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OZONIOTERAPIA NA MEDICINA VETERINÁRIA E SUA AÇÃO ANTIBACTERIANA NA CISTITE CANINA

TESE DE DOUTORADO

Santa Maria, RS
2023

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Tese apresentada ao Curso de Pós-Graduação em Medicina Veterinária, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do título de Doutora em Medicina Veterinária.

Orientadora: Profª Drª Cinthia Melazzo de Andrade

Santa Maria, RS
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Aprovado em 27 de março de 2023:

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RESUMO

OZONIOTERAPIA NA MEDICINA VETERINÁRIA E SUA AÇÃO ANTIBACTERIANA NA CISTITE CANINA

AUTORA: Ana Martiele Engelmann
ORIENTADORA: Cinthia Melazzo de Andrade

Esta tese de doutorado foi dividida em três partes, resultando em dois manuscritos e um artigo científico. O primeiro manuscrito constituiu em verificar as principais prescrições de tratamentos com ozônio medicinal na clínica de pequenos animais, além de verificar o perfil do profissional que a prescreve. Para tanto, um questionário estruturado foi disponibilizado em plataforma digital durante o mês de abril e o mês de maio de 2021. A divulgação da pesquisa foi realizada por meio das redes sociais e encaminhamento aos Programas de Residência e de Pós-Graduação em Medicina Veterinária dos três estados do sul do Brasil (Rio Grande do Sul, Santa Catarina e Paraná), além de veterinários de clínicas e hospitais e que prestam atendimento domiciliar. Dentre os 213 participantes da pesquisa, 98,1% afirmaram utilizar ao menos uma terapia integrativa na prática clínica, sendo que as mais utilizadas foram a fisioterapia (81,7%), a acupuntura (80,3%) e a ozonioterapia (68,5%). Dentre os participantes, 72% afirmaram aplicar ou recomendar a ozonioterapia (OZT). As principais enfermidades tratadas com a OZT foram a cicatrização de feridas abertas (91,1%), as dermatites (63,7%), a assepsia de feridas contaminadas (46,6%) e a cicatrização pós-cirúrgica (49,7%). Dentre os participantes que não aplicam/recomendam a OZT, os principais motivos foram a falta de comprovação da eficácia (38,6%) e falta de conhecimento técnico do profissional (35,1%). O segundo manuscrito teve como objetivo avaliar a atividade antimicrobiana *in vitro* da solução salina ozonizada (SSO_3) sobre cepas padrão e sobre isolados bacterianos resistentes e com perfil de multirresistência comumente associados a cistite em cães. Para isto, utilizou-se o método *plating* para avaliar a atividade antimicrobiana da exposição por 60 segundos de SSO_3 a 78 $\mu g\ mL^{-1}$ sobre as cepas padrão (*Staphylococcus aureus* e *Escherichia coli*) e isolados multirresistentes (*Proteus mirabilis*, *Klebsiella* sp. e *Enterococcus* sp.), e o teste de microdiluição em caldo para determinar a concentração inibitória e bactericida mínima para as cepas de *S. aureus*, *E. coli* e *Pseudomonas aeruginosa* dessa mesma solução. O *plating* foi o único ensaio em que o tratamento com a SSO_3 resultou em atividade bactericida, promovendo uma redução superior a 99% das unidades formadoras de colônia para a maioria das cepas testadas, exceto para o *P. mirabilis*. O terceiro estudo, teve como objetivo relatar a eficácia de dois protocolos diferentes de irrigação da bexiga com SSO_3 em um canino paraplégico com cistite bacteriana recorrente causada por *Proteus* spp. A primeira abordagem terapêutica aplicada, a qual consistiu em repetidas instilações de 60 mL de SSO_3 a 59 $\mu g\ mL^{-1}$, uma vez ao dia, por três dias consecutivos, não foi efetiva. Já, o segundo protocolo utilizado, que consistiu de sucessivas lavagens em um único dia, respeitando-se um intervalo de 2 horas entre as mesmas, resultou em uma redução significativa da presença de bactérias na sedimentoscopia da urina e na urocultura. De forma geral, os dados deste estudo forneceram um leque de usos potenciais da ozonioterapia na clínica médica de pequenos animais que poderá ser explorado principalmente pela comunidade científica para traçar estudos experimentais futuros, buscando a consolidação do conhecimento acerca do ozônio medicinal na medicina veterinária.

Palavras-chave: Mistura oxigênio-ozônio. Terapia integrativa. Infecção do trato urinário. Efeito bactericida. Solução fisiológica ozonizada.

ABSTRACT

OZONE THERAPY IN VETERINARY MEDICINE AND ITS ANTIBACTERIAL ACTION IN CANINE CYSTITIS

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This doctoral thesis was divided into three parts, resulting in two manuscripts and a scientific article. The first manuscript consisted of verifying the main prescriptions of treatments with medicinal ozone in the small animal clinic, in addition to verifying the profile of the professional who prescribes. To this end, structured questionnaire was made available on a digital platform during April and May 2021. The research was carried out through social networks and forwarded to the Residency and Postgraduate Programs in Veterinary Medicine in the three southern states of Brazil (Rio Grande do Sul, Santa Catarina and Paraná), as well as veterinarians from clinics and hospitals who provide home care. Among the 213 research participants, 98.1% claimed to use at least one integrative therapy in clinical practice, with the most used being physiotherapy (81.7%), acupuncture (80.3%) and ozone therapy (OZT) (68.5%). Among the participants, 72.0% claimed to apply or recommend ozone therapy. The main diseases treated with OZT were healing of open wounds (91.1%), dermatitis (63.7%), asepsis of contaminated wounds (46.6%) and post-surgical healing (49.7%). Among the participants who do not apply/recommend it, the main reasons were the lack of proof of effectiveness (38.6%) and the lack of technical knowledge of the professional (35.1%). The second manuscript aimed to evaluate the in vitro antimicrobial activity of the ozonized saline solution (O_3SS) over standard strains and on resistant or multi-drug resistant isolates commonly associated with cystitis in dogs. For this, the plating method was used to evaluate the antimicrobial activity of exposure for 60 seconds of O_3SS at 78 μg mL^{-1} on standard strains (*Staphylococcus aureus* and *Escherichia coli*) and multiresistant isolates (*Proteus mirabilis*, *Klebsiella* sp. and *Enterococcus* sp.), and the broth microdilution test to determine the minimum inhibitory and bactericidal concentration for strains of *S. aureus*, *E. coli* and *Pseudomonas aeruginosa* in the same solution. Plating was the only assay in which treatment with O_3SS resulted in bactericidal activity, promoting a reduction greater than 99% of colony forming units for most strains collected, except for *P. mirabilis*. The third study aimed to report the effectiveness of two different protocols of bladder irrigation with O_3SS in a paraplegic canine with recurrent bacterial cystitis caused by *Proteus* spp. The first treated approach applied, which consisted of repeated instillations of 60 mL of O_3SS at 59 μg mL^{-1}, once a day, for three consecutive days, was not effective. On the other hand, the second protocol used, which consisted of successive washes in a single day, respecting an interval of 2 hours between them, resulted in a significant reduction in the presence of bacteria in urine sedimentoscopy and in urine culture. In general, the data from this study provide a range of potential uses of OZT in the medical clinic of small animals that can be explored mainly by the scientific community to outline future experimental studies, seeking to consolidate knowledge about medicinal ozone in veterinary medicine.

Keywords: Oxygen-ozone mixture. Integrative therapy. Urinary tract infection. Cystitis. Bactericidal effect. Ozonized physiological solution.

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ARTIGO 1

LISTA DE ABREVIATURAS E SIGLAS

ATP	Adenosina trifosfato
CAT	Catalase
CEP	Comitês de Ética em Pesquisa
$\bullet CO_3$	Radical carbonato
COX	Ciclooxigenase
DNA	Ácido desoxirribonucleico
2,3-DPG	2,3-difosfoglicerato
ERA	Elementos de resposta antioxidante
ERO	Espécie reativa de oxigênio
GSH	Glutationa reduzida
GPx	Glutationa peroxidase
GR	Glutationa redutase
GST	Glutationa S-transferase
HClO	Hipoclorito
H_2O	Água
H_2O_2	Peróxido de hidrogênio
HO-1	Heme oxigenase-1
$HO_2\bullet$	Radical hidroperóxido
HSP	Proteínas de choque térmico
IL-1β	Interleucina 1 *beta*
IL-6	Interleucina 6
IL-8	Interleucina 8
IL-10	Interleucina 10
IP3	Trifosfato de inositol
NOS	Óxido nítrico sintetase
ITU	Infecção do trato urinário
IM	Intramuscular
IR	Insuflação retal
INFγ	Inteferon-*gama*
iNOS	Óxido nítrico sintase induzível
Keap 1	Proteína 1 associada à ECH semelhante a Kelch

LOPs	Produtos da peroxidação lipídica
MPO	Mieloperoxidase
MAF	Fibrossarcoma musculoaponeurótico
MOO	Mistura oxigênio-ozônio
NADPH	Nicotinamida adenina dinucleotídeo fosfato
NADH	Nicotinamida adenina dinucleotídeo
NFAT	Fator nuclear
NF-κB	Fator nuclear *kappa* B
NLRP3	Pirina da família NLR contendo receptor 3
$\bullet NO_2$	Dióxido de nitrogênio
NO	Óxido nítrico
NQO-1	Quinquinona-oxido redutase
Nrf2	Fator nuclear eritróide tipo 2
O_2	Oxigênio
O_3	Ozônio
•OH	Radical hidroxila
$\bullet O_2^-$	Radical superóxido
$ONOO^-$	Peroxinitrito
OZT	Ozonioterapia
PNPIC	Política Nacional de Práticas Integrativas e Complementares
Proteína ASC	Proteína *speck-like* associada à apoptose adaptadora
PUFA	Ácidos graxos poli-insaturados
SOD	Superóxido dismutase
SSO_3	Solução salina ozonizada
SUS	Sistema Único de Saúde
TCR	Receptor de antígeno da célula T
TNF-α	Fator de necrose tumoral *alpha*
Trx	Tiorredoxina
TrxR	Tiorredoxina redutase
VEGF	Fator de crescimento endotelial vascular
PDGF	Fator de crescimento derivado de plaqueta
TGF-β	Fator transformador de crescimento beta

SUMÁRIO

1. INTRODUÇÃO

Atualmente, tem se notado o retorno para a visão milenar e ancestral de cuidar de forma integral do ser humano, percebendo-se não somente os aspectos relacionados à saúde física, mas também mental do indivíduo, visto que o ser humano é um ser biopsicossocial (GALLI et al., 2012). Sabe-se que aspectos emocionais, psicossociais, afetivos e espirituais são fatores que afetam a saúde proporcionando o aparecimento de doenças no corpo físico (ANDRADE; ALMEIDA DA COSTA, 2010). Desta forma, é crescente o interesse pelas terapias integrativas/complementares como métodos para auxiliar o indivíduo no seu processo de adoecimento e cura (GALLI et al., 2012).

As terapias Integrativas e Complementares, também denominados pela Organização Mundial da Saúde (OMS) como pertencentes à Medicina Tradicional e complementar/alternativa (MT/MCA), compreendem um grupo de práticas de atenção à saúde não alopáticas e englobam atividades como a acupuntura, naturopatia, fitoterapia, meditação, reiki, terapia floral, dentre outras (OMS, 2001). A medicina integrativa não rejeita a medicina convencional, mas integra a ela as terapias alternativas ou complementares, com o objetivo de auxiliar no processo de recuperação da saúde (CASPI et al., 2003; KLIEGER et al., 2004).

