UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

EFEITOS DA CASTRAÇÃO QUÍMICA COM CLORETO DE CÁLCIO ASSOCIADO COM DMSO SOBRE A ESPERMATOGÊNESE E FERTILIDADE DE RATOS

CRISTIANE SELLA PARANZINI

Botucatu - SP
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CRISTIANE SELLA PARANZINI

Tese apresentada ao Programa de Pós Graduação em Biotecnologia Animal da Faculdade de Medicina Veterinária e Zootecnia da Universidade Estadual Paulista como pré-requisito para obtenção do título de Doutora

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Palavras-chave: Agente esclerosante; Biomarcador de fertilidade; Infertilidade Masculina; Orquite Aguda; Proteômica.

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Àqueles que deram a vida pelo avanço da ciência ...

“Não sei se a vida é curta ou longa para nós, mas sei que nada do que vivemos tem sentido, se não tocarmos o coração das pessoas.
Muitas vezes basta ser: colo que acolhe, braço que envolve, palavra que conforta, silencio que respeita, alegria que contagia, lágrima que corre, olhar que acaricia, desejo que sacia, amor que promove.
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Cora Coralina

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LISTA DE ABREVIATURAS E SIGLAS

µg	Microgramas
µL	Microlitros
Ca^{+}	Cálcio
$CaCl_2$	Cloreto de cálcio
Conc.	Concentração
CTR	Grupo controle
DMSO	Dimetilsulfoxido
EROs	Espécies Reativas ao Oxigênio
FDA	US Food and Drug Administration
FSH	Hormônio Folículo Estimulante
GnRH	Hormônio Liberador da Gonadotrofina
IL1	Interleucina 1
IL6	Interleucina 6
LH	Hormônio Luteinizante
Mot.	Motilidade
NaCl	Cloreto de sódio
TNFα	Fator de necrose tumoral

RESUMO

PARANZINI, C. S. EFEITOS DA CASTRAÇÃO QUÍMICA COM CLORETO DE CÁLCIO ASSOCIADO COM DMSO SOBRE A ESPERMATOGÊNESE E FERTILIDADE DE RATOS. Botucatu – SP. 2019. 108p. Tese (Doutorado em Biotecnologia Animal) – Faculdade de Medicina Veterinária e Zootecnia, Campus Botucatu, Universidade Estadual Paulista.

Este estudo teve como objetivo avaliar os efeitos da ação imediata e por 10 dias consecutivos, da injeção intratesticular de 20% de $CaCl_2$ associada a 0,5% de DMSO nas características espermáticas, na temperatura, biometria e histologia testicular, nas alterações macroscópicas do escroto e tecidos subjacentes, na proteômica do líquido epididimal e do espermatozóides, e nos efeitos tardios sobre a fertilidade em ratos Wistar. Foram utilizados 96 ratos machos e 24 fêmeas, com 80 a 90 dias de idade, respectivamente. Os machos foram divididos em dois grupos (controle e tratado), pesados e, as temperaturas corporal e escrotal aferidas (tempo 0). Os ratos receberam uma injeção intratesticular de 0,1 mL de NaCl a 0,9% (controle/CTR; n = 6) ou 0,1 mL de 20% de $CaCl_2$ associado com 0,5% de DMSO (grupo tratado; n = 90); o grupo tratado foi dividido em 15 subgrupos, de acordo com o momento da eutanásia (2, 4, 8 e 12 h; D1-D10 e D100; n = 6/grupo). A temperatura corpórea e testicular, parâmetros seminais, biometria testicular, dor, avaliação testicular macroscópica e histológica foram realizadas às 2, 4, 8 e 12 h, a cada 24 h por 10 dias consecutivos e aos 100 dias. A análise proteômica dos espermatozóides foi realizada 2 h e D1, enquanto a proteômica do fluído epidimal às 2 h, D1, D5, D7 e D10. Aos 80 dias, os machos (CTR e tratado D100) foram pareados com 3 fêmeas para o teste de fertilidade. O tratamento com 20% de $CaCl_2$ associado com 0,5% de DMSO prejudicou os parâmetros seminais com mínimos efeitos sistêmicos e aumentou a temperatura escrotal as 2, 4, 8 e 12 h no grupo tratado. No D100 do grupo tratado, houve azoospermia, atrofia testicular e o teste de fertilidade foi negativo. Os ratos do grupo CTR no D100 eram normospérmicos e férteis. Adicionalmente, a avaliação protêmica forneceu a descrição e quantificação das proteínas do líquido epididimário e das células espermáticas de amostras de ratos férteis e após a indução da orquite. A injeção intratesticular de 20% de $CaCl_2$ associado com 0,5% de DMSO prejudicou imediatamente a função testicular (2 h) e causou reação inflamatória testicular intensa que prejudicou a fertilidade, mas não interferiu no bem-estar animal. Além disso, proteínas identificadas na proteômica poderiam ser usadas como biomarcadores de fertilidade/infertilidade ou alvo das vacinas imunocontraceptivas.

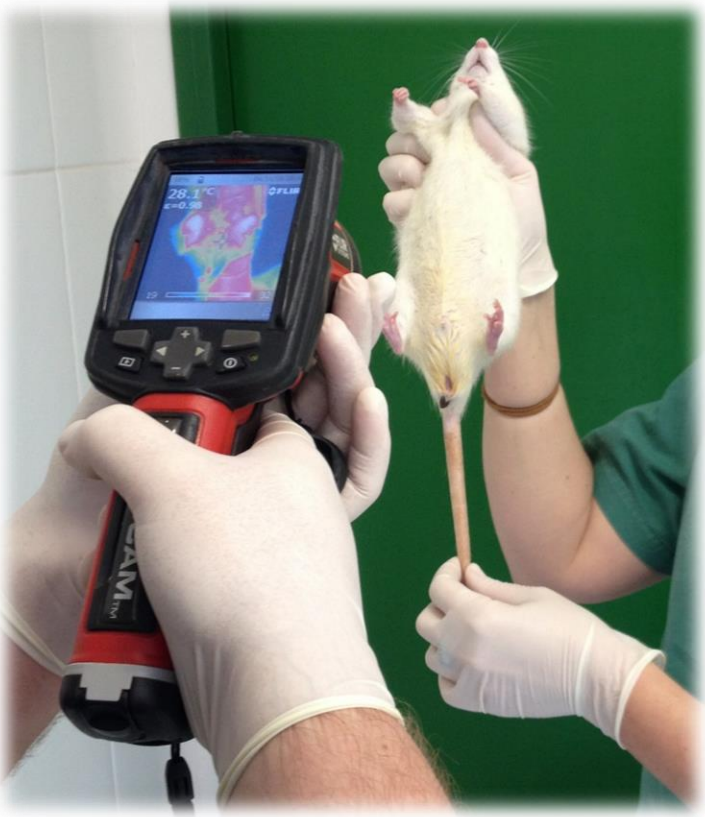
Palavras chave: Infertilidade masculina, Orquite aguda, Agente esclerosante, Biomarcador de fertilidade, Proteômica

ABSTRACT

PARANZINI, C. S. EFFECTS OF CALCIUM CHLORIDE WITH DMSO INJECTION ON SPERMATOGENESIS AND FERTILITY IN RATS. 2019. 108p. Thesis (PhD Degree in Animal Biotecnology) – Faculdade de Medicina Veterinária e Zootecnia, Campus Botucatu, Universidade Estadual Paulista.

This study aimed to evaluate immediate effect of intratesticular injection of $CaCl_2$ 20% associated with DMSO 0.5% on sperm characteristics, testicular temperature, biometry and histology, macroscopic scrotal and adjacent tissue changes, epididymal fluid and spermatozoa proteomics, and the late effect of fertility on Wistar rats. We used 96 male and 24 female Wistar rats, 80 to 90 days old respectively. Males were divided into two groups (control and treated); weighed, and body and scrotal temperatures measured (time 0). Rats received an intratesticular injection of 0.1 mL of NaCl 0.9% (control/CTR; n = 6) or $CaCl_2$ 20% associated with DMSO 0.5% (treated group; n = 90); treated group was divided into 15 subgroups depending on euthanasia time (2, 4, 8 and 12 h; D1–D10 and D100; n = 6). Body and testicular temperature, seminal characteristics, testicular biometry, pain, macroscopic and histological testicular evaluations were performed at 2, 4, 8 and 12 h, every 24 h for 10 consecutive days and at 100 days. Spermatozoa proteomics analysis was done 2 h and D1 while epididymal fluid at 2 h, D1, D5, D7 and D10. At 80^{th} day, male (CTR and treated D100) were couple with 3 female. The treatment with CaCl2 20% associated with DMSO 0.5% impaired seminal parameters with minimal systemic effects and increased scrotal temperature at 2, 4, 8 and 12 h in the treated group. At D100 was azoospermia, testicular atrophy and negative fertility test. Rats in the CTR group at D100 were normospermic and fertile. The proteomic evaluation provided epididymal fluid and sperm cell quantity and baseline proteins description of samples with normal seminal parameters and after acute testicular inflammatory reaction. Intratesticular injection of $CaCl_2$ 20% with DMSO 0.5% immediately (2 h) impaired testicular function and caused testicular inflammatory reactions that harmed fertility, but did not interfere with animal welfare. Furthermore, the presented proteins can be used as fertility/infertility biomarker or immunocontraceptive vaccine target.

Keywords: Male infertility, Acute orchitis, Sclerosing agent, Fertility biomarker, Proteomic



CAPÍTULO I

1. INTRODUÇÃO E JUSTIFICATIVA

Métodos de contracepção são estudados em diferentes espécies, incluindo humanos, espécies de animais de produção, silvestres e animais de companhia. Porém, apesar de diversos estudos e das preocupações com o bem-estar animal, o desafio atual é encontrar método contraceptivo eficiente, sem efeitos adversos, de baixo custo e com manejo reduzido.

Com esse propósito, estudos focados na esterilização de machos, utilizando agentes esclerosantes intratesticulares se intensificaram nos últimos 10 anos. É um método de fácil utilização, custo relativo baixo e de única aplicação em alguns casos, com mínimos efeitos adversos para esterilização de machos errantes e ferais. De fato, alguns produtos já estudados ocasionam lesões ulcerativas testiculares importantes e dor, e em vista disso, o cloreto de cálcio ($CaCl_2$) associado com outros produtos tem sido recomendado para esterilização animal, sem efeitos adversos. Este composto químico causa reação inflamatória testicular, orquite, que leva a lesão tecidual por necrose e peroxidação lipídica devido à produção de radicais livres (JANA; SAMANTA, 2011), acarretando em infertilidade permanente (PARANZINI et al., 2018; SILVA et al., 2018).

Os relatos de literatura disponíveis citam as alterações causadas pela epidídimo-orquite aguda, de maneira tardia (LUDWIG, 2008; RUSZ et al., 2012; AZENABOR; EKUN; AKINLOYE, 2015). Assim, em vista da necessidade de maior entendimento da fisiopatogenia da inflamação testicular e dos mecanismos moleculares envolvidos na infertilidade/fertilidade masculina, este estudo tem como objetivo avaliar os efeitos da orquite aguda imediatamente e por 10 dias consecutivos, após a indução da reação inflamatória local pela aplicação intratesticular de $CaCl_2$ 20% associado com DMSO 0,5% sobre as características das células espermáticas, temperatura testicular, biometria testicular, alterações macroscópicas, histologia, proteínas do fluído epididimário e espermatozoides e, sobre os efeitos tardios na fertilidade em ratos Wistar.

2. REVISÃO DE LITERATURA

2.1. Sistema Reprodutivo Masculino e a Espermatogênese

De forma elipsoidal e localizado dentro do escroto nos ratos, estão os testículos, que possuem função de produção de gametas masculinos e hormônios sexuais. Os testículos estão envolvidos pela túnica albugínea (cápsula espessa), dividido em lóbulos e possuem dois compartimentos funcionais, o intersticial e o tubular. Ademais, o epidídimo e ducto deferente, que fazem parte dos ductos genitais, junto com os testículos e outras glândulas anexas ao sistema reprodutivo, compõem o órgão sexual masculino interno (RAVINDRANATH, NEELAKANTA DETTIN; DYM, 2003; SCHILLO, 2009).

No espaço intersticial dos testículos há predominância das células de Leydig que produzem os hormônios testosterona e diidrotestorona. Nos mamíferos, os hormônios androgênicos são responsáveis pela masculinização fetal, pelas características sexuais secundárias e pelo espermatogênese (SCHILLO, 2009). O espaço intersticial também contém células do sistema imune (macrófagos e linfócitos), vasos sanguíneos e linfáticos, nervos, fibroblastos e tecido conjuntivo frouxo. A proliferação e regulação das células de Leydig são estimuladas pelo LH e por citocinas liberadas pelos macrófagos (WEINBAUER et al., 2001).

O espaço tubular compõe 60 a 80% do volume testicular e é o local que ocorre a espermatogênese (WEINBAUER et al., 2001). Este espaço é composto por túbulos seminíferos que contém dois tipos de células somáticas: as células germinativas e as de Sertoli. As células de Sertoli circundam as células germinativas desde a lâmina basal até o lúmen dos túbulos seminíferos e tem como função dando suporte (SCHILLO, 2009) e secreta uma variedade de fatores reguladores, como proteínas, citocinas, fator de crescimento, opioides, esteroides, prostaglandinas, moduladores de divisão celular, entre outros. Esses fatores são responsáveis por coordenar o ciclo espermatogênico que dura 12 a 13 dias no rato. As células de Sertoli também constituem a barreira hemato-testicular. Por volta de 90% do fluído produzido por essas células, é secretado no lúmen tubular. A barreira hemato-testicular impede a reabsorção do fluido secretado, o qual irá transportar o espermatozoide até a *rete testis*. Então, além de funcionar como isolamento físico antigênico, a barreira hemato-testicular prepara o lúmen tubular com fluídos de suas células para o transporte e o desenvolvimento espermático (WEINBAUER et al., 2001).

A espermatogênese ocorre dentro dos túbulos seminíferos. É um processo complexo e bem organizado para a formação do espermatozoide, e dura em média de 40

a 60 dias na maioria dos mamíferos (RAVINDRANATH; DETTIN; DYM, 2003) e nos ratos, é descrita entre 51 a 53 dias (WEINBAUER et al., 2001). A produção de espermatozoides pelos testículos é constante e atribuída a grande número de espermatogônias presentes na membrana basal dos túbulos seminíferos. Conforme características morfológicas e funcionais, a espermatogênese pode ser dividida em 3 fases: fase proliferativa e meiótica na qual ocorre a duplicação do DNA, a recombinação gênica e duas divisões reducionais que dão origem, sucessivamente aos espermatócitos II e às espermátides arredondadas; e fase de diferenciação, quando as espermátides arredondadas passam por drásticas alterações morfológicas e funcionais, como a formação do acrossoma e do flagelo, e condensação nuclear, resultando em uma célula altamente especializada, o espermatozoide (RAVINDRANATH; DETTIN; DYM, 2003; SCHILLO, 2009). Nos roedores, a forma da cabeça do espermatozoide é de gancho (SCHILLO, 2009), mas independente da forma, sua composição básica e função, são as mesmas de outros mamíferos (RAVINDRANATH; DETTIN; DYM, 2003).

A regulação endócrina da função testicular é conduzida pelo eixo hipotálamo-hipófise-gonadal. O hipotálamo libera GnRH em pulsos pelo sistema portal, que irá estimular a produção e liberação dos hormônios gonadotrópicos, LH e FSH, pela hipófise anterior, e são transportados pela corrente sanguínea, irão agir diretamente nas gônadas para o controle da esteroidogênese e espermatogênese, respectivamente. Estimuladas pelo LH, as células de Leydig produzirão testosterona, que também funcionará como regulador local da espermatogênese. Tanto LH quanto FSH são regulados pelo hipotálamo e hipófise, por *feedback* negativo (WEINBAUER et al., 2001).

Após a espermiação, os espermatozoides chegam ao epidídimo via *rete testis*, que o qual está ligado ao testículo e possui três regiões distintas: cabeça, corpo e cauda. A cabeça do epidídimo está envolvida na absorção de fluídos dos testículos resultando em aumento da concentração espermática, e a cauda é responsável pelo armazenamento, proteção e transporte do espermatozoide, durante ejaculação. A passagem do espermatozoide da cabeça do epidídimo para o corpo e cauda é facilitada pelas contrações rítmicas da lâmina muscular da mucosa epididimária, enquanto que da cauda para ductos deferentes, durante o estímulo sexual. A principal função do epidídimo é criar um ambiente luminal propício para maturação, motilidade e fertilização espermática. Devido à barreira hemato-epididimária, o controle do microambiente é realizado por atividades absortivas e secretórias de íons, moléculas orgânicas e proteínas do epitélio epididimário.

A exposição da célula espermática aos componentes do fluído epididimário resulta em modificações estruturais, metabólicas e bioquímicas tornando o gameta masculino apto para fertilização (CORNWALL, 2003).

Após a espermatogênese, o espermatozoide deixa o testículo morfologicamente completo. Conforme transita pelo epidídimo e entra em contato com o fluído epididimal, há a remodelação da membrana plasmática, com alterações nos lipídios, perda ou modificação de glicoproteínas preexistentes e incorporação de novas glicoproteínas, tornando-o apto a fertilização do ovócito, processo denominado de maturação espermática (FOUCHECOURT et al., 2000). O ambiente luminal do epidídimo, o qual envolve os espermatozoides, é complexo e a remodelação da membrana espermática, ocorre desde a cabeça até a cauda, local no qual as células permanecem estocadas até o momento da ejaculação (HU et al., 2018). Neste último compartimento, os mecanismos envolvidos na longevidade das células, não são totalmente compreendidos, porém, o espermatozoide, após ser ejaculado pode sobreviver de 12 a 24 horas em ratos, enquanto que na cauda do epidídimo, por 10 dias (SULLIVAN, 2004; HU et al., 2018). O tempo estimado que o espermatozoide leva da cabeça ao corpo do epidídimo é de 2 a 4 dias e para atravessar a cauda do epidídimo, é de 4 a 6 dias, porém, pode ser influenciada pela frequência ejaculatória (CORNWALL, 2003).

Durante a maturação epididimária, sob o controle de hormônios androgênicos, o epitélio secreta proteínas no seu compartimento intraluminal, de maneira sequencial, que interagem com o espermatozoide (HU et al., 2018).

2.2. Células Espermáticas e suas Funções

A célula espermática é composta por uma cauda que produz movimento, peça intermediária que contém mitocôndrias geradoras e fornecedoras de energia e cabeça, contendo nucléolo altamente condensado e DNA compactado. Esta célula tem como função o transporte e transferência do material genético masculino para a formação do embrião (DORUS; KARR, 2009).

A motilidade é propulsionada pela cauda do espermatozoide que contém várias proteínas estruturais como as tubulinas, actinas e dineínas. A composição comum do flagelo é de 9 pares mais 1 par (central) de axonemas de microtúbulos, e estruturas vizinhas, fibras densas externas, que funcionam fornecendo estabilidade e força necessárias para flexão flagelar e movimentos rítmicos coordenados (DORUS; KARR,

2009). Dentre as proteínas que compões a cauda do espermatozoide e estão envolvidas tanto na estruturação como no movimento, podemos citar a proteína de fibra densa (ODF) (PETERSEN; FÜZESI; HOYER-FENDER, 1999),

A energia necessária para prover os movimentos da cauda é fornecida pelo ATP produzido pelas mitocôndrias localizadas na peça intermediária, imediatamente caudal ao corpo basal, estrutura que liga a cauda a cabeça do espermatozoide (DORUS; KARR, 2009).

A cabeça do espermatozoide possui uma membrana especializada denominada acrossoma que além da actina contém diversas proteínas associadas que regulam a polimerização. Em muitas espécies a reação do acrossoma é uma característica essencial para fertilização bem-sucedida. O DNA contido na cabeça do espermatozoide é altamente compactado, e as histonas somáticas, as quais são proteínas nucleares, são substituídas por protaminas, durante a espermatogênese. Além de conter o genoma paterno haploide, a cabeça do espermatozoide, é especializada no reconhecimento e na fusão com o ovócito (DORUS; KARR, 2009).

Estas modificações adquiridas durante a espermatogênese e na maturação espermática permitem que o gameta masculino realize a capacitação no sistema reprodutivo feminino (VISCONTI; KOPF, 1998). Fatores de decapacitação impedem que os espermatozoides iniciem a capacitação no sistema reprodutor masculino ou precocemente no feminino. Durante a capacitação, esses fatores são removidos das células permitindo a fertilização. Alguns fatores de decapacitação são adquiridos no epidídimo, e outros durante a ejaculação, provenientes das secreções das glândulas acessórias (COOPER; YEUNG, 2001). Estes fatores são em sua maioria proteínas que causam alterações no efluxo de colesterol e fosfolipídios da membrana espermática como por exemplo, a albumina sérica (THÉRIEN; MOREAU; MANJUNATH, 2005).

Durante a capacitação, a hiperativação espermática ocorre e o espermatozoide atravessa as células do *cumulus*. O processo subsequente é a reação do acrossoma, o qual tem dois objetivos, atravessar a zona pelúcida e expor a região equatorial para interação com proteínas específicas do ovócito. Tanto a hiperativação espermática como a reação do acrossoma estão associados a fosforilação de proteínas por proteína quinases ativadas (COOPER; YEUNG, 2001; SCHILLO, 2009). A capacitação espermática está associada ao influxo de íons de cálcio, aumento dos níveis de adenosina 3’,5’-monofostato cíclica

(cAMP), aumento na fluidez de membrana devido alteração lipídica e mudanças nas atividades enzimáticas na superfície da membrana (LAMIRANTE; GAGNON, 1993).

