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Biomodelos e simuladores: da pesquisa ao ensino

Andreia Nobre Anciuti

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Dedicatória

“Dedico esta tese aos meus pais, meus irmãos e à minha vó que sempre estiveram ao meu lado, comemorando e trocendo por mim”

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Resumo

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O período de doutoramento é um momento em que o futuro doutor deve experimentar os três pilares da educação: pesquisa, extensão e ensino. A pesquisa com fitoterapia se destaca no cenário científico, porém muitos produtos não têm sua eficácia e segurança validadas. Neste sentido, foram estudados os efeitos nos parêmetros espermáticos do uso tópico de pomada de óleo de copaíba (OC), usada para o tratamento de lesões inflamatórias na pele. Animais tratados com OC a 0,01% e 0,1% por 7 dias aumentaram o número de células com membranas lesadas (79,6% e 68,9%) quando comparados ao grupo controle (43%). Após 14 dias (D14) de tratamento, foi observado um aumento na porcentagem de células com DNA lesado nos grupos tratados com copaíba a 0,01% (27,8%) e 0,1% (23,7%) comparados ao grupo controle (0%). Mostrando que uso tópico de OC pode diminuir a qualidade espermática no período e nas concentrações estudadas. No entanto, a identificação precoce da toxicidade de novas substância através do desenvolvimento de métodos alternativos a uso de animais é uma busca constante, norteada pelos Princípios do 3R's. Foi proposto um ensaio de triagem para identificação de toxicidade de diferentes doses de ATP com célula espermática suína comparando com células da linhagem ZFL, pela avaliação de cinética, estrutura e metabolismo celular. Os espermatozoides da espécie suína demonstraram uma alta sensibilidade aos efeitos tóxicos do agente testado, assim foi possível detectar alterações de membrana plasmática e de produção de espécies reativas de oxigênio, dados que não foram encontrados nas avaliações da linhagem celular. No âmbito da educação, o desenvolvimento do projeto de ensino "Produção de recurso didático alternativo para o curso de Medicina Veterinária", foi elaborado de um manequim canino capaz de tornar possível a palpação abdominal e vaginal de uma fêmea gestante. Esse material enriqueceu as aulas práticas da disciplina de Obstetrícia e Glândula Mamária do curso de Medicina Veterinária, proporcionando maior segurança para os alunos realizarem a manobra em um futuro paciente. Sendo uma alternativa viável ao uso de animais. Quanto à atividade de extensão universitária, o I Ciclo de Palestras em Pré-Natal de Pequenos Animais, teve como principal objetivo disseminar e propagar informações dos mais diferenciados temas acerca da obstetrícia em pequenos animais e como superar os obstáculos que ainda persistem sobre esse processo. As palestras foram ministradas por médicos veterinários especialistas expondo divergentes visões a respeito da complexidade do tema abordado.

Palavras-chave: toxicidade; bioensaio; material didático; reprodução; espermatozoide

Abstract

ANCIUTI, Andreia Nobre **Biomodels and simulators: since research to teaching** 2019. 103f. Thesis (Doctor degree in Sciences) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2019.

The doctoral period is a time when the future doctor must experience the three pillars of education: research, extension and teaching. Phytotherapy research stands out in the scientific scenario, but many products do not have their efficacy and safety validated. In this sense, the effects on sperm parameters of the topical use of copaiba oil (OC) ointment, which is used for the treatment of inflammatory skin lesions, were studied. Animals treated with 0.01% and 0.1% OC for 7 days increased the number of damaged membrane cells (79.6% and 68.9%) when compared to the control group (43%). After 14 days (D14) of treatment, an increase in the percentage of damaged DNA cells was observed in the 0.01% (27.8%) and 0.1% (23.7%) treated groups with copaiba. control group (0%). Showing that topical use of OC can decrease sperm quality over the period and concentrations studied. However, early identification of the toxicity of new substances through the development of alternative animal methods is a constant pursuit, guided by the 3R's Principles. A screening assay has been proposed to identify the toxicity of different doses of swine sperm cell ATP compared to cells of the ZFL strain by assessing cell kinetics, structure and metabolism. Swine sperm were shown to have a high sensitivity to the toxic effects of the tested agent, so it was possible to detect changes in plasma membrane and reactive oxygen species production, data not found in cell line evaluations. In the field of education, the development of the teaching project "Production of alternative didactic resource for the Veterinary Medicine course" was elaborated from a canine dummy capable of making possible the abdominal and vaginal palpation of a pregnant female. This material enriched the practical classes of the Obstetrics and Mammary Gland discipline of the Veterinary Medicine course, providing greater security for students to perform the maneuver in a future patient. Being a viable alternative to the use of animals. Regarding the university extension activity, the First Cycle of Prenatal Lectures on Small Animals, had as its main objective to disseminate and propagate information about the most differentiated themes about obstetrics in small animals and how to overcome the obstacles that still persist about this process. The lectures were given by expert veterinarians exposing divergent views on the complexity of the topic addressed.

Keywords: toxicity; bioassay; courseware; reproduction; sperm

Lista de Figuras

Artigo 1

Artigo 2

Artigo 3

Artigo 4

Lista de Tabelas

Lista de Abreviaturas e Siglas

AO	*Acridine Orange*
ATCC	*American Type culture collection*
ATP	Adenosina trifosfato
Bracvam	Centro Brasileiro para Validação de Métodos Alternativos
BSA	Albulmina sérica bovina
BTS	*Beltsville Thawing Solution*
CASA	*Computer Assisted Sperm Analysis*
CB2R	*Cannabinoid Receptors Type 2*
CC	*Negative Control*
CDMSO	*Positive Control*
CE	*Cooling Extender*
CEEA-UFPel	*Ethics and Animal Experimentation Committee of Federal University of Pelotas*
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CO	*Copaifera Oil*
CONCEA	Conselho Nacional de Controle de Experimentação Animal
D14	14 dias
D21	21 dias
D7	7 dias
DCF-DA	*2',7'-Dichlorofluorescin diacetate*
DMC	Diabetes Mellitus Canino
DMSO	*Dimetilsulfoxide*
DNA	Ácido Desoxirribonucleico
ECVAM	Centro de Validação de Métodos Alternativos da Europa
EDTA	*Ethylenediamine Tetraacetic Acid*
FBS	*Fetal Bovine Serum*
FE	*Freezing Extender*

IP	*Propidium Iodide*
LPO	Lipoperoxidação
M540	*Merocianine 540*
MEM	*Minimal Essential Medium*
MF	*Membrane Fluidity*
MIF	*Mitochondrial Function*
OECD	*The Organization for Economic Co-operation and Development*
PBS	*Phosphate-Buffered Saline*
ReproPel	Núcleo de Ensino e Pesquisa em Reprodução Animal
ROS	Espécies reativas de oxigênio
SE	*Standard Error*
SPTZ	Espermatozoide
UFPel	Universidade Federal de Pelotas
UFRJ	Universidade Federal do Rio de Janeiro
UV	Ultra-Violeta
ZFL	Linhagem de células hepáticas de *zebrafish*

Lista de Símbolos

β	Beta
g	Grama
°C	Graus Celsius
>	Maior
±	Mais ou Menos
®	Marca Registrada
<	Menor
≤	Menor ou Igual
µL	Microlitro
mg	Miligrama
mg/mL	Miligrama por Mililitro
mL	Mililitro
mm^2	Milímetro Quadrado
mM	Milimolar
min	Minuto
nm	Nanômetro
-	Negativo
n	Número Amostral
mL^{-1}	Por Mililitro
%	Porcento
kg	Quilograma
:	Razão
rpm	Revoluções por Minuto
s	Segundos
×	Vezes
v/v	Volume por Volume

Sumário

1 Introdução

A utilização de animais tanto para o ensino quanto para pesquisa é um tema complexo e conflitante. Por um lado, considerada como a principal responsável pelos avanços da saúde humana e animal, por outro, criticada pela extrapolação interespecífica de dados. Anualmente milhões de vertebrados são utilizados em protocolos de experimentação e ensino. E entre os mais utilizados, podemos citar camundongos, ratos, coelhos e cães (Regis, 2012)

A busca por alternativas ao uso dos animais remonta o século XVIII com James Ferguson (1710-1776), que criticou o sofrimento dos animais utilizados em experimentos sobre a respiração. Em suas demonstrações públicas, usou um modelo de balão para simular os pulmões. Já em 1835, Marshall Hall (1790-1857), médico neurologista, estabeleceu uma série de regras éticas a serem adotadas para o bem-estar dos animais de experimentação científica. Assim, ele sugeria a realização de experimento com animais, I) somente em casos em que não fosse possível obter a informação pela simples observação; II) toda experiência só deveria ser realizada se tivesse um objetivo definido; III) é dever de todo cientista estar informado sobre seus predecessores e colegas para evitar a repetição desnecessária de um experimento; IV) experimentos justificáveis deveriam minimizar ao máximo a dor/sofrimento dos animais; V) e os experimentos deveriam ser muito bem delineados para fornecer resultados claros, para que não precisem ser repetidos (Simões, 2011)

Em meados do século seguinte, Russel e Burch publicaram o livro "*The Principles of Humane Experimental Technique*" ("Princípios da técnica experimental humana") no qual estabeleceram o princípio dos três R's da pesquisa em animais: substituir (*replace*), reduzir (*reduce*) e refinar (*refine*). Nesse contexto, substituir indica que se deve procurar substituir a utilização de vertebrados por outros métodos que utilizem outros materiais, não sencientes, o que pode incluir plantas, microorganismos, entre outros. Reduzir indica que se deve procurar reduzir o número de animais utilizados no experimento, o que é possível, por exemplo, com uma "escolha correta das estratégias". E refinar indica, entre outras medidas, que se deve minimizar o desconforto ou sofrimento animal (utilização de drogas anestésicas ou analgésicas,

manejo adequado) (Russel e Burch, 1959). Nesse sentido também a ciência estaria se beneficiando com melhores delineamentos experimentais. Esses princípios deram o impulso inicial para o conceito de “alternativas ao uso de animais” (Tréz, 2015).

A substituição animal é uma alternativa que segue uma corrente mundial para o desenvolvimento de técnicas que não incluam o uso de animais em experimentos e no ensino. Muitas instituições foram fundadas no intuito de validar métodos, de modo a legalizar e harmonizar o uso de tais testes (Presgrave, 2002), como por exemplo o Centro de Validação de Métodos Alternativos da Europa (ECVAM) e o Centro Brasileiro para Validação de Métodos Alternativos (Bracvam). A substituição pode ser classificada em quatro categorias. A substituição direta prevê a utilização de um sistema para fornecer respostas que sejam mais próximas possíveis de um modelo animal. A substituição indireta, sistema em que o resultado experimental se dá através da dosagem ou reação de algum mediador que produz a resposta no sistema *in vivo* (Presgrave, 2002). A substituição total, quando a informação que se deseja pode ser obtida sem uso de animais experimentais (Duvall, 2011). Ou, ainda a substituição parcial, substituição ao uso de animais é feita parcialmente, como no caso de cultivo de células, órgãos isolados ou uso de preparações subcelulares. Algumas das técnicas alternativas importantes são culturas de células e tecidos, relações estrutura-atividade e bancos de dados, modelagem computacional, uso de cortes de tecido e uso de organismos inferiores como substitutos para vertebrados em testes de toxicidade. Dentre os protocolos experimentais, os mais difíceis de serem substituídos são os ensaios para identificação de toxicidade, pois muitos efeitos tóxicos são descobertos apenas nas fases clínicas I e II dos estudos de toxicidade (Adler, 2011).

Os alvos de agente tóxicos mais preocupantes são o fígado, o coração, o sistema nervoso e o sistema reprodutivo. Dentre estes, o sistema reprodutivo é um dos primeiros alvos de agentes tóxicos, já que este sistema não é necessário para sobrevivência do organismo como as células nervosas e cardíacas (Redfern et al., 2010). As células deste sistema respondem rapidamente as agressões sofridas com alterações de cinética, estrutura e metabolismo celular, inclusive, algumas susbtâncias podem levar a danos no material genético. Nos casos de testes com xenobióticos, quando a via de contato com o agente prevê ingestão oral ou a penetração cutânea é considerável, se tem a necessidade de buscar dados/informações sobre carcinogenicidade, toxicidade e toxicocinética reprodutiva, além de genotoxicidade. A toxicidade reprodutiva envolve diversas fases do ciclo

reprodutivo incluindo fertilidade, comportamento sexual, implantação de embriões, desenvolvimento embriofetal, parto, adaptação pós-natal e subsequente crescimento e desenvolvimento na maturidade sexual. Sabendo que essas células são as responsáveis pela geração de novos indivíduos e com isso a perpetuação da espécie, o estudo toxicidade que avaliam potencial efeitos secundários em órgãos do sistema reprodutor são relevantes. Assim, vários testes *ex vivo* e *in vitro* foram propostos para estudar a toxicidade nos processos de reprodução e desenvolvimento (Adler, 2011).

Ensaios *in vitro* com cultivos primários e linhagens estabelecidas de hepatócitos vem sendo desenvolvidos no intuito de identificar substâncias potencialmente tóxicas. Há um consenso na comunidade científica de que as células hepáticas são as melhores fontes de enzima na realização de testes de triagem de biotransformação (Gómez-Lechón et al., 2003; Houston and Galetin 2008; Riley and Kenna, 2004). Isso porque esta é a principal rota de eliminação de químicos orgânicos, sendo 70-80% dos fármacos completa ou parcialmente eliminados pelo metabolismo (Zanger et al. 2008.). O peixe *Danio rerio* (*zebrafish*) tem demontrado ser um ótimo modelo para estudos de toxicidade, pois muitos mecanismos fisiológicos são altamente conservados entre ele e os mamíferos (Hernández and Allende, 2008). A linhagem celular de hepatócitos de *zebrafish* (ZF-L) foi isolada e descrita por Collodi e colaboradores (1992) e detalhadas por Gosh e colaboradores (1994). Desde então tem sido utilizada para estudos de toxicologia como transcrição e expressão gênica após exposição a diferentes íons metálicos (Sandrini et al., 2009), bem como teste de novos fármacos (Chan, 2006; Pomati et al., 2007), e estudos metabolômicos após exposição a estrogênios (Teng et al., 2013). Por outro lado, essa linhagem de células expressa níveis detectáveis menores de alguns genes envolvidos na identificação de sobstâncias tóxicas em comparação com o cultivo primário de hepatócitos de *zebrafish* machos e fêmeas (Eide and Rutsen, 2014).