Essa busca pelo bem-estar próprio também vem se estendendo aos animais de companhia, de forma que é crescente o interesse dos tutores por terapias integrativas com intuito terapêutico aos seus *pets* (MENON et al., 2016). Dessa forma, a medicina veterinária também vem buscando constantemente terapias integrativas e/ou alternativas capazes de auxiliar na resolução das enfermidades animais, principalmente quando há ineficácia nos tratamentos convencionais ou ocorrem efeitos adversos, ou ainda, quando há a resistência microbiana (MEMON et al., 2016; SHMALBERG; MEMON, 2015). São várias as terapias complementares já inseridas na medicina veterinária (CARSON; HAMMER; RILEY, 2006; SHMALBERG; MEMON, 2015; MEMON et al., 2016), no entanto, muitas ainda aguardam regulamentação. Um exemplo disso é a regulamentação, apenas em 2020, do uso do ozônio medicinal ou ozonioterapia (CFMV, 2020), o qual vem ganhando adeptos na medicina humana e animal devido aos relatos de eficácia.

A ozonioterapia é uma modalidade de terapia não farmacológica e de baixo custo, baseada na capacidade indutora antioxidante do ozônio (SCASSELLATI et al., 2020). Um dos maiores usos tanto em humanos quanto em animais está no tratamento de feridas e aceleração da cicatrização. Além das infecções de feridas de difícil resolução, outro desafio na prática

clínica é a resistência bacteriana. A ozonioterapia já se provou um antisséptico potente contra a maioria dos microrganismos envolvidos nas infecções cutâneas e também em casos de cistites bacterianas (LOGAN, 2018; SONG et al., 2018; ROTH et al., 2020; TASDEMIR et al., 2013; VILELA, 2015).

Os relatos de uso na medicina veterinária da ozonioterapia estão em constante crescimento numérico seja em grandes animais, pequenos, e até mesmo selvagens (FACO; XAVIER; CAZATI, 2012; MURPHY et al., 2016; RODRÍGUEZ et al., 2017; TEIXEIRA et al., 2013). Relatos de caso descrevendo os protocolos utilizados e o sucesso das terapias são amplamente disponíveis em bases científicas. No entanto, é necessário aprofundar os estudos visando investigar os reais mecanismos de ação dessa terapia em nível celular e sistêmico, criando informações de base para o entendimento científico, os mecanismos de ação, além de possíveis aplicações em patologias de forma a resguardar os profissionais que a prescrevem.

Diante desse cenário, por acreditarmos que é dever da pesquisa fornecer o conhecimento científico e técnico referente a novas formas de tratamento e a comprovação destas, objetivamos investigar o efeito bactericida da ozonioterapia no tratamento da infecção do trato urinário inferior de cães de forma clínica e *in vitro*, além de relatar as principais prescrições do ozônio medicinal recomendadas pelos médicos veterinários aos seus pacientes.

2. REVISÃO BIBLIOGRÁFICA

2.1 BREVE HISTÓRICO DA OZONIOTERAPIA

A primeira menção sobre o ozônio (O_3) na literatura científica foi feita pelo físico holandês Mark Van Marumom, em 1785, durante experimentos em uma instalação de eletrificação, onde observou o aparecimento de uma substância gasosa, incolor e com odor característico quando um arco elétrico atravessava o ar (SCHWARTZ; MARTÍNEZ-SÁNCHEZ, 2012). Essa substância fora nomeada de ozônio que é derivada da palavra grega *ozein*, cujo significado é "cheiro" (SRIKANTH et al., 2013).

O primeiro registro bibliográfico da utilização da ozonioterapia (OZT) na Medicina data da primeira guerra mundial, quando o Dr. Albert Wolf aplicou essa terapia no tratamento antimicrobiano e na cicatrização de feridas de soldados durante a grande guerra (BOCCI, 2005). No entanto, o precursor do uso do O_3 de forma "medicinal" foi Werner von Siemens, que em 1857 construiu o primeiro tubo de indução para produzir ozônio a partir de oxigênio (VERANES et al., 1999). Este primeiro gerador fora utilizado para investigação das propriedades físico-químicas do O_3, dentre elas seu enorme potencial de destruição de microrganismos patogênicos. Com ele também se fez as primeiras insuflações e experiências de uso do O_3 em contato com as mucosas, tanto em animais como em humanos (RODRÍGUEZ et al., 2017).

Nas últimas três décadas, o desenvolvimento médico científico da OZT passou a ser um fenômeno cada vez mais crescente, o que acabou por culminar no aprofundamento da farmacologia e dos mecanismos moleculares do O_3 no uso médico (VIEBAHN-HAENSLER; LEÓN FERNÁNDEZ, 2021). Nas décadas de 1980 e 1990, foi demonstrada sua influência no metabolismo das hemácias com uma melhora na liberação de oxigênio como resultado final (WASHÜTTL; VIEBAHN, 1986), enquanto o impacto do O_3 nas células imunocompetentes foi o principal assunto da pesquisa de Bocci na década de 1990 (BOCCI; PAULESU, 1990; BOCCI et al., 1993). Já, o primeiro artigo que descreveu o efeito regulador do O_3 sobre o equilíbrio redox em nível celular foi publicado por León e seu grupo em 1998 (LÉON et al, 1998). Todos estes efeitos da OZT vêm sendo amplamente investigados em todo o mundo até os dias de hoje (VIEBAHN-HAENSLER; LEÓN FERNÁNDEZ, 2021).

2.2 CONCEITOS BÁSICOS NA PRODUÇÃO NATURAL E ARTIFICIAL DO OZÔNIO

O O_3 é um componente natural da atmosfera gerado principalmente por meio da ação de descargas elétricas e da luz ultravioleta (CENCI et al., 2022; SCIORSCI et al., 2020). No entanto, sua distribuição varia entre a parte superior (estratosfera) e inferior (troposfera) da atmosfera (SCIORSCI et al., 2020). Na estratosfera, o ozônio forma uma indispensável camada que filtra as radiações ultravioletas (HÄNNINEN, 2019). Já na troposfera, o O_3 é considerado um poluente gerado a partir da reação entre os óxidos de nitrogênio, os quais são liberados de fontes como escapamento de automóveis, emissões de usinas de energia e de incêndios florestais, e luz solar (LANGE et al., 2018).

Sua molécula está organizada em uma estrutura cíclica composta por três átomos de oxigênio elementar (BOCCI, 2005; SCHWARTZ; KONTORCHINIKOVA; MALESNIKOV, 2011). À temperatura ambiente, o gás apresenta coloração incolor, odor forte e pungente característico da substância. Devido à presença de estados mesoméricos em sua estrutura molecular, o O_3 é uma molécula altamente reativa e instável, sendo dez vezes mais solúvel em água e no plasma do que seu alótropo diatômico (oxigênio - O_2) (BOCCI, 2011; ELVIS; EKTA, 2011; ZANARDI et al., 2016). Além disso, sua potência oxidante é maior que a do O_2, constituindo-se no terceiro oxidante mais potente depois do flúor e do persulfato (BOCCI, 2011).

O O_3 usado para fins medicinais consiste em uma mistura gasosa de O_2 e O_3, obtida por meio do contato entre o oxigênio puro e um alto gradiente de voltagem (NOGALES et al., 2008). As concentrações clínicas típicas de O_3 variam de 10 a 80µg/mL (µg de O_3 / mL de O_2), sendo o volume total da mistura 5 a 0,05% correspondente ao gás ozônio e 95-99% ao oxigênio e, por este motivo, outro termo utilizado para se referir ao O_3 medicinal é “mistura oxigênio-ozônio” (MOO) (SCHWARTZ-TAPIA; MARTÍNEZ-SÁNCHEZ; SABAH, 2015; TRAVAGLI, 2020; ZANARDI et al., 2016). No entanto, embora mais de 95% da mistura gasosa seja sempre O_2, pequenas variações no conteúdo de O_3 alteram seus efeitos potenciais (CLAVO et al, 2019).

Devido à estrutura instável do O_3, a obtenção de altas concentrações da molécula tende a ser difícil de se alcançar (SMITH et al., 2017). O O_3 se decompõe em oxigênio molecular e atômico com uma meia-vida de 40 minutos a 20°C e aproximadamente 140 minutos a 0°C (ELVIS; EKTA, 2011).

Os dispositivos geradores de O_3 baseiam-se em três diferentes sistemas de geração: sistema ultravioleta, sistema de plasma frio e o sistema de descarga de corona - também conhecido como descarga de barreira dielétrica (NOGALES et al., 2008). Atualmente, os dispositivos que empregam a descarga de corona são os mais comumente utilizados, uma vez que são aparelhos de fácil manuseio e que permitem o controle da taxa de produção de O_3 (KOGELSCHATZ, 2003; KOMALI, 2012; PARK et al., 2006).

2.3 VIAS DE ADMINISTRAÇÃO DO OZÔNIO MEDICINAL

Diferentes vias podem ser utilizadas para a administração do O_3 medicinal, sendo estas classificadas em vias locais ou sistêmicas (BOCCI, 2005). A administração local pode ser executada por meio da aplicação intramuscular, intradiscal e paravertebral, oral, conjuntival, vaginal, vesical, pleural e peritoneal ou diretamente sobre a pele (RODRIGUEZ et., 2017). São consideradas vias sistêmicas a auto-hemoterapia com O_3 (SAGAI; BOCCI, 2011) e a insuflação do gás por via intrarretal e intraperitoneal (RODRIGUEZ et al., 2017). A escolha da via de administração é dependente das características do processo patológico e da condição geral do paciente (RODRÍGUEZ et al., 2017).

As vias locais de aplicação são principalmente utilizadas na odontologia em casos de cáries e infecções da raiz dentária (ALMAZ; SONMEZ, 2013; HALBAUER et al., 2013; SHILPA REDDY et al., 2013), e também na dermatologia em casos de feridas contaminadas e de difícil resolução (SHAH et al., 2011). O uso sobre a pele se dá por meio da administração da MOO no local de interesse, na forma de "*baggins*", lavagens com água ozonizada, solução salina ozonizada ou com aplicação de óleos vegetais ozonizados (XIAO et al., 2017; VIEBAHN-HAENSLER; LEÓN FERNÁNDEZ, 2021). Essa forma de aplicação local pode necessitar de períodos de tratamento um pouco mais longos, provavelmente devido ao menor poder de oxigenação ou ao efeito sistêmico inferior (SCHWARTZ; KONTORSCHIKOVA; MALESNIKOV, 2011). No entanto, uma das vantagens que esse método terapêutico proporciona é a possibilidade de aplicar a terapia a domicílio, sem a presença física dos pacientes no local onde os tratamentos com ozônio na forma de gás são realizados (SCHWARTZ; KONTORSCHIKOVA; MALESNIKOV, 2011). Além disso, esse método pode ser combinado com outras vias de aplicação de O_3 (SCHWARTZ; KONTORSCHIKOVA; MALESNIKOV, 2011).

Na via de aplicação tópica conjuntival utiliza-se o óleo ozonizado colírio no saco conjuntival afetado a cada oito horas (RODRÍGUEZ et al., 2017). Esse método tem sido bastante utilizado nos casos de ceratoconjuntivite causada por clamídias e pelo vírus do herpes em felinos, além do uso em transplantes de córnea, nas ceroconjuntivites e endoftalmites em coelhos e nas ceratoconjuntivites secas e infecciosas em cães (LAKE et al., 2004; RODRÍGUEZ et al., 2017).