2.3. Proteômica Espermática

Durante décadas, foram utilizados métodos tradicionais de avaliação seminal para detecção da infertilidade nos machos, porém o valor clínico destes testes, sempre foi questionado, pois alguns indivíduos inférteis podem apresentar parâmetros espermáticos (concentração, motilidade e morfologia) dentro da normalidade (DORUS; KARR, 2009; RAHMAN et al., 2013). Assim, estudos moleculares das células e fluídos, são essenciais para o melhor entendimento nos mecanismo envolvidos na reprodução (NAZ, 2011, 2014), pois podem apontar as falhas no funcionamento destes mecanismos (DORUS; KARR, 2009).

Nesse sentido, em todos os processos fisiológicos do espermatozoide, as principais macromoléculas envolvidas são proteínas e por isto estudos têm relacionado as funções espermáticas com a proteômica (RAHMAN et al., 2013). O proteoma abrange todas as proteínas que são expressas em uma célula ao mesmo tempo, incluindo modificações proteicas pós-traducionais e isoformas, é dinâmico, temporal e responde a fatores externos (RAPPSILBER; MANN, 2002; CECILIANI et al., 2014). As proteínas da membrana espermática são essenciais para fertilidade e alterações na sua composição, seja o aumento, diminuição ou ausência de expressão, contribuem para infertilidade (NOWICKA-BAUER; KURPISZ, 2013). Assim, a identificação de proteínas marcadoras da fertilidade/infertilidade, associadas ou não com injúrias testiculares pode determinar a potencialidade reprodutiva do indivíduo (RAHMAN et al., 2013).

Sem dúvida, a identificação da proteômica seminal permite melhor compreensão da fisiologia espermática e da capacidade fertilizante do espermatozoide, bem como num futuro próximo, pode auxiliar no diagnóstico de alterações reprodutivas. Apesar de ser um campo recente de estudo, pela compilação de 30 publicações sobre a proteômica espermática humana, usando a abordagem shotgun e espectrometria de massas em tandem (LC-MS/MS), de 2005 a 2012, Amaral et al. (2014) listaram 6.198 diferentes proteínas de homens férteis e inférteis. As proteínas identificadas como mais abundantes são moléculas que formam o citoesqueleto do espermatozoide, principalmente, a cauda, dentre as quais, podem ser citadas proteínas comuns a flagelos como a tektina, tubulina e actina, mas também proteínas específicas da cauda do espermatozoide como a fibrous sheat e outer dense fiber protein (ODF) (NOWICKA-BAUER; KURPISZ, 2013). Essas

duas últimas proteínas, além de contribuir para qualidade do movimento, protegem o flagelo contra forças de cisalhamento do movimento espermático. Indivíduos com alterações do movimento, como a astenozoospermia, apresentam diminuição na expressão de ODFs (ZHAO et al., 2007, 2018).

Além das proteínas da cauda do espermatozoide, proteínas envolvidas em vias glicolíticas e de sinalização das células, tem influência direta na motilidade espermática, que é primordial para que ocorra a fertilização. Os canais de Ca+ (Catsper) dependentes de progesterona e prostaglandina, participam da regulação da motilidade. O influxo de Ca+ no trato reprodutivo feminino ativa a capacitação do espermatozoide (LISHKO; MANNOWETZ, 2018). O aumento do nível intracelular de cálcio, não é a única condição necessária para hipermotilidade e reação do acrossoma bem-sucedida. É crucial que a CABYR, proteína reguladora da fosforilação da tirosina cálcio dependente, crie um complexo proteico juntamente com a proteína de ancoragem à quinase A (AKAP) e roporina (NAABY-HANSEN et al., 2002; LI et al., 2011; YOUNG et al., 2016a).

Para o bom funcionamento metabólico celular e motilidade, é necessária a produção e disponibilidade de energia. Várias proteínas associadas com a produção e transporte de energia, já foram identificadas. Uma destas proteínas é a triosephosphate isomerase, lactate dehydrogenase C, acyl-coenzyme A thioesterase 8, carnitina O-acetyltransferase, glutatione S-transferase Mu-1 (ODET et al., 2008; WANG et al., 2009; VANI et al., 2010; CAO et al., 2018).

Outras proteínas já mencionadas estão envolvidas na interação espermatozoide-ovócito, *turnover* e presentes no núcleo dos espermatozoides, tais como a CRISP2, (YOUNG et al., 2016b), HYAL5, SPAM1 (KIMURA et al., 2009), PH20 (AITKEN; BAKER, 2008), heat shock proteins (LIMA et al., 2006; WALSH et al., 2008; HOSSEINIFAR et al., 2013) e histonas (DE MATEO et al., 2011).

Já na medicina veterinária, o maior interesse é estudar proteínas associadas com a célula espermática com o propósito de investigar biomarcadores de fertilidade, assim como de infertilidade, para seleção de animais de produção (CECILIANI et al., 2014). Dentre estas a IZUMO1 (AITKEN; YOUNG; BAKER, 2015), SP22 (KLINEFELTER et al., 1997), osteopontina (NOVAK et al., 2010), SPATA, CRISP3, ENO1 (DACHEUX; DACHEUX; DRUART, 2016), ODF2, tubulins, Catsper (MOURA; MEMILI, 2016), são alguns exemplos apontados na literatura como possíveis biomarcadores de fertilidade em diferentes espécies.

Devido ao auto potencial reativo e interação com anticorpos anti-espermático, as proteínas do sistema reprodutivo, despertam interesse para produção de métodos contraceptivos imunológicos. Os estudos dessas vacinas estão em andamento e vem sendo facilitado pelos avanços no campo da proteômica. Já se tem publicado estudos com proteínas como a CatSper, SPAM1, Izumo, YLP12 e proteínas da zona pelúcida, como potenciais para produção dessas vacinas (NAZ; ALEEM, 2007; NOWICKA-BAUER; KURPISZ, 2013; NAZ, 2014).

O aprimoramento e desenvolvimento das técnicas de identificação e quantificação das proteínas, permitirá que num futuro próximo, seja possível avaliar de maneira mais ampla o potencial reprodutivo, identificar a possível causa de infertilidade e desenvolver métodos contraceptivos seguros nas diferentes espécies.

2.4. Métodos Contraceptivos em Machos

A superpopulação animal é um problema mundial e os custos de programas de controle de natalidade e os prejuízos econômicos causados a sociedade são elevados (BOWEN, 2008). Diante dos riscos de saúde pública, contaminação ambiental, perturbação da ordem pública (ex. lixões, acidentes automobilísticos, ataque a humanos, comportamento sexual, vocalizações noturnas), riscos a outros animais por meio de transmissão de doenças e quando representam ameaça à vida selvagem pela predação, é necessário um gerenciamento de controle populacional emergencial e eficiente (ICAM COALITION, 2007, 2011).

A escolha de um método de controle de fertilidade para o manejo de animais errantes é uma decisão complexa, pois inclui a aceitação social, o bem-estar animal, a eficácia, a legalidade, a viabilidade e sustentabilidade (MASSEI; MILLER, 2013). Entre as alternativas de controle populacional, o método cirúrgico é o mais utilizado. São citados também métodos hormonais (progestágenos e andrógenos), imunológicos (vacinas anti-GnRH e antígenos espermáticos), químicos com agonistas (deslorelina, nafarelina e acetato de leuprolida) e antagonistas do GnRH, e agentes esclerosantes de aplicação testicular (gluconato de zinco, $CaCl_2$, salina hipertônica, nitroso, clorexidina, entre outras) (BUTTLER, 2015).

A castração cirúrgica é outra opção, que inclui a orquiectomia e deferentectomia. Nestes casos, o animal deve ser anestesiado e passar por um período de recuperação mais longo, comprometendo o bem-estar animal. Além disso, complicações pós-cirúrgicas são

descritas em várias espécies e podem comprometer a saúde animal. Apesar disso, é um método definitivo, mas de custo mais elevado e que pode requerer a internação dos animais (LEVY et al., 2008).

A castração química com hormônios, similares ou inibidores hormonais tem sido descrita em muitas espécies, incluindo animais de companhia e silvestres. Apesar de, na maioria, ser um método seguro, requer aplicações extras e não é considerado permanente (LEVY et al., 2004; PURSWELL; KOLSTER, 2006; MASSEI; MILLER, 2013).

Já a castração química com agentes esclerosantes atua sobre o epitélio seminífero ou epidídimo e pode levar a inflamação, com consequente subfertilidade, infertilidade ou esterilidade, condições que podem ser temporárias ou irreversíveis. Dependendo do grau de acometimento leva a degeneração testicular que geralmente progride para atrofia e fibrose do testículo (PINEDA et al., 1977; KOGER, 1978; IJAZ; ABALKHAIL; KHAMAS, 2000; RINGLER, 2000; NASCIMENTO; SANTOS, 2003; KWAK; LEE, 2013; FAGUNDES et al., 2014). Alguns agentes esclerosantes ainda alteram a androgênese, que também contribuem com a azoospermia e diminuição da produção de hormônios androgênicos (AIUDI et al., 2010; OLIVEIRA et al., 2012ª; PARANZINI et al., 2018; SILVA et al., 2018).

O efeito irritante de produtos esclerosantes é que determina o grau de severidade e extensão da lesão (RINGLER, 2000). O $CaCl_2$, já pesquisado anteriormente e constatado seguro para uso intratesticular (KOGER, 1978), é um agente esclerosante capaz de promover lesões degenerativas testiculares por necrose e fibrose dos túbulos seminíferos, das células germinativas e das células intersticiais (Leydig), além da produção de radicais livres que promovem a peroxidação lipídica, acarretando em azoospermia permanente (JANA; SAMANTA; GHOSH, 2002; JANA; SAMANTA; GOSH, 2005; JANA; SAMANTA, 2006). Ainda que controversa, a diminuição da produção de testosterona (JANA; SAMANTA, 2007, 2011, LEOCI et al., 2014a, 2014b; PARANZINI et al., 2018; SILVA et al., 2018) é descrita em alguns estudos. Essas alterações testiculares associadas com a manutenção da testosterona em níveis basais causa queda da motilidade espermática, oligozoospermia, necrospermia ou azoospermia (LEOCI et al., 2014b; PARANZINI et al., 2018).

Os veículos associados com agentes esclerosantes também são alvos de pesquisas, já que os mesmos podem diminuir a quantidade de reações adversas e potencializar a ação do fármaco, conferindo melhor mecanismo de ação do agente esclerosante (LEOCI et al.,

2014ª). O DMSO já foi estudado como veículo para o $CaCl_2$ e também para o gluconato de zinco (SOTO et al., 2009; VANNUCCHI et al., 2015; PARANZINI et al., 2018) por ser solvente, é frequentemente utilizado em estudos biológicos. Devido as características físico-químicas da sua molécula [(H3C)2SO], apresenta grande capacidade de penetração e transporte de membranas (WILLSON; BROWN; TIMMENS, 1965; SANTOS et al., 2003). Também, possui potente ação anti-inflamatória, analgésica, e bacteriostática, interfere na deposição de colágeno nos tecidos, potencializa a ação de fármacos associados, promove vasodilatação (JACOB; HERSCHLER, 1986) e apresenta baixa toxicidade (JACOB; WOOD, 1967; JACOB; HERSCHLER, 1986).

Em ratos, doses de 10, 15 e 20 mg de $CaCl_2$ causam fibrose testicular, em diferentes graus, 21 dias após injeção intratesticular. Em felinos domésticos adultos, $CaCl_2$ 20% associado a lidocaína 1% causou necrose testicular completa do epitélio germinativo com presença de tecido fibrótico e hialínico 60 dias após a injeção intratesticular. Também, 60 dias após injeção intratesticular de $CaCl_2$ 7,5 mg associado ao DMSO 0,5% em cães, é relatado degeneração testicular intensa, fibrose com infiltrado mononuclear nos túbulos seminíferos e nas células de Leydig (SILVA et al., 2018), já em gatos, além da degeneração dos túbulos seminíferos, nota-se hiperplasia compensatória das células de Leydig 80 dias após injeção intratesticular de $CaCl_2$ 20% associado ao DMSO 0,5% (PARANZINI et al., 2018).

Além da reação inflamatória causada no testículo, compostos químicos podem prejudicar a espermatogênese em qualquer estádio da proliferação espermática e os vários tipos de células (células de Leydig, Sertoli e germinativas), em vista da liberação de compostos tóxicos relacionadas com a morte celular ou dano celular sub-letal. Células danificadas morrem dentro do epitélio ou são lançadas no lúmen do túbulo seminífero. A morte celular *in situ* pode ocorrer por necrose, lise não controlada e derrame não específico de conteúdo celular; ou apoptose, processo fisiológico de morte celular programada no qual uma célula se decompõe em corpos apoptóticos, que são fagocitados pelas células de Sertoli. A apoptose é um dos principais mecanismos de ação diante da exposição às toxinas testiculares (BRINKWORTH; HANDELSMAN, 2001).

Outro fator que pode ser afetado devido à toxidade local das substâncias químicas, é a neuromotilidade epididimal, causando tanto aceleração assim como estase do espermatozoide no epidídimo e interfere na secreção do fluído epididimal. Substâncias químicas também apresentam efeitos específicos nos espermatozoides do epidídimo de

ratos, causando perda completa da motilidade e, portanto, esterilidade (BRINKWORTH; HANDELSMAN, 2001).

2.5. Inflamação testicular: Orquite e Degeneração Testicular

Apesar da castração química ser considerada um método contraceptivo seguro e que preserva o bem-estar animal, a reação inflamatório testicular é intensa e alguns estudos mencionam a ulceração da bolsa testicular e dor, nos animais submetidos a este métodos de contracepção (WANG, 2002; FDA, 2003).

Dentre os principais químicos usados para castração química, o gluconato de zinco é descrito por induzir a dor após a aplicação (OLIVEIRA et al., 2012b). Ao contrário, no $CaCl_2$ os estudos não mencionam a dor nos animais tratados (JANA; SAMANTA, 2007; ABUAHMED, 2015; LEOCI et al., 2019). A ulceração escrotal é relacionada ao extravasamento do produto durante a aplicação ou retirada da agulha do tecido testicular, no caso do gluconato de zinco (CEDILLO; VARGAS; MONROY, 2006). Já $CaCl_2$, relata-se o crescimento tumoral em animais tratados, porém que já apresentavam a neoplasia anterior ao tratamento (SILVA et al., 2018). Como já mencionado, a associação com agentes anti-inflamatórios, como o DMSO, pode reduzir tais efeitos.

Estes estudos referem o intenso edema testicular, nos primeiros dias após a aplicação dos produtos, o que torna importante o estudo da fisiopatogenia da reação inflamatório relacionada com o tratamento. Os sinais clínicos da orquite, a inflamação testicular, geralmente, se caracterizam por aumento do volume testicular acompanhado de dor, calor, rubor, porém pode cursar de maneira assintomática. O tratamento da orquite é difícil, a menos que a causa possa ser identificada. Mesmo assim, pode evoluir para degeneração testicular (SCHUPPE et al., 2017).

Independente da natureza do estímulo lesivo, a resposta inflamatória ocorre inicialmente de maneira padronizada, assim o processo inicial agudo, se manifesta localmente de forma uniforme. Muito embora a reação inflamatória se manifeste localmente, ela envolve o organismo como um todo, com a participação dos sistemas nervoso e endócrino na regulação do processo, e o aparecimento de manifestações gerais dentre as quais febre, leucocitose, taquicardia, fibrinólise e alterações na bioquímica sanguínea são relatadas (BECHARA; SZABÓ, 2006ª).

Mediadores químicos são produzidos localmente em resposta a injúria, e recrutam e ativam células do sistema imunológico (reconhecimento, liberação e recrutamento),

proteínas e mensageiros químicos (HACKETT, 2011). Imediatamente após a injúria, há isquemia temporária devido a intensa vasoconstrição. Inicia-se imediatamente a diapedese e migração leucocitária, com notoriedade nos primeiros 15 a 30 minutos de reação inflamatória, atingindo seu pico entre 6 a 9 horas e devendo finalizar 24 horas após a injúria (ALLISON; SMITH; WOOD, 1955), momento no qual ocorre o pico do edema (Figura 1) (GURTNER; CALLAGHAN; LONGAKER, 2006; HÄGGSTRÖM, 2014).

Em menos de 10 minutos após a injúria ocorre dilatação das veias na área e aceleração do fluxo sanguíneo, que é notada pelo rubor e calor local. Dependendo da gravidade da lesão, a estase vascular e a hemorragia também podem ocorrer. O aumento na permeabilidade vascular resulta em edema local devido ao extravasamento do exsudato rico em proteínas. O aparecimento e a duração do edema, também estão associados ao tipo e severidade da injúria (RYAN, 1976). Adicionalmente a inflamação provoca dor e a longo prazo, pode levar a perda da função do órgão (BECHARA; SZABÓ, 2006b).

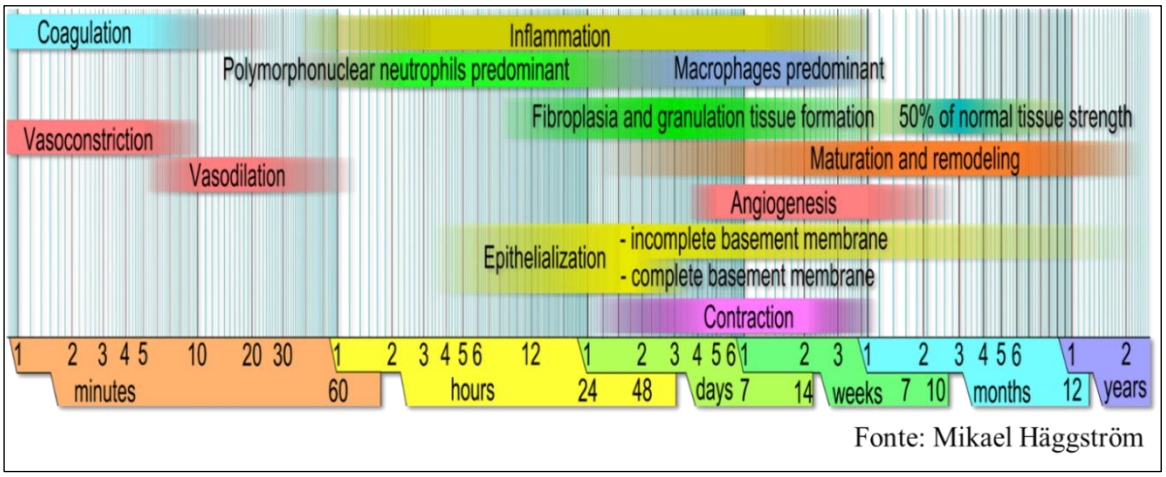


Figura 1. Fases da cicatrização de feridas. Os limites variam dentro de intervalos de menor intensidade de cor, principalmente pelo tamanho da ferida e condições de resolução da injúria. A imagem não inclui grandes deficiências que causam feridas crônicas (HÄGGSTRÖM, 2014).

Durante este processo, os sinalizadores celulares, químicos e proteicos estimulam a cascata pró-inflamatória e desencadeiam a cascata anti-inflamatória simultaneamente, que atua para limitar o processo à área afetada. Se tudo funcionar corretamente, ocorre a resolução do processo, os tecidos danificados são reestabelecidos, a resposta inflamatória ficará confinada à área local e o órgão reestabelece sua função (remoção, resolução e

restauração), caso contrário, ocorrerá cicatrização, com substituição tecidual e possível perda da função (HACKETT, 2011).

No compartimento intersticial, células residentes ou circulantes do sistema imunológico podem ser encontradas em números consideráveis, porém, em condições fisiológicas, não se encontram nos túbulos seminíferos devido a presença da barreira hemato-testicular (SCHUPPE; MEINHARDT, 2005; CHENG; MRUK, 2011; ZHAO et al., 2014). As células do sistema imunológico presentes no compartimento intersticial, têm atividades pro-inflamatórias reduzidas que são importantes nas funções imuno-reguladoras, pois apresentam preferência por citocinas imunossupressivas (ex. IL10 e fatores de crescimento transformador beta) (HEDGER, 2011).

Já a barreira hemato-testicular, visa impedir a invasão de patógenos e que mecanismos autoimunes interfiram na produção do espermatozoide. A quebra desta barreira geralmente resulta em orquite imunomediada, fator etiológico frequente da infertilidade em machos (SCHUPPE; MEINHARDT, 2005; CHENG; MRUK, 2011; ZHAO et al., 2014). Por outro lado, um processo inflamatório testicular promove o declínio na resistência elétrica transepitelial e, a redução e redistribuição da claudina nas junções *tight* das células de Sertoli aumentando a permeabilidade da barreira hemato-testicular e propiciando a infiltração de células inflamatórias. Isto resulta em descamação das células germinativas nos túbulos seminíferos e o aumento da apoptose (PÉREZ et al., 2014) interferindo diretamente na espermatogênese (SCHUPPE; MEINHARDT, 2005; CHENG; MRUK, 2011).

Diferentemente do epitélio testicular, a barreira epididimal é menos eficiente e células do sistema imunológico podem ser comumente observadas no epitélio e lúmen do epidídimo. Este órgão é mais susceptível a injúrias que os testículos, e em humanos, a epididmite é mais frequente que orquite. Mesmo assim, ocorre que lesões nesses órgãos promovem, a perda da tolerância imunológica e propicia a formação de anticorpos espermáticos (HEDGER, 2011).