Para produção de cultivo celular primário, é necessário realizar a eutanásia dos animais para retirada dos tecidos, fato que aumente o número de animais utilizados na pesquisa. Nesse sentido, a utilização de espermatozoides da espécie suína, é uma nova proposta, como um método de rápida identificação de substâncias potencialmente tóxicas para células de mamíferos (Vicent-Carrillo et al., 2015). Considerando que, a colheita das células na espécie suína é realizada de maneira rotineira em centrais de inseminação artificial, tornando-se assim, um material de baixo custo, quando comparado à métodos *in vitro*, além de não ser necessária a

realização da eutanásia dos animais para tal procedimento. Estudos relatam que as células espermáticas suínas podem ser utilizadas eficientemente como um biosensor para avaliação toxicologia de toxinas microbianas (Mikkola et al., 2004, 2007; Kruglov et al., 2009).

Já para o ensino, o uso de animais como modelo didático no ensino superior enfrenta várias críticas desde seus pressupostos éticos e morais, além dos problemas de ordem psicológica que podem ocasionar. Com o objetivo de melhorar a qualidade da educação e assegurar que animais não sejam utilizados de forma danosa, os métodos alternativos surgem como resposta a uma exigência crescente por parte de professores e pincipalmente de alunos. Assim, a Resolução Normativa nº 38 de 17 de abril de 2018 do Conselho Nacional de Controle de Experimentação Animal - CONCEA, a qual entrou em vigor em abril de 2019, restringe o uso de animais no ensino apenas para aquelas atividades que “objetivem desenvolver habilidades psicomotoras e competências dos discentes envolvidos”. As atividades didáticas que utilizem animais para fins demonstrativos ou observacionais devem ser integralmente substituídas por vídeos, modelos computacionais, ou outros recursos providos de conteúdo e de qualidade suficientes para manter ou para aprimorar as condições de aprendizado (BRASIL, 2019).

Embora existam algumas restrições, há uma grande variedade de métodos alternativos didáticos que podem ser utilizados no ensino. O estudo de inseto é indispensável e insere-se em diversos campos por serem organismos-modelo, atendendo disciplinas amplas dentro da biologia, para isso uma alternativa à captura ds invertebrados é a confecção de coleções entomológicas em cerâmica fria (Andrade et al, 2018). Outro método é a utilização de filmes e vídeos que apresentam baixo custo e apesar de passivo apresentam uma importante ferramenta para procedimentos que antecedem, por exemplo, procedimento cirúrgicos. Nesse sentindo, há, ainda, as simulações as quais integram um sistema de aprendizagem multimídia (textos, sons, animações e vídeos) permitindo a interação do aluno. Manequins e simuladores são importantes opções para o contato inicial do estudante com técnicas e procedimentos, podendo este praticar no seu próprio ritmo, aprendendo com seus erros e repetindo os processos até obter segurança. O próximo passo para o desenvolvimento do aprender é a utilização de cadáveres eticamente adquiridos, e por fim a aplicação do conhecimento em pacientes que necessitem de atendimento clínico (Lima, 2018).

Por outro lado, alguns criticam o uso de alternativas no ensino, afirmando que tais técnicas não reproduzem inteiramente os aspectos e condições encontrados na utilização de um animal vivo, já que não mostram a dinâmica da interação entre os sistemas. Algumas pesquisas apontam que métodos de ensino obsoletos e ineficientes, os quais não são suficientes para motivar os estudantes, estão associados com altos níveis de evasão escolar e reprovação. Os processos modernos de construção de conhecimento exigem uso combinado de sentidos como visão e toque, interpretação e comunicação. O melhor desempenho dos métodos alternativos se deve ao fato de que há a possibilidade de repetição, economia de tempo (não é necessário a preparação de experimentos com animais).

2 Objetivos

Utilizar biomodelos animais como instrumento para pesquisa, ensino e extensão.

2.1 Objetivos específicos:

- Avaliar o efeito de um fármaco a base de óleo de copaíba de uso tópico nas células espermáticas de ratos;
- Propor um protocolo utilizando células espermáticas suínas para identificação precoce de agentes/doses tóxicas como um método alternativo à utilização de animais;
- Desenvolver um manequim didático para palpação abdominal e da vaginal de pequenos animais;
- Avaliar, por meio de pesquisa de satisfação, a qualidade do I Ciclo de palestras sobre pré-natal em pequenos animais organizado durante o desenvolvimento do projeto.

3 Artigos

3.1 Artigo 1

Sperm Parameters Reflect the Effects of Topical Use of Copaifera Oil in Wistar Rats

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Sperm parameters reflect the effects of topical use of copaifera oil in wistar rats

Parâmetros espermáticos refletem os efeitos do uso tópico de óleo de copaíba em ratos wistar

Abstract

Copaiba resin oil (CO) is used in the treatment of inflammatory skin injuries in humans. This study assessed the effects of topical use of CO ointment on sperm parameters. Sixty-three Wistar male rats were anesthetized and two wounds were inflicted on the back of each rat. The healing injuries were treated daily with Petroleum jelly (control group), 0.01%, and 0.1% of CO ointment until the day they were euthanized: seven, fourteen, or twenty-one days later (D7, D14, or D21, respectively). Each group contained seven animals and the traits assessed in terms of sperm quality were sperm motility, integrity of plasma membrane, mitochondrial function, DNA integrity, and histopathological characteristics. Animals treated with CO at 0.01% and 0.1% for D7 had an increase in the number of cells with injured membranes (79.6% and 68.9%, respectively) and nonfunctional mitochondria (44.8% and 48.5%, respectively) when compared to the control group (43% of injured membrane and 19% of nonfunctional mitochondria). After D14 of treatment, we observed an increase ($P<0.05$) in the percentage of cells with damaged DNA and injured acrosome in the Copaiba-treated groups at 0.01% (27.8% and 43.8%, DNA and acrosome respectively) and 0.1% (23.7% and 41.4%, DNA and acrosome respectively), when compared to the control group (0% and 19.2%, DNA and acrosome respectively). We concluded that the topical use of CO decreased sperm quality in the period and at the concentrations studied.

Keywords: Sperm quality, Sperm motility, Mitochondria, DNA damage, Herbal medicine

Resumo

O óleo de copaíba (OC) é usado para o tratamento de lesões inflamatórias na pele em humanos. Este estudo avaliou os efeitos do uso tópico da pomada contendo OC sobre os parâmetros espermáticos. Sessenta e três ratos machos *Wistar* foram anestesiados e duas lesões foram feitas sobre o dorso de cada animal. As lesões em cicatrização foram tratadas diariamente com vaselina (grupo controle) e pomada de OC nas concentrações de 0,01% e 0.1% até o dia em que os animais foram eutanasiados: sete, quatorze ou vinte e um dias após o tratamento (D7, D14 ou D21, respectivamente). Cada grupo continha sete animais e foram analisadas as características sobre os parâmetros de qualidade espermática: motilidade espermática, integridade de membrana plasmática, funcionalidade de mitocôndria, integridade de DNA e histopatologia. Os animais tratados com OC a 0,01% e 0,1% por D7 tiveram um aumento no número de células com membranas lesadas (79,6% e 68,9%, respectivamente) e mitocôndrias não-funcionais (44,8% e 48,5%, respectivamente) quando comparados ao grupo controle (43% de membranas lesadas e 19% de mitocôndrias não-funcionais). Após o D14 de tratamento, foi observado um aumento ($P<0,05$) na porcentagem de células com DNA e acrossoma lesionados nos grupos tratados com copaíba a 0,01% (27,8% para o DNA e 43,8% para o acrossoma) e 0,1% (23,7% para o DNA e 41,4% para o acrossoma) quando comparados ao grupo controle (0% para o DNA e 19,2% para o acrossoma). Concluímos que o uso tópico de OC diminuiu a qualidade espermática no período e nas concentrações estudadas.

Palavras-chave: Dano ao DNA, medicamento fitoterápico, motilidade espermática, mitocôndria, qualidade espermática

Introduction

Copaiba trees are native from Latin America and Western Africa. In Brazil, there are more than twenty species and the oil-resin is obtained by tapping the trunk of Copaifera trees. The oil-resin from Copaifera plants has biological properties, such as anti-inflammatory (VEIGA JUNIOR et al., 2007), antimicrobial (TOBOUTI et al., 2017), healing (DIAS-DA-SILVA et al., 2013), analgesic (CARVALHO et al., 2005), antibacterial (PIERI et al., 2012), antifungal (DEUS et al., 2011; SVETLICHNY et al., 2015), antitumor (LIMA et al., 2003). The use of copaifera oil-resin in herbal medicine is approved by Brazil's National Health Surveillance Agency (ANVISA), which recommends the external use of 10% Copaifera oil ointment in skin wounds three times a day on the affected area (BRASIL, 2011).

This oil is made up of different sesquiterpenes and diterpenes according to the Copaifera species. The main sesquiterpene found in Copaifera sp. is β-caryophyllene. This compound has spicy properties and it is commonly ingested with vegetables in an estimated daily intake of 10-200 mg (GERTSCH et al., 2008). Moreover, it has antileishmanial (DE ALBUQUERQUE, et al., 2017), antimicrobial (PIERI et al., 2012), antioxidant, anti-inflammatory (CARVALHO et al., 2005) and anticarcinogenic (LIMA et al., 2003) properties.

The β-caryophyllene compound is supposed to be effective in the treatment of endometriosis (ABBAS et al., 2013). However, this compound negatively affects sperm counts, motility and morphology, but it does not affect histological or ultrastructural features of testis and tail of epididymis of rats treated orally (AL-ALAMI et al., 2015).

As SANTANA et al. (2014) observed, in the Amazonian region 41% of the elderly interviewed used this oil as a healing agent. They also noted that respondents use the oil cautiously in low doses, reporting that the use in high doses can bring on health damages. Up until now, no study has reported the effect of topical use of copaiba oil-based products on sperm

quality in vivo. Knowing that the recruitment of cells from stem cell lines occurs every 12.9 days (DU and DIANJUN, 2013; FRANÇA et al., 1998), the aim of this study was to assess the effects of topical use of Copaifera oil-ointment on sperm parameters of rats after 7, 14, and 21 days of treatment.

Materials and Methods

Collection and characterization of Copaifera oil

The copaifera resin oil (Copaifera sp.) was collected through puncture in the trunk of the tree Copaifera sp. (Herbarium – HFSL:6726). The collection was performed by Fundação Universidade Federal de Rondônia, in Rondônia State (RO-463, Theobroma, RO, 2.4 Km- NE- 10.294235, -62.404860). After oil acquisition and botanical characterization, the chemical characterization was performed through gas chromatography (model GC/MS-QP 2010SE Shimadzu, Japan) using an auto-injector equipment (AOC-20i). The compound identification was determined by mass spectrometry using the library NIST 8 of GC/MS that stocked information of compounds previously identified and the quantity established by normalized area.

Dose setting up

Doses were defined from previous studies by our research group (unpublished data). Briefly, a cytotoxicity assay with Vero cells (African green monkey Kidney cells) was performed to determine which doses should be used in in vivo assay. Cells were cultured in minimal essential medium (MEM), containing 1% antibiotic solution and 10% fetal bovine serum (FBS) and kept in a humidified incubator at 37 ºC with 5% atmospheric CO_2. After confluent monolayer formation, aliquots were collected to perform the subculture at the bottom

of a 96-well plate, in order to perform the cytotoxic effect test after 48h through MTT (3- (4,5-dimethylthiazol-2-yl bromide) -2,5-diphenyltetrazolium). For cell treatment, the MEM was emulsified in dimetilsulfoxide solution (DMSO), thus allowing penetration of the copaiba oil resin into the cells at concentrations of 10%, 5%, 1%, 0.1%, 0.01%, 0.001%, 0.0001%, 0.00001%. Then, plates were stored and kept in a humidified incubator at 37ºC with 5% atmospheric CO2. The assay was performed in triplicates using MEM as negative control (CC) and MEM emulsified in 1:200 DMSO as positive control (CDMSO). Cell viability was calculated using the formula: (mean absorbance of treated group/mean absorbance of control group) x 100 (WANG et al., 2011).

Animals

After the determination of doses in the in vitro assay, 63 Wistar male rats (90 days old) from the Central Vivarium of Federal University of Pelotas (UFPel) were used for in vivo assay. The rats were housed in pairs or threes in standard plastic cages with stainless-steel covers and wood shavings as bedding, and kept under controlled temperature (23 °C, ± 2 ºC), relative humidity (maximum 70%), and a 12:12 h photoperiod with lights turned on at 07:00 a.m. A standard commercial diet for laboratory rats (Nuvilab CR-1® - Nuvital Curitiba-Brazil) and tap water were provided ad libitum. Rats were acclimatized for ten days before the experiment. The research protocol was approved by the Ethics and Animal Experimentation Committee of Federal University of Pelotas (CEEA-UFPEL), Nº. 9226.

Experimental design

Sixty-three male rats were divided into nine groups of seven animals each, according to the treatment received and the day of euthanasia. First, they were anesthetized with ketamine (100 mg kg^{-1}) and xylazin (10 mg kg^{-1}) for dorsum trichotomy and antisepsis, then two injuries

were performed on the back of each rat using a punch number 8, according to CAPELLA et al. (2016) (Figure 1). Rats were provided with a heated bed and received care until full recovery. The healing injuries were treated daily during three different periods: 7 (D7), 14 (D14), and 21 (D21) days, with liquid Petroleum jelly (control group), 0.01% Copaifera oil (Herbário-HFSL:6726) ointment (0.01% Copaifera group), and 0.1% Copaifera oil ointment (0.1% Copaifera group). At the end of each experimental period, rats were euthanized with an overdose of ketamine and xylazin followed by cardiac exsanguination. Afterwards, the testicles of each animal were collected and stored at 10% formalin solution. The epididymal tail was collected and stored in phosphate-buffered saline (PBS) preheated up to 37°C. Sperm was collected through incisions with a hypodermic needle (40X12) in order to assess sperm parameters (OYEYIPO et al., 2018).

Sperm motility

To determine the sperm motility (0-100%), a droplet of the collected sperm was placed between a slide and cover slip and visualized under an optical microscope with a phase contrast and a heated plate at 37 °C (Olympus BX41-PH-III America Inc., São Paulo, Brazil) and 200X magnification in duplicates. Motility assessment ranged from zero to 100%.

Sperm concentration

To determine sperm concentration, 900 µL of formol saline was added to a 100 µL of sperm aliquot. The suspension was mixed thoroughly and placed into a Neubauer counting chamber. The total sperm count was determined by counting cells inside five 1 mm^2 squares and multiplying by 5×10^4, in order to express the number of spermatozoa per mL.

Microscopical analyses of cell structure

Samples were incubated for 5 minutes in the dark, and 200 cells were observed by epifluorescence microscopy (Olympus BX 51, América Inc., São Paulo, SP). Analyses that were performed are as described according to Table 1.