A via tópica vesical consiste na instilação direta de um volume que varia conforme a capacidade do animal em 5-50mL da MOO e/ou de solução salina ozonizada (RODRÍGUEZ et al., 2017). Há vários trabalhos que descrevem a utilização da solução salina ozonizada para o tratamento de cistite, os quais demonstraram resultados promissores como a redução significativa da quantidade de mastócitos e leucócitos e a manutenção da integridade da mucosa (BAYRAK et al., 2014; NEIMARK et al., 2014; TASDEMIR et al., 2013). Bonforte e colaboradores (2013) trataram mulheres com infecção urinária recorrente provocadas por *Morganella morganni* e *Escherichia coli* através de instilações de solução salina ozonizada intravesicais em volumes crescentes. Houve completa remissão dos sintomas com resultados de cultura negativos. Além disso, neste mesmo estudo, também se utilizou a aplicação tópica vaginal por meio de lavagens com solução salina ozonizada para tratar um caso de vulvovaginite por *Candida* sp., no qual houve remissão dos sinais clínicos após 6 dias de tratamento. Assim como a via vesical, a via intravaginal consiste em passar um fluxo constante de água ou solução salina ozonizada, no interior da vagina, utilizando-se uma cânula (RODRÍGUEZ et al., 2017).

A aplicação intra-articular da MOO tem sido amplamente estudada em casos clínicos humanos (CALUNGA et al., 2012; CARDELLI et al., 2008; DAIF, 2012; MISHRA et al., 2011) e consiste na injeção direta do gás na articulação afetada (RODRÍGUEZ et al., 2017). Essa via é amplamente utilizada como opção terapêutica adjuvante para promover analgesia e melhorar a funcionalidade em pacientes humanos com lombalgia e osteoartrite de joelho (DE SIRE et al., 2021).

Em relação as vias sistêmicas de aplicação, a auto-hemoterapia maior e menor são métodos de ozonização extracorpórea, nos quais o sangue é misturado ao O_3 e novamente injetado no paciente (BORRELLI et al., 2012; FOGLIENI et al., 2011; HENSLER et al., 2009; VIEBAHN-HÄNSLER; LEON FERNANDEZ; FAHMY, 2012). A auto-hemoterapia maior ozonizada foi descrita pela primeira vez em 1974 por Wollf e consiste na retirada de sangue periférico em recipiente com anticoagulante citrato de sódio, sua ozonização e reinfusão

endovenosa (BOCCI, 2006). Na auto-hemoterapia maior a quantidade de sangue coletada varia de 5 a 150 mL de sangue de acordo com o peso do animal. É indicada para tratamento de desordens circulatórias arteriais, infecções, artrite reumática, imunoestimulação e tratamento de carcinoma em pacientes geriátricos (RODRÍGUEZ et al., 2017). Outra modalidade, a auto-hemoterapia menor, tem o mesmo princípio da auto-hemoterapia maior, mas o volume de sangue colhido é menor, geralmente no volume de 1mL/10kg de peso corpóreo do animal, e a reinfusão do sangue é intramuscular (IM) (RODRÍGUEZ, 2017; VIEBAHN-HÄNSLER; LEON FERNANDEZ; FAHMY, 2012). É indicada para tratamento de alergias, furunculoses, e adjuvante no tratamento do câncer (NOGALES et al., 2008).

A insuflação retal (IR) é classificada como uma técnica de administração sistêmica devido à alta irrigação sanguínea da qual esta região anatômica é composta, o que favorece a absorção de qualquer substância (RODRÍGUEZ, 2011). A IR de O_3 foi descrita pela primeira vez por Aubourg (1940) para tratamento de colite crônica e fístulas (BOCCI et al., 2012). A partir de então, essa técnica começou a ser amplamente utilizada por ser de fácil aplicação e baixo custo (RODRÍGUEZ et al., 2017). De acordo com Rodríguez et al. (2017), essa técnica consiste da introdução de cerca de 5cm de uma sonda retal lubrificada no animal através do esfíncter anal. Em seguida, o ozônio é carregado em uma seringa no volume de 5mL de gás/kg de peso corporal e então insuflado de forma lenta (RODRÍGUEZ et al., 2017).

Apesar de Bocci e colaboradores (2012) questionarem a eficácia e segurança da IR de O_3, uma vez que alguns fatores como quantidade de material fecal no reto e a ocorrência de flatulência durante a aplicação acaba-se por invalidar a técnica, pois não é possível garantir que a dose aplicada seja realmente absorvida, muitos estudos têm demonstrado resultados promissores acerca dessa via de aplicação. De forma geral, essa via é indicada para desordens circulatórias arteriais, imunoestimulação, adjuvante na terapia contra o câncer (CALUNGA et al., 2009; MARTÍNEZ-SÁNCHEZ et al., 2005; NOGALES et al., 2008; ZAKY et al., 2011), e também para promover efeito analgésico como em estados pós operatórios, doenças articulares e no alívio de queimaduras cutâneas (RODRÍGUEZ et al., 2017; TEIXEIRA et al., 2013; VIEBAHN-HAENSLER; LEÓN FERNÁNDEZ, 2021).

A via intraperitoneal é nova e pouco utilizada e consiste em injetar um volume determinado do gás por meio de uma agulha acoplada a uma seringa (RODRÍGUEZ et al., 2017). Sempre que aplicada, o controle da pressão intra-abdominal deve ser realizado pois, o aumento da mesma pode conduzir à síndrome de hipertensão compartimental com consequente falência múltipla dos órgãos e morte súbita do animal (RODRÍGUEZ et al., 2017). Apesar

disso, existem vários estudos experimentais de choque séptico em modelos animais (ratos e camundongos) que demonstraram o efeito modular da resposta inflamatória sistêmica e o reestabelecimento do equilíbrio redox induzido por esta via (RODRÍGUEZ et al., 2009; SILVA et al., 2009; SOUZA et al., 2010). Por fim, a via IM, consiste na infusão de um volume entre 1 a 1,5 mL da MOO na concentração entre 10 e 25 mg/L, na área dos músculos paravertebrais e/ou bíceps femorais (RODRÍGUEZ et al., 2017).

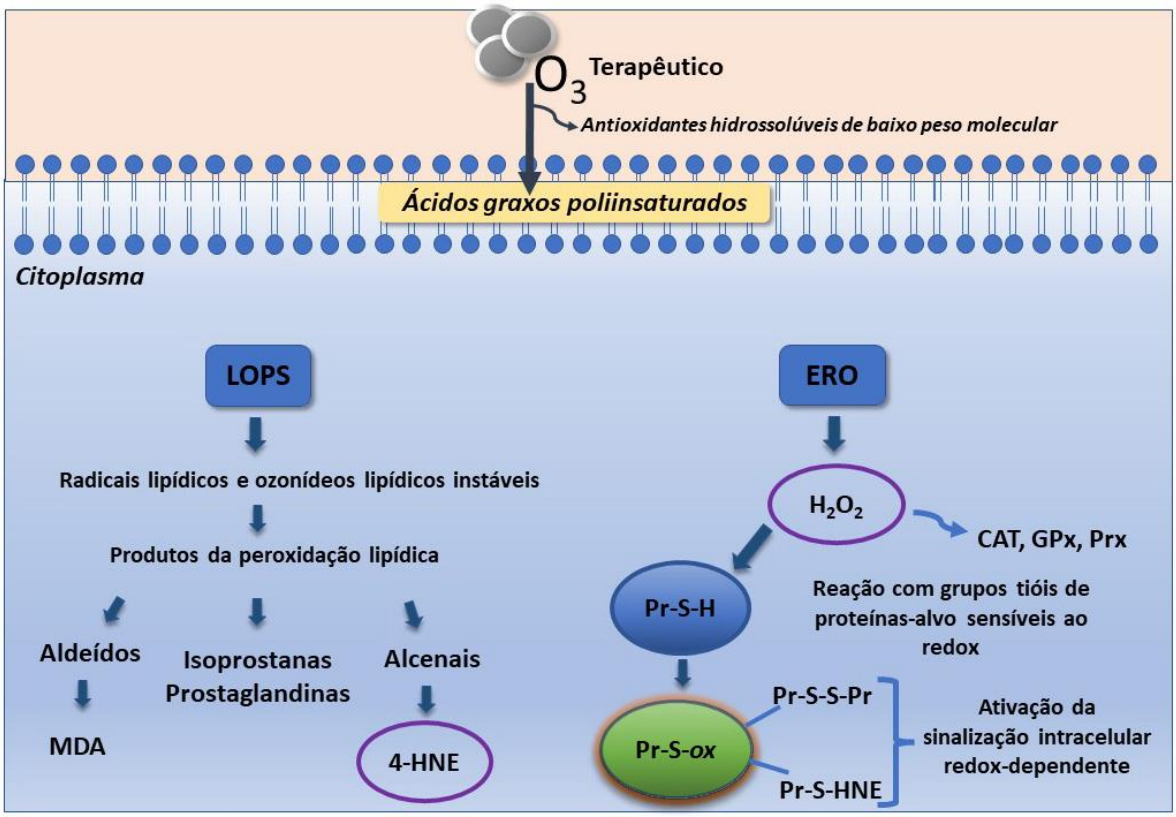
A única rota impraticável para aplicação médica de O_3 é a inalação, devido à sua toxicidade pulmonar (NOGALES et al., 2008). A capacidade de oxidar compostos orgânicos do O_3 somada ao sistema antioxidante deficiente do trato respiratório, faz com que esse gás, quando inalado, seja tóxico às células das vias aéreas superiores e inferiores (BOCCI, 2006). Epífora, rinite, tosse, dor de cabeça e, às vezes, náusea e vômito são os efeitos colaterais típicos da terapia com O_3 (NOGALES et al., 2008). O O_3 não deve ser administrado pelas vias sistêmicas em condições como gravidez, favismo, hipertireoidismo, miastenia grave e anemia (NOGALES et al., 2008). Além disso, jamais deve-se injetar diretamente a MOO pela via intravenosa devido ao risco de provocar embolia por O_2, uma vez que 95% da mistura gasosa é composta por este gás (BOCCI, 2006).

2.4 EFEITOS BIOLÓGICOS DA OZONIOTERAPIA

A alta solubilidade do O_3 na presença de água (H_2O), 10 vezes mais solúvel que o O_2, permite a rápida difusão deste gás nos fluidos biológicos (BATTINO; RETTICH; TOMINAGA, 1983). Essa propriedade do O_3 permite que seu principal subproduto oxidativo, o radical hidroxila (•OH), reaja imediatamente com antioxidantes hidrossolúveis (ácido ascórbico, ácido úrico, glutationa) e lipídios presentes nos tecidos biológicos (PRYOR et al, 1985; BOCCI; ZANARDI; TRAVAGLI, 2011). Dentre os lipídios, os ácidos graxos poli-insaturados (PUFA) representam o principal alvo do O_3 gerando os produtos da peroxidação lipídica (LOPs) e o peróxido de hidrogênio (H_2O_2) (Figura 1) (CENCI et al., 2022).

Devido à meia vida longa, os LOPs levam mais tempo para atingirem a parede vascular e interagirem com os tecidos onde desencadeiam sua ação biológica, sendo responsáveis, portanto, pelos efeitos tardios do ozônio (CENCI et al, 2022). Ao contrário, o H_2O_2 tem um tempo de meia vida mais curto e é responsável pelos primeiros efeitos biológicos nas células sanguíneas (eritrócitos, leucócitos e plaquetas), onde modula a sinalização intracelular (Figura 1) (CENCI et al, 2022).