Não só a inflamação local (epidídimo-orquite), mas também as respostas sistêmicas inflamatórias, tem ação inibitória na função reprodutiva. A ativação do sistema imune desencadeia a produção de citocinas, interleucina, fatores tumorais, espécies reativas de oxigênio (EROs), óxido 13itroso e corticoesteróides no organismo, que irão interagir nos vários níveis do eixos hipotálamo-hipófise-Leydig, causando efeito negativo na produção e maturação espermática (HEDGER, 2011). Ainda, todo o tecido testicular respondem

aos mediadores inflamatórios endógenos, principalmente a IL1, TNFα, óxido nitroso e intérferons, pois regulam a mitose e meiose da espermatogênese, as atividades da célula de Sertoli e como já mencionado anteriormente, as junções da barreira hemato-testicular (HEDGER, 2011).

Nada obstante, as EROs, que são geradas pelo metabolismo normal dos gametas masculinos e exercem papel fundamental na regulação da atividade funcional do espermatozoide como maturação, capacitação e hiperativação, reação do acrossoma e fusão dos gametas. Ocorre que durante a inflamação, as espécies reativas de oxigênio (EROs) são produzidas em concentrações elevadas. Com a produção excessiva de EROs e/ou uma deficiência no sistema de defesa celular, é gerado um quadro de estresse oxidativo e como consequência, a peroxidação dos ácidos graxos poli-insaturados. Por ser formada por altas concentrações de ácidos graxos poli-insaturados, a membrana plasmática do espermatozoide de mamíferos é suscetível ao processo de peroxidação lipídica, que leva a danos irreversíveis, podendo inclusive resultar em infertilidade (NOGUEIRA et al., 2014).

As EROs alteram a fluidez e integridade da membrana, causando desde falha na manutenção de suas funções, a alteração no movimento flagelar (ZALATA; HAFEZ; COMHAIRE, 1995; AITKEN et al., 2010), pois resulta em rompimento do gradiente eletroquímico e fosforilação oxidativa nas mitocôndrias, causando depleção de ATP e consequente queda da motilidade do espermatozoide (DIEMER et al., 2003). Também, altera a capacidade fusogênica da membrana, ativa a reação do acrossomo e prejudica a atividade da acrosina (ZALATA; HAFEZ; COMHAIRE, 1995; AITKEN et al., 2010). As células espermáticas, por serem desprovidas de abundante citoplasma e pobres em enzimas antioxidantes em seu citosol (DIEMER et al., 2003) são dependentes dos fatores antioxidantes fornecidos pelo meio extracelular no epidídimo, para sua sobrevivência e longevidade (AITKEN *et al.*, 2007).

Ainda, a fim de preservar a espermatogênese dentro dos parâmetros fisiológicos, o testículo conta com dois sistemas termorregulatórios para manutenção de uma temperatura fisiologicamente mais baixa: a transferência do calor para o meio externo através da fina pele do escroto e pelo plexo pampiniforme. No caso de inflamação testicular, a alteração da vascularização pode levar ao aumento da temperatura escrotal, que prejudica a função espermatogênica do testículo. O mecanismo celular e molecular envolvidos no prejuízo da espermatogênese, pelo calor, é desconhecido, o que se sabe é

que o aquecimento testicular inibe a expressão da proteína G, a qual é essencial para a transdução do sinal endócrino localizada no testículo, prejudicando a espermatogênese (BRINKWORTH; HANDELSMAN, 2001; WEINBAUER et al., 2001).

Na literatura, os relatos que se tem publicado referente a implicação da epidimo-orquite aguda sobre parâmetros espermáticos e nos órgãos do sistema reprodutivo, geralmente utilizam análise de dados tardias. Isto ocorre devido à dificuldade em se conseguir amostra espermática durante a inflamação, a duração do ciclo espermático, e dependendo da intensidade e magnitude da afecção as alterações podem ter a longo prazo (LUDWIG, 2008; RUSZ et al., 2012; AZENABOR; EKUN; AKINLOYE, 2015). As alterações mais comuns relatadas nos casos de epididymo-orquite aguda são a redução do volume, da concentração, motilidade espermática e apoptose espermática, lesão do acrossomo e fragmentação do DNA (SCHUPPE et al., 2008, 2017), (HAIDL; ALLAM; SCHUPPE, 2008). Importante ressaltar que danos do DNA têm sido associado a baixas taxas de implantação e gravidez após fertilização *in vitro* (FIV) e injeção intracitoplasmática de espermatozoides (ICSI) (HAIDL; ALLAM; SCHUPPE, 2008).

Esclarecer os mecanismos complexos relacionados com a inflamação do trato genital masculino, bem como seus impactos nos parâmetros seminais e na fertilidade, são pré-requisitos para o desenvolvimento de ferramentas de diagnóstico inovadoras e estratégias terapêuticas baseadas em evidências (FIJAK et al., 2018). Mais importante ainda, pesquisas futuras precisam ir além de estudos puramente descritivos da produção e ação de citocinas/proteínas locais. A adoção de estudos mais funcionais usando animais, particularmente modelos de inflamação testicular e ativação imune, devem incorporar uma análise dos eventos, incluindo a quantificação das citocinas e proteínas, além da identificação e das alterações produzidas nos parâmetros endócrinos e da espermatogênese (HEDGER; MEINHARDT, 2003).

2.6. Termografia Infravermelha para Detectar Orquite

Por mudar a dinâmica vascular, a reação inflamatória pode ser detectada pela termografia infravermelha (PARANZINI et al., 2018), que é uma técnica não invasiva de diagnóstico e monitoramento, que detecta a radiação (calor) naturalmente emitida pelo objeto/organismo. Este método não é invasivo, é seguro ao operador e paciente, e adequado para uso prolongado e repetitivo (AMMER; RING, 2012).

Todos os objetos com a temperatura acima de zero absoluto (0 Kelvin = -273,15°C) emitem radiação infravermelha de sua superfície (BOUZIDA; BENDADA; MALDAGUE, 2009). Para o registro da temperatura, a termografia utiliza essencialmente a radiação infravermelha natural emitida pela superfície da pele, e fornece medidas objetivas das alterações clinicamente significativas (AMMER; RING, 2012). Quanto mais alta a temperatura do objeto, maior será a radiação infravermelha emitida. Os sensores geram imagens de alta resolução e permitem definir, por meio de mapeamento térmico, o estado fisiológico do tecido ou órgão examinado, a qual pode contribuir para o diagnóstico médico com medições precisas (AMMER; RING, 2012; MEIRA et al., 2014).

Nesta técnica, padrões anormais de temperatura, podem ser facilmente detectados pela imagem térmica, indicando alterações fisiopatológicas locais ou sistêmicas. Também tem sido utilizado com sucesso na detecção e monitoramento de enfermidades como reumatismo, doenças inflamatórias dos membros, varicocele testicular, lesões esportivas, tumor de mama em humanos e laminite em equinos (PEREIRA et al., 2018ª) dentre outras.

A imagem termográfica pode detectar as mínimas alterações da temperatura superficial da pele devido a mudanças na dinâmica vascular do tecido subjacente causado por qualquer distúrbio, pois apresenta sensibilidade de até 0,07 ºC (AMMER; RING, 2012), tornando-se uma ferramenta eficiente na detecção e monitoramento da inflamação escrotal por agentes esclerosantes intratesticulares como previamente descrito (PARANZINI et al., 2018). Os sinais clínicos de orquite e epididimite estão associados com a intensidade da assimetria térmica no parênquima testicular, medidos na termografia infravermelha, e podem ser precocemente detectadas por estas imagens em humanos. A acurácia da termografia para diagnóstico de inflamação testicular é de 100% (TIKTINSKIĬ, O.L.; MELNIKOVA, V.P.; MOSHKALOV; NOVIKOV, 1989).

2.7. Perspectivas Futuras

Culturalmente, a infertilidade de um casal é na maioria dos casos, atribuída a mulher, o que permitiu que as técnicas de diagnósticos e terapias para infertilidade do sexo feminino, fossem bem desenvolvidas. Apesar do diagnóstico de infertilidade no homem ser relativamente mais acessível, a maioria dos casos permanecem sem explicações (SULLIVAN, 2004). Em animais, principalmente os de produção, a rotina de

diagnóstico de infertilidade masculina, é corriqueira. Com isso, os estudos da infertilidade masculina nas diversas espécies, estão contribuindo para o diagnóstico de alterações espermáticas, estabelecimento do prognóstico e desenvolvimento de estratégias contraceptivas mais modernas em homens e animais (SULLIVAN, 2004).

Técnicas de prevenção da infertilidade, encontra-se em um futuro muito distante (AITKEN et al., 2006), razão pela qual estudos envolvendo a avaliação do potencial de fertilização de espermatozoides são importantes, pois determinam como as influências genéticas, epigenéticas e ambientais interagem na fertilidade relativa de indivíduos. Por consequência, para o desenvolvimento de testes de diagnósticos rápidos, baratos e padronizados, o conhecimento de fundamento bioquímicos e moleculares pelos quais as qualidades funcionais dos espermatozoides possam ser determinadas, é primordial. Um dos avanços mais significativos nessa área foi o recente reconhecimento das alterações espermáticas correlacionadas com o estresse oxidativo e o desenvolvimento de métodos de análises químicas/moleculares da célula espermática para detecção de lesões no DNA como, o ensaio da estrutura da cromatina espermática (SCSA), eletroforese em gel de célula única (COMETA), método dUTP mediada por *desoxinucleotidil-transferase-nick end-terminal* de rotulagem (TUNEL), eletroforese em gel acalino e PCR quantitativa de DNA nuclear e mitocondrial (qPCR) (AITKEN et al., 2006).

As mesmas técnicas moleculares que auxiliam os estudos relacionados com a infertilidade, também contribuem para o desenvolvimento de métodos contraceptivos. Como por exemplo, vacinas anti- GnRH, contra proteínas específicas da zona pelúcida (JOHNSTON; RHODES, 2015) e contra proteínas espermáticas que impeçam a atuação dos gametas masculinos como Izumo (NAZ, 2008) e a YLP12 (NAZ, 2005; NAZ; ZHU; KADAM, 2005). Como vantagem, as vacinas contra proteínas espermáticas apresentam alta especificidade pelo sistema reprodutivo sem qualquer efeito adverso conferindo proteção total contra gestação. Como desvantagens, essas vacinas contraceptivas dependem da variabilidade individual da resposta imune para o seu sucesso, sendo necessárias pesquisas adicionais para estabelecimento de uma dose eficiente. Um ponto importante que deve ser considerado para controle populacional em massa, é a reversibilidade e necessidade de dose de reforço (NAZ, 2005, 2008; HAY; LI; GUO, 2018). O desenvolvimento de vacinas contraceptivas é de abordagem multidisciplinar que requer experiência em diferentes áreas, incluindo produção de vacinas, imunologia, biologia molecular, biologia celular e endocrinologia reprodutiva tornando seu

desenvolvimento mais desafiador do que de outras vacinas. Estudos dos mecanismos moleculares relacionados com a função das proteínas na cascata de fertilização são necessários para elaboração de melhores estratégias vacinais (NAZ, 2011).

Dentre os métodos de controle populacional permanente disponíveis, a castração cirúrgica de machos e fêmeas é o método que apresenta maior vantagem e aceitação social, porém o custo global do procedimento (pessoal, material, espaço e equipamentos) e o tempo necessário para recuperação cirúrgica torna o método impraticável em locais remotos. Outros métodos contraceptivos como os antagonistas hormonais não são permanentes, possuem efeitos iniciais indesejados e alto custo relativo, não sendo a primeira opção de escolha para o uso em campanhas de controle populacional em massa. Já a castração química, apesar de ser um método não disponível para fêmeas, é específico para o sistema reprodutor, possui baixo custo de produção e aplicação e é menos invasivo que o método cirúrgico, porém apresentam resistência social devido a fatores culturais e ainda não está totalmente regulamentado pelo ministério da saúde.

Diante desta atual realidade e das recentes conquistas médicas, uma das técnicas mais avançadas na atualidade, em ciências biomédicas é a terapia gênica e o silenciamento gênico, o que permite a idealização de um novo modelo para esterilização cães e gatos. A contracepção vetorizada, como é denominada, pode ser feita pela terapia gênica que consiste na expressão de uma proteína que interfira nos mecanismos reprodutivos, ou pelo silenciamento gênico, que irá inibir a expressão de proteínas essenciais à reprodução. Primeiramente, para que este método se torne possível, é necessário a identificação de proteínas ou toxinas específicas que possam suprimir a reprodução, fazer um vetor viral que transporte o gene dessa substância para as células, e que faça a expressão ou supressão, de maneira sistêmica ou local nas gônadas. Este método é pouco invasivo, uma única aplicação intramuscular ou subcutânea, para machos e fêmeas, podendo durar por toda vida do animal (JOHNSTON; RHODES, 2015). As pesquisas de esterilização vetorizadas, não se concentram apenas em animais de companhia, mas sim em animais de fazenda, zoológico e selvagens (LEWIS, 2018).

Lewis (2018) cita como um exemplo da esterilização vetorizada a produção de anticorpos monoclonais, que interfiram na cascata de sinalização hormonal (do hipotálamo para hipófise, e consequentemente nas gônadas), interrompendo a produção das células reprodutivas em machos e fêmeas. Fármacos ou transgenes (cápsulas virais) indutores de anticorpos monoclonais, e no caso da contracepção vetorizada, seriam

responsáveis por introduzir instruções genéticas para às células-alvo, produzindo anticorpos continuamente. Ainda aponta alguns cenários possíveis, como ligação do anticorpo ao GnRH no hipotálamo impedindo a estimulação da produção de hormônios gonadotrópicos pela hipófise; ligação dos anticorpos ao LH e o FSH não permitindo sua interação com as gônadas; ligação com o hormônio anti-mülleriano bloqueando a maturação dos folículos nos ovários e produção de testosterona nos testículos e; bloqueio de parte das proteínas de receptores da zona pelúcida, impedindo a ligação ovócito-espermatozoide. Acrescenta ainda a possibilidade de alvos como moléculas envolvidas na maturação espermática, e outras que permitem a fixação do embrião no útero.

Recentemente, Pépin et al. (2018) relataram o sucesso da terapia gênica pela produção de anticorpos contra GnRH e hormônio anti-mülleriano de ratas e gatas. Mesmo que em fase inicial de pesquisa, foi possível obter supressão da ovulação em gatas por 56 dias, sem efeitos adversos aparentes. Acredita-se que as gatas desenvolveram uma reação imune ao material genético sintético (transgene) inserido no músculo, o que limitou o tempo de ação do tratamento. Os autores desse trabalho acreditam que esterilização permanente pela contracepção vetorizada, poderá ser alcançada na segunda geração de vetores que serão construídos de acordo com o sequenciamento gênico da espécie em que será utilizada.

Com base nos diversos relatos, terapia gênica parece simples, mas levou muitos anos para que fosse desenvolvida e que fosse considerada segura para uso clínico. O futuro da terapia gênica é potencialmente promissor tanto para contracepção quanto para o combate a infertilidade (KOJIMA et al., 2008), porém pode enfrentar a percepção pública negativa dos transgênicos (LEWIS, 2018).

Do mesmo modo que diferentes técnicas de controle populacional são criadas com o passar dos anos, percebe-se a contínua tendência na volta de estudos de métodos já anteriormente consagrados (30 – 40 anos atrás), implementados pelo uso de ferramentas modernas de análises. O emprego de biotecnologias na pesquisa científica permite a ampliação e eficiência dos resultados de métodos contraceptivos já existentes, através de obtenção de substâncias mais modernas, seja em sua composição, meio de aplicação e minimização de efeitos adversos. Apesar de toda mistificação em torno da dor causada pela injeção intratesticular, fica evidente, por todos os estudos publicados, que quando utilizada na concentração e dose recomendada para espécie, é uma ótima opção frente as alternativas disponíveis hoje. Além da técnica, a castração química com $CaCl_2$ associada

ao DMSO é eficiente, de baixo custo, rápida aplicação e técnica simples, pouco invasiva, sendo possível o controle de seu efeito adverso, a inflamação testicular, portanto, indicada.

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4. HIPÓTESES

- $CaCl_2$ 20% associado com DMSO 0,5% intratesticular causa reação inflamatória testicular e sistêmica em ratos.
- $CaCl_2$ 20% associado com DMSO 0,5% intratesticular promove alteração das funções testiculares em ratos.

5. OBJETIVOS

5.1. Objetivo Geral

- Estudar os efeitos da castração química sobre a fertilidade causadospela injeção intratesticular de $CaCl_2$ 20% associado a 0,5% de DMSO no rato.

5.2. Objetivos Específicos

- Estudar os efeitos aplicação intratesticular de $CaCl_2$ 20% associado a 0,5% de DMSO, pela termografia infravermelha, biometria e histologia testicular.
- Estudar os efeitos imediatos da aplicação intratesticular de $CaCl_2$ 20% associado a DMSO 0,5% nas características do ejaculado.
- Estudar a fertilidade dos animais tratados 100 dias após a aplicação do castrador químico.
- Estudar a proteômica dos espermatozoides e fluído epididimário após aplicação intratesticular de $CaCl_2$ 20% associado a 0,5% de DMSO.

CAPÍTULO II

Artigo redigido de acordo com as normas da revista Laboratory Animals (SAGE PUBLISHING) (fator de impacto 1,450) com exceção da numeração das páginas, da disposição das figuras e tabelas. https://us.sagepub.com/en-us/sam/journal/laboratory-animals#submission-guidelines

(SUBMETIDO)

INSIGHTS ON INFLAMMATORY REACTION CAUSED BY CHEMICAL CASTRATION ON TESTICULAR FUNCTION AND FERTILITY IN RATS

TESTICULAR INFLAMMATORY REACTION IMPAIRS FERTILITY

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ABSTRACT

This study aimed to evaluate the immediate inflammatory effects of chemical castration on testicular function and fertility of rats. We used 96 male and 24 female Wistar rats, 80 to 90 days old. Males were divided into two groups (control and treated); weighed, and body and scrotal temperatures were measured (time 0). Rats received an intratesticular injection of 0.1 mL of 0.9% NaCl (control/CTR; n = 6) or 20% $CaCl_2$ with 0.5% DMSO (treated group; n = 90); treated group was divided into 15 subgroups depending on euthanasia time (2, 4, 8 and 12 h; D1–D10 and D100; n = 6). Body and testicular temperature, seminal parameters, testicular biometry, pain, macroscopic and histological testicular evaluations were performed at 2, 4, 8 and 12 h, every 24 h for 10 consecutive days and at 100 days. *In vivo* Fertility test was done 80 days after intratesticular injection. The treatment impaired seminal parameters with minimal systemic effects and increased scrotal temperature at 2, 4, 8 and 12 h in the treated group. At D100 after intratesticular injection, there was azoospermia, testicular atrophy and negative fertility. Rats in the CTR group at D100 were normospermic and fertile. Intratesticular injection of $CaCl_2$ with DMSO immediately impaired testicular function and caused testicular inflammatory reactions that harmed fertility, but did not interfere with animal welfare.

Keywords: Male; Infertility; Orchitis; Epididymitis, Mass population control.

1. INTRODUCTION

Recently, the search for a low-cost and non-invasive spaying method for animal population control has intensified. Intratesticular injection of a sclerosing agent has been proposed; these injections cause interruption of sperm production and may or may not interfere with testosterone output (GARDE et al. 2016; JANA et al. 2005; LEOCI et al. 2019; OLIVEIRA et al. 2013; SILVA et al. 2018). In several species, various chemicals have been tested, including zinc gluconate, calcium chloride, sodium chloride, cadmium, chlorhexidine; studied animals include bulls, rats, dogs, cats and black bears (CANPOLAT et al., 2006; JANA; SAMANTA, 2006, 2011; AIUDI et al., 2010; BRITO et al., 2011; CANPOLAT; KARABULUT; EROKSUZ, 2016; CAVALIERI, 2016; KARMAKAR; DAS, 2017). Studies reports good tolerance to the procedure, requiring only mild sedation. Doses of $CaCl_2$ lower than 20% concentration causes slight increases in testicular firmness at palpation and swelling during the first 24–72 hours after injection, possibly causing discomfort during the first 3 days, with progressive remission in the subsequent week. Long term, these injections decrease sperm count, cause testicular fibrosis, resulting in reduction of testicular volume and size. No scrotal skin reaction has been reported with the use of $CaCl_2$ (JANA; SAMANTA; GHOSH, 2002; JANA; SAMANTA, 2006; LEOCI et al., 2014a).

The combination of $CaCl_2$ with other substances is more effective and promotes long-term azoospermia with durable reduction of testosterone levels and reduced aggressive and sexual behavior (LEOCI et al., 2014a, 2019; SEN et al., 2017). Previous studies in dogs, cats and bulls showed that the association of $CaCl_2$ with 0.5% DMSO was more effective for chemical castration than with 0.9% saline solution or 1% lidocaine, and it was safe for animals as an alternative to surgical castration (PARANZINI et al., 2018; PEREIRA et al., 2018b; SILVA et al., 2018).

