UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE CIÊNCIAS BIOMÉDICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM IMUNOLOGIA E PARASITOLOGIA APLICADAS

Avaliação dos efeitos *in vitro* dos antígenos solúveis de *Neospora caninum* e *Toxoplasma gondii* na qualidade do sêmen bovino

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UBERLÂNDIA-MG

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Tese apresentada ao Colegiado do Programa de Pós-Graduação em Imunologia e Parasitologia Aplicadas como requisito parcial para obtenção do título de Doutor.

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Orientador: Prof. Dr Marcelo Emílio Beletti

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Em 29 de agosto de 2024, às 8 horas, reuniu-se por Webconferência, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação, composta pelos seguintes membros titulares: André Belico Vasconcelos - UNIUBE; Letícia Zoccolaro Oliveira - UFMG; Neide Maria da Silva - ICBIM/UFU; Caroline Martins Mota - IBTEC/UFU e pelo presidente, Prof. Marcelo Emilio Beletti - ICBIM/UFU orientador(a) do(a) candidato(a).

Iniciando os trabalhos o(a) presidente da mesa, Dr(a). Marcelo Emílio Beletti, apresentou a Comissão Examinadora e o candidato(a), agradeceu a presença do público, e concedeu ao discente a palavra para a exposição do seu trabalho. A duração da apresentação do discente e o tempo de arguição e respostaforam conforme as normas do Programa.

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DEDICATÓRIA

Aos meus avós que sempre estarão presentes em meu coração

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RESUMO

Toxoplasma gondii é um parasita intracelular obrigatório que afeta humanos e vários hospedeiros vertebrados; este protozoário tem um ciclo de vida complexo no qual gatos e outros membros da família Felidae participam como hospedeiros definitivos. As infecções por este parasita são prevalentes em humanos e animais em todo o mundo, tanto os seres humanos quanto outras espécies são infectados via transplacentária durante a gravidez, ou após o nascimento, ao ingerir cistos teciduais de carne malcozida, alimentos e bebidas contaminados com oocistos ou oocistos do ambiente acidentalmente. Atualmente, esta doença é considerada uma importante causa de perdas reprodutivas em ovelhas, cabras, vacas e porcos criados em fazendas. Já o *Neospora caninum* é um parasita distribuído mundialmente; este protozoário tem um ciclo semelhante ao do *T. gondii* no qual os cães participam como hospedeiros definitivos. Este protozoário pode ser transmitido através da ingestão de alimentos contaminados ou por via transplacentária durante a gravidez. A infecção por esse parasita é um importante fator que desafia o desenvolvimento saudável da pecuária causando grandes perdas econômicas em todo o mundo devido ao aborto, morte fetal e doenças do sistema nervoso em bovinos. Sabe-se que animais infectados eliminam DNA, proteína e mesmo formas infectantes de *N. caninum* e *T. gondii* no sêmen, contudo não se sabe exatamente qual o efeito do contato de debris celulares deste parasita sobre a qualidade do sêmen. Este estudo teve como objetivo avaliar os efeitos *in vitro* dos antígenos solúveis de *N. caninum* e *T. gondii* na qualidade do sêmen bovino. Os espermatozoides foram tratados com antígenos de *N. caninum*, em dupla diluição seriada classificada em dose alta, média e baixa (8, 4, 2µg/ml) em meio "TALP-SPERM e TALP-FERT". Após os tratamentos, os espermatozoides foram avaliados quanto a motilidade, alterações da cromatina, integridade da membrana citoplasmática e do acrossoma e atividade mitocondrial. Os resultados mostraram que os antígenos solúveis de *T. gondii* e *N. caninum* afetam a velocidade e angulação média da trajetória dos espermatozoides na ausência e presença de fluxo em um ambiente simulado da tuba uterina bovina. Foi demonstrado que o tratamento com antígenos de ambos os parasitas causa alterações na integridade da cromatina espermática, danos severos à membrana e ao acrossoma dos espermatozoides, bem como diminui a atividade mitocondrial. Finalmente, foi avaliada a taxa de fertilização com a utilização da dose média de antígenos como tratamento nos espermatozoides a ser utilizada na produção *in vitro* de embriões (PIVE). No caso do tratamento com antígenos

solúveis de *N. caninum*, os resultados mostraram que foram afetadas a taxa de clivagem e de blastocisto, e com o tratamento de antígenos solúveis de *T. gondii*, só foi afetada a taxa de blastocisto. Considerando todos os resultados, conclui-se que touros após serem infectados por estes parasitas podem apresentar alterações em seus espermatozoides, o que pode comprometer o processo de fertilização e o desenvolvimento embrionário, afetando sua fertilidade.

Palavras-chaves: *T. gondii, N. caninum,* antigenos, fertilidade, espermatozoide.

ABSTRACT

Toxoplasma gondii is an obligate intracellular parasite that affects humans and several vertebrate hosts; this protozoan has a complex life cycle in which cats and other members of the Felidae family participate as definitive hosts. Infections by this parasite are prevalent in humans and animals worldwide; both humans and other species are infected transplacentally during pregnancy, or after birth, by ingesting tissue cysts from undercooked meat, food, and beverages contaminated with oocysts, or oocysts from the environment accidentally. This disease is currently considered an important cause of reproductive losses in sheep, goats, cows, and pigs raised on farms. *Neospora caninum* is a parasite distributed worldwide; this protozoan has a cycle like that of *T. gondii* in which dogs participate as definitive hosts. This protozoan can be transmitted through the ingestion of contaminated food or transplacentally during pregnancy. Infection by this parasite is an important factor that challenges the healthy development of livestock, causing great economic losses worldwide due to abortion, fetal death, and diseases of the nervous system in cattle. It is known that infected animals eliminate DNA, protein and even infective forms of *N. caninum* and *T. gondii* in semen, however, it is not known exactly what the effect of contact with cellular debris of this parasite on semen quality is. This study aimed to evaluate the in vitro effects of soluble antigens of *N. caninum* and *T. gondii* on the quality of bovine semen. Sperm were treated with *N. caninum* antigens in double serial dilution classified as high, medium and low doses (8, 4, 2 µg/ml) in "TALP-SPERM and TALP-FERT" medium. After treatments, sperm were evaluated for motility, chromatin alterations, integrity of the cytoplasmic membrane and acrosome and mitochondrial activity. The results showed that soluble antigens of T. gondii and N. caninum affect the speed and mean angle of sperm trajectory in the absence and presence

of flow in a simulated environment of the bovine fallopian tube. It was demonstrated that treatment with antigens of both parasites causes changes in sperm chromatin integrity, severe damage to the sperm membrane and acrosome, as well as decreased mitochondrial activity. Finally, the fertilization rate was evaluated using the mean dose of antigens as treatment in sperm to be used in in vitro embryo production (IVEP). In the case of treatment with soluble antigens of N. caninum, the results showed that the cleavage and blastocyst rates were affected, and with treatment with soluble antigens of T. gondii, only the blastocyst rate was affected. Considering all the results, it is concluded that bulls after being infected by these parasites may present changes in their sperm, which may compromise fertilization and embryonic development, affecting their fertility.

Keywords: *T. gondii*, *N caninum,* antigens, fertility, sperm.

LISTA DE ABREVIATURAS E SIGLAS

NLA- *N. caninum* antigen lysate

TEM - Transmission electron microscopy

DNA- Deoxyribunuclei acid

PSA- Pisum sativum lectins

PI- Propidium iodite

TA- Toulidine blue

PBS- Buffer fosfato

HOS- hypo-osmotic swelling

ZP- Zone pellucida

ATP- Adenosine triphosphate

OS- Oxidative stress

IVP- *In vitro* embryo production

HD- Definitive host

GPI- Glycosyl-phosphatidylinositol

SAGs-Surface antigens

MIC- Microneme complexes

MJ-Moving junction

RON- Rhoptry neck

ROP- Rhoptry antigens

PV- Parasitophorous vacuole

PVM- Parasitophorous vacuole membranes

IFAT- Immunifluorescent antibody test

Nc-p- Surface protein of *N. caninum* tachyzoites

ISCOMS- Immunostimulating complexes

SDS-PAGE- Polyacrylamide-sodium dodecyl sulfate

Ca++- Calcium channels

SAAF- Sperm activating and attracting factor

PAN- Atrial natriuretic peptide

SAP- Sperm activating peptide

GC- Guanylyl Cyclas

SUMÁRIO

APRESENTAÇÃO

Este trabalho está estruturado por capa, dedicatória, agradecimentos, resumo, lista de abreviaturas e siglas, resumo, capítulo número I (revisão bibliográfica, justificativa do trabalho, objetivos gerais e específicos, referências) capítulo número II (dois artigos anexados submetidos para publicação)

CAPÍTULO I

Revisão bibliográfica

INTRODUÇÃO

Características gerais de *T. gondii*

Este parasita é um protozoário intracelular obrigatório que pertence ao filo Apicomplexa, (Blader et al., 2015). Apresenta um ciclo de vida complexo no qual, animais praticamente de sangue quente inclusive humanos podem atuar como hospedeiros intermediários. (Fais et al., 2020). O ciclo de vida de *T. gondii* inclui uma fase assexuada e sexuada, (Dubey, 1998; Dubey, 2020). A fase sexuada, que ocorre nos hospedeiros definitivos, começa quando ocorre a ingestão de cistos contendo bradizoítos pelos felinos, levando a liberação de parasitos na mucosa gástrica que migram para o epitélio intestinal, onde se replicam por esquizogonia e formam esquizontes. O núcleo dos esquizontes inicia lentamente sua individualização através da divisão da membrana plasmática, originando os merozoítos. Estes, por sua vez, dão origem aos gametas, e havendo fecundação, formam-se oocistos. Após cair na luz intestinal, os oocistos são liberados para o meio ambiente juntamente com as fezes destes animais. A ingestão de alimentos contaminados, tanto com cistos quanto com oocistos, pode levar a infecção de hospedeiros intermediários, pois estes liberam bradizoítos e esporozoítos, respectivamente, que em seguida vão se transformar em taquizoítos capazes de invadir e se disseminar pelas células do indivíduo, ocorrendo a fase assexuada (Montoya; Liesenfeld, 2004; Robert-Gangneux, 2014; al-Malki, 2021).

Já se passaram 100 anos desde a descoberta e nomeação do *T. gondii*. O parasita foi encontrado pela primeira vez em animais de laboratório. Sua importância médica permaneceu desconhecida até 1939, quando o *T. gondii* foi identificado conclusivamente em tecidos de uma criança infectada congenitamente na cidade de Nova York, EUA, e sua importância veterinária tornou-se conhecida quando se descobriu que causava aborto em ovelhas em 1957 na Austrália (Dubey e Jones., 2008).

As infecções por *T. gondii* são prevalentes em humanos e animais em todo o mundo. Os felídeos são as espécies animais-chave no ciclo de vida deste parasita porque são os hospedeiros que podem excretar o estágio ambientalmente resistente, o oocisto. Os seres humanos são infectados após o nascimento ao ingerir cistos teciduais de carne malcozida, consumir alimentos ou bebidas contaminados com oocistos ou ingerir acidentalmente oocistos do ambiente. No entanto, apenas uma pequena porcentagem de humanos adultos expostos ou outros animais desenvolvem sinais clínicos da doença. Não se sabe se a

gravidade da toxoplasmose em hospedeiros imunocompetentes se deve à cepa do parasita, variabilidade do hospedeiro ou outros fatores. Recentemente, a atenção tem sido focada na variabilidade genética entre isolados de *T. gondii* de hospedeiros aparentemente saudáveis e doentes. (Dubey e Jones., 2008).

1.2. Características gerais de *N. caninum*

N. caninum é um protozoário intracelular obrigatório, detectado em 1984 no sistema nervoso central e músculo esquelético de cães na Noruega (Duarte et al., 2023). Os cães e os coiotes são os hospedeiros definitivos devido a liberação de oocistos nas fezes. Este parasita compartilha muitas características morfológicas e biológicas com seu parente próximo *T. gondii* (Dubey et al., 2007; Dubey e Schares, 2011).

O *N. caninum* tem um ciclo de vida heteróxeno (o ciclo só é completado quando possuem dois ou mais hospedeiros) com estágio sexual ocorrendo no intestino do hospedeiro definitivo (HD), podendo ser transmitido por meio das fezes, (Dias et al,.2014). Antes de 1988, *N. caninum* foi diagnosticado erroneamente como *T. gondii* devido à sua semelhança estrutura. No entanto, devido a diferenças ultraestruturais distintas, como a espessura da parede do cisto e o número de róptrias, e diferenças a nível genético foi possível distinguir essas duas espécies uma da outra. O cultivo *in vitro* também recentemente forneceu informações detalhadas sobre o processo de adesão e invasão de células endoteliais da aorta bovina por taquizoítos de *N. caninum*, modificações químicas e enzimáticas das superfícies das células endoteliais e parasitárias revelaram que o contato entre *N. caninum* e células endoteliais como o próprio processo de invasão foi um evento mediado por receptores, a natureza substancial deste receptor-ligante, foi baseado em um sistema de interações proteína-proteína, em vez de interações proteína-carboidrato. Assim, em analogia ao *T. gondii* taquizoítos, proteínas de superfície do parasita de *N. caninum* tem um papel crucial durante a adesão e penetração do hospedeiro na membrana celular (Hemphill e Gottstein, 1996).

Este parasita tem distribuição global e causa aborto e mortalidade neonatal em bovinos, em cães se apresenta como uma doença neuromuscular grave, e no caso de ovinos e caprinos, já foram relatados aborto, natimortos e filhotes debilitados. Apesar da neosporose não ser considerada zoonose, existem evidências sorológicas divergentes em humanos e um relato molecular em sangue do cordão umbilical de crianças recém-nascidas (Duarte et al., 2023).

1.3. Antígenos de *T. gondii*

Os antígenos de *T. gondii* são encontrados na superfície da membrana celular do parasito e no citosol, bem como em organelas secretoras, como róptria, micronemas e grânulos de alta densidade liberados durante a invasão do parasita na célula hospedeira. Os antígenos de *T. gondii* também preenchem o interior de um vacúolo parasitóforo e cisto tecidual (Ferra, Holec-Gąsior e Grąźlewska, 2020). Em *T. gondii*, a superfície do estágio de taquizoíto é coberta por uma família de pelo menos oito antígenos ancorados a glicosil-fosfatidilinositol (GPI) chamados SAGs (antígenos de superfície) e SRSs (sequências relacionadas a SAG1) (Hehl, Krieger e Boothroyd, 1997, (Boothroyd *et al.*, 1998 Manger et al., 1998).

A produção dos antígenos MIC depende da concentração intracelular de íons cálcio. Estes antígenos formam complexos adesivos ou ocorrem na forma de proteínas únicas e são secretados por organelas secretoras especializadas chamadas micronemas. As funções desses antígenos incluem adesão e perturbação da integridade da membrana da célula hospedeira para facilitar a penetração do parasita dentro das células hospedeiras. MICs possuem motivos adesivos geralmente encontrados em proteínas eucariotas superiores (Tomley et al., 2001).

Além disso, desempenham um princípio funcional na fixação da célula hospedeira, motilidade, invasão e têm um papel sinérgico no processo infeccioso. Três complexos distintos de proteínas micronemas foram identificados até o momento, MIC6-MIC1-MIC4, MIC8-MIC3 e MIC2-M2AP. Os complexos MIC6-MIC1-MIC4 e MIC8-MIC3 são responsáveis pela adesão direcionada aos receptores de superfície das células hospedeiras, criando uma conexão entre o parasita e a célula hospedeira (Ferra, Holec-Gąsior e Grąźlewska, 2020). As proteínas micronemas que constroem o complexo MIC2-M2AP também desempenham um papel fundamental no movimento e penetração do parasita nas células hospedeiras. Outro antígeno importante é o AMA1, que, junto com as proteínas rhoptry neck (RON), forma uma conexão móvel, o chamado complexo “moving junction” (MJ). (Carruthers et al., 2007).

No caso do pescoço de róptria (RON), e antígenos de róptria (ROP) são um grupo de proteínas secretadas pelo pescoço ou bulbo de róptria que facilitam o dobramento coronal da célula hospedeira e a formação do vacúolo parasitóforo (PV). Todos os ROPs

contêm um peptídeo sinal e muitas têm pelo menos um domínio transmembranar previsto ou âncora GPI, sugerindo associação com membranas. Além disso, a análise proteômica da róptria levou à caracterização de proteínas localizadas especificamente no pescoço da róptria, ou seja, as RONs. Muitos ROPs e RONs contêm motivos repetidos que podem estar envolvidos nas interações proteína-proteína. Ressaltando que quase todas as informações sobre o conteúdo ou funções das roptrias vêm de estudos sobre o estágio de taquizoíta do *T. gondii* (Camejo *et al.*, 2014, Besteiro *et al.*, 2009). Praticamente, apenas uma ROP específica para bradizoítos foi descrita até o momento, sendo associados os antígenos ROP principalmente com a formação de PV e membranas de vacúolos parasitóforos (PVM), enquanto as proteínas RON estão principalmente envolvidas na criação do complexo MJ; sendo os complexos mais importantes AMA1-RON2-RON4/5/8, (Ferra, Holec-Gąsior e Grążlewska, 2020).

1.4. Antígenos de *N. caninum*

Foi demonstrado no passado que muitos dos antígenos identificados em parasitas apicomplexos relacionados estão associados a estruturas superficiais ou são encontrados localizados dentro das organelas secretoras, nomeadamente micronemas, roptrias e grânulos densos. Estas localizações implicam que algumas das moléculas correspondentes podem desempenhar funções importantes durante a entrada na célula hospedeira ou o desenvolvimento intracelular destes parasitas (Hemphill et al., 1999). A invasão celular é resumida em três etapas, sendo a primeira quando ocorre a interação inicial entre a célula hospedeira e o parasita, esta acontece sem qualquer orientação e envolve antígenos de superfície imunodominates (NcSAG1 e NcSRA2), (Ribeiro, 2008)

Estudos têm sido realizados para identificar e caracterizar em nível molecular componentes antigênicos específicos de *N. caninum*, a fim de melhorar o diagnóstico sorológico e aprimorar a visão atual sobre as muitas questões em aberto sobre a biologia celular desse parasita e suas interações com o hospedeiro a nível imunológico e celular (Hemphill *et al.*, 1999). Foram Identificamos duas proteínas de superfície de 29 e 35 kDa (designadas Ncp29 e Ncp35, respectivamente) de taquizoítos de *N. caninum* que são os antígenos predominantes reconhecidos por anti-soros de animais infectados por *Neospora*. Estudos de localização e marcação de superfície com biotina demonstraram que Ncp29 e Ncp35 são associados à membrana e exibidos na superfície do parasita (Howe *et al.*, 1998).

Além das proteínas anteriormente mencionadas, outra proteina que foi identificada como principal proteína de superfície dos taquizoítos de *N. caninum* foi A Nc-p43, conforme demonstrado por eletroforese em gel de poliacrilamida-dodecil sulfato de sódio (SDS-PAGE), Nc-p43 apresenta uma massa molecular de 43 kDa e anticorpos purificados por afinidade direcionados contra Nc-p43 não reagem com nenhum *T. gondii* (RHcepa). Essas proteínas associadas à superfície celular de *Neospora spp* desempenham um papel crucial durante a adesão e penetração da membrana da célula hospedeira (Hemphill, 1996). Esforços iniciais para desenvolver um ELISA de *N. caninum* foram prejudicados por problemas com baixa sensibilidade e especificidade quando comparado com o teste de referência IFAT. Esses problemas foram reduzidos quando foi extraído o detergente de antígenos de taquizoítos incorporados em complexos imunoestimulantes (iscoms), em vez de extratos de taquizoítos brutos foram usados como antígeno. Iscoms são estruturas semelhantes a gaiolas de 40 nm formadas por interação hidrofóbica. *N. caninum* iscom foi encontrado para consistir em um número restrito de proteínas em comparação com o taquizoíto bruto (Björkman e Hemphill, 1998).

1.5. Presença de *T. gondii e N. caninum* em sêmen de mamiferos

O primeiro relato do isolamento de *T. gondii* no sêmen foi no ano 1978 de um estudo feito em 1971 onde conseguiram recuperar este agente de amostras seminais de três entre 125 homens com infecção toxoplásmica natural. Mais tarde em 1982, 1988 e 2001 diferentes pesquisas reportaram a presença deste parasita em sêmen de ovinos, caprinos, bovinos e suínos infectados experimentalmente (Moura *et al.*, 2007). Estudos em várias dessas espécies forneceram resultados diferentes sobre os efeitos do *T. gondii* na qualidade do sêmen, relatando que a toxoplasmose afetou variáveis no sêmen de ratos machos (Terpsidis et al., 2009). observando em estudos mais atuais que poderia existe uma ligação entre a toxoplasmose e o número de espermatozoides morfologicamente anormais (Saki et al., 2020). Quanto ao *N. caninum,* foi demonstrado que é detectável no sêmen de touros infectados, mas, novamente, há pouca evidência epidemiológica sugerindo que esta é uma importante via de transmissão (Ortega-Mora et al., 2003). Uma vez que o DNA de *N. caninum* foi relatado em sêmen fresco e congelado de touros naturalmente e experimentalmente infectados, parece que *N. caninum* pode infectar órgãos reprodutivos (Serrano-Martinez et al. 2007). No caso de estudos feitos em carneiros, foi detectado o DNA de *N. caninum* em 34,4% das amostras de sêmen de

carneiros infectados experimentalmente (Amdouni *et al.*, 2019). Em outro estudo desenvolvido para avaliar lesões reprodutivas em camundongos machos BALB/c infectados com *N. caninum,* os achados demonstram que esse parasita podem induzir graus variados de danos espermatozoides e outras células dos camundongos machos, causar desequilíbrio hormonal, inibir a produção de espermatozoides e afetar a capacidade reprodutiva (Li *et al.*, 2021).

1.6. Impacto econômica

Nenhum estudo específico estimou ainda o impacto econômico global gerado pela neosporose no gado, dados regionais escassos indicam que perdas financeiras de custos de abortos e assistência médicas veterinária podem chegar a várias centenas de milhões de dólares por ano, mostrando que as perdas globais só pela infecção ou aborto por *N. caninum* podem chegar a US $ 2,38 bilhões anualmente (Reichel et. al 2013). No entanto, a infecção por *T. gondii* gera perdas econômicas na pecuária devido a diversos distúrbios reprodutivos, como morte embrionária precoce, aborto, natimorto e morte neonatal, principalmente em pequenos ruminantes (Buxton et al., 2007) (Dubey; Jones, 2008). Não está bem compreendido se esses distúrbios reprodutivos são devidos à contaminação externa por *T. gondii,* pela ingestão de oocistos ou por sêmen infectado. Tudo o que sabemos é que a presença de *T. gondii* já foi detectada no sistema genital masculino e no sêmen de varrascos e carneiros experimentalmente infectados (Moura et al., 2007), Terpsidis et al., 2009).

1.7. Espermatozoide

A célula germinativa masculina é produzida na gônada (testículo) por um processo permanente de divisão das células germinativas ou espermatogônias. O processo de divisão meiótica chamada espermatogênese é controlada hormonalmente pelo eixo Hipófise-hipotálamo- gônada. De cada espermatogônia se produzem quatro espermatócitos haploides que continuarão ligados uns aos outros por pontes citoplasmáticas e ao mesmo tempo está em comunicação com a célula de Sertoli; esta célula, a partir de moléculas sinalizadoras induzem o processo chamado de espermiogênese ou metamorfose, que irá converter os espermátides em espermatozoides (Olivera et al., 2006). Os espermatozoides são células polarizadas com uma cabeça, um flagelo e uma peça intermediária. A cabeça do espermatozoide pode ser subdividida em

quatro regiões: apical, pré-equatorial, equatorial e pós-equatorial. A cabeça do espermatozoide, envolvida na interação espermatozoide-ovócito, a peça intermediária com mitocôndrias, envolvidas na produção de energia e o flagelo, envolvido na motilidade. (Flesch e Gadella, 2000). O espermatozoide de mamíferos é 10 vezes menor quando comparado à célula somática, e por isso a organização do seu material genético é única, tornando sua cromatina altamente condensada e compactada (Miller et al., 2018).