Figura 1 – MODULAÇÃO INTRACELULAR DO OZÔNIO MEDICINAL



FONTE: (Adaptado de CENCI et al., 2022). O O_3 dissolve-se rapidamente nos fluidos biológicos, reagindo com antioxidantes hidrossolúveis de baixo peso molecular como o ácido ascórbico, ácido úrico e glutationa, e com os ácidos graxos poli-insaturados (PUFA) presentes na membrana celular. Em sua reação com os PUFA, o O_3 dá origem a uma cascata de reações gerando os produtos resultantes da peroxidação lipídica (LOPS) e as espécies reativas de oxigênio (ERO), sendo o peróxido de hidrogênio (H_2O_2) a ERO mais abundante. Os LOPS incluem os radicais lipídicos, os ozonídeos lipídicos instáveis, o malondialdeído (MDA), os isoprostanos, as prostaglandinas e o alcenal 4-hidroxinonenal (4-HNE). A sobrecarga intracelular de H_2O_2 é evitada pela atividade antioxidante das enzimas catalase (CAT), glutationa peroxidase (GPX) e peroxiredoxina (PRX). O 4-HNE e H_2O_2 oxidam os grupos tióis críticos de proteínas alvo redox-sensíveis (PR-S-H) induzindo sua oxidação (PR-S-OX) com a formação de pontes dissulfetos reversíveis com outros grupos tióis (PR-S-S-PR) ou adutos irreversíveis covalentes (S-HNE), respectivamente. Essas modificações dos grupos tióis alteram a estrutura e a atividade das enzimas redox-dependentes, ativando a sinalização intracelular e desencadeando a expressão de genes citoprotetores e de sobrevivência com o objetivo de potencializar a proteção contra o estresse oxidativo.

É importante ressaltar que os efeitos benéficos da OZT são baseados na capacidade do O_3 de atuar através de um mecanismo hormético de dose-resposta, sendo capaz de promover a geração de mediadores bioquímicos capazes de induzir efeitos benéficos de estresse oxidativo leve (BOCCI; ZANARDI; TRAVAGLI, 2011; GALIÈ et al., 2019). Portanto, quando administrada de forma regular e dentro da janela terapêutica, a qual varia entre 10 e 80 µg/mL (0,21-1,68 µmol/mL) de O_3 por mL de sangue, a OZT possui um efeito hormético sobre o

organismo, ou seja, provoca um pré-condicionamento oxidativo, o que acaba por gerar respostas adaptativas contra os danos causados pelos agentes oxidantes no organismo (LEÓN et al., 1998). Por outro lado, quando utilizado em concentrações maiores que o limite superior da janela terapêutica, o estresse oxidativo induzido pode ultrapassar a capacidade antioxidante do organismo, tornando-se, desta forma, um pró-oxidante e promotor de alteração do estado *redox* e de danos oxidativos intracelulares (BOCCI; ZANARDI; TRAVAGLI, 2011; CENCI et al., 2022). É importante salientar que quando administrado em concentrações inferiores a 10 μg/ml, o tratamento é biologicamente ineficiente por não atingir o limiar terapêutico, uma vez que o gás é prontamente neutralizado pelo sistema antioxidante do sangue (SAGAI; BOCCI, 2011).

2.5 CAPACIDADE ANTIOXIDANTE DO OZÔNIO

O princípio da terapia com O_3 é provocar no indivíduo um estresse oxidativo agudo, moderado e controlado, a fim de modificar a resposta biológica do organismo com consequente ativação e estimulação do sistema antioxidante (BOCCI; ZANARDI; TRAVAGLI, 2011). Os primeiros efeitos antioxidantes do O_3 foram explorados pelos estudos do grupo de Bocci mostrando que, ao usar doses abaixo do limiar superior da janela terapêutica da MOO, ou seja, abaixo da concentração de 80 μg/mL, o sangue total foi protegido da hemólise e sua reatividade foi controlada em cerca de 20% a 40% pelo sistema antioxidante do plasma, evitando os efeitos tóxicos observados em concentrações mais elevadas, como os detectados nos eritrócitos (peroxidação lipídica, níveis de meta-hemoglobina e hemólise) (BOCCI et al., 2009; TRAVAGLI et al., 2007).

Ao entrar em contato com o plasma sanguíneo ou com as soluções de cloreto de sódio a 0,9% e de ringer lactato, o O_3 se dissolve em poucos minutos e desaparece, gerando mensageiros essenciais para a produção das espécies reativas de oxigênio (ERO) (SAGAI; BOCCI, 2011). O peróxido de hidrogênio (H_2O_2) é o principal mensageiro produzido, sendo responsável pelos efeitos oxidativos/imediatos (SAGAI; BOCCI, 2011). Os segundos mensageiros produzidos pela MOO com as biomoléculas são os produtos derivados da peroxidação de ácidos graxos poli-insaturados - também conhecidos como produtos derivados da peroxidação lipídica (LOPS) - representados pelos radicais lipoperoxil, hidroperóxidos, malondialdeído (MDA), isoprostanos, ozoneto e o alcenal 4-hidroxinonenal (4-HNE), sendo estes responsáveis pelos efeitos tardios e indicadores da oxidação das membranas celulares (SAGAI; BOCCI, 2011).

O estresse oxidativo moderado causado pela MOO aumenta a ativação do fator transcricional que medeia o fator 2 relacionado ao fator nuclear-eritróide 2 (Nrf2) (INAL et al., 2011; SMITH et al., 2017). O domínio do Nrf2 é responsável pela ativação da transcrição de elementos de resposta antioxidante (ERA) (INAL et al., 2011).

Em condições normais, o Nrf2 é expresso em baixos níveis, sendo encontrado no citoplasma ligado ao seu inibidor específico, a proteína 1 associada à ECH semelhante a Kelch (Keap1), a qual promove sua degradação pela via da ubiquitina (BOCCI et al., 2009; ITOH et al., 1999). Foi relatado que o O_3 ativa o Nrf2 de maneira dose-dependente (GALIÈ et al., 2019) e, embora esse mecanismo ainda precise ser esclarecido, os alcenais derivados do O_3 demonstraram dissociar o complexo Nrf2-Keap1, ativando o Nrf2 (Figura 2) (JANSSEN-HEININGER et al., 2008; LI et al., 2008). A proteína Keap1 tem dois grupos tióis que sofrem oxidação mediada por LOPs alterando a sua conformação e liberando Nrf2 (JANSSEN-HEININGER et al., 2008; LI et al., 2008). A proteína então se acumula no núcleo, onde interage com o fator de transcrição fibrossarcoma musculoaponeurotico (MAF) formando um heterodímero que se liga às regiões do DNA que contêm os elementos da resposta antioxidante (JANSSEN-HEININGER et al., 2008; LI et al., 2008). Essa ligação induz a ativação transcricional de mais de 200 genes com ação antioxidante, como superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx), glutationa redutase (GR), glutationa-S-transferase (GST), enzima nicotinamida adenina dinucleotídeo fosfato (NADPH) quinona oxidoredutase 1 (NQO1), hemeoxigenase (HO-1) e o sistema tiorredoxina (Trx)/tiorredoxina redutase (TrxR) (GALIÈ et al., 2019; RE et al., 2014).

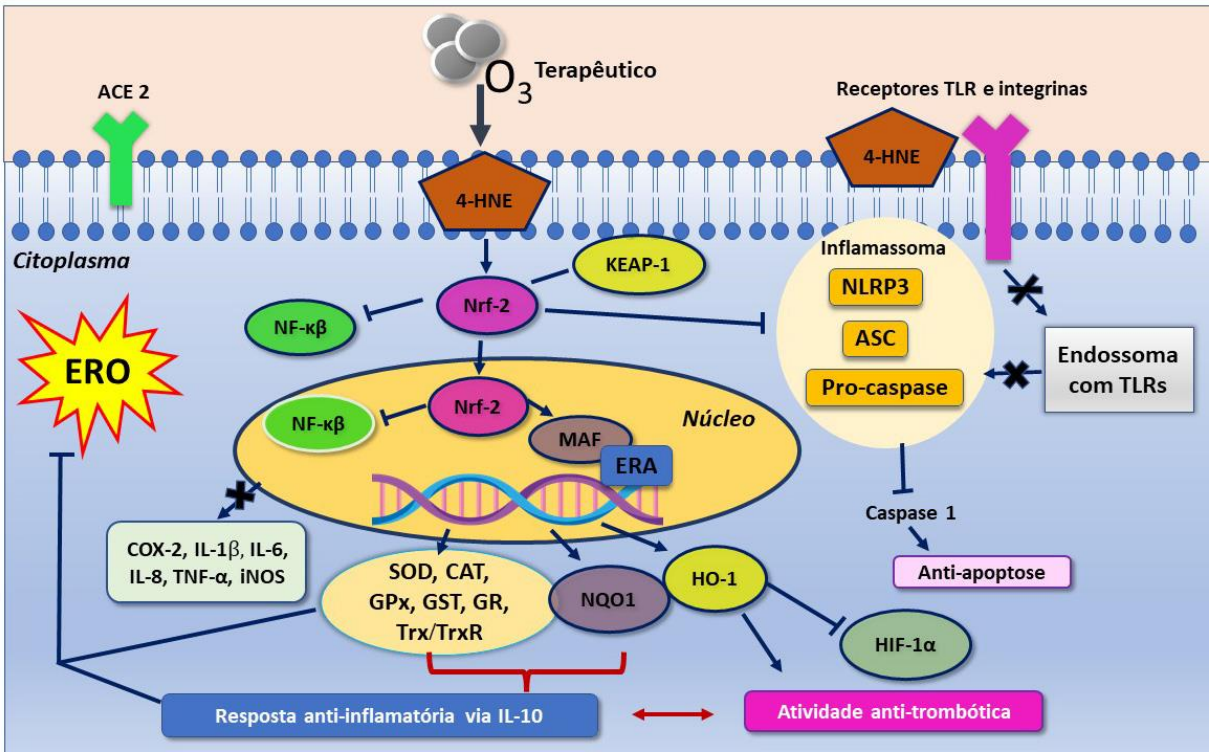
A ação dessas enzimas antioxidantes neutraliza a das enzimas pró-oxidantes ligadas à inflamação, como a NADPH oxidase, oxido nítrico sintase, xantina oxidase, lipoxigenase, ciclooxigenase (COX) e mieloperoxidase (MPO) (CENCI et al., 2022). Essas enzimas pró-oxidantes - as quais produzem e liberam altos níveis de radicais livres derivados de oxigênio e nitrogênio (superóxido - O_2•, hidroxila - •OH, carbonato - •CO_3, dióxido de nitrogênio - •NO_2) e espécies reativas não radicalares (H_2O_2 e peroxinitrito - $ONOO^-$) - desempenham um papel fundamental em condições inflamatórias caracterizadas pela ativação de células endoteliais da parede vascular, células musculares lisas vasculares, fibroblastos e células sanguíneas (incluindo células fagocíticas, plaquetas e eritrócitos) (JANSSEN-HEININGER et al., 2008; MITTAL et al., 2014).

Essas espécies reativas produzidas por fagócitos durante a inflamação, são potencialmente capazes de induzir dano celular irreversível por meio da modificação pós-

transcricional de proteínas ligadas à sinalização intracelular (JANSSEN-HEININGER et al., 2008; MITTAL et al., 2014). O hipoclorito (HClO) formado pela MPO liberada pelos fagócitos ativados pode oxidar as lipoproteínas plasmáticas e favorecer sua ligação a receptores específicos na superfície plaquetária, favorecendo indiretamente as funções pró-inflamatórias e pró-coagulantes das plaquetas (CENCI et al., 2022).