Histological analysis

Testes samples were stored in 10% formalin and sent to the Laboratory of Histology of the Department of Animal Pathology, UFPel. Tissue fragments were processed, embedded in paraffin, subsequently cut into five-micron-thick sections, and stained with hematoxylin and eosin. Tissues were assessed for the presence/absence of degenerative, inflammatory, and proliferative wounds.

Statistical Analysis

Data are presented as the mean ± standard error (SE). Data normality and homogeneity of variances were assessed using the Shapiro-Wilk test. Mean values of dependent variables with a normal distribution were subjected to analysis of variance (ANOVA) followed by Tukey's test. All analyses were performed using Statistix 9.0® software.

Results and Discussion

Routinely, products of topical application are used indiscriminately, with the belief that they are not capable of carrying systemic consequences to the body. However, the absorption of a drug depends mainly on the vehicle used for emulsion. Petroleum jelly, an oily solution, which is indicated for preparations with terpenes, such as copaiba oil, showed absorption in all layers of the whole skin in just one hour (CAL, 2006). The topical use of copaiba oil ointment on open wounds, even at a lower dose than that recommended by ANVISA, was toxic to sperm

cells of rats under the studied conditions. The literature points out the cytotoxic potential of copaiba oil against microorganisms such as *Leishmania amazonensis* (DE ALBUQUERQUE et al., 2017; SOARES et al., 2013), *Streptococcus* spp. (DIEFENBACH et al., 2018), *Staphylococcus* spp. (DIAS et al., 2015), *Pseudomonas aeruginosa* and *Escherichia* spp. (MENDONÇA and ONOFRE, 2009). In addition, the resin oil also exhibits larvicidal (SILVA et al., 2007) and acaricidal (FERNANDES et al., 2016) activities. Some of its compounds are said to be responsible for this capacity, such as sesquiterpenes caryophyllene (DIEFENBACH et al., 2018) and humulene (GOVINDARAJAN and BENELLI, 2016).

In the chromatographic assessment the following compounds were identified: caryophyllene (78.6%), humulene (11%), bergamotene (2.9%), copaene (2%), muurolene 4%), elemene (1.2%), cadinene (1%) and 1H-cycloprop [e] azulene (0.2%). The cytotoxicity assay with MTT Copaifera sp. demonstrated that the doses presented viability equal to or less than 0.01%. In the present study, we chose to use the first inoculum dose (0.01%) and the first cytotoxic group (0.1%).

Although histological differences were not observed in the studied groups, the topical use of Copaifera oil in both concentrations showed toxic effects to rat spermatozoa at the three observation times. After D7 of treatment with Copaifera oil ointment in the groups at 0.01% and 0.1% concentrations, the motility of the sperm cells were 38.0% and 41.6%, respectively, exhibiting a significant decrease in the percentage of mobile cells when compared to the control group (67.1%) ($P<0.05$). However, after D14 and D21 of treatment, the motility of sperm cells of Copaifera group 0.01% were 42.5% and 42.0%, respectively, and values were equal to those of the control group (56.6% and 54.6%, respectively) ($P<0.05$). Meanwhile, the motility of sperm cells of Copaifera group at 0.1% (23.3% and 17.5%, respectively) continued to decrease ($P<0.05$). The values of sperm motility are shown in Figure 2.

In the sperm concentration analysis, we observed that after D7 of treatment, the control group (157.4×10^4 sptz mL^{-1}) had a significant increase in cell concentration when compared to the Copaifera-treated groups: 0.01% group with 65×10^4sptz mL^{-1}; and 0.1% group with 81.5×10^4 sptz mL^{-1} ($P<0.05$). This trend was inverted after D14 of treatment, when the Copaifera groups (0.01%, 126.7×10^4 sptz mL^{-1}; and 0.1%, 100.8×10^4 sptz mL^{-1}) presented a significant increase when compared to the control group (9.33×10^4 sptz mL^{-1}; $P<0.05$). After D21 of treatment, 0.1% Copaifera group exhibited a higher sperm concentration (167.9×10^4 sptz mL^{-1}) when compared to control (74.8×10^4 sptz mL^{-1}) and 0.01% Copaifera (69.9×10^4 sptz mL^{-1}) groups ($P<0.05$) (Figure 3).

Data published by AL-ALAMI et al. (2015) showed that caryophyllene, the main compound found in the resin oil used in this study (78.6%), adversely affects sperm count, motility and morphology, without affecting the testicular structure and the tail of the epididymis. In the present study, it was also possible to observe a negative effect on motility, especially in the treatment with higher concentration of resin oil, and sperm concentration after D14 of treatment. Caryophyllene has agonistic activity on cannabinoid receptors type 2 (CB2R), present in T cells of the immune system and testicular germ cells and, when activated, they are involved in the process of spermatogenesis. CB2R is highly found in cells at differentiation stages, which are poorly found in spermatids and undetectable in spermatozoa, and have pro-differentiation properties (AL-ALAMI et al., 2015; GRIMALDI et al., 2009).

Regarding the impact of the CO in cell membranes, the Copaifera group at 0.01% and 0.1% after D7 of treatment had a significantly increase in the number of cells with injured membranes (43.0%, 79.6%, and 68.9%, respectively) when compared to the control group. Moreover, with respect to the injured membrane, no statistical significant difference was observed between the studied groups after D14 of treatment ($P>0.05$). When animals were treated to D21, there was a significant decrease in the percentage of injured membrane cells of

0.1% Copaifera group (48.1%) when compared to the control (70.6%) and 0.01% Copaifera groups (68.8%; P<0.05) (Figure 4).

With respect to mitochondrial function, the Copaifera oil component β–caryophyllene caused an alteration in the mitochondrial potential of the protozoan Trypanosoma cruzi, decreasing the functionality of this organelle (DE ALBUQUERQUE et al., 2017; IZUMI et al., 2012). In our study, the control group exhibited a decreased percentage of cells with nonfunctional mitochondria (19.0%) when compared to 0.01% Copaifera group (44.8%; P<0.05) after D7 of treatment (P<0.05). Moreover, the control group presented 25.2% of cells with nonfunctional mitochondria and the 0.1% Copaifera group presented 40.2% after D14 of treatment. At D21 after treatment, the 0.1% Copaifera group presented a significant increase in the percentage (59.9%) of cells with nonfunctional mitochondria when compared to the other groups (P<0.05) (Figure 5). These results corroborate the previous statement of possible mitochondrial alteration. CASTRO-E-SILVA et al. (2004) demonstrated that Copaifera resin oil could uncouple oxidative phosphorylation in the mitochondrial respiratory chain and increase the rate of basal cellular respiration. However, the compound responsible for this action has not been established yet. These results agree with sperm motility findings, since the energy required for cell movement is stored in the mitochondria, which synthesizes ATP through the electron transport chain. We observed that the alterations in mitochondrial function are reflected in the motility changes.

Studies have shown that along with mitochondrial function and sperm motility, acrosome integrity is essential for fertilization (KASAI et al., 2002; URIÓSTEGUI-ACOSTA et al., 2014). The Copaifera group at 0.01% exhibited a higher percentage of cells with injured acrosomes. After D7 of treatment, we observed a significant increase in the percentage of cells with injured acrosomes in the Copaifera 0.01% and 0.1% groups (43.8% and 41.4%, respectively) when compared to the control group (19.2%) (P<0.05). After D21 of treatment,

both groups treated with Copaifera (0.01% and 0.1%) had a significantly increased percentage of cells with injured acrosome, 59.2% and 49.6% respectively ($P<0.05$) (Figure 6).

In the DNA integrity assessment, we found no statistical significant difference between the studied groups after D7 of treatment ($P>0.05$) (Figure 2A). After D14 of treatment, we observed a significant increase in the percentage of cells with injured DNA in the Copaifera groups at 0.01% and at 0.1% (27.75% and 23.67%, respectively), when compared to the control group (0%; $P<0.05$), although there was no significant statistical difference. After D21 of treatment, the Copaifera group at 0.1% presented a significant increase in the percentage of cells with injuries in the DNA (20.0%; $P<0.05$) (Figure 7). DAHHAM et al. (2015) demonstrated in vitro that colorectal cancer cells exposed to β–caryophyllene had their nuclear morphology altered using the DNA fragmentation analysis. At 10 μM concentration, this compound caused significant nuclei condensation after 6 h of treatment, indicating apoptosis. This compound activated caspase-3 in tumor cell lines. Induction of caspase-3 activity in turn leads to chromatin condensation, degradation and dissolution (AMIEL et al., 2012).

The damage in the sperm cell DNA along with mitochondria and acrosome changes can lead to decreased sperm motility, reducing the capacity of semen fertilization. The recruitment of committed cells from stem-cell line occurs every 12.9 days (DU and DIANJUN, 2013; FRANÇA et al., 1998), and our results demonstrated that the topical use of Copaifera oil can cause damages to sperm cells. These changes stimulated the production of sperm cells after both D14 and D21 after exposure to Copaifera resin oil. These findings can be the responses of the organism against the toxic effects such as compensation.

This study showed that the use of copaiba ointment at 0.01% and 0.1% concentrations may lead to poor sperm quality both in the acrosome and mitochondria, and at the DNA level. Thus, it is necessary to evaluate a possible grace period for the use of this formulation, in order to obtain an herbal product with efficacy and safety.

Conclusion

The topical use of Copaifera oil decreased sperm quality at the studied concentrations. Our results showed that exposure to Copaifera oil at 0.01% and 0.1% for up to D14 caused alterations in differentiated sperm cells. Moreover, after D21, we noted that the use of 0.1% Copaifera oil ointment caused changes in structures formed during initial spermatogenesis, such as DNA and mitochondria.

Conflicts of interest

The authors have declared that there are no conflicts of interest.

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Table 1. Assessments in sperm cell structures (integrity of plasma membrane, mitochondrial function, acrosome integrity, and DNA integrity) using fluorescent probes, observed by epifluorescence microscopy and its observation parameters (emission, excitation, and magnification).

Assessment	Fluorescent probe	Classification	Fluorescence emission	Emission	Excitation	Magnification	Reference
Integrity of Plasma membrane	Carboxyfluorescein diacetate (C4916 – 25 mg, Sigma Chemical Company, St. Louis, MO, USA)	Intact	Green fluorescence	516 – 617 nm	450 – 490 nm	400X	HARRISON and VICKERS (1990)
		Injured	Red fluorescence				
Mitochondrial function	Rhodamine 123 (R8004-5mg, Sigma Chemical Company, St. Louis, MO, USA),	Functional	Mid-piece intense green fluorescence	516 – 617 nm	450 – 490 nm	400X	EVENSON et al. (1982)
		Non-functional	Mid-piece with few or without green fluorescence				
Acrosomal integrity	Conjugate of Arachis hypogaea lecithin-FITC	Intact	Acrosome with green fluorescence and normal form	520 nm	450 – 490 nm	1000X	KAWAMOTO et al. (1999)
		Injured	Acrosome without green fluorescence or abnormal form				
DNA integrity	Acridine orange (Molecular Probes Inc., Eugene, OR, USA)	Normal	Green fluorescence	520 nm	450 – 490 nm	1000X	(EVENSON et al., 1982)
		Injured	Red or yellow fluorescence				

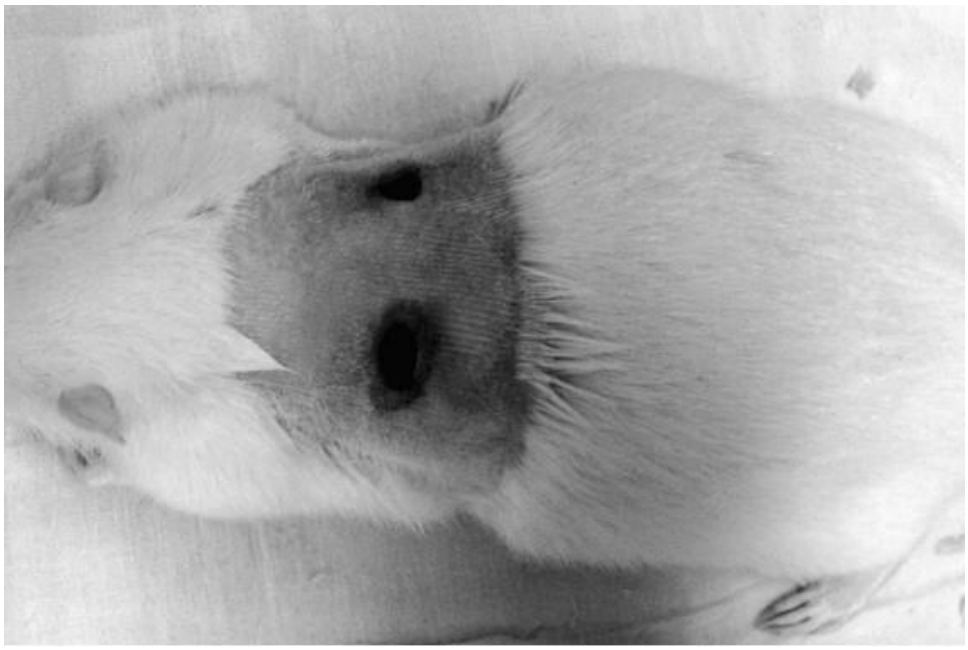

Figure 1. Rat with two punch wounds (blue staining) of 8mm length on the back (dorsal view).

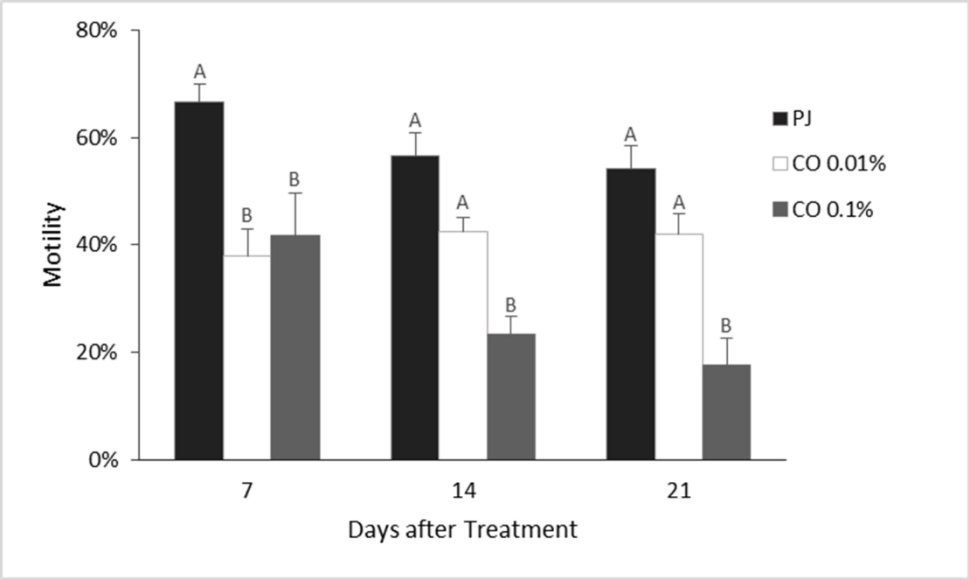

Figure 2. Effect on the motility of the different concentrations of Copaiba oil (0.01% and 0.1%) in the ointment used in the injuries after 7, 14 and 21 days of administration. Petroleum jelly was used as control vehicle. Petroleum jelly: D7 n=6, D14 n=6, D21 n=12; Copaiba 0.01%: D7 n=5, D14 n=4, D21 n=11; Copaiba 0.1%: D7 n=6, D14 n=6, D21 n=12; $p<0.01$. Different letters represent significative diferences between treatments in the observed dates ($p<0.05$).