Morphological changes on histology are dose-dependent because of the intensity of the inflammatory reaction that causes tissue disruption by lipid peroxidation and necrosis (JANA; SAMANTA; GHOSH, 2002; JANA; SAMANTA, 2011). Testicular atrophy can be noted within 3 months (LEOCI et al., 2019). Despite the various publications on this subject, the immediate effect of inflammatory reactions on testicular tissue, function and late on fertility remain unclear. Therefore, we aimed to evaluate the immediate and late effects of intratesticular injection of 20% $CaCl_2$ with 0.5% DMSO on testicular histology, function and fertility in rats.

2. MATERIAL AND METHODS

2.1. Ethical aspects, animals and groups

The project was evaluated and approved by the Institutional Animal Use Ethics Committee (CEUA) on April 4th, 2016 (registration number 46/2016).

Ninety-six male and 24 female Wistar rats (*Rattus norvegius*), ranging from 80 (male) to 90 (female) days old, were used. The rats were kept in appropriate boxes (49 x 34 x 16 cm), controlled temperature (at 23º C), humidity and light. They had free access to water and commercial food. The rats were randomly divided into 2 groups, control (n = 6) and treated (n = 90) divided in 15 subgroups (n=6/subgroup, Table S1), weighed and identified. Temperature of the body (digital clinical thermometer) and scrotum (infrared thermograph) were measured (time 0, D0).

The control group (CTR) received 0.9% NaCl intratesticularly and was evaluated for body temperature and testicular thermography (2, 4, 8 and 12 h, every 24 h for 10 consecutive days and at 100 days). The treated group received 20% $CaCl_2$ associated with 0.5% DMSO, and the rats were evaluated and euthanized at 2, 4, 8 and 12 h, every 24 h for 10 days (times D1 to D10), and at 100 days (D100). Eighty days after intratesticular injection, males from the CTR (n = 6) and treated groups (n = 6) were kept individually in a box containing two females, for 20 days to test fertility. Females and males were euthanized at D100.

2.2. Testicular thermography

Testicular thermography was performed with a specific device (Flir, IRC57, InfraCAM SD ™ Thermal Imaging Camera, Boston, Massachusetts, USA). A minimum distance of 60 cm was maintained between the camera and the testicles. Measurements were evaluated in a single opportunity with *Flir quick report software*® (2009) and we used a rectangular tool to determine maximal and minimal scrotal infrared thermal images, providing a mean temperature in °C.

2.3. Pain evaluation

Subjective pain was assessed daily and described as the National Research Council (US) Committee on Recognition and Alleviation of Pain in Laboratory Animals. Rats were observed for postural changes such as reluctance to move, teeth grinding, decreased

appetite and self-mutilation. In addition, palpation of the injection site, body temperature monitoring and weight were used as parameters to demonstrate pain signs; if identified, rescue therapy with 2.5 mg/kg morphine was used.

Table S1. Experimental design. Distribution of groups for times and procedure identification.

EVALUATION MOMENTS/GROUPS																
0h	**2h**	**4h**	**8h**	**12h**	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**	**D7**	**D8**	**D9**	**D10**	**D100**	**CTRL**

Weighing. testicular thermography. rectal temperature. clinical evaluation and of $CaCl_2$ 20% + DMSO 0.5% ITJ

Testicular thermography. rectal temperature. clinical evaluation

Weighing. testicular thermography. rectal temperature. clinical evaluation. blood collection. euthanasia. testicular collection(measurement. weighing and macroscopic evaluation). semen collection from epididymis tail (evaluation)

Animal breeding: 2 females : 1 male - fertility test

2.4. Intratesticular injection and euthanasia

For chemical castration, rats were anesthetized with morphine (3 mg/Kg, intraperitoneally) and isoflurane. Intratesticular injection was performed with 1 mL syringe and a 26-G needle. The needle was inserted in the distal (ventro-caudal) region of the testicle, laterally to the epididymal tail, to the cranial region of the testis, without crossing the tunica vaginalis in the contralateral region. The volume administered was 0.1 mL in each testicle.[2] For injection, previous scrotum antisepsis with alcoholic chlorhexidine was performed. A similar procedure was conducted in the CTR group with 0.1 mL of 0.9% NaCl injection.

Euthanasia was performed with deep inhalation anesthesia.

2.5. Testicular biometry, seminal collection and evaluation

After euthanasia, scrota were sectioned and adjacent tissue was observed for macroscopic changes. Then, the testicles were removed and separated from the epididymis. The testicles were weighed, measured for length (L) and width (W) with a digital caliper (Digimess, São Paulo, Brazil), sectioned longitudinally and stored for histological evaluation. The formula ($L \times W^2 \times 0.52$) was used to calculate testicular volume as proposed by Mbaeri et al.[23], and the results were expressed in mL.

The epididymides were deposited on a petri dish and soon after, the vas deferens was compressed in 100 μL 0.9% NaCl for sperm collection. Spermatozoa were subjectively evaluated for motility, vigor, concentration and morphology.

For sperm motility and vigor evaluations, one aliquot of epididymal fluid, containing spermatozoa, was diluted (1:10) in 0.9% NaCl and subjectively assessed at 200X magnification under a microscope. An aliquot was diluted in formol-saline (1:50) and samples were used to evaluate sperm concentration (millions spermatozoa/mL) in a Neubauer chamber, and to count sperm morphological defects, using differential interference contrast microscopy (DIC, Leica DM 2500, Leica Microsystems, São Paulo, Brazil). Morphological alterations were classified as head, intermediate piece and cauda defects. Results were expressed as percentages.

2.6. Testicular histological evaluation

Histopathology of the testicle was performed on paraffin-embedded tissue. Sections were obtained from longitudinal cuts stained with hematoxylin-eosin. For histological analysis, quantitative evaluation of tissue alterations including necrosis, inflammatory infiltrates, edema, calcification, fibroplasia and neovascularization were performed, separately considering the tubular and interstitial compartments. Lesion scores were 3 when above 50% of tissue; 2 between 25 and 50%; and 1 for 25% or less. Inflammatory infiltrates were evaluated as discrete peripheral (1), peripheral intense (2) and peripheral and central (3). Granulomatous infiltrates and abscess foci were considered peripheral intensity (2). For neovascularization, we only considered the interstitial compartment.

2.7. Statistical analysis

Variables were expressed as mean and standard error (SEM). Normality was

assessed by the Shapiro-Wilk test. Variables with normal distribution were evaluated by the t-test, otherwise by the Mann-Whitney test. Testicular weight and volume, sperm characteristics and body weight were compared to the CTR group D100 *vs* each time-point for the treated group. Each time-point for the CTR group for scrotal and body temperature was compared with each time of the treated group using the t-test or Mann-Whitney test. In each group, we compared temperature at D0 with each time-point using the paired t-test. Histological scores were expressed as frequencies and were analyzed using the Tukey test. Analyses were performed using *SigmaPlo*t® 11.0 software (2008) and the significance level was $P \leq 0.05$.

3. RESULTS

3.1. Clinical findings, testicular thermography and behavioral assessment

A total of 861 scrotal thermal pictures were analyzed. Scrotal temperature increased between 8 and 12 h in both groups. From 12 h, there was a gradual decrease, with oscillations until the last evaluation (Figs. 1 and 2).

There were no changes in body temperature in either group. Mean ± SEM was 37.3 ± 0.1º C and 37.1 ± 0.1º C, in the treated and CTR groups, respectively.

No behavior changes were noticed, except at 48 h after intratesticular injection in one rat, who presented with teeth gridding, coined and aggressiveness. On clinical exam, we observed intense scrotal edema, priapism and scrotum painful to palpation. This rat was euthanized. In all other rats, a slight sensitivity was observed at D1 and D2 after intratesticular injection.

There was no significant weight loss; however, we observed weight gain. The CTR group presented initial (D0) weights of 0.31 ± 0.04 kg and final weight (D100) of 0.46 ± 0.07 kg ($P < 0.001$). The treated group was 0.28 ± 0.04 kg and 0.46 ± 0.04 kg ($P < 0.001$), respectively. There was no significant difference in weight gain between the CTR and treated groups.

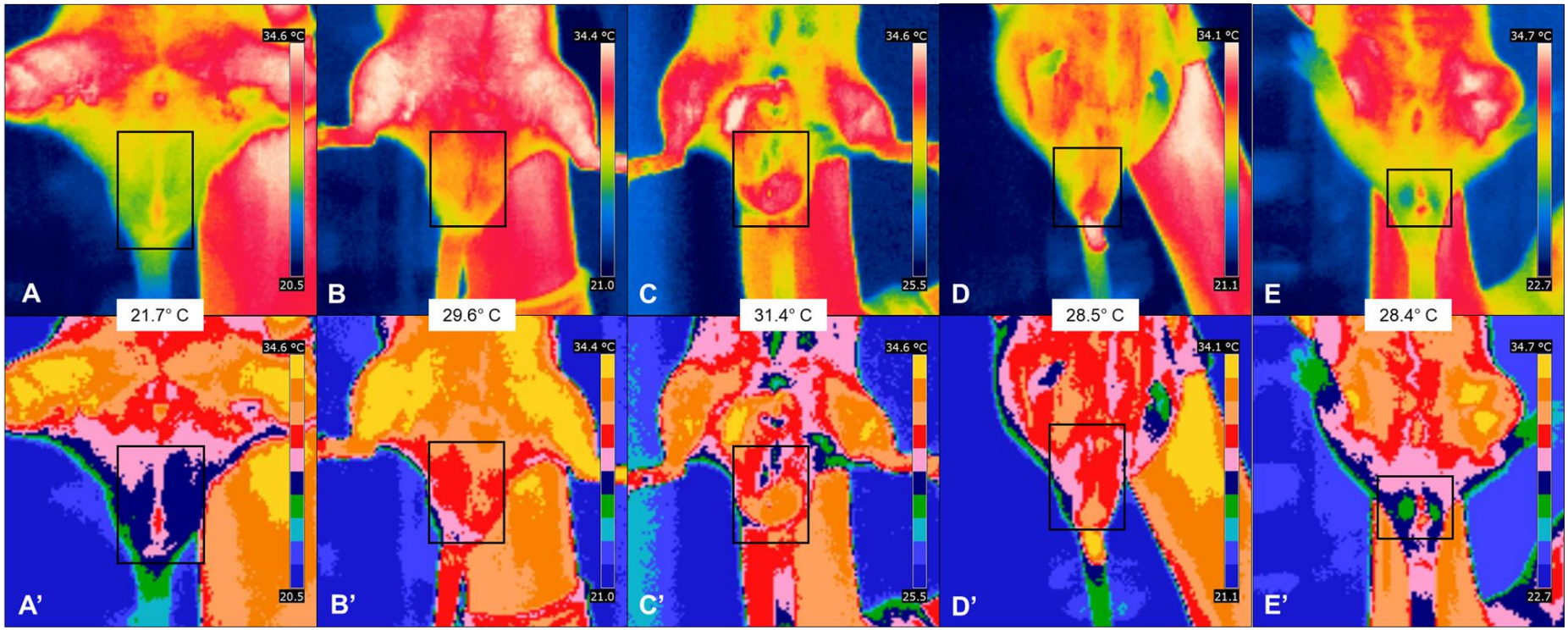

Figure 1 Infrared testicular thermographic picture (A to E) and medical pallet representation (A' to E') from the same time, showing mean temperature measured at times 0 (A and A'), 2 h (B and B'), 8 h (C and C'), D9 (D and D') and D100 (E and E') of the treated group.

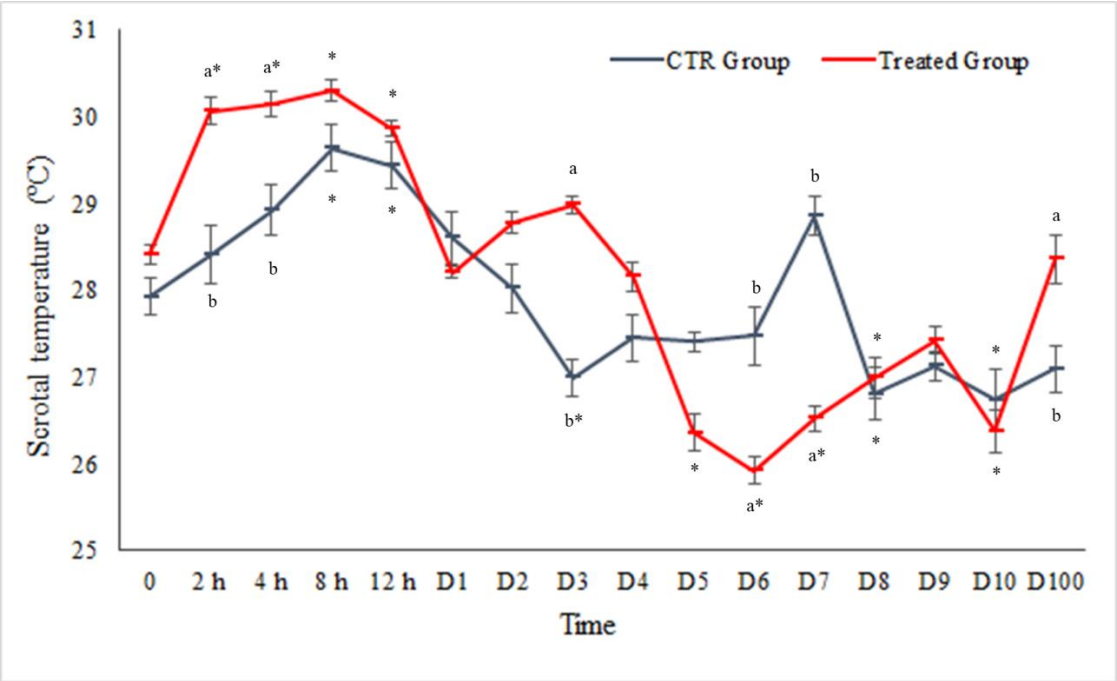

Figure 2 Scrotal temperature of the CTR and treated groups measured by infrared thermography. Different letters indicate significant differences between groups at each time-point. Symbol (*) indicates significant difference between 0 and other times in the same group.

3.2. Weight and volume of testicles

The weights and volumes of testicles are presented in Figure 3. All times of the treated group showed testicular weight lower than those of the CTR (D100) group. Regarding testicular volume, there was less in the treated group from D2 to D6, D9 and D100 than in the CTR (D100) group. In the treated group, reduction of testicular volume occurred progressively, 30% at D5 and 75% at D100, while testicular weight reduction occurred from 2 h to D10 and ranged from 13% and 43%, to 83% at D100.

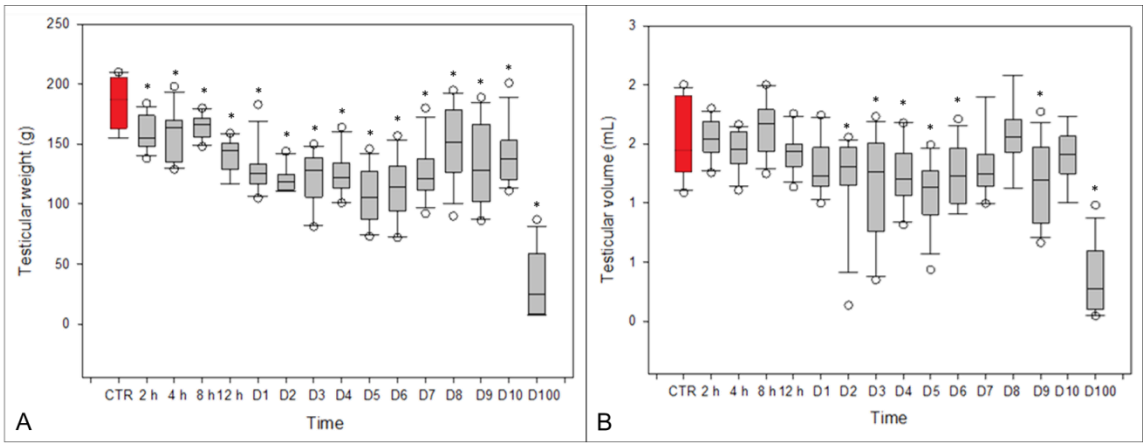

Figure 3 Boxplot of weight and volume of testicles from treated and CTR rats (D100 – red box). Symbol (*) indicates significant difference between CTR (D100 – red box) and each treated group. Circle (O) on top of the box represents the outliers.

3.3. Sperm analysis

At all times in the treated group, sperm characteristics were lower than those of the CTR (D100 – Table 1). Spermatozoa were found in the epididymides of treated rats until D9. At D10, only one testis from one rat even presented cells. At D100, there were no spermatozoa in the epididymides of the treated group. The morphological defect percentage showed a progressive rise, reaching 86% at D10, and primary defect at all times was detached spermatic head.

TABLE 1 Mean and standard error of epididymal spermatozoa characteristics from CRT and treated group.

Group	Time	Motility (%)	Vigor (0 to 5)	Concentration x10^6/mL	Total morphological defects (%)
CTR (D100)		50.0 ± 6.0	2.7 ± 0.2	187.5 ± 44.7	5.4 ± 1.1
TREATED	2h	0*	0*	32.0 ± 3.7*	12.3 ± 2.6*
	4h	10.8 ± 2.7*	1.0*	22.8 ± 4.0*	7.6 ± 1.9
	8h	20.0 ± 2.6*	0.8 ± 0.2*	48.0 ± 4.4*	7.6 ± 1.8
	12h	10.8 ± 3.8*	0.9 ± 0.2*	28.0 ± 3.0*	5.8 ± 1.4
	D1	5.8 ± 4.2*	0.2 ± 0.2*	42.7 ± 5.5*	24.1 ± 4.5*
	D2	15.8 ± 5.4*	1.0 ± 0.2*	19.0 ± 6.0*	9.1 ± 5.7
	D3	6.7 ± 4.0*	0.7 ± 0.3	0*	14 ± 4.5*
	D4	0*	0*	0*	31 ± 14.9*
	D5	0*	0*	1.9 ± 7.0*	48.7 ± 14.3*
	D6	0*	0*	6.6 ± 2.4*	82.4 ± 8.3*
	D7	0.4 ± 0.4*	0*	7.7 ± 2.6*	42.1 ± 17.4*
	D8	0*	0*	3.3 ± 1.3*	52.1 ± 16.7*
	D9	0*	0*	2.2 ± 1*	41 ± 9.2*
	D10	0*	0*	0.1*	86 ± 0*
	D100	--	--	--	--

Symbol (*) indicates difference between CTR (D100) and each treated group.

3.4. Fertility test

None of the females paired for 20 days with the treated group (D100) became pregnant. By contrast, all the females mating with rats in the CTR group (D100) became pregnant. In this group, the mean number of cubs/female was 12.

3.5. Macroscopic testicular evaluation

On palpation of the testicle, we noted a progressive hardening of testicular tissue and macroscopic changes observed during necropsy (Fig. 4).

At 2, 4, 8 and 12 h in the treated group, there was mild edema of the tunica that involved the testicles (Fig. 4C). There was also intense testicular hyperemia and a hemorrhagic point (suffusion) at the injection site, as well as edema and hyperemia of the pampiniform plexus (Fig. 4D). The testicles were firm, with distended capsules. There was intense scrotal and adjacent tissue swelling at D1 and D2 (Fig. 4E and 4F). At this point, tunica vaginalis, spermatic cord and adjacent tissue presented hemorrhage and

edema (Fig. 4G and H).

The scrotal swelling reduced progressively from D3 to D8; however, spermatic cords remained swollen and firm. The testicular capsules were thick (Fig. 4I) and the testicles were friable. There was testicular adhesion to the tunica vaginalis (Fig. 4J) at D3–D5. Testicular vascularization was decreased at D6–D10 (Fig. 4K and L); however, the scrota presented normal size. The testicular parenchyma was pale and firmer, with the friable capsule containing whitish spots at D10 (Fig. 4M and N). Finally, at D100, scrota appeared to be normal and testicles were difficult to palpate. After opening the scrotum, the testicles were small, firm, rough in appearance and poorly vascularized (Fig. 4N band O), and the majority presented diffuse calcification.

3.6. Testicular histology

Histological evaluation of tubular compartment showed that tissular edema did not differ at any time-points in the treated CTR groups, therefore apoptosis/necrosis were evident from D2, inflammatory infiltrates from D3, calcification increased at D7, D8 and D10, with fibroplasia at D8, D9, D10 and D100. Regarding the interstitial compartment, increases in tissue edema and apoptosis/necrosis were noticed starting at D1, inflammatory infiltrates at D3, calcification at D6, fibroplasia at D7 and neovascularization only at D100. The frequency score of tissue injury is shown in Table S2.