As já mencionadas enzimas controladas pela resposta antioxidante contribuem para restaurar a homeostase redox intracelular alterada pelo estresse oxidativo derivado da inflamação por meio da: (i) eliminação dos oxidantes produzidos em excesso, (ii) restauração dos níveis de antioxidantes de baixo peso molecular (glutationa, biliverdina), (iii) manutenção dos grupos tiol em sua forma reduzida adequada para sinalização celular, e (iv) promoção da redução de compostos geradores de ERO, como as quinonas (CENCI et al., 2022). Em particular, a NADPH NQO1 é uma enzima desintoxicante que protege as células do estresse oxidativo, diminuindo as ERO e protegendo o ácido desoxirribonucleico (DNA), as proteínas e os lipídios dos danos mediados pela hiperóxia (BURKE et al., 2021).

Figura 2 - EFEITO ANTIOXIDANTE DO O_3



FONTE: (Adaptado de CENCI et al., 2022). O 4-HNE formado após o tratamento com a MOO interage no citoplasma diretamente com o Nrf-2, que foi liberado da inibição com a proteína 1 associada à ECH semelhante a Kelch (Keap-1). O Nrf-2, após a sua translocação para o núcleo, além de inibir a atividade do fator nuclear kappa

B (NF-kβ), também atua como um segundo mensageiro ligando-se ao fator de transcrição do fibrossarcoma musculoaponeurótico (MAF) e às regiões dos elementos responsivos aos antioxidantes (ERA). Este processo permite a transcrição de enzimas antioxidantes, como superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx), glutationa-S-transferase (GST), glutationa redutase (GSH), e o sistema tiorredoxina/tiorredoxina redutase (Trx/TrxR), NADPH quinona oxidoredutase 1 (NQO1) e hemeoxigenase (HO-1), todos destinados a neutralizarsuperprodução de espécies reativas de oxigênio (ERO) e nitrogênio (ERN) e restauração fisiológica do equilíbrio redox. A formação do complexo Nrf2/MAF/ERA também resulta na inibição da transcrição de enzimas pró-oxidantes, como a cicloxigenase (COX) e a isoforma induzível da óxido nítrico sintase (iNOS), e citocinas pró-inflamatórias, como IL-1β, IL-6, IL-8 e TNF-α. A síntese de HO-1 é impulsionada pelo aumento mediado pelo 4-HNE dos níveis de Nrf2. A HO-1 reduz o estresse oxidativo inibindo o alto nível de expressão do fator 1 α induzido por hipóxia (HIF-1α), um sensor molecular de O_2 que é ativado durante a inflamação. Por meio da ativação do Nrf2, o 4-HNE inibe a atividade do inflamassoma, um complexo pró-inflamatório proteico formado pelo domínio da pirina contendo o receptor 3 (NLP3), juntamente com a proteína *speck-like* associada à apoptose adaptadora (proteína ASC) e a proteína efetora caspase 1. A inibição do inflamassoma bloqueia a ativação da caspase 1 e de interleucinas, favorecendo por sua vez a produção de citocinas anti-inflamatórias e consequentemente a regulação negativa das vias apoptóticas. A resposta antiinflamatória da via IL-10, estimulada pela reação antioxidante do O_3, contribui para a diminuição da atividade trombótica e prevenção de lesões vasculares.

2.6 MODULAÇÃO VASCULAR E HEMATOLÓGICA DO OZÔNIO

O O_3 estimula o fluxo transmembranar de O_2, uma vez que a maior parte da MOO é composta por O_2 (SMITH et al., 2017). O aumento dos níveis de O_2 dentro da célula secundário à terapia com O_3 torna a cadeia respiratória mitocondrial mais eficiente (MADEJ et al., 2007). Nos eritrócitos, a auto-hemoterapia ozonizada pode aumentar a atividade da fosfofrutoquinase, aumentando a taxa de glicólise (BOCCI, 2002; BOCCI et al., 2009). Ao aumentar a taxa glicolítica, há um aumento de adenosina trifosfato (ATP) e 2,3-difosfoglicerato (2,3-DPG) na célula e, consequentemente, devido ao efeito de Bohr, há um deslocamento para a direita na curva de dissociação da oxiemoglobina, permitindo que a hemoglobina ligada ao oxigênio seja descarregada mais facilmente nos tecidos isquêmicos (BOCCI; ZANARDI; TRAVAGLI, 2011). Além disso, a auto-hemoterapia ozonizada aumenta a perfusão sanguínea para a área isquêmica uma vez que estimula a atividade da enzima óxido nítrico sintase (BOCCI; ZANARDI; TRAVAGLI, 2011) e aumenta os níveis de prostaciclina, um conhecido vasodilatador (ELVIS; EKTA, 2011).

O O_3, em baixas doses, também pode proteger a célula sanguínea do estresse oxidativo, induzindo a expressão de enzimas antioxidantes, conforme descrito anteriormente (WANG et al., 2014). O tratamento repetido com ozônio induz a produção dos LOPs, os quais, quando em quantidade suficiente, atingem a medula óssea e favorecem, de forma sutil, a eritrogênese (BOCCI, 2002). Além disso, o O_3 também causa uma redução de nicotinamida adenina

dinucleotídeo (NADH) e auxilia na oxidação do citocromo c (BRIGELIUS-FLOHÉ; FLOHÉ, 2011; ELVIS; EKTA, 2011).

2.7 EFEITOS ANTI-INFLAMATÓRIOS E IMUNOMODULADORES DO OZÔNIO

Além do estímulo antioxidante, o O_3 também tem efeito sobre a resposta imune (SAGAI; BOCCI, 2011). Por esse motivo, estudos foram realizados para avaliar as aplicações sistêmicas de O_3 e suas implicações na resposta imune de humanos e animais (BOCCI, 1996; VAN HOOF et al., 1997; ZAMORA et al., 2005). Foi demonstrado que a exposição ao O_3 pode tanto suprimir quanto estimular o sistema imunológico, de modo que, baixas doses são capazes de induzir a resposta imune enquanto altas doses a inibem (BOCCI, 1996; JAKAB et al., 1995).

Quando o receptor de antígeno da célula T (TCR) reconhece qualquer antígeno estranho, a fosfolipase C é ativada (SAGAI; BOCCI, 2011). Depois disso, a fosfolipase hidrolisa fosfatidilinositol-4,5-bifosfato, um lipídio de membrana, para produzir os mensageiros secundários, trifosfato de inositol (IP3) e diacilglicerol (SAGAI; BOCCI, 2011). O IP3 induz a liberação de Ca2+ do retículo endoplasmático para o citosol, que desfosforila as células T ativadas por fator nuclear (NFAT) e o transporta para o núcleo (SAGAI; BOCCI, 2011). O NFAT então induz a transcrição de citocinas, como interleucina (IL)-2, fator de necrose tumoral alfa (TNFα), IL-6 e interferon (IFNγ), e elementos de resposta imune no DNA, que são traduzidos em suas respectivas proteínas (SAGAI; BOCCI, 2011). O estresse oxidativo leve induzido pela terapia com O_3, dentro da "janela terapêutica", pode ativar o NFAT seguido por funções imunes (ELVIS; EKTA, 2011; SAGAI; BOCCI, 2011).

Além disso, a ativação da via de sinalização Nrf2-ERA provocada pelo estresse oxidativo moderado causado por doses baixas a moderadas de O_3, também tem papel importante no alívio da inflamação por meio da modulação do fator nuclear kappa B (NF-κB) (CLAVO et al., 2019. Essa via ativa a liberação de citocinas pró-inflamatórias como: TNFα, INFγ, IL-1β, IL-6, IL-8, além de genes pró-inflamatórios como ciclooxigenase-2 (COX-2) e óxido nítrico sintase induzível (iNOS) (AHMED et al., 2017). Quando ativada, a via de sinalização Nrf2-ERA suprime a degradação do IκBα, proteína inibitória que impede a translocação do fator NF-κB para o núcleo da célula, impedindo assim a ativação desta via pró-inflamatória (CLAVO et al., 2019; ORAKDOGEN et al., 2016). Em contraste, uma alta dose de O_3 promove inflamação pela ativação da via NF-κB (KAFOURY et al., 2007). Como resultado, a dose administrada na

terapia de ozônio e sua resposta hormética têm um papel crucial para gerenciar as respostas de equilíbrio anti-inflamatório/pró-inflamatório (CLAVO et al., 2019).

Outra ação chave imunomoduladora do O_3 consiste na inibição do domínio pirina da contendo receptor 3 (NLRP3) (WANG et al., 2018) (Figura 2). O NLRP3 é um sensor intracelular pertencente ao complexo inflamassoma que medeia a maturação de caspases pró-inflamatórias as quais levam à ativação de citocinas pró-inflamatórias. Este complexo proteico é expresso principalmente no citosol de células imunes e inflamatórias inatas, como monócitos circulantes, macrófagos teciduais, células dendríticas e neutrófilos (SWANSON et al., 2019). O NLP3 é capaz de reconhecer vários sinais em resposta ao ataque microbiano, como os chamados padrões moleculares associados a patógenos que induzem danos nos tecidos e os padrões moleculares associados ao perigo, ativando e montando o inflamassoma (SWANSON et al., 2019). O inflamassoma é, portanto, um complexo proteico formado por NLP3, juntamente com a proteína *speck-like* associada à apoptose adaptadora (proteína ASC também chamada de PYCARD) e a proteína efetora caspase 1 (SWANSON et al., 2019).

A caspase 1, uma vez ativada pelo corte proteolítico ASC, dá origem a uma série de reações resultando na produção de IL-1β e IL-18 que promovem a inflamação sistêmica (CENCI et al., 2022). Recentemente, observou-se que a proteína NLRP3 é expressa em níveis elevados na doença renal crônica progressiva (DRC) (YU et al., 2017). O mesmo grupo de pesquisadores desmonstrou, que ratos com DRC tratados com insuflação intraretal de O3 tiveram uma redução estatisticamente significativa na expressão e na produção da proteína NLRP3, ASC1 e caspase 1, bem como, na expressão das citocinas inflamatórias IL-1β, TNFα e IL-6, em relação aos controles, demonstrando assim a ação anti-inflamatória do O_3.

O O_3 também tem efeito sobre outros mediadores imunológicos, como o fator de crescimento endotelial vascular (VEGF), fator de crescimento derivado de plaquetas (PDGF) e o fator transformador de crescimento beta (TGF-β) (ZHANG et al., 2014). Doses baixas de O_3 também aumentaram a secreção de macrófagos e leucócitos e inibiram a síntese de prostaglandinas e a liberação de bradicinina, como demonstrado em estudo realizado com ratos (ORAKDOGEN et al., 2016). Além disso, a regulação das concentrações endógenas de óxido nítrico (NO) promovida por baixas doses de O_3 também é um passo importante, pois o NO desempenha um papel fundamental no controle fisiológico da resposta imune (RE et al., 2008).

2.8 AÇÃO MICROBICIDA DO OZÔNIO

Graças ao seu alto poder oxidante, à sua alta solubilidade em água e rápida transformação em O_2, o O_3 é largamente aplicado em diversos processos industriais, tais como, desinfeção de água (água engarrafada, redes de água potável e piscinas), ar, madeira, frutas e legumes, além de superfícies que se encontram em contato direto com alimentos (VALLONE; STELLA, 2014). Em 2001, o U.S. Food and Drug Administration aprovou o O_3 como desinfetante para superfícies de contato com alimentos e aplicação direta para produtos alimentícios (U.S. FOOD AND DRUG ADMINISTRATION, 2001) e água (LEZCANO et al., 2001).

A eficácia antimicrobiana do O_3 é influenciada pelas condições ambientais, principalmente temperatura, umidade e valores de pH (VALLONE; STELLA, 2014). A melhor ação microbicida é obtida em ambientes com baixas temperaturas e presença de água ou alta umidade relativa (VALLONE; STELLA, 2014).