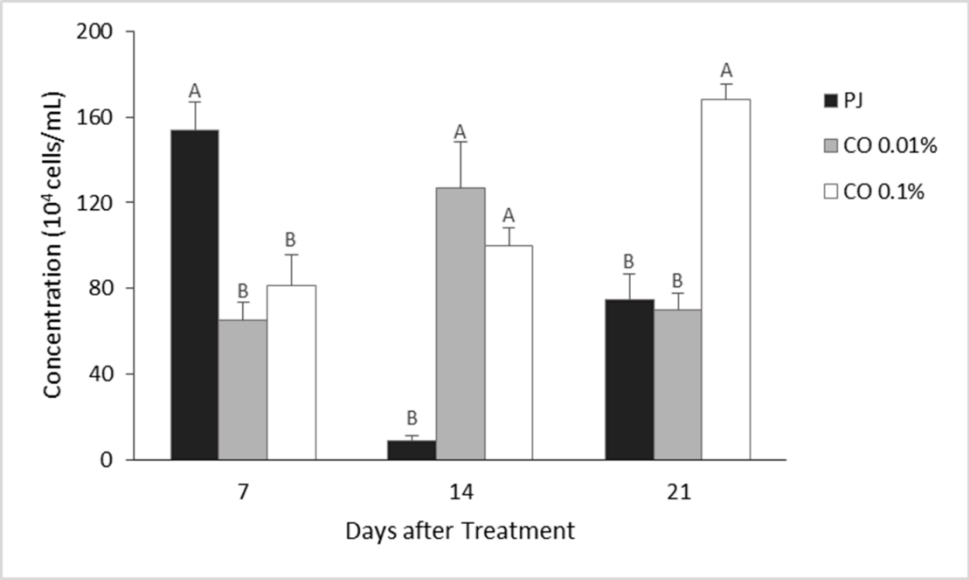

Figure 3. Effect on the sperm concentration of the different concentrations of Copaiba oil (0.01% and 0.1%) in the ointment used in the injuries after 7, 14 and 21 days of administration. Petroleum jelly was used as control vehicle. Petroleum jelly: D7 n=6, D14 n=6, D21 n=12; Copaiba 0.01%: D7 n=5, D14 n=4, D21 n=11; Copaiba 0.1%: D7 n=6, D14 n=6, D21 n=12; $p<0.01$. Different letters represent significative diferences between treatments in the observed dates ($p<0.05$).

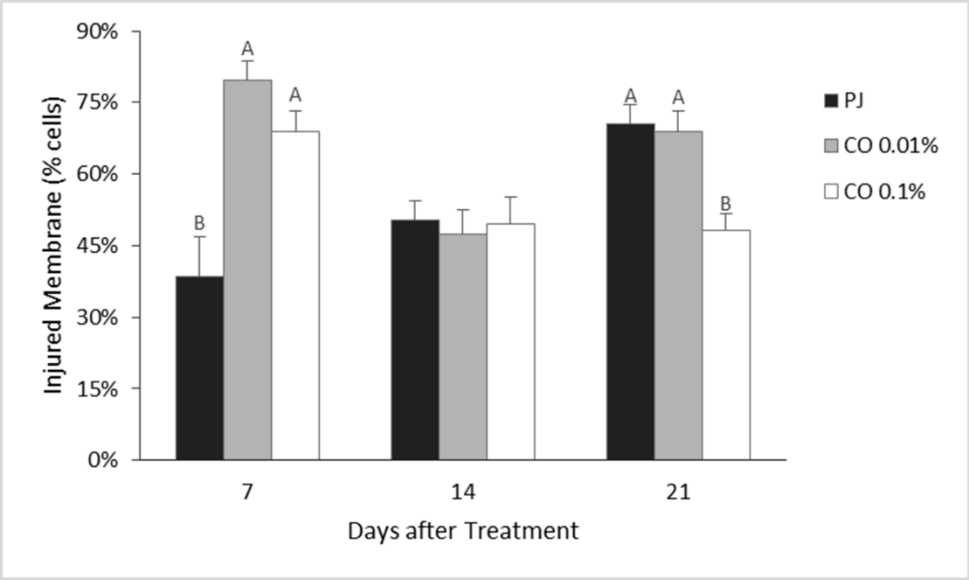

Figure 4. Effect on injured membrane of the different concentrations of Copaiba oil (0.01% and 0.1%) in the ointment used in the injuries after 7, 14 and 21 days of administration. Petroleum jelly was used as control vehicle. Petroleum jelly: D7 n=6, D14 n=6, D21 n=12; Copaiba 0.01%: D7 n=5, D14 n=4, D21 n=11; Copaiba 0.1%: D7 n=6, D14 n=6, D21 n=12; $p<0.01$. Different letters represent significative diferences between treatments in the observed dates ($p<0.05$).

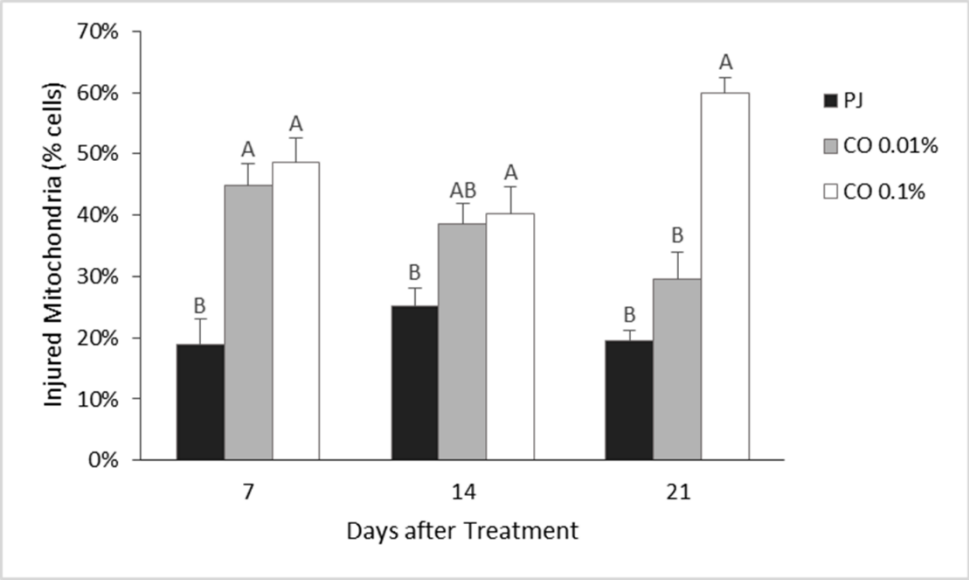

Figure 5. Effect on mitochondria of the different concentrations of Copaiba oil (0.01% and 0.1%) in the ointment used in the injuries after 7, 14 and 21 days of administration. Petroleum jelly was used as control vehicle. Petroleum jelly: D7 n=6, D14 n=6, D21 n=12; Copaiba 0.01%: D7 n=5, D14 n=4, D21 n=11; Copaiba 0.1%: D7 n=6, D14 n=6, D21 n=12; $p<0.01$. Different letters represent significative diferences between treatments in the observed dates ($p<0.05$).

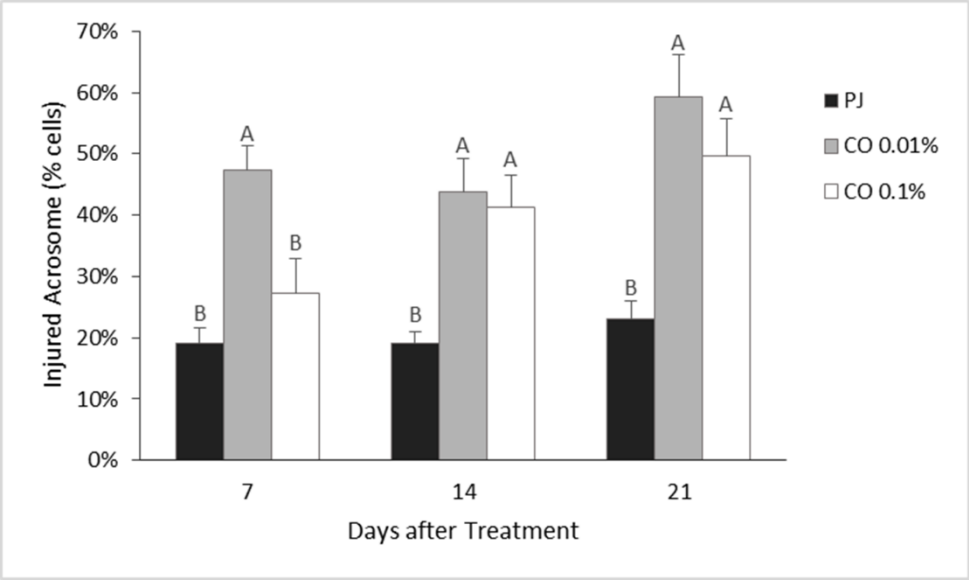

Figure 6. Effect on acrosome of the different concentrations of Copaiba oil (0.01% and 0.1%) in the ointment used in the injuries after 7, 14 and 21 days of administration. Petroleum jelly was used as control vehicle. Petroleum jelly: D7 n=6, D14 n=6, D21 n=12; Copaiba 0.01%: D7 n=5, D14 n=4, D21 n=11; Copaiba 0.1%: D7 n=6, D14 n=6, D21 n=12; $p<0.01$. Different letters represent significative diferences between treatments in the observed dates ($p<0.05$).

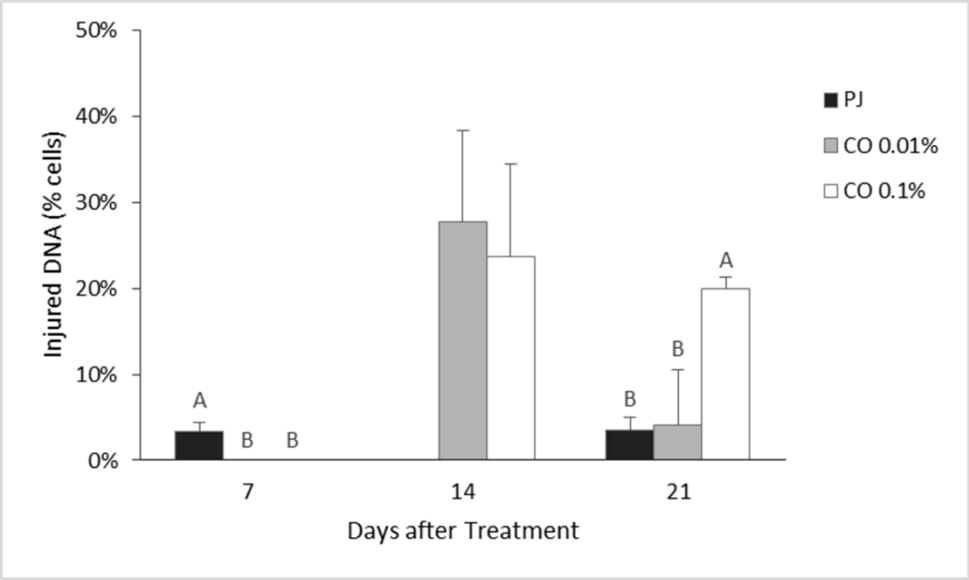

Figure 7. Effect on DNA of the different concentrations of Copaiba oil (0.01% and 0.1%) in the ointment used in the injuries after 7, 14 and 21 days of administration. Petroleum jelly was used as control vehicle. Petroleum jelly: D7 n=6, D14 n=6, D21 n=12; Copaiba 0.01%: D7 n=5, D14 n=4, D21 n=11; Copaiba 0.1%: D7 n=6, D14 n=6, D21 n=12; $p<0.01$. Different letters represent significative diferences between treatments in the observed dates ($p<0.05$)

3.2 Artigo 2

Sperm cells as an alternative trial to toxic substances testing

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Sperm cells as an alternative trial to toxic substances testing

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Abstract

The development of alternative methods to the use of animals in toxicity tests is a constant quest, guided by the Three R's Principles. In this study we proposed a trial assay to observe the influence of exogenous substances in boar sperm cells and compared the results with the ones obtained in ZFL cells lineage by assessing kinematic parameters, structure, and metabolism of the sperm cells. Both cell types were exposed to five different concentrations of ATP for 2 hours at ideal temperature for each cell type. Swine sperm cells exhibited high sensitivity to the toxic effects of the agent tested, since it was possible to detect alterations in the plasma membrane and also the production of reactive oxygen species, which was not detectable in the ZFL lineage. Therefore, it was demonstrated the potential use of sperm cells as a model for toxicology assay, presenting itself as a promising alternative as a trial of exogenous substances through kinematic, cell structure, and metabolism assessments.

Keywords: spermatozoa, cell culture, toxicology.

1 – Introduction

In the current scenery of animal experimentation, there is a constant search for the introduction and development of alternative methods to their use. Since 1959 there is a compromise of the Scientific Community to follow up "The Three R's Principles" (Replacement, Reduction, and Refinement) in animal experimentation, stipulated by Russell et al. (1959). Whenever substitution is not possible, it is necessary to find means to reduce the number of animals used and search for techniques that provide more comfort and well-being to those animals (Adler et al., 2011). There several methods to substitute animals in experimentation, and among them there are computational models, physical-chemical systems that mimic biological functions, tests with microorganisms and organs and tissues isolated from animals. Estimates point out that between 50 and 100 million animals are used to perform toxicity tests and basic and biomedical research annually (Bhanushali et al., 2010). It is believed that

toxicity tests for chemical substances are likely to be the most difficult to substitute since exogenous agents may act in different pathways according to the environment they are inserted.