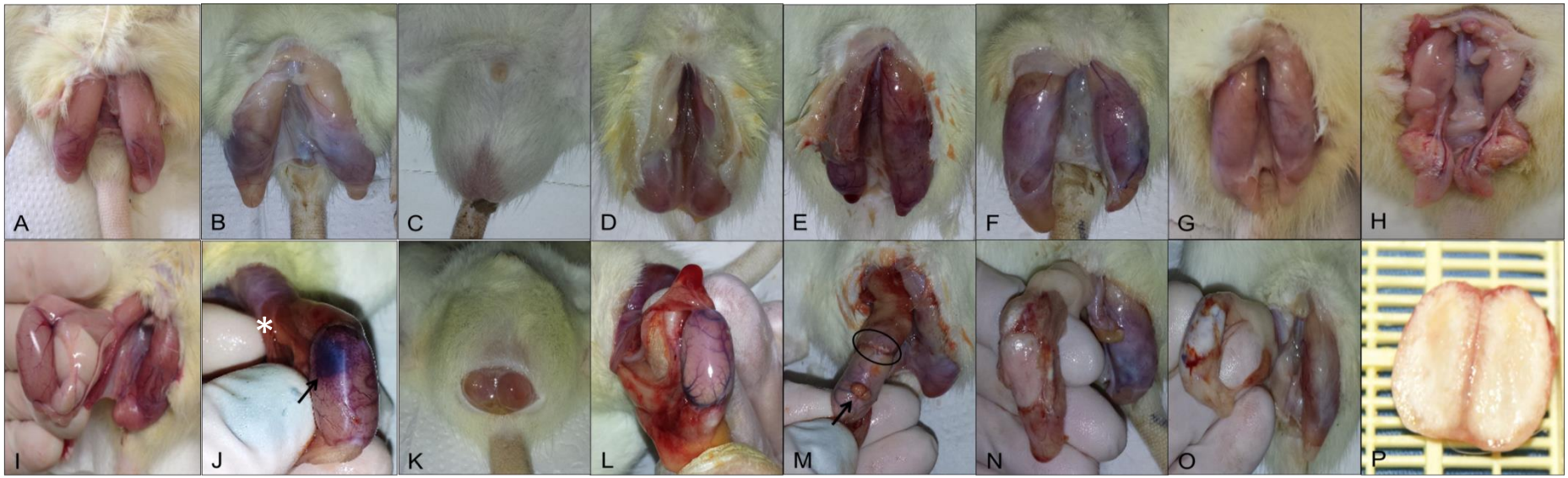

Figure 4 Macroscopic testicular evaluation of rats. **CTR group: A.** Normal testicle inside tunica vaginalis; **B.** Normal testis (note normal size, shape and vascularization); **Treated group - times: C.** Testicular tunica vaginalis edema and testicular hyperemia (2 to 12 h); **D.** Hyperemic testis with suffusion (arrow) and pampiniform plexus vessels hyperemia and edema (*) (2 to 12 h); **E.** Swollen scrotal sac (D1 and D2); **F.** Subcutaneous edema (D1 and D2); **G.** Subcutaneous and tunica edema around the testicles (D1 and D2); **H.** Testicular and adjacent tissue hyperemia observed (D1 and D2); **I.** Testicular and adjacent structures hyperemia (D3 to D5); **J.** Rupture of the testicular capsule with exposure and friable testicular tissue (arrow) and fibrin deposit s(inside the circle) (D3 to D5); - **K. M.** Scrotal area tissue with less edema and hemorrhage (D6 to D10); **L. N.** Testicular size reduction (atrophy), soft and diminished vascularization (D6 to D10) - **O. P.** Atrophied testicle with rough and pale appearance and firm consistency (D100).

Table S2 Injury frequency (%) classified for histological intensity score in the testicles before and after intratesticular injection.

			SEMINIFEROUS COMPARTMENT					INTERSTITIAL COMPARTMENT					
GROUPS	TIME	SCORE	NEC	INF	EDE	CAL	FIBR	NEC	INF	EDE	CAL	FIBR	NEO
CTR	D100	0	100	100	0	100	100	100	100	0	100	100	100
		1	0	0	91.6	0	0	0	0	91.6	0	0	0
		2	0	0	8.3	0	0	0	0	8.3	0	0	0
		3	0	0	0	0	0	0	0	0	0	0	0
TREATED	2H	0	41.6	91.6	50.0	100	100	41.6	50.0	0	100	100	100
		1	58.3	8.3	50.0	0	0	58.3	50.0	66.6	0	0	0
		2	0	0	0	0	0	0	0	33.3	0	0	0
		3	0	0	0	0	0	0	0	0	0	0	0
	4H	0	91.6	91.6	0	91.6	100	91.6	91.6	0	100	100	100
		1	0	8.33	91.6	8.33	0	0	0	25.0	0	0	0
		2	0	0	8.3	0	0	0	8.33	66.6	0	0	0
		3	8.33	0	0	0	0	8.33	0	8.33	0	0	0
	8H	0	100	100	0	100	100	100	75.5	0	100	100	100
		1	0	0	83.6	0	0	0	25.5	33.3	0	0	0
		2	0	0	16.3	0	0	0	0	50.0	0	0	0
		3	0	0	0	0	0	0	0	16.6	0	0	0
	12H	0	25.0	100	0	100	100	25.0	75	0	100	100	100
		1	66.6	0	66.6	0	0	66.6	25	8.3	0	0	0
		2	8.3	0	33.3	0	0	8.3	0	75	0	0	0
		3	0	0	0	0	0	0	0	16.6	0	0	0
	D1	0	8.3	25	0	91.6	100	16.6	16.6	0	91.6	100	58.3
		1	16.6	58.3	33.3	8.3	0	8.3	58.3	16.3	8.3	0	41.6
		2	33.3	8.3	58.3	0	0	83.3	8.3	16.3	0	0	0
		3	41.6	16.6	16.6	0	0	0	16.6	75	0	0	0
	D2	0	0	50.0	0	83.3	100	0	8.3	0	75	66.6	75.0
		1	0	41.6	91.6	16.6	0	0	50	16.3	25	33.3	25.0
		2	0	8.3	8.3	0	0	0	25	66.6	0	0	0
		3	100	0	0	0	0	100	16.3	16.3	0	0	0
	D3	0	16.6	16.6	0	83.3	75	16.6	8.3	0	75	66.6	83.3
		1	8.3	58.3	75	16.6	25	8.3	8.3	25	16.6	25	8.3
		2	0	16.6	25	0	0	16.6	50.0	41.6	8.3	8.3	8.3
		3	75	8.3	0	0	0	58.3	33.3	33.3	0	0	0
	D4	0	8.3	8.3	8.3	58.3	100	0	0	0	58.3	66.6	75
		1	0	75	66.6	33.3	0	8.3	8.3	0	16.6	33.3	25
		2	0	8.3	25	8.3	0	16.6	58.3	66.6	25	0	0
		3	91.6	8.3	0	0	0	75	33.3	33.3	0	0	0
	D5	0	0	16.6	16.6	50	100	0	0	0	75	100	100
		1	0	83.3	83.3	50	0	0	25	8.3	25	0	0
		2	0	0	0	0	0	0	50	91.6	0	0	0
		3	100	0	0	0	0	100	25	9	0	0	0
	D6	0	0	0	0	50	91.6	0	0	0	0	33.3	100
		1	0	58.3	75	41.6	0	0	33.3	33.3	75	50	0
		2	0	33.3	25	8.3	0	0	33.3	58.3	25	16.6	0
		3	100	8.3	0	0	8.3	100	33.3	8.3	0	0	0
	D7	0	0	0	0	0	50	0	0	0	0	8.3	100
		1	0	41.6	83.3	91.6	50	0	8.3	0	50	66.6	0
		2	0	50	16.6	8.3	0	0	33.3	100	41.6	16.6	0
		3	100	8.3	0	0	0	100	58.3	0	8.3	8.3	0
	D8	0	0	0	0	9.1	0	0	0	0	18.2	9.1	90.9
		1	0	54.5	45.5	63.6	45.5	0	0	9.1	81.8	36.3	0
		2	0	36.3	54.5	27.7	54.5	0	45.5	81.8	0	36.3	9.1
		3	100	9.1	0	0	0	100	54.5	9.1	0	18.1	0
	D9	0	0	0	0	0	58.3	0	0	0	66.6	0	91.6
		1	0	83.3	83.3	66.6	33.3	0	41.6	33.3	33.3	58.3	8.3
		2	0	16.6	16.6	33.3	8.3	0	41.6	58.3	0	25	0
		3	100	0	0	0	0	100	16.6	8.3	0	16.6	0
	D10	0	0	0	0	25	16.6	0	0	0	16.6	0	41.6
		1	0	41.6	41.6	66.6	66.6	0	8.3	75	75	33.3	33.3
		2	0	25	50	8.3	8.3	0	33.3	25	8.3	33.3	0
		3	100	33.3	8.3	0	8.3	100	58.3	0	0	33.3	8.3
	D100	0	0	22.2	0	55.5	11.1	0	0	0	77.7	0	0
		1	0	44.4	44.5	44.5	55.5	0	66.6	77.7	20.3	22.2	11.1
		2	22.2	22.2	55.5	0	11.1	11.2	33.3	22.3	0	11.1	88.8
		3	77.8	11.1	0	0	22.2	88.8	0	0	0	66.6	0

NEC. = Necrosis, INF. = Inflammatory infliltrate, EDE. = Edema, CALC. = Calcification, FIBR. = Fibroplasia, NEO. = Neovascularization; Score: 0 = no injury, 1 = mild injury (up to 25%), 2 = moderate injury (25 to 50%), 3 = pronounced injury (over 50%).

4. DISCUSSION

We evaluated the effects of intratesticular injection of 20% $CaCl_2$ with 0.5%

DMSO immediately and at 100 days using scrotal temperature, pain (behavior), testicular weight and volume, seminal analysis, testicular macroscopic changes and histology. To our knowledge, there has been no prior study analyzing the immediate effects of a testicular sclerosing agent on seminal parameters and testicular histology.[5,8,10,14,15,24–29]

In the design of this study, we chose only to have one control group, to reduce the number of animals otherwise, we had used double, unnecessarily. We can see that this option was efficient in checking data for all variables collected. Regarding body and testicular temperature, the control was the temperature before and at the correlated moments of the animals of CTR group. For these measures, such control was necessary because it could receive internal and external interference. Already, in relation to testicular biometry, seminal parameters, macroscopic and histological changes, this study efficiently showed the consequences caused by a mild (CTR) in comparison to an intense inflammatory reaction (treated group). In addition to that, normal parameters data are available in the literature for consultation.

Scrotal temperature sharply increased until 2 h followed by progressive rise until 8 h (peak) and slight decrease to D1. Within minutes after injury, an acute inflammatory reaction take place, altering vascular and cellular dynamics.[30] Inflammation modifies blood flow, and generates heat that can be measured by a temperature gradient. The diagnosis and monitoring of testicular inflammatory reactions can performed using infrared thermography, a reliable method that efficiently detects temperature changes on skin surface due to underlying pathological processes.[4,29,31]

By macroscopic evaluation, intense testicular hyperemia was observed from 2 h to D2. The infrared thermography failed to detect raised testicular temperature on D1 and D2 because of the intense subcutaneous edema that promoted a thick layer between the testicle and skin with fluid accumulation. As soon as the edema decreased, the detection of testicular temperature normalized. At D10, testicular temperature was restored to its initial (time 0) level.

Gonadal temperature elevation causes degeneration of the germinal epithelium and Sertoli cells, depresses spermatogenesis, and thereby decreases sperm output, increases morphological defects and alters motility parameters. Furthermore, thermal stress causes disruption of epididymal absorptive and secretory epithelium, changing protein composition of intraluminal fluid, causing reduction of its storage capacity. Depending on the temperature intensity, sperm impairment can be noted within 4 to 8 days after thermal stress.[32,33]

Interestingly, 2 h after intratesticular injection in treated group, we observed absence of sperm motility. The sperm concentration was 6 times lower than that of the CTR group, although the injection site was not at the epididymal cauda. $CaCl_2$ caused toxic injury (osmolarity, temperature and others) to the sperm cells stored in the epididymis and induced necrospermia. Decreased sperm concentration can be caused by sperm cell apoptosis due to toxicity; however, it remains a puzzle to be solved.

Even though it was not the objective of this study, we could see that after injecting a dye into the testicle, it immediately distributed through the parenchyma, reaching neighbor structures and lymphatic vessels (Figure S3). It is possible to note dye at the epididymal cauda within seconds after injection, where the sperm is stored.

In treated group, slight increases in sperm motility were noted at 4 h, but no more than 20% when compared with CRD D100. There is a greater chance that the most part of the substance was already absorbed and removed by the lymphatic vessels, and the organism was trying to restore homeostasis. Necrospermia was present again from D4–D10. Studies reported motility of epididymal spermatozoa from rats ranging between 61% and 89% (34) and sperm count between 238.1 ± 50.5 to 122.6 ± 11.6 x 10^6/mL (35), higher than that found in the CTR group. This may be because they were maintained for 20 days with females and seminal evaluation was performed without resting time. Nevertheless, the *in vivo* fertility was 100% in the CTR group.

Morphological defects increased progressively, reaching near 90% at D10 in the treated group. Morphological defect cut-off values reported in the literature are 10% for subjective evaluation.[36] At 2 h, there were 13% increase on morphological defects and the majority were coiled tail. From 4 to 12 h, morphological values recovered to normal, rising again at D1 (24%). From D4 to D10, the majority of sperm defects were head-tail detachments.

Spermatogenesis and sperm maturation are well orchestrated mechanisms, and any interference in the testis or epididymis can lead to sperm impairment. For the first 12 h, scrotal temperature increased at least 2 °C, caused by increased blood flow due to the inflammatory process. In the present study, the majority of morphological defects at D4 to D10 were detached head; this agrees with results of Rocha et al.[37] who reported morphological defects four days after testicular insulation in rams.

The sperm abnormalities described from 4 h to D10 in the treated group can be classified as oligo-astheno-teratozoospermia (OAT) syndrome, which was described in an infertile man.[38] Asthenozoospermia is a particular characteristic of epididymal dysfunction, as it affects the microenvironment and interferes with protein secretion,

impairing sperm maturation and consequently motility.[39] Other alterations in sperm parameters result from lipid peroxidation that prejudices the sperm cell, causing increased morphological defects, interference with motility, and damage to the seminiferous tubule, impairing spermatogenesis.[40]

Inflammation also alters cellular metabolic functioning by fluid extravasation, cell recruitment, secretion of mediators and other signaling molecules.[30] Despite the role of sperm modulation and anti-oxidative function, serum cytokines increase, causing worsening of seminal quality, generated by increased lipid peroxidation reported in men with idiopathic infertility or genital inflammation.[20,41]

Body temperature was assessed in the same way as scrotal thermography. Both parameters were measured at the same time. The normal body temperature variation described in literature for *Rattus novergicus* is 35.9 to 37.5º C. The circadian rhythm temperature amplitude for rats is 2.1 ºC,[42] which was the variation observed in our study. Temperature increases could be systemic signs of inflammation, because the release of acute phase proteins produced by the liver stimulates and facilitates the production of prostaglandins, responsible for the onset of symptoms (e.g. pain and fever) through their effects on the central nervous system.[43]

Despite the patchy distribution of the lesions, testicular inflammation was associated with disruption of testicular function, i.e., spermatogenesis.[44] In the present study, we verified an immediate effect of the sclerosing agent on testicular tissue by histological evaluation.

Every substance can cause tissue irritation, the intensity of which varies depending on its basic ion valence.[45] The valence of NaCl is zero (1.7 mEq) while that of $CaCl_2$ is -2 (1.8 mEq). NaCl was the substance used in the CTR group, and its valence is lower than that of $CaCl_2$. Even with a valence of zero, it caused a slight inflammatory reaction and changes in scrotal temperature as detected by infrared thermography. No histological changes were detected in the CTR group.

The association of $CaCl_2$ with other substances can enhance its effect on testicular tissue.[15] DMSO is an efficient solvent, usually used as a vehicle.[46] It is an anti-inflammatory drug that facilitates membrane penetration and increases the effectiveness of other substances.[47] Concentrations lower than 10% have low systemic toxicity.[46] In the present study, the anti-inflammatory properties of DMSO were not evaluated.

Analyzing histological scores, injuries to testicular tissue of both compartments (tubular and interstitial) at various times in the treated group were equally involved. Inflammatory infiltrates were noted after 2 h of intratesticular injection; however,

important histological changes started after the inflammatory edema peak (D1). By contrast to the acute inflammation that usually leads to recovery after the elimination of the irritant, the 20% $CaCl_2$ associated with to 0.5% DMSO caused a chronic orchitis and destroyed the testicular parenchyma architecture. At D100, fibroplasia and neovascularization indicated a repair process with tissue replacement and function loss. Testicular atrophy and azoospermia was expected at D100, corroborating the findings of Paranzini et al. (2018), 80 days after intratesticular injection of 20% $CaCl_2$ with 0.5% DMSO in cats.

Reduction of testicular weight and volume were not uniform between the rats during the first 10 days; however, at D100, we observed reductions of 83% and 75%, respectively.

No signs of distress or body weight loss were presented during the first 10 days; however, it is important to note that, at D1 and D2, the rats presented scrotum volume enlargement and protective reaction to palpation due to the edema caused by the local inflammatory reaction. Edema starts 3 to 5 hours after tissue injury, becoming maximal at 24 hours and regressing at about 7 days.[48,49] The pain produced was considered slight because of the absence of association with others clinical signs or food intake reduction. In dogs, scrotal swelling was reported from 24 to 72 h after intratesticular injection of $CaCl_2$,[24] and in donkeys after 48 h.[50]

In summary, 20% $CaCl_2$ with 0.5% DMSO caused toxic injury to sperm cells, causing necrospermia and oligospermia 2 h after intratesticular injection. Furthermore, it produced acute and intense testicular inflammatory reactions, altering sperm parameters 4 h after intratesticular injection, histological changes after D2 and infertility at 100 days. According to the lesion characteristics seen on histological evaluation, we believe that testicular changes caused by this substance are irreversible. No signs of systemic involvement were noted. This is efficient method for mass population control, without prejudice to animal welfare.

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6. DISCLOSURES

The authors have no competing interests to disclose.

7. AUTHOR CONTRIBUTIONS

Paranzini, Lima, Martins and Souza designed the study. Paranzini, Camargo, Yamada, Ruediger and Ramirez Neto conducted the study and sample analysis. Ferreira supervised the findings. Honsho processed the histological samples. Macedo performed the histological evaluations. Paranzini drafted the early manuscript that was later critically appraised and improved by all authors.

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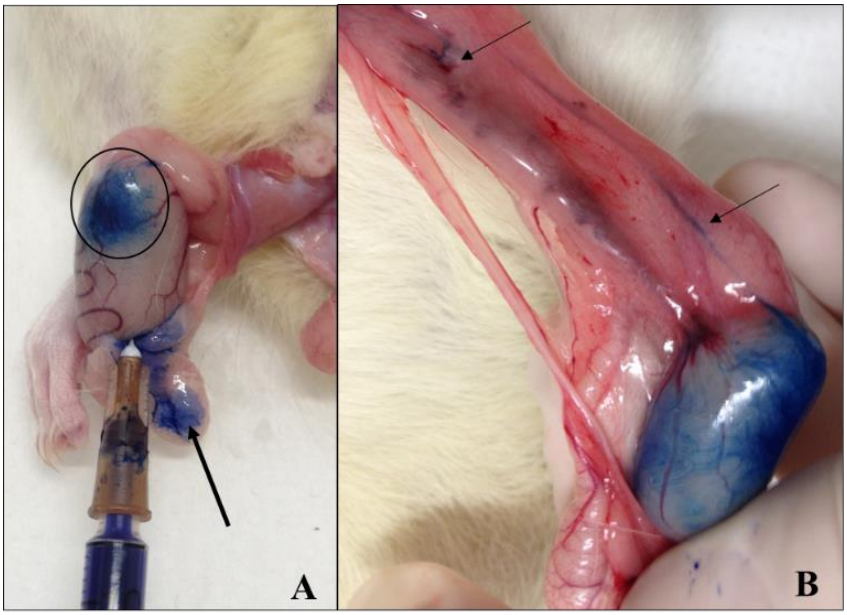

Figure S3. Picture taken immediately after blue dye injection into rat testicle. **A.** Coloration is noted at the site where substance deposition started (circle), with rapid distribution to the epididymis cauda (arrow) and **B.** lymphatic vessels (arrow).

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PROTEOMIC OF SPERMATOZOA AND EPIDYDIMAL FLUID AFTER ACUTE TESTICULAR INFLAMMATION IN RATS (*Rattus norvegicus*)

SEMINAL PROTEOMIC APPROACH AFTER ACUTE ORCHITIS IN RATS

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ABSTRACT

The aim was to study the proteomic of spermatozoa and epididymal fluid after intratesticular injection with $CaCl_2$ 20% associated with DMSO 0.5%, to potentially identify infertility biomarkers and immune contraceptive vaccine targets. The study was carried out using 36 (n=6) male mature Wistar rats (*Ratus norvegius*) after orchitis induced by 0.1 mL of $CaCl_2$ 20% associated with DMSO 0.5%, a testicular sclerosing agent, used for chemical castration. Fluid was recovered from the cauda epididymis and *vas deferens* at 2 h (2h), 1 day (D1), 5 days (D5), 7 days (D7) and 10 days (D10) (treated group). Spermatozoa were separated from epidydimal fluid by centrifugation, and samples were processed and digested for nano-LC–ESI-Q-TOF mass spectrometry analysis. For statistical analysis, samples of spermatozoa and epididymal fluid from the control group were compared with the samples taken after intratesticular injection, based on total spectra, using Scaffold software. Some proteins, identified as fertility biomarkers by other studies, were downregulated in the epididymal fluid-treated group (infertile samples) and this is in agreement with other studies. Some other proteins, identified as fertility biomarkers in previous studies, are upregulated in the sperm protein extract treated group due to a compensatory mechanism in maintaining homeostasis of acute involvement. A targeted follow-up study to confirm biomarkers of spermatozoa infertility is strongly recommended. Proteins related with the inflammatory process were upregulated in treated group epididymal fluid and can be used to diagnose acute orchitis. Additionally, a list of proteins found in fertile sperm were provided, and serve as immunocontraceptive targets.