Além de seu uso em alimentos e água, a atividade oxidante do O_3 é útil para destruir paredes bacterianas, membranas citoplasmáticas de fungos e inativar leveduras, protozoários e vírus (SCIORSCI et al., 2020). A atividade antimicrobiana do O_3 tem sido explorada como coadjuvante na antibioticoterapia, proporcionando benefícios no tratamento de doenças infecciosas. O potencial antimicrobiano do O_3 vem sendo investigado a fim de reduzir a carga bacteriana em feridas e acelerar a cicatrização. Quando combinado com clorexidina, o O_3 melhora a atividade antibacteriana e o efeito fungicida (BORGES et al., 2017). O tratamento com O_3 reduziu efetivamente inclusive a colonização de *Staphylococcus aureus* resistente a drogas em cultura de placas (DYAS; BOUGHTON; DAS, 1983; YAMAYOSHI; TATSUMI, 1993). Silva e colaboradores (2009) avaliaram a aplicação intraperitoneal de O_3 na forma gasosa de ratos e relataram que houve inibição potente do crescimento bacteriano.

O efeito bactericida do O_3 se baseia no ataque direto aos microrganismos por oxidação de suas membranas biológicas (THANOMSUB et al. 2002). Uma vez adicionado em soluções aquosas, o O_3 decompõe-se rapidamente, gerando ERO como os radicais $\bullet O_2^-$, $HO_2\bullet$ e $OH\bullet$ (TOMIYSU; FUKUTOMI; GORDON, 2015). Essas ERO formadas atacam a superfície da célula bacteriana oxidando principalmente dois grupos: os PUFA e aminoácidos de peptídeos, enzimas ou proteínas. Uma vez danificada pela oxidação, esses radicais livres penetram pela membrana da célula microbiana, provocando danos ao DNA ou às proteínas intracelulares, o

que impacta na reparação e transcrição, podendo assim, resultar em lise ou morte celular (PATIL et al., 2011).

Além disso, ao contrário do que ocorre com os antibióticos, as bactérias não têm a capacidade de desenvolver resistência ao modo de ação do ozônio (ROWEN, 2019), evitando assim a formação de genes de resistência e a disseminação de bactérias resistentes a antibióticos (SCIORCI et al. 2020). Um estudo conduzido por Stange e colaboradores (2019) demonstrou que, mesmo em baixa concentração de 1 mg/L, o O_3 removeu mais de 99% das bactérias resistentes a antibióticos e dos genes de resistência a antibióticos.

O O_3 também tem potencial para inativar partículas virais (BOLTON; ZEE; OSEBOLD, 1982; ROY et al., 1981). A inativação das partículas virais pelo O_3 ocorre principalmente por meio da peroxidação lipídica e da carbonilação proteica, aumentando a sensibilidade, em especial, dos vírus envelopados (MURRAY et al., 2008; WELLS et al., 1991). Os fosfolipídios do envelope contêm muitos pontos de insaturação ao longo de suas cadeias de hidrocarbonetos; o O_3 oxida essas ligações, levando a danos estruturais (SCIORCI et al. 2020). O vírus da imunodeficiência humana tipo 1 foi inativado pelo tratamento com O_3, mesmo em concentrações não citotóxicas (CARPENDALE; FREEBERG, 1991; WELLS et al., 1991).

A carbonilação de proteínas desempenha um papel fundamental na inativação de vírus não envelopados (SCIORCI et al. 2020). Foi verificado que o H_2O_2 mediado pelo O_3 pode interagir com proteínas e causar danos e destruição na estrutura do capsídeo (MURRAY et al., 2008). Um estudo conduzido por Thurston-Enriquez e colaboradores (2005) revelou que a água ozonizada pode inativar os vírus não envelopados calicivírus felino e o adenovírus tipo 40, usando 0,30 e 0,06 mg/L de O_3, respectivamente. No entanto, além de danificar a proteína do capsídeo do vírus, a ozonização também danifica o ácido nucleico viral. De acordo com Jiang et al. (2019), a inativação do poliovírus 1 pelo O_3 está associada a danos ao genoma viral em vez das proteínas do capsídeo.

A ação fungicida do O_3, assim como ocorre com as células bacterianas, está fundamentada na oxidação da membrana celular destes microrganismos (SCIORCI et al. 2020). O O_3, em suas formas gasosa e oleosa, tem sido utilizado em três gêneros comuns de dermatófitos (*Epidermophyton*, *Microsporum* e *Trichophyton*), revelando seus efeitos fungicidas e inibitórios na esporulação (OUF et al., 2016). Além disso, o O_3 também atua reduzindo a produção das enzimas necessárias para as interações fungo-hospedeiro e fungo-fungo, que normalmente são produzidas para facilitar sua multiplicação dentro do hospedeiro

(OUF et al., 2016). Em relação às leveduras, um estudo conduzido por Zargaran et al. (2017) demonstrou os efeitos fungicidas do O_3 em diferentes formas de *Candida albicans*.

A ação do O_3 contra protozoários foi demonstrada *in vitro* com diferentes parasitas, como *Leishmania*, *Giardia*, *Cryptosporidium* e *Microsporidium*, usando óleo ozonizado e água ozonizada (RAJABI et al., 2015; KHALIFA; EL TEMSAHY; ABOU EL NAGA, 2001).

2.9 OZONIOTERAPIA NA MEDICINA

Apesar de ser usada em todo o mundo, a terapia com o O_3 medicinal ainda não foi aceita como medicina ortodoxa em todos os países. Atualmente, a OZT é reconhecida na Bulgária, Cuba, República Checa, França, Alemanha, Israel, Itália, México, Romênia e Rússia (ARAUJO, 2006). No presente momento, a prática da OZT não encontra-se regulamentada pelo Conselho Federal de Medicina no Brasil, sendo um procedimento que somente pode ser utilizado em experimentação clínica de acordo com os princípios éticos do sistema dos Comitês de Ética em Pesquisa (CEP) (BRASIL, 2018a). Entretanto, apesar de sua prática ainda não ser regularizada na medicina, a OZT está inserida na Política Nacional de Práticas Integrativas e Complementares (PNPIC) do Sistema Único de Saúde (SUS), pela Portaria n° 702/2018 do Ministério da Saúde (BRASIL, 2018b). A partir de sua inserção às PNPIC do SUS, os Conselhos de Classe buscaram compreender e regulamentar a OZT no âmbito de atuação de cada profissão, estando a sua prática, atualmente, regulamentada pelos conselhos que regem o exercício profissional de enfermeiros, cirurgiões dentistas, biomédicos, fisioterapeutas e farmacêuticos (ABOZ, 2019).

Na medicina, a OZT é empregada para o tratamento de várias patologias, tais como abscessos, acne, eczema, psoríase, vírus da imunodeficiência humana e síndromes de imunodeficiência adquirida, fibromialgia, artrite, asma, cânceres, inflamações, doenças cardíacas, distúrbios hepáticos, uveítes, cistite, feridas crônicas, dislipidemia, osteomielite, doença de Raynaud, doença de Parkinson, sepse, sinusite, cárie dentária, infecções da cavidade oral e pé diabético (BAYSAN; LYNCH, 2005; MERHI et al., 2019; RE et al., 2008; ZHANG et al. , 2014).

2.10 OZONIOTERAPIA NA MEDICINA VETERINÁRIA

Em outubro de 2020 o Conselho Federal de Medicina Veterinária, por meio da Resolução nº 1364, regulamentou a prática da OZT em animais como uma atividade privativa do Médico Veterinário, podendo ser empregada de forma isolada, adjuvante ou complementar (CFMV, 2020). Os relatos de uso na medicina veterinária da OZT estão em constante crescimento numérico, sendo descritos em pequenos e grandes animais, e também nos animais selvagens (FACO; XAVIER; CAZATI, 2012; MURPHY et al., 2016; RODRÍGUEZ et al., 2017; TEIXEIRA et al., 2013). Em cães, há relatos que demonstraram a eficácia da utilização do ozônio como uma alternativa terapêutica ao uso de antibióticos para o tratamento de dermatites de etiologia bacteriana ou de feridas contaminadas (BORGES et al., 2019). Essa atividade bactericida do ozônio também foi evidenciada no estudo realizado por Marchegiani et al. (2019), cujos resultados demonstraram que a limpeza da pele periocular com óleo ozonizado e a instilação de colírio ozonizado no saco conjuntival foi capaz de reduzir a microbiota bacteriana pré-operatória dessas regiões com efeito equivalente à solução de iodopovidine 5%.

A terapia com ozônio também tem demonstrado resultados bastante promissores na medicina equina, sobretudo no tratamento de feridas. Garcia et al. (2010), demonstraram que o uso do tratamento local com água e azeite ozonizados e sistêmico de insuflação retal de O_3 associados, foi eficaz no tratamento da habronemose cutânea de um equino de 15 anos de idade. Em um estudo utilizando cinco equinos hígidos, os quais foram submetidos a realização de uma ferida induzida experimentalmente, foi comparada a ação da solução isotônica ozonizada, do óleo de andiroba puro e do óleo de andiroba ozonizado durante o tratamento das mesmas. O grupo tratado com solução isotônica ozonizada obteve resultados inferiores aos dos grupos controle, tratado com óleo de andiroba puro e tratado com óleo de andiroba ozonizado. O grupo trato com óleo de andiroba ozonizado obteve um grau de retração da ferida superior aos demais, além de apresentar analgesia e menor taxa de formação de tecido de granulação exuberante (ARAÚJO, 2014).

Outro estudo mais recente, demonstrou que a auto-hemoterapia maior pode ser utilizada para aprimorar o condicionamento de equinos de alta performance submetidos a exercícios físicos intensos, uma vez que, melhora a capacidade antioxidante desses animais (TSUZUKI et al., 2015). A OZT, mais especificamente a administração intravenosa de 500 mL de solução

salina ozonizada concentração de 50 µg/mL, também pode ser efetiva na atenuação dos efeitos de lesões de reperfusão intestinal em equinos (ALVES et al., 2004).

Em relação ao uso da terapia com O_3 em animais de produção, há vários estudos principalmente na área da obstetrícia e ginecologia veterinária, os quais objetivaram aumentar a performance reprodutiva e produtiva dos animais. Na bovinocultura leiteira, a OZT também tem sido útil no tratamento de vacas com mastite clínica aguda (OGATA; NAGAHATA, 2000; OHTSUKA et al., 2005). A OZT também apresentou efeitos satisfatórios no tratamento de mastites clínicas e subclínicas em rebanhos leiteiros por meio da insuflação direta do gás no canal galactóforo dos quartos afetados (OGATHA; NAGAHATA, 2000; PEREIRA; RIBEIRO; CARVALHO, 2003). Além disso, a terapia com o O_3 não impede a utilização do leite pela indústria ou na alimentação, ao contrário do que ocorre com o leite proveniente de vacas tratadas com antibióticos, o qual deve ser prontamente descartado durante o tratamento e durante o período de carência (PEREIRA; RIBEIRO; CARVALHO, 2003).

Zobel e colaboradores (2012), demonstraram que a OZT oferece uma opção prática e relativamente econômica para o tratamento de urovagina devido a sua aplicação única, ação não espermicida e ausência de efeitos negativos no hospedeiro em relação aos resíduos quando comparado ao tratamento convencional com o uso de antibióticos. Ainda, Djuricic et al. (2012), constataram que a aplicação intrauterina preventiva de O_3 durante o período puerperal precoce, especialmente na forma de espuma (*spray*), resultou em efeitos positivos sobre o desempenho reprodutivo de vacas leiteiras. Da mesma forma, a aplicação intrauterina de O_3 na forma de spray demonstrou-se eficaz para o tratamento de retenção da membrana fetal em cabras leiteiras e de retenção de placenta e de possíveis infecções uterinas após partos distócicos em ovelhas, com resultados semelhantes e superiores, respectivamente, a antibioticoterapia clássica (DJURICIC; VALPOTIC; SAMARDZIJA, 2015; DJURICIC et al., 2016).