Currently, tests for new drugs are performed accordingly to the OECD (The Organization for Economic Co-operation and Development) Guidelines for the Testing of Chemicals, which conducts in vitro and in vivo testing. To do so, several animals are employed seeking out for toxicity of new drugs in cardio, neuro, and hepatic systems, which sometimes are detected only in Clinical Assays of Phase I or II (Adler et al., 2011; Dykens and Will, 2007). The main and most relevant mechanism of toxicity during the development of a new drug is the mitochondrial function disturbance, observed in liver and heart cells during the in vitro tests with culture cells. These assays are performed both with primary cell cultures (tissue isolated from an animal, with the need of euthanasia subsequently) and in already-established cell culture lineage (cells purchased from specialized companies and kept in culture media).

In this context, some cell lineages are very useful for toxicological studies, such as the hepatocytes derived from the fish Danio rerio named ZFL, initially described by Collodi et al. (1992) and detailed by Ghosh et al. (1994). They form a cell culture of easy management, cheaper when compared to a primary cell culture (Schneider et al., 2009), evidencing it is an excellent model able to detect cytotoxic and genotoxic xenobiotics, demonstrating similar results seen in primary cultures (Eide et al., 2014; Goulart et al., 2015; Sandrini et al., 2009).

On the other hand, some studies indicate that boar sperm cells are able to identify toxic agents, such as the cell cultures mentioned above, with the benefit of being closer to the organism physiology (Andersson et al., 2010; Vicente-Carrillo et al., 2015; Vicente-Carrillo, 2018). The cytotoxic assays performed on sperm cell does not need euthanasia of the sperm donor, since sperm collection is routinely done for commercial purposes (artificial insemination). Besides, these cells respond rapidly to external stimuli, easily detected by sperm kinematic analysis, making them a low-cost, easy management, and fast-response method when compared to other in vitro methods (Rodriguez-Martinez and Wallgren, 2011). Therefore, the objective of this study is to propose a trial assay for chemical substances using sperm cells and compare their responses to the responses obtained in ZFL lineage cells, through kinematic, structural, and metabolic assessments.

2 – Material and Methods

2.1 - Assay with boar sperm cells

Thirty samples of boar semen doses, obtained from a commercial boar stud, from different boar donors, with total motility at least 70% for each dose, were used.

2.1.1 – Cooling and freezing rate

Doses were kept at 21°C for 10 min and afterwards they were placed in an air conditioned box for boar semen at 17°C for 90 min, for stabilization of the cells with the diluent. Subsequently, samples were fractioned in 15 mL conical tubes and centrifuged at 800 × g for 10 min, to remove the supernatant (seminal plasma and Beltsville Thawing Solution [BTS]) and the pellet (sperm cells) was re-suspended in two thirds of the semen straw volume with the cooling extender (CE) (80% of the volume with 11% lactose solution [Sigma-Aldrich® Chemical Company, St. Louis, MO – USA] and 20% egg-yolk [v/v]). Samples were then transferred into Eppendorf tubes and stored in an air conditioned box for boar semen at 5°C for 90 min. Then, the remaining one-third of the straw final volume was completed with freezing extender (FE), added to the samples (85.9% of CE, 1.5% Equex-Paste® [Nova Chemical Sales, MA – USA], and 9% glycerol). Total volume was 0.5 mL, and the final cell concentration was 1×10^9 sperm cells/mL, which was stored in 0.5 mL semen straws, properly identified and sealed with polyvinyl alcohol. Straws were positioned horizontally in liquid nitrogen vapor (N2L) at 5 cm above the level, for 20 min. Afterwards, samples were plunged in N2L and stored in a nitrogen container until thawing process.

2.1.2 – ATP exposure

Straws were thawed in water bath at 37°C, for 20 s, and 100 µL of each semen sample was diluted in 900 mL of BTS + Bovine Serum Albumine (BSA) + ATP, previously heated (3 mg of BSA/mL BTS). After dilution, the samples were fractioned in (1) 0.8 mL, for incubation in a heated plate at 37°C for 10 min, and then sperm kinematic parameters were assessed; and (2) 0.2 mL, stored at 5°C, for latter analysis of sperm cells structure and metabolism.

2.1.3 – Kinematic analysis of sperm cells

Due to the motility characteristics of sperm cells we performed kinematic analysis, since it constitutes an important parameter of assessment. Post-thaw semen was assessed regarding total and progressive motilities by Computer-Assisted Sperm Analysis system (CASA) (Sperm Vision 3.5, Minitub®). In this analyses, we used 3 µL of the previously diluted and incubated semen sample loaded into a pre-heated sperm counting chamber, as indicated by the manufacturer, and 6 fields were visualized to analyze at least 1000 sperm cells. Assessments were performed in three moments: 10, 60, and 120 min after incubation in a heated plate.

2.2 Somatic cells assay – zebrafish liver cells (ZFL)

2.2.1 Cell culture

We used established cultures of zebrafish hepatocytes (ZFL) obtained from the American Type Culture Collection (ATCC) and deposited in the Cell Bank of Rio de Janeiro - UFRJ, kept in culture bottles at a temperature of 28°C, in RPMI 1640 medium (Sigma, Aldrich) supplemented with fetal bovine serum (10%), antibiotic (penicillin and streptomycin) and antimycotic (1%). Cells were re-plated once or twice a week.

Prior to plate assembly, we assessed cell viability through trypan blue dye exclusion test (0.08%) and afterwards we proceeded only when cell viability was higher than 95%. Then, cells were prepared at a concentration of 2×10^6 cells/mL in a plate of 96 wells, at least 72 hours before the experiment, to ensure cell adhesion to the plate at the time of the assay.

2.2.2 ATP exposure

We removed standard culture media prior to ATP exposure and added 100 µL of ATP diluted in culture media, in the concentrations aimed, keeping it for 120 min. After the exposure period, we removed media containing treatments and added 50 µL of trypsin. The plate was kept in dry bath at 37°C for as long as the cells were not

adhered to the plate. Afterwards, we added 50 µL of standard culture. The content in the wells was retrieved to perform the analyses of cell structure and metabolism.

2.4 ATP concentrations studied

The ATP concentrations to which both sperm cells and ZFL lineage were exposed were: 0 mM (control group), 0.025 mM, 0.25 mM, 2.5 mM, and 25 mM.

2.5 Flow cytometry

Cell structures were assessed by flow cytometer (Attune® Cytometer Version 2.1.0 of software – Life Technologies), equipped with blue laser (Argon 488 nm) and violet laser (UV 405 nm). In all assessments we used 10 µL of sperm cell samples and 10 µL of fluorescent probe, specific for each structure assessed, and they were incubated for 10 min at 37°C. Subsequently, 20 µL of Hoechst 33342 solution (10 mg/mL) was added to each sample, except for the DNA fragmentation analysis. Lastly, 500 µL of phosphate buffered saline (PBS) containing EDTA was added to detect 10,000 cells through the equipment. Data were expressed in percentage.

As for ZFL cells, in all assessments 5 µL of sample and 10 µL of fluorescent probe were used, specific for each structure assessed, and incubated for 10 min at 37°C. Subsequently, 15 µL of Hoechst 33342 solution (10 mg/mL) was added to each sample, except for the DNA fragmentation analysis. Lastly, 400 µL of phosphate buffered saline (PBS) containing EDTA was added to detect cells through the equipment. Data were expressed in fluorescence intensity.

The assessments performed, the probes and concentrations used, the classification, and other cytometry parameters are summarized on Table 1.

2.6 Statistical analysis

Samples underwent normality analysis through Shapiro-Wilk's test and transformed when necessary. Results were compared between treatments within respective times through Kruskal-Wallis' test. Means were compared through Dunn's test using Statistix 13® software, and P-value was significant when ≤ 0.05.

3 Results

According to the results obtained, sperm cells exhibited higher sensitivity against the exogenous substances when compared to the zebrafish liver cells. The assay in which the ATP was used, demonstrated to be toxic to the sperm cells, which exhibited structural, metabolic, and kinematic changes when compared to the control group. When ZFL cells were exposed to the ATP treatments, we did not detect differences between treated groups and control groups, though they were cited as being cells sensitive to toxic substances (Sequero, 2014).

3.1 Sperm kinematic analysis

After 10 min of incubation, the results obtained in the assessment demonstrated that group 1 (control) exhibited motility values (Figures 1 and 2) statistically equal to group 2 and superior when compared to groups 3, 4, and 5; however, the progressive motility observed in group 1 was higher when compared to other groups (Figure 2). After 60 min of incubation, only in group 4 total and progressive motility values (Figures 1 and 2) were inferior when compared to the other groups. After 120 min of incubation, total motility values in groups 1, 2, and 3 did not differ statistically between one another; however, they were statistically different when compared to groups 4 and 5. When the progressive motility was analyzed (Figure 2), best results were obtained in treatment 2; groups 1 and 3 did not differ from one another; group 4 differ from the other groups; and group 5 exhibited inferior values when compared to the other groups.

3.2 Flow cytometry

3.2.1 Sperm cells

The results of membrane fluidity, mitochondrial function, lipid peroxidation, and DNA fragmentation index are displayed on Table 2.

When ROS were analyzed, we detected that values of fluorescence intensity in group 5 were statistically different from the other groups in incubation times 10 min (92553$\pm$12132), 60 min (82925$\pm$11713) and 120 min (72337$\pm$10522) (Figure 4).

3.2.2 ZFL cells

We did not observe statistical differences between treatments in the assessments performed on ZFL cells (Table 3).

4 Discussion

In this study we demonstrated that boar sperm cells used in toxicology trial tests are sensitive to the exposure of toxic agents/doses, displaying a rapid response, easy-to-evaluate, and of low cost when compared to ZFL cell lineage. We observed that kinematic, structure, and metabolism assessments exhibited a rapid in vitro response to the different concentrations of ATP, a substance that is familiar to the cell and with energetic function. These results were validated when structure and metabolism parameters were compared to a traditional cytotoxicity test using cells from ZFL lineage. b

Sperm cells are highly sensitive in detecting toxins that lead to the disruption of ionic homeostasis, energy generation, and mitochondrial function in mammal cells (Andersson et al., 2006), besides displaying similar results to somatic cells regarding cytotoxicity detection (Severin et al., 2005). Our results corroborates the hypothesis tested by Andersson et al. (2010) and Vicente-Carrillo et al. (2015), that demonstrated that in vitro assays that used boar sperm cells were an important potential model to test cytotoxicity, being advantageous when compared to other in vitro methods used routinely.

The motility parameters found in our study reflect the sensitivity of these cells and the analysis for identification of potentially toxic agents/doses. We observed that boar sperm cells are remarkable for exhibiting rapid responses, when facing eight mito-toxic substances and 130 compounds and/or drugs of pharmaceutical use with other mechanisms of toxicity (Vicente-Carrillo et al., 2015). Besides, these cells can be assessed more easily through sperm kinematic parameters, such as total and progressive motilities, which are assessed through an optical microscope coupled or not to a semi-automated software of semen analysis (CASA) (Castagnoli et al., 2018). Albeit these analyses are highly sensitive to ATP exposure that inhibit cell cation homeostasis, we could not detect whether the agent caused damage to nuclei acids

and/or proteins, being necessary and important to use protocols of analysis of cell structure, for instance, membrane fluidity and DNA fragmentation assessments.

In this context, we observed that the membrane structure of sperm cells suffered a modification after the exposure to high doses of ATP. Boar sperm cells have a relatively high content of ethanolamine phosphoglycerides in the plasma membrane, which are necessary to settle protein content in the plasma membrane, therefore increasing the probability of alterations in protein function and disturbances in the bilayer during the exposure to toxic substances (Jaaskelainen et al., 2003; Parks and Lynch, 1992). Therefore, these cells stand out for being more sensitive when compared to bovine and ovine sperm cells (Eskov et al., 2008; Eskov et al., 2007) when facing injuries; besides, the average volume per ejaculate is 500 mL, at a concentration of 120×10^9 viable cells/mL, which surpass the variables obtained for the other species cited (Vicente-Carrillo, 2018), being possible to perform several tests with few ejaculates. It is noteworthy that boar sperm cells are advantageous in terms of quality and homogeneity when compared to human sperm (World Health Organization., 2010). Regarding the rodents, the most beneficial factor is the non-necessity of euthanasia to obtain the sperm cells, besides of being a painless method (Duselis and Vrana, 2007), aspects that make the use of sperm cells for the identification of toxic substances a promising bioassay. Nonetheless, the current method indicated to perform cytotoxicity are cell cultures, such as primary cultures of hepatocytes of zebrafish (more sensitive) or yet from established lineages, such as the ZFL.

To perform tests with sperm cells it is dispensable the euthanasia, since semen collection on boar is performed aiming commercial artificial insemination. At boar studs, the collection is performed by the gloved-hand method, or through semi-automated methods such as the BoarMatic® (Minitube, Tiefenbach, Alemanha) and Collectis® (Genes Diffusion, Douai, França) that are painless and avoid any discomfort to the animal (Aneas et al., 2008). To obtain primary culture of hepatocytes of zebrafish (adult animals) it is essential to perform the euthanasia in order to isolate the liver tissue (Eide et al., 2014), which is against the Three R's Principles (Russell et al., 1959). These concepts are highly encouraged in the scientific community, and they claim for reduction, replacement, and refinement in the protocols that use animals, highlighting the importance of the development of alternatives to toxicity studies, since they seem to be burdensome to the well-being of the animals (Adler et al., 2011). The

cultivation of already established cell lineages may be an alternative to the use of primary cultures, which can be obtained from specialized companies.

The cultivation of ZFL lineage have an increased sensitivity when exposed to silver nanoparticles when compared to other cell lineages, exhibiting changes in the mitochondria activity and in the membrane integrity, even in low doses (Sequero, 2014). Our results demonstrated that within 2 hours (at 37°C) of the exposure to the toxic agent/dose, it is likely to observe alterations in the plasma membrane of the sperm cells, which did not occur to the ZFL lineage. Albeit other studies demonstrated that the alterations in these cells occur when they are exposed to toxic substances, the necessary time of contact to identify such changes ranged from 12 to 48 hours at 28°C in dark chamber (Sandrini et al., 2009), 24 hours at 27°C under agitation at 100 rpm (Sequero, 2014), and 24 to 96 hours at 28°C (Yang and Chan, 2015). It should also be noted the ZFL lineage is derived from liver cells, which play an important role in detoxification, which can cover up possible alterations caused by the toxic agent when the cytotoxic potential of chemical substances and new drugs are studied (Eide et al., 2014). Therefore, the assay we proposed in this study is economically and logistically less expensive to the laboratory, since there is no need to have equipment to maintain cell cultures (Gomez-Lechon et al., 2003). Besides, the use of boar semen has the advantage of having homogenous samples and with the viability being assessed immediately after collection and dilution, since only the best breeders remain in the boar studs and in case the immediate motility assessment is less than 70% it is a parameter for culling (Knox, 2016).