1.8. Motilidade Espermática

A motilidade espermática é desencadeada por alterações no meio iônico extracelular, pela interação com ligantes específicos e pela glicose, presente no líquido seminal e no trato reprodutor feminino; essas alterações induzem sinais citosólicos flagelares, através da fosforilação de proteínas, canais de Ca++ e vias dependentes de nucleotídeos cíclicos (cGMP e cAMP). Os ligantes específicos mais conhecidos são: a progesterona e o esteroide sulfatado SAAF (Sperm Activating and Attracting Factor) que induzem a entrada de Ca++; peptídeo ativador de espermatozoides (SAP) e peptídeo natriurético atrial (PAN), que atuam através de um receptor de membrana ou por ativação direta da guanilil ciclase (mGC). Outros ligantes específicos são os fatores do tipo olfativo e odorante (hOR17-4) produzidos pelo oócito para induzir a quimiotaxia espermática (Olivera et al., 2006).

O padrão de movimento dos espermatozoides varia entre as espécies e de acordo com o ambiente físico em que os espermatozoides nadarem. É melhor definir a motilidade hiperativada em comparação com motilidade ativada para cada espécie em particular. A motilidade ativada é definida como se segue os espermatozoides no epidídimo, são inativos ou apenas fracamente móveis. Quando são lançados em plasma seminal in vivo ou em meio fisiológico *in vitro*, eles rapidamente começam a nadar vigorosamente em uma linha quase reta trajetória. A motilidade hiperativada é definida como a padrão de natação mostrado pela maioria dos espermatozoides recuperados de a ampola ovidutal na fertilização. Os flagelos de espermatozoides hiperativados são lançados em curvas mais profundas e sua batida é geralmente menos simétrica do que a de flagelos de espermatozoides ativados (Ho e Suarez, 2001)

1.9. Cromatina

A estrutura e a atividade genética do genoma são moduladas por dois grupos distintos de proteínas cromossômicas. As histonas, um grupo bem definido de proteínas

básicas ricas em arginina, lisina e histidina, ligam-se ao DNA por meio de interações hidrofóbicas e eletrostáticas que empacotam o DNA em a forma conhecida como cromatina. (Balhorn, 1982). Durante a espermiogênese dos mamíferos, as histonas somáticas, proteínas nucleares básicas, seriam substituídas, em parte ou totalmente, por nucleoproteínas específicas dos espermatozoides, denominadas protaminas ou proteínas queratinosas, as quais possuem um caráter mais básico, ricas em arginina e cisteína oxidada. Estudos mostraram que a permanência de histonas somáticas ou a ocorrência de anormalidades nas protaminas poderiam levar à formação de distúrbios de condensação da cromatina (complexo DNA-proteína) dos espermatozoides, a qual se torna frouxa com repercussões sobre a fertilidade (Beletti, 2013). Destacando que a unidade básica da cromatina de espermatozoides é o toroide de protamina, essa estrutura é formada por cerca de 50.000 pares de base de DNA firmemente enrolados pelas protaminas, formando uma estrutura em forma de "donut" (rosca) ou toroide. Essa constituição provavelmente é importante para a proteção e manutenção da estabilidade do DNA (Miller, 2018).

1.10. Relação integridade da cromatina e fertilidade

Estudos têm mostrado que qualquer forma de anormalidade da cromatina espermática ou dano ao DNA pode resultar em infertilidade masculina, e para fortalecer esta conclusão, foi observado que a fecundação in vivo diminui progressivamente quando mais do que 30% dos espermatozoides são identificados com danos no DNA (Agarwal, 2003). Esses espermatozoides que apresentam graves alterações cromatínicas podem apresentar alterações na forma da cabeça e, consequentemente, na sua hidrodinâmica, isso acaba por interferir na motilidade e no processo de fecundação. Outras alterações espermáticas menos graves poderiam não interferir na motilidade e consequentemente no processo de fecundação, porém possuiriam danos no DNA que impossibilitariam a união dos pró-núcleos masculino e feminino, por conseguinte, inviabilizariam o zigoto. Alterações ainda mais leves poderiam não interferir na fecundação e no desenvolvimento embrionário inicial, mas poderiam interferir em etapas posteriores do desenvolvimento embrionário o que poderia levar à morte embrionária com consequente reabsorção fetal ou aborto. Ainda em situações menos frequentes, a gestação poderia chegar a termo, mas os neonatos possuiriam alterações genéticas de diversos tipos e intensidades (Beletti, 2013; Martins et al., 2021).

1.11. Membrana Plasmática

Vários estudos destacam o importante papel da membrana plasmática do espermatozoide na fertilização, sendo esta uma estrutura muito dinâmica e levando em consideração que a membrana plasmática é essencial para que ocorra a interação espermatozoide-óvulo, não pode ser considerada esta estrutura apenas como a borda do espermatozoide. Também destacando que durante o trânsito do espermatozoide através do epidídimo, a membrana plasmática apresenta alterações, por exemplo, modificação e adsorção de proteínas e lipídios. O papel dessas alterações de superfície não é totalmente compreendido, embora algumas proteínas adsorvidas estejam envolvidas na ligação espermatozoide-oócito (Flesch e Gadella, 2000). A integridade das membranas e a estabilidade de seu aspecto semipermeável são pré-requisitos para a viabilidade do espermatozoide. Além disso, se a membrana plasmática está intacta, mas funcionalmente instável, o espermatozoide não é capaz de interagir com o ambiente do trato genital feminino e consequentemente fertilizar o ovócito (Batista et al, 2010).

JUSTIFICATIVA

N. caninum é um protozoário intracelular obrigatório que é parasita em muitas células de mamíferos (por exemplo, gado, ovelhas e cães). Este parasita causa principalmente aborto, morte fetal ou discinesia neonatal e doenças do sistema nervoso em fêmeas (por exemplo, cães e gado) *N. caninum* pode ser transmitido verticalmente, causando os mais graves danos ao gado e tornou-se um dos principais fatores que desafiam o desenvolvimento saudável da indústria pecuária (Jia et al., 2020). Quanto ao *T. gondii*, pertence ao filo Apicomplexa e à ordem dos coccídeos, sendo um protozoário intracelular obrigatório que afeta humanos e vários hospedeiros vertebrados. Como não existem espécies conhecidas de mamíferos ou aves resistentes à infecção por esse agente, a toxoplasmose é considerada uma das zoonoses mais difundidas no mundo (Lopes et al., 2013). Por outro lado, no que se refere à parte reprodutiva e principalmente às afetações espermáticas de acordo com as evidências relatadas, existe uma ligação entre a toxoplasmose e o número de espermatozoides morfologicamente anormais (Saki et al., 2020).

A possibilidade de transmissão de *N. caninum* através do sêmen pode implicar profundas repercussões no comércio de sementes de gado. A inseminação artificial é um procedimento importante para a melhoria da produção bovina e milhões de doses de sêmen bovino congelado são trocadas anualmente em todo o mundo aumentando a possibilidade de espalhar diferentes doenças entre as populações de gado, mesmo existindo pouca evidência epidemiológica sugerindo que esta é uma importante via de transmissão para *N. caninum* (Ortega-Mora et al., 2003). As estatísticas mostram que as perdas econômicas globais para o gado causadas pela infecção ou aborto por *N. caninum* podem chegar a US $ 2,38 bilhões anualmente (Reichel et al., 2013). No entanto, a infecção por *T. gondii* gera perdas econômicas na pecuária devido a diversos distúrbios reprodutivos, como morte embrionária precoce, aborto, natimorto e morte neonatal, principalmente em pequenos ruminantes (Buxton et al., 2007)(Dubey; Jones, 2008). Não está bem compreendido se esses distúrbios reprodutivos são devidos à contaminação externa por *T. gondii,* pela ingestão de oocistos ou por sêmen infectado. Tudo o que sabemos é que a presença de *T. gondii* já foi detectada no sistema genital masculino e no sêmen de varrascos e carneiros experimentalmente infectados (Moura et al., 2007; Terpsidis et al., 2009).

Já foi relatado a presença de DNA de *N. caninum* em sêmen fresco e congelado de touros naturalmente e experimentalmente infectados (Serrano et al., 2007). Assim como formas infecciosas de *T. gondii* no sêmen de humano, bovinos, caninos e ovelhas, (Moraes et al., 2010; Koch et al., 2016). Apesar da já comprovada existência desses parasitas na genitália de diferentes mamíferos e que animais infectados podem eliminar DNA, proteína e mesmo formas infectantes de *N. caninum e T. gondii*, não se sabe exatamente qual o real efeito do contato de debris celulares deste parasita sobre a qualidade do sêmen. Por tanto, este estudo teve como objetivo avaliar as alterações causadas pelos antígenos solúveis de *N. caninum e T. gondii* na qualidade do espermatozoide bovino.

OBJETIVOS

3.1. Objetivo geral

Avaliar os efeitos *in vitro* dos antígenos solúveis de *N. caninum* e *T. gondii* na integridade do espermatozoide bovino

3.2. Objetivos Específicos

- Avaliar as alterações da motilidade e angulação espermática causado por antígenos solúveis de *N. caninum e T. gondii.* na ausência e nas diferentes condições de fluxo em medio TALP-Sperm e Talp-Fert em um ambiente simulado da tuba uterina bovina;
- Identificar possíveis alterações na qualidade da cromatina espermática causado por antígenos solúveis de *N. caninum e T. gondii;*
- Avaliar alterações na integridade da membrana espermática espermática causado por antígenos solúveis de *N. caninum e T. gondii;*
- Identificar possíveis alterações na integridade do acrossoma espermático espermática causado por antígenos solúveis de *N. caninum e T. gondii;*
- Avaliar alterações na atividade mitocondrial espermática causado por antígenos solúveis de *N. caninum e T. gondii;*
- Analisar o efeito dos antígenos solúveis de *N. caninum e T. gondii* na capacidade de fecundação dos espermatozoides na FIVI.

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CAPÍTULO I

ARTIGO 1

The quality and characteristics of bovine sperm are compromised by *Toxoplasma gondii* antigens, impacting in *in vitro* bull fertility

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Abstract

Studies in various species have demonstrated different results on the effects of *T. gondii* infection on sperm quality. It has also been demonstrated that in some stages of the disease, there is elimination of cellular debris or even the intact parasite in the semen. The present work aimed to evaluate the *in vitro* effects of the presence of soluble *T. gondii* antigens in bovine semen on sperm integrity. The spermatozoa were treated with *T. gondii* antigens in double serial dilutions classified as high, medium and low doses (8, 4, 2 µg/ml) in "TALP-Sperm" and "TALP-Fert" media. The results showed that *T. gondii* antigens affect sperm motility and mitochondrial activity, and cause changes in sperm chromatin integrity, as well as damage to the sperm membrane and acrosome. Finally, spermatozoa treated with *T. gondii* antigens were evaluated in the *in vitro* production of embryos (IVEP). The use of semen contaminated with antigens in IVEP routines did not lead to a decrease in the fertilization of oocytes, as sperm undergo selection before *in vitro* fertilization, which eliminates the most altered sperm. However, early embryonic development was affected, probably by structural changes that were not eliminated in the selection process. The results demonstrated that the presence of soluble *T. gondii* antigens in bovine semen alters sperm integrity and vital characteristics for the fertilization process and embryonic development and therefore causes fertility problems in males.

Keywords: Toxoplasmosis; sperm; *in vitro* fertilization; cattle

1. Introduction

T. gondii is an obligate intracellular protozoan that belongs to the apicomplexan phylum and the order of coccidia (Blader et al., 2015). There are no known species of mammals or birds resistant to infection by this agent, and toxoplasmosis is considered one of the most widespread zoonoses in the world (Lopes et al., 2013). This parasite has a complex life cycle, in which the definitive host is felines and warm-blooded animals, including humans, can act as intermediate hosts. (Fais et al., 2020).

It has been 100 years since the discovery and naming of *T. gondii,* and its veterinary importance became known when it was found to cause abortion in sheep in 1957 in Australia (Dubey and Jones, 2008). This protozoan causes economic losses in livestock farming due to several reproductive disorders, such as early embryonic death, abortion, stillbirth, and neonatal death, mainly in small ruminants (Buxton et al., 2007; Dubey and Jones, 2008). It is not well understood whether these reproductive disorders are due to external contamination by *T. gondii,* by ingestion of oocysts, or by infected semen. The presence of *T. gondii* has been detected in the male genital system and inthe semen of pigs (Moura et al., 2007), rats (Terpsidis et al., 2009) and sheep (Moraes et al., 2010; Lopes et al., 2013). Forms of this parasite have also been identified in the semen of rabbits (Liu et al., 2006), cattle (Scarpelli et al., 2009), humans (Flegr et al., 2014), goats (Wanderley et al. 2015), and canines (Koch et al., 2016).

Studies on the effects of *T. gondii* on sperm quality in various species have shown different results. Terpsidis (2009, pp238–241) reported severe changes in the semen of experimentally infected rats. A link between toxoplasmosis and the number of morphologically abnormal sperm has also been observed in mice (Saki et al., 2020).

Hlaváčová et al. (2021, pp854–862) suggested that latent toxoplasmosis affects certain parameters of human semen (sperm count and motility) but does not appear to affect semen morphology and volume.

DNA of *T. gondii* has been found in the semen of some animals, without intact parasites being found, which suggests that cellular debris from this parasite may exist in the semen in certain stages of toxoplasmosis (Scarpelli et al., 2009; Bezerra et al., 2014; Wanderley et al., 2015). However, it is not known exactly how the presence of this cellular debris in semen affects various sperm characteristics, such as membrane and acrosome integrity, mitochondrial activity, chromatin compaction, and sperm motility, especially rheotaxic behavior. Recent work has shown that this behavior is very important for male fertility, and is important for directing the sperm to the oocyte in the fallopian tube. This behavior is even used to select sperm for the *in vitro* production of bovine embryos, leading to better early embryonic development (Yaghoobi et al., 2024). This study aimed to evaluate changes in these characteristics of bovine sperm caused by soluble *T. gondii* antigens.**2. Materials and methods**

2.1. Semen samples

Frozen semen samples were used from a single ejaculate of a Nellore bull, acquired from a semen processing center. This ejaculate has already been tested in the Reproduction Biology Laboratory at the Federal University of Uberlândia (UFU), demonstrating excellent results in the *in vitro* production of embryos.

2.2. *T. gondii* antigen production

Soluble *T. gondii* antigen (STAg) was produced as described in previous studies, with minor modifications. Briefly, suspensions of *T. gondii* strain RH obtained from cell culture were adjusted to 1×10^8 tachyzoites/mL and subjected to freeze-thaw cycles and sonication in the presence of protease inhibitors. The preparation was centrifuged at 10,000 × g for 30 min at 4°C; the supernatants were collected, and aliquots were stored at -20°C until use in different procedures (Santana et al., 2021).

2.3. Semen dilution media

The dilution and maintenance of spermatozoa were performed with sperm capacitation medium (TALP-Fert) and sperm dilution medium (TALP-Sperm), which are routinely used in laboratories for *in vitro* production of bovine embryos. To verify the effects on spermatozoa, frozen bovine semen samples were diluted (10 to 20 million spermatozoa/mL) in TALP-Sperm or TALP-Fert medium supplemented with *T. gondii* antigens via serial double dilution (8 µg/ml), (4 µg/ml), or (2 µg/ml) or as a control medium (without antigen) and subsequently incubated for one hour at 37 ℃, 5% CO2 and saturated humidity.

Since it is almost impossible to be certain of a physiological dose or level of circulating antigens during T. gondii infection, since the parasite burden in the testicles are not well defined up to now and the parasitemia levels are variable between in the different stages of the infection, the parasite dose and strain virulence. We chose to use a concentration of the antigen that ranged around the most common dose used in the literature – 5 ug/ml. This dose has been used to stimulate different cell types and tissues, including studies on congenital infections (Vallochi et al., 2001, Scanga et al., 2002, Gómez-Chávez et al., 2019, Gómez-Chávez et al., 2020).

2.4. Flow chamber

The flow chamber was constructed of glass and silicone with the following dimensions: 32 mm × 1.5 mm × 0.2 mm (length × width × height). The base is a common glass microscope slide, and the sides are two halves of a coverslip (24 mm × 32 mm) fixed with silicone on the slide and maintaining a central distance of 1.5 mm. An entire coverslip was also glued onto this set with silicone. Thus, considering that the coverslip is 0.17 mm high and the silicone layer is approximately 0.02 mm, there will be a tunnel with a rectangular profile measuring approximately 0.3 mm^2 (Figure 1). For flow control, a piece of filter paper (20 mm × 30 mm) moistened with the same medium as the tested sample (TALP-Sperm or TALP-Fert) was placed at each end of the chamber. After homogenization, the bovine semen samples were placed in the flow chamber until they were completely filled. After complete stabilization of the mixture inside the chamber, the flow was controlled by dripping the medium onto the ends of the paper.

Before using the flow chambers we built, several tests were carried out which demonstrated the repeatability of the analyses (coefficient of variation between replicates of around 4.0%). In this study, the coefficient of variation in the control group was around 6.0%. However, this coefficient increased in the treated groups, reaching around 19%. These data show that our device has sufficient repeatability to be used in scientific experiments.

2.5. Sperm image acquisition

The behavior of bull spermatozoa in the presence or absence of flow was recorded on a Leica DM500 microscope coupled to a Leica ICC50 image capture system with a 10X objective. Images were acquired in AVI format and stored for further processing and quantitative analysis.

2.6. Motility pattern analysis

ImageJ FIJI software (version 1.53q) was used for image processing. Using the Manual Tracking plugin, the sperm trajectories were manually traced frame by frame, with a human operator marking the position of the sperm on the X/Y axis in each recording frame. The generated data were processed by an algorithm developed in the MATLAB programming environment. In an environment with flow, only spermatozoa that presented rheotaxis were considered. In an environment without flow, progressively motile spermatozoa were analyzed. The flow velocity, $\vec{v}_F$, was estimated by following the particle trajectories (cell debris and dead spermatozoa) at the same focal level of spermatozoa. For greater precision, this velocity was estimated for several particles along the same video sequence to obtain an interpolated vector field $\vec{v}_F(d)$, defined in terms of the distance d from the flow chamber wall. The velocity of each sperm, $\vec{v}_S$,, was estimated following the center of mass of the respective image of the sperm head. The two absolute velocities $\vec{v}_F$ and $\vec{v}_S$, were computed considering the subsequent frames by calculating the first order derivative referring to the displacement concerningn relation to time. The relative sperm velocity was then obtained by $\vec{v}_{R=}\vec{v}_S + \vec{v}_F(d)$, where d is the distance of the specific sperm to the flow chamber wall. All speeds were measured as the number of pixels that each structure covered in one second (pixel/s).

2.7. Toluidine blue staining technique for analysis of sperm chromatin integrity

Semen smears were taken from all treatment groups and fixed in 3:1 (v/v) ethanol/acetic acid for one minute and then in 70% ethanol for three minutes. After fixation, the smears were subjected to acid hydrolysis in 4 N hydrochloric acid for 25 minutes and subsequently washed in distilled water. After drying, the smears were stained by placing a

drop of 0.025% toluidine blue (TA) solution in citric acid-phosphate buffer (McIlvaine buffer) at pH 4.0. Immediately after, a cover slip was placed on the slide, and the slide was sealed with nail polish. After 3 minutes, digital images were captured, totaling at least 100 spermatozoa per slide. For this purpose, a Leica DM500 light microscope coupled to a Leica ICC50 camera (Leica Microsystems Inc., Buffalo Grove, IL, USA) was used with a 100x objective (immersion).

2.8. Computational analysis of images

The computerized image analyses were divided into two stages: segmentation of the heads and quantitative analysis of the percentage decompression and mean chromatin heterogeneity of each head. For segmentation of the heads, software developed in the MATLAB programming environment and executed by the Octave program was used (Hiraiwa et al., 2015). To evaluate the mean intensity of chromatin decompaction and heterogeneity, software developed in the SCILAB programming environment (Beletti et al., 2005) was used.

2.9. Hyposmotic test

The test consisted of incubating a solution consisting of 75 µL of semen after the four different treatments and 475 µL of hyposmotic solution (MLQ water) in a water bath at 37 ℃ for 30 minutes. Wet preparations were made from each of the samples, and 200 cells were evaluated. Sperm with an intact membrane were considered those with a curled tail and were considered to have damaged membrane, those spermatozoa that remained with straight tails. The evaluations were performed with a Leica DM500 light microscope (Leica Microsystems Inc., Buffalo Grove, IL, USA) using a 40x objective. The hyposmotic test was performed according to Papa et al., (2014).

2.10. Transmission electron microscopy

The samples of each treatment were centrifuged at 50 × g, the supernatant was discarded, and a 2.5% glutaraldehyde fixative solution was added to 0.1 M phosphate buffer (pH 7.4) for approximately 4 hours. After fixation, the supernatant was discarded to remove the fixatives, the volume was replaced with phosphate buffer solution (pH 7.4), and the microtube was shaken. The sample was again subjected to centrifugation at 50 × g for 5 minutes, after which the supernatant was discarded. The procedure was repeated three times. After the last wash, 500 μL of 2% osmium tetroxide solution and 500 μL of phosphate buffer solution (pH 7.4) were added to the microtubes for 30 minutes. Again, centrifugation was performed at 50 × g for 5 minutes, after which the supernatant was discarded.

Subsequently, the removal of osmium tetroxide was carried out with a phosphate buffer solution in a way similar to that used for the removal of glutaraldehyde. After the last wash, 2.5% agar at 50°C was added to the cell precipitate, which was mixed to introduce the agar between the cells. The cells were kept at the bottom of the microtube and placed in refrigerator (4°C) until solidification. The solidified agar was removed from the microtube and broken into 1 mm3 fragments. The fragments were placed in glass flasks and dehydrated in baths of increasing concentrations of alcohol followed by three baths of propylene oxide. After discarding the propylene oxide from the last dehydration step, the fragments were finally added to Epon resin. After solidification (polymerization) in the oven, the blocks were trimmed, and cuts were made with a Reichert-Jung ultramicrotome (Leica Microsystems Inc., Buffalo Grove, IL, USA) with a thickness of 60 to 90 nm. The blocks were subsequently placed on 200 mesh copper grids and stained with uranyl acetate and lead citrate as described by Bozzola and Russel (1998, pp670). The sections were analyzed and imaged witha Hitachi HT 7700 electron microscope, and digital images of the spermatozoa heads were obtained.

2.11 Analysis of the integrity of the plasma membrane

The sperm were incubated in TALP-Sperm medium supplemented with soluble *T. gondii* antigens at three concentrations (8, 4 and 2 µg/ml) and medium only (no antigen) as a control at 37°C, 5% CO2 and saturated humidity. After one hour of incubation, centrifugation was performed at 16 × g for 5 minutes, with subsequent disposal of the supernatant. Fifteen microliters of the cell precipitate suspension were placed in 200 µL of phosphate buffer (PBS) heated to 37°C with 5 µL of propidium iodide (PI) stock solution (50 ml of PBS in 0.01 g of PI). This solution was incubated for 5 minutes at room temperature. For the evaluation, a drop of the solution was placed on slides preheated to 37°C and covered with a coverslip. Then, images were captured for a period of 5 minutes to avoid false positives due to temperature changes. The evaluations were performed with an Olympus FSX100 fluorescence microscope using a 40x objective. Those sperm with a red fluorescent head were considered to have membrane lesions.