Keyword: Fertility biomarker, Oxidative stress, Asthenozoospermia, Acute orchitis, Infertility

INTRODUCTION

The study of the composition of sperm, physiology and translational changes is essential to understand the factors involved in fertility (Ceciliani, Eckersall, Burchmore, & Lecchi, 2014). Protein analysis methods, such as antibody binding or mass spectrometry, have been key techniques for the study of individual proteins and whole proteomes. The proteome includes all the proteins that are expressed in a cell in a specific moment, including isoform modifications. The proteome is dynamic over time and can be modified in response to external factors (Rappsilber & Mann, 2002). Because the spermatozoa are highly specialized cells, easily obtained and purified, they are very suitable for proteomic studies (Rahman, Lee, Kwon, & Pang, 2013).

Sperm cells, prior to translocation to the epididymis, have accomplished morphological maturity. During transit, sperm is in direct contact with epididymal fluid, which elicits modifications through a series of biochemical reactions. This causes plasma membrane remodeling, acquisition of motility and the ability to fertilize the oocyte (Fouchecourt, Metayer, Locatelli, Dacheux, & Dacheux, 2000). Moreover, those biochemical reactions modulate protein expression, including proteins relevant for fertility. Any abnormal protein levels such as absence or up/downregulation of the sperm plasma membrane or epididymal fluid can affect the spermatozoa's fertilizing ability (Chakradhar, 2018; Nowicka-Bauer & Kurpisz, 2013).

Among the most common causes of male infertility, genital inflammation of diverse etiologic origin has high incidence, causing modulations of protein expression in sperm and impairing male reproductive function (Pilatz et al., 2015). The inflammatory reaction stimulates reactive oxygen species (ROS), resulting in lipid peroxidation and acting directly on membrane fluidity; leading to membrane damage, increasing apoptosis, decreasing motility, causing acrosome reaction and DNA fragmentation (Haidl, Allam, & Schuppe, 2008; Mc Entee, 1990; Schuppe et al., 2017).

Chemical castration caused by intratesticular injection of a sclerosing agent leads to male subfertility/infertility (Jana & Samanta, 2006). One example is calcium chloride ($CaCl_2$) associated with DMSO; the chemicals cause a local inflammatory reaction, promoting testicular degenerative injuries, necrosis and fibrosis of the seminiferous tubules, germ cells and interstitial cells (Paranzini et al., 2018; Pereira et al., 2018; Silva et al., 2018). Furthermore, this also causes lipid peroxidation, which results in irreversible azoospermia associated with the production of free radicals (Kutzler, 2015; Oliveira et al., 2012; Paranzini et al., 2018; Samanta, 1998).

Infertile individuals may present normal seminal characteristics (concentration, motility and morphology), thus, molecular studies can be used to indicate failures in functional mechanisms (Dorus & Karr, 2009; Martínez-Heredia, Estanyol, Ballescà, & Oliva, 2006; Nowicka-Bauer & Kurpisz, 2013). The utilization of proteomics could provide insights into the molecular basis of sperm function after injuries, such as inflammatory processes, and also facilitate the identification of specific cell targets for the diagnosis of male infertility (Baker, Hetherington, Reeves, Müller, & Aitken, 2008). This is because the unknown causes of infertility are often attributed to a lack of knowledge of spermatozoa protein composition (Martínez-Heredia et al., 2006). Therefore, our objective was to study the proteomics of spermatozoa and epididymal fluid after intratesticular injection of a sclerosing agent ($CaCl_2$ 20% associated with DMSO 0.5%) for the identification of possible infertility biomarkers and immune contraceptive vaccine targets.

MATERIAL AND METHODS

Reagents

All reagents used in the present study were of the highest purity and obtained from Sigma-Aldrich (St. Louis, MO, USA), Promega (Madison, WI, USA), GE Healthcare Life Sciences (São Paulo, São Paulo, Brazil), Waters Corp. (Barueri, São Paulo, Brazil) and Thermo Fisher Scientific (São Paulo, São Paulo, Brazil), unless otherwise cited.

Ethical aspects

The study was conducted in accordance with the ethical guidelines recommended by the National Council for the Control of Animal Experimentation and the College of Animal Experimentation, and was approved by the Institution's Animal Care and Experimentation Ethics Committee on August 04, 2016, registered under protocol number 46/2016. Other results related to this study have already been published (submitted article).

Animals and experimental groups

Thirty-six male rats (*Ratus norvegius*, Wistar), 70 days-old, were used. The animals were kept in appropriate boxes (49 x 34 x 16 cm), with specific shaving substrate, a density of 3 rats/box and free access to water and commercial food. Prior to the study, they were identified and randomly divided into 2 groups: control (CTR) and treated. The treated group was evaluated over 5 time points (n=6/time point): 2 h (2h), 1 day (D1), 5 days (D5), 7 days (D7) and 10 days (D10) after intratesticular injection of $CaCl_2$ associated with DMSO. The CTR group received 0.1 mL of 0.9% NaCl solution (intratesticular) and was evaluated at 100 days (D100). Epididymal fluid and sperm were collected from the treated groups in each time point (2h, D1, D5, D7 and D10) and from the control group at 100 days.

Treatments, epididymal fluid and sperm collection

Males were anesthetized with morphine (3 mg/Kg, intraperitoneally) and isoflurane on an anesthetic-soaked cotton inside an inhalation chamber. Then, 0.1 mL of 20% $CaCl_2$ associated with 0.5% DMSO (treated group) or 0.9% NaCl solution (CTR group) were injected into each testicle. The injection was administered with a 1 mL syringe and a 27½ G hypodermic needle. The needle was inserted into the distal testis, 0.5 cm laterally to the tail of the epididymis, and was advanced into the cranial region of the testis. The substance was deposited slowly across the longitudinal extent of the testis, as the needle was being removed, as an infusion.

Regarding the protocol for euthanasia, rats were anesthetized with isoflurane and the cauda epididymis was collected. Immediately after, the cauda epididymis and vas deferens were compressed and the content was collected and diluted in 100 µL of 0.9% NaCl solution to carry out the proteomic assays.

Proteomic analysis of seminal fluid and sperm cells

Due to the small amount of recovered material, a pool was made which contained the samples of each group. The fluid recovered from the cauda epididymis and vas deferens was centrifuged at 800 g for 10 min to separate the spermatozoa. The supernatant (epididymal fluid) was collected and re-centrifuged at 10,000 g for 30 min at 4° C and immediately stored at – 20° C. The pellet (spermatozoa) was washed 3 times (800 g for 5 min) in 50 mM TRIS-HCl pH 7.2 containing protease inhibitors (0.8 mM EDTA, 1 µg/mL aprotinin, 1 µg/mL leupeptin and 35 µg/mL PMSF). Subsequently, the cells were maintained in an ice bath and diluted in modified RIPA extraction buffer (150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS in 50 mM TRIS-HCl pH 7.5, (Peach, Marsh, & MacPhee, 2012). Then the samples were sonicated according to Souza

et al. (2009) with a 3.0 mm probe in a 20% amplitude for 30 seconds on ice bath, repeated 10 times at one minute intervals between sets. After sonication, the samples were centrifuged at 15,000 g at 4° C for 30 min. The supernatant (protein extract) was collected and frozen at -20° C.

The samples, sperm protein extract and epididymal fluid, were thawed in an ice bath and the total protein concentration was determined by the Bradford method (Sigma-Aldrich, São Paulo, Brazil) using a spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Ultrospec 2000 UV/VIS Spectrophotometer, Uppsala, Sweden). The results were based on a standard curve made of known concentrations of bovine serum albumin.

Proteins from epididymal fluid were digested in solution using the procedures previously reported by Codognoto *et al.* (2018). Briefly, an aliquot containing 50 μg of total protein was added to urea (8 M), followed reduction with DTT (5 mM) and alkylation with iodoacetamide (14 mM). The quench was performed by adding DTT (5 mM). Digestion was completed with trypsin (ratio of 1:50 enzyme:substrate) and the enzymatic action was stopped with trifluoroacetic acid (0.4%). Desalting of peptides was conducted in reverse phase columns (SepPack C18 WAT054955, Waters Corporation, Milford, MA, USA), according to the manufacturer's instructions. Thus, the volume was reduced (~1 μL) in a vacuum concentrator (SPD1010 Integrated SpeedVac ™ Systems, Thermo Fisher Scientific Inc., Waltham, MA, USA) and the samples were stored at −20° C until mass spectrophotometry.

Samples containing 50 μg of sperm protein extract were run in 12% SDS-PAGE, until entering the separation gel, to remove the extraction buffer compounds. The gel was stained in colloidal Coomassie G-250 (Luo, Wehr, & Levine, 2006; Neuhoff, Stamm, & Eibl, 1985; Neuhoff, Arold, Taube, & Ehrhardt, 1988). Tryptic digestion was performed according to a modified version of Shevchenko, Wilm, Vorm, & Mann (1996). In each

pool the unique band formed was cut, distained (50% acetonitrile and 100% acetonitrile), reduced with DTT (20 mM), alkylated with iodoacetamide (55 mM) and dehydrated with 100% acetonitrile. The digestion was conducted with trypsin (ratio of 1:50 enzyme:substrate) and stopped with 50% acetonitrile and 5% formic acid; samples were then centrifuged and the supernatant was reduced in a vacuum concentrator and stored at −20° C prior to mass spectrophotometry.

For nano-LC–ESI-Q-TOF mass spectrometry analysis, epididymal fluid and sperm protein extract samples were thawed, diluted in formic acid 0.1% in the proportion of 0.7 μg/μL, homogenized and centrifuged at 1,100 g for 5 min. The supernatant was removed (20 μL) and deposited in glass vials (Clear glass 12 × 32 mm screw neck total recovery vial, Waters Corporation, Milford, MA, USA). Proteomic analysis was performed according to Aragão *et al.* (2012). An aliquot of 4.5 μL was separated by column C18 (100 μm × 100 mm) RP nano UPLC (NanoAcquity, Waters Corporation, Milford, MA, USA) coupled to the Q-Tof Premier mass spectrometer (Waters Corporation, Milford, MA, USA) with nanoelectrospray at a flow rate of 0.600 μL/min. The gradient was 2% to 90% acetonitrile with 0.1% formic acid for 45 min. The voltage of the nanoelectrospray was 3.5 kV with a voltage cone of 30 V at 100 μC. The apparatus was operated in the top three mode in which an MS spectrum was acquired followed by MS/MS of the three most intense peaks detected. After MS/MS fragmentation, the ion was placed on the exclusion list for 60 s. Endogenous cleavage peptides were analyzed by using real-time deletion. Spectra were acquired by using MassLynx v.4.1 software, and raw data files were converted to a peak list format (.mgf) without adding the scans from the Mascot Distiller software v.2.3.2.0, 2009 (Matrix Science Ltd., Boston, MA, USA) with carbamidomethylation with fixed modifications, oxidation in methionine with variable modification, a trypsin cleavage, and tolerance of 0.1 Da for the precursor ions of

fragments. The relative quantification of each protein in the mixture was determined by total spectra obtained by Mascot Distiller software (Matrix Science Inc., Boston, MA USA). Mascot was set up to search the Uniprot_SProt_release_Jan_2016 database (selected for Mammalia, 65090 entries).

For protein identification, Scaffold software (version Scaffold_4.8.7, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, Keller, Kolker, & Aebersold, 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. For statistical analysis, the control group was compared with the samples after intratesticular injection (control *vs* treated), based on total spectra, using the Fisher exact test ($P < 0.05$) in Scaffold software.

RESULTS

Proteomic analyses for sperm extract protein found 2.160 spectra and identified 63 proteins by mass spectrometry (Fig. 1 and 2A). According to statistical analyses, the comparison between control (CTR) and treated samples taken at 2h resulted in 13 up- and 8 downregulated proteins in the treated group. When CTR was compared with D1 samples, this resulted in 15 proteins up- and 6 downregulated in the treated group (Table 1). The proteins were classified according to their functions in 5 categories (Table 1):

movement and structure (13 proteins); energy and metabolism (25 proteins); signaling, transport and turn over (8 proteins); stress response and antioxidant activity (6 proteins); and cell recognition and sperm-egg interaction (11 proteins). Furthermore, twenty-six proteins were present in the treated group and absent in CTR (Table 2).

In epididymal fluid samples 4.171 spectra were found, and 70 proteins were identified (Fig. 1 and 2B). The comparison of protein amount between the CTR group and the times 2h, D1, D5, D7 and D10 of the treated group resulted in proteins 23 up- and 31 downregulated in the treated group (Table 3). Of the 23 proteins upregulated in the treated group, 21 are involved in the inflammatory pathway/cascade. Sixteen proteins were present in the treated group and were absent in CTR (Table 2).

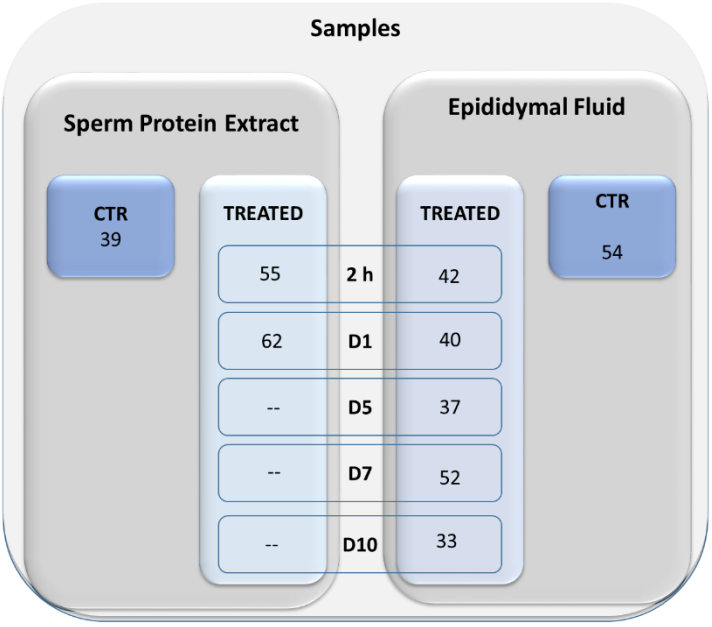

Figure 1. Protein number found in CTR group and each time of treated group of sperm proteins extract and epididymal fluid.

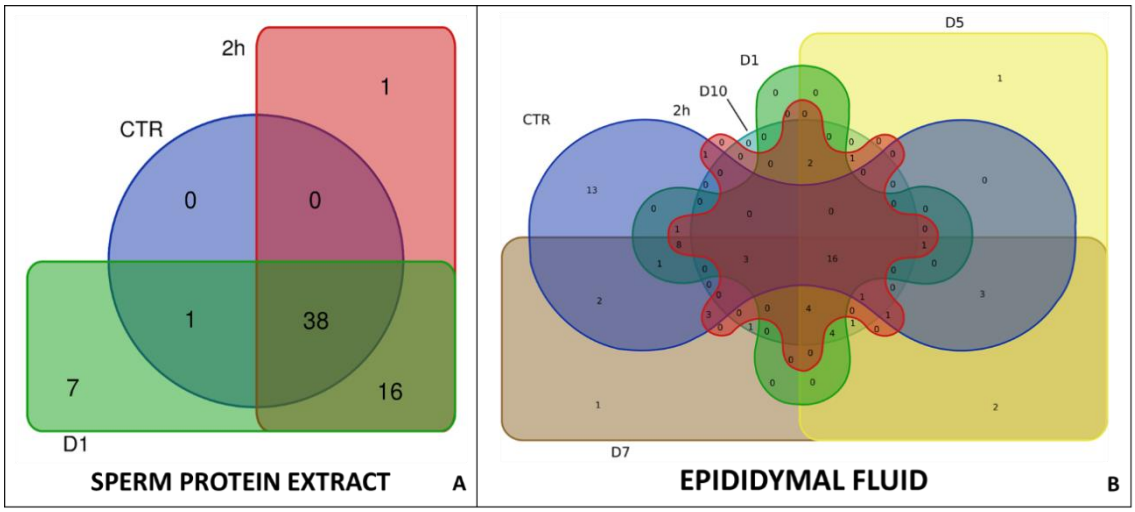

Figure 2. Venn diagram of the proteins identified in mass spectrometry of sperm protein extract (A) and epididymal fluid (B) of CTR and treated groups (http://bioinformatics.psb.ugent.be/webtools/Venn/ access 01.09.2019).

Table 1. Amount of sperm protein extract on CTR group and at 2h and D1 of treated group.

	SPERM PROTEIN EXTRACT IDENTIFIED	PROTEIN ID	GENE	CTR	TREATED	
					2H	1D
TREATED DOWN REGULATED	**Category: Movement and structural organization**					
	Outer dense fiber protein	Q6AYX5	Odf2	62 [a]	26 [b]	13 [b]
	Keratin, type II cytoskeletal 8	Q10758	Krt8	33 [a]	15 [b]	14 [b]
	Keratin, type I cytoskeletal 19	Q63279	Krt19	28 [a]	15 [b]	21 [b]
	Keratin, type II cytoskeletal 1	Q6IMF3	Krt1	15 [a]	11 [b]	6 [a]
	Category: Energy and metabolism					
	Alpha-enolase	P04764	Eno1	59 [a]	40 [b]	44
	Glutamine synthetase	P09606	Glul	9 [a]	0 [b]	2 [b]
	Aldehyde dehydrogenase, mitochondrial	P11884	Aldh2	25 [a]	31 [a]	15 [b]
	Fatty acid-binding protein 9	P55054	Fabp9	28	26	21
	Carbonyl reductase [NADPH] 1	P47727	Cbr1	12	8	9
	Aspartate aminotransferase, mitochondrial	P00507	Got2	6	6	2
	Phosphatidylethanolamine-binding protein 1	P31044	Pebp1	5	3	4
	Prostaglandin G/H synthase 2	P35355	Ptgs2	4	1	1
	Category: Signaling, transport and turnover					
	Isoaspartyl peptidase/L-asparaginase	Q8VI04	Asrgl1	16	16	12
	Category: Cell recognition and Sperm-egg interaction					
	Actin, cytoplasmic 1	P60711	Actb	31 [a]	23 [b]	14 [b]
	Beta-actin-like protein 2	D3ZRN3	Actbl2	14 [a]	3 [b]	3 [a]
	Actin, aortic smooth muscle	P62738	Acta2	19	18	7
TREATED UP REGULATED	**Category: Movement and structural organization**					
	Tubulin beta-4B chain	Q6P9T8	Tubb4b	19 [a]	65 [b]	62 [b]
	A-kinase anchor protein 4	O35774	Akap4	18 [a]	180 [b]	134 [b]
	Tubulin beta-4A chain	B4F7C2	Tubb4a	13 [a]	48 [b]	41 [b]
	Tubulin beta-6 chain	Q4QQV0	Tubb6	4 [a]	22 [b]	27 [b]
	Tubulin alpha-3 chain	Q68FR8	Tuba3a	13 [a]	17 [a]	27 [b]
	Ropporin-1	Q4KLL5	Ropn1	2	3	7

Table 1. *continued*

	SPERM PROTEIN EXTRACT IDENTIFIED	PROTEIN ID	GENE	CTR	TREATED	
					2H	1D
	Category: Energy and metabolism					
TREATED UP REGULATED	ATP synthase subunit alpha, mitochondrial	P15999	Atp5a1	0 [a]	8 [b]	10 [b]
	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	Q9ESV6	Gapdhs	3 [a]	20 [b]	12 [b]
	Hexokinase-1	P17710	Hk1	1 [a]	9 [b]	10 [b]
	Cytochrome c, testis-specific	P10715	Cyct	0 [a]	10 [b]	3 [b]
	ATP synthase subunit beta, mitochondrial	P10719	Atp5b	15 [a]	24 [b]	19 [a]
	Mitochondria-eating protein	Q6AYL6	Spata18	11 [a]	5 [b]	18 [a]
	Triosephosphate isomerase	P48500	Tpi1	3 [a]	19 [b]	10 [a]
	Cytochrome c oxidase subunit 5A, mitochondrial	P11240	Cox5a	0 [a]	12 [b]	10 [a]
	L-lactate dehydrogenase C chain	P19629	Ldhc	14 [a]	29 [a]	47 [b]
	Cytochrome c oxidase subunit 6B2	Q6YFQ1	Cox6b2	0 [a]	5 [a]	11 [b]
	Succinyl-CoA:3-ketoacid coenzyme A transferase 2A, mitochondrial	Q5XIJ9	Oxct2a	0 [a]	1 [a]	9 [c]
	Malate dehydrogenase, mitochondrial	P04636	Mdh2	0 [a]	0 [a]	8 [c]
	Glucose-6-phosphate 1-dehydrogenase	P05370	G6pd	0 [a]	0 [a]	7 [c]
	Saccharopine dehydrogenase-like oxidoreductase	Q6AY30	Sccpdh	5	6	3
	ATP synthase subunit delta, mitochondrial	P35434	Atp5d	0	6	4
	L-lactate dehydrogenase A chain	P04642	Ldha	0	3	3
	Glycerol-3-phosphate dehydrogenase, mitochondrial	P35571	Gpd2	0	0	4
	Category: Signaling, transport and turn over					
	Hemoglobin subunit beta-1	P02091	Hbb	0 [a]	0 [a]	6 [b]
	Solute carrier family 2, facilitated glucose transporter member 3	Q07647	Slc2a3	9	18	14
	Protein MENT	Q5X162	Ment	6	8	6
	Voltage-dependent anion-selective channel protein 2	P81155	Vdac2	4	9	11
	Normal mucosa of esophagus-specific gene 1 protein	Q5RK28	Nmes1	4	6	4
	Voltage-dependent anion-selective channel protein 3	Q9R1Z0	Vdac3	2	9	6