2.11 CISTITE BACTERIANA EM CÃES

A cistite, também denominada infecção do trato urinário (ITU), comumente ocorre por meio da ascensão de microrganismos pela uretra com consequente invasão da vesícula urinária (BARTGES, 2015). Essa doença é causada principalmente por bactérias provenientes do intestino ou da pele que circunda o períneo (SMEE; LOYRD; GRAUER, 2013). Em cães, essa afecção acomete com maior frequência fêmeas devido às diferenças na anatomia uretral e à proximidade entre uretra e ânus (LAMOUREUX et al., 2019; SMEE; LOYRD; GRAUER,

2013). Em contrapartida, os machos são menos predispostos a desenvolver ITU devido ao maior comprimento da uretra e à secreção de zinco no fluído prostático, o qual possui efeito bacteriostático (LAMOUREUX et al., 2019; SMEE; LOYRD; GRAUER, 2013).

A ITU inferior pode ser classificada como cistite bacteriana esporádica (não complicada) ou recorrente (complicada) (WEESE et al., 2011; WEESE et al., 2019). Cães acometidos pela cistite bacteriana esporádica normalmente apresentam polaquiúria, disúria e/ou estrangúria como sinais clínicos e apresentam de um a dois episódios ou suspeita de cistite em um período de 12 meses (WEESE et al., 2019). A cistite bacteriana recorrente, por sua vez, caracteriza-se pela ocorrência de três ou mais episódios de cistite clínica em um prazo de 12 meses ou 2 ou mais episódios em um período de seis meses (WEESE et al., 2019). Apesar de ser denominada como cistite bacteriana recorrente, essa terminologia também inclui reinfecções, podendo ser pelo mesmo ou outro agente (WEESE et al., 2019). Além disso, este tipo de cistite pode estar associado a uma doença ou causa subjacente identificável, como a presença simultânea de prostatite, cálculos urinários, bexiga neurogênica, cistite polipoide, diabetes melito, imunodeficiência ou de alguma anormalidade estrutural ou funcional (WEESE et al., 2011; WEESE et al., 2019).

A cistite canina bacteriana esporádica constitui-se em uma causa comum de morbidade, afetando aproximadamente 14% dos cães atendidos em hospitais veterinários (LING, 1984). Estima-se que de 5 a 27% dos cães apresentarão ITU em alguma fase de sua vida (BUSH, 1976).

A ITU de etiologia bacteriana é um problema clínico comum em cães e está entre os principais motivos para o uso de antimicrobianos (LING, 1984; WEESE, 2019). A urocultura combinada ao teste de suscetibilidade a antibióticos (antibiograma) constitui a base laboratorial para o diagnóstico de ITU, sendo o melhor instrumento para orientar as decisões de tratamento (BALL et al., 2008; BARTGES et al., 2004). No entanto, comumente os clínicos iniciam o tratamento antimicrobiano empírico com base apenas no diagnóstico presuntivo, antes mesmo de obter resultado de cultura e antibiograma (BALL et al., 2008). O custo e o tempo necessários para a cultura bacteriana têm sido apontados como possíveis empecilhos para a realização deste exame na prática clínica (DE BRIYNE et al., 2013; JESSEN et al., 2017).

A administração empírica de antimicrobianos é digna de preocupação, uma vez que essa prática pode acarretar na seleção de organismos resistentes a múltiplas drogas (RMD) ou alterar a flora normal, predispondo a outras infecções (WILCOX, 2009). O tratamento de infecções causadas por bactérias RMD é muito difícil, podendo causar morbidade significativa aos

animais, além de tornar-se oneroso financeiramente aos tutores (JOHNSTONE, 2019). Além da seleção de populações bacterianas resistentes, o uso inadequado de antimicrobianos também pode causar efeitos adversos ao paciente (WONG et al., 2015). A necessidade de iniciar o tratamento para resolver o desconforto do paciente e a pressão dos tutores tem sido sugerida como uma possível razão para a adesão aos tratamentos empíricos em casos de ITU (SØRENSEN et al., 2018).

A resistência bacteriana a antimicrobianos é um problema de saúde pública emergente, e a transmissão de genes de resistência entre patógenos infectantes de animais de estimação e seres humanos já foi documentada (DAMBORG et al., 2016; PITOUT; DEVINNEY, 2017). Apesar de algumas destas bactérias procederem do ser humano, a possibilidade de os animais de estimação atuarem como reservatórios das mesmas é cada vez mais reconhecida (BALL et al. 2008; GIBSON et al. 2008; GUARDABASSI; LOEBER; JACOBSON 2004; JOHNSTONE, 2019).

Foi demonstrado que os padrões de resistência bacteriana a antibióticos variam de acordo com a localização geográfica, porém, o seu aumento é global (SHEPHERD; POTTINGER, 2013). Segundo a Organização Mundial da Saúde (OMS) (2019), atualmente, 700.000 pacientes morrem anualmente no mundo devido à resistência microbiana e à ineficácia nos tratamentos. Ainda, de acordo com essa mesma agência, a previsão é de que esse número seja crescente diante da seleção de organismos RMD com o uso cada vez maior de antimicrobianos de forma indiscriminada.

Com a emergência da resistência bacteriana aos antimicrobianos, as opções de tratamento das ITUs e de outras infecções tornam-se cada vez mais limitadas. Em função disso, tratamentos não convencionais como imunoestimulantes orais como o extrato de *Escherichia coli* M-39 (Uro-Vaxom), suplementos de *cranberry*, probióticos orais e vaginais, ervas ou preparações à base de plantas, manose, acupuntura, estrógenos vaginais e orais, e mais recentemente, a OZT têm sido propostos e estudados na medicina humana (AL-BADR; AL-SHAIKH, 2013; BEEREPOOT et al., 2013; GEERLINGS; BEEREPOOT ; PRINS, 2014; HEAD, 2008; LOGAN, 2018; SHEPHERD; POTTINGER, 2013; VILELA, 2015). Dessa forma, pesquisas com o ozônio na medicina veterinária devem ser investigadas com o intuito de buscar novas alternativas de tratamento complementar na rotina clínica de pequenos animais, inclusive nos casos de cistite, uma vez que, trata-se de uma das principais afecções na qual faz-se o uso significativo e, por vezes, inadequado de antimicrobianos.

3. OBJETIVOS

3.1 OBJETIVO GERAL

Investigar os potenciais de aplicação da ozonioterapia como terapia integrativa na medicina veterinária, verificar os principais usos do ozônio medicinal na clínica médica de pequenos animais, além de testar os efeitos antibacterianos sobre algumas das principais bactérias causadoras de cistite em cães.

3.2 OBJETIVOS ESPECÍFICOS

Manuscrito 1: Relatar as principais prescrições de ozônio medicinal recomendadas aos pacientes por médicos veterinários, auxiliando na tomada de decisões terapêuticas dos colegas de profissão

Manuscrito 2: Avaliar a atividade antimicrobiana *in vitro* da solução salina ozonizada sobre cepas padrão e sobre isolados bacterianos resistentes e com perfil de multirresistência comumente associados à cistite em cães.

Artigo 1: Relatar a eficácia de dois diferentes protocolos de instilação da vesícula urinária com solução salina ozonizada em um canino paraplégico com cistite bacteriana recorrente causada por *Proteus* spp.

4. MANUSCRITO 1 - PRESCRIPTION OF MEDICINAL OZONE IN SMALL ANIMALS' VETERINARY PRACTICE

Manuscrito submetido para publicação no periódico:

Research in Veterinary Science

Prescription of medicinal ozone in small animals' veterinary practice

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Abstract

Veterinary medicine is constantly looking for alternatives capable of helping to resolve animal diseases, especially when conventional treatments are ineffective or adverse effects and microbial resistance occur. Ozone therapy has been gaining supporters in both human and animal medicine due to the effects reported by professionals who use it. Thus, the objective of this work was to verify the main prescriptions of treatments with medicinal ozone in the small animal clinic, in addition to verifying the profile of the professional who prescribes it. Among the 213 research participants, (98.1%) claimed to use at least one integrative therapy in clinical practice, with the most used being physiotherapy (81.7%), acupuncture (80.3%) and ozone therapy (68.5%). Among the participants, 72.0% claimed to apply or recommend ozone therapy. Among the participants who do not apply/recommend it, the main reasons were the lack of proof of effectiveness (38.6%) and the lack of technical knowledge of the professional (35.1%). As for the results with the use of ozone therapy, 90.0% reported good or excellent results. Even among those who apply/recommend therapy, 87.0% said they use it associated with conventional treatment. The main diseases treated were healing of open wounds (91.1%), dermatitis (63.7%), asepsis of contaminated wounds (46.6%) and post-surgical healing (49.7%). It is concluded from this study that the use of medicinal ozone is therapy with high expansion potential, and requires experimental studies to strengthen scientific evidence for an effective and safe clinical use in the routine of small animals.

Keywords: Analgesia. Antimicrobial effect. Company animals. Integrative treatment. Ozone therapy.

Introduction

Increasingly, integrative or alternative therapies have been gaining ground as recommendations in the treatment of companion animals and the acceptance of tutors (Shmalberg and Memon, 2015). These therapies contribute to the quality of life and are therapeutic resources for various diseases, especially when conventional treatment is flawed or with excessive adverse effects (Shmalberg and Memon, 2015). As frustration with conventional medicine deepens, people tend to seek new approaches to the care of their health and that of their pets (Memon et al., 2016). Many alternative therapies are not only comparatively inexpensive and readily available, but they also offer owners the opportunity to actively contribute to the health of their pets. Elderly animals, with chronic, neurological, and orthopedic diseases (Di Mauro et al., 2019; Shmalberg and Memon, 2015), with resistant infections and with difficult-to-resolve wounds (Anzolin et al., 2020; Roth et al., 2020; Rowen, 2018; Smith et al., 2017) are strong candidates for integrative treatments. There are several therapies already inserted in veterinary medicine, such as acupuncture, physiotherapy, homeopathy, phytotherapy, floral remedies, aromatherapy, and laser therapy, among others (Carson et al., 2006; Memon et al., 2016; Shmalberg and Memon, 2015); however, many are still awaiting regulation. Recently, ozone therapy in veterinary treatments was regulated in Brazil (CFMV, 2020a).

Oxygen-ozone (O_2-O_3) therapy is a non-invasive, easy-to-obtain, non-pharmacological and low-cost procedure based on the antioxidant-inducing capacity of O_3 (Anzolin et al., 2020; Scassellati et al., 2020). Reports of ozone therapy use in veterinary medicine are constantly increasing in number, having already been reported in horses, ruminants and dogs (de Souza et al., 2021; Murphy et al., 2016; Rodríguez, 2017; Teixeira et al., 2013). Some evidence points to mechanisms involving immunomodulation, antimicrobial, anti-inflammatory, analgesic and

healing action (de Souza et al., 2021; Smith et al., 2017; Viebahn-Haensler and León Fernández, 2021).

The hope surrounding alternative veterinary medicine is understandable, but for most modalities there is little evidence to support efficacy, and some pose risks to humans and companion animals. Although the range of randomized scientific studies with medicinal ozone is so far scarce and superficial (Shmalberg and Memon, 2015), this article aims to present the prescriptions of ozone therapy by veterinarians for the treatment of dogs and cats. Based on the clinical success reported by them, this study may help colleagues in the profession in making therapeutic decisions with the use of this therapy. In addition, from this study, the profile of professionals who use ozone therapy will be verified so far.