In this study we used ATP, which is a molecule of purine, familiar to the cell metabolism and the mechanism of action has been quite studied in cancer cell cultures (Ma et al., 2014; Mello et al., 2014). High concentrations of ATP induce the formation of pores in the plasma membrane of the cell, which leads to an increase in the free Calcium ions in the cytosol and the induction of cell death (Castagnoli et al., 2018). However, the effect of lower concentrations varies according to the type of cell studied, stimulating or inhibiting their growth (Burnstock and Di Virgilio, 2013; Ma et al., 2014). Toxic substances act in different ways in different cell types and normally are dose- and time-dependent. Therefore, possible toxic alterations should be assessed as well in physiologically healthy cells, such as reproductive cells. The toxicity in this type of cell is remarkable due to its complexity, being engaged in the mechanisms of male and female fertility and also in the process of development during prenatal life. Besides, it

is problematic to predict the impact that reproductive disturbances may have during adult life.

When 150 pharmaceutical compounds were identified as being toxic for humans during the clinical trials, the assays performed in rodents and non-rodents predicted 71% of toxicity in such cases. On the other hand, when considering only the assays performed on rodents, the prediction of toxicity dropped to 43% (Olson et al., 2000). Therefore, it is necessary to perform a broad and complete assessment of new drugs potentially therapeutic, seeking for answers in different physiological systems and also following the Three R's Principles.

The development of alternative methods to the use of animals is an ongoing objective. The search for assays that are sensitive and that enable an early identification of toxic substances, allows the reduction of the number of animals used in preclinical and clinical trials of new drugs. The assay we proposed in this study, in addition to meeting this theme, has advantages that must be considered, such as easy management/labor, low-cost, rapid cell response, homogeneity among samples, and high sensitivity.

5 Conclusion

In this study we demonstrated the potential use of sperm cells as a model for toxicology assay, presenting itself as a promising trial method for testing toxic substances, through assessments such as kinematic, structure, and metabolism of sperm cells.

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List of Figures:

List of Tables

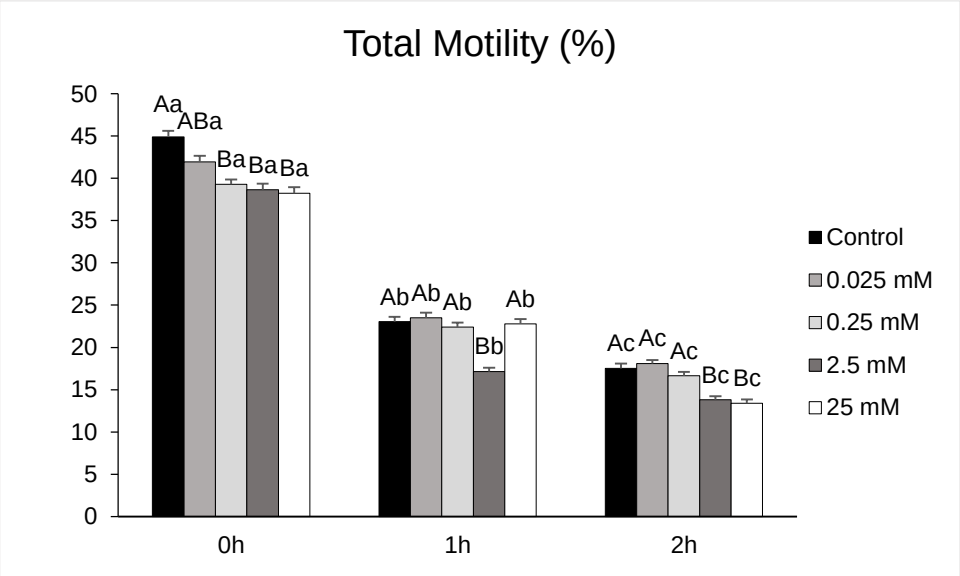

Figure 1. Data of total motility in groups that were exposed to ATP at concentrations of 0 mM (Control group), 0.025 mM, 0.25 mM, 2.5 mM, and 25 mM, throughout different times (10, 60, and 120 min). Uppercase letters represent significant statistical differences between treatments in the same time. Different lowercase letters indicate significant statistical differences between each treatment in the times analyzed.

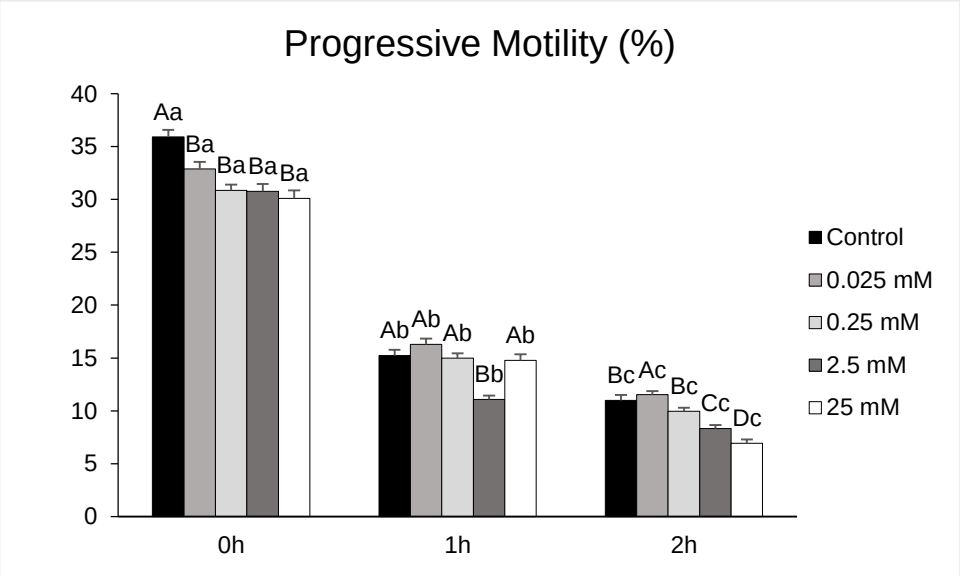

Figure 2. Data of progressive motility in groups that were exposed to ATP at concentrations of 0 mM (Control group), 0.025 mM, 0.25 mM, 2.5 mM, and 25 mM, throughout different times (10, 60, and 120 min). Uppercase letters represent significant statistical differences between treatments in the same time. Different lowercase letters indicate significant statistical differences between each treatment in the times analyzed.

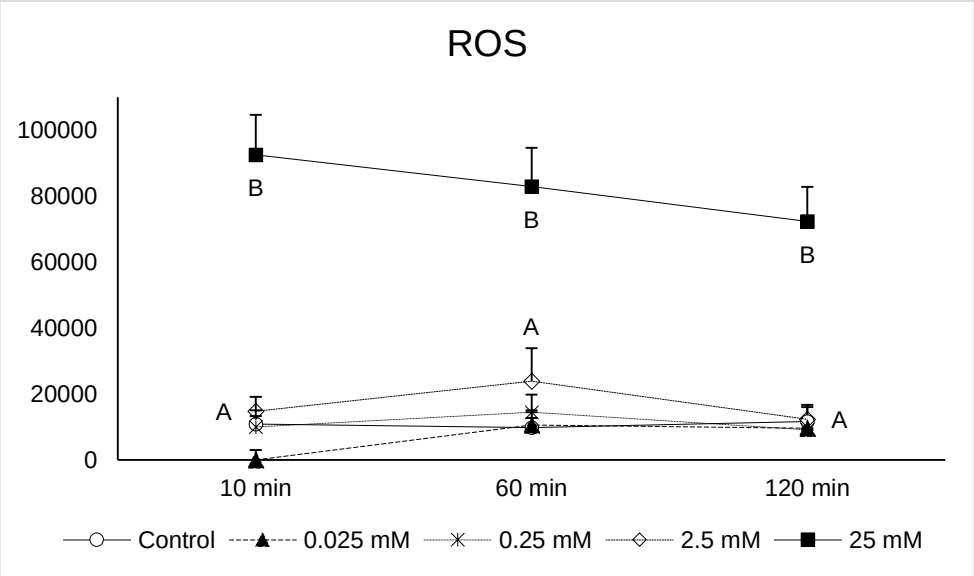

Figure 3. Reactive oxygen species output in groups which were exposed to ATP at concentrations 0 mM (Control group), 0.025 mM, 0.25 mM, 2.5 mM, and 25 mM, throough different times (10, 60, and 120 min). Different letters represent statistical differences between treatments ($p < 0.05$).

Table 1: Cell structures analyses, probes and concentrations used, classification, fluorescence type, filters, and detectors used in the flow cytometry on boar sperm cells (SPTZ) and zebrafish hepatocytes (ZFL).

Analysis	Probe concentrations by cell type	Fluorescent probe	Classification	Fluorescence Emission	Detector	Reference
Membrane Fluidity	SPTZ*: 2 µL YoPro; 5 µL M540; 993 µL PBS ZFL: 3% YoPro; 6% M540; 91% MCC	Merocianine 540 (M540) (Invitrogen - Eugene, OR, EUA) YOPRO-1 (Invitrogen - Eugene, OR, EUA)	High Fluidity Low Fluidity Whole Injured	> Intense Orange Fluorescence < Intense Orange Fluorescence <Intense Green Fluorescence > Intense Green Fluorescence	Photodetector BL2, filter 574/26 Photodetector BL1, filter 530/30	(Fernandez-Gago et al., 2013)
Mitochondrial Function	SPTZ*: 2% Rh123; 2% IP; 96% PBS ZFL: 10% IP; 20% Rh123; 70% MCC	Rhodamine 123 (R8004-5mg, Sigma Chemical Company, St. Louis, MO, USA) Propidium Iodide (IP)	High Functionality Low Functionality Viable	> Green Fluorescence in the Middle Piece < Green Fluorescence in the Middle Piece No Fluorescence	Photodetector BL1, filter 530/30	(He and Woods, 2004)

			Non-Viable	Red Fluorescence	Photodetector BL3, filter 640LP	
Reactive Oxygen Species (ROS)	SPTZ*: 1% DCF; 2% IP; 97% PBS ZFL: 10% IP; 10% DCF; 80% MCC	2',7'-Dichlorofluorescin diacetate (DCF-DA) Propidium Iodide (IP)	Viable Non-Viable	Green Fluorescence No Fluorescence Red Fluorescence	Photodetector BL1, filter 530/30 Photodetector BL3, filter 640LP	(Dominguez-Rebolledo et al., 2011)
Lipid peroxidation (LPO)*	-	4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (BODIPY® 581/591 C11	Non-peroxidized Peroxidized	Red Fluorescence Green Fluorescence		(Hagedorn et al., 2012)
DNA Fragmentation	SPTZ and ZFL: 5 µL AO; 10 µL Triton X-100; 5 µL TNE (0.01 M Tris-HCl; 0.15 M NaCl; 0.001 M EDTA; pH 7.2)	Acridine Orange (AO)	Intact DNA Injured DNA	Green Fluorescence Orange Fluorescence		(Evenson et al., 1994; Jenkins et al., 2015)

Table 2. Means and standard error means of membrane fluidity (MF), mitochondrial function (MIF), lipid peroxidation (LPO), and index of DNA fragmentation (DNA) assessments in sperm cells in groups treated with ATP in the following concentrations: 0mM (Control), 0.025 mM, 0.25 mM, 2.5 mM, and 25 mM. Data are presented as in percentage of cells.

Treatment	10 min				60 min			120 min		
	MF	MIF	LPO	DNA	MF	MIF	LPO	MF	MIF	LPO
0 mM (Control)	36.19 ±4.81[a]	21.74 ±2.73[a]	40.02 ±3.20[a]	0.036 ±0.008[a]	38.81 ±3.76[a]	15.01 ±2.14[a]	44.28 ±3.70[a]	46.99 ±3.70[a]	12.39 ±1.74[a]	49.61 ±4.03[a]
0.025 mM	36.28 ±3.31[a]	19.24 ±2.44[ab]	39.99 ±3.61[a]	0.044 ±0.014[a]	44.75 ±3.80[a]	13.84 ±1.94[a]	38.45 ±4.27[a]	52.51 ±3.64[a]	11.81 ±1.86[a]	46.28 ±3.99[a]
0.25 mM	34.01 ±3.90[a]	21.59 ±2.43	39.95 ±3.29[a]	0.035 ±0.009[a]	46.89 ±3.33[a]	15.65 ±2.22[a]	37.51 ±3.76[a]	50.16 ±3.04[a]	11.01 ±1.67[a]	44.21 ±4.05[a]
2.5 mM	40.44 ±4.16[a]	20.76 ±2.61[a]	39.39 ±3.40[a]	0.043 ±0.010[a]	46.71 ±3.25[a]	15.68 ±2.34[a]	37.51 ±3.66[a]	47.30 ±3.26[a]	12.75 ±1.84[a]	45.00 ±4.10[a]
25 mM	41.13 ±3.59[b]	10.80 ±1.55[b]	39.82 ±3.59[a]	0.046 ±0.016[a]	53.21 ±4.13[b]	10.22 ±1.65[a]	42.48 ±4.07[a]	57.75 ±2.86[b]	14.05 ±2.71[a]	46.28 ±3.95[a]

* Different letters indicate statistical differences within columns ($p < 0.05$).

Table 3. Means and standard error means of membrane fluidity (MF), mitochondrial function (MIF), lipid peroxidation (LPO), index of DNA fragmentation (DNA), and reactive oxygen species (ROS) assessments in ZFL cells in groups treated with ATP in the following concentrations: 0mM (Control), 0.025 mM, 0.25 mM, 2.5mM, and 25mM. Data are expressed in fluorescence intensity.