Table 1. *continued*

	SPERM PROTEIN EXTRACT IDENTIFIED	PROT ID	GENE	CTR	TREATED	
					2H	1D
	Category: Stress response and antioxidant activity					
TREATED UP REGULATED	Glutathione S-transferase Mu 5	Q9Z1B2	Gstm5	13 [a]	39 [b]	26 [a]
	Cytosolic 5'-nucleotidase 1B	A0A0G2JX78	Nt5c1b	0 [a]	0 [a]	5 [b]
	Phospholipid hydroperoxide glutathione peroxidase, mitochondrial	P36970	Gpx4	26	32	21
	Redox-regulatory protein FAM213A	Q6AXX6	Fam213a	12	25	27
	Glutathione S-transferase omega-2	Q6AXV9	Gsto2	1	7	4
	Category: Cell recognition and Sperm-egg interaction					
	Histone H2A type 2-C	D4ACV3	Hist2h2ac	0 [a]	6 [b]	1 [a]
	Histone H4	P62804	Hist1h4b	0 [a]	15 [b]	4 [a]
	Hyaluronidase PH-20	Q62803	Spam1	0 [a]	11 [b]	23 [c]
	Ras-related protein Rab-2A	P05712	Rab2a	1 [a]	5 [a]	11 [b]
	Acrosin-binding protein	Q6AY33	Acrbp	0 [a]	6 [a]	14 [b]
	Calcium-binding tyrosine phosphorylation-regulated protein	M0R8P3	Cabyr	0 [a]	0 [a]	6 [b]
	Sperm surface protein Sp17	Q9Z1K2	Spa17	0	4	0

Different letters indicate statistical differences between groups ($p < 0.05$)

Table 2. Proteins that are present on treated group (after oxidative stress) and are absent on CTR group

SPERM EXTRACT PROTEIN	EPIDIDYMAL FLUID PROTEIN
10 kDa heat shock protein, mitochondrial	Alpha-1-inhibitor 3
78 kDa glucose-regulated protein	Alpha-1-macroglobulin
Acrosin-binding protein	Apolipoprotein A-I
A-kinase anchor protein 3	Apolipoprotein E
ATP synthase subunit alpha, mitochondrial	Ceruloplasmin
ATP synthase subunit delta, mitochondrial	Complement C3
Calcium-binding tyrosine phosp.-regulated protein	Complement C4
Clusterin	Haptoglobin
Cytochrome c oxidase subunit 5A, mitochondrial	Hemopexin
Cytochrome c oxidase subunit 6B2	Ig gamma-1 chain C region
Cytochrome c, testis-specific	Ig gamma-2A chain C region
Cytosolic 5'-nucleotidase 1B	Ig kappa chain C region, B allele
Glucose-6-phosphate 1-dehydrogenase	Pregnancy zone protein
Glycerol-3-phosphate dehydrogenase, mitochondrial	Superoxide dismutase [Cu-Zn]
Hemoglobin subunit beta-1	T-kininogen 1
Histone H2A type 2-C	T-kininogen 2
Histone H4	
Hyaluronidase PH-20	
Izumo sperm-egg fusion protein 1	
L-lactate dehydrogenase A chain	
Malate dehydrogenase, mitochondrial	
Phospholipid hydroperoxide glutathione peroxidase, mitochondrial	
Sperm surface protein Sp17	
Succinyl-CoA:3-ketoacid coenzyme A transferase 2A, mitochondrial	
Zona pellucida-binding protein 1	

Table 3. Amount of epididymal fluid spectra proteins that presented down and upregulation when the CTR group was compared with the times 2h, D1, D5, D7 and D10 of treated group.

	EPIDIDYMAL FLUID PROTEINS IDENTIFIED	PROTEIN ID	GENE	CTR	TREATED				
					2H	1D	5D	7D	10D
TREATED DOWNREGULATED	**Category: Movement and structural organization**								
	Tubulin beta chain (*Sus scrofa*)	P02554	-	25	1*	0*	0*	2*	0*
	Tubulin beta-4A chain	Q9D6F9	Tubb4a	20	3*	2*	0*	3*	0*
	Tubulin beta-6 chain (*Mus muscullus*)	Q922F4	Tubb6	14	0*	0*	0*	0*	0*
	Tubulin alpha-1B chain	Q6P9V9	Tuba1b	11	0*	0*	0*	0*	0*
	L-lactate dehydrogenase C chain	P19629	Ldhc	6	0*	0*	0*	0*	0*
	Keratin, type II cytoskeletal 6A	Q4FZU2	Krt6a	5	0*	0*	0*	3	0*
	Keratin, type II cytoskeletal 1	Q6IMF3	Krt1	4	0*	0*	0*	0*	0*
	Carboxylesterase 5A	Q5GRG2	Ces5a	44	32	25	0*	15*	0*
	Category: Energy and metabolism								
	Carbonyl reductase [NADPH1]	P47727	Cbr1	122	54*	35*	21*	103*	29*
	Creatine kinase B-type	P07335	Ckb	8	4	4	4	4	5
	Aldose reductase-related protein 2 (*Mus muscullus*)	P45377	Akr1b8	3	0	0	2	2	0
	Phosphatidylethanolamine-binding protein 1	P31044	Pebp1	95	17*	13*	0*	13*	0*
	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	Q9ESV6	Gapdh	20	9*	4*	0*	9*	5*
	Phosphoglycerate mutase 2	P16290	Pgam	17	6*	5*	0*	2*	0*
	Glucose-6-phosphate isomerase	Q6P6V0	Gpi	11	0*	4*	0*	3*	0*
	Aldo-keto reductase family 1 member B10	Q6AY99	Akr1b10	7	0*	0*	2*	2*	0*
	Glyceraldehyde-3-phosphate dehydrogenase, muscle	P04797	Gapdh	13	8	4*	0*	6	3*
	Fructose-bisphosphate aldolase A	P05065	Aldoa	12	7	0*	0*	2*	0*
	Phosphoglycerate kinase 2 (*Sus scrofa*)	Q6RI85	PGK2	48	38	35	0*	1*	0*
	Malate dehydrogenase, cytoplasmic	O88989	Mdh	4	1	1	0	3	0
	Alpha-enolase	P04764	Eno1	4	1	0	2	2	0
	Fatty acid-binding protein, epidermal O	P55053	Fabp5	2	3	2	3	5	0

Table 3. *continued*

	EPIDIDYMAL FLUID PROTEINS IDENTIFIED	PROTEIN ID	GENE	CTR	TREATED				
					2H	1D	5D	7D	10D
TREATED DOWNREGULATED	**Category: Signaling, transport and turn over**								
	Elongation factor 1-alpha 1	P62630	Eef1a1	3	0	0	0	0	0
	Epididymal-specific lipocalin-5	P06911	Lcn5	48	19*	14*	0*	23	0*
	Protein deglycase DJ-1	O88767	Park7	34	12*	8*	0*	0*	0*
	Prostaglandin reductase 1	P97584	Ptgr1	21	0*	0*	2*	16	0*
	Prostaglandin-H2 D-isomerase	P22057	Ptgds	12	0*	0*	0*	0*	0*
	Catechol O-methyltransferase	P22734	Comt	4	1	0	0	1	0
	14-3-3 protein zeta/delta	P63102	Ywhaz	9	2	0*	0*	0*	0*
	Category: Stress response and antioxidant activity								
	Cysteine-rich secretory protein 1	P12020	Crisp1	59	49	41	0*	12*	0*
	Glutathione S-transferase Mu 1	P04905	Gstm1	47	8*	2*	7*	19*	6*
	Glutathione S-transferase Mu 7	A0A0G2K4U2	Gstm7	16	0*	0*	0*	0*	0*
	Glutathione S-transferase Mu 3	Q9WU21	Gstm3	2	0	0	0	0	0
	Heat shock cognate 71 kDa protein	P63018	Hspa8	7	0*	0*	0*	5	0*
	78 kDa glucose-regulated protein	P06761	Hspa5	7	0*	0*	0*	0*	0*
	Category: Cell recognition and Sperm-egg interaction								
	Actin, cytoplasmic 1	P60711	Actb	44	21	8*	13	34	29
	Actin, alpha cardiac muscle 1	P68035	Actc1	27	17	8*	10	26	24
	Category: Inflammatory response								
	Fibrinogen alpha chain	P06399	Fga	8	0*	0*	0*	0*	0*
	Fibrinogen beta chain	P14480	Fgb	8	0*	0*	0*	0*	0*
	Aspartyl aminopeptidase	Q9Z2W0	Dnpep	3	0	0	0	0	0
TREATED UPREGULATED	**Category: Signaling, transport and turn over**								
	Apolipoprotein A-I	P04639	Apoa1	0	0	0	0	2	2
	Apolipoprotein E	P02650	Apoe	0	2	0	2	0	5*
	Clusterin	P05371	Clu	8	2	3	0*	20*	6
	POTE ankyrin domain family member F	**RGD	Potef	12	11	7	6	14	13
	Category: Stress response and antioxidant activity								
	Ceruloplasmin	P13635	Cp	0	0	0	6*	2	0
	SH3 domain-binding glutamic acid-rich-like protein 3	B2RZ27	Sh3bgrl3	2	3	3	4	13*	4
	Superoxide dismutase [Cu-Zn]	P07632	Sod1	0	0	0	0	4*	0

Table 3. *continued*

	EPIDIDYMAL FLUID PROTEINS IDENTIFIED	PROTEIN ID	GENE	CTR	TREATED				
					2H	1D	5D	7D	10D
	Category: Inflammatory response								
TREATED UPREGULATED	Hemoglobin subunit alpha-1/2	P01946	Hba1	19	29*	41*	42*	19	38*
	Hemoglobin subunit beta-1	P02091	Hbb	17	38*	55*	43*	17*	31*
	Serotransferrin	P12346	Tf	10	24*	35*	53*	27*	55*
	Ig gamma-2A chain C region	P20760	Igg-2a	0	11*	15*	21*	21*	15*
	Hemopexin	P20059	Hpx	0	6*	6*	20*	9*	10*
	Complement C3	P01026	C3	0	4*	5*	27*	1*	1*
	Hemoglobin subunit beta-2	P11517	-	10	9	18*	15*	6	14*
	T-kininogen 2	P08932	-	0	0	10*	43*	12*	18*
	T-kininogen 1	P01048	Map1	0	0	8*	33*	10*	15*
	Alpha-1-inhibitor 3	P14046	A1i3	0	3	6*	12*	0	9*
	Ig gamma-1 chain C region	P20759	Ighg	0	2	5*	14*	0	5*
	Haptoglobin	P06866	Hp	0	0	4*	16*	5*	6*
	Alpha-2-HS-glycoprotein	P24090	Ahsg	3	2	6	13*	3	10*
	Alpha-1-macroglobulin	Q63041	A1m	0	0	1	5*	10*	2
	Peptidyl-prolyl cis-trans isomerase A	P10111	Ppia	5	3	0	1	17*	7
	Ig kappa chain C region, B allele	P01835	-	0	0	0	2	1	4*
	Hemoglobin subunit beta (*Balaenoptera acutorostrata*)	P18984	HBB	7	6	7	8	4	6
	Hemoglobin subunit beta-C (*Ammotragus lervia*)	P68058		7	6	7	8	4	6
	Hemoglobin subunit epsilon (*Lagothrix lagotricha*)	P51440	HBE1	7	6	7	8	4	6
	Macrophago migration inhibitor factor	P30904	Mif	6	8	7	4	7	2
	Pregnancy zone protein	**RGD:628643	Pzp	0	0	0	2	1	0
	Complement C4	P08649	C4	0	0	0	2	0	0

For variables with asterisk (*) the difference is statistically significant when compared to CTR group.
** RGD IDENTIFICATION (Rat Genome Database). Not found at UNIPROT

DISCUSSION

To our knowledge, there is no study that has previously compared the proteomics of sperm protein extract and epididymal fluid pre- and post-testicular induced inflammatory reaction in rats (immediate analysis). Thus, this study identified and quantified proteins present in sperm protein extract and epididymal fluid before and during acute orchitis. We have also discussed some recently discovered functions of sperm extracts proteins, and the relationship with other proteins (supplementary table S1).

In this study, the inflammatory testicular reaction was caused by a sclerosing agent, already tested in other species (Paranzini et al., 2018; Pereira et al., 2018; Silva et al., 2018), to promote chemical sterilization, which causes intense local inflammation (epididymo-orchitis) as an adverse effect. The testicular inflammation was previously described and detected by infrared thermography in cats (Paranzini et al., 2018; Paranzini et al., 2019), or by studying the clinical signs, seminal characteristics and histological changes in rats, bulls, donkeys and dogs (Ibrahim, Ali, Abou-khalil, & Ali, 2016; Jana & Samanta, 2006; Leoci et al., 2014; Pereira et al., 2018).

Although the experimental design had fixed the sperm sample collection, at 2h, D1, D5, D7 and D10 from the treated groups, from D5 onwards a drastic decrease in sperm production was observed. This drastically reduced the amount of sperm cells and, unfortunately, not enough cells were available for proteomic analysis. Therefore, only the 2h and D1 time points from treated groups were considered for proteomic studies of sperm protein extract. However, from epididymal fluid, measurements were conducted normally as described above (2h, D1, D5, D7 and D10).

Sperm characteristics were evaluated; however, the data is not discussed in this article. For illustration, the results were described in another study (Paranzini et al., 2018b), which described the clinical and reproductive characteristics. Sperm cell characteristics presented as oligo-astheno-teratozoospermia parameters from 2h to D4

and oligo-necro-teratozoospermia from D5 to D10. All males from the control group were fertile at D100 after NaCl 0.9% intratesticular injection (Paranzini et al., 2019 – article submitted).

Our results for sperm protein extracts are in accordance with previous human and boar studies (Pixton et al., 2004 and Kwon et al., 2015, respectively) in which infertile patients showed a higher number of proteins expressed when compared to normal fertile controls. Interestingly in our study, sperm protein extract samples, proteins involved in movement and structural organization, such as keratin, type I cytoskeletal 19 (Q6AYX5), outer dense fiber protein 2 (Q6AYX5) and others (see table 1), were downregulated in treated groups (2h and D1) and corroborate with literature. In contrast, the A-kinase anchor protein 4 (O35774), tubulin beta-4B chain (B4F7C2), tubulin beta-4A chain (Q6P9T8) and tubulin beta-6 chain (Q4QQV0) were upregulated in treated groups. Usually these proteins are often described to be downregulated in asthenozoospermic and infertile semen samples. The protein downregulated, keratin , outer dense fiber are structural. The other ones (as A-kinase, Tubulin and so on), require tyrosine phosphorylation, a post-translational modification necessary for sperm hyperactivation, to activate sperm motility. Typically, in infertile men, tyrosine phosphorylation is displayed at lower levels and the upregulation of these proteins in this study, may be compensatory.

Recently, it was proposed that a downregulation of either subunits of the ATP complex results in decreased sperm motility. Thus, immotile sperm have a lower expression of ATP (Colagar, Karimi, & Jorsaraei, 2013), in contrast to the observed in this study. The proteins responsible for maintaining cellular energy and metabolism, such as aldehyde dehydrogenase, mitochondrial (P11884), Alpha-enolase (P04764) and others were downregulated, but the majority (16/25) as ATP (P15999), Hezokinase-1 (P17710)

and others (see Table 1), were upregulated in sperm protein extracts from treated groups. Furthermore, a study indicated that immotile sperm are still capable of generating ATP, but not of utilizing it for forward motility. The high ATP concentration may also be a reflection of the inability of certain motile sperm to effectively utilize available ATP for motility (Du Toit, Bornman, Van Der Merwe, Du Plessis, & Oosthuizen, 1993). Recently, a compensatory effect was suggested. When oxygen became scarce due to the inflammatory reaction, in order to maintain energy production an anaerobic glycolysis metabolism is used to generate ATP. Since the mechanism of anaerobic glycolysis is less efficient than aerobic glycolysis, the amount of energy and metabolic protein may be elevated to enhance the rate of glycolysis (Valvona, Fillmore, Nunn, & Pilkington, 2016). Furthermore, *Gapdhs* (Q9ESV6) is an immunogenic protein and was suggested as a potential target for immunocontraception and as a biomarker for fertility (Suryawanshi, Khan, Gajbhiye, Gurav, & Khole, 2011). Contradictorily to which was already publish, in this study, the level of *Gapdhs* is raised in the sperm protein extracts of treated groups (infertile sample). Therefore, in this case, it is not indicated as a fertility predictor following.

Suryawanshi et al. (2011) (Suryawanshi et al., 2011)(Suryawanshi et al., 2011)(Suryawanshi et al., 2011) and Hashemitabar et al. (2015) described an upregulation of Isoaspartyl peptidase/L-asparaginase (Aslgr1) in asthenozoospermic semen samples and proposed ASRGL1 epitopes as an infertility biomarker and candidate for contraceptive purposes. However, they also suggested additional studies to elucidate the exact location and role of ASRGL1 in sperm (Hashemitabar et al., 2015). Interestingly, in this study, the value of Aslgr1 did not differ between groups and, therefore, does not have value for diagnosing fertility/infertility.

Furthermore, in our results, Hspe1 and Hspa5 were upregulated in sperm protein extracts from treated groups. However, in epididymal fluid from treated groups, Hspa8 was downregulated. Hspe1, Hspa5 and Hspa8 belong to the heat shock protein family (HSP) and are part of mammalian cell strategies to adapt to environmental changes, responding to heat, oxidative stress, glucose and oxygen deprivation and their levels raise to confer a protective function in sperm cells (Subjeck & Shyy, 2017). Two other enzymes with similar functions were observed in our study: Glutathione S-transferase omega-2 (Q6AXV9) was upregulated in sperm protein extracts from treated groups whereas Cluster of Glutathione S-transferase *Mu* (P04905) was downregulated in epididymal fluid from treated groups. The high production of antioxidant proteins is associated to a compensating effect due an increase in ROS production(Kwon et al., 2015). Usually, subfertile/infertile in males has a high production of ROS and these finding corroborate with literature (Kwon et al., 2015). Furthermore, calcium ionophores and other agents are also recognized to induce a rise in these stress proteins (Subjeck & Shyy, 2017). Regarding the down expression observed in the epididymal fluid of treated groups, this suggests that the pathophysiological mechanisms involved in heat stress and its dysfunctional consequences may have surpassed the protective action on cellular autoregulation (Lima et al., 2006).

Although, the presence of ROS is often considered negative, physiological levels are necessary to trigger tyrosine phosphorylation and initiate the cascade that induces capacitation and the acrosome reaction (Sikka, 1996). The high ROS quantity generated by the inflammatory reaction activates tyrosine phosphorylation by the protein kinases (PTK) at a higher level, leading to capacitation and the acrosome reaction. Furthermore, we infer that, in an attempt to avoid capacitation, alpha-2-HS-glycoprotein O (P24090),

which encodes PTK inhibitor, was over-expressed in the epididymal fluid of treated groups.

Another indication of this fact is that most of the proteins involved in capacitation and the acrosome reaction (the ones classified in the cell recognition and sperm-egg interaction category) are upregulated in the sperm protein extract of treated groups. According to Kwon et al. (2015) Acrosin-binding protein (Q6AY33) and Ras- related protein (P05712) hypothetically control male fertility through interactions with a variety of factors. In fact, elevated ROS levels have been linked to release of cytochrome C (P10715) that is upregulated in sperm protein extracts of treated groups. Cytochrome activates apoptotic reactions, and is increased in patients with male factor infertility (Wang et al., 2009).

The protein Clusterin (P05371), which can be found in seminal fluid and on plasma membranes, was upregulated in treated groups (both in sperm protein extracts and epididymal fluid), in accordance with other reported findings. For example, Clusterin has higher expression in sperm with low motility (Hashemitabar et al., 2015), and in oligospermic samples (Sharma, Agarwal, Mohanty, Du Plessis, et al., 2013; Sharma, Agarwal, Mohanty, Jesudasan, et al., 2013) as compared to control groups. However, further studies are necessary to clarify the upregulation, since it correlates positively with motility and maturity (Ibrahim, Gilbert, Loseth, & Crabo, 2000).

In this study, we observed that cluster of protein deglycase levels (DJ-1, also knowns in human as SP22) are downregulated in epididymal fluid from treated groups and absent from D5 to D10 samples. DJ-1 is an antioxidant protein that prevents oxidative stress reactions to avoid sperm cell damage (Yasuda et al., 2013). A reasonable explanation is that the ROS produced by the immediate inflammatory reaction consumed all the DJ-1 available, and the physiological disruption caused by the testicular

inflammatory reaction inhibits its further expression. With the absence of protective proteins and rising levels of ROS, it is expected that sperm cell damage increases (Hosseinifar et al., 2013). Other studies correlate low or an absence of DJ1 expression in asthenozoospermic patient (Hosseinifar et al., 2013; Wang et al., 2009). When the spermatozoa reaches the epididymis, it is largely devoid of cytoplasm and heavily dependent on the extra-cellular milieu provided by the epididymis for protection from various kinds of attack, including oxidative stress (Aitken et al., 2007). Epididymal fluid is an important source of antioxidants in semen (Zini & Agarwal, 2018) and contains unique proteins necessary for sperm function and survival that play a variety of roles such as protecting the sperm and supporting capacitation, acrosome reaction and sperm-egg fusion. It also modulates the immune response in male and female reproductive tracts, ensuring that the most competent spermatozoa meet the oocyte during fertilization (Sharma, Agarwal, Mohanty, Jesudasan, et al., 2013). Likewise, most of the proteins upregulated in epididymal fluid from treated groups are related to the inflammatory phases. These are upregulated already at 2h, with a progressive increase until D10. Therefore, an up-expression of inflammatory proteins was expected in this study.