2.12. Acrosome integrity analysis

The spermatozoa were incubated in TALP-Sperm medium at 37°C, 5% CO2 and saturated humidity with soluble *T. gondii* antigens at three concentrations (8, 4 and 2 µg/ml), and as a control, only medium (without antigen) was used. After one hour of incubation, centrifugation was performed at 16 × g for 5 minutes, with subsequent disposal of the supernatant. Fifteen microliters of the cell precipitate suspension were placed in 200 µL of phosphate buffer (PBS) heated to 37°C with 5 µL of the solution for use of Pisum Sativum Lectins associated with fluorescein (PSA/FITC) (stock solution: 2 mg total vial content in 2 ml of PBS). The solution used was 100 µL of stock solution in 900 µL of PBS. This solution was incubated for 5 minutes at room temperature. The evaluations were performed with an Olympus FSX100 fluorescence microscope using a 40x objective. Spermatozoa with acrosome injury were identified by showing green fluorescence in the acrosomal region.

2.13. Analysis of mitochondrial activity.

The test consisted of incubating spermatozoa in TALP-Sperm medium supplemented with soluble *T. gondii* antigens at three concentrations, namely, high (8 µg/ml), medium (4 µg/ml), low (2 µg/ml) and as control, only medium (without antigen), at 37°C, 5% CO2 and saturated humidity. After one hour of incubation, centrifugation was performed at 16 × g for 5 minutes, with subsequent disposal of the supernatant. Fifteen microliters of the cell precipitate suspension was placed in 200 µL of phosphate buffer (PBS) heated to 37°C with 10 µL of the stock solution of MitoTracker Green (2.8 µg/ml of anhydrous dimethylsulfoxide (DMSO)). This solution was incubated for 30 minutes at 37 °C, 5% CO2 and saturated humidity. The evaluations were performed with an Olympus FSX100 fluorescence microscope using a 40x objective. Sperm with mitochondrial activity were identified by having the middle piece of the tail fluorescing green.

2.14. *In vitro* production of embryos

Eight *in vitro* embryo production routines were carried out in which the days were denominated as -1, 0, 1, 2 and 3, as described in Table 1; on day 0, the spermatozoa were treated with the antigen *T. gondii* solubles.

The routines were performed according to the protocol already established at the Laboratory of Biology of Reproduction at UFU and described by Lucio et al. (2016).

The cleavage rate was evaluated 48 hours after *in vitro* fertilization by evaluating the structures that had two or more cells. Seven days after fertilization, the percentage of blastocysts was evaluated based on the number of structures that reached this stage of development, without differentiating among initial, expanded or hatched blastocysts.

2.15. Statistical analysis

To identify possible differences between the conditions tested in the analyses, the Kolmogorov-Smirnov test was performed to verify the distribution of the data; subsequently, one-way ANOVA was used to compare the treatments, and Fisher's exact test was used for comparisons among the three treatments. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Soluble *T. gondii* antigens decrease the average trajectory speed of capacitated sperm at different concentrations in environments with and without flow.

We evaluated the average velocity of capacitated spermatozoa treated with soluble *T. gondii* antigens in a simulated bovine fallopian tube environment when appropriate in a flow environment. The average speed of spermatozoa was altered, decreasing at the three concentrations tested (8.4.2 μg/ml), compared to that of the control (medium) (****$p < 0.0001$) (Fig. 2A).

In our experiment to evaluate the average speed of capacitated sperm under the same conditions in an environment without flow, we observed that the average speed of capacitated sperm was affected, being significantly decreased in all conditions tested compared to the control (medium) (*** *$p < 0.0001$). Regarding the difference between conditions, treatments with a high dose (8 μg/ml) and low dose (2 μg/ml) had lower speeds than treatment with a medium dose (4 μg/ml) (**** $p < 0.001$) (Figure 2B).

3.2. The average trajectory speed of noncapacitated spermatozoa in environments with and without flow was altered by soluble *T. gondii* antigens.

To evaluate the effect of soluble *T. gondii* antigens on the average trajectory speed of uncapacitated spermatozoa in a simulated flow bovine fallopian tube environment (Fig. 3A). Bovine semen samples were diluted in TALP-Sperm medium and treated with different

concentrations (8, 4 and 2 µg/ml) of *T. gondii* antigens. The results showed that the average speed of spermatozoa was affected, decreasing the speed at the high dose (8 µg/ml) in relation to that of the control (**p < 0.001). Regarding the difference between the conditions evaluated, treatment with a medium dose (4 µg/ml) or low dose (2 µg/ml) had greater effects than treatment with a high dose (8 µg/ml), did (**** p< 0.00001). We then evaluated the average speed of noncapacitated sperm in an environment without flow. The average speed of sperm decreased in all conditions tested compared to that in the control group (****p < 0.00001). According to the differences between the concentrations evaluated, treatment with a high dose (8 µg/ml) or low dose (2 µg/ml) had lower speeds than treatment with a medium dose (4 µg/ml), (*p < 0.05). (Fig. 3B).

3.3. Treatment with soluble *T. gondii* antigens changed the angle of the trajectory of capacitated and noncapacitated spermatozoa in a flow environment.

With the aim of understanding the influence of *T. gondii* soluble antigens on rheotactic behavior, we began to evaluate whether antigen treatments changed the average angle of sperm trajectory in a simulated environment of the bovine fallopian tube in a flow environment. This effect was observed both in capacitated sperm (Fig. 4A) and in noncapacitated sperm (Fig. 4B). The angulation of the sperm trajectory at all concentrations tested (8, 4 and 2 µg/ml) was significantly lower than that of the control group (*p < 0.05).

3.4. Soluble *T. gondii* antigens alter the compaction of bovine sperm chromatin.

To evaluate possible changes in chromatin, the first technique was computational image analysis of semen smears stained with toluidine blue (pH 4.0) after acid hydrolysis (Fig. 5A). The results showed that the high and medium dose treatments (8.4 µg/ml) resulted in greater number of sperm chromatin decompaction than didthe control treatment (*p < 0.05).

As a complementary assessment of changes in bovine sperm chromatin compaction, we used transmission electron microscopy (TEM). In this analysis, three chromatin classifications were considered: intact, with mild changes (points of decompression) and with severe changes (areas of decompression). First, the percentage of sperm with intact chromatin did not significantly differ from that the control group (NS) (Fig. 6A, 6B). Regarding the classification of mild changes, the low dose (2 µg/ml) of antigen showed fewer changes than did the control (medium) (***$p < 0.0005$). (Fig. 6C, 6D). According tothe results of the severe changes (Fig. 6E, 6F), all antigen concentrations evaluated (8.4.2 µg/ml) increased the amount of this type of chromatin damage in relation to that in the control (*** $p < 0.0005$) (**$p < 0.005$).

3.5. Soluble *T. gondii* antigens damage the integrity of the plasma membrane of bovine sperm.

To evaluate changes in the integrity of the plasma membrane, the first assessment was carried out using a hypoosmotic test. The main objective of this test was to distinguish between sperm with intact membranes and those with damaged membranes (Fig. 7A, 7B). There was a percentage difference but not a statistically significant difference compared with the control at any of the concentrations used (NS). To obtain better results in evaluating the sperm plasma membrane, transmission electron microscopy (TEM) was subsequently used. As a first step, three classifications of damage to the sperm plasma membrane were considered: intact membrane, membrane with mild damage, and totally damaged membrane. According to the results of the evaluations of the percentage of spermatozoa with an intact membrane, all antigen treatments (8.4.2 µg/ml) reduced the percentage of spermatozoa with an intact membrane compared to that of the control group (medium) (***$p < 0.0001$). (Fig. 7C, 7D). In the following classification of mild membrane damage, there was no significant

difference in any of the concentrations evaluated in relation to the control (NS). (Fig. 7E, 7F). Regarding the classification of total membrane damage, all antigen treatments (8.4.2 µg/ml) increased the amount of this type of damage in relation to that of the control (***p < 0.0005) (**p < 0.005). (Fig. 7G, 7H). To confirm our results in the assessment of damage caused to the sperm plasma membrane by these antigens, propidium iodide was used as a complementary technique, as if there was a loss of membrane integrity, the incorporation of the dye into these cells would be observed. (Fig. 8A, 8B). The integrity of the sperm membrane was significantly affected by the antigens at the three concentrations evaluated (8, 4 and 2 µg/ml) compared with that of the control (*p < 0.05). (***p < 0.0001).

3.6. Bull sperm treated with different concentrations of soluble *T. gondii* antigens show damage to the acrosome.

Transmission electron microscopy (TEM) was used to evaluate the effects of antigens on the bull sperm acrosome. First, two classifications of damage to the sperm acrosome were considered: intact acrosome and damaged acrosome. The percentage of sperm with an intact acrosome, and the concentrations of antigens at low and medium doses (2.4 µg/ml) were lowerin the classification of this type of acrosome compared to the control group (*p < 0 .05). (Fig. 9A, 9B). Finally, in the classification of damaged acrosomes, there was no statistically significant difference in relation to the control in any of the treatments used (NS). (Fig. 9C, 9D). As a second evaluation to obtain better results in evaluating the integrity of the sperm acrosome in our research, Pisum Sativum Lectins (PSA) associated with fluorescein were used as acrosomal markers. The results showed that soluble *T. gondii* antigens altered the integrity of the bovine sperm acrosome at the three concentrations evaluated in relation to the control (***p < 0.0001). (Fig. 10A, 10B, 10C, 10D)

3.7. Soluble *T. gondii* antigens affect the mitochondrial activity of bovine spermatozoa

To evaluate whether these antigens affect the mitochondrial activity of sperm, the mitochondrial labeling technique with MitoTracker was used. (Fig. 11A, 11B). The results demonstrated that these antigens affect the mitochondrial activity of sperm. There was a significant difference in the three conditions evaluated (8, 4 and 2 μg/ml) in relation to the control (***$p < 0.0005$).

3.8. Soluble *T. gondii* antigens affect the blastocyte rate in *in vitro* embryo production (IVP).

The effect of a medium dose (4 μg/ml) of soluble *T. gondii* antigens on the cleavage and blastocyte rates in PIVE was evaluated. In the case of the cleavage rate, the results showed that in the treatment with treated sperm there was no statistically significant difference compared to the control (NS). (Fig. 12A). However, the blastocyte rate was consequently reduced compared to that in the control group (*$p < 0.05$) (Fig. 12B)

4. Discussion

This study aimed to evaluate changes in these characteristics of bovine sperm caused by soluble *T. gondii* antigens, emphasizing membrane and acrosome integrity, mitochondrial activity, chromatin compaction, and sperm motility, especially rheotaxic behavior.

Evaluating our findings, it is clear that the presence of soluble antigens of this parasite in the Talp-Sperm and Talp-Fert media affected the behavior of sperm in terms of average swimming speed (positive rheotaxis), both in the absence and under flow conditions. The average angulation of the trajectory of rheotactic spermatozoa and the presence of these antigens in the Talp-Sperm and Talp-fert media under different flow conditions showed that the angulation of capacitated and noncapacitated spermatozoa was also affected. In both natural production and assisted reproduction biotechnologies, motility is an important factor for reproductive efficiency (Zaferani and Abbaspourrad, 2018). In mouse sperm there is a link between toxoplasmosis and the number of morphologically abnormal sperm (Saki et al.,

2020). In the case of cattle, there are few studies evaluating what changes *T. gondii* antigens cause in motility, especially in the average swimming speed and angulation of the trajectory of bovine spermatozoa that present rheotaxis in a flow environment. Studies with male rats provided results on the effects of *T. gondii* on sperm quality, where after evaluation of the percentage of sperm motility, the average number of progressively motile spermatozoa was significantly decreased in the infected group compared to the control group (Terpsidis et al., 2009). In another investigation, acute *T. gondii* infection in mice significantly decreased several reproductive parameters, such as the concentration, motility and number of normal sperm, compared to those in the control group. It is important to highlight that the studies cited evaluated the effect of infection with *Toxoplasma gondii* on semen and the present study specifically evaluated the *in vitro* effects of the antigens of this protozoan on spermatozoa.

We must consider that in species with internal fertilization, such as mammals, spermatozoa face a selective environment within the uterine tube that recognizes them as foreign agents, preventing a large percentage of them from reaching the site of fertilization. To highlight, only ~0.004% (~250 sperm) of all inseminated motile sperm reach the fallopian tubes in humans, and only 50% of them end up in the ovulatory fallopian tubes to meet an egg (Fitzpatrick and Lüpold, 2014). This selective environment begins with a retrograde flow of fluid to its destination, caused by muscle contraction and ciliary beats present in the bovine uterine tube, where hyperactivated spermatozoa are able to maintain increased swimming speed but change the swimming pattern to linear (El-Sherry et al., 2014; Johnson et al., 2017). The tendency of a cell to swim against flow has been reported as positive rheotaxis, gaining attention in the last 50 years as an orientation mechanism of great importance in fertilization. (Miki and Clapham, 2013; Johnson et al., 2017).). In this natural process called fertilization, thousands of sperm must travel through their bodies in a

complicated environment with flow of viscous liquid and the presence of immune cells that eliminate a significant amount of them. Ultimately, few sperm can cross the fallopian tube, but at the same time, these processes can result in the selection ofcells with better reproductive characteristics, in this case, better morphology and healthy mitochondria, increase the possibility of selecting a desirable male gamete (Zhang et al., 2016; De Martin et al., 2017). Therefore, these disorders in motility and rheotaxis caused by soluble *T. gondii* antigens may be associated with fertility problems both *in vitro* and *in vivo*.

To evaluate the compaction of sperm chromatin, there are different methods or techniques, including the toulidine blue (AT) method and transmission electron microscopy (TEM), were used in our study. Our results using both techniques revealed that soluble *T. gondii* antigens contribute to changes in bovine sperm chromatin. It is important to highlight that there are mechanisms responsible for inducing damage to sperm DNA, including abortive apoptosis, defective protamination, a defective epididymal microenvironment and oxidative stress. In addition, the loss of sperm DNA integrity can also occur during spermatogenesis, during spermiogenesis, during transit through the epididymis or after ejaculation. (Da Silva, 2022). According to our TEM evaluation, the decompression points present in sperm chromatin after treatment with soluble *T. gondii* antigens increased in diameter, indicating that chromatin changes became more intense in the presence of *T. gondii* antigens. These results are relevant, as it has been demonstrated in other investigations that, depending on the intensity of the chromatin alteration, sperm that present severe damage would have abnormalities in the shape of the head, causing problems in their hydrodynamics, leading to inadequate motility, and directly interfering in the process of fertilization (Beletti, 2013). However, less severe sperm changes might not interfere with motility or therefore the fertilization process. However, by fertilizing the oocyte and causing damage to the DNA, it

would be impossible for the male and female pronuclei to unite and, as a consequence, would make the zygote unviable. Furthermore, these slight changes in sperm chromatin could also interfere with later stages of embryonic development, which could lead to embryonic death with consequent fetal reabsorption or abortion. In certain situations, pregnancy can reach term, with the possibility that newborns may present genetic problems of different types and magnitudes (Beletti, 2013; Martins et al., 2021). Therefore, any form of sperm chromatin abnormality or DNA damage can result in male fertility problems without being able to guarantee the maintenance of good health in future generations (Agarwal and Tamer, 2003). Our results demonstrate that the presence of soluble *T. gondii* antigens in semen can lead to changes in sperm chromatin, with serious consequences for the reproductive process.

Our results from the hypo-osmotic swelling test (HOS, HOST test) showed that there was no significant difference in the number of sperm with damaged membranes compared to the control. However with the use of TEM, the results showed that these antigens alter the integrity of the plasma membrane of bovine sperm. Studies carried out in sheep under conditions of induced experimental infection have shownthe harmful effect of this parasite on the integrity of the sperm membrane (Fais et al., 2020). To evaluate whether the soluble antigens of *T. gondii* affect the functional integrity of the membrane in bovine spermatozoa, the hypo-osmotic swelling test (HOS) was used, and as complementary tests seeking to have greater safetyin our evaluations, transmission electron microscopy (TEM) and propidium iodide were used. The hypo-osmotic swelling test (HOS, HOST test) is widely used to evaluate the functional integrity of sperm plasma membranes in humans and many animal species (Prochowska et al., 2022) and has shown good results in cattle, equines, dogs and pigs (Lodhi et al., 2008).

As with chromatin, the antigens intensified the membrane lesions observed via TEM, as the number of spermatozoa classified as having mild damage decreased and the number of spermatozoa with a completely damaged membrane increased.

In the case of the evaluation carried out with propidium iodide, it was also observed that the antigens affected the integrity of the plasma membrane in all the treatments evaluated, highlighting that this intercalating agent is used to stain nucleic acids and is capable of binding to the DNA molecules between their bases, and in the case of a loss in the integrity of the membrane, the incorporation of the dye into the cells will be observed.

The importance of our results is focused on the damage caused by these antigens to the sperm membrane, and as already demonstrated by several authors, membrane integrity is related to decreased sperm motility and increased DNA damage (Padrik et al., 2012). Importantly, for spermatozoa to carry out vital processes such as capacitation, acrosome reactions, connection to the zona pellucida and fertilization, they require intact and active membranes (Fais et al., 2020). Therefore, our results demonstrate that the presence of soluble *T. gondii* antigens in semen can cause damage to the sperm cell membrane, which can lead to male fertility problems.

Our results revealed that soluble *T. gondii* antigens cause significant changes in the integrity of the sperm acrosome. It has been observed that damage to certain parts of the male reproductive system is associated with *T. gondii* infection, as a large number of tachyzoites are found in the semen of infertile patients leading to an increased rate of sperm deformation (Zheng et al., 2019). In male mice infected with *T. gondii*, an increase in abnormal sperm morphology was also observed (Taherimoghaddam et al., 2021). Despite the existence of studies showing the influence of this parasite on the abnormal sperm morphology, no studies have evaluated the influence of *T. gondii* on the integrity of the sperm acrosome. Fertilization

in mammals is not a simple process (Storey et al., 1984) because of the complex set of molecular events that allow the sperm to recognize and bind to the extracellular lining of the egg, the zona pellucida. (ZP). (Tulsiani et al., 1998). In this process, the acrosome plays an important role at the sperm-zona pellucida binding site (Abou-Haila andTulsiani, 2000). There is evidence showing that the sperm-ZP interaction is a carbohydrate-mediated receptor-ligand binding event that initiates a signal transduction pathway resulting in the exocytosis of acrosomal contents (Tulsiani et al., 1998). This exocytic event, called the acrosome reaction, is induced by the influx of calcium, the purpose of which is to allow sperm to penetrate the zona pellucida. For this process to be successful, sperm must have an intact acrosome (Fierro et al., 2013). This highlights the importance of our results, as described in the study by Schill et al. (1988). The presence of sperm with defective acrosomes is a parameter that represents a decrease in male fertility.

To expand and deepen our research, studies were also carried out to identify the mitochondrial activity of spermatozoa subjected to treatments with soluble *T. gondii* antigens. Our analysis used MitoTracker as a marker of mitochondrial activity and revealed that soluble *T. gondii* antigens affect the mitochondrial activity of bovine sperm.

Mitochondria surround the sperm midpiece and produce adenosine triphosphate (ATP) for the axoneme; this ATP is used to provide flagellar propulsion to sperm (Folgerø et al., 1993). Recent research on animal and human sperm physiology has led to increasing interest in mitochondria as biomarkers of sperm health and fertility. The role of mitochondria in male fertility is directly linked to motility, but these organelles are also crucial for hyperactivation, capacitation, acrosin activity, acrosome reactions and sperm DNA integrity (Madeja et al., 2021). Given that impaired mitochondrial function seriously compromises the maintenance of energy production necessary for sperm motility and other functions

mentioned above, this anomaly is one of the main causes of male fertility problems (Bonanno et al., 2016). Our results demonstrate yet another way in which *T. gondii* can interfere with male fertility.

Fertilization in mammals is not a simple process of collision between sperm and eggs (Storey et al., 1984). To do this, the sperm must make a tortuous journey of more than 1,000 times its length to the site of fertilization in the ampulla of the oviduct (Romero et al., 2018). Millions or billions of fertilizing sperm are deposited in the uterine tract by mating or artificial insemination. During their passage through the bovine uterine tube, selection occurs, but successful spermatozoa interact with the luminal fluid and epithelia, avoiding destruction by the immune system and responding to rheotactic, chemical and adhesive stimuli to undergo functional changes and reach the site of fertilization (Miller, 2018). Fertility parameters are normally linked to sperm quality, and this phenomenon depends on the ability of sperm to fertilize the oocyte and allow the development of the zygote (Alomar et al., 2006). Our results revealed changes in chromatin integrity, membrane integrity, motility and sperm angulation caused by soluble *T. gondii* antigens. Accordingto an *in vitro* study, sperm are not exposed to the complex environment of the fallopian tube, which can lead to different behaviors. To evaluate how these antigens affect assisted reproduction biotechniques, *in vitro* embryo production routines were carried out using spermatozoa that had or had not come into contact with *T. gondii* antigens. We observed that after exposing sperm to treatment with antigens, the cleavage rate did not significantly differ from that of the control, but the blastocyst rate did. This means that the *in vitro* fertilization capacity of the sperm that came into contact with the antigens was little affected. The decrease in the blastocyst rate demonstrated that early embryonic development was affected. This effect may be related to mild chromatin changes that cannot interfere with fertilization, but rather

interfere with early embryonic development (Beletti, 2013). According to Jeyendran (1984, pp219–228) for successful fertilization and embryonic development, sperm structures must be in perfect condition. It is important to highlight that in IVEP, sperm undergo selection using a Percoll gradient, which must eliminate sperm whose fertilization capacity is affected.

Conclusion

The present study revealedthat the presence of soluble *T. gondii* antigens in bovine semen alters sperm integrity and vital characteristics for the fertilization process and embryonic development, such as chromatin, sperm membrane integrity, mitochondrial activity and motility, which certainly interfere with male fertility.

The use of semen contaminated with antigens in PIVE routines does not lead to a reduction in the fertilization of oocytes, as sperm undergo selection before *in vitro* fertilization. However, early embryonic development is affected, probably by structural changes that are not eliminated in the selection process.

Credit authorship contribution statement

Kelvin Espinoza Blandon and Marcelo Emilio Beletti: designed the experiments, performed the experiments, analyzed the data, and wrote the manuscript. Bruno Augusto Nassif Travençolo: Software development for data analysis and image analysis. Aryani Felixa Fajardo Martínez: performed the experiments and writing. Yulizabeth Daniela Pinto Rojas: performed the experiments and wrote the manuscript. Muller Carrara Martins and Kamila Alves Fontoura: carried out the IVEPs. Tiago Wilson Patriarca Mineo: production of soluble antigens of *Toxoplasma gondii*. All authors approved the final version of the manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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Figure Legends:

Figure 1. The flow chamber was used to measure the behavior of bull sperm in the presence or absence of flow. In the central part, the duct where the spermatozoa were placed was observed, and at the ends, the pieces of filter paper fulfilled the function of causing flow.

Figure 2. Bull sperm were treated with soluble *T. gondii* antigens at different concentrations in TALP-Fert medium for 1 h, and bull semen was used as a control in antigen-free medium. Motility tests were performed, and sperm velocity is represented as the average speed of the trajectory of capacitated sperm in a counterflow (A) no-flow (B) environment. The results are expressed as the mean ± standard deviation of three independent experiments performed

with three replications. Significant differences were detected by one-way ANOVA with Tukey's multiple comparison post hoc test when appropriate. ****P < 0.00001. ****P < 0.00001.