Treatment	120 min				
	MF	**MIF**	**LPO**	**DNA**	**ROS**
0 mM (Control)	16367 ±543.31[a]	85691 ±3837.1[a]	41.400 ±2.1500[a]	0.0617 ±0.0004[a]	15739 ±1254.60[a]
0.025 mM	15100 ±419.52[a]	89943 ±7327.1[a]	43.700 ±3.0600[a]	0.0621 ±0.0004[a]	16441 ±931.53[a]
0.25 mM	15744 ±404.77[a]	78742 ±3202.6[a]	40.725 ±1.7010[a]	0.0611 ±0.0004[a]	16253 ±837.26[a]
2.5 mM	15220 ±885.90[a]	76481 ±3758.7[a]	41.237 ±1.1590[a]	0.0620 ±0.0005[a]	16456 ±758.46[a]
25 mM	15340 ±406.88[a]	80807 ±1301.9[a]	40.975 ±1.4568[a]	0.0617 ±0.0005[a]	16241 ±1042.30[a]

3.3 Artigo 3

Material didático: Manequim para palpação abdominal e vagina de pequenos animais

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Didactic Materials: Mannequin for Canine Abdominal and Vaginal Palpation

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Abstract

Techniques and imaging modalities employed in the canine veterinary obstetric routine are considerably complex and delicate, and demand high precision and accuracy both by professionals and students. Compared to the veterinary education system decades ago, ethical and welfare questions are of major relevance in the current scenario. Therefore, certain learning practices involving *in vivo* animal studies are no longer performed. To overcome this limitation, the use of mannequins has emerged as an interactive and immersive method of learning that simulates a real situation. Furthermore, these provide the trainees with a margin of error without the risk of injuring or damaging the patient and thus considered to be highly useful. The aim of the present study was to develop mannequins to teach obstetrics to veterinary students. Here, we used a plastic canine prototype to simulate the abdominal and vaginal palpation technique. An abdominal incision was made, filled with synthetic fiber, and a fictional uterus was placed. In the same mannequin, an orifice was created that correspond to the vagina. This model greatly helped in training students in performing palpation techniques, conducting the static evaluation, and assessing maternal-fetal ratio. Moreover, the obstetric prototype made possible the demonstration of manometry of abdominal and vaginal palpation. This practice of

vaginal palpation was found to be of great value since several students could easily take the examination and identify different stages of canine gestation, which could hardly be achieved using animals. Mannequins have received good student acceptance owing to their safety and ease to use and resulted in a better understanding of the procedure to perform future *in vivo* animal studies.

Keywords: Canine prototype teaching approaches; animal simulator; dog pregnancy; palpation examination, hands-on learning

Introduction

The past years have witnessed a tremendous advancement in the learning and education method. The development of audiovisual and tactile resources has immensely contributed to technological evolution. These novel education modalities also provide students with the necessary abilities, knowledge, and comprehension required in the veterinary profession. A remarkable advancement is encouraging the adoption of alternative resources to minimize the use of animals *in vivo* in practical classes (Martinsen, 2005). This has become an important principle of veterinary practice to protect animals. However, during veterinary medicine study, undergraduates are faced with difficult situations and involving excessive manipulation. These challenges often make the study of this specialty not very attractive and involve considerable ethical and moral aspects (Fletcher, 2012).

The predilection for didactic materials in experimentation and other teaching activities is attributed to increased ethical consideration of animal welfare, students' ethical development, and the availability of new alternative methods that satisfactorily imitate structures and circumstances involved in clinical and surgical professional routines. It is especially important for those students who feel insecure while performing maneuvers for the first time in living models. In this way, the use of model animals has become an optional and less determinant situation for providing quality education in certain subjects of the veterinary medicine course (Martinsen, 2005).

Since the 1960s, the premise of "3 R's Principles on Animal Experimentation (Replacement, Reduction, and Refinement)" determined by Russel and Burch, laid the foundation for mimicking animal application, using mannequins, prototypes, and other devices. In addition, recognizing the importance of practical teaching activities that

favor adherence of knowledge to the perspective of meaningful learning, the National Council for Control of Animal Experimentation (CONCEA) in Brazil, within its competencies, encourages the introduction of alternative methods in place of animals in both research and teaching (Marques, 2005; Rivera, 2006). Therefore, ideal replacement methods for teaching practices involve model animals for training and after that, skills can be improved with animals that need intervention (Martinsen, 2005).

Prototypes and mannequins include synthetic objects designed to replicate or simulate organs (limbs or whole animals) and physiological functions to teach clinical skills, including those required in critical care (Lima, 2018). In this context, the present work is a part of the teaching project “production of alternative didactic resource for the veterinary medicine course” of the Federal University of Pelotas and aims to describe a canine mannequin capable of mimicking the abdominal and vaginal palpation of a pregnant female, thus enriching the practical class with the subject “Obstetrics and Mammary Gland.”

Methods

To simulate the abdominal and vaginal palpation technique, a plastic canine clothing mannequin was employed in which an abdominal opening was created (Figure 1). Subsequently, the internal space was filled with synthetic fiber (Figure 2) to the point where the fictitious uterus (Figure 3) reached the surface to facilitate the sensitivity of the “fetuses.” Simulators of uterus and fetuses were created for various stages of fetal development. Next, the region was topped with a TNT fabric square. An orifice was also created in the region corresponding to the vagina. To simulate the vestibular mucosa, a “finger of procedure glove” was used by inverting and fixing it to the interior of the mannequin, providing training to students in palpation techniques, static assessment, and knowledge of maternal–fetus ratio.

Results and Discussion

The present study describes the use of mannequins to train students in the field of obstetrics and mammary gland as part of their practical veterinary course. The undergraduates expressed both a feeling of curiosity and security toward this approach. This prototype allowed “patient care” to be a significant attribute of the existing medical clinic routine. Thus, training and improvement of abdominal and

vaginal palpation techniques, as well as general and specific examination of the female reproductive tract, were presented in theoretical classes.

The main motivation for developing this mannequin was to provide students with an opportunity to practice abdominal and vaginal palpation in a pregnant canine female in different stages of embryonic development and evaluate the fetal statics. This increased confidence among students, and consequently, prepared them to perform a gestation diagnosis as part of dog clinical veterinary routine. These procedures provided technical advancements in gestation diagnosis, identification of gestational stage, and prenatal follow-up. Abdominal palpation is one of the first methods among the possible procedures used in gestational diagnosis, followed by radiographic and ultrasonographic examinations and regulation of plasma relaxin. The palpation pregnancy diagnosis is always indicated because it is an early, safe, and cheap method; however, it requires extreme professional precision (Concannon, 2001; Johnston, 2001).

The use of alternative didactic materials has increased the interest and curiosity of veterinary students, who reported a greater comprehension of procedures and safety in performing future *in vivo* animal studies. Students require practical experience to deal with living tissues and animals. To achieve this, the development of methodologies that bring the undergraduates closer to reality such as consultations, simulations, and the introduction of tactile resources, such as a mannequin, attract the attention of students as compared to videos and other audiovisual resources. It is an important training tool that enables students to feel more secure (Martinsen, 2005)

Plastic models are used to demonstrate internal structures, such as morphology and orthopedics, to exemplify fracture types. Mannequins are increasingly used as a clinical training tool to perform palpation, blood collection, intubation, thoracentesis, and cardiopulmonary resuscitation. Simulators, in turn, are developed to facilitate the learning of surgical techniques, critical care, and clinical practices (Martinsen, 2005). These are alternatives to provide greater skill and safety to undergraduates.

Mannequins have found more frequent use in human medicine. The first prototype marketed in veterinary medicine was the “Jerry Dog for Critical Care,” a perfect model for emergency care training. It integrates a digital simulator of breathing and heart sounds, as well as opportunities to practice intubation, cardio-respiratory resuscitation, intravenous accession, and other skills (Fletcher, 2012). The use of static mannequins for teaching provides a safe learning environment, reduces animal

suffering, decreases stress and/or fears of students, prepares students for future patient procedures, and reproduces structures and situation in a real way. The absence of emotions, such as anxiety, stress, and insecurity may be beneficial to students; emotional states induced by harmful practices can directly affect learning and memory (Marques, 2005)

Conclusions

In conclusion, in addition to being economically viable, elaboration of mannequins using simple and easy to execute techniques makes the practical application of these models more palpable, dynamic, interesting, and readily available to students. Moreover, it does not require a pregnant animal to perform the techniques. These resources are expected to accurately train veterinary students to achieve greater self-confidence while performing future *in vivo* procedures.

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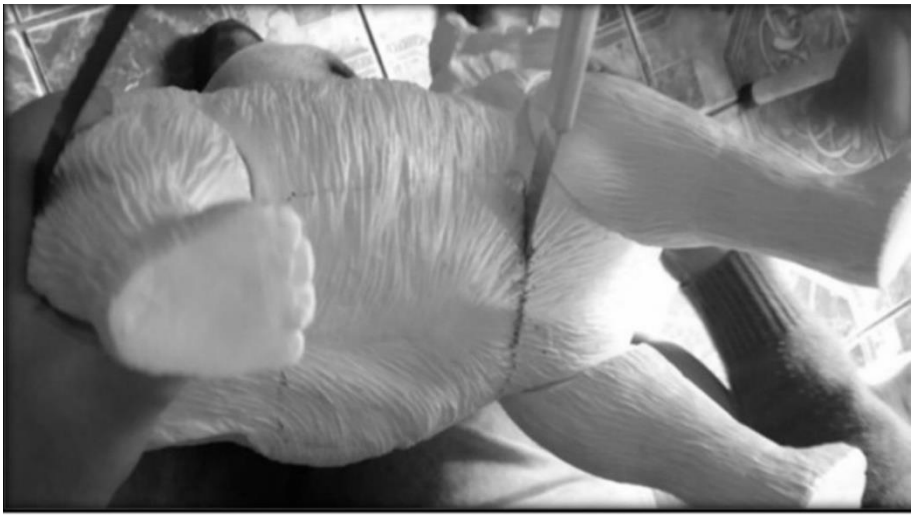

Figure 1: Abdominal opening with knife.

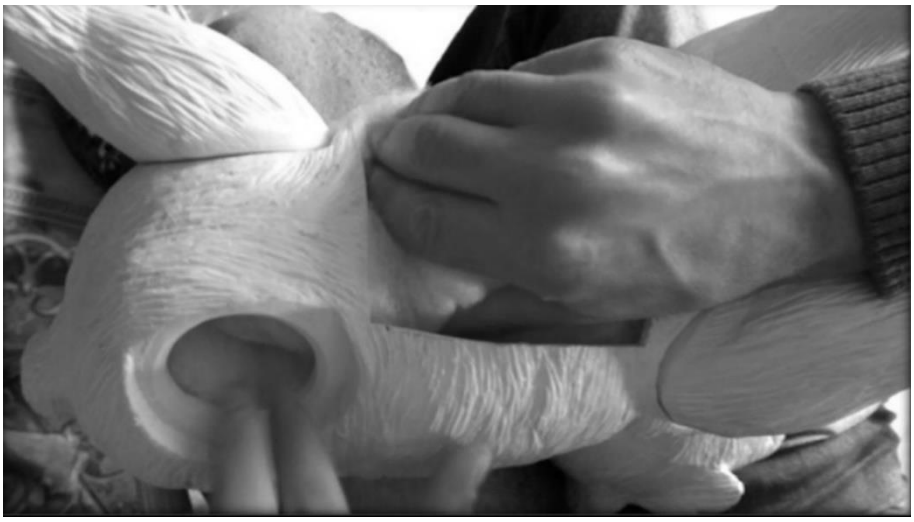

Figure 2: Abdominal space filled with synthetic fiber.

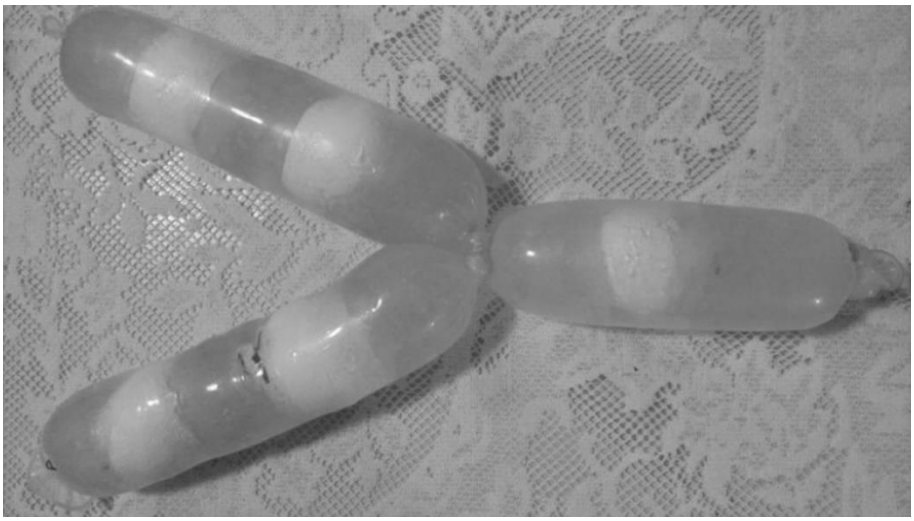

Figure 3: Fictitious uterus filled by mucilage interspersed with ballon balls filled with modeling mass.

3.4 Artigo 4

I Ciclo de Palestras em Pré-Natal de Pequenos Animais

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I CICLO DE PALESTRAS EM PRÉ-NATAL DE PEQUENOS ANIMAIS

RESUMO

A neonatologia tem despertado o interesse de diversos médicos veterinários, principalmente daqueles que prestam assistência à gatis ou canis. O I Ciclo de Palestras em Pré-Natal de Pequenos Animais, realizado no período de 19 a 23 de junho do ano de 2017 pelo Núcleo de Ensino e Pesquisa em Reprodução Animal (ReproPEL) da Universidade Federal de Pelotas, teve como principal objetivo disseminar e propagar informações dos mais diferenciados temas acerca da obstetrícia em pequenos animais e como superar os obstáculos que ainda persistem sobre esse processo. As palestras foram ministradas por médicos veterinários especialistas em medicina felina, endocrinologia, reprodução, imagenologia e anestesiologia, expondo divergentes visões a respeito da complexidade do tema abordado colaborando para disseminação e conhecimento na área.

Palavras-chave: Neonato; Obstetrícia; Pré-natal; Pequenos Animais; Bem-estar.

ABSTRACT

Neonatology has instigated the interest of veterinarians, especially those who provide assistance to kennels. The I Ciclo de Palestras em Pré-Natal de Pequenos Animais, held in the period from June 19 to 2, 2017 by Núcleo de Ensino e Pesquisa em Reprodução Animal (ReproPEL) of Federal University of Pelotas, aimed to disseminate and propagate a lot of information by small animals obstetric and how to overcome the obstacles that persist on this process. The speeches teach by veterinarian's specialists in feline medicine, endocrinology, reproduction, imaging and anesthesiology, exposed divergent views on the complexity of the topics collaborating for dissemination and knowledge in the area.

Key-words: Neonate; Obstetric; Prenatal; Small Animals; Welfare.

1 INTRODUÇÃO

A neonatologia tem despertado o interesse de diversos médicos veterinários, principalmente daqueles prestam assistência à gatis ou canis, já que o acompanhamento da gestante e o cuidado pré-natal adequado estão intimamente relacionados ao nascimento de

filhotes sadios e à redução da mortalidade neonatal. Por muito tempo, a busca por informações nesse âmbito foi negligenciada pela carência de estudos referentes a esta etapa da vida da fêmea e dos neonatos e hoje, nos deparamos com cenários que revelam os benefícios que a intervenção veterinária pode realizar na saúde e bem-estar da mãe e dos filhotes. Neste contexto, observa-se a possível insegurança do profissional veterinário frente às necessidades básicas destas categorias e a quadros de enfermidades durante o período gestacional e neonatal, notando-se a grande importância da realização do I Ciclo de Palestras em Pré-natal de Pequenos Animais.

Portanto, este projeto tem como propósito atualizar os profissionais e a comunidade acadêmica sobre problemas referentes ao estágio gestacional da fêmea como diagnóstico de gestação, acompanhamento de gestantes obesas e diabéticas, procedimentos anestésicos para esta categoria, suplementações vitamínicas e hormonais, assim como novos métodos de intervenção alimentar para a gestação e nascimento de um filhote saudável, estabelecendo diferenças entre espécies (caninos e felinos). Assim, este trabalho tem como objetivo descrever a metodologia empregada e os resultados alcançados com a promoção do I Ciclo de Palestras em Pré-Natal Pequenos Animais.

2 METODOLOGIA

O I Ciclo de Palestras em Pré-natal de Pequenos Animais foi idealizado pelo Núcleo de Ensino e Pesquisa em Reprodução Animal (ReproPEL) da Universidade Federal de Pelotas (UFPel). A organização do evento teve início com a decisão dos temas a serem abordados bem como dos profissionais ministrantes, abordando cinco palestras, distribuídas na semana de 19 a 23 de junho de 2017, perfazendo uma carga horária de cinco horas. A divulgação deste contou com a elaboração de um banner que foi exposto em murais da Faculdade de Medicina Veterinária e compartilhado nas redes sociais (Figura 1). As inscrições ocorreram por meio de preenchimento de formulário *online*. No decorrer do evento, a organização foi realizada pelos graduandos colaboradores do grupo, contando, também, com o auxílio de pós-graduandos e docentes envolvidos na idealização do projeto. Os ministrantes convidados exibiram suas palestras em apresentações digitais em um tempo médio de uma hora, permitindo a abertura de discussões e sanamento de dúvidas na sequência.

Figura 1. Arte de divulgação criada para o evento, demonstrando toda a tenção e o carinho que temos que ter com a mãe e o filhote de pequenos animais.

A frequência de cada inscrito era controlada a partir de um sistema de *check-in* com auxílio de planilhas administradas pela equipe organizadora (Figura 2). Ao final do evento, realizou-se a pesquisa de satisfação por meio da distribuição de fichas para que os participantes pudessem fazer sua avaliação quanto aos temas abordados, palestrantes escolhidos, grau de importância pessoal sobre a temática abordada e organização do ciclo. Além destes pontos, o público pôde dissertar sobre pontos fortes e fracos, bem como fazer sugestões para uma possível segunda edição do evento.

Figura 2. Equipe responsável pela preparação, execução e avaliação do evento.

3 RESULTADOS E DISCUSSÃO

Durante a gestação, o organismo da fêmea é moldado para o evento do nascimento. Por este fato, cada momento de cada ciclo reprodutivo é importante, bem como o exame clínico, exames laboratoriais e de imagem para acompanhar o desenvolvimento natural do feto**.** O evento colocou em pauta tópicos particulares, como as peculiaridades da gestação de felinos, haja vista as disparidades de aspectos fisiológicos e, especialmente, etológicos, entre as espécies canina e felina, além de dissertar sobre possiveis fatores que podem levar a distocia que podem ocorrer durante ou após o parto e como evita-los, como diabetes melittus, hipocalcemia, doenças endócrinas, dentre outras.

A diabetes mellitus canino (DMC), uma endocrinopatia comum rotina clínica com origem multifatorial, como genética, autoimune e um ambiente diabetogênico. A DMC ocorre principalmente em fêmeas no período gestacional, devido ao predomínio da progesterona e influência da prolactina (PÖPPL & GONZÁLEZ, 2009; NELSON, 2010). Outro distúrbio endócrino importante neste meio, é a hipocalcemia puerperal, que pode ocorrer em cadelas (particularmente de raças pequenas com ninhadas numerosas), sendo causada por um aumento súbito no requerimento de cálcio para a produção de colostro e leite (NELSON, 2010**).** Não

obstante, a pseudogestação clínica, associada a afecções endócrinas, é uma síndrome observada em cadelas não gestantes, 6 a 14 semanas após o estro, caracterizada por sinais clínicos e mimetização dos comportamentos pré, peri e pós-parto (MARTINS & LOPES, 2005).

Ocorrência de parto anormal, quando há falha em iniciar o parto no momento correto, ou quando há problema na expulsão normal dos fetos, uma vez que o parto tenha iniciado (LUZ, 2004). Os exames complementares como o ultrassom são uteis no diagnóstico dos partos anormais e malformações fetais, bem como no acompanhamento dos batimentos cardíacos fetais pré parto. Existem outros exames complementares como a avaliação laboratorial por meio de hemograma e dosagens de cálcio, glicose, uréia e creatinina, embora apenas a realização de hemograma seja rotina nas clínicas. (JOHNSTON et al., 2001).

Outro tema de grande relevância abordado foi a intervenção durante a distocia, com a realização de manobras obstétricas digitais para a retirada de fetos que estejam obstruindo o canal do parto (LUZ, 2004; LINDE-FORSBERG & ENEROTH, 1998; ENEROTH et al., 1999). Têm-se as opções, também do tratamento medicamentoso e cirúrgico, mas caso haja comprometimento materno, a cesariana deve ser imediatamente realizada, associada à terapêutica complementar (JOHNSTON et al., 2001).

Além disso, o uso seguro de medicações pré-anestésicas e anestésicas deve ser feito com cautela, ressaltando os opioides butorfanol, tramadol e meperidina, e os tranquilizantes como a acepromazina que devem ser utilizados apenas para fêmeas muito agitadas. Dentre os agentes para indução anestésica, tem-se o propofol, o tiopental, a cetamina e deve-se associar a cetamina aos benzodiazepínicos, para minimizar a rigidez muscular e possíveis convulsões. É importante ressaltar que o uso da anestesia epidural é considerada uma boa opção, principalmente para fêmeas menos agitadas, já que os filhotes nascem vigorosos e rapidamente apresentam reflexo de sucção (MUIR & HUBBELL, 2001; MASTROCINQUE, 2002).

A primeira edição do evento contabilizou um público geral de cerca de 50 pessoas. Os pontos supracitados foram difundidos, ao longo do evento, firmando a ideia de que é uma área que necessita de atenção já na graduação, ressaltando a importância da especialidade, onde o profissional da área deve ter conhecimento sobre os eventos normais do parto, a necessidade de uma cesariana e as particularidades do paciente neonatal, além de realizar um exame clínico completo para que seja capaz de identificar algumas alterações. O interesse pelo conhecimento geral ou pela especialização na área foi despertado, cumprindo com o objetivo do referido projeto. O conteúdo das fichas foi cautelosamente registrado e interpretado, constituindo o *feedback* fundamental para a organização e realização dos ciclos subsequentes.

4 CONSIDERAÇÕES FINAIS

O I Ciclo em pré-natal de Pequenos Animais colaborou com a disseminação de conhecimento acerca dos cuidados que devem ser administrados com as fêmeas gestantes e seus recém-nascidos, respeitando as peculiaridades em nível de fisiologia, metabolismo e comportamento da categoria. Desta forma, pôde-se despertar o interesse por parte de graduandos e profissionais da medicina veterinária na referida especialidade, agregando benefícios ao público e, também, à equipe de palestrantes e organizadores de forma a impulsionar o aumento de estudos nessa área e minimizar a mortalidade materna e fetal.

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4 Considerações Finais

- Eventos de ensino como o I Ciclo em Pré-Natal de Pequenos Animais colabora com a disseminação de conhecimento acerca dos cuidados que devem ser administrados com as fêmeas gestantes e seus recém-nascidos. Despertando o interesse por parte de graduandos e profissionais da medicina veterinária.
- A elaboração de métodos paradidáticos para o ensino é economicamente viável, a partir de técnicas simples e de fácil execução. Tornando as atividades práticas didáticas mais palpáveis, dinâmicas, interessantes e disponíveis aos discentes.
- As células espermáticas suínas demonstraram potencial para utilização como modelo de ensaio toxicológico, apresentando-se como um promissor método de triagem de substâncias tóxicas, através de análises como da cinética, estrutura e metabolismo celular.
- O uso tópico do óleo de copaíba nas concentrações estudadas diminui a qualidade espermática. Nossos resultados mostram que o óleo de copaíba a 0,01% e 0,1% por 14 dias causou alterações em células espermáticas diferenciadas. Além disso, após 21 dias, notamos que o uso de óleo de copaíba 0,1% causou alterações em estruturas formadas na fase inicial da espermatogênese, como o DNA e a mitocôndria.

Dessa forma, no período de doutoramento no Programa de Pós-Graduação em Veterinária foi possível realizar atividades nos três pilares da educação: extensão, ensino e pesquisa. Foram realizados trabalhos de divulgação de conhecimento para aos graduandos em medicina veterinária e para os médicos veterinários já formados, com atividade de ensino e extensão. Ainda, no ensino, foram desenvolvidos materiais paradidáticos, manequins, para aulas práticas da disciplina de Obstetrícia e Glândula Mamária, despertando interesse dos discentes, para proporcionar melhor entendimento e memorização do conteúdo ensinado. Por fim, no âmbito da pesquisa, foi possível propor um método alternativo ao uso de animais para rápida identificação de substâncias tóxicas.

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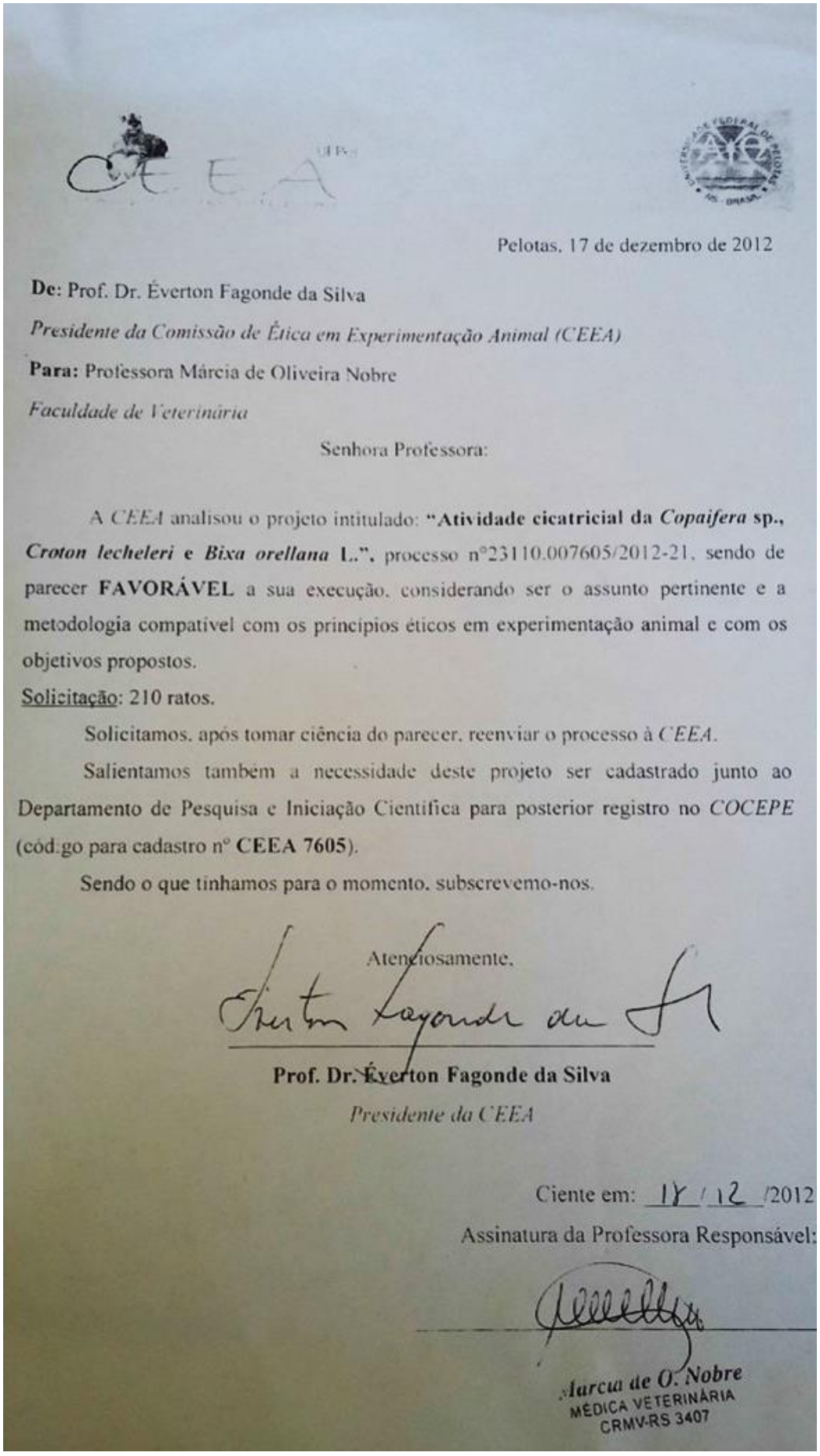
Anexos

Anexo I - Documento da Comissão de Ética e Experimentação Animal



CEEA UFPel

Pelotas, 17 de dezembro de 2012

De: Prof. Dr. Éverton Fagonde da Silva

Presidente da Comissão de Ética em Experimentação Animal (CEEA)

Para: Professora Márcia de Oliveira Nobre

Faculdade de Veterinária

Senhora Professora:

A *CEEA* analisou o projeto intitulado: "**Atividade cicatricial da *Copaifera* sp., *Croton lecheleri* e *Bixa orellana* L.**", processo nº23110.007605/2012-21, sendo de parecer **FAVORÁVEL** a sua execução, considerando ser o assunto pertinente e a metodologia compatível com os princípios éticos em experimentação animal e com os objetivos propostos.

Solicitação: 210 ratos.

Solicitamos, após tomar ciência do parecer, reenviar o processo à *CEEA*.

Salientamos também a necessidade deste projeto ser cadastrado junto ao Departamento de Pesquisa e Iniciação Científica para posterior registro no *COCEPE* (código para cadastro nº **CEEA 7605**).

Sendo o que tinhamos para o momento, subscrevemo-nos.

Atenciosamente,

Prof. Dr. Éverton Fagonde da Silva

Presidente da CEEA

Ciente em: 18 / 12 /2012

Assinatura da Professora Responsável:

Márcia de O. Nobre
MÉDICA VETERINÁRIA
CRMV-RS 3407