In epididymal fluid, some proteins suggested by other researchers as fertility biomarkers, were downregulated in treated groups (infertile samples) in this study. This reinforces their possible role as fertility biomarkers and as immunocontraceptive targets. These proteins include the Tubulins (Moura & Memili, 2016), Enolase, Cysteine-rich secretory protein (Dacheux, Dacheux, & Druart, 2016) and SP22 (Klinefelter, Laskey, Ferrell, Suarez, & Roberts, 1997). Furthermore, we observed that some epididymal fluid proteins, such as Albumin, L-lactate dehydrogenase C chain, Glyceraldehyde-3-phosphate dehydrogenase and Phosphoglycerate kinase 2 presented an upregulated pattern after the testicular inflammatory reaction. An interesting study (Intasqui, Agarwal,

Sharma, Samanta, & Bertolla, 2018) suggested several of these proteins as infertility biomarkers of diverse factors. Other upregulated proteins are involved in the inflammatory process, mainly in the acute phase, and thus indicate that the inflammatory process is in progress.

The differences between this study and other proteomic studies published with asthenozoospermic/infertile models, is the time in which seminal protein samples are analyzed, which is usually after remission of clinical signs or after treatment (late analysis) (Hashemitabar et al., 2015; Huang et al., 2018; Ko, 2014; Kwon et al., 2015; Pixton et al., 2004; Siva et al., 2010). This study evaluated sperm protein extracts and epididymal fluid within 24 hours after injury. Thus, amounts of proteins that have been reported to be downregulated in asthenozoospermic/infertile models, including humans, are upregulated. The compensatory effect, as already mentioned, is a reasonable explanation for the difference between this study and others.

Even testicular oxidative stress seems to be a common feature in the pathophysiology of male infertility (Turner & Lysiak, 2008), still, there are many more points to be studied regarding the understanding and prevention of male infertility; however, studies like this generate new insights into biochemical pathways that are possibly associated with infertility. Additionally, this study linked the expression of seminal proteins with fertility in rats. Consequently, it also assists the development of fertility/infertility biomarkers and future development of contraceptive methods.

The ability to predict male fertility is of paramount importance for animal breeding industries and for human reproduction (Kwon et al., 2015). Seminal proteomic evaluation is essential to evaluate sperm functional competence, even in patients within the normal range of seminal parameters. This study successfully provided a description of quantity/baseline protein expression in epididymal fluid and sperm cell samples with

normal seminal parameters, and after initiation of an acute testicular inflammatory reaction caused by intratesticular injection of $CaCl_2$ 20% associated with 0.5% DMSO . In addition, the obtained data indicate the ability of proteomics analyses to detect proteins in epididymal fluid that are involved in the testicular inflammatory reaction, thus confirming orchitis.

Our vision for fertility/infertility biomarkers is that the establishment of a proteomic panel would give more accurate and reliable information. Furthermore, it would help in infertility treatment, since downregulated proteins can be collected from a fertile donor and added to the seminal plasma or to dilution medium of an infertile patient, for fertility reestablishment.

DECLARATION OF INTEREST

The authors have no competing interests to disclose.

AUTHOR'S ROLE

Paranzini, Camargo and Souza designed the study. Paranzini, Calman, Camargo, Yamada and Ruediger conducted the study. Paranzini, Calman and Scott processed proteomic samples. Paranzini interpreted proteomics analysis. Souza supervised the study and findings. Paranzini, Souza and Rizzoto drafted the early manuscript that was later critically appraised and improved by all authors.

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Supplementary Table S1. Proteins detected in sperm protein extract for CTR and treated group, biological function/process with importance on reproduction and mainly interactions proteins. The sperm protein extract are separated by categories.

SPERM PROTEINS EXTRACT	UNIPROT ID	GENE SYMBOL	PROTEIN CLASS*	FUNCTION/BIOLOGICAL PROCESS	PROTEIN INTERACTION **
CATEGORY: Movement and structural organization					
A-kinase anchor protein 3	Q66HC6	Akap3	Kinase activity	Sperm tail fibrous sheath component; motility control. Suppression of Akap phosphorylation impairs the induction of hyperactivated movement leading, ultimately, to a loss of fertility (Miki *et al.*, 2002)	Ropn1; Spa17; Tssk3
Cluster of A-kinase anchor protein 4	O35774	Akap4	Cilium biogenesis/ degradation	Major structural component of sperm fibrous sheath; flagellated sperm motility; associated with male infertility. It is a scaffold protein and without Akap4, transduction signal and glycolytic enzymes fail to become associated with the fibrous sheath, hence fail to regulate flagellar function. It is essential for sperm motility (Miki *et al.*, 2002)	Fsip2; Fsip1; Spag6; ENSRNOG00000025787; Tnp2; Ropn1; Tnp1
Clusterin	P05371	Clu	Chaperone	It have a role in the lipid exchange accompanying the remodeling of the sperm plasma membrane during sperm maturation (Griffiths *et al.*, 2009)	Cb8; Vegfa; App; Serping; Timp1
Keratin, type I cytoskeletal 9	Q8CIS9	Krt9		Mutant male mice for Krt9 expression can display sperm tail deformity and altered pattern motility (Rivkin *et al.*, 2005)	Krt77; Krt7; Krt8; Krt10; Krt5; Krt19; Kg23; Krt4; Krt75; Krt72
Keratin, type I cytoskeletal 10	Q6IFW6	Krt10		Protein keratin appears in the sperm as the ODFs are formed. It is one of the proteins that form one subclass of intermediate filaments in living cells (Lindemann and Kanous, 1989)	Krt77; Dmd; Krt15; Krt7; Krt5
Cluster of Keratin, type I cytoskeletal 19	Q63279	Krt19			
Keratin, type II cytoskeletal 8	Q10758	Krt8			

Supplementary Table S1. *continued*

Cluster of Keratin, type II cytoskeletal 1	Q6IMF3	Krt1		Regulate the activity of kinases such as PKC and SRC via binding to integrin beta-1 (ITB1) and the receptor of activated protein C kinase 1 (RACK1)	
Mitochondria-eating protein	Q6AYL6	Spata18		It has a role in spermiogenesis and, particularly, in maturation of spermatids into spermatozoa; In addition a portion of this protein is retained in spermatozoa as a flagellar component after spermiation, and might serve as a structural constituent in the flagella (Iida *et al.*, 2006; Kaneko *et al.*, 2010)	
Outer dense fiber protein 2	Q6AYX5	Odf2	Developmental protein	Major component of sperm tail outer dense fibers, is located on the outside of the axoneme in the midpiece and principal piece of the mammalian sperm tail, and provide tensile strength that is necessary to protect the sperm tail against shearing forces encountered during epididymal transport and especially during ejaculation. It modulate sperm motility and is important for sperm morphology and function (Petersen *et al.*, 1999); The lack of Odf2 is associated with axoneme defects.	Cntrl; Cep164 ;Pcnt; Ccp110; Cep83; Cep97; Cep135; Cep89; Fbf1; Sclt1
Ropporin-1	Q4KLL5	Ropn1		Important for male fertility. With ROPN1L, involved in fibrous sheath integrity and sperm motility, plays a role in PKA-dependent signaling processes required for spermatozoa capacitation	Rhpn1; Akap3; **Cabyr;** Spa17; Asrgl1; Akap4; Fsip1; Acrbp; Prkar2a
Tubulin alpha-3 chain O	Q68FR8	Tuba3a	Tubulin	Major constituent of microtubules	Tubb5; Tubb3; Tubb2a; Tubb4b; Tubb4a; Tubb1; Tubb6
Cluster of Tubulin beta-4B chain	Q6P9T8	Tubb4b	Tubulin	Major constituent of microtubule	Tubb5; Tubb4; Tubb1a; Ckap5; Mapre1; Tubgcp2; Tubgcp2; Tubgcp6

Supplementary Table S1. *continued*

CATEGORY: Energy and metabolism					
Aldehyde dehydrogenase, mitochondrial	P11884	Aldh2	Oxidoreductase	Protect the mitochondria during cell respiration and in the maintenance of sperm motility (Gibb *et al.*, 2016)	
Aspartate aminotransferase, mitochondrial	P00507	Got2	Transaminase enzyme	Involved in aspartate metabolic process; cellular amino acid metabolic process; dicarboxylic acid metabolic process; Participates in gluconeogenesis pathway	Ldhc
ATP synthase subunit alpha, mitochondrial	P15999	Atp5a1	ATP synthase	ATP metabolic process (binding and synthase)	Atp5b; Atp5d
ATP synthase subunit beta, mitochondrial	P10719	Atp5b	ATP synthase	ATP metabolic process	
ATP synthase subunit delta, mitochondrial	P35434	Atp5d	ATP synthase	ATP metabolic process	
Carbonyl reductase [NADPH] 1	P47727	Cbr1	Oxidoreductase	Steroid catabolic process. Participates on sperm capacitation (Botto *et al.*, 2010)	
Cluster of Alpha-enolase	P04764	Eno1	Lyase	Glycolitic pathway, can interact can interact with tubulin and microtubules and is distributed mostly in the tail of cauda epididymal rat sperm (Ijiri *et al.*, 2011)	
Cluster of L-lactate dehydrogenase C chain	P19629	Ldhc	Oxidoreductase	Glycolysis pathway; Flagellated sperm motility; Male infertility. Is responsible for most of the lactate dehydrogenase activity in sperm. With the lack of Ldhc, the sperm do not acquire hyperactivate motility, and is unable to penetrate the zona pellucida in vitro, and faile to undergo the phosphorylation events characteristic of capacitation. It's most abundant on principal piece (Odet *et al.*, 2011)	
Cytochrome c oxidase subunit 5A, mitochondrial	P11240	Cox5a	Oxidase	Oxidative phosphorylation pathway	
Cytochrome c oxidase subunit 6B2	Q6YFQ1	Cox6b2	Oxidase	Oxidative phosphorylation pathway	

Supplementary Table S1. *continued*

Cytochrome c, testis-specific	P10715	Cyct	Signaling molecule	Positive regulation of intrinsic apoptotic signaling pathway. ATP synthesis	
Fatty acid-binding protein 9	P55054	Fabp9	Lipid binding	Protein that exhibits lipid binding Involved in acrosome assembly. Citoplasmatic and acrossomal proteins (Chmurzyńska, 2006)	Mtl5; Akap4; Ropn1
Glucose-6-phosphate 1-dehydrogenase	P05370	G6pd	Dehydrogenase	Main function of this enzyme is to provide reducing power (NADPH) and pentose phosphates for fatty acid and nucleic acid synthesis, but participate on cellular response to oxidative stress	Hk2
Glutamine synthetase	P09606	Glul	Ligase	Metabolic process - provide a precursor for nucleic acid synthesis (Kvidera and Carey, 1994)	
Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	Q9ESV6	Gapdhs	Dehydrogenase	Flagellated sperm motility; glycolytic process. Required for sperm motility and male fertility	
Glycerol-3-phosphate dehydrogenase 2, mitochondrial	P35571	Gpd2	Dehydrogenase	Sperm Motility and Hyperactivation. GPD2 is involved in sperm capacitation (Kota *et al.*, 2010)	
Hexokinase-1	P05708	Hk1	Kinase Transferase	Catabolic process; ATP activity; response to ischemia	
Malate dehydrogenase, mitochondrial	P04636	Mdh2	Oxidoreductase	Involved in malate metabolic process; NADH metabolic process; oxaloacetate metabolic process; Participates in citric acid cycle pathway	Cs; Fh; Mdh1; Pc; Got2; Got1; Me2; Me1; Cyc1; Me3
Phosphatidylethanolamine-binding protein 1	P31044	Pebp1		ATP binding; kinase binding; mitogen-activated protein kinase binding	
Prostaglandin G/H synthase 2	P35355	Ptgs2	Oxigenase	Is involved in the pathway prostaglandin biosynthesis, which is part of lipid metabolism	
Saccharopine dehydrogenase-like oxidoreductase	Q6AY30	Sccpdh	Oxidoreductase activity	Glycolipid biosynthetic process	Act1
Succinyl-CoA:3-ketoacid coenzyme A transferase 2A, mitochondrial	Q5XIJ9	Oxct2a	Transferase	Ketone bodies metabolic pathway; found in mitochondrion and probably play and important roles in the energy metabolism of spermatozoa	

Supplementary Table S1. *continued*

Triosephosphate isomerase	P48500	Tpi1	Isomerase	Gluconeogenesis; carbohydrate biosynthesis	Gpi; Aldoa; Aldoa2; Aldoc; Aldob; Gapdh; Pgk1; Pgk2; Eno1; RGD1560402
CATEGORY: Signaling and transport					
Hemoglobin subunit beta-1	P02091	Hbb	Oxidoreductase activity	Oxygen transport. Regulator of pain and inflammation (EBI, 2019)*	
Isoaspartyl peptidase/L-asparaginase	Q8VI04	Asrgl1	Metabolic process	The ASRGL1 epitopes act as autoimmunity antigens and may impair fertility. This protein was found to be upregulated in ashenozoospermic human sperm among with CLU e KRT1 (Hashemitabar *et al.*, 2015; Cao *et al.*, 2018)	Ropn1; Aspg; Rcc2
Normal mucosa of esophagus-specific gene 1 protein	Q5RK28	Nmes1			
Protein MENT	Q5XI62	Ment		Involved in control of cellular proliferation	Ccin; Ar; Gnrh1; Rps16; Mnt
Ras-related protein Rab-2A	P05712	Rab2a	Transport	Required for protein transport from the endoplasmic reticulum to the Golgi complex. Rab family mediated signaling pathway. Is involved in acrosome formation and regulates vesicular transport and membrane fusion (Kwon *et al.*, 2015)	Rab14; Rab11a; Rab5b; Gorasp2; Rab18; Rab6a; Blzf1; Rab6b; Rab11b; Rab36
Solute carrier family 2, facilitated glucose transporter member 3	Q07647	Slc2a3		Participates in facilitative sugar transporter mediated glucose transport pathway	Hk2
Voltage-dependent anion-selective channel protein 2	P81155	Vdac2	Hezokinase	Exhibits voltage-gated anion channel activity; Involved anion transport; binding of sperm to zona pellucida	
Voltage-dependent anion-selective channel protein 3	Q9R1Z0	Vdac3		Voltage-gated anion channel activity; Participate in calcium/calcium-mediated signaling pathway	

Supplementary Table S1. *continued*

CATEGORY: Stress response and antioxidant activity					
10 kDa heat shock protein, mitochondrial	P26772	Hspe1	Chaperone	Its expression was identified over the apical domain of the sperm acrosome in capacitated mouse spermatozoa. There is an association with ATP binding, but anti-HSPE1 is able to suppress sperm-zona pellucida interaction in a significant dose-dependent manner, without compromising either sperm viability or motility (Walsh *et al.*, 2008)	Hspe9; Hspd1
78 kDa glucose-regulated protein O	P06761	Hspa5	Chaperone		
Cluster of Phospholipid hydroperoxide glutathione peroxidase, mitochondrial	P36970	Gpx4	Peroxidase	Essential antioxidant peroxidase; Required for normal sperm development and male fertility. The lack of this protein was identified in idiopatic asthenozoospermic human male (Vernet *et al.*, 2004; Nayak *et al.*, 2019)	
Cytosolic 5'-nucleotidase 1B	A0A0G2JX78	Nt5c1b	Adenosine metabolic process	Helps to regulate adenosine levels. Autoimmune infertility-related protein	
Glutathione S-transferase Mu 5	Q9Z1B2	Gstm5	Transferase	Glutathione transferase activity; protein homodimerization activity	Gstm7; Gstm4
Glutathione S-transferase omega-2	Q6AXV9	Gsto2	Oxidoreductase Transferase	Antioxidant activity; oxidoreductase activity	Gstt1; Gstm1; Gstm2; Gstp1; Gsta2; Gstm7; Gsta1
Redox-regulatory protein FAM213A	Q6AXX6	Fam213a	Antioxidant	Involved in redox regulation of the cell. Acts as an antioxidant	Hsp90ab1; Gusb; Actb; Gapdh
CATEGORY: Cell recognition and Sperm-egg interaction					
Acrosin-binding protein	Q6AY33	Acrbp	Proteinase	Sperm capacitation. Proacrosin/acrosin binds non-enzymically to ZP glycoproteins an enable penetration begins (Howes and Jones, 2002) Its absence or reduce activity may cause male infertility (Zalata *et al.*, 2004)	Acr; Izumo1; Ropn1; Zpbp

Supplementary Table S1. *continued*

Calcium-binding tyrosine phosphorylation-regulated protein	M0R8P3	Cabyr		Involved on sperm Capacitation. Associated to male infertility. Pyruvate hydrogenase (Nayak *et al.*, 2019)	Fscb; Ropn1l; Akap3; Ropn1; ENSRNOG00000022406; Spa17; Zpbp2; Tcp11l2; Fsip1
Cluster of Actin, cytoplasmic 1	P60711	Actb	Actin	ATP binding; protein kinase binding; regulate gene transcription and motility and repair of damaged DNA. Involved in capacitation phosphorylation (Nayak *et al.*, 2019)	
Histone H2A type 2-C	D4ACV3	Hist2h2ac	Histone		Hist2h2be; Hist1h2bo; Hist1h2bf; Hist2h2ab; LOC690131; Hist1h2bk; Hist1h2ba; Hist3h2bb; Hist3h2ba; Hist1h2ao
Histone H4	P62804	Hist1h4b	Histone	Transcription regulation, DNA repair DNA replication and chromosomal stability. Involved in the process of chromatin condensation during transformation of spermatozoa in the epididymis (Marushige and Marushige, 1975; Chapman and Michael, 2003)	Hist1h3f; H3f3c; Hist1h3c; Hist1h2bk; ENSRNOG00000050098; Hist1h2bh; Hist1h2ai; Hist1h2an; Hist3h2ba; Crebbp
Hyaluronidase PH-20	Q62803	Spam1	Hydrolase	Involved in sperm-egg adhesion; aids in penetrating the layer of cumulus cells by digesting hyaluronic acid	Arsb; Gusb; Idua; Adam2; Spesp1; Acrv1; Zp3r; Adam7; Adam3; Rnf123

Supplementary Table S1. *continued*

Izumo sperm-egg fusion protein 1	Q6AY06	Izumo1	Protein binding	Essential sperm cell-surface protein required for fertilization by acting as a ligand for IZUMO1R/JUNO receptor on egg	Cd9; Izumo3; Adam2; Tss6; ENSRNOG00000045902; Izumo2; Acrbp; Clgn; Izumo4
Sperm surface protein Sp17	Q9Z1K2	Spa17	calmodulin binding	Function in binding zona pellucida. Removal of surface-associated proteins from incapacitated spermatozoa plasma membrane lead to an immediate increase in fertilizing ability, as determined by a rise in the proportion of fertilized eggs (Hernández-Silva and Chirinos, 2019)	Akap14; Spesp1; Crisp4; Ropn1|; Igsf8; Ropn1; Efcab7; Adam15; Akap3; Cabyr;
Zona pellucida-binding protein 1	Q6AXU2	Zpbp		Binding sperm to zona pellucida; integral component of membrane	Acrbp; Zp1; Odf3; Lcn9; Acr; Rbm44; Hesx1; Tex11; Tekt2; Rgs3

All the information above were extracted from UNIPROT database, Panther Classification System and Rat Genome Database (Access January, 2019), otherwise, reference is cited / *PROT. (protein) / **PROT. INT (Interaction with protein, according to STRING: functional protein association networks, with reproduction interest).

CAPÍTULO III

CONSIDERAÇÕES FINAIS

Esta revisão de literatura teve como objetivo central resumir brevemente o funcionamento do sistema reprodutivo masculino interno e os possíveis mecanismos envolvidos na infertilidade masculina após a injeção intratesticular do $CaCl_2$, que está sendo utilizado em pesquisas de controle populacional em massa de cães e gatos errantes. Com isso, considera-se que o objetivo principal desta revisão, foi alcançado.

Com relação aos objetivos secundários, a revisão de literatura foi consistente com o tema que incluiu conceitos importantes da patofisiologia da reprodução do macho, mecanismos da inflamação testicular e consequências sobre a fertilidade, do método de diagnóstico da orquite empregado neste estudo, a importância do estudo molecular e perspectivas futuras para o controle populacional diante dos avanços tecnológicos.

Com toda esta pesquisa de doutaramento, foi possível identificar e entender melhor as implicações clínicas imediatas da injeção intratesticular do $CaCl_2$ 20% associado com DMSO 0,5% no testículo, sobre a célula espermática e sobre a fertilidade de ratos. Também foi importante no apontamento dos efeitos tóxicos imediatos do composto químico na célula espermática. Outro ponto importante do estudo foi em relação a alteração proteômica da célula espermática e fluído epididimal após a inflamação testicular aguda.

Espera-se que esse estudo tenha contribuído para o melhor entendimento dos mecanismos que influenciam a alteração da capacidade fertilizante do espermatozoide e que no futuro, estudos como estes permitam o desenvolvimento de técnicas diagnósticas precoces da inflamação testicular reduzindo impacto econômico e no desenvolvimento de métodos contraceptivos mais efetivos.