Figure 3. Bovine spermatozoa were treated with soluble *T. gondii* antigens at three different concentrations for 1 h in Talp-Sperm medium. Bovine semen and medium without antigens were used as control group. The motility test was carried out, and the sperm velocity is represented as the average velocity of the trajectory of noncapacitated spermatozoa against flow (A) without flow (B) in a simulated environment of the bovine fallopian tube when appropriate. The results are expressed as the mean ± standard deviation of three independent experiments performed with three replications. Significant differences were detected by one-way ANOVA with Tukey's multiple comparison post hoc test when appropriate. *P < 0.01. ***P < 0.0001. ****P < 0.00001.

Figure 4. Assessment of the average angulation of the trajectory of capacitated and noncapacitated spermatozoa in a simulated bovine fallopian tube environment. Bull spermatozoa were exposed to various concentrations of soluble *T. gondii* antigens for 1 h in TALP-Fert and TALP-Sperm media. As a control, sperm were exposed to the same conditions but without antigen treatment. Sperm angulation is represented as the average angulation in the trajectory of capacitated (A) and noncapacitated (B) spermatozoa against the flow. The results are expressed as the mean ± standard deviation of three independent experiments performed with three replications. When significant differences were detected, analyses were carried out using one-way ANOVA with Tukey's multiple comparison post hoc test, when appropriate. *P < 0.05.

Figure 5. Changes in chromatin compaction in bovine sperm were identified through the use of the toluidine blue (AT) method. Bull sperm were treated for 1 h with soluble *T. gondii*

antigens in Talp-Sperm medium at three concentrations. As a control, bovine semen was used under the same conditions in medium without antigens. The intensity of chromatin decompaction is represented as the average intensity of sperm chromatin decompaction in each spermatozoon. The results are expressed as the mean ± standard deviation of three independent experiments. Significant differences were detected by one-way ANOVA with Tukey's multiple comparison post hoc test when appropriate. *P < 0.05.

Figure 6. Chromatin decompaction was evaluated via transmission electron microscopy (TEM). Bovine spermatozoa in Talp-Sperm medium were treated with soluble *T. gondii* antigens for 1 h at three concentrations. As a control, spermatozoa were cultured in noncapacitated medium without antigens. Spermatozoa with intact chromatin (A). rank represents the total number of sperm with intact chromatin per condition compared to the control. Reference image showing sperm with intact chromatin. (B) Significant differences not detected (NS). The sperm exhibited slight chromatin decompression. This classification was represented as the total number of spermatozoa with chromatin decompaction points in the different conditions versus the control (C). A representative image of sperm with slight chromatin decompression is shown (D). Significant differences were detected by Fisher's exact test, and when appropriate, ***P < 0.0001. Sperm with severe chromatin damage (E). This classification was represented as the total number of sperm with areas of chromatin decompaction per condition versus the control. Reference image of sperm with severe chromatin damage (F). Significant differences were detected by Fisher's exact test, when appropriate. **P < 0.001. ***P < 0.0001.

Figure 7. Assessment of sperm membrane integrity. Bull spermatozoa in Talp-Sperm medium were treated for 1 h with soluble *T. gondii* antigens at different concentrations. As a control, sperm were exposed to the same conditions without antigen treatment. For evaluation

carried out using the Hypo-Osmotic test, the integrity of the sperm membrane was represented as the average number of sperm with damaged membranes per condition versus the control. Representative image of spermatozoa in the Hypo-Osmotic test (A), showing spermatozoa with damaged membranes that remained with straight tails and with integrated membranes that showed bending in the tails (B). The results are expressed as the mean ± standard deviation of three independent experiments performed with three replications, and significant differences were not detected (NS). The TEM method was implemented to classify different types of damage to the sperm membrane of bovine sperm. The intact sperm membrane (C) classification represents the total number of sperm with intact membranes per condition versus the control. A representative image of a sperm sample with an intact membrane is shown (D). Significant differences were detected by Fisher's exact test, and when appropriate, ***P < 0.0001. For sperm with mild membrane damage (E), in this classification, mild damage was represented as the total number of sperm with slightly damaged membranes per condition versus the control. Image showing slight damage to the sperm membrane (F). No significant differences were observed (NS). The number of sperm with a totally damaged membrane (G), classified as the total number of sperm with a totally damaged membrane per condition versus the control. Reference image of sperm with a completely damaged membrane (H). Significant differences were detected by Fisher's exact test, when appropriate. **P < 0.001. ***P < 0.0001.

Figure 8. Assessment of sperm membrane integrity.

Bovine spermatozoa were treated for 1 h with soluble *T. gondii* antigens at different concentrations in Talp-Sperm medium. In the case of the control, sperm with only medium (without antigen) were used. For the propidium iodide test, (A) Sperm membrane integrity is represented as the average number of sperm with damaged membranes per condition versus

the control. The image shows spermatozoa with red heads with damaged membranes and spermatozoa with integral membranes that showed no red color in the head (B). Significant differences were detected by one-way ANOVA with Tukey's multiple comparison post hoc test when appropriate. *P < 0.05. ***P < 0.0001.

Figure 9. Evaluation of the sperm acrosome using TEM. Bovine spermatozoa in Talp-Sperm medium were treated with soluble *T. gondii* antigens at different concentrations for 1 h. As a control, semen was subjected to the same conditions without antigen treatment. The classification of intact acrosomes (A) is represented as the total number of sperm with an intact acrosome per condition versus the control. Image showing the intact sperm acrosome (B). Significant differences were detected by Fisher's exact test, when appropriate. *P < 0.05. The number of damaged acrosomes (C) was calculated as the total number of sperm with damaged acrosomes per condition versus the control. Image of the damaged sperm acrosome (D). Significant differences were not detected (NS).

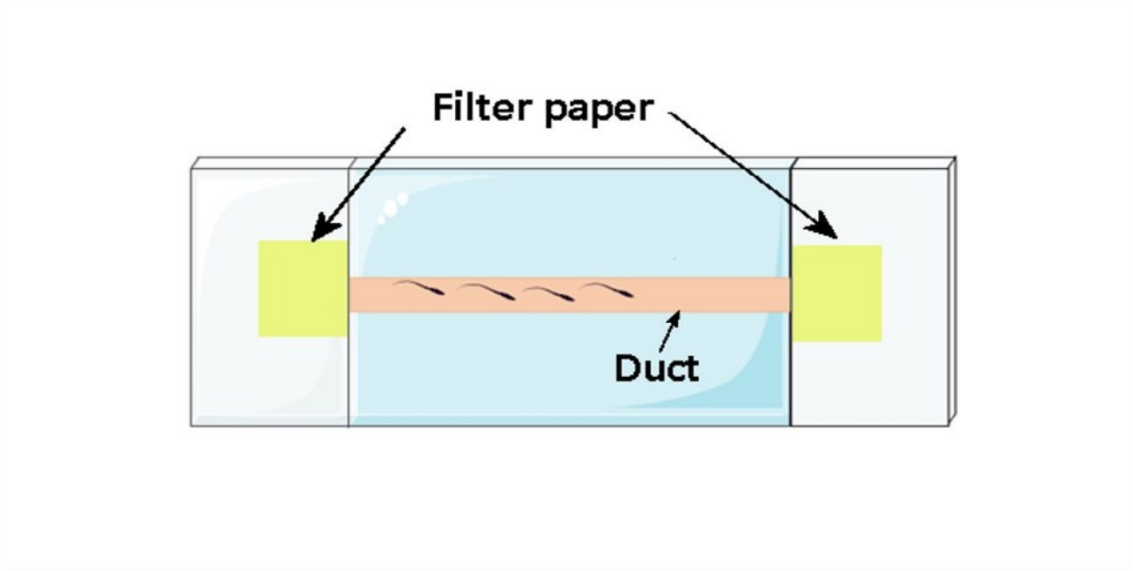
Figure 10. Assessment of the sperm acrosome. Bull spermatozoa were treated with soluble *T. gondii* antigens for 1 h in Talp-Sperm medium at three concentrations. As a control, spermatozoa were subjected to the same conditions without the presence of antigens. This test was carried out with *Pisum sativum* lectins (PSAs) combined with fluorescein as an acrosomal marker (A). Acrosome integrity is represented as the average number of sperm with injured acrosomes per condition versus the control. Representative images of sperm with damaged acrosome (B, C) and sperm with intact acrosomes (D). Significant differences were detected by one-way ANOVA with Tukey's multiple comparison post hoc test when appropriate. ***P < 0.0001.

Figure 11. Assessment of mitochondrial activity. Soluble *T. gondii* antigens at different concentrations were used to treat bovine spermatozoa in Talp-Sperm medium for 1 h. As a

control, sperm with only medium (without antigen) were used. In this analysis, MitoTracker was used as a marker of mitochondrial activity (A). This activity was represented as the average number of spermatozoa with the presence of mitochondrial activity per condition versus the control. Reference image of sperm with and without mitochondrial activity (B). The intermediate pieces of sperm with mitrocindial activity are marked in green, and the intermediate piece of sperm without mitochondrial activity is not marked. Significant differences were detected using one-way ANOVA with Tukey's multiple comparison post hoc test when appropriate. ***$P < 0.0001$.

Figure 12. Cleavage rate and blastocysts. Bull spermatozoa were treated with soluble *T. gondii* antigens for 1 h in Talp-Sperm medium, using only medium-dose concentrations of antigens (4 μg/ml). In the case of the control, sperm were processed in the same way without antigen treatment. Eight *in vitro* embryo production routines (IVPs) were performed. The cleavage rate was assessed 48 hours after *in vitro* fertilization by counting embryos that had two or more cells (A) (cleavage rate = number of embryos/number of initial oocytes). Significant differences were not detected. The blastocyst rate (B) was determined on the seventh day after fertilization in relation to the number of oocytes that initiated cleavage and reached this stage of development (blastocyst rate = no. of blastocyst/no. of initial oocytes). Significant differences were detected by Fisher's exact test, when appropriate. *$P < 0.05$.

Figure 1



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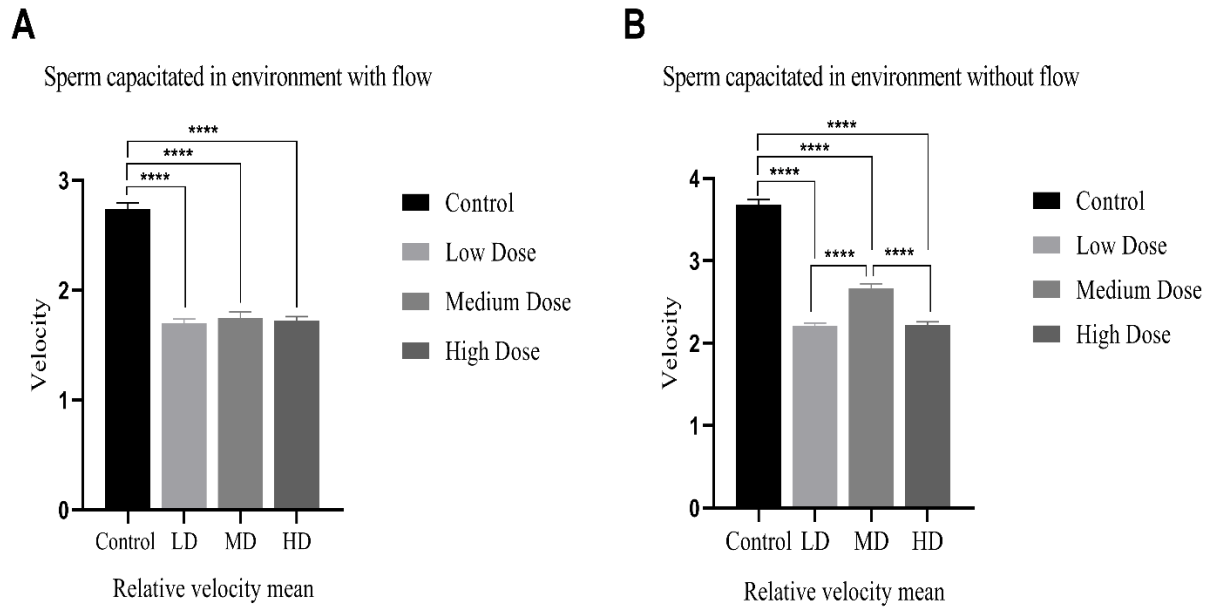

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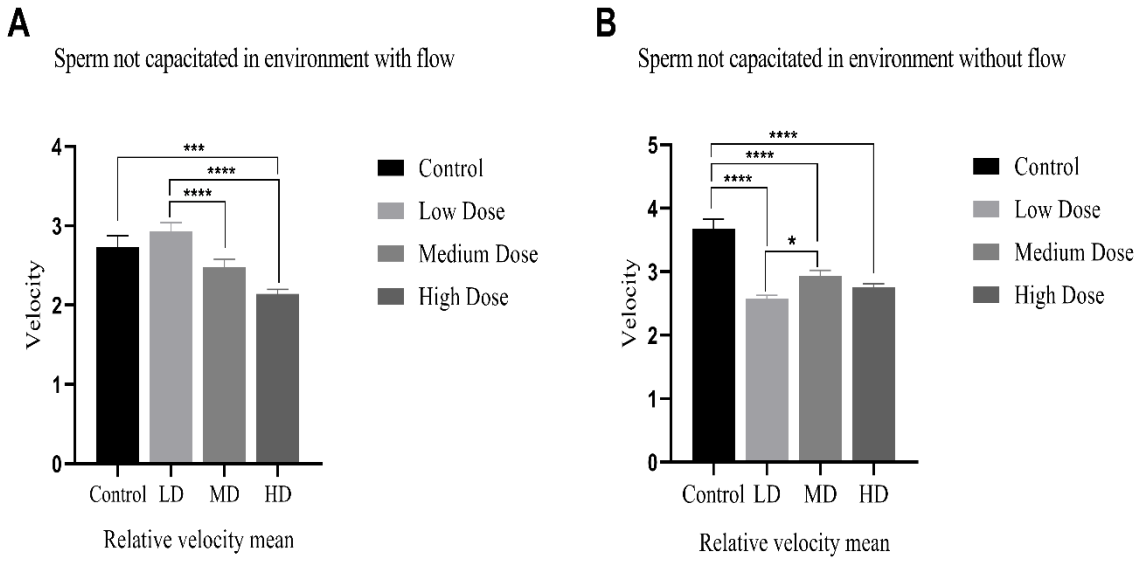

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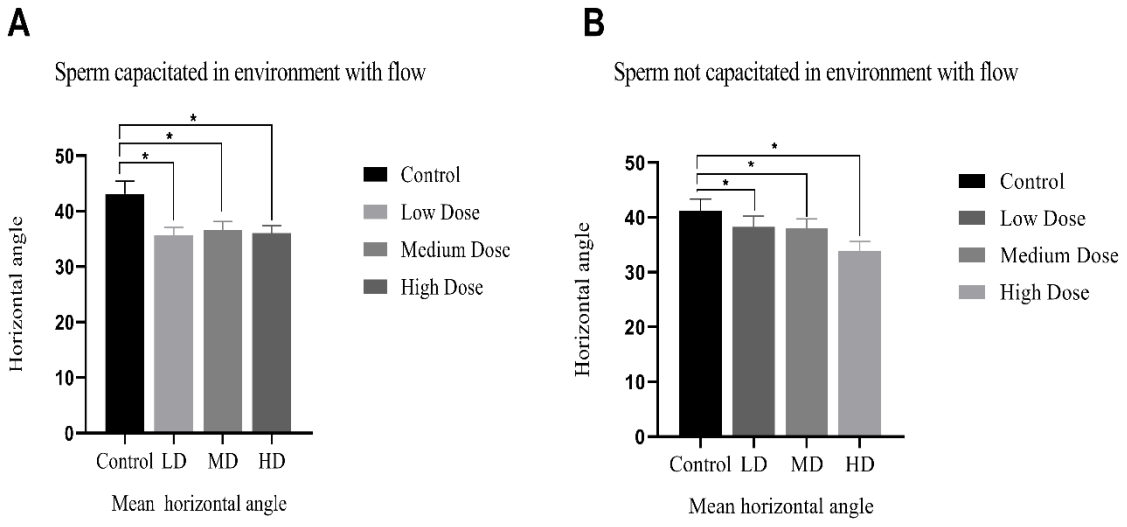

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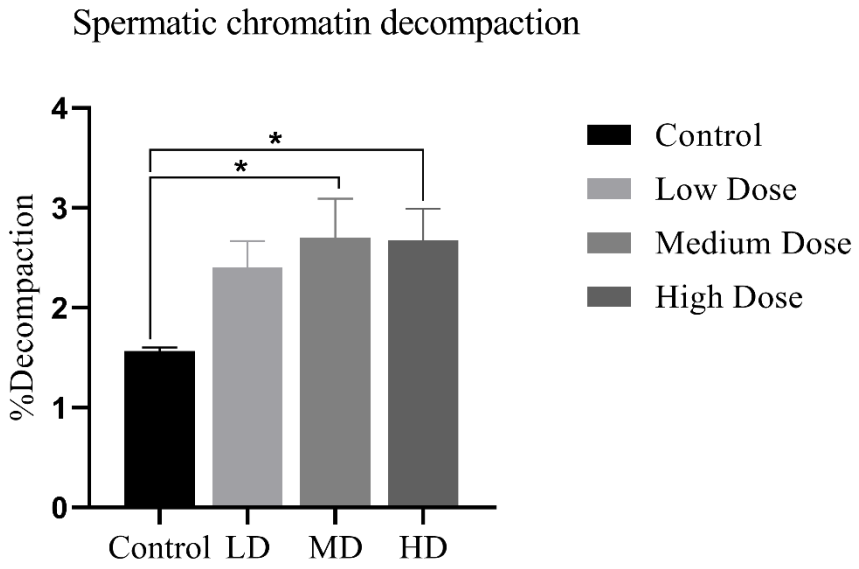

Sperm contamined with soluble *T. Gondii* antigens

Figure 6

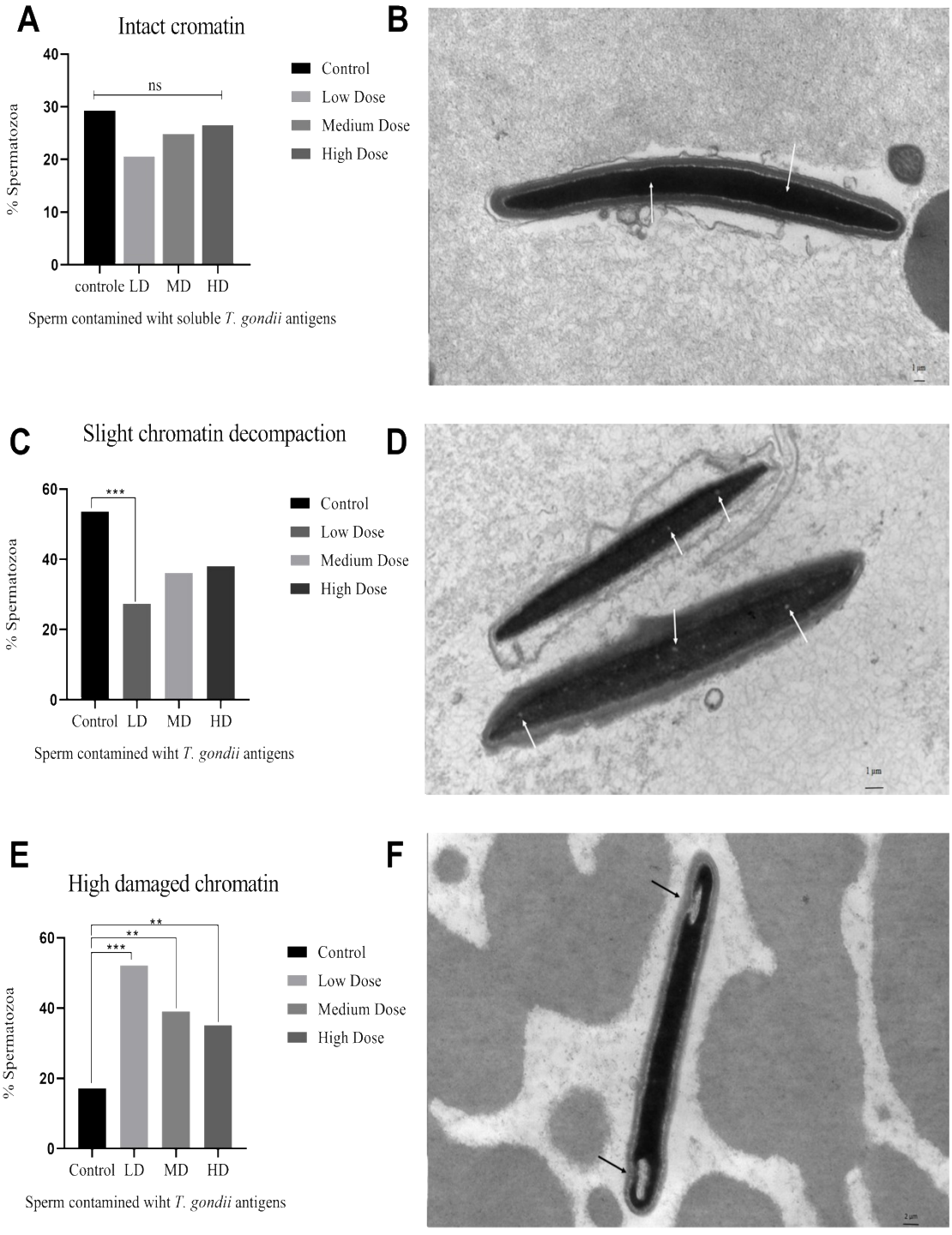
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Figure 7

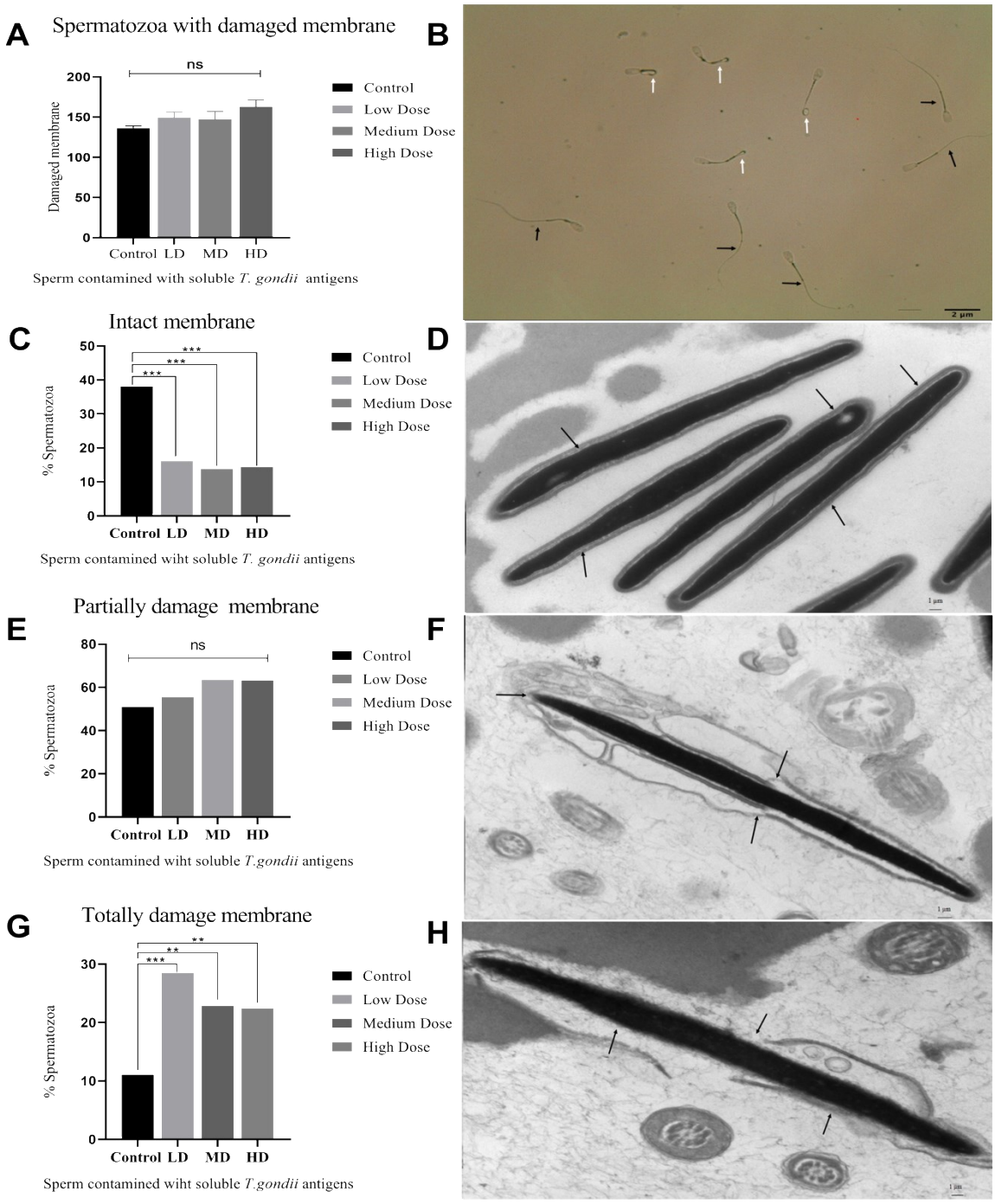
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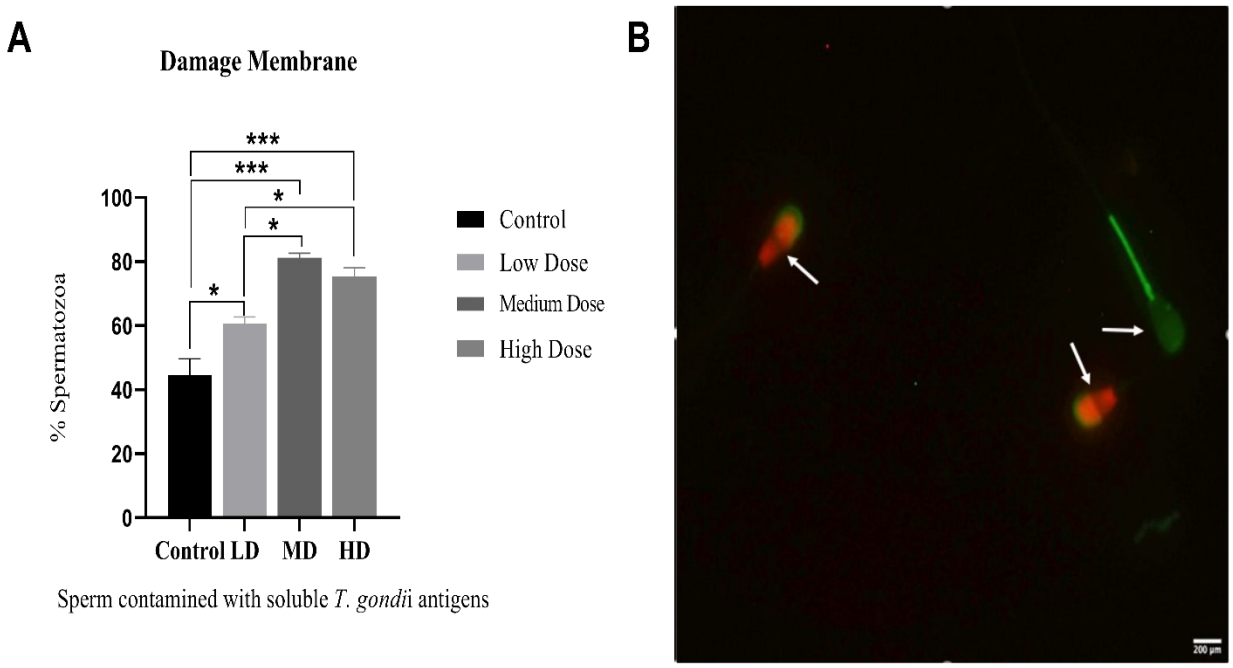
Figure 8



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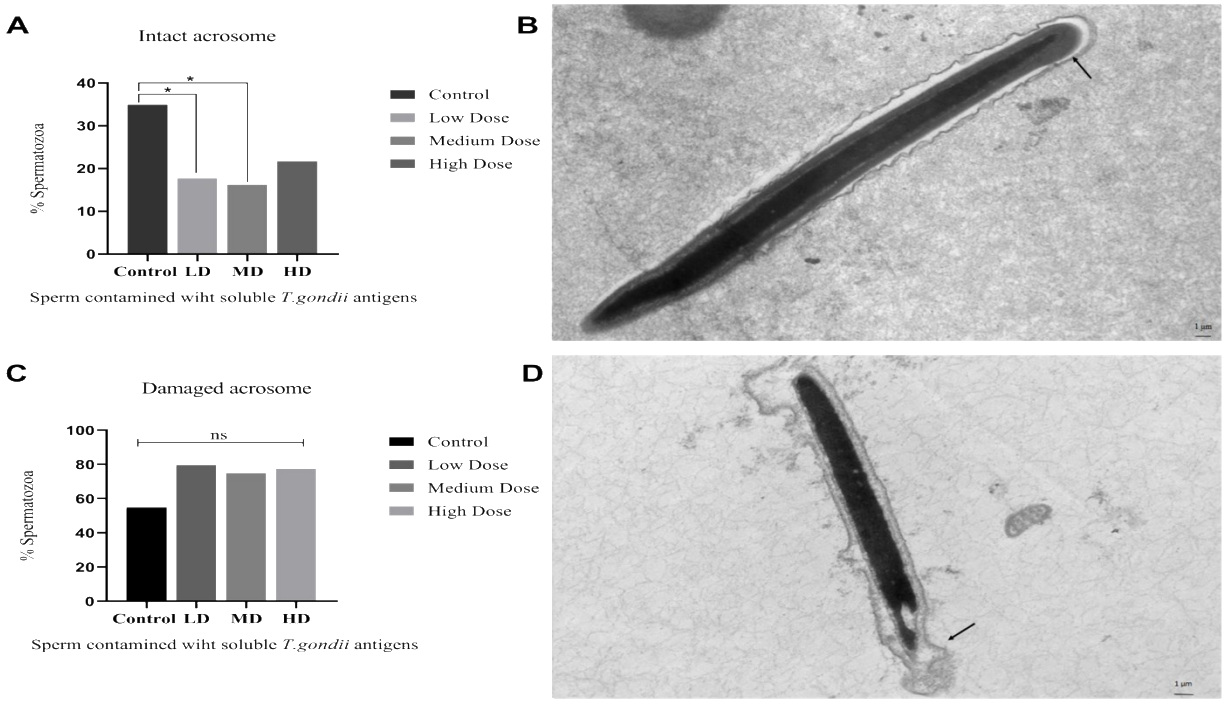

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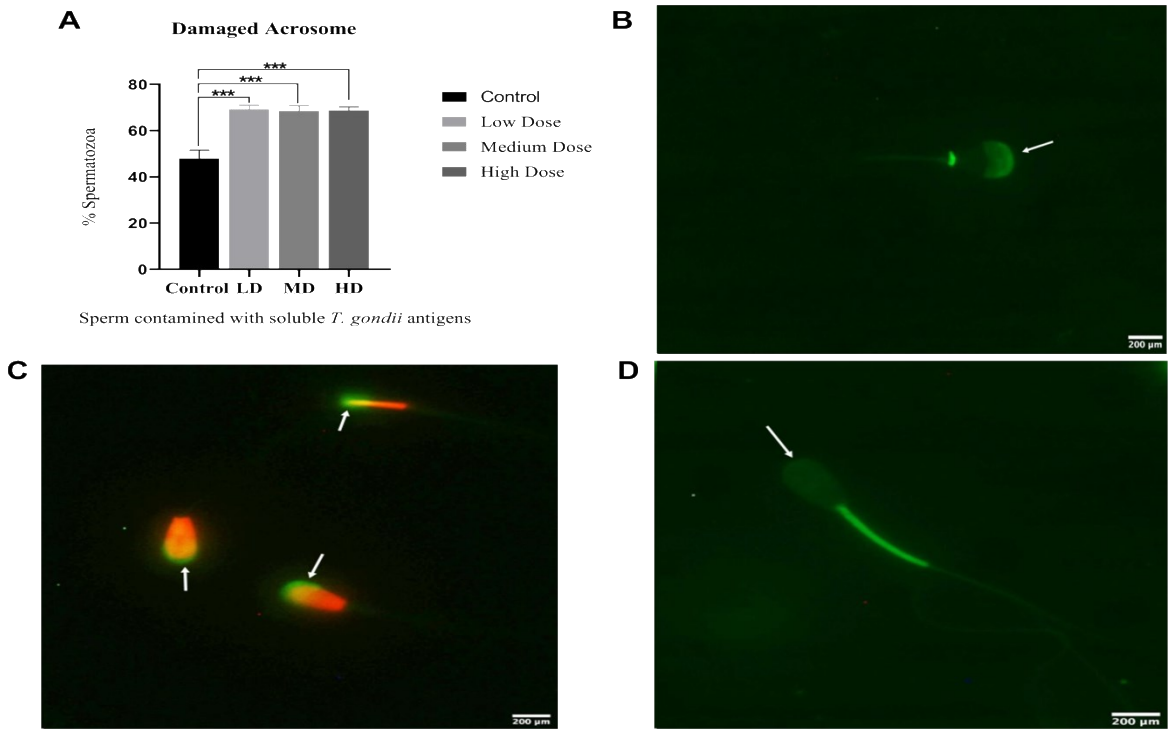

Figure 11

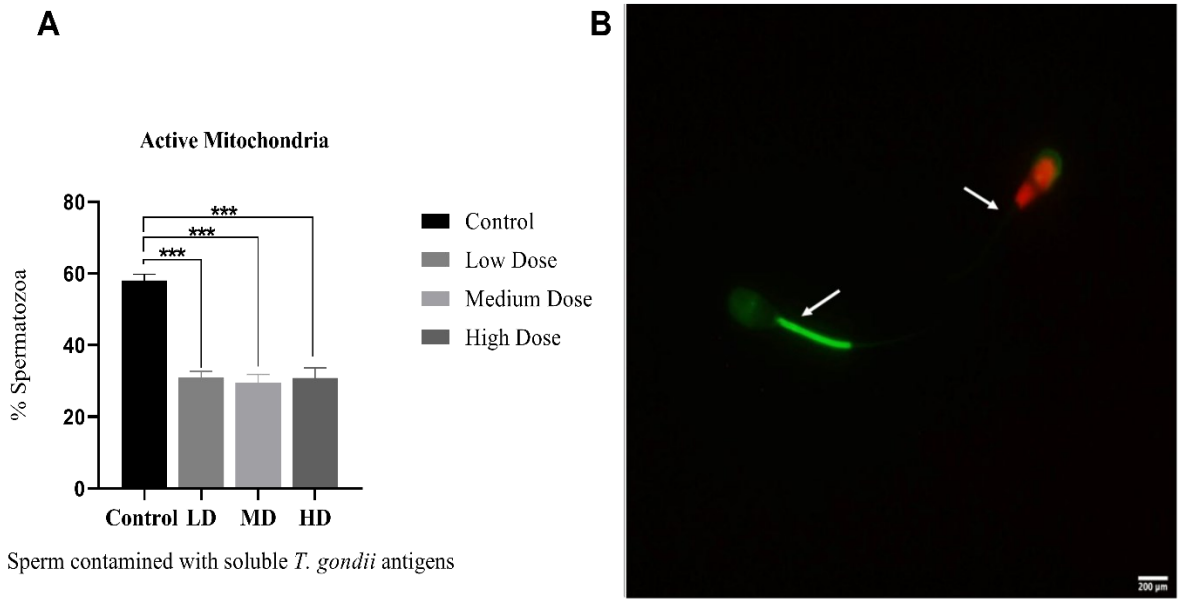

Figure 12

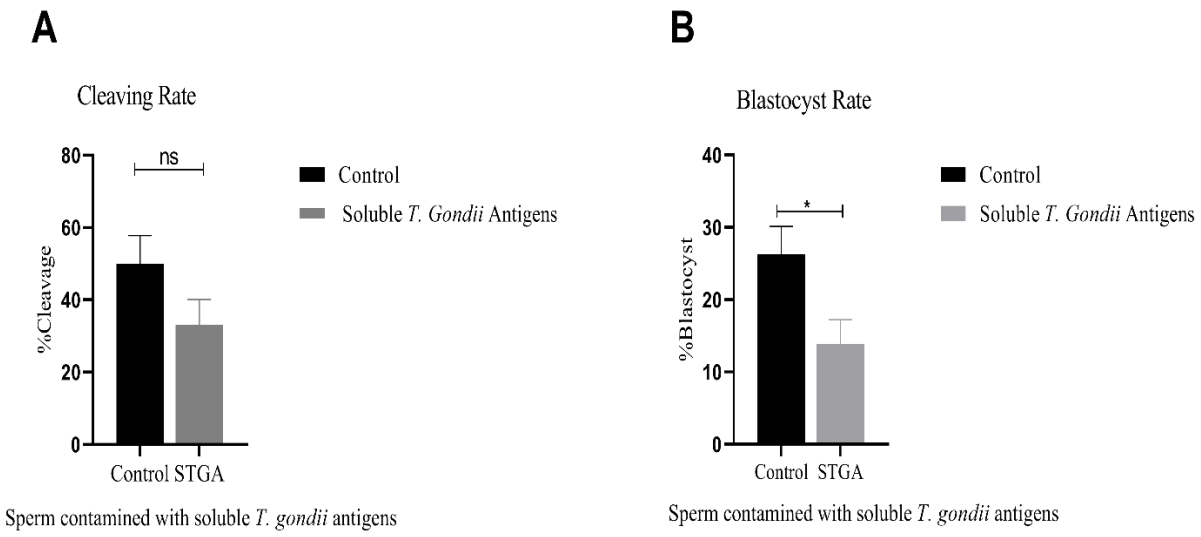

Table 1. Dynamics of in vitro production of bovine embryos.

Days	ACTIVITIES
-1	Post-mortem follicular aspiration In vitro selection and maturation of oocytes (IVM)
0	Contamination of spermatozoa in Sperm-TALP medium
0	In vitro fertilization (IVF) of matured oocytes (after 22 hours of maturation)
1	Stripping (from 18 to 22 hours after fertilization) and beginning of in vitro culture of probable zygotes
2	Observation of cleavage rate (48h after IVF)
3	Morula count, initial, expanded and hatched blastocysts.

ARTIGO 2

The soluble antigens of *Neospora caninum* affect the integrity and vital characteristics of bovine sperm, compromising *in vitro* fertilization and embryonic development

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Abstract
N. caninum is a parasite found worldwide that can be transmitted through contaminated food or from an infected mother to her offspring during pregnancy. This protozoan is a major threat to the healthy development of livestock, causing significant economic losses due to abortion, fetal death, and nervous system diseases in cattle. The effect of contact with cell debris from *N. caninum* on semen quality is not known, although infected animals shed DNA, protein, and even infective forms of the parasite. This study aimed to evaluate the effects of soluble *N. caninum* antigens on bovine semen quality *in vitro*. The spermatozoa were subjected to serial double dilutions of *N. caninum* antigens at high, medium, and low doses (8, 4, 2 µg/ml) using TALP-Sperm and TALP-Fert medium. Results indicated that soluble *N. caninum* antigens can affect the velocity and angle of sperm trajectory, both in the presence and absence of flow, within a simulated fallopian tube environment. In addition, several techniques, including Toulidine Blue, Propidium Iodide, transmission electron microscopy, Pisum Sativum Lectins, and mitochondrial labeling with MitoTracker, have been used to demonstrate that treatment with soluble *N. caninum* antigens can affect the integrity of sperm chromatin and cause severe damage to the sperm membrane and acrosome. Finally, the spermatozoa were treated with antigens before *in vitro* embryo production, which impacted the cleavage and blastocyst rates. After being infected with *N. caninum*, bulls may experience changes in their sperm, which can compromise fertilization process and embryonic development, affecting their fertility.

Keywords: *N. caninum*, semen quality, sperm motility, sperm membrane, acrosome, chromatin.

2. Introduction

N. caninum is an obligate intracellular protozoan that belongs to the phylum Api-complexa and the order of coccidia. This parasite is one of the main pathogens responsible for economic losses in livestock due to the high rate of abortions and stillbirths in different animals. According to statistics, the global economic losses caused by *N. caninum* infection or abortion in livestock can reach $2.38 billion annually (1). There are also additional costs associated with professional help, diagnosis, rebreeding, loss of milk production, and replacement of cull cows (2). This pathogen can infect various mammalian cells, including those of bovines, sheep and dogs (3). Dogs and coyotes are the definitive hosts of this pathogen (4). Neosporosis is a disease with a worldwide distribution and has been recognized as one of the main reproductive problems in cattle since 1991 in different countries, such as Australia, the United States, Brazil, Great Britain, Holland, Spain, France and New Zealand. The disease leads to abortion rates of approximately 21%, 34%, and 42%, respectively, in these countries (5-10). This protozoan can be transmitted horizontally or laterally postnatally. It can be ingested via contaminated food or drinking water containing tachyzoites, tissue cysts, sporulated oocysts, or transplacentally from an infected mother to the fetus during pregnancy (4). The parasite *N. caninum* can be detected in the sperm of infected bulls. This raise concerns that the parasite could be transmitted through semen, which can have serious consequences for the cattle semen industry. Artificial insemination is a widely used method for improving bovine production, and millions of doses of frozen bovine semen are sold annually around the world. This increases the risk of spreading various diseases, including neosporosis, among bovine populations (11). *N. caninum* DNA can be found in the fresh and frozen semen of bulls that are naturally or experimentally infected (12).

Although this parasite has been found in the reproductive organs of different mammals, and infected animals can eliminate DNA, protein, and even infective forms of *N. caninum*, the real effect of exposure to cellular debris from this parasite on semen quality is not well understood. Therefore, further research is needed to gain more knowledge about how such exposure affects sperm characteristics (13). The objective of this study was to evaluate the changes in the quality of bovine semen caused by soluble antigens of *N. caninum*.

2. Materials and methods

2.1. Semen samples

Frozen semen samples were obtained from a Nellore bull at a semen processing center. These samples were taken from a single ejaculate and were previously tested in the Reproduction Biology Laboratory at the Federal University of Uberlândia (UFU). The results of the test were excellent, particularly for the *in vitro* production of embryos.

2.2. *N. caninum* antigen production

N. caninum antigen lysate (NLA) was prepared using methods previously described by (14). Parasite suspensions were diluted in phosphate buffer solution (PBS) and treated with protease inhibitors (Complete Mini, Roche, Germany). The suspensions were then subjected to rapid freezing and thawing cycles and sonicated on ice. The resulting lysates were centrifuged (10,000 × g, 30 min, 4°C), and the supernatant was collected. The protein concentration was quantified using Bradford's method (15). Aliquots of the NLA were stored at -20°C until use in different procedures.

2.3. Semen dilution media

The dilution and maintenance of spermatozoa were performed with sperm capacitation medium (TALP-Fert) and sperm dilution medium (TALP-Sperm), which are routinely used in laboratories for *in vitro* production of bovine embryos. To examine the effects on spermatozoa, frozen bovine semen samples were diluted to 10 to 20 million spermatozoa per mL in TALP-Sperm or TALP-Fert medium supplemented with *N. caninum* antigens in serial double dilutions of 8 µg/ml, 4 µg/ml, and 2 µg/ml. As a control, medium without antigen was used. The samples were then incubated for one hour at 37 ℃, 5% CO_2, and saturated humidity.

2.4. Flow chamber

The flow chamber was made of glass and silicone and had the following dimensions: 32 mm in length, 1.5 mm in width, and 0.2 mm in height. The base is made of a standard glass microscope slide, while the sides consist of two halves of a coverslip (24 mm × 32 mm) joined together with silicone on the slide. This design ensures that the distance between the two halves is maintained at 1.5 mm. Additionally, an entire coverslip was fixed onto this set using silicone. Thus, considering that the laminula is 0.17 mm high and that the silicone layer is approximately 0.02 mm, there will be a tunnel with a rectangular profile measuring approximately 0.3 mm2 (Figure 1). For flow control, a piece of filter paper (20 mm × 30 mm) moistened with the same medium as the tested sample (TALP-Sperm or TALP-Fert) was placed at each end of the chamber. After homogenization, the bovine semen samples were placed in the flow chamber until they were completely filled. After complete stabilization of the mixture inside the chamber, the flow was controlled by dripping the medium onto the ends of the paper.

2.5. Sperm image acquisition

The study recorded the actions of bull spermatozoa with and without flow on a Leica DM500 microscope coupled to a Leica ICC50 image capture system with a 10X objective. Images were acquired in AVI format and stored for further processing and quantitative analysis.

2.6. Motility pattern analysis

ImageJ FIJI software (version 1.53q) was used for image processing. Using the Manual Tracking plugin, the sperm trajectories were manually traced frame by frame, with a human operator marking the position of the sperm on the X/Y axis in each frame of the recording. The generated data were processed by an algorithm developed in the MATLAB programming environment. In an environment with flow, only spermatozoa that presented rheataxis were considered. In an environment without flow, progressively motile spermatozoa were analyzed. The flow velocity, $\vec{v}_F$, was estimated by following the particle trajectories (cell debris and dead spermatozoa) at the same focal level of spermatozoa. For greater precision, this velocity was estimated for several particles along the same video sequence to obtain an interpolated vector field $\vec{v}_F(d)$, defined in terms of the distance d from the flow chamber wall. The velocity of each sperm, $\vec{v}_S$,, was estimated following the center of mass of the respective image of the sperm head. The two absolute velocities $\vec{v}_F$ and $\vec{v}_S$, were computed considering the subsequent frames by calculating the first order derivative referring to the displacement of the sperm in relation to time. The relative sperm velocity was then obtained by $\vec{v}_{R=}\vec{v}_S + \vec{v}_F(d)$, where d is the distance of the specific sperm to the flow chamber wall. All speeds were measured as the number of pixels that each structure covered in one second (pixel/s).

2.7. Toluidine blue staining technique for analysis of sperm chromatin integrity

Semen smears were taken from all treatment groups and fixed in 3:1 (v/v) ethanol-acetic acid for one minute and then in 70% ethanol for three minutes. After fixation, the smears were subjected to acid hydrolysis in 4N hydrochloric acid for 25 minutes and subsequently washed in distilled water. After drying, the smears were stained by placing a drop of 0.025% toluidine blue (TBO) solution in citric acid-phosphate buffer (McIlvaine buffer) at pH 4.0. Immediately after, a cover slip was placed on the slide, and the slide was sealed with enamel. After 3 minutes, digital images were captured, totaling at least 100 spermatozoa per slide. For this purpose, a Leica DM500 light microscope coupled to a Leica ICC50 camera (Leica Microsystems Inc., Buffalo Grove, IL, USA) was used with a 100x objective (immersion).

2.8. Computational analysis of images

The analysis of computerized images involved two stages. First, the software segmented the heads, and then the percentage of decompression and mean chromatin heterogeneity of each head were quantified. For segmentation of the heads, we used software developed in the MATLAB programming environment and executed by the Octave program to evaluate the mean chromatin unpacking and heterogeneity, we used software developed in the SCILAB programming environment (16).

2.9. Hyposmotic test

The experiment involved incubating a mixture containing 75 µL of semen and 475 µL of hyposmotic solution (MLQ water) after administering four different treatments. The mixture was kept in a water bath at 37 ℃ for 30 minutes. Wet preparations were made

from each of the samples, and 200 cells were evaluated. Spermatozoa with intact membranes were considered those that presented folding in the tail and were considered to have damaged membranes, those spermatozoa that remained with straight tails. The evaluations were performed with a Leica DM500 light microscope (Leica Microsystems Inc., Buffalo Grove, IL, USA) using a 40x objective. The hyposmotic test was performed according to (17).

2.10. Transmission Electron Microscopy (TEM)

The samples from each treatment were centrifuged at 50 × g, the supernatant was discarded, and a 2.5% glutaraldehyde fixative solution was added to 0.1 M phosphate buffer (pH 7.4) for approximately 4 hours. After the fixation process, the supernatant was discarded to remove the fixatives, the volume was readjusted with PBS (pH 7.4), and the microtube was shaken. The sample was again subjected to centrifugation at 50 × g for 5 minutes, after which the supernatant was discarded. The procedure was repeated three times. After the last wash, 500 µL of 2% osmium tetroxide solution and 500 µL of PBS (pH 7.4) were added to the microtubes for 30 minutes. Again, centrifugation was performed at 50 × g for 5 minutes, after which the supernatant was discarded.

Subsequently, the removal of osmium tetroxide was carried out with a PBS in a way like that used for the removal of glutaraldehyde. After the last wash, 2.5% agar at 50°C was added to the cell precipitate and mixed to introduce the agar between the cells. The cells were kept at the bottom of the microtube and taken to the refrigerator (4°C) until solidification. The solidified agar was removed from the microtube and broken into 1 mm 3 fragments. The fragments were placed in glass flasks and dehydrated in baths of increasing concentrations of alcohol followed by three baths of propylene oxide. After

discarding the propylene oxide from the last dehydration step, the fragments were finally included in Epon resin. After solidification (polymerization) in the oven, the blocks were trimmed, and cuts were made with a Reichert-Jung ultramicrotome (Leica Microsystems Inc., Buffalo Grove, IL, USA) with a thickness of 60 to 90 nm. The blocks were subsequently placed on 200 mesh copper grids and stained with uranyl acetate and lead citrate as described by (18). The sections were analyzed and documented photographically in a Hitachi HT 7700 electron microscope, and digital images of the spermatozoa heads were obtained.

2.11 Analysis of the integrity of the plasma membrane

The sperm cells were placed in TALP-Sperm medium and incubated with soluble *N. caninum* antigens at three different concentrations (8, 4, and 2 µg/ml) and medium only (no antigen) as a control at 37 ℃, 5% CO_2 and saturated humidity. After one hour of incubation, centrifugation was performed at 16 × g for 5 minutes, with subsequent disposal of the supernatant. Fifteen microliters of the cell precipitate suspension was placed in 200 µL of PBS heated to 37°C with 5 µL of propidium iodide (PI) stock solution (50 ml of PBS in 0.01 g of PI). This solution was incubated for 5 minutes at room temperature. For the evaluation, a drop of the solution was placed on slides preheated to 37°C and covered with a coverslip. Then, images were captured for a period of 5 minutes to avoid false positives due to temperature changes. The evaluations were performed using an Olympus FSX100 fluorescence microscope with a 40x objective. The sperm with a red fluorescent head were considered to have membrane lesions.

2.12. Acrosome integrity analysis

The experiment involved incubating spermatozoa in TALP-Sperm medium at 37 ℃, 5% CO2 and saturated humidity with varying concentrations of soluble *N. caninum* antigens (8, 4 and 2 µg/ml), while a control group was only incubated in medium without any antigen. After an hour of incubation, centrifugation was performed at 16 × g for 5 minutes, followed by disposal of the supernatant. Fifteen microliters of the cell precipitate suspension was placed in 200 µL of phosphate buffer heated to 37°C with 5 µL of the solution for use of Pisum Sativum Lectins associated with fluorescein (PSA/FITC) (stock solution: 2 mg total vial content in 2 ml of PBS). The solution used was 100 µL of stock solution in 900 µL of PBS. This solution was incubated for 5 minutes at room temperature. The evaluations were performed with an Olympus FSX100 fluorescence microscope using a 40x objective. The damage to the acrosome of sperm cells was detected by observing green fluorescence in the acrosomal region.

2.13. Analysis of mitochondrial activity.

The experiment involved placing spermatozoa in TALP-Sperm medium supplemented with varying concentrations of soluble *N. caninum* antigens - high (8 µg/ml), medium (4 µg/ml), low (2 µg/ml) - and a control medium with no antigen. The mixture was then incubated at 37 ℃, 5% CO_2 and saturated humidity for one hour. After that, the mixture was centrifuged at 16 × g for 5 minutes, and the supernatant was discarded. Fifteen microliters of the cell precipitate suspension was placed in 200 µL of PBS heated to 37°C with 10 µL of the stock solution of MitoTracker Green (2.8 µg/ml of anhydrous dimethylsulfoxide (DMSO)). This solution was incubated for 30 minutes at 37 ℃, 5% CO_2 and saturated humidity. The evaluations were performed with an Olympus FSX100 fluorescence microscope using a 40x objective. The sperm with active

mitochondria were identified by observing the green fluorescence in the middle section of their tail.

2.14. *In vitro* production of embryos

Eight *in vitro* embryo production routines were carried out with designated days labeled -1, 0, 1, 2, and 7 (Table 1). On day 0, the spermatozoa were treated with soluble *N. caninum* antigens. The routines were performed following the protocol established at the Laboratory of Biology of Reproduction at UFU and described by (18).

The cleavage rate was evaluated 48 hours after *in vitro* fertilization by examining structures that had two or more cells. Seven days after fertilization, the rate of blastocyst formation was evaluated by counting structures that had reached this stage of development, regardless of whether they were initial, expanded, or hatched blastocysts.

2.15. Statistical analysis

To determine any differences between the conditions tested in the analyses, the Kolmogorov–Smirnov test was performed to confirm the data distribution; later, one-way ANOVA was used to compare the treatments, and Fisher's exact test was used to compare the three treatments. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Soluble *N. caninum* antigens altered the angulation and average swimming speed of the trajectory of capacitated and noncapacitated spermatozoa in environments with and without flow.

First, the average velocity of capacitated spermatozoa contaminated with soluble *N. caninum* antigens was evaluated in a simulated environment of a bovine fallopian tube

with flow. The mean sperm velocity was reduced at the three concentrations evaluated compared to the control (medium) (****$p < 0.00001$). Regarding the difference between the conditions, treatments with a medium (4 µg/ml) and low dose (2 µg/ml) showed lower rates compared to treatment with a high dose (8 µg/ml) (*** $p < 0.0001$) (Figure 2A). In our experiment to measure the average velocity of capacitated spermatozoa in a no-flow environment, we found that the velocity of capacitated spermatozoa was lower in all tested conditions compared to the control (medium) (****p< 0, 00001) (Figure 2B). Afterwards, we conducted tests to determine whether certain antigens affected the speed of noncapacitated spermatozoa in a simulated environment of a bovine uterine tube with flow. To do this, various assays were performed on bovine semen samples. These samples were diluted in TALP-Sperm medium and then treated with different concentrations (8, 4 and 2 µg/ml) of *N. caninum* antigens. The results showed that soluble *N. caninum* antigens caused a reduction in the mean sperm velocity at medium and high concentrations (8 and 4 µg/ml) compared to the control group. The difference was significant (****$p < 0.0001$), as shown in (Figure 3A). In addition, we conducted a study to assess the average speed of noncapacitated spermatozoa in a flow-free environment. The results showed that the average speed of spermatozoa decreased at different concentrations (8, 4 and 2 µg/ml) compared to the control (****$p < 0.0001$). We also observed that treatment with a low dose (2 µg/ml) resulted in a higher speed compared to the medium and high concentration treatments (8 and 4 µg/ml) (***$p < 0.0001$) (Figure 3B). Subsequently, to assess whether soluble *N. caninum* antigens have a direct effect on rheotaxic behavior, we evaluated whether antigen treatments affect the average angulation in the trajectory of capacitated and noncapacitated spermatozoa in a simulated bovine fallopian tube environment with flow. The average angulation of capacitated spermatozoa

in the high and low dose conditions (8 and 2 µg/ml) was significantly lower than that in the control group. (**p < 0.005). (*p < 0.05). (Figure 4A). In the case of noncapacitated spermatozoa, there was no significant difference in relation to the control (Figure 4B). Notably, the changes caused in sperm were dose independent. Then, with the aim of deepening our studies, we began to evaluate the effect of soluble *N. caninum* antigens on sperm chromatin.

3.2. *N. caninum* soluble antigens promote the decompression of bovine sperm chromatin.

To assess chromatin alterations, the first method used was computer image analysis of semen smears stained with toluidine blue (pH 4.0) following acid hydrolysis (Figure 5A). The analysis revealed that the high-dose treatment (8 µg/ml) resulted in a greater number of sperm chromatin alterations compared to the control treatment (**p < 0.005). Moreover, there was a significant difference between the high and low doses (8.2 µg/ml) (*p < 0.05) under different conditions.

For a second evaluation of bovine sperm chromatin decompression, we used TEM. Three classifications of chromatin were considered in this analysis: intact, with mild alterations (points of decompression) and with severe alterations (areas of decompression). The analysis showed that compared with the control treatment, the high-dose treatment (8 µg/ml) decreased the percentage of spermatozoa with intact chromatin (*p < 0.05) (Figure 6A, 6B).

In the case of minor alterations, it was observed that a low dose (2 µg/ml) of the antigen caused fewer changes compared to tratamentos of 8 µg/ml and 4 µg/ml (***p < 0.0001) (as shown in (Figures 6C and 6D). However, for severe alterations (Figure 6E and

6F), all concentrations of the antigen tested (8 µg/ml, 4 µg/ml and 2 µg/ml) resulted in an increase in the amount of chromatin damage compared to the control group (****$p < 0.00001$). In terms of the comparison of different concentrations, treatment with a low dose (2 µg/ml) showed a higher number of alterations in comparison to the treatment with a medium dose (4 µg/ml) (*$p < 0.05$). As a subsequent evaluation, the effect of these antigens on the integrity of the sperm membrane was assessed.

3.3. *N. caninum* soluble antigens disrupt bovine sperm membrane integrity.

To determine the impact of antigens on the plasma membrane of bull spermatozoa, a hyposmotic test was conducted. This test helps in identifying spermatozoa with intact membranes and damaged membranes (Figure 7A, 7B). The test results revealed that there was a percentage difference, but it was not statistically significant for any of the concentrations used in comparison to the control (NS). We conducted transmission electron microscopy to evaluate the sperm plasma membrane and obtain better results. We classified membrane damage into three categories: intact membrane, mildly damaged membrane, and completely damaged membrane. According to the analysis of the percentage of sperm with intact membranes, all antigen treatments (8, 4, and 2 µg/ml) decreased the percentage of sperm with intact membranes compared to the control group (***$p < 0.0001$), as shown in (Figures 7C and 7D). However, in the membrane classification with light damage, there was no significant difference in any of the evaluated concentrations in relation to the control (NS), as illustrated in (Figures 7E and 7F).

Finally, in the classification of totally damaged membranes, all concentrations (8, 4, and 2 µg/ml) of the antigen significantly increased the amount of damage compared to

the control group (***p < 0.0001). This is illustrated in (Figures 7G and 7H). Furthermore, when comparing the different doses, the medium and low doses were found to cause more damage to the membrane than the high dose did (*p < 0.05).

As a complementary test to confirm the results of our evaluation of the effect of soluble *N. caninum* antigens on sperm membrane integrity, we used propidium iodide. This helps verify the viability of the plasma membrane. Loss of membrane integrity led to the incorporation of the dye into the cells, as shown in (Figures 8A and 8B). These antigens significantly impacted the integrity of the spermatic membrane. There was a noticeable difference in all three concentrations evaluated (8, 4 and 2 µg/ml) in comparison to the control (***p < 0.0001). Additionally, we evaluated the effect of these antigens on the integrity of the sperm acrosome, which is essential for the fertilization process.

3.4 Sperm exposed to various concentrations of soluble *N. caninum* antigens exhibit acrosomal damage.

This study aimed to evaluate the impact of antigens on bull sperm acrosome using TEM. The acrosome was classified into two types: intact and damaged. The findings revealed in the case of the first classification that all antigen concentrations used decreased the percentage of sperm with intact acrosomes compared to the control (****p < 0.00001). This result is evident in (Figures 9A and 9B). According to the second classification, compared with the control, the antigen doses (4 and 8 µg/ml) significantly increased the number of sperm with acrosome damage (*p < 0.05). This result is evident in (Figures 9C and 9D).

To improve the evaluation of sperm acrosome integrity, Pisum Sativum Lectins (PSA) combined with fluorescein were used as acrosomal markers. The analysis demonstrated that the soluble antigens of *N. caninum* affect the acrosome integrity of bovine spermatozoa (Figures 10A, 10B, 10C, 10D). The results showed that the treatments with high and medium doses (8 and 4 µg/ml) had greater effects than the control. (**$p < 0.005$). Moreover, compared with the low dose (2 µg/ml), the high and medium doses had significantly different effects on acrosome integrity (*$p < 0.05$). The next assessment will focus on analyzing the effect of these antigens on mitochondrial activity.

3.5. *N. caninum* soluble antigens affected mitochondrial activity in bovine sperm at varying concentrations.

This study aimed to investigate the effect of soluble antigens of *N. caninum* on the mitochondrial activity of bull spermatozoa. The researchers employed the mitochondrial labeling technique with MitoTracker and observed that the antigens had a significant impact on the mitochondrial activity of the spermatozoa (Figures 11A, 11B, 11C and 11D). The study showed a substantial difference in the results of the three evaluated conditions (8, 4, and 2 µg/ml) when compared to the control group (***$p < 0.0001$). Researchers have further examined the effect of these antigens on the fertilization process and embryonic development by conducting *in vitro* embryo production (IVP) experiments.

3.6. Cleavage and blastocyst rates are impacted by the presence of soluble *N. caninum* antigens during *in vitro* embryo production (IVEP).

We conducted a study to determine how the mean dose (4 µg/ml) of soluble *N. caninum* antigens impacts the cleavage and blastocyst rates in IVEP. We found that the use of spermatozoa infected with antigens during fertilization significantly reduced the cleavage rate (***$p < 0.0001$) as shown in (Figure 12A) and subsequently the blastocyst rate compared to those of the control group (*$p < 0.05$) as shown in (Figure 12B).

4. Discussion

Neospora caninum is a type of parasite that lives inside host cells and belongs to the Apicomplexa phylum and the coccidia order. It has been observed that infected bulls can carry *N. caninum* in their semen, which can lead to problems in the quality of their sperm (11). However, it is not clear whether these issues are caused by defects in spermatogenesis or the direct impact of parasite antigens on the existing spermatozoa. This is the rationale behind conducting this research.

However, how *N. caninum* antigens affect the average swimming speed and trajectory angle of bovine spermatozoa that show rheotaxis (counterflow movement) in a simulated bovine fallopian tube environment has not yet been investigated. Low sperm motility is the main cause of male infertility. (19). Another aspect that has not been studied is the rheotaxic behavior (counterflow movement) and the angle of the sperm trajectory on its journey to find the oocyte. Our research group has demonstrated that even before sperm capacitation, spermatozoa exhibit rheotaxic behavior with slight lateral angulation in relation to the flow trajectory. This behavior causes the spermatozoon to reach the limit of its environment, which in this case is the wall of the fallopian tube. This location is where final sperm capacitation occurs (20).

Sperm motility and angulation are crucial factors to consider when evaluating sperm characteristics. The results of the present study revealed that the presence of soluble *N. caninum* antigens in TALP-Sperm and TALP-Fert medium affected sperm behavior, specifically in terms of the mean swimming speed (positive rheotaxis) and angulation. This impact was observed in both the absence and presence of flow. However, angulation was only affected in capacitated sperm. It is important to note that spermatozoa must travel long distances through viscous fluid and against a flow of fluid to reach the ovum, which is typically located in the center of the ampulla of the bovine fallopian tube; therefore, when these functions are affected, the angulation and speed may not be enough for spermatozoa to be able to overcome the tubal flow and rise to meet the oocyte without missing the ideal moment of fertilization. These changes caused by the presence of *N. caninum* antigens may lead to fertility problems both in vitro and in vivo.

These results are consistent with those obtained by (13) who analyzed the sperm parameters of samples obtained from the epididymis of bulls not infected or infected with *N. caninum* and found that the sperm motility rate decreased significantly ($p = 0.037$). Another study by (21) reported that *N. caninum*-seropositive bulls had significantly lower concentrations, viability, and motility of epididymal spermatozoa than seronegative bulls. Similarly, a study on male BALB/c mice infected with *N. caninum* by (22) also revealed that this parasite has a negative effect on sperm motility.

It is worth noting that research has been conducted to assess the impact of *N. caninum* on the quality of sperm in various species; however, no significant changes were found. Nevertheless, these studies were unable to determine the stage of infection, meaning it was not possible to determine if and when the animals began to eliminate the parasites in their semen. For instance, (23) evaluated the sperm quality of seropositive and

seronegative Belga Blue bulls at an artificial insemination center in Belgium. They concluded that the presence of *N. caninum* antibodies in these bulls did not negatively affect their semen quality, including morphology, total motility, progressive motility, and fresh motility. It is important to note that only the bulls that tested positive or negative for neosporosis through six antibody tests were included in the final evaluation during the one-year research period.

There are several methods for evaluating sperm chromatin compaction, including toluidine blue, which allows the simultaneous evaluation of sperm morphometry (16), and transmission electron microscopy, which is defined by (24-26) as the best method for exploring morphology, sperm pathologies, and the characterization of ultrastructural defects of the acrosome, nucleus, chromatin, intermediate piece, mitochondria, axonemal and peraxonemal structures of spermatozoa. Our results demonstrated by both techniques that the soluble antigens of *N. caninum* promote the decompression of bovine sperm chromatin. Emphasizing the observations made with the use of TEM, after treatment with antigens, the points of slight decompression existing in the sperm chromatin became large areas of decompression, suggesting that the different concentrations used increased the intensity of decompression of the sperm chromatin in bovine sperm.

Previous studies have shown that any form of sperm chromatin abnormality or DNA damage can result in male infertility, and in support of this conclusion, it has been observed that *in vivo* fertilization progressively decreases when more than 30% of spermatozoa are identified with DNA damage (27). When referring to chromatin, the differences in decompression by what little they are of great importance. Such changes in chromatin could interfere with male fertility in different ways depending on the intensity of the change. Severe chromatin alterations in spermatozoa cause alterations in the shape

of the head and, therefore, in its hydrodynamics, which can lead to inadequate motility, directly interfering with the fertilization process. Other less severe sperm alterations might not interfere with motility and, consequently, with the fertilization process, but damage to the DNA would make it impossible for the male and female pronuclei to unite and, therefore, would make the zygote unfeasible. Even milder alterations might not interfere with fertilization and initial embryonic development but could interfere with later stages of embryonic development, which could lead to embryonic death with consequent fetal resorption or abortion. Even in less frequent situations, pregnancy can reach term, but neonates can experience genetic alterations of different types and intensities (28,29). The integrity of DNA in sperm is essential for the transmission of genetic information and, in turn, the maintenance of good health in future generations (27).

The conventional parameters used for semen evaluation have limited application, and it is necessary to consider that each sperm cell consists of multiple subcellular compartments with different functions, which must be intact for successful fertilization (30). Currently, to improve male fertility assessments, the integrity of the sperm plasma membrane has become a very important factor to be evaluated, as it is fundamental in the fertilization process (31). One of the tests used to assess membrane integrity in bovine spermatozoa in our study was the hypo-osmotic swelling test, which was originally developed with the aim of evaluating the membrane of human spermatozoa (32). After its development, this test was considered a simple and accessible method (30), starting to be used to evaluate the integrity of the spermatic membrane in other species, showing good results in cattle, horses, dogs and cats. pigs (31). Using this method, our results showed that there was a percentage increase in spermatozoa with damaged membranes, but there was no significant difference in relation to the control.

As a second test, TEM was used to analyze the integrity of the sperm membrane, and our results revealed that the soluble antigens of *N. caninum* cause damage to the integrity of the plasma membrane of bull spermatozoa, considering that in spermatozoa that presented partially damaged membrane, after being subjected to treatment with soluble antigens of *N. caninun*, the damage in the membranes of the bovine spermatozoa increased, becoming completely damaged. In addition, as a complementary test for greater safety in our evaluations, propidium iodide, which is an intercalating agent that is used to stain nucleic acids and is capable of binding to DNA molecules between their bases, was used; one of its applications is the evaluation of the viability of the plasmatic membrane because, if the integrity of the membrane decreases, the incorporation of the dye in the cells will increase. Our results showed that all antigen conditions tested affected sperm membrane integrity compared to the control. It can be concluded from our observations that soluble *N. caninum* antigens cause and increase sperm membrane damage in bull spermatozoa.

We highlight the importance of our results, considering that the integrity of the membrane and the stability of its semipermeable aspect are prerequisites for sperm viability. Furthermore, if the plasma membrane is intact but functionally unstable, the sperm cannot interact with the environment of the female genital tract and consequently fertilize the ovum (33). That is, some injuries to the sperm membrane can prevent vital processes from being fulfilled, highlighting that the integrity and functional activity of sperm are highly important for carrying out metabolism, fertilization, training, acrosome reaction and sperm binding to the zona pellucida; these processes require intact and active membranes (34).

Following the trend of deepening our study, we started to evaluate the sperm acrosome as a fundamental part of the fertilization process. Mammalian sperm undergo many changes during their formation, with new spermatozoa becoming immobile and unable to attach to the extracellular lining of the egg, the zona pellucia (ZP) and fertilizing the egg. During epididymal transit, sperm undergo a series of biochemical processes and functional changes, collectively referred to as epididymal maturation, and during capacitation in the female genital tract. Only capacitated sperm are capable of binding to the ovum with the zona intact and undergoing an acrosomal reaction (35). The acrosome plays an important role at the sperm-zone (egg) binding site during the fertilization process. This egg-sperm interaction is a specific event mediated by carbohydrate species, an event that initiates a signal transduction cascade resulting in the exocytosis of the acrosomal content of the sperm, that is, the acrosomal reaction. This step is believed to be a prerequisite that allows acrosome-reacted spermatozoa to penetrate the zona pellucida and fertilize the ovum (36).

In this sense, based on our results, we observed that the soluble antigens of *N. caninum* significantly affected the integrity of the spermatic acrosome both in analyses using TEM and in those made with Lectins Pisum Sativum associated with fluorescein. Since this type of glycoprotein is specific for binding to terminal sugars located in cell structures, recognizing the mannose, glucose and glucosamine terminails of glycoconjugates located in the acrosomal matrix, when associated with fluorescein, it can identify spermatozoa with injured or absent acrosomes (37). It should be considered that defective acrosomes in a sperm population indicate a greater chance of male infertility, highlighting that the functional integrity of the acrosome is essential for fixation, species-specific binding, penetration and gamete fusion (38).

There is little or no information from investigations that evaluated the influence of *N. caninum* on the integrity of the sperm acrosome, highlighting that several studies have made more general evaluations. In the case of bacteria, there are reports demonstrating that the invasion of these microorganisms can contribute to the deterioration of sperm quality, which is visible in routine semen analysis, especially in infertile men, where sperm morphology and impairment of acrosomal reactions are the alterations most frequently revealed in spermatozoa (39).

For sperm to be able to perform their functions and reach their ultimate goal, which is fertilization, they need energy (40), with mitochondria responsible for approximately 90% of the production of this cellular energy from the generation of adenosine triphosphate (ATP), thereby enabling cell movement. (41). Our analysis used MitoTracker, which is capable of passively crossing the membrane of viable cells and reacting with protein and peptide thiols to form an aldehyde-fixable conjugate (42), as a marker of mitochondrial activity. Therefore, highlighting the importance of our results regarding how the soluble antigens of *N. caninum* affect the mitochondrial activity of bovine spermatozoa, we can infer that if mitochondrial activity is affected, ATP is not produced, which interferes with sperm function, as it affects the movement of flagella, impairing sperm motility and consequently the fertilization process (43). Highlighting that mitochondrial dysfunction that has been implicated in the pathogenesis of seminal oxidative stress (OS), is a key element responsible for many cases of male infertility, leading to the conclusion that the role of mitochondria in male fertility is directly linked to sperm motility (44), and this lack or low sperm motility is the main cause of male infertility (19). These results corroborate with the results found from the rheotaxis analyses.

Among the main factors that compromise oocyte fertilization, gamete transport and embryonic and fetal viability in cattle are genetic, metabolic, environmental, nutritional and infectious factors (45). Despite the fact that the correlation between fertility parameters and sperm quality is not always very clear, this phenomenon is very complex and depends on the capacity of the spermatozoa to fertilize the oocyte and allow the development of the zygote. To provide more information on the functional state of the spermatozoa that participate in the in vitro fertilization process, with the aim of obtaining better rates in this process, it is necessary to evaluate parameters related to motility and specifically progressive motility, the integrity of the sperm membrane that reflects sperm viability, and the integrity of the sperm acrosome that is necessary for sperm penetration through the zona pellucida (46). Finally, after evaluating different sperm parameters, such as the integrity of the different components that make up the sperm, our results demonstrated that the soluble antigens of *N. caninum* affect the cleavage and blastocyst rate in the *in vitro* embryo production.

In this case, infectious factors could compromise the process of fertilization of oocytes, in addition to embryonic development. Therefore, it is necessary to consider that to carry out the fertilization process, the structures of the spermatozoa must be in perfect condition (32). As already demonstrated in our study, important sperm parameters and components, such as motility, chromatin compaction, the plasma membrane and the acrosome, were affected. However, spermatozoa contaminated with these antigens were able to carry out the fertilization process, but with a low percentage of cleavage and blastocyst rate compared to the control. In the case of blastocysts that managed to develop, they could have slight chromatin alterations, which could interfere with later stages of embryonic development, which could lead to embryonic death or abortion (28).

Conclusion

In conclusion, it was observed that the presence of soluble antigens of *N. caninum* in bovine semen alters motility, mitochondrial activity, chromatin, membrane integrity and sperm acrosome, these characteristics being essential for the fertilization process and embryonic development, interfering with male fertility.

In the in vitro embryo production routines, semen contaminated with antigens was used, observing that the fertilization and embryonic development process was affected, highlighting that the sperm were subjected to a selection process using a Percoll gradient, with the aim of eliminating sperm whose fertilization capacity was affected

Author contributions

KB and MB: designed the experiments, performed the experiments, analyzed the data, and wrote the manuscript. BT: Software development for data analysis and analysis. AF: performed the experiments and writing. YP: performed experiments and writing. MM: performed the experiments and writing. KF: performed experiments. TM: production of soluble antigens of *Neospora caninum*. All authors approved the final version of the manuscript.

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Abbreviations list

ATP = adenosine triphosphate

CO2 = carbon dioxide

HOS = hypo-osmotic swelling test

IVP = *in vitro* embryo production

NLA = *N. caninum* antigen lysate

PBS = phosphate buffer solution

PI = propidium iodide

PSA/FITC = pisum sativum lectins associated with fluorescein

TBO= toluidine blue

TALP-Sperm = sperm dilution medium

TALP-Fert = sperm capacitation medium

TEM = transmission electron microscopy

OS = oxidative stress

ZP = zona pellucia

Declaration of competing interest

The authors declare that they have no known personal, business, or financial relationships that could be construed as a potential conflict of interest that could have influenced the work presented in this article.

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Figure Legends:

Figure 1. The flow chamber was used to measure the behavior of bull sperm in the presence or absence of flow. In the central part, the duct where the spermatozoa were

placed was observed, and at the ends, the pieces of filter paper fulfilled the function of causing flow.

Figure 2. Bovine spermatozoa in TALP-Fert medium were treated with soluble *N. caninum* antigens at different concentrations for 1 h, and bovine semen in medium without antigens was used as a control. In the motility tests, the sperm velocity is represented as the average trajectory velocity of capacitated spermatozoa in a against flow environment (A) without flow (B). The results are expressed as the mean ± standard deviation of three independent experiments performed with three replications. Significant differences were detected by one-way ANOVA with Tukey's multiple comparison post hoc test, when appropriate. ****P < 0.00001. ***P < 0.0001.

Figure 3. Bovine spermatozoa were treated with soluble *N. caninum* antigens at different concentrations in TALP-Sperm medium for 1 h, and bovine semen and antigen-free medium were used as controls. The motility assay was performed, and sperm velocity is represented as the average trajectory velocity of noncapacitated spermatozoa against flow (A) and without flow (B) in a simulated bovine fallopian tube environment when appropriate. The results are expressed as the mean ± standard deviation of three independent experiments performed with three replications. Significant differences were detected by one-way ANOVA with Tukey's multiple comparison post hoc test, when appropriate. ****P < 0.00001. ***P < 0.0001.

Figure 4. Average angulation of capacitated and noncapitated sperm trajectories in a simulated bovine fallopian tube environment. Bovine spermatozoa were treated for 1 h with various concentrations of soluble *N. caninum* antigens in TALP-Fert and TALP-Sperm medium. As a control, spermatozoa were subjected to the same conditions but without the presence of antigens. Sperm angulation is represented as the average

angulation in the trajectory of capacitated (A) and noncapacitated (B) spermatozoa against the flow. The results are expressed as the mean ± standard deviation of three independent experiments performed with three replications. When significant differences were detected, the analyses were performed using one-way ANOVA with Tukey's multiple comparison post hoc test, when appropriate. **P < 0.001. *P < 0.01.

Figure 5. Identification of bovine sperm chromatin alterations. Bovine spermatozoa were treated with three concentrations of soluble *N. caninum* antigens for 1 h in TALP-Sperm medium. Spermatozoa and antigen-free noncapacitated medium (A) were used, with the implementation of the toluidine blue method. Chromatin unpacking was represented as the average sperm chromatin unpacking percentage. Results are expressed as mean ± standard deviation of three independent experiments. Significant differences detected by one-way ANOVA with Tukey's multiple comparison post-test, when appropriate. *P < 0.01. **P < 0.001.

Figure 6. Evaluation of different classifications of chromatin decompression with the implementation of the TEM technique in bovine spermatozoa treated with soluble antigens of *N. caninum* for 1 h. As a control, spermatozoa in TALP-Sperm medium without the presence of antigens. Sperm with intact chromatin (A). This classification was represented as the total number of spermatozoa with intact chromatin per condition versus control. A representative image of sperm with intact chromatin is shown (B). Significant differences were detected by Fisher's exact test; when appropriate, *P < 0.01. **P < 0.001. (C), Spermatozoa with slight chromatin unpacking. The classification is represented as the total number of spermatozoa with chromatin decompression points under the different conditions versus the control. A reference image showing slight chromatin decompression (D). Significant differences were detected by Fisher's exact test,

when appropriate. ***P < 0.0001. (E), Sperm with severe chromatin damage. The chromatin damage score is represented as the total number of sperm with areas of chromatin decompression per condition versus the control. (F) Representative image of sperm with severe chromatin damage. Significant differences were detected by Fisher's exact test, when appropriate. ***P < 0.0001. *P < 0.01.

Figure 7. Sperm membrane integrity. Spermatozoa were treated with soluble *N. caninum* antigens at different concentrations for 1 h in TALP-Sperm medium. As a control, spermatozoa were subjected to the same conditions without antigen treatment. In the case of the analysis performed for the Hypo-Osmotic test (A), sperm membrane integrity was represented as the average of spermatozoa with damaged membrane per condition versus the control. Representative image of the Hypo-Osmotic test, the black arrows show spermatozoa with intact membrane that presented folding in the tail and the white arrows those with damaged membrane that presented straight tails (B). Significant differences were not detected. Different types of sperm membrane damage were classified by implementing the TEM method. Intact sperm membrane (C) is represented as the total number of sperm with intact membrane per condition versus control. A representative image of spermatozoa showing an intact sperm membrane is shown (D). Significant differences were detected by Fisher's exact test, when appropriate. ***P < 0.0001.*P < 0.01. Sperm with mild membrane damage (E), mild damage in this classification was represented as the total number of sperm with slightly damaged membrane per condition versus controle. Reference image showing slight damage to the sperm membrane (F). Significant differences not observed. Completely Membrane Damaged Sperm (G), this membrane score was represented as the total number of fully membrane damaged sperm per condition versus control. Representative image of spermatozoa with completely

damaged membrane (H). Significant differences detected by Fisher's exact test, when appropriate. ***P < 0.0001. *P < 0.01.

Figure 8. Sperm membrane integrity. The sperm were treated with soluble *N. caninum* antigens at different concentrations for 1 h in TALP-Sperm medium. As a control, spermatozoa with only medium (no antigen) were used. For this propidium iodide test, (A), sperm membrane integrity was plotted as the mean of sperm with damaged membrane per condition versus the control. Representative image considering with damaged membrane the spermatozoa with red head color, and spermatozoa with integral membrane those that presented absence of the red color in the head (B). Significant diferences were detected by one-way ANOVA with Tukey's multiple comparison post hoc test, when appropriate. ***P < 0.0001.

Figure 9. Evaluation of the sperm acrosome. Spermatozoa were treated with soluble *N. caninum* antigens at different concentrations for 1 h in TALP-Sperm medium. As a control, spermatozoa were subjected to the same conditions without the presence of antigens. The evaluations were made using the TEM method. The intact sperm acrosome classification (A) is represented as the total number of spermatozoa with intact acrosome per condition versus the control. Image showing the intact spermatic acrosome (B). Significant differences were detected by Fisher's exact test, when appropriate. ****P < 0.00001. *P < 0.01. In the case of damaged acrosome (C), this classification was represented as the total number of spermatozoa with damaged acrosome per condition versus the control. A representative image of a damaged sperm acrosome (D). Significant differences were detected by Fisher's exact test, when appropriate. *P < 0.01.

Figure 10. Evaluation of the sperm acrosome. The sperm were treated with soluble *N. caninum* antigens at different concentrations for 1 h in TALP-Sperm medium. As a

control, spermatozoa with only medium (no antigen) were used. This analysis was carried out using *Pisum* sativum lectins combined with fluorescein as an acrosomal marker (A). The acrosome integrity is represented as the average of spermatozoa with damaged acrosome per condition versus the control. Reference image considering spermatozoa with damaged acrosome (B, C) and spermatozoa with intact acrosome (D). Significant differences were detected by one-way ANOVA with Tukey's multiple comparison post hoc test, when appropriate. **P < 0.001. *P < 0.05.

Figure 11. Assessment of mitochondrial activity. Bovine spermatozoa were treated with soluble *N. caninum* antigens at different concentrations for 1 h in TALP-Sperm medium. As a control, spermatozoa were subjected to the same conditions without the presence of antigens. In this analysis, MitoTracker was used as a marker of mitochondrial activity (A). This activity was represented as the mean number of spermatozoa with presence of mitochondrial activity per condition versus control. Representative image of sperm without mitochondrial activity (B), and sperm with mitochondrial activity C, D). Significant differences detected by one-way ANOVA with Tukey's multiple comparison post-test, when appropriate. ***P < 0.0001.

Figure 12. Cleavage rate and blastocysts. Bovine spermatozoa were treated with soluble *N. caninum* antigens in TALP-Sperm medium for 1 h, in this case, only the the medium dose concentration of antigens (4 µg/ml) was used. As a control, spermatozoa were processed in the same way without the presence of parasite antigens. Eight in vitro embryo production routines were carried out. The cleavage rate was evaluated 48 hours after in vitro fertilization by counting the embryos that had two or more cells (A) (cleavage rate = number of embryos/number of initial oocytes). Significant differences were detected by Fisher's exact test, when appropriate. ****P < 0.00001. The blastocyst

rate (B) was determined on the seventh day after fertilization, in relation to the number of oocytes that initiated cleavage and reached this stage of development (blastocyst rate = number of blastocysts/number of initial oocytes). Significant differences were detected by Fisher's exact test; when appropriate, *$P < 0.01$.

Figure 1

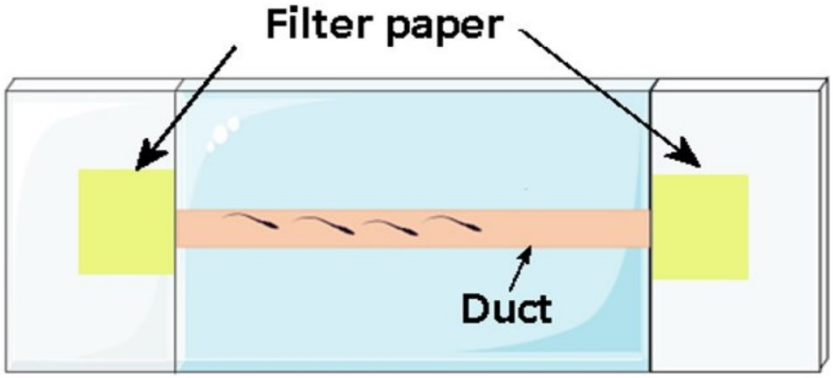
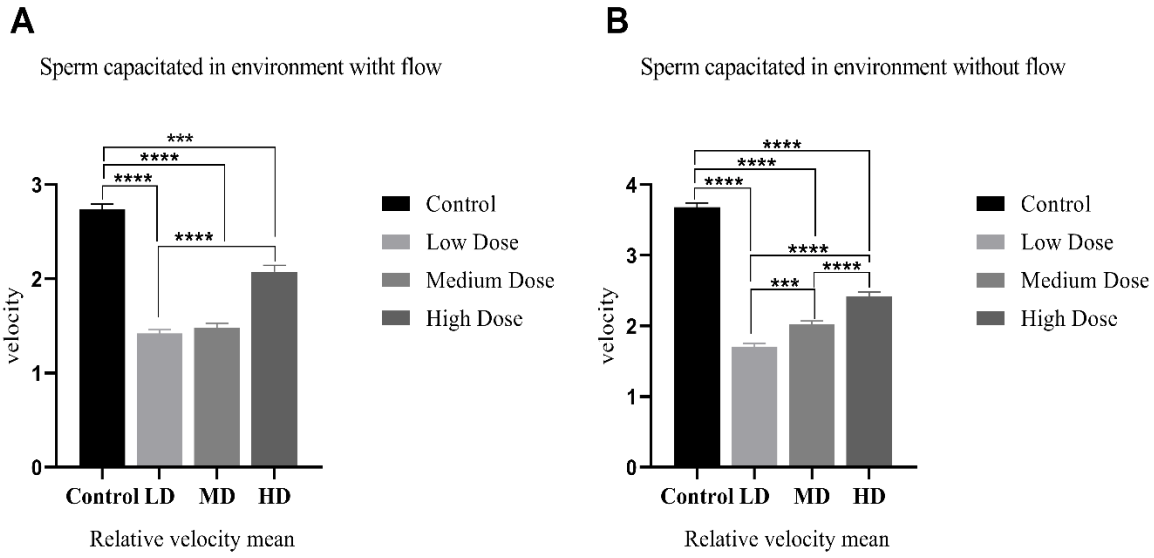


Figure 2



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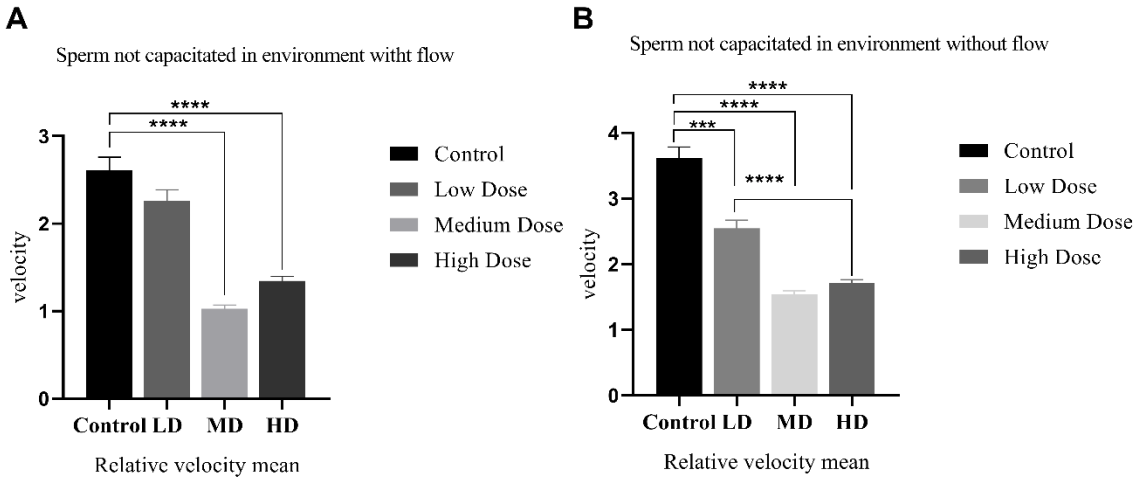

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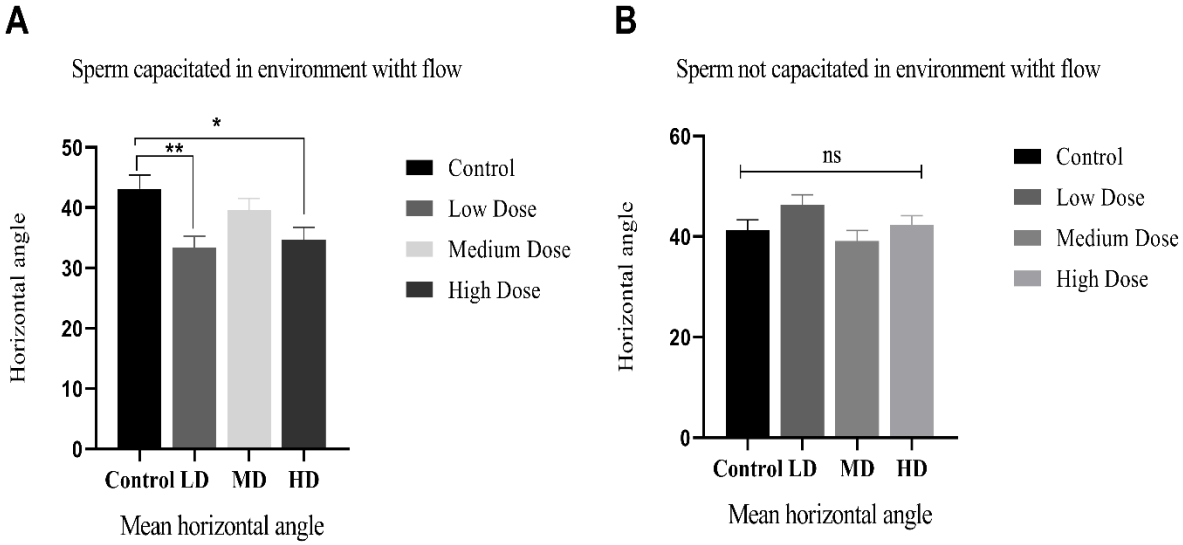

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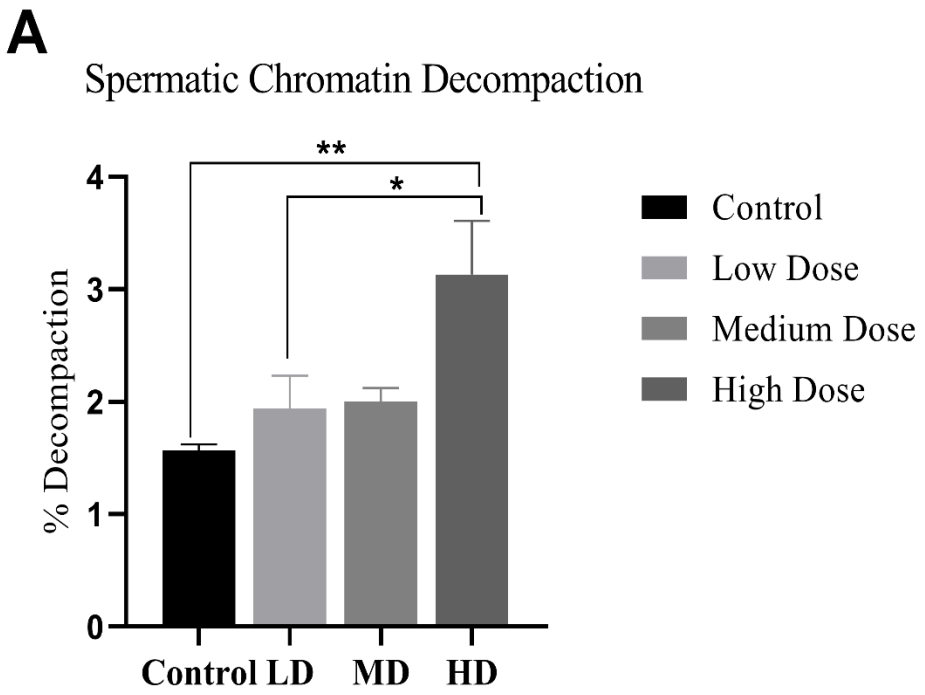


Sperm contamined with soluble N. caninum antigens

Figure 6

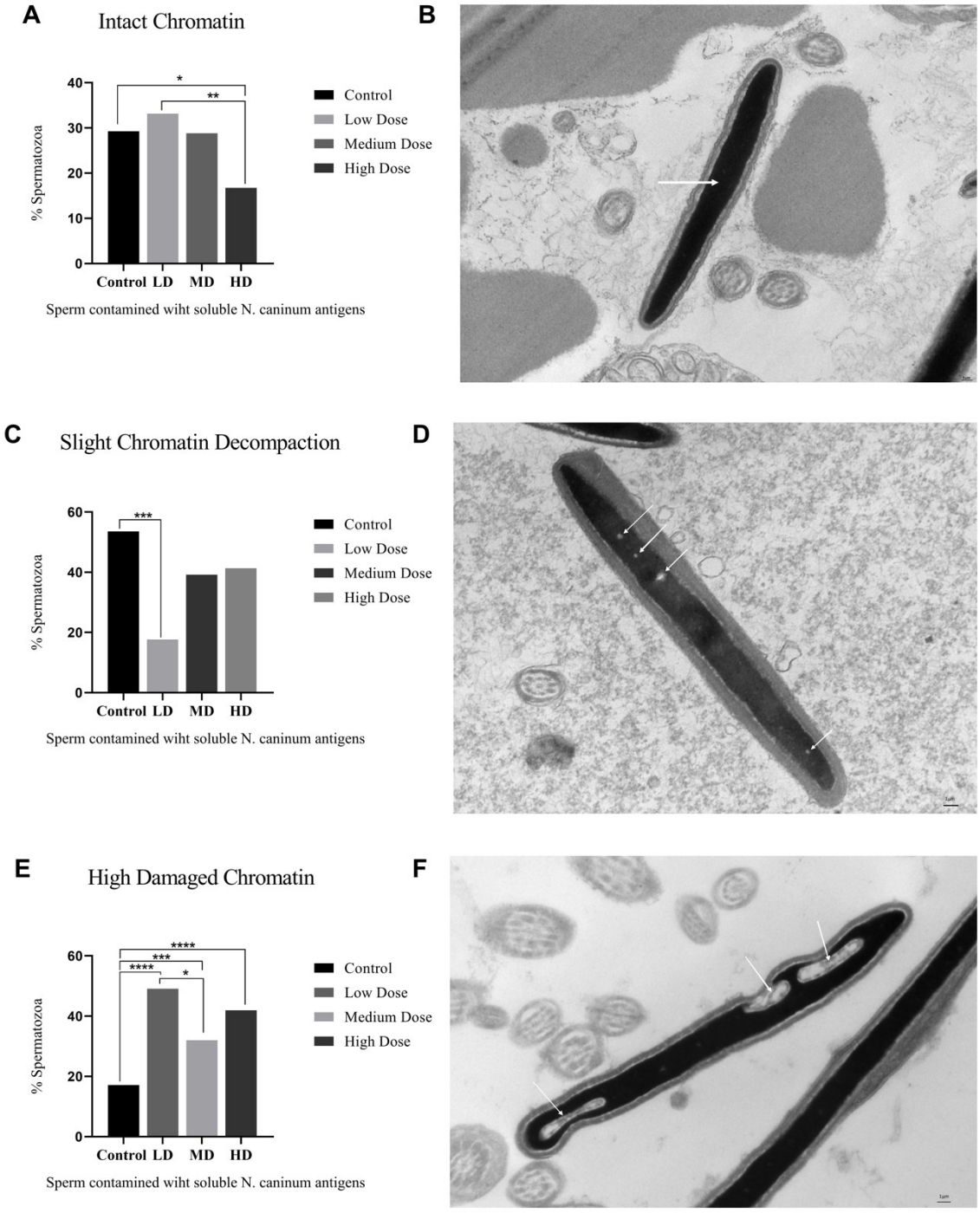

A
Intact Chromatin
% Spermatozoa
40
30
20
10
0
*
**
Control LD MD HD
Control
Low Dose
Medium Dose
High Dose
Sperm contamined wiht soluble N. caninum antigens
B
C
Slight Chromatin Decompaction
% Spermatozoa
60
40
20
0
***
Control LD MD HD
Control
Low Dose
Medium Dose
High Dose
Sperm contamined wiht soluble N. caninum antigens
D
E
High Damaged Chromatin
% Spermatozoa
60
40
20
0
****
***
****
*
Control LD MD HD
Control
Low Dose
Medium Dose
High Dose
Sperm contamined wiht soluble N. caninum antigens
F

Figure 7

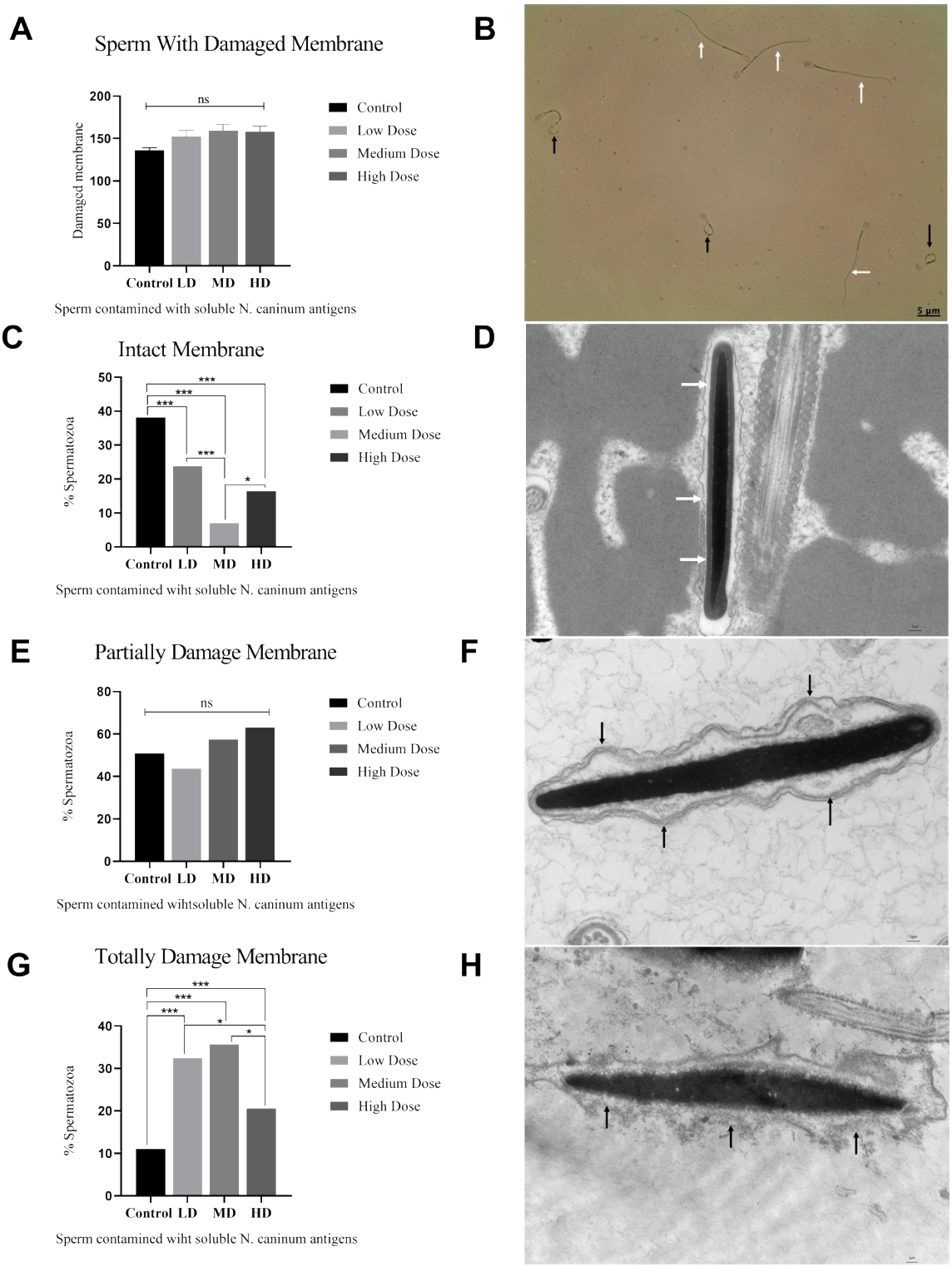
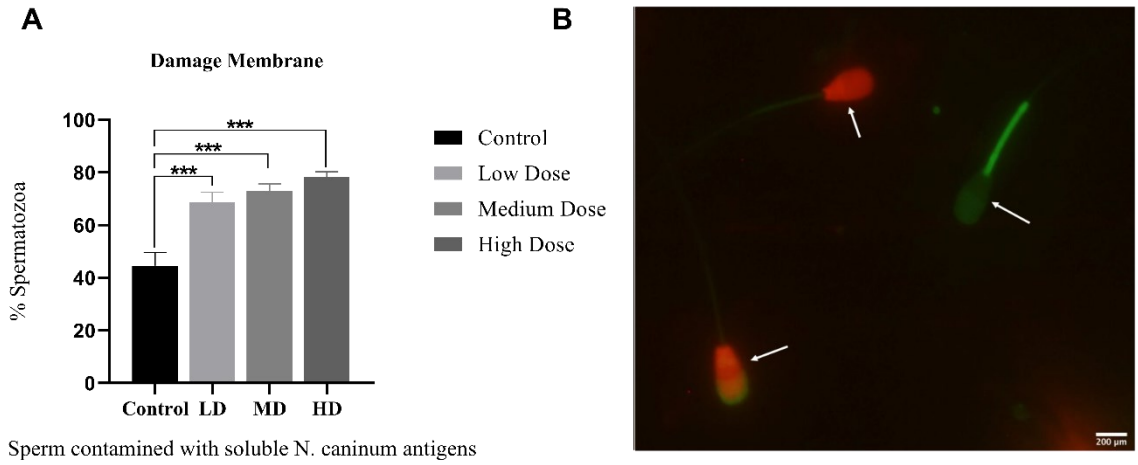

A
Sperm With Damaged Membrane
Damaged membrane
200
150
100
50
0
ns
Control LD MD HD
Control
Low Dose
Medium Dose
High Dose
Sperm contamined with soluble N. caninum antigens
B
5 µm
C
Intact Membrane
% Spermatozoa
50
40
30
20
10
0
***
***
***
***
*
Control LD MD HD
Control
Low Dose
Medium Dose
High Dose
Sperm contamined wiht soluble N. caninum antigens
D
E
Partially Damage Membrane
% Spermatozoa
80
60
40
20
0
ns
Control LD MD HD
Control
Low Dose
Medium Dose
High Dose
Sperm contamined wihtsoluble N. caninum antigens
F
G
Totally Damage Membrane
% Spermatozoa
40
30
20
10
0
***
***
***
*
*
Control LD MD HD
Control
Low Dose
Medium Dose
High Dose
Sperm contamined wiht soluble N. caninum antigens
H

Figure 8



Sperm contamined with soluble N. caninum antigens

Figure 9

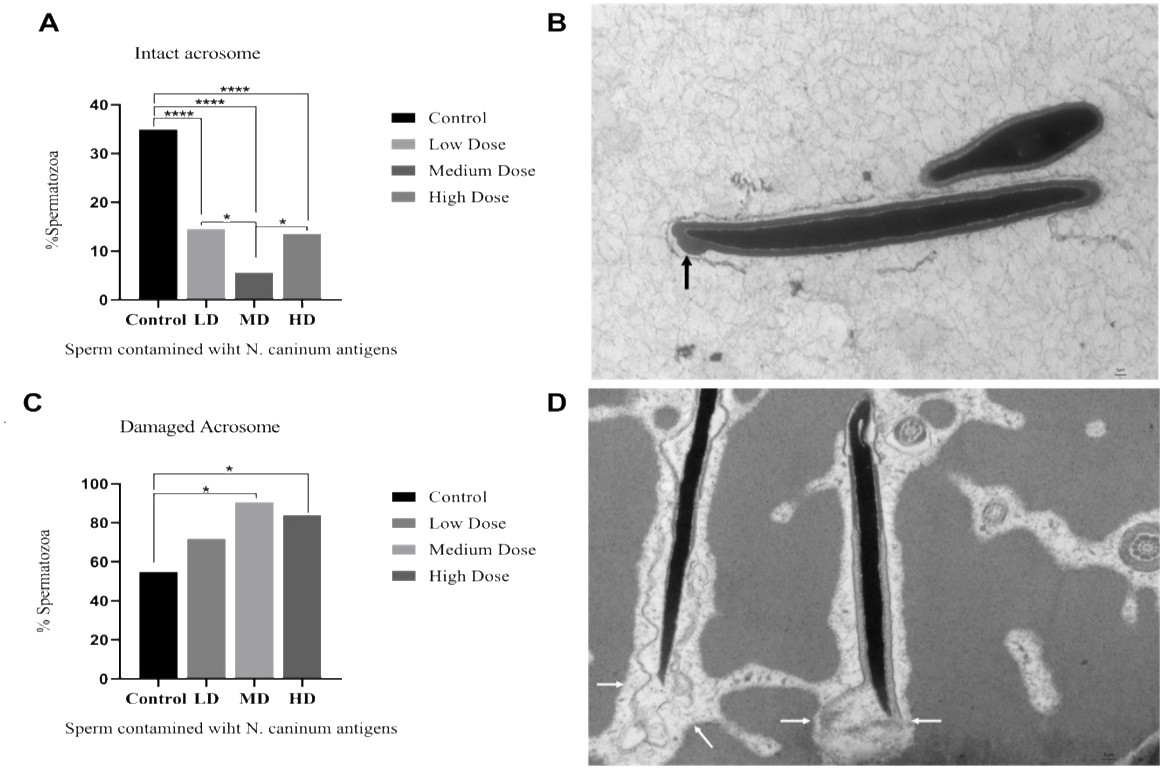

A
Intact acrosome
%Spermatozoa
40
30
20
10
0
****
****
****
*
*
Control LD MD HD
Sperm contamined wiht N. caninum antigens
Control
Low Dose
Medium Dose
High Dose
B
C
Damaged Acrosome
% Spermatozoa
100
80
60
40
20
0
*
*
Control LD MD HD
Sperm contamined wiht N. caninum antigens
Control
Low Dose
Medium Dose
High Dose
D

Figure 10

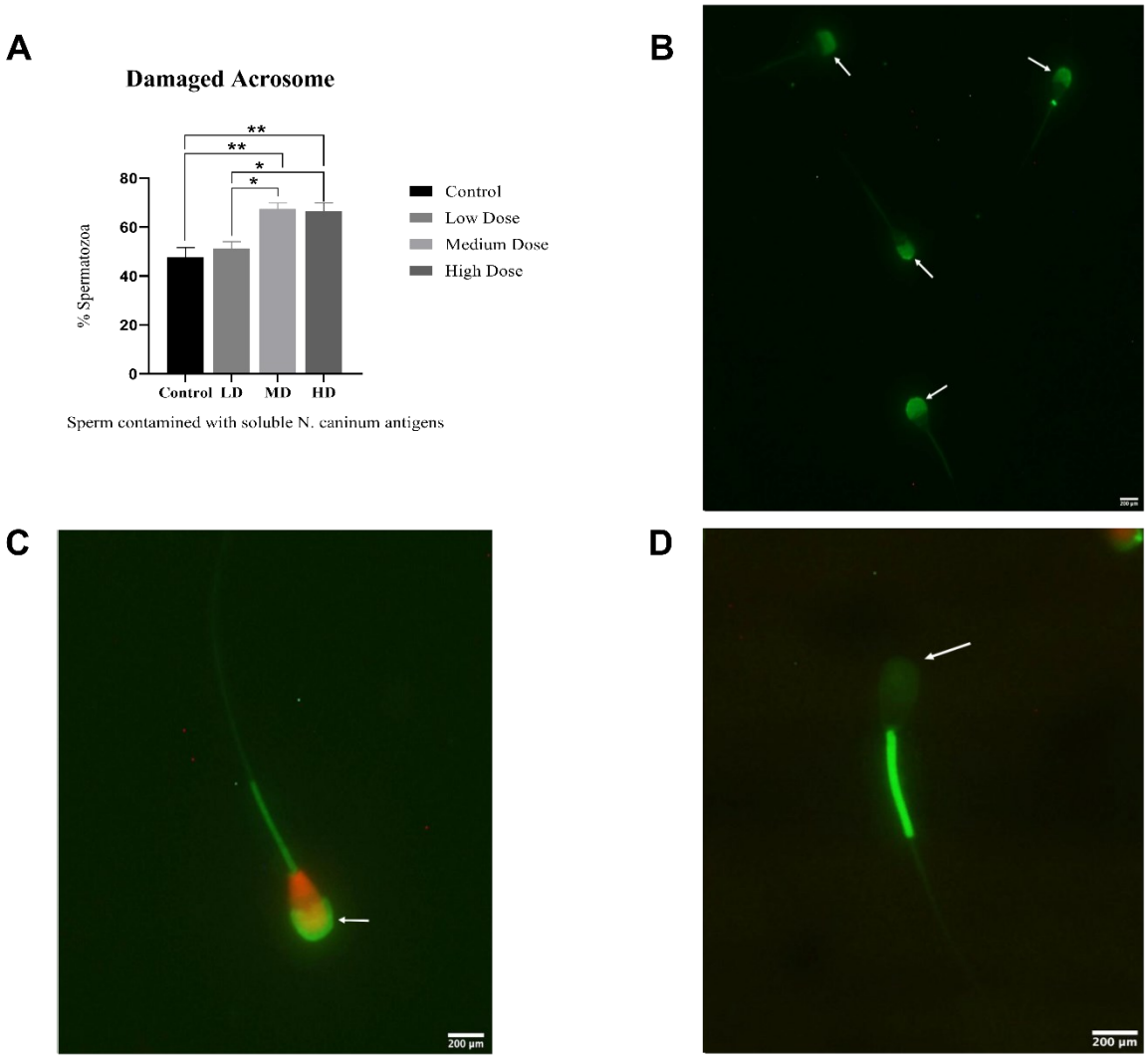

A
Damaged Acrosome
% Spermatozoa
80
60
40
20
0
**
**
*
*
Control
Low Dose
Medium Dose
High Dose
Control LD MD HD
Sperm contamined with soluble N. caninum antigens
B
C
200 µm
D
200 µm

Figure 11

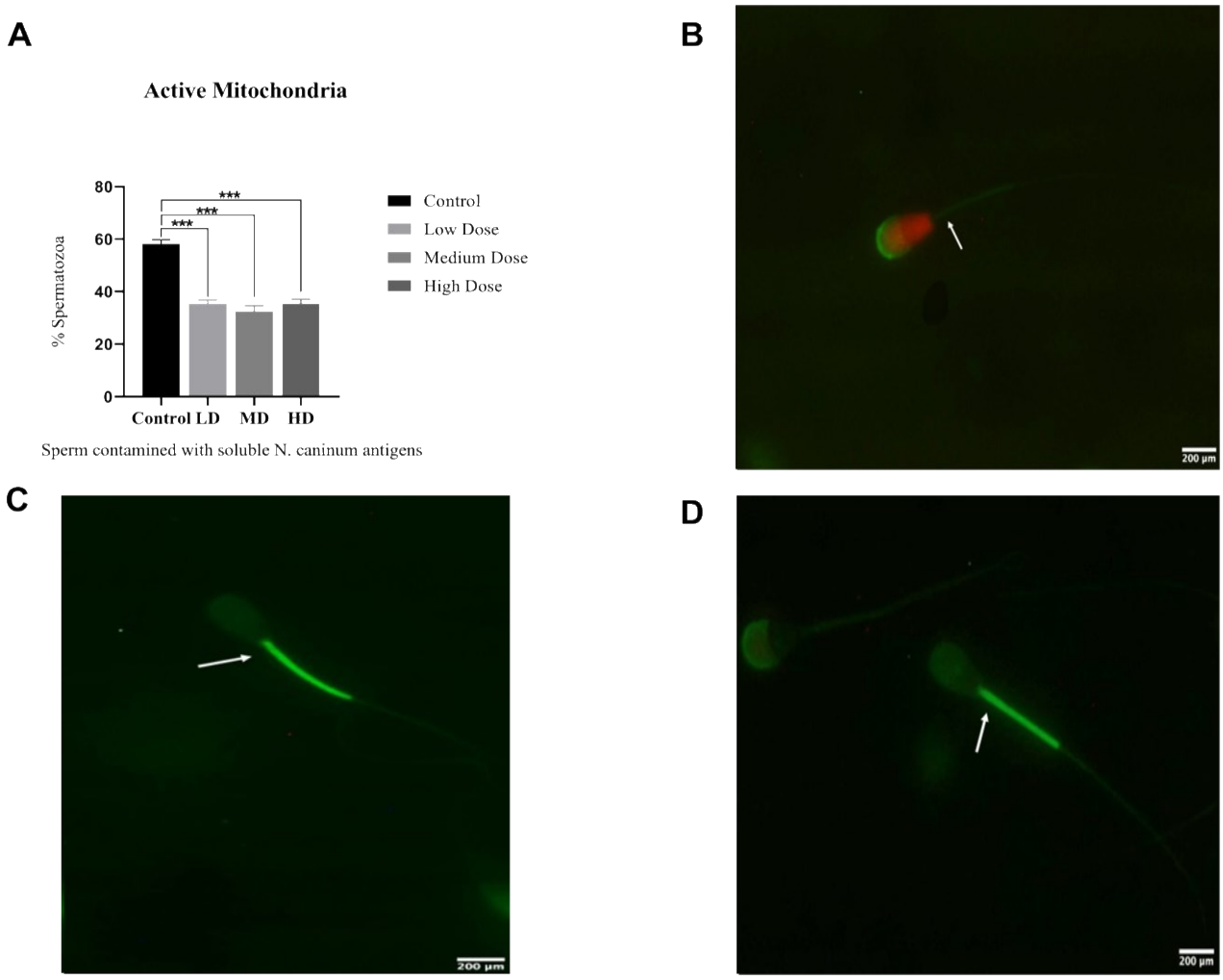

A
Active Mitochondria
% Spermatozoa
80
60
40
20
0
***
***
***
Control LD
MD
HD
Control
Low Dose
Medium Dose
High Dose
Sperm contamined with soluble N. caninum antigens
B
200 µm
C
200 µm
D
200 µm

Figure 12

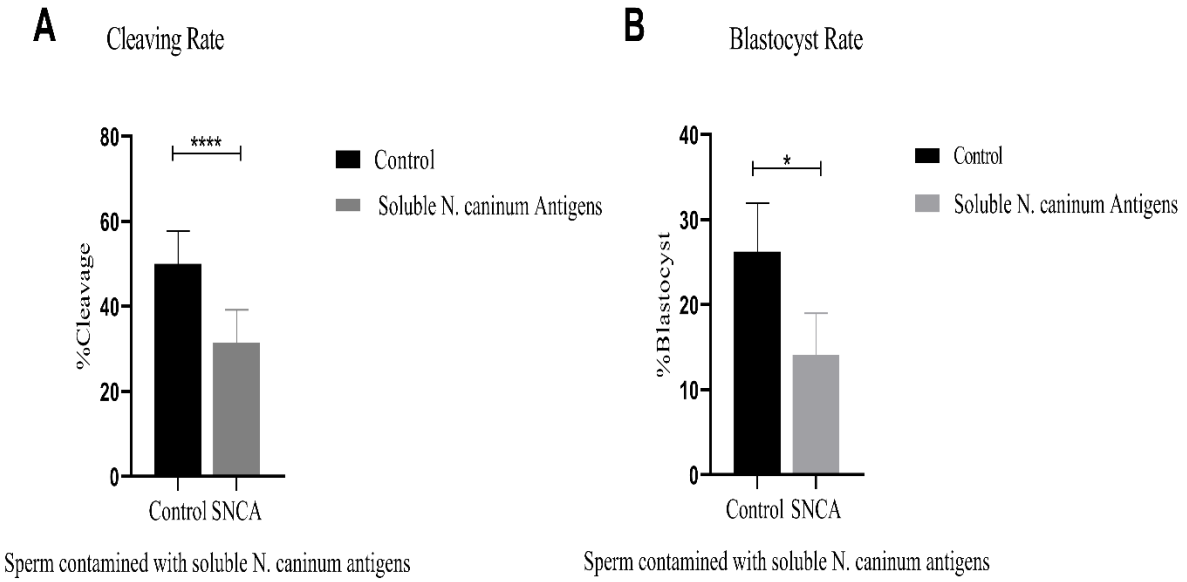


Table 1. Dynamics of in vitro production of bovine embryos.

Days	ACTIVITIES
-1	Post-mortem follicular aspiration
	In vitro selection and maturation of oocytes (IVM)
0	Contamination of spermatozoa in Sperm-TALP medium
0	In vitro fertilization (IVF) of matured oocytes (after 22 hours of maturation)
1	Stripping (from 18 to 22 hours after fertilization) and beginning of in vitro culture of probable zygotes
2	Observation of cleavage rate (48h after IVF)
3	Morula count, initial, expanded and hatched blastocysts.