluated have no positive effect for IVEP. *There was no statistical difference compared to the control without antioxidant.

For more specific methods, quantification of apoptotic cells using the terminal deoxynucleotidyl transferase dUTP Nick end labeling assay (TUNEL assay) is used widely because ROS can cause DNA damage (NGUYEN et al., 2017). Another employed technique is the analysis of gene expression, especially of enzymes involved in the cellular antioxidant system such as catalase, superoxide dismutase, and glutathione peroxidase, and genes related to apoptotic pathways (ESHTIYAGHI et al., 2016; MESALAM et al., 2017).

Moreover, quantification of ROS levels using a fluorescent marker (2′, 7′-dichlorodihydrofluorescein diacetate) allows specific evaluation of the antioxidant ability to reduce intracellular ROS (AMBROGI et al., 2017). In addition, it is also possible to check the levels of glutathione, an antioxidant produced by the cell. Some antioxidant compounds also act by stimulating the production of intracellular glutathione to act against ROS and thus reduce oxidative stress (SOVERNIGO et al., 2017). Therefore, this analysis is performed using the fluorescent label 4-chloromethyl-6, 8-difluoro-7-hydroxycoumarin (KWAK et al., 2012). Another in use is the measurement of mitochondrial membrane potential in gametes and embryos, as increased activity of this organelle can increase the ROS production (MOJICA-VILLEGAS et al., 2014; AMBROGI et al., 2017).

Therefore, non-specific parameters are very important to predict the efficiency of the culture system as well as the quality and competence of the gametes and embryos used for the experiments. However, these parameters are used in association with specific methods to obtain more consistent and accurate data, which allow a more direct evaluation of antioxidant activity and contribute to understanding the possible mechanisms of action of the tested compounds.

Natural antioxidants

There are several methods for obtaining essential oils, extracts, and isolation of specific bioactives. The most traditional and commonly used methods for obtaining essential oils are hydrodistillation, steam distillation, solvent extraction, enfleurage, cohobation, and maceration (HESHAM et al., 2016). For extracts, the most common techniques are maceration, decoction, infusion, and percolation, with variations in the solvents used (AZWANIDA, 2015). One limitation for the use of such compounds is their chemical composition which may vary according to the extraction method and solvent, as well as the place of origin of the plant, the

time of the year, and time of the day. This fact may hinder the reproducibility of results. In contrast, isolated substances allow greater control over the bioactive that will be tested. For this, chromatographic techniques, especially high performance liquid chromatography, and immunoassays have been used (SASIDHARAN et al., 2011).

Isolated substances/compounds

Among isolated compounds with their antioxidant action evaluated in IVEP, resveratrol is certainly one of the most studied (SALZANO et al., 2014). This polyphenol (non-flavonoid) is naturally present in several plant species with a protective function and when isolated, shows several therapeutic properties, such as anticancer (JANG et al., 1997), anti-diabetic (PALSAMY; SUBRAMANIAN, 2008), and cardioprotective properties (SZMITKO; VERMA, 2005) including antioxidant activity (BHAT et al., 2001). Therefore, application of resveratrol in IVEP showed good results in relation to protection against oxidative stress, decreasing ROS levels, and increasing GSH levels (KWAK et al., 2012; WANG et al., 2014). Wang et al. (2014) observed higher maturation rate (93.4%), increased quality (cells/blastocyst: 118.7) and rates of bovine embryo development (30.0%) after addition of 1.0 μM of resveratrol during IVM, compared to 0, 0.1, and 10.0 μM. At the same IVEP step with porcine oocytes, supplementation of the medium with 2.0 μM of resveratrol improved oocyte quality (88.3%), monospermal fertilization (54.1%) and embryo development (62.1%) (KWAK et al., 2012). In sperm capacitation, resveratrol was beneficial for stimulation of sperm activity, the protection against lipid peroxidation, and the maintenance of fertility [cattle (TVRDÁ et al., 2015), murine (MOJICAVILLEGAS et al., 2014), human (GARCEZ et al., 2010)]. In addition, when used in IVD medium, this antioxidant at a concentration of 0.5 μM, could improve the cryotolerance of vitrified embryos with an increased rate of hatching (58.9%) after warming (SALZANO et al., 2014).

Another polyphenol that has been studied in IVEP is quercetin, which can be found at high concentrations in black tea, red wine, fruits, and vegetables (GUEMRA et al., 2013). This flavonoid exerts various pharmacological effects such as anti-inflammatory and antioxidant activities (DAVIS et al., 2009). Due to its antioxidant and metal chelating potential, supplementation with quercetin (2.0 μM) in IVM of bovine oocytes induced improvement in the blastocyst rate (59.5%), which was higher than that obtained with cysteamine (50.4%) and

in the control without the antioxidant (42.3%) (GUEMRA et al., 2013). In porcine oocytes during IVM, treatment with 1.0 µg/mL of quercetin reduced the intracellular ROS levels and significantly improved blastocyst development (15.8%) (KANG et al., 2013). Moretti et al. (2012) evaluated quercetin, rutin, naringenin and epicatechin, all flavonoids found naturally in plants, of which only quercetin (30.0 µM) and rutin (30.0 µM) conferred protection against lipid peroxidation.

Recently, crocin and crocetin, which are carotenoids responsible for the antitumor and antioxidant activity of saffron extract (*Crocus sativus* L.), were evaluated in IVEP. Maleki et al. (2014) evaluated high concentrations [0, 50.0, 100.0, 400.0 µg/mL] and Maleki et al. (2016) used low concentrations [0, 5.0, 10.0 µg/mL] of crocin in the maturation medium of murine oocytes and obtained better rates of maturation (75.0%), cleavage (47.0%), blastocysts (43.0%), and increased GSH concentration when they used 10.0 µg/mL of this antioxidant. Moreover, when crocin was added to the medium of bovine sperm capacitation, satisfactory results obtained both in sperm quality parameters (motility, intact acrosome, acrosome reaction, and DNA fragmentation) as well as in IVF and IVD using these gametes (SAPANIDOU et al., 2015). In this study, the blastocyst rate (54.2%) was significantly higher with the addition of 1.0 mM of crocin in sperm capacitation compared to the control without antioxidant (37.5%) (SAPANIDOU et al., 2015). Crocetin was used in the IVD medium, where a concentration of 1.0 µM could significantly improve the blastocyst rate (46.1%) and embryo quality by decreasing the percentage of apoptotic cells and increasing cryotolerance (ZULLO et al., 2016b).

L-ergothioneine is a thiol compound synthesized by fungi and bacteria that can be easily found in some types of mushrooms, and has chemical properties and physiological functions suitable for an *in vitro* cytoprotectant (CHEAH; HALLIWELL, 2012). This compound when isolated shows antioxidant activity, which was evidenced in IVEP studies in sheep, and was used as a supplement in IVM and IVD medium (10.0 mM) resulting in increased maturation (80.4%), cleavage (29.9%), and morula (6.5%) rates (ÖZTÜRKLER et al., 2010). In addition, in the IVD medium of bovine embryos, L-ergothioneine (0.1 mM) improved the quality and cryotolerance and decreased the apoptosis rate in blastocysts (ZULLO et al., 2016a).

Another interesting compound of sustainable origin studied at IVEP is verbascoside (polyphenol), which is present in olive oil, has anti-inflammatory activity and was obtained from wastewater from the production of this oil. However, despite good results in relation to the reduction of ROS levels in cell culture (CARDINALI et al., 2012), when verbascoside was used in IVM by Dell'aquila et al. (2014), no improvement was observed in any of the development parameters evaluated. In this study, the 1.03 µM concentration was similar to the control without antioxidant on the blastocyst rate (31.2 vs. 24%), but larger amounts (2.06 and 4.11 µM) of verbascoside showed a negative effect (11.5 and 11.9%). Furthermore, for unknown reasons, a significant increase was observed at the ROS levels (DELL'AQUILA et al., 2014). Nevertheless, Martino et al. (2016) noted that 1 nM of verbascoside had a beneficial effect on embryo development to the blastocyst stage (37.8 vs. 27.3%) and blastocyst quality by acting as an antioxidant molecule, as it reduced oocyte ROS and maintained mitochondrial activity at basal levels.

Recently, 2-methoxystypandrone, a naphthoquinone purified from *Polygonum cuspidatum* (known as "Huzhang"), which is used in food, cosmetics, and pharmaceuticals, was evaluated for the first time during IVM of bovine oocytes at concentrations of 0.1, 0.5, 1, and 1.5 µM. It was observed that 1.0 µM of this substance increases the blastocyst rate and the number of total cells, decreases apoptosis and ROS levels, and influences the expression of genes important for embryonic development (MESALAM et al., 2017).

Another substance called anthocyanin, which also has various biological activities, was extracted from *Ipomoea batatas* (purple sweet potato) and evaluated (0.1, 1.0, 10.0 µg/mL) during IVD in cattle (SAKATANI et al., 2007). It was observed that low concentration (0.1 µg/mL) of anthocyanin has a similar effect to control under normal conditions, but under conditions of heat shock, anthocyanin protects embryos by improving development, reducing oxidative stress, and increasing the intracellular GSH levels. You et al. (2010) used anthocyanin at 0.1 µg/mL during IVM and/or IVD for parthenogenetic activation (PA) and cloning by somatic cell nuclear transfer (SCNT) in swine. In this study, oocytes treated with anthocyanin during IVM showed higher rates of blastocyst formation after PA (55.7 vs. 44.9%) and SCNT (32.2 vs. 16.1%) compared to untreated oocytes. Moreover, in PA and SCNT embryos, anthocyanin treatment during IVM or IVD significantly increased the intracellular GSH level resulting in reduced ROS levels (YOU et al., 2010).

Green tea polyphenols have been shown to be useful anti-diabetic, antitumor and antioxidant agents and were also evaluated in IVEP of cattle as natural antioxidants. Initially, Wang et al. (2007) evaluated these compounds during IVM and obtained better rates of maturation (86.1%), fertilization (79.9%), and embryonic development (38.1%) at a concentration of 15.0 µM, correlating this effect with the higher level of intracellular GSH in treated oocytes. Afterwards, Wang et al. (2013) also evaluated the molecular parameters of bovine embryos and transfer to synchronized recipients after IVM and IVD with green tea polyphenols. In this study, the expression of antioxidant enzyme genes (superoxide dismutase, catalase, and glutathione peroxidase) was increased and the apoptosis rate was decreased; the pregnancy rate after 30 and 60 days was also higher with the addition of 15.0 µM of the antioxidant in relation to the control without antioxidant.

Considering these results, these substances show remarkable potential for use with gametes and embryos at all steps of IVEP. Since there is a growing interest in increasing the rates of embryonic development for all species, it is important to know that there are natural compounds that can contribute to these improvements with high efficiency. Many of these antioxidant substances have been discovered through studies of mixtures such as extracts and essential oils from plants in cell culture and pharmacological studies (CARDINALI et al., 2012; MALEKI et al., 2014). Thus, the effect of these mixtures has also been studied in IVEP biotechnology.

Essential oils and extracts

In general, extracts and essential oils are rich in phenolic compounds, carotenoids, terpenes, and other organic substances that have varied therapeutic properties (PEREIRA et al., 2012b) such as antioxidant activity (ESHTIYAGHI et al., 2016). Some of these compounds have also been studied in IVEP as antioxidants though they were not purified and of variable composition. The aqueous extract of *Crocus sativus* L. or saffron was evaluated during the IVM of murine oocytes and presented a beneficial effect increasing the rates of oocyte and embryo development (TAVANA et al., 2012; MALEKI et al., 2014). In studies evaluating this extract, concentrations ranging from 5-40 µg/ mL were tested; Tavana et al. (2012) obtained better blastocyst rate (29.8%) with a concentration of 5.0 µg/mL whereas Maleki et al. (2014)

obtained better rates of maturation (76.0%), cleavage (78.0%), and blastocyst (38.0%) with 40.0 µg/mL of the extract.

The ethanolic extract of *Papaver rhoeas* L. was evaluated during the IVM of murine oocytes at low concentrations (0.0, 10.0, 15.0, 20.0, and 25.0 µg/ mL) and high concentrations (0.0, 50.0, 100.0, and 200 µg/mL). Among the concentrations used, 100.0 µg/mL promoted an increase in the maturation rate (70.7 vs. 56.3%) and developmental competence of embryos (34.8 vs. 18.8%) compared to those in control group. In addition, the *P. rhoeas* extract at concentration of 50.0 µg/mL also showed a positive effect on the maturation rate of sheep oocytes compared to the control group (70.1 vs. 54.7%) (RAJABI-TOUSTANI et al., 2013).

Recently, the essential oil of *Lippia origanoides* (wild oregano), which is rich in carvacrol and thymol, was evaluated in the IVM medium of bovine and buffalo oocytes. However, at the concentrations tested (2.5, 5.0, and 10.0 µg/mL), no differences were observed in the rates of embryonic development (26-35%) (SOLLECITO et al., 2016). In addition, there was no difference in the H2O2 concentrations in the media of the evaluated treatments. The essential oil of *Thymus munbyanus* (thyme) was evaluated in the capacitation of human sperm but no improvement in viability and motility was observed (CHIKHOUNE et al., 2015).

Finally, royal jelly, a highly nutritious blend with antioxidant properties, was also evaluated (2.5, 5.0, and 10.0 mg/mL) during the IVM of ovine oocytes. In this study, 10.0 mg/mL of royal jelly showed better rates of development of oocytes (85.6%) and embryos (39.6%), increased expression of antioxidant enzyme genes (superoxide dismutase, catalase and glutathione peroxidase) in cumulus cells and increased intracellular GSH levels (ESHTIYAGHI et al., 2016).

Conclusion

Considering the need for antioxidant compounds to reduce oxidative stress during IVEP, studies aiming to establish this type of supplement are essential, since each step presents different requirements. In this context, natural antioxidants are a viable low cost alternative with very promising results. These compounds have been used in isolated form and as mixtures with results highly dependent on the concentrations used in the medium. Thus, the main

challenges for the use of natural compounds as antioxidants during IVEP are to prove their efficiency against ROS and to determine the best concentration at each step. In addition, in relation to essential oils and extracts, reproducibility of the results may be difficult due to variation in composition according to the season and origin of the plant. Therefore, it is expected that these and other natural antioxidants will be studied during IVEP and their mechanisms of action will be deciphered, thus contributing to greater success of this technique. Finally, such studies may also contribute to research with *in vitro* cell cultures under oxidative stress and to studies in the field of pharmacology.

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CAPÍTULO 3 – SUPLEMENTAÇÃO COM ÓLEO ESSENCIAL DE *Syzygium aromaticum* DURANTE A MATURAÇÃO *IN VITRO* DE OÓCITOS BOVINOS MELHORA O DESENVOLVIMENTO DE EMBRIÕES PARTENOGENÉTICOS

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Syzygium aromaticum essential oil supplementation during *in vitro* bovine oocyte maturation improves parthenogenetic embryonic development

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Abstract

The use of natural antioxidants in culture media can be an alternative to minimize the negative effects of oxidative stress produced by culture conditions. Essential oil from *Syzygium aromaticum* (EOSA) has therapeutic properties, including antioxidant activity in different cell types, and could be an interesting antioxidant agent during *in vitro* maturation (IVM) of bovine oocytes. Therefore, we sought to evaluate the antioxidant effect of the EOSA on bovine IVM, levels of reactive oxygen species (ROS), mitochondrial membrane potential ($\Delta\Psi m$), and subsequent preimplantation embryonic development. Then, viable oocytes were matured *in vitro* under five sets of conditions: EOSA0 (without antioxidants), EOSA10 (10 µg/mL of EOSA), EOSA15 (15 µg/mL of

EOSA), EOSA20 (20 μg/mL of EOSA), and CYS (100 μM of cysteamine). These oocytes were used in three experiments. In the first experiment, oocytes were evaluated for IVM according to the expansion and viability of *cumulus* cells, the presence of the first polar body, and metaphase II. In the second experiment, denuded oocytes were evaluated for an antioxidant effect by labeling them with H_2DCFDA (ROS levels) and MitoTracker Red (ΔΨm). In the third experiment, denuded matured oocytes were artificially activated and embryos were cultured for eight days. In the first experiment, no difference was observed in the IVM rates ($P > 0.05$). Nevertheless, EOSA15, EOSA20, and CYS improved the viability of *cumulus* cells after MIV, with EOSA20 viability higher than that of EOSA0 ($P < 0.05$). In the second experiment, although no difference has been observed for ROS levels ($P > 0.05$), oocytes derived from the EOSA15, EOSA20, and CYS groups showed significantly lower ΔΨm compared to the EOSA0 group. In the third experiment, although no difference in cleavage rates was observed, EOSA20 improved the blastocyst/total oocyte and blastocyst/cleavage oocyte rates when compared to EOSA0 ($P < 0.05$). Moreover, the rates of the EOSA20 group were similar to that of the CYS group ($P > 0.05$). Additionally, embryos derived from EOSA15 and EOSA20 showed a higher number of cells when compared to those derived from EOSA0 ($P < 0.05$). Therefore, EOSA, at 20 μg/mL, increased the viability of *cumulus* cells, promoted a reduction of in ΔΨm, and improved embryonic development in bovine oocytes. In conclusion, EOSA, added to the IVM medium, could be an interesting alternative for the reduction of damage caused by the oxidative stress in bovine oocytes.

Keywords: Cattle; *In vitro* embryo production; Oxidative stress; Natural antioxidant; Clove bud.

1. Introduction

In vitro maturation (IVM) of bovine oocytes is a determining step for the success of assisted reproductive techniques such as *in vitro* fertilization (IVF) [1], intracytoplasmic sperm injection (ICSI) [2], and somatic cell nuclear transfer (SCNT) [3] cloning. All of these reproductive techniques require successful IVM, which occur with the acquisition of meiotic and cytoplasmic oocyte competence necessary for embryonic development

[4]. Nevertheless, oocytes matured *in vivo* have greater competence and quality compared to oocytes matured *in vitro* [5], as the culture systems decrease the quality of these gametes, and, subsequently, reduce the rate of early embryonic development [6].

Normally, the oxygen tension used during IVM (~20% O_2), which is greater than that found in the female reproductive system (3–9% O_2) [7], causes an increase in reactive oxygen species (ROS) production that exceeds the cellular antioxidant defense capacity [8]. ROS excess can oxidize cellular molecules, such as lipids, carbohydrates, amino acids, and nucleic acids, modifying their functions and compromising cell viability [9]. Thus, due to *in vitro* culture conditions and the reduction of maternal antioxidant protection after COC removal from the ovarian follicle [10], it is necessary medium with antioxidant substances to prevent the formation of ROS, avoid oxidative stress [11], and enhance oocyte competence for embryonic development [12].

Although cysteamine has been widely used as a synthetic antioxidant for IVM [13] with blastocyst rates above 30% [14, 11], there have been promising results with compounds of a natural origin, both purified and un-purified [15]. During IVM of bovine oocytes, isolated substances, such as resveratrol [16] and 2-Methoxystypandrone [12], improved embryonic development with blastocyst rates higher than 30%. Moreover, *Phellodendron amurense* (0.01 µg/mL) and *Humulus japonicus* (0.01 µg/mL) extracts used during the bovine embryonic development increased the blastocyst rate (34–35%) and quality related to reduced ROS and apoptosis levels [17]. Therefore, investigating new antioxidant agents may optimize embryo production in cattle.

The plant *Syzygium aromaticum*, known as clove, has antioxidant activity associated with the presence of phenolic compounds [18]. In reproductive studies, hexane extract from cloves (15 mg/kg body weight/day) increased murine sperm motility and secretory activity of the epididymis and seminal vesicles after *in vivo* treatment [19]. Furthermore, Baghshahi et al. [20] evaluated clove extracts (35 and 75 µg/mL) in sheep semen diluent and observed a beneficial effect on motility, membrane integrity, and sperm viability. Additionally, the primary component of clove essential oil, known as eugenol,

demonstrated an ability to inhibit lipid peroxidation, decrease protein damage, prevent DNA fragmentation, and reduce generation of free radicals in murine macrophages [21].

Therefore, owing to the antioxidant properties and positive effects established for reproduction, as well as the large quantities of eugenol in essential oil from *S. aromaticum* (EOSA), this oil may be an alternative for antioxidant supplementation in IVM medium. In addition, for initial studies, the use of an essential oil allows for a neater combination of bioactive compounds without the interference and variation of solvents [22].

Thus, the aim of this study was to evaluate the antioxidant effect of EOSA on bovine IVM, ROS levels, ΔΨm, and subsequent early embryonic development after artificial oocyte activation.

2. Materials and methods

All experiments were conducted in accordance with the Animal Ethics Committee of the Federal Rural University of Semi-Arid (Opinion Nº 23091.002360/2016-17). Unless otherwise stated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.1. Extraction and chemical analysis of EOSA

S. aromaticum flower buds were collected in Salvador-BA (12°58'1311S, 38°30'45''W), located in Northeastern Brazil. EOSA was extracted by hydrodistillation of fresh buds using a Clevenger-type apparatus [23], then analyzed with a Shimadzu QP-2010 instrument under the following conditions: column: SH-Rtx-5 (5% diphenyl and 95% dimethyl polysiloxane) fused silica capillary column (30 m length x 0.25 mm diameter x 0,25 µm film diameter); carrier gas: He (1.78 mL. min^{-1}, in constant linear velocity mode); injector temperature: 250°C, in split mode (1:10); detector temperature: 250°C; column temperature programming: 40–180°C at 4°C min^{-1}, 180–280°C at 20°C min^{-1}, and then 280°C for 10 min; mass spectra: electron impact 70 eV. The injected sample volume was 1 µL (1.0 mg of oil in 1000 µL of 99.9% pure dichloromethane).

The constituents were identified by their gas chromatography (GC) retention times relative to known compounds and expressed in terms of the Kovat Index (KI), which were calculated by linear regression and comparison of their mass spectra (MS) with those published spectra [24]. Thus, the chemical composition and retention indices of EOSA constituents were: eugenol (68.38%; KI: 1359), *β*-cariophylene (19.65%; KI: 1419), and acetyl eugenol (11.97%; KI: 1522).

2.2. Recovery of cumulus-oocyte complexes (COCs) and IVM

Bovine ovaries were collected in slaughterhouses and transported to the laboratory in saline solution (NaCl, 0.9%) supplemented with 0.05 mg/mL penicillin at 35–37°C within 1 h after slaughter. In the laboratory, all visible follicles with a 2–8 mm diameter were aspirated for the oocyte recovery using a 21 G needle attached to a 5.0 mL syringe containing oocyte recovery medium (ORM), which consisted of TCM199 (Gibco-BRL, Carlsbad, CA, USA) supplemented with 0.2 mM sodium pyruvate, 10% fetal bovine serum (FBS, Gibco-BRL, Carlsbad, CA, USA), and 1% antibiotic–antimycotic solution.

Oocytes were classified with a stereomicroscope and only COCs with more than one layer of *cumulus* cells and homogeneous cytoplasm were used for IVM. Thus, COCs were divided in drops (15–30 COCs per 100 µL) covered with mineral oil and incubated 24 h at 38.5°C in a humidified atmosphere with 5% CO_2. The IVM medium consisted of ORM plus 20 µg/mL FSH/LH (Pluset®, Hertape Calier, Juatuba, MG, Brazil) and antioxidant according to experimental design. EOSA dilution was performed with 0.25% dimethylsulfoxide in IVM medium [25].

2.3. Evaluation of cumulus cells

After IVM, *cumulus* cell expansion was evaluated with a stereomicroscope and COCs presenting expanded *cumulus* cells were considered matured. For cell viability assessment, *cumulus* cells were removed by successive pipetting, and the obtained cellular suspension was stained with trypan blue (0.2%) and visualized. Viable (non-stained) and non-viable (stained in blue) cells were counted in the 4 outer quadrants of the Neubauer chamber [26].

2.4. Assessment of nuclear maturation

Initially, we evaluated the presence of the first polar body (1PB) in denuded oocytes under a stereomicroscope. Those presenting 1PB were considered matured. To evaluate the nuclear stage, denuded oocytes were fixed in 4% paraformaldehyde in PBS for 30 min. Then, cells were stained with Hoechst 33342 (10 µg/mL) for 15 min. The oocytes were transferred to glass slides and visualized with a fluorescent microscope. Finally, oocytes presenting a nucleus in metaphase II (metaphase plate, MII) and 1PB were considered matured and those in other nuclear phases, as prophase I, metaphase I, anaphase I, and telophase I, were considered immature.

2.5. Quantification of intracellular ROS levels by dichlorofluorescein assay

Intracellular ROS levels in oocytes were quantified after IVM using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Invitrogen, Carlsbad, CA, USA), according to Ambrogi et al. [8]. Denuded oocytes were washed twice in PBS and placed into dishes containing 500 µL of 5 µM H_2DCFDA. The oocytes were incubated at 38.5°C in 5% CO_2 for 30 min. Stained oocytes were washed twice in PBS, placed on glass slides, photographed with a fluorescence microscope (Olympus BX51TF, Tokyo, Japan), and the fluorescence signal intensity (pixels) was quantified. Images obtained were evaluated using the ImageJ software. The background signal intensity was subtracted from the values obtained for the treatment images. The group without antioxidant supplementation (EOSA0) was assessed as the calibrator and the measured value of each treatment micrograph was divided by the mean of the calibrator to generate relative expression levels (arbitrary fluorescence units).

2.6. Assessment of ΔΨm and cytoplasmic maturation

The ΔΨm was assessed using the fluorescent probe MitoTracker Red® (CMXRos, Invitrogen, Carlsbad, CA, USA) at 500 nM [8]. The procedure, incubation, and evaluation of images were performed as described for the quantification of ROS (item 2.5).

Additionally, labeled oocytes were evaluated for cytoplasmic maturation according to the mitochondrial distribution. Thus, oocytes were classified as peripheral distribution

(immature oocytes), dispersed distribution (mature oocytes), and mitochondria in transition (between peripheral and dispersed distributions; partially mature oocytes) [8].

2.7. Artificial activation and in vitro development (IVD) of embryos

After IVM, *cumulus* cells were removed with hyaluronidase (0.1%) and gentle pipetting for up to 2 min. Oocytes with 1PB were activated with 5 µM ionomycin (I24222; Gibco) in ORM for 4 min at 37°C. Then, oocytes were washed and transferred to drops of 2 mM 6-dimethylaminopurine (6-DMAP) in ORM for 3 h at 38.5°C and 5% CO_2. Subsequently, the structures were washed and incubated in 50 µL drops of IVD medium covered with mineral oil. Synthetic oviductal fluid (SOF) supplemented with 0.2 mM sodium pyruvate, 0.2 mM L-glutamine, 0.34 mM sodium citrate, 2.8 mM myo-inositol, 2% essential amino acid solution, 1% non-essential amino acid solution, 1% antibiotic–antimycotic solution, 5 mg/mL fraction V bovine serum albumin (BSA), and 2.5% FBS were employed as IVD medium. The day of activation was considered D0 and culture was carried out until D8. On D3, 50% of the culture medium was exchanged for fresh medium.

2.8. Counting the total number of blastocyst cells

After eight days of IVD, the blastocysts expanded, and hatching/hatched cells were labeled with Hoechst 33342 (10 µg/mL) for 15 min, washed twice in PBS, and placed on glass slides. Then, embryos were visualized individually via fluorescence microscopy and images were utilized for further counting via ImageJ software.

2.9. Experimental Design

To evaluate the influence of EOSA on IVM, ROS levels, ΔΨm, and preimplantation embryonic development, viable oocytes were randomly divided into five groups according to the supplementation of the IVM medium. These groups consisted of EOSA0 (without antioxidants), EOSA10 (10 µg/mL of EOSA), EOSA15 (15 µg/mL of EOSA), EOSA20 (20 µg/mL of EOSA), and CYS (100 µM of cysteamine). Considering that the EOSA consists of 68.38% eugenol, oil concentrations were defined as described by Mahapatra et al. [21], who evaluated the effect of different concentrations (1, 5, 10, 15, and 20 µg/mL) of eugenol on oxidative stress induced in murine macrophages *in*

vitro. In this study, 15 µg/mL was the most efficient eugenol concentration. From this, higher (20 µg/mL) and a lower (10 µg/mL) EOSA concentrations were defined for evaluation in the present work.

2.9.1. Experiment 1: effects of EOSA on cumulus cells, nuclear and cytoplasmic maturation of bovine oocytes

After 24 h of IVM, nine replicates were performed to assess the expansion and viability of *cumulus* cells. The meiotic competence was measured by visualization of the 1PB and nuclear stage (MII). Additionally, three replicates were carried out to evaluate cytoplasmic maturation by mitochondrial distribution using MitoTracker Red®.

2.9.2. Experiment 2: effect of EOSA on intracellular ROS levels and ΔΨm in bovine oocytes

After 24 h of IVM, oocytes were labeled with the fluorescent probes H_2DCFDA and MitoTracker Red to quantify ROS (nine replicates) and assess of ΔΨm (three replicates), respectively. For each replicate, each individual stained oocyte functioned as the experimental unit for each group.

2.9.3. Experiment 3: effect of EOSA on development of parthenogenetic bovine embryos

After 24 h of IVM, oocytes were artificially activated and cultured for eight days. On day 3 of IVD, the total cleavage rate and number of embryos were quantified that contained 2 cells, 3 to 7 cells, or 8 or more cells. On days 7 and 8 of IVD, the total blastocyst rate and the developmental phase [initial blastocyst (iB), blastocyst (B), expanded blastocyst (eB) and blastocyst hatching/hatched (hB)] were quantified. To evaluate the quality, the total numbers of cells were counted (eight replicates).

2.10. Statistical analysis

All data are expressed as the mean ± standard error and were analyzed using the StatView 5.0 (SAS Institute Inc., Cary, NC, USA). Normality of all results were verified with the Shapiro–Wilk test and homoscedasticity was verified with the Levene's test. ROS and ΔΨm levels, as well as the number blastocysts, were altered with arcsine and analyzed by variance analysis (ANOVA) followed by a Tukey test. All

other data were compared with a chi-squared test. Statistical significance was set at $P < 0.05$.

3. Results

A total of 416 ovaries were used to acquire 1567 viable immature oocytes (Fig. 1A) that were selected (3.8 viable oocytes/ovary) and distributed for all experiments.

3.1. Experiment 1: effects of EOSA on cumulus cells, nuclear and cytoplasmic maturation of bovine oocytes

The evaluation of *cumulus* cells and nuclear maturation are shown in Table 1. Initially, no difference was observed in the IVM rates obtained from both 1PB (Fig. 1B) and MII (Fig. 2A and B) evaluations. Nevertheless, EOSA15, EOSA20, and CYS improved the viability of *cumulus* cells after MIV ($P < 0.05$, Table 1). Moreover, EOSA20 significantly improved this viability compared to EOSA0 ($P < 0.05$, Table 1). Also, for *cumulus* cell expansion, the EOSA concentrations were similar to that of EOSA0 and CYS (Table 1). No difference was observed in the IVM rates for cytoplasmic maturation evaluated in terms of mitochondrial distribution (Table 2, Fig. 2C and D).

3.2. Experiment 2: effect of EOSA on intracellular ROS levels and ΔΨm in bovine oocytes

Although no difference has been observed in the ROS levels for all groups (Fig. 3A1–A5 and 3B), when evaluating ΔΨm (Fig 1A6–A10 and 3C), oocytes derived from the EOSA15 (0.73 ± 0.01), EOSA20 (0.72 ± 0.03), and CYS (0.80 ± 0.06) groups showed reduction of in ΔΨm when compared to the EOSA0 group (1.00 ± 0.04, $P < 0.05$). Additionally, only the EOSA20 group showed statistical different with EOSA10 (0.94 ± 0.00, $P < 0.05$).

3.3. Experiment 3: effect of EOSA on development of parthenogenetic bovine embryos

The assessment of embryonic development is delineated in Table 3. The cleavage rate (Fig. 1C), after 3 days of IVD, was similar in the evaluated groups, ranging from 81.2% to 87.9% ($P > 0.05$). No difference was observed among groups in the blastocyst/total

oocyte rates on D7 (Table 3). Nevertheless, EOSA20 improved the blastocyst/total oocyte and blastocyst/cleavage oocyte rates on D8 (Fig. 1D) when compared to EOSA0 and EOSA10 *(P* < 0.05). Moreover, the EOSA20 rates were similar to those of CYS (*P* > 0.05).

In relation to embryo development kinetics, or the proportion of embryos at different stages of development, there was no significant difference among the groups in the percentage of embryos classified with 2, 3 to 7, or ≥ 8 cells (Fig. 4A). On D8, the percentage of embryos classified as iB, B, or hB were similar among the treatments studied (*P* > 0.05). Nevertheless, the CYS group had a higher percentage of expanded blastocysts (EB; Fig. 1E) compared to EOSA0. EOSA10, EOSA15, and EOSA20, percentages were similar to CYS and EOSA0 (Fig. 4B). Finally, EOSA, at 15 µg/mL or 20 µg/mL, promoted significantly better embryo quality as assessed by the comparison of cell counts (Fig. 1F and 4C) those observed in the EOSA0 group.

4. Discussion

We demonstrated, for the first time, that IVM with EOSA (20 µg/mL) supplemented medium significantly increased the viability of *cumulus* cells, promoted a reduction in ΔΨm, and improved embryonic development and quality in artificially activated bovine oocytes when compared to those without EOSA supplementation. Moreover, EOSA20 presented results similar to those obtained with the CYS, which is currently the most used antioxidant agent during IVM [15].

Cumulus cells have a fundamental role in oocyte competence, since they allow oocyte communication with the external environment, nutrient passage, hormone passage, and regulatory factor passage [27]. In this study, the expansion of the *cumulus* cells in groups supplemented with EOSA was similar to that of the EOSA0 and CYS groups. Pereira [28], using *Lippia origanoides* essential oil as a possible antioxidant agent, did not observe a difference in *cumulus* cell after IVM of bovine (92.7–95.0%) and buffalo (73.3–78.9%) oocytes. Nevertheless, in terms of *cumulus* cell viability, media supplemented with 20 µg/mL of EOSA was able to maintain a higher percentage of viable cells after IVM compared to EOSA0, EOSA10, and EOSA15. Since *cumulus*

cells are more frequently exposed to *in vitro* manipulation [26], it is believed that these cells are more susceptibility to ROS damage to the plasma membrane. Therefore, a higher rate of *cumulus* cell viability may contribute to the maintenance of oocyte quality and competence.

In the present work, the nuclear maturation, evaluated in terms of 1PB and MII, was not affected by the addition of EOSA or CYS to the IVM medium. Studies show that the addition of antioxidants during maturation does not directly influence bovine meiotic progression in bovine (green tea polyphenols [29]; quercetin [30]; quercetin, cysteamine, carnitine, vitamin C, and resveratrol [11]). Nevertheless, antioxidants improve the intrinsic competence of the oocyte, which can be proven during embryonic development [11, 29, 30] as demonstrated in this study.

During oocyte development, mitochondria play a key role in adenosine triphosphate (ATP) generation for maturation, fertilization, and embryonic development [31]. Mitochondria move to the sites requiring more energy, migrating from the periphery of immature oocytes towards the center of oocytes that resume meiosis but showing a dispersed and homogeneous distribution at the end of maturation [8, 32]. In the present study, we observed a pattern of distribution similar to that described for bovine oocytes by Ambrogi et al. [8], who did not observe a significant difference when comparing a combination of cysteine, cysteamine, and catalase to cultures without antioxidant supplementation. Similarly, supplementation with different concentrations of EOSA or CYS did not influence cytoplasmic maturation in relation to the EOSA0.

The complex mechanism of ATP production by mitochondria involves the electron transport chain. Higher $\Delta\Psi m$ indicates higher mitochondrial ATP synthesis [33]. Moreover, ROS generation depends on $\Delta\Psi m$. Therefore, a high $\Delta\Psi m$ mitochondrial respiratory chain becomes a significant ROS producer [34]. This is due to the leakage of electrons from the mitochondrial membrane that can bind to O_2 forming ROS [35]. Based on this process, and their own data, Ambrogi et al. [8] suggest that higher $\Delta\Psi m$ may lead to higher ROS production in bovine oocytes. In the present study, $\Delta\Psi m$ was significantly lower in groups EOSA15, EOSA20, and CYS when compared to EOSA0,

suggesting a mechanism for decreasing ROS levels. These data suggest that, at concentrations of 15 and 20 μg/mL, EOSA has an antioxidant effect for bovine oocytes similar to that observed with CYS.

Additionally, Sovernigo et al. [11] observed that different antioxidants can act through different mechanisms. Cysteamine, for example, showed ROS levels similar to that of the control, as in the present work, but was able to increase the synthesis of the important intracellular antioxidant glutathione. Vitamin C, resveratrol, and quercetin decreased ROS levels but were unable to increase glutathione synthesis. Thus, further studies with EOSA are necessary to understand the mechanisms of its antioxidant effects in oocytes. Nevertheless, in *in vitro* somatic cell studies, eugenol and β-caryophyllene demonstrated the ability to significantly increase both the activity and levels of glutathione [21, 36]. Therefore, as these are the main constituents of EOSA, the positive results observed in the present study could be attributed to this mechanism.

As for embryo development, the cleavage rate and the number of embryos with 2, 3 to 7, or ≥ 8 cells were similar among all experimental groups. This result agrees with other work that evaluated antioxidant substances and did not observe a difference in this parameter [1, 11, 12], probably because this evaluation occurs prior to activation of the embryonic genome [37]. Differences in the blastocyst percentage were observed on D8, with a greater percentage of blastocysts in the EOSA20 group compared to that without antioxidant supplementation. Additionally, when the rate of blastocysts/cleaved cells was calculated, the EOSA20 group stood out from the other concentrations of oils tested and the CYS group. Finally, different EOSA concentrations were similar to both the CYS and EOSA0 groups.

Efficient antioxidants, in adequate concentrations, can improve the embryo development and quality [1, 11, 12]. In this study, in addition to the blastocyst rate, the media supplemented with EOSA15 and EOSA20 produced better quality embryos, as determined by the number of blastomeres. In general, a higher number of blastocysts cell is related to a low rate of apoptosis [1, 38]. This criterion is very important because of its direct influence on normal fetal development [39]. EOSA may elicit these positive

effects due to the rich eugenol composition, which has strong antioxidant activity demonstrated in biochemical and cellular tests [21, 40]. In addition, the other constituents of clove essential oil obtained in the present work, β-cariophylene and acetyl eugenol, also exhibit antioxidant properties [36, 41]. Therefore, it is possible there is a synergic effect among these bioactive compounds that improves EOSA performance.

In conclusion, IVM medium supplementation with 20 μg/mL of EOSA improves the viability of *cumulus* cells, possibly through antioxidant effects due to the decreased oocyte ΔΨm. Also, EOSA20 can significantly improve the rate of development and quality of bovine parthenogenetic blastocysts. The results for EOSA20 are comparable to those with cysteamine in terms of most parameters evaluated. Finally, this is the first report to demonstrate the efficiency of the natural antioxidant mixture obtained from clove in bovine oocytes, which is inexpensive and can be easily obtained. EOSA may be an accessible alternative to combat oxidative stress in IVEP, and further studies should assess its mechanism of action.

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Table 1. Influence of EOSA during bovine IVM on evaluation of *cumulus* cells and nuclear maturation.

Group	Evaluation of *cumulus* cells		Nuclear maturation	
	Expansion, %	Viability, %	1PB, %	MII, %
EOSA0	92.5 ± 2.7 (160/173)[b]	54.1 ± 2.8 (1334/2466)[bc]	69.1 ± 3.4 (114/165)[a]	69.5 ± 6.2 (66/95)[a]
EOSA10	95.9 ± 1.6 (163/170)[a,b]	56.3 ± 2.8 (1080/1919)[b]	74.7 ± 4.7 (124/166)[a]	76.5 ± 4.9 (78/102)[a]
EOSA15	96.0 ± 1.7 (166/173)[a,b]	53.0 ± 3.2 (934/1763)[c]	66.2 ± 3.8 (102/154)[a]	67.9 ± 3.4 (57/84)[a]
EOSA20	95.9 ± 1.3 (165/172)[a,b]	57.2 ± 3.9 (938/1639)[a]	66.9 ± 5.4 (105/157)[a]	70.9 ± 3.6 (56/79)[a]
CYS	97.7 ± 1.1 (168/172)[a]	55.9 ± 2.9 [a,b,c] (1045/1870)	66.1 ± 4.3 (109/165)[a]	70.4 ± 3.8 (69/98)[a]

[a,b,c]: Values with different superscript letters within columns are significantly different ($P < 0.05$). MII, metaphase II; 1PB, first polar body.

Table 2. Cytoplasmic maturation evaluated by the cytoplasmic distribution of mitochondria in matured oocytes in the absence and presence of EOSA and CYS.

Group	No. oocytes	Peripheral, %	Transition, %	Dispersed, %
EOSA 0	29	0.0 ± 0.0 (0)	34.5 ± 7.2 (10)	65.5 ± 7.2 (19)
EOSA 10	28	0.0 ± 0.0 (0)	32.1 ± 2.4 (9)	67.9 ± 2.4 (19)
EOSA 15	29	0.0 ± 0.0 (0)	27.6 ± 1.9 (8)	72.4 ± 1.9 (21)
EOSA 20	27	0.0 ± 0.0 (0)	22.2 ± 4.9 (6)	77.8 ± 4.9 (21)
CYS	29	0.0 ± 0.0 (0)	31.0 ± 1.4 (9)	69.0 ± 1.4 (20)

No differences were observed among treatments ($P > 0.05$).

Table 3. Development of parthenogenetic bovine embryos derived from oocytes matured in the absence and presence of EOSA and CYS.

Group	Cleavage D3, %	Blastocyst D7, %	Blastocyst D8, %	Blastocyst /Cleaved, %
EOSA0	86.6 ± 6.0[a] (84/97)	22.7 ± 5.5[a] (22/97)	24.7 ± 5.2[b] (24/97)	28.6 ± 6.7[b] (24/84)
EOSA10	87.9 ± 5.3[a] (87/99)	19.4 ± 4.3[a] (19/98)	22.5 ± 4.9[b] (22/98)	25.3 ± 4.8[b] (22/87)
EOSA15	81.2 ± 6.9[a] (82/101)	21.8 ± 4.1[a] (22/101)	25.7 ± 4.1[ab] (26/101)	31.7 ± 6.8[b] (26/82)
EOSA20	83.9 ± 5.7[a] (94/112)	30.4 ± 5.6[a] (34/112)	37.5 ± 6.7[a] (42/112)	44.7 ± 8.1[a] (42/94)
CYS	85.9 ± 4.9[a] (85/99)	28.6 ± 4.8[a] (28/98)	30.6 ± 5.8[ab] (30/98)	35.3 ± 8.2[ab] (30/85)

[a,b]: Values with different superscript letters within columns are significantly different ($P < 0.05$).

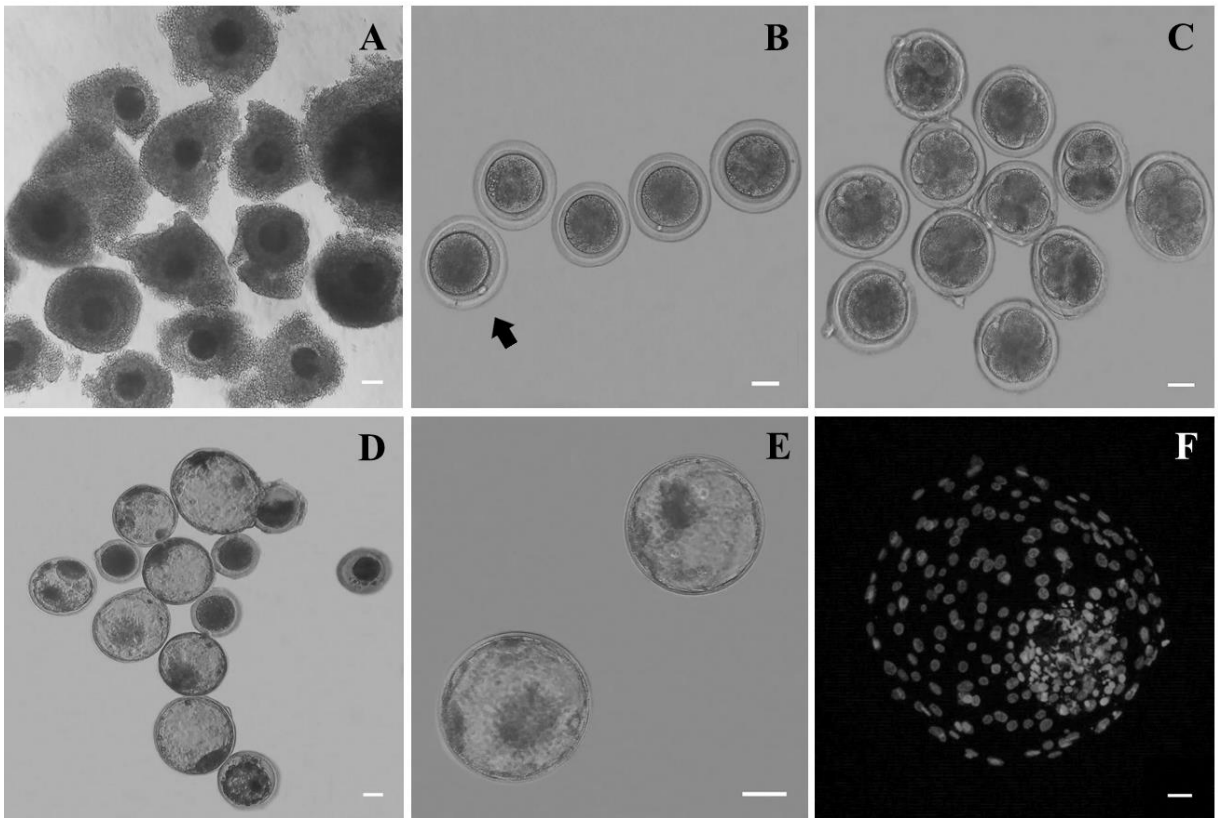

Fig. 1. Representative images of bovine oocytes before and after IVM with different concentrations of EOSA, cleavage, development and quality of embryos after artificial activation. (A) Immature oocytes. (B) Oocytes with a polar body (arrows) after 24 h of IVM. (C) Cleavage after 72 h of culture. (D) Embryo development after eight days in culture. (E) Expanded blastocysts. (F) Expanded blastocyst stained with Hoechst 33342 for counting the total number of cells viewed under a fluorescence microscope. Scale bars = 200 µm in A and D; 150 µm in C; 100 µm in B, E and F.

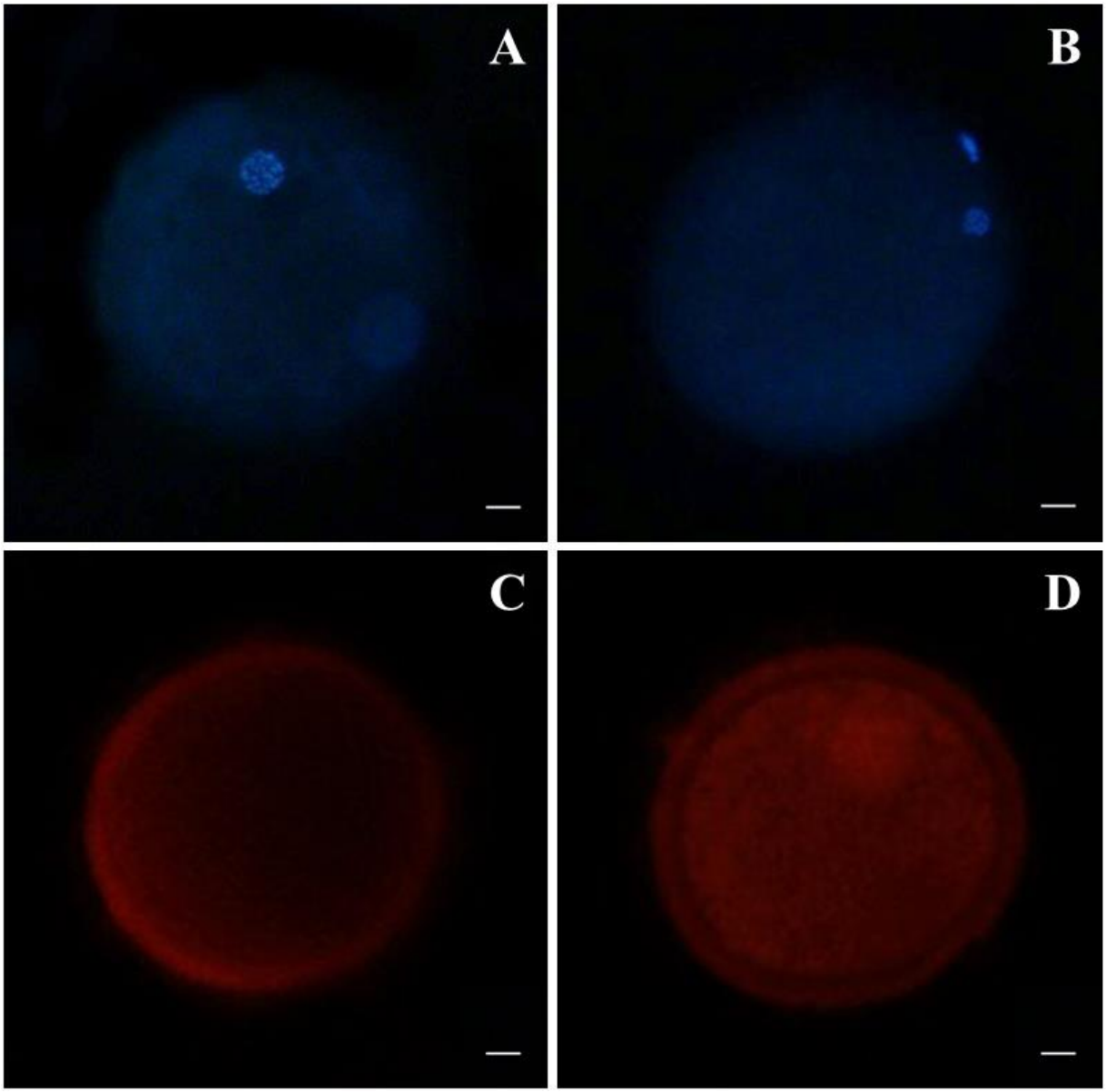

Fig. 2. Representative images of nuclear maturation (presence of metaphase plate and first polar body) and cytoplasmic maturation (cytoplasmic distribution of mitochondria) of oocytes matured with different concentrations of EOSA. (A) Immature oocyte in the stage of germ vesicles and (B) oocyte matured in metaphase II; (C) Oocyte with mitochondrial distribution in transition (immature) and (D) dispersed (mature). Scale bars = 50 µm.

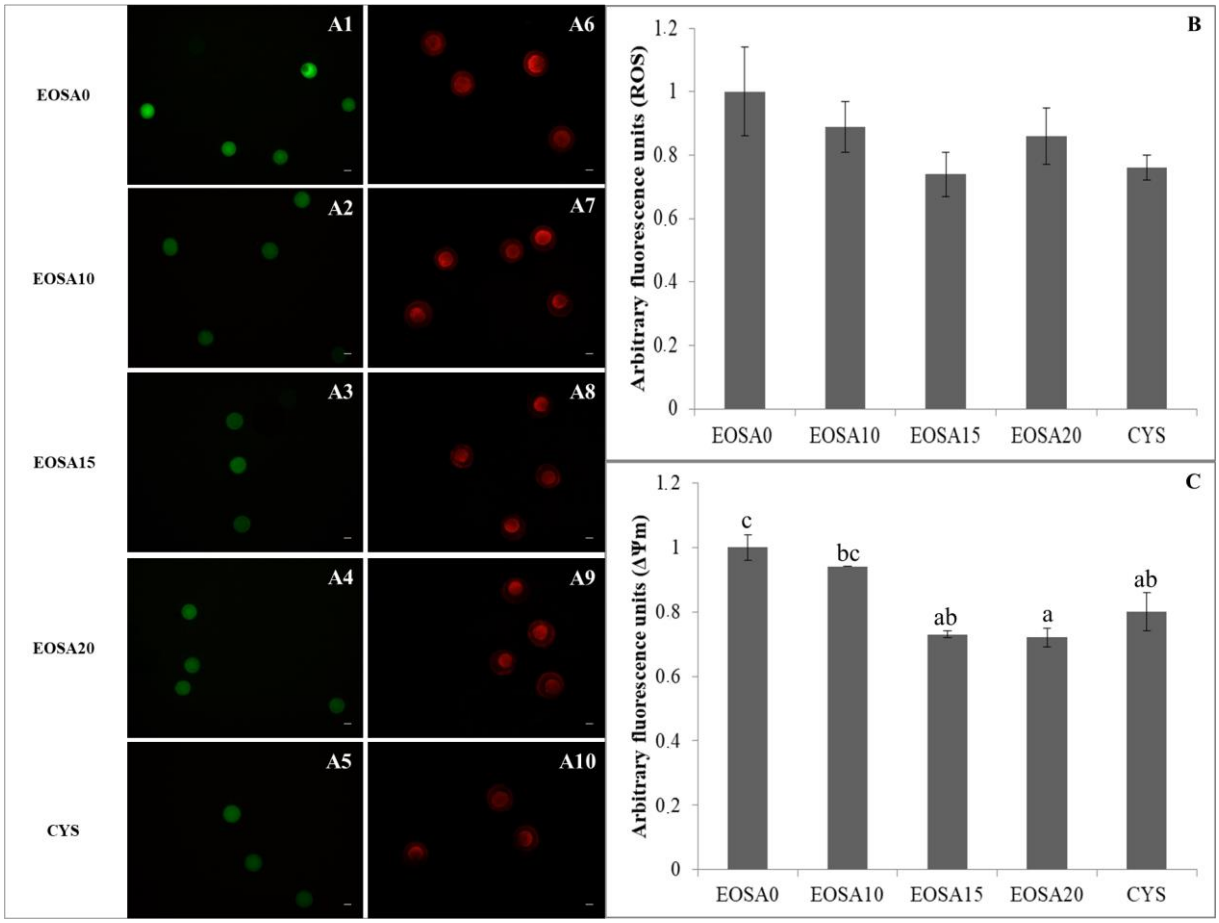

Fig. 3. Intracellular concentration of reactive oxygen species (ROS) and mitochondrial membrane potentials (ΔΨm) of bovine oocytes matured with different concentrations of EOSA. (A1–A5) Oocytes labeled with H_2DCFDA for ROS quantification. (A6–A10) Oocytes labeled with MitoTracker Red® for mitochondrial membrane potential quantification. (B) ROS: no differences were observed among treatments ($P > 0.05$). (C) ΔΨm: different letters indicate significant statistical difference among treatments ($P < 0.05$). Scale bars = 150 µm.

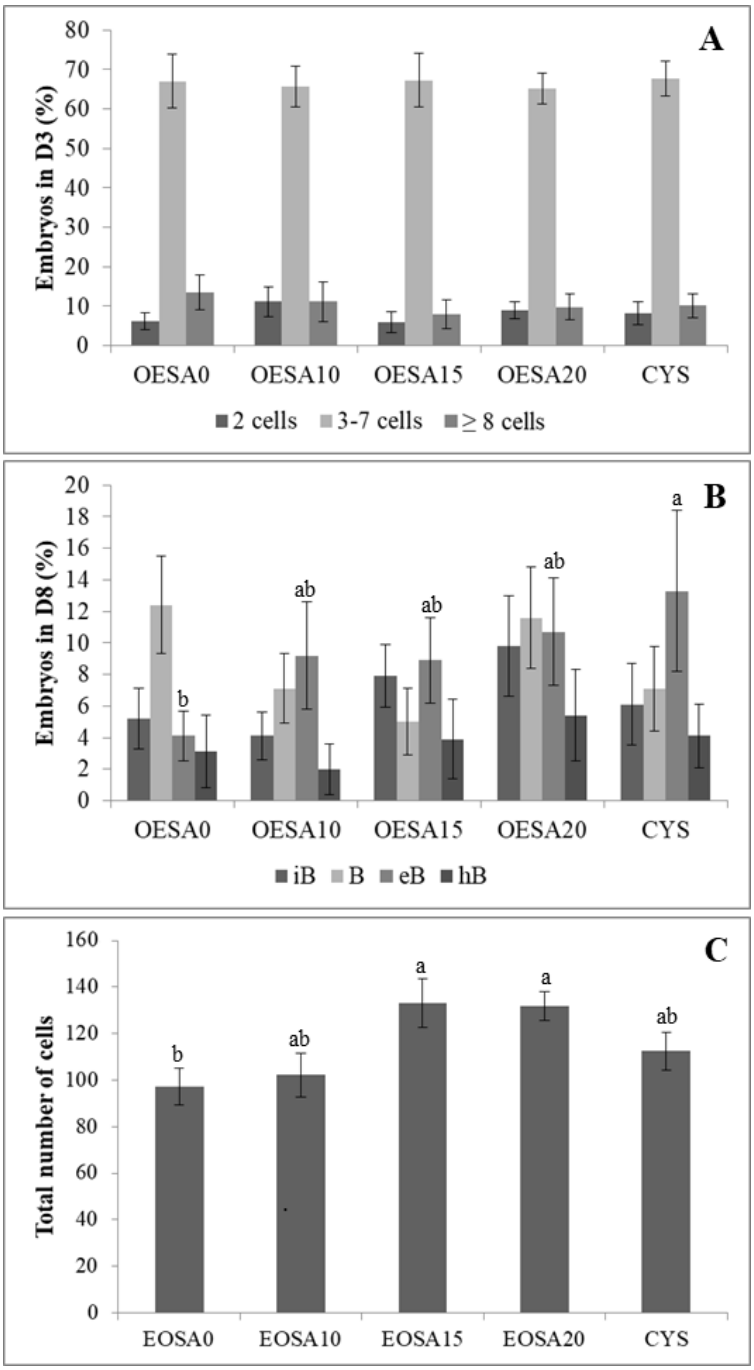

Fig. 4. Kinetics of embryonic development during IVD and blastocyst quality after maturation of bovine oocytes with different concentrations of EOSA. (A) Percentage of embryos in D3 with 2 cells, 3 to 7 cells and 8 cells. (B) Percentage of embryos in D8 classified as initial blastocysts (iB), blastocysts (B), expanded blastocysts (eB) and blastocysts hatching or hatched (hB). (C) Counting the total number of blastocyst (eB and hB) cells labeled with Hoechst 33342. Different letters above the columns indicate a statistically significant difference among treatments ($P < 0.05$).

CAPÍTULO 4 – EFEITO BENÉFICO DO ÓLEO ESSENCIAL DE *Syzygium aromaticum* SOBRE ESPERMATOZOIDES EPIDIDIMÁRIOS BOVINOS

Artigo Experimental N° 02: The beneficial effect of essential oil of *Syzygium aromaticum* on bovine epididymal sperm.

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The beneficial effect of essential oil of *Syzygium aromaticum* on bovine epididymal sperm

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Running title: Effects of *S. aromaticum* essential oil on bovine spermatozoa

Contents

Aiming its application toward reproductive biotechniques, we evaluated the effect of essential oil of *S. aromaticum* (EOSA) on bovine epididymal spermatozoa quality parameters such as morphology, membrane functional, and structural integrity, motility, metabolic activity and reactive oxygen species (ROS) levels. Spermatozoa derived from eight males were incubated for 1 h and 6 h according to the following groups: EOSA0 (without EOSA), EOSA10 (10 μg/mL of EOSA), EOSA15 (15 μg/mL of EOSA), and EOSA20 (20 μg/mL of EOSA). The spermatozoa evaluated immediately after recovery were considered to be the fresh control. No difference in sperm morphology was observed among the groups ($P > 0.05$), but the spermatozoa incubated in the presence of EOSA showed preservation of quality parameters over time, especially functional and structural integrity of the plasma membrane, mitochondrial activity, curvilinear velocity, and metabolic activity ($P < 0.05$). Moreover, spermatozoa derived from the EOSA15 group maintained similar results to the fresh control group for medium velocity (after 6 h), functional integrity of the membrane, percentage of damaged plasma membrane, and inactive mitochondria ($P < 0.05$). Additionally, no difference was observed for ROS levels among incubated groups ($P > 0.05$). These results indicate the first stage of systematic characterization of the effects of EOSA on bovine spermatozoa. In conclusion, EOSA protects bovine epididymis spermatozoa up to 6 h, especially at a concentration of 15 μg/mL, and improves the quality of these gametes.

Keywords: Cattle, clove buds, sperm analysis, oxidative stress.

Introduction

Spermatozoa are susceptible to damage caused by reactive oxygen species (ROS) due to the composition of their plasma membrane, which is rich in polyunsaturated fatty acids (Vernet, Aitken, & Drevet, 2004). Moreover, spermatozoa have long been known as ROS producers, which act to transduce early signals that lead to capacitation (Aitken, Paterson, Fisher, Buckingham, & Duin, 1995). Normally, *in vivo* conditions have powerful and sophisticated antioxidant strategies capable of maintaining the ideal microenvironment for spermatozoa (Vernet et al., 2004). However, for *in vitro*

fertilization (IVF), and intracytoplasmic sperm injection (ICSI), spermatozoa suffer from external factors such as oxygen tension, light and heat interference, and medium constituents which may accentuate ROS production and cause oxidative stress (Agarwal, Said, Bedaiwy, Banerjee, & Alvarez, 2006).

Therefore, the addition of antioxidants *in vitro* may be an interesting tool to maintain sperm fertility and achieve greater fertilization success (Sapanidou et al., 2016). Several natural substances have been studied for antioxidant treatment of bovine spermatozoa (Santos, Borges, Queiroz Neta, Bertini, & Pereira, 2018), both as isolated substances (crocetin, Sapanidou et al., 2016, resveratrol, Tvrdá, Kováčik, Tušimová, Massányi, & Lukáč, 2015, curcumin, Tvrdá et al., 2016), or as essential oils and extracts (extract of *Vitis vinifera*, Sapanidou et al., 2014).

In general, organic compounds such as plant essential oils and extracts have played an important role in the discovery of substances with medicinal properties, such as antioxidants (Santos et al., 2018). Previous studies were performed using an extract derived from *Syzygium aromaticum* on sperm cells of murine (Mishra & Singh, 2013), and sheep sperm (Baghshahi, Riasi, Mahdavi, & Shirazi, 2014). This plant, commonly called clove, is known for its flower bud which is considered a strong antioxidant and whose efficacy is associated with a composition rich in phenolic compounds, especially eugenol (El-Maati, Mahgoub, Labib, Al-Gaby, & Ramadan 2016). It was previously reported that the hexane extract of clove increased murine sperm motility and secretory activity of the epididymis and seminal vesicle after oral administration through gavage for 35 days (Mishra & Singh, 2013). Already, Baghshahi et al. (2014), using clove extract in semen extender for cryopreservation, observed a beneficial effect of the extract on sheep sperm motility, membrane integrity, and viability.

Chemically obtaining an essential oil involves more complex procedures with respect to the extracts but it allows a cleaner combination of bioactives without the interference that different solvents can provide (El-Maati et al., 2016). Therefore, we aimed to evaluate the antioxidant effects of this oil on bovine epididymal spermatozoa quality parameters and ROS levels.

Materials and methods

Bioethics and chemicals

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The experiments were conducted in accordance with the Animal Ethics Committee (Opinion CEUA/UFERSA No. 23091.002360/2016-17).

Extraction and chemical analysis of EOSA

Flower buds of *S. aromaticum* were collected in Salvador-BA (12°58'1311S, 38°30'45''W), situated in northeastern Brazil. EOSA was extracted by hydrodistillation of the flower buds using a Clevenger-type apparatus (Craveiro, Matos, & Alencar, 1976). The chemical analysis of EOSA was performed using a gas chromatography coupled to mass spectroscopy (GCMS-QP2010 Plus, Shimadzu, Quioto, Japan), containing a capillary SH-Rtx-5, and with helium as the entrainment gas, flowing at 1.78 $mL.min^{-1}$. Thus, 1.0 μL of the diluted sample (1.0 mg of oil in 1000 μL of 99.9% dichloromethane) was injected in split mode (1:10). The column temperature program started 40°C followed by an increase 4ºC/min up to 180°C, and then an increase of 20°C/min up to 280°C maintained for 10 min.

Finally, the chemical constituents (Table 1) were identified by their gas chromatography retention times, relative to known compounds and expressed by Kovat Index (KI), which were calculated by linear regression and by comparison of their mass spectra with published spectra (Adams, 2001).

Recovery of epididymal sperm

Eight testicle-epididymis complexes derived from slaughterhouse adult males were used for sperm collection utilizing a retrograde washing method. Each testicle-epididymis complex was dissected to isolate the tail of the epididymis and the vas deferens. This was followed by insertion of a 26-gauge needle, connected to a 5 mL sterile syringe filled with saline solution warmed to 37°C, into the vas deferens. The expelled fluid was collected and evaluated immediately. The sperm concentration was determined using a

Neubauer counting chamber, and the study was only conducted when sperm cells had more than 60% motility. Finally, the sperm solution was centrifuged (300 g for 10 min) and suspended in sperm Tyrode's Albumin Lactate Pyruvate (Sperm-TALP) medium (Sapanidou et al., 2016).

Experimental design and *in vitro* incubation

Initially, a sample of spermatozoa was evaluated immediately after centrifugation, being denominated the fresh control group. The other groups were subjected to incubation for 1 h, and 6 h: EOSA0 (without EOSA), EOSA10 (10 μg/mL of EOSA), EOSA15 (15 μg/mL of EOSA), and EOSA20 (20 μg/mL of EOSA). EOSA was diluted with dimethylsulfoxide (DMSO) for easy diffusion of EOSA into the Sperm-TALP medium, in the addition of up 0.25% DMSO (Avery, & Greve, 2000).

The sperm concentration was adjusted with Sperm-TALP medium to 20 x 10^6 sptz/mL in a final volume of 500 μL. This suspension was incubated in tubes with the lid ajar to allow contact of the suspension with the humid atmosphere of 5% CO_2 at 38.5°C. After 1 h of incubation, 250 μL of each treatment group was withdrawn for evaluation and, after 6 h of incubation, the remainder of the suspension of each treatment group was also evaluated, resulting in the comparison of nine groups.

Sperm evaluation

After sperm collection and each incubation period, samples were evaluated for sperm morphology, plasma functional integrity, plasma membrane integrity and mitochondrial activity, motility, metabolic activity, and ROS levels.

For morphological analysis, Bengal Rose-stained (Cromato®, SP, Brazil) smears were prepared and evaluated using light microscopy (1000×). One hundred cells per slide were counted. Morphologic defects were classified according to the region of the sperm: head, midpiece (including droplets), or tail defects (Mondal, Karunakaran, Lee, & Rajkhowa, 2010).

Functional integrity of the sperm membrane was verified by evaluating the sperms' osmotic response under a hypo-osmotic swelling test using 9 g fructose and 4.9 g sodium citrate per liter of distilled water (100 mOsm/L) as the hypo-osmotic solution (Sarıözkan, Bucak, Tuncer, Büyükleblebici, & Cantürk, 2014). A volume of 5 µL of semen was added to 95 µL of a hypo-osmotic solution and incubated at 37°C for 60 min. After incubation, slides were mounted and visualized under a phase contrast microscope. Sperm with swollen or coiled tails were recorded.

To evaluate plasma membrane integrity and mitochondrial activity of the sperm, an aliquot (10 µL) of sperm was incubated at 37°C for 10 min with 2 µL Hoechst 33342 (H; 40 µg/mL). After incubation, 3 µL of propidium iodide (PI; 0.5 mg/mL) and 5 µL of chloromethyl-X-rosamine (CMXRos, Mito Tracker Red®, 500 µM) were added to each sample and incubated at 37°C for 8 min. The samples were evaluated through an epifluorescence microscope (400×; Olympus BX51TF, Tokyo, Japan) and 100 cells were counted. Sperm marked in blue (H) were classified as having an intact sperm membrane and those marked in red (PI) were classified as non-intact. Sperm with a midpiece marked in red were classified as showing mitochondrial activity (Celeghini, Arruda, Andrade, Nascimento, & Raphael, 2007).

Sperm motility was assessed by computer assisted sperm assessment (CASA; IVOS 12.0, Hamilton-Thorne, Beverly, USA) using 5 µL of sample and a setup previously adjusted for the analysis of bovine spermatozoa, according to the manufacturer's instructions. The following parameters were analyzed: motile spermatozoa (%), progressive spermatozoa (%), average pathway velocity (VAP, µm/s), straightline velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm), beat/cross-frequency (BCF, Hz), straightness (STR, %) and linearity (LIN, %). The overall sperm population was subdivided in four categories: rapid, medium, slow, and static.

For the evaluation of metabolic activity, 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. MTT (10 µL; 5 mg/mL) was added to 100 µL of sperm suspension and incubated in tubes for 2 h at 38.5°C and 5%

CO_2 (Tvrdá et al., 2015). After incubation, the formazan crystals were dissolved in 900 µL of DMSO and the optical density was determined in a spectrophotometer the absorbance at a wavelength of 595 nm. The fresh control group was considered to have 100% metabolic activity.

Finally, the intracellular ROS levels of spermatozoa were determined according to Pang et al. (2016). A volume of 5 µL of 2′,7′-dichlorofluorescein diacetate (H_2DCFDA; 5 mM) was added to 100 µL of each sperm suspension and incubated at 37˚C for 30 min. Sperm cells were then incubated with 5 µL of PI for 10 min and washed twice in phosphate buffer solution. Sperm were analyzed under an epifluorescence microscope to determine ROS levels. The fluorescence intensity of the images was quantified using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). The fresh control group was used as a calibrator and the measured value of each treatment micrograph was divided by the mean of the calibrator to generate relative expression levels (arbitrary fluorescence units).

Statistical analysis

All data were expressed as mean ± standard error (eight males/eight repetitions) and analyzed using the StatView 5.0 (SAS Institute Inc., Cary, NC, USA). All results were verified for normality by the Shapiro–Wilk test and for homoscedasticity by Levene's test. All data were transformed in arcsine and analyzed by one-way variance analysis (ANOVA), followed by post-hoc (Fisher's protected least significance difference or Tukey test). Statistical significance was set at $P < 0.05$.

Results

No difference was observed among treatment groups for sperm morphology (Figure 1). The functional integrity of the sperm membrane (Table 2) was maintained better during the 6 h incubation when the medium was supplemented with EOSA ($P > 0.05$). In addition, the functional integrity of the sperm membrane observed in the EOSA15 group after 1 h of incubation was similar to that observed in the fresh control group's spermatozoa ($P > 0.05$).

The percentage of spermatozoa showing an intact membrane and mitochondrial activity (greater viability, Figure 2A and 3A) in the EOSA0 group showed a significant decrease after 6 h of incubation when compared to 1 h of incubation ($P < 0.05$). However, when the media was supplemented with EOSA, sperm viability was better maintained during the incubation times studied. As for the spermatozoa with damaged membranes and lack of mitochondrial activity (non-viable) (Figure 2B, and 3B–C), a lower percentage was observed in the EOSA15 group compared to the EOSA0 group after 6 h ($P < 0.05$). Additionally, this number of non-viable spermatozoa increased in the EOSA0 group after 6 h of incubation compared to the time of 1 h ($P < 0.05$).

The results of motile evaluation at CASA are shown in Table 3. Total motility was highest in the fresh control group but there was very little variability observed among groups after incubation. In progressive motility and VAP, both the EOSA15 group and the EOSA0 group showed identical results to the fresh control group after 1 h of incubation. In relation to VCL, the only group that was not able to maintain the same value as the fresh control group was EOSA0 incubated for 6 h ($P < 0.05$). In the category of spermatozoa with medium motility, the EOSA15 group incubated for 6 h maintained the best percentage and was most similar to the fresh control group ($P > 0.05$).

It was determined that the presence of EOSA in the medium maintained sperm metabolic activity better than the medium without EOSA (Table 4). Additionally, no difference was observed for ROS levels among incubated groups (Figure 4).

Discussion

This study evaluated, for the first time, the effects of EOSA on the parameters of quality and oxidative stress in bovine epididymis spermatozoa. It was observed that the addition of this oil to Sperm-TALP medium promotes the maintenance of sperm quality over a 6 h incubation time, especially for functional and structural integrity of the plasma membrane, mitochondrial activity, curvilinear velocity, and sperm metabolic activity. Additionally, the EOSA15 treatment group showed more promising results regarding the percentage of membrane functional integrity, percentage of spermatozoa with

damaged membranes and inactive mitochondria, spermatozoa with medium motility after 6 h, and ROS levels. This effect of EOSA becomes important in *in vitro* reproductive biotechniques because they require several hours of incubation for fertilization to occur (Pang et al., 2016). In cattle, the IVF time varies according to sperm concentration and can range from 6 to 18 h, which may or may not include an additional time of 1 h for sperm capacitation before IVF (Kochhar, Kochhar, Basrur, & King, 2003; Pang et al., 2016). For this reason, incubation times of 1 h and 6 h were evaluated in the present study. Additionally, we chose the epididymal spermatozoa model due to the availability in slaughterhouses and a similar quality and resemblance resistance to ejaculated spermatozoa (Cunha et al., 2016).

The antioxidant and protective effects of EOSA have been especially attributed to eugenol, which is the component with the highest concentration in clove (Lee & Shibamoto, 2001). It was previously reported that this terpenoid may be incorporated into the membrane due to its hydrophobic nature (Nagababu & Lakshmaiah, 1992) and may inhibit free radical attack by decreasing lipid peroxidation (Kumaravelu, Subramaniyam, Dakshinamoorthy, & Devaraj 1996). In fact, it was reported that eugenol may reduce ROS levels, decreasing lipid peroxidation, and DNA damages (Mahapatra, Chakraborty, Majumdar, Bag, & Roy 2009). In the present study, most likely due to these properties, media supplemented with EOSA demonstrated protection of the membrane functional integrity over the 6 h incubation time. In addition, the group with 15 μg/mL of EOSA was similar to the fresh control group after 1 h.

Another method of evaluating the viability used in this work was fluorescent marking for the identification of membrane damage and mitochondrial activity. It was observed that only the media with EOSA retained sperm viability after 6 h of incubation. In a previous study, extract of clove added during the cryopreservation of ram spermatozoa showed a positive effect on membrane viability and integrity after thawing (Baghshahi et al., 2014). However, the best concentration of EOSA observed in that study (75 μg/mL) was higher than the EOSA amounts tested in the present study. This is probably due to the extreme conditions sperm encounter during cryopreservation that causes severe damages.

The viability and fertility of sperm are compromised when the membrane is damaged and/or the mitochondria are inactive (Celeghini et al., 2007). In the category of spermatozoa that had these two characteristics (the lowest viability), the EOSA0 group at 6 h incubation showed a significant increase in percentage when compared to 1 h incubation. Furthermore, the EOSA15 group showed a significantly lower percentage than the EOSA0 group after 6 h incubation. Therefore, EOSA prevents the percentage of non-viable sperm from increasing over time, especially at the concentration of 15 μg/mL.

Motility is considered one of the most important factors for assessing sperm quality and functionality (Baghshahi et al., 2014). In the present study, despite subtle differences found in motility, the results are promising to establish the use of EOSA for the antioxidant treatment of spermatozoa in *in vitro* biotechniques. The value of VCL has been correlated to a higher rate of fertility and conception (Marshburn et al., 1992) and a larger subpopulation of spermatozoa with medium velocity that is directly related to the value of VAP (Goovaerts et al., 2006). Previously, Baghshahi et al. (2014) used clove extract and obtained positive results in sheep sperm motility characteristics after cooling and freeze-thaw processes. In this referenced study, the addition of 35 μg/mL and 75 μg/mL of clove extract to the cryoprotectant medium increased the characteristic of sperm motility evaluated by CASA. However, a concentration of 115 μg/mL of extract decreased the values for most sperm motility parameters. Therefore, this result emphasizes the importance of establishing the optimal concentration of antioxidant substances to ensure the best results.

Several studies have used the MTT test to study metabolic activity of spermatozoa treated with antioxidants (Tvrdá et al., 2015; Tvrdá et al., 2016). In this study, no differences in metabolic activity were observed among the EOSA concentrations evaluated. However, once again, the medium without EOSA was unable to maintain similar metabolic activity between the 1 h and 6 h incubation periods. Our results lead us to conclude that sperm viability decreases over time and the addition of EOSA, as an antioxidant, maintains this important characteristic.

Clove essential oil has already demonstrated strong ROS-sequestering activity in both biochemical and cellular assays (Baghshahi et al., 2014). This activity is related to eugenol and other components of its composition, such as eugenyl acetate (Lee & Shibamoto, 2001). Our results show a significant decrease in ROS levels in all incubated groups, with the EOSA15 group presenting lower levels after 1 h of incubation. Therefore, the positive results found in motility and viability parameters can also be attributed to the antioxidant effect of EOSA.

In conclusion, these results indicate the first stage of a systematic characterization of the effects of EOSA on bovine spermatozoa. EOSA protects epididymis spermatozoa over time up to 6 h and maintains sperm viability as evidenced in this study by the analysis of membrane integrity, mitochondrial activity, curvilinear velocity, and metabolic activity. In addition, 15 μg/mL of clove oil improved the quality parameters of these gametes and, therefore, we conclude that EOSA could be an agent of interest for the maintenance of sperm quality during *in vitro* reproductive biotechniques.

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Conflict of interest

None of the authors have conflict of interest.

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Table 1 Composition of the essential oil of *S. aromaticum*.

Compounds	KI	Relative %	Molecular formula
Eugenol	1359	68.38	$C_{10}H_{12}O_2$
β-cariophylene	1419	19.65	$C_{15}H_{24}$
Acetyl eugenol	1522	11.97	$C_{12}H_{14}O_3$

KI: Kovat Index.

Table 2 Functional integrity of the bovine sperm membrane with a hypoosmotic swelling after treatment with different concentrations of EOSA for 1 h and 6 h.

Treatments	Incubation time	
Control	78.3 ± 3.2^{a}	
	1 h	*6 h*
EOSA0	53.9 ± 6.4^{bA}	36.1 ± 5.5^{bB}
EOSA10	55.0 ± 6.1^{bA}	41.0 ± 6.2^{bA}
EOSA15	64.0 ± 5.9^{abA}	52.0 ± 6.9^{bA}
EOSA20	56.4 ± 5.4^{bA}	42.9 ± 6.3^{bA}

^{a,b}: Lowercase letters in the same column indicate differences among treatments ($P < 0.05$). ^{A,B}: Uppercase letters on the same line indicate differences among incubation times ($P < 0.05$).

Table 3 Motility and kinetic parameters of bovine epididymal spermatozoa after treatment with different concentrations of EOSA for 1 h and 6 h.

	Incubation time								
		1 h				6 h			
CASA	Control	EOSA0	EOSA10	EOSA15	EOSA20	EOSA0	EOSA10	EOSA15	EOSA20
Motility (%)	71.5 ± 3.08[a]	43.0 ± 5.8[b]	33.3 ± 6.2[b]	42.1 ± 6.5[b]	38.0 ± 5.9[b]	29.4 ± 6.4[b]	34.5 ± 5.7[b]	38.6 ± 7.2[b]	38.3 ± 8.7[b]
P. motility (%)	27.6 ± 5,1[a]	15.3 ± 4.4[ab]	13.0 ±5.0[b]	15.5 ± 5.2[ab]	13.0 ±4.7[b]	8.3 ±3.8[b]	5.4 ±1.9[b]	7.6 ±2.4[b]	10.4 ± 4.1[b]
VAP (µM/sec)	70.9 ± 2.9[a]	63.2 ± 3.7[ab]	59.9 ± 4.2[b]	62.5 ± 4.0[ab]	60.2 ± 3.9[b]	55.6 ± 3.2[b]	55.6 ± 3.1[b]	58.2 ± 3.3[b]	59.0 ± 4.1[b]
VSL (µM/sec)	50.7 ± 2.7[a]	48.3 ± 3.8[ab]	45.7 ± 4.4[ab]	47.8 ± 3.9[ab]	45.8 ± 3.8[ab]	39.3 ± 2.6[b]	37.1 ± 2.3[b]	40.4 ± 2.4[b]	40.0 ± 3.8[b]
VCL (µM/sec)	136.6 ± 5.2[a]	126.2 ± 5.6[ab]	124.3 ± 6.9[ab]	126.6 ± 6.4[ab]	122.0 ± 6.6[ab]	112.9 ± 8.6[b]	118.1 ± 5.3[ab]	119.9 ± 7.1[ab]	125.4 ± 7.9[ab]
ALH (µM)	7.7 ± 0.2	7.0 ± 0.2	7.1 ± 0.4	6.7 ± 0.3	6.6 ± 0.3	7.6 ± 1.4	7.3 ± 0.3	7.6 ± 0.4	7.1 ± 0.4
BCF (Hz)	22.7 ± 1.1	24.6 ± 1.1	24.4 ± 0.9	24.9 ± 1.1	25.3 ± 1.0	24.9 ± 2.6	21.8 ± 1.0	22.6 ± 0.8	24.3 ± 1.4
STR (%)	70.8 ± 1.5	74.9 ± 1.7	74.4 ± 2.8	75.4 ± 1.2	75.1 ± 1.5	72.3 ± 3.9	67.6 ± 1.1	70.0 ± 2.2	68.0 ± 2.3
LIN (%)	37.4 ± 0.8	39.1 ± 1.4	37.0 ± 2.3	38.5 ± 1.3	38.5 ± 1.2	37.8 ± 2.1	33.1 ± 1.1	35.6 ± 1.3	32.9 ± 1.4
Rapid (%)	44.3 ± 6.4[a]	20.3 ± 4.9[b]	16.4 ± 5.8[b]	20.9 ± 6.6[b]	16.8 ± 5.8[b]	13.3 ± 4.9[b]	11.0 ± 3.4[b]	16.1 ± 4.4[b]	20.4 ± 7.7[b]
Medium (%)	9.9 ± 1.5[a]	6.3 ± 0.7[b]	4.9 ± 0.5[b]	5.8 ± 1.0[b]	5.4 ± 0.8[b]	4.6 ± 1.3[b]	6.3 ± 1.2[b]	6.6 ± 0.9b[a]	4.9 ± 0.8[b]
Slow (%)	17.5 ± 2.9	17.0 ± 2.3	11.8 ± 0.8	15.3 ± 1.3	15.9 ± 1.9	11.5 ± 1.5	16.9 ± 1.7	15.9 ± 2.3	13.0 ± 2.1
Static (%)	28.5 ± 3.1[a]	57.0 ± 5.8[b]	66.8 ± 6.2[b]	57.9 ± 6.5[b]	62.0 ± 5.9[b]	70.6 ± 6.4[b]	65.5 ± 5.7[b]	61.4 ± 7.2[b]	61.8 ± 8.7[b]

[a,b]: Lowercase letters in the same line indicate differences among treatments ($P < 0.05$).

Table 4 Metabolic activity of bovine epididymal sperm after treatment with different concentrations of EOSA for 1 h and 6 h.

Treatments	Incubation time	
Control	100.0 ± 0.0^{a}	
	1 h	*6 h*
EOSA0	88.2 ± 4.6^{bA}	71.2 ± 5.8^{bB}
EOSA10	84.8 ± 2.7^{bA}	70.1 ± 8.5^{bA}
EOSA15	77.9 ± 4.5^{bA}	74.8 ± 8.1^{bA}
EOSA20	79.3 ± 6.9^{bA}	76.1 ± 5.4^{bA}

[a,b]: Lowercase letters in the same column indicate differences among treatments ($P < 0.05$). [A,B]: Uppercase letters on the same line indicate differences among incubation times ($P < 0.05$).

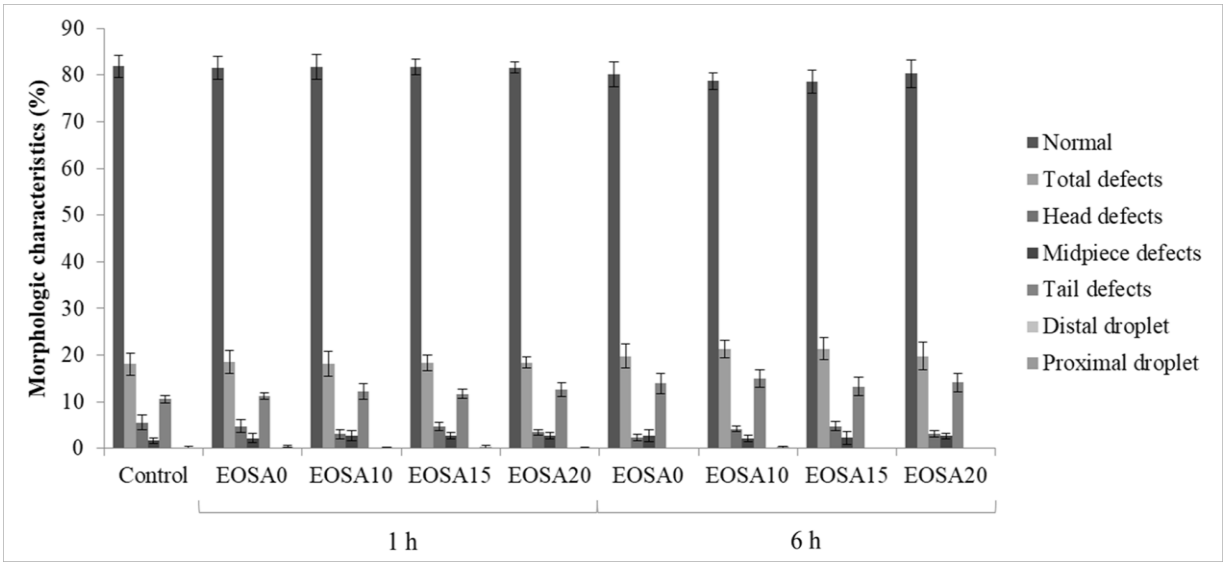

Figure 1 Morphology of bovine epididymal spermatozoa after treatment with different concentrations of EOSA for 1 h and 6 h ($P > 0.05$).

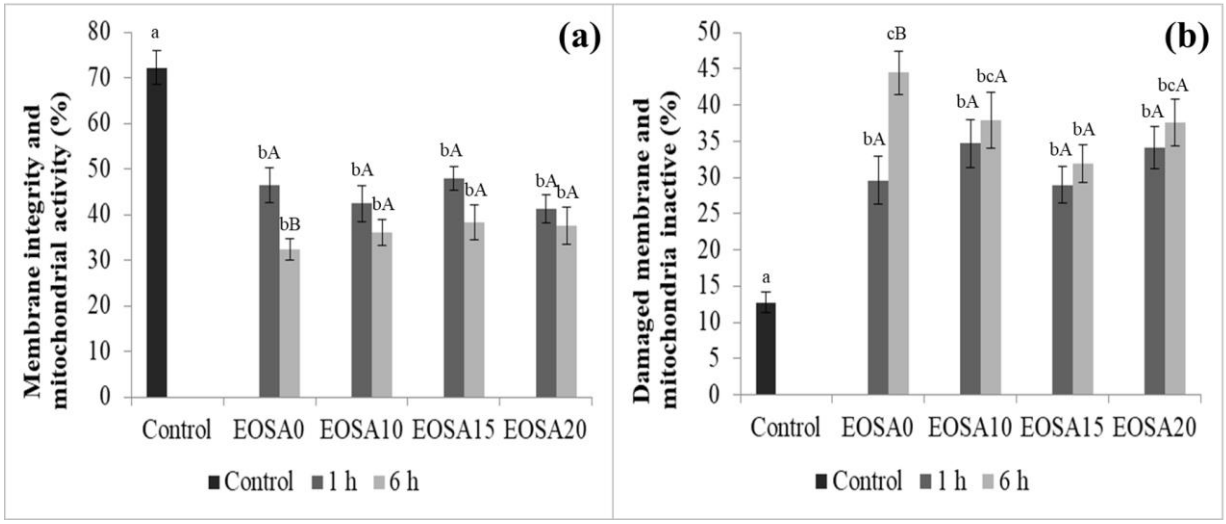

Figure 2 Percentage of bovine epididymal sperm with plasmatic membrane integrity and mitochondrial activity after treatment with different concentrations of EOSA for 1 h and 6 h. [a,b]: Lowercase letters indicate differences among treatments in the same time ($P < 0.05$). [A,B]: Uppercase letters indicate differences among incubation times ($P < 0.05$).

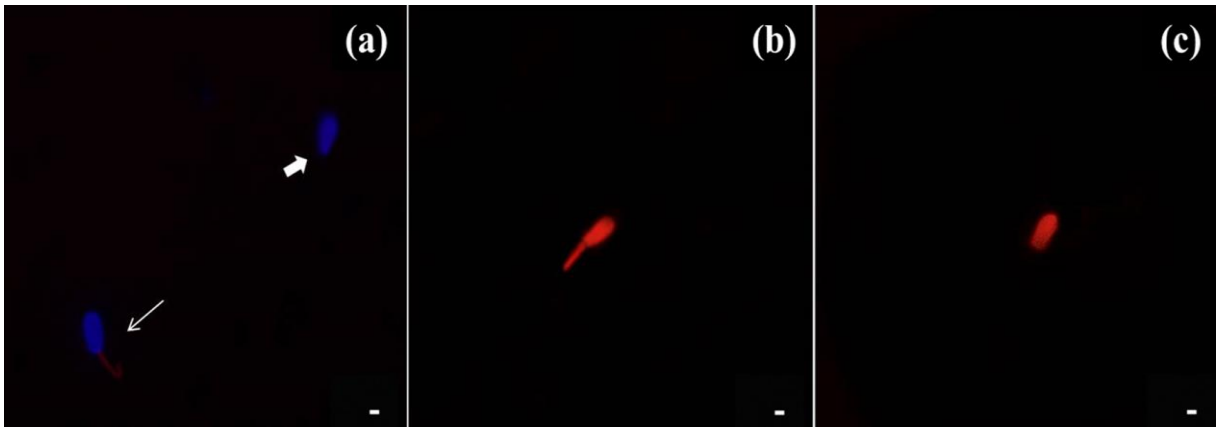

Figure 3 Representative images of spermatozoa classified according to membrane integrity and mitochondrial activity. (A) Thin arrow: membrane integrity and mitochondrial activity; thick arrow: membrane integrity without mitochondrial activity. (B) Damaged membrane and mitochondrial activity. (C) Damaged membrane and inactive mitochondria. Scale bars = 200 µm.

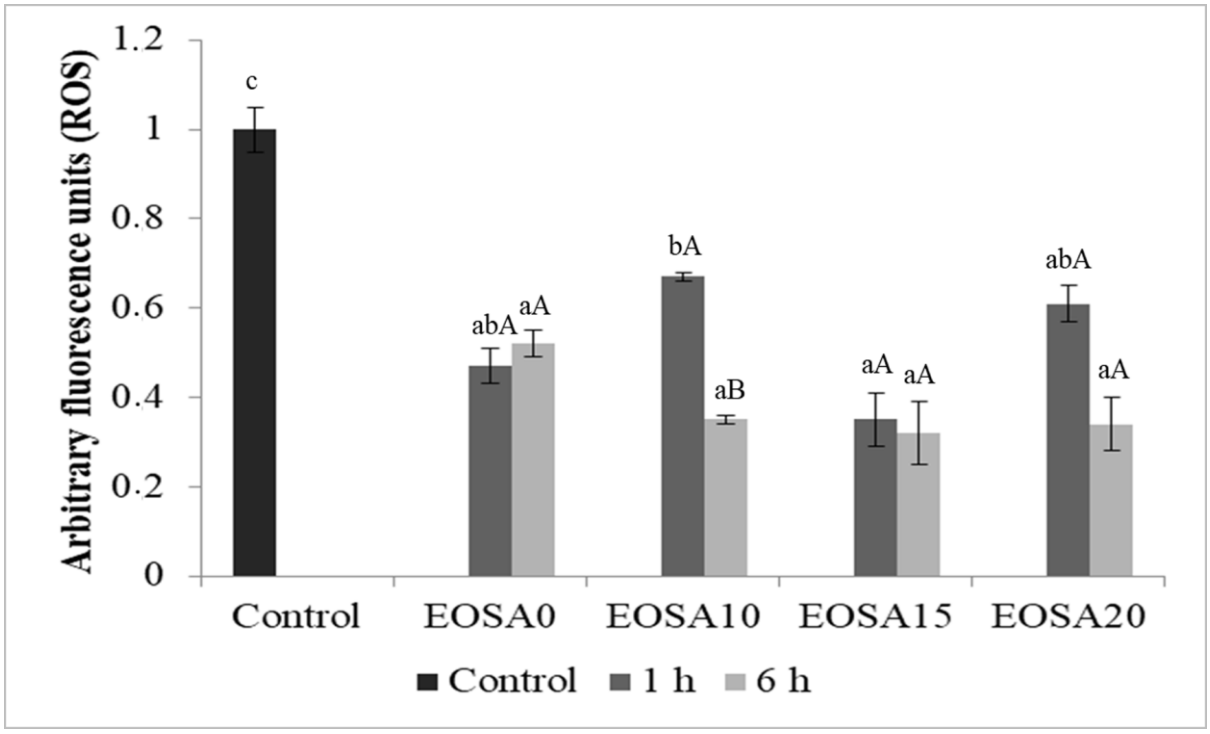

Figure 4 Reactive oxygen species (ROS) production by bovine epididymal sperm after treatment with different concentrations of EOSA for 1 h and 6 h. [a,b]: Lowercase letters indicate differences among treatments in the same time ($P < 0.05$). [A,B]: Uppercase letters indicate differences among incubation times ($P < 0.05$).

CONSIDERAÇÕES FINAIS E PERSPECTIVAS

O presente trabalho descreveu pela primeira vez os efeitos positivos do OESA como antioxidante durante a MIV e tratamento espermático bovino. A suplementação com 20 µg/mL OESA no meio de MIV manteve uma maior viabilidade das células do *cumulus*, com possível efeito antioxidante devido à diminuição de ΔΨm nos oócitos, maior taxa de desenvolvimento e qualidade de blastocistos partenogenéticos bovinos. Além disso, a adição do OESA, especialmente na concentração de 15 µg/mL, protegeu os espermatozoides epididimários ao longo de 6 h de incubação *in vitro*, mantendo a velocidade média, integridade funcional da membrana e percentual de membrana danificada e mitocôndria inativa.

Portanto, a suplementação de meios com OESA pode ser uma alternativa interessante para a manutenção da qualidade de gametas bovinos *in vitro*, com potencial para ser um novo composto a ser empregado na produção *in vitro* de embriões bovinos. Os resultados encontrados neste trabalho podem ser utilizados como parâmetros para o estabelecimento de OESA como antioxidante natural acessível e de fácil obtenção. Além disso, uma vez que as propriedades terapêuticas do óleo essencial são atribuídas aos seus principais constituintes, estudos posteriores podem ser realizados para avaliar tais compostos isoladamente e entender os mecanismos de ação.

ANEXOS

ANEXO A – COMPROVANTE DE SUBMISSÃO DO ARTIGO: *Syzygium aromaticum* ESSENTIAL OIL SUPPLEMENTATION DURING *IN VITRO* BOVINE OOCYTE MATURATION IMPROVES PARTHENOGENETIC EMBRYONIC DEVELOPMENT À REVISTA : THERIOGENOLOGY 02 – JUN – 2018

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ANEXO B – COMPROVANTE DE SUBMISSÃO DO ARTIGO: THE BENEFICIAL EFFECT OF ESSENTIAL OIL OF *Syzygium aromaticum* ON BOVINE EPIDIDYMAL SPERM À REVISTA: REPRODUCTION IN DOMESTIC ANIMALS 06 – JUN – 2018

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APÊNDICES

APÊNDICE A: Resumo científico apresentado no Encontro de Biotecnologia do Nordeste – RENORBIO, realizado em Natal-RN, de 8 a 11 de agosto de 2017.

Avaliação do óleo essencial de cravo-da-índia (*Syzygium aromaticum*) na maturação *in vitro* de oócitos bovinos

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A produção *in vitro* de embriões (PIVE) vem sendo amplamente utilizada em programas de produção bovina com eficiência variável. O estresse oxidativo é um dos fatores que influencia nessa eficiência, pois pode causar alterações sobre as características dos gametas. Nesse contexto, o uso de antioxidantes naturais em meios de PIVE, especialmente na etapa da maturação *in vitro* (MIV), pode ser uma alternativa para minimizar os efeitos negativos do estresse oxidativo e aumentar as taxas de oócitos maturados. Assim, o óleo essencial de *Syzygium aromaticum* (OESA) apresenta grande potencial para essa finalidade. Portanto, o objetivo do presente trabalho foi analisar o efeito de OESA em diferentes concentrações (10, 15 e 20 µg/mL) sobre a taxa de maturação de oócitos bovinos avaliada pela presença do primeiro corpúsculo polar (1CP) e placa metafásica. Para tanto, ovários foram obtidos em abatedouro local e transportados até o laboratório (NaCl 0,9% a 35–37ºC por 1 h) onde foram submetidos a aspiração folicular usando com agulha (21G) e seringa (5,0 mL). Em seguida, oócitos foram recuperados e apenas àqueles apresentando uma ou mais camadas de células do *cumulus* compactas e citoplasma homogêneo foram selecionados para a MIV. Os oócitos foram divididos aleatoriamente em quatro grupos experimentais: controle com antioxidante sintético, a cisteamina (CC), 10 µg/mL de OESA (OESA10), 15 µg/mL de OESA (OESA15) e 20 µg/mL de OESA (OESA20). Para a MIV, oócitos foram incubados por 24 h (5% de CO_2 e atmosfera úmida) em meio TCM 199 com 2,2 g/L de bicarbonato de sódio, 0,2 mM de piruvato de sódio, 1% de solução de antibiótico-antimicótico, 20 µg/mL de FSH/LH, 10% de soro fetal bovino e acrescido de antioxidante de acordo com cada grupo. Após a MIV, as

células do *cumulus* foram removidas por sucessivas pipetagens e foi realizada a análise do 1CP nos oócitos em estereomicroscópio. Posteriormente, oócitos foram fixados em paraformaldeído (4%), marcados com Hoechst 33342 (1,0 µg/mL; 15 min) e em microscópio de fluorescência foi identificada a presença ou ausência da placa metafásica. Os dados foram expressos como média percentual ± erro padrão e analisados pelo teste exato de Fisher ($P < 0,05$). Após três repetições, um total de 82 ovários foi obtido, resultando em 304 oócitos viáveis recuperados e perfazendo uma média de 3,7 oócitos viáveis por ovário. Quanto à taxa de maturação pela avaliação do 1CP, não houve diferença entre os grupos CC (65,6% ± 3,5), OESA10 (69,6% ± 14,0), OESA15 (60,7% ± 6,2) e OESA20 (60,0% ± 15,1). Em relação à presença de placa metafásica com a marcação fluorescente, os grupos também foram estatisticamente semelhantes (CC: 69,4% ± 2,9; OESA10: 78,0% ± 9,8; OESA15: 66,7% ± 3,7; OESA20: 65,6% ± 8,6). Portanto, pode-se concluir que, independente da concentração, o óleo essencial de cravo-da-índia apresentou efeito similar à cisteamina quanto à maturação nuclear de oócitos bovinos.

Suporte financeiro: CNPq e CAPES.

Palavras Chaves: Produção *in vitro* de embriões, antioxidantes naturais, maturação nuclear.

APÊNDICE B: Resumo científico apresentado no XXIII Seminário de Iniciação Científica da UFERSA – SEMIC, realizado em Mossoró-RN, de 11 a 12 de dezembro de 2017.

Avaliação da eficiência antioxidante do óleo essencial do cravo (*Syzygium aromaticum*) sobre a viabilidade das células do *cumulus* de oócitos maturados bovinos

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A produção *in vitro* de embriões (PIVE) é comumente utilizada para melhorar aspectos científicos e comerciais da produção animal. Apesar disso, essa biotécnica ainda apresenta fatores limitantes, como o estresse oxidativo que ocorre devido às condições *in vitro*. Para evitar os danos que podem ser causados por espécies reativas de oxigênio tem sido realizada a suplementação dos meios, especialmente na etapa de maturação *in vitro* (MIV), com compostos antioxidantes. Nesse contexto, um antioxidante natural que apresenta grande potencial para uso na PIVE é o óleo essencial de cravo-da-índia (*Syzygium aromaticum*). Portanto, o objetivo do trabalho foi avaliar o efeito de diferentes concentrações do óleo essencial de *Syzygium aromaticum* (OESA) sobre a maturação de oócitos bovinos e viabilidade das células do *cumulus* (CCs). Para tanto, oócitos viáveis recuperados foram divididos aleatoriamente para a MIV em cinco grupos experimentais: controle com antioxidante sintético, cisteamina (CC), controle sem OESA (OESA0), 10 µg/mL de OESA (OESA10), 15 µg/mL de OESA (OESA15) e 20 µg/mL de OESA (OESA20). Após a MIV, oócitos foram avaliados quanto à presença do primeiro corpúsculo polar (1CP) e viabilidade das CCs. Para a análise estatística, todos os dados foram expressos como média percentual ± erro padrão e as diferenças consideradas significativas quando $P<0,05$. Para a presença de 1CP foi utilizado o teste exato de Fisher e para a viabilidade das CCs foi utilizado o teste do chi-quadrado. Após três repetições, um total de 82 ovários foi obtido, resultando em 304 oócitos viáveis recuperados e perfazendo uma média de 3,71 oócitos viáveis por ovário.

Quanto à presença do 1CP, não foi observada diferença entre as suplementações (CC: 65,6% ± 3,5; OESA0: 68,4% ± 3,4; OESA10: 69,6% ± 14,0; OESA15: 60,7% ± 6,2; e OESA20: 60,0% ± 15,1). Já a viabilidade das CCs foi significativamente inferior para os grupos OESA10 (49,4% ± 6,1) e OESA15 (49,7% ± 8,5), quando comparado aos grupos cisteamina (58,3% ± 4,9), OESA0 (58,5% ± 9,3) e OESA20 (58,6% ± 10,0). Em conclusão, pode-se afirmar que a presença de OESA a 20 µg/mL durante a MIV de oócitos bovinos contribuiu para taxas de maturação semelhantes à cisteamina, antioxidante sintético.

Agência Financiadora: CNPq. UFERSA. CAPES.

Palavras-chave: Produção *in vitro* de embriões; oócit
ovinos.