Material and Methods

Study Design

This study was carried out in the three southern Brazilian states– Paraná (PR), Santa Catarina (SC) and Rio Grande do Sul (RS). Adherence to the study was voluntary. The application of the questionnaire took place in April and May 2021, through the online platform Google Forms. Therefore, this work was previously approved by the Ethics Committee on Human Beings linked to the Federal University of Santa Maria. To participate, veterinarians had to previously agree to the Free and Informed Consent Form.

Target sample

The social networks Instagram and WhatsApp were used to disseminate the questionnaire to veterinarians working in clinics, hospitals and self-employed. In addition, the questionnaire was sent via e-mail to 80 teaching institutions that have a Veterinary Medicine

course and/or Residency and Graduate Program in PR, SC and RS, seeking wide dissemination to professors. area and students who graduated from the institutions.

Collected information

Personal information was requested regarding age, gender, training time, registration number with the veterinary medical class council, state and city of operation. As for integrative therapies, participants were asked to point out all of which they recommend or apply to their patients. Specifically, about ozone therapy, participants were asked about their knowledge of it; when positive affirmation, if the participants recommend or apply the therapy; and in cases where they do not recommend or do not apply, for what reason.

Regarding the workplace, it was asked whether there is a qualified professional who applies ozone therapy or whether a referral is made to an external professional; also, for how many years the participant has applied or recommended the therapy. Veterinarians who claimed to apply medicinal ozone to their patients were asked whether they had participated in a training course to do so. It was asked about which species professionals apply/recommend therapy and the main diseases for which they recommend/apply ozone therapy. Professionals were asked to evaluate the results of using medical ozone; as well as if the therapy is recommended/applied in a prophylactic/preventive way. Those who stated to apply/recommend were also asked when they decided to use it (if as a last option, or first option exclusively, or associated with conventional treatment); if the professional has already applied or recommended antibacterial treatments and in cases of bacterial resistance to conventional drugs, and these cases, if the treatment was successful; and if you have already recommended/applied ozone therapy with an analgesic effect. Finally, it was asked whether veterinarians believe that ozone therapy will be expanding in the coming years.

To those who said they did not recommend/apply the therapy, five responses were suggested to justify the decision: lack of proof of efficacy; lack of technical knowledge (courses/specialization); non-acceptance by animal tutors for use; lack of necessary equipment or lack of professional interest in working in this area.

Exclusion criteria

Questionnaires in which participants claimed to be from other states, to be under 22 years of age, or to have graduated less than six months ago were excluded from the study (since we considered that they did not have enough experience to answer our questionnaire). Questionnaires answered more than once by the same participant were also excluded.

Statistical analysis

The results of the questionnaire were analyzed descriptively, in addition to testing the correlations between variables using the Chi-square test of independence and Fisher's exact test. For the correlation tests, a significance level (α) of 5% was adopted. The data were tabulated in a Microsoft Office Excel spreadsheet and the tests performed were developed in the R environment (v-4.2.0).

Results

Descriptive analysis

There was a total of 218 participants who signed the informed consent and responded to the questionnaire, however, 5 were disregarded for meeting the exclusion criteria. The participants were from 80 different cities of the southern Brazilian states. All questionnaire

results are represented in Table 1. The most used integrative therapies are shown in Figure 1. Table 2 presents the main diseases treated with the therapy.

Statistical analysis: Chi-square and Fisher's test

When testing the association between sex and the number of integrative therapies that are used, a statistical difference was obtained (P = 0.001), with females using and recommending more integrative therapies than males; similarly, women were the ones who most applied/recommended medical ozone therapy (P = 0.008).

There was no statistical association between the age of the participants and whether or not they knew about ozone therapy (P = 0.546); between the age of the participants and recommending/applying the therapy in a prophylactic/preventive way to diseases in the animals (P = 0.167) and between the age and replacing or not the use of ozone with antibiotics (P = 0.163). However, it was found that the longer the time using ozone therapy, the greater the recommendation of the technique to obtain an analgesic effect in patients (P = 0.007).

There was no association between the time of training in veterinary medicine and the number of integrative therapies that are recommended by professionals (P = 0.080). There was also no association between time using ozone therapy and the number of illnesses treated with medical ozone (P = 0.276). Similarly, the duration of therapy use did not affect the results declared by the participants (fair, good, excellent) in use (P > 0.842); nor was it an association factor for success or failure in the antibacterial use of medicinal ozone (P = 0.379). The age range of professionals and the number of years using ozone therapy was associated (P = 0.008), and the older the professional, the longer the time of use of the technique. Similarly, time in veterinary training and time using ozone therapy was associated (P = 0.012), with the longer the training period, the longer the time of use by professionals.

The performance status was not associated with the number of integrative therapies recommended by the professionals (P = 0.607), with the fact of knowing or not the therapy (P = 0.583), or even with the fact of applying/recommending/not applying and not recommend therapy (P = 0.506). There was also no association between the performance status and the time of use of ozone therapy (P = 0.549). However, there was an association between the state of work and the reason stated by professionals who do not apply or recommend ozone therapy, and professionals from RS declared the lack of scientific evidence as the main reason, while professionals from PR and SC claimed not to have technical knowledge (course/specialization) to work in this area (P = 0.038).

Discussion

This is the first time that a study has been carried out to obtain a profile of veterinary medical professionals who use integrative therapies, especially ozone therapy, as well as to verify the prescription for the use of the therapy. Studies such as this are of paramount importance as an indicator of therapy success, since so far very little is known about the effects and benefits of medicinal ozone. The most publications about this therapy are case reports and in vitro tests. Randomized studies (with untreated control groups and a control group treated with an established conventional therapy), using a significant sample of animals, are rare.

Despite being old, including having been successfully used in the antimicrobial treatment and wound healing of soldiers during the First World War (Stoker, 1916), medical ozone therapy was long forgotten. In human medicine and Brazil, only in 2018 was ozone therapy included in the list of integrative therapies to be used in the National Policy for Integrative and Complementary Practices - PNPIC (BRASIL, 2018), while its release for use in animals took place in 2020 (CFMV, 2020a). Despite being recently regulated, it was verified

in this study that ozone therapy is among the integrative therapies most recommended by professionals, behind only the most renowned and ancient therapies, such as physiotherapy and acupuncture. This fact seems to indicate that the therapy has been gaining ground, quite possibly due to the positive results reported in clinical routine (Orlandi et al., 2021; Sciorsci et al., 2020).

The vast majority of participants declared knowing or having heard of ozone therapy. In addition, the fact of knowledge about ozone therapy was not predominant according to the age of the participants, which means that both older and younger professionals know, apply or similarly recommend this therapy. That is, they knew of it a long time ago (since it is quite old), and since then, they possibly persisted using it due to the results they obtained. in their treatments during their career, even if not yet regulated by the Profession Counsil (CFMV, 2020a).

The fact that almost two-thirds of the veterinary professionals in this study recommend ozone therapy to another professional who does it, reveals a market possibility for professionals who are interested in specializing in the area. This idea is reinforced by the information that half of the establishments do not have a professional who applies the technique, needing to seek external professionals. Fortunately, most professionals who claim to apply ozone therapy are properly qualified after completing courses or specializations. Use by a non-qualified professional presents risk to the patient, as they do not have control over the concentrations and application times, routes, or knowledge of contraindicated diseases and situations (Di Mauro et al., 2019; Viebahn-Haensler and León Fernández, 2021). As demonstrated for other medical gases used in clinical practice (O_2, NO, and CO), ozone can be toxic or safe as a real drug, depending on its dosage, exposure time, and antioxidant capacity of the exposed tissue (Di Mauro et al., 2019; Kowaltowski et al., 2009).

In this study, most veterinarians reported using ozone therapy in both pet species, while a second group used it only in dogs. Cats are more susceptible to damage by oxidative stress, especially if used systemically, due to the complexity of their erythrocyte sulfhydryl groups (Webb et al., 2003) that are easily oxidized and damaged. Therefore, the medicinal ozone should be applied with caution in this animal species. The professionals in this study (the vast majority qualified by courses/specialization in the area) may have such knowledge, using it predominantly in dogs and with caution in cats; or even that dogs are predominant in their care routine, as seen in cases of care at the small animal clinic (Bergmann et al., 2019; Xavier, 2012).

In the present study, the main therapeutic purposes of medicinal ozone were the healing of open wounds first, dermatitis second, and contaminated wounds of difficult healing, third, and fourth, the promotion of post-surgical healing. Several studies and reports have demonstrated the effectiveness of medicinal ozone in curing skin disorders (Anzolin et al., 2020; Smith et al., 2017; Xiao et al., 2017). The use on the skin takes the form of 'Baggins', washes with ozonized water, ozonized saline solution, or with the application of ozonized vegetable oils (Viebahn-Haensler and León Fernández, 2021; Xiao et al., 2017). Ozonated oil has been shown to significantly decrease the area of skin lesions and accelerate healing through increased fibroblast migration and decreased inflammation (Xiao et al., 2017). The effects extend to reducing microbial infection, debridement effect, modulating the inflammatory phase, stimulating angiogenesis, as well as biological and enzymatic reactions that favor oxygen metabolism, improving wound healing (Anzolin et al., 2020). It is known that skin diseases are highly prevalent in small animal clinics, being the most frequent reason for animals to be taken to the veterinarian (Scott et al., 1996). This fact may also contribute to the predominant use in these situations.

The decision of most professionals not to recommend replacing antibiotics/antifungals with ozone therapy is pertinent due to the lack of in vivo studies. However, from what is already known, there seems to be an effective antibacterial action of medicinal ozone, since many veterinarians questioned in this study claimed to use it in the treatment of dermatitis, contaminated wounds, and otitis. Ozone therapy has been validated in the alternative antiseptic treatment against most microorganisms involved in skin infections and contaminated wounds (Borges et al., 2019; Marchegiani et al., 2019; Roth et al., 2020). The bactericidal effect of O_3 is based on the direct attack on microorganisms by oxidizing their biological membranes, followed by DNA or intracellular proteins damage, thus resulting in cell lysis or death (Rangel et al., 2021; Patil et al., 2011; Thanomsub et al., 2002). Unlike antibiotics, bacteria cannot develop resistance to the ozone mode of action (Rowen, 2018), thus preventing the formation of resistance genes and the spread of antibiotic-resistant bacteria (Sciorsci et al., 2020; Song et al., 2018).

Additionally, ozone therapy can work synergistically with traditional antibiotic treatments when used in combination therapy. One of the mechanisms of bacterial resistance to antibiotics is the development of changes in the cell membrane and, therefore, preventing the antibiotic from entering the cell. By oxidizing the outer layer of bacteria, ozone can eliminate this barrier and allow antibiotics to enter the bacterial cell, making it effective (Roth et al., 2020). In addition to damaging and inactivating pathogens, activation of the local immune system can contribute to the positive outcome of infection (Smith et al., 2017).

These results are promising and stimulate further investigation into the use of gaseous ozone in low concentrations as a disinfectant or antiseptic (Rangel et al., 2021). In the present study, one-third of the professionals who recommend/apply ozone therapy applied the therapy in cases of antimicrobial resistance; of these, most reported efficacy in the treatment, which

seems to credit the antibacterial effect of ozone. Antimicrobial resistance and the decrease in the pipeline of drugs to treat resistant strains are considered by the World Health Organization as one of the ten global threats to public health (WHO, 2021). Resistance situations are frequently faced in human and animal medicine and are seen in difficult to resolve wounds. A paradigm shift in the treatment of infectious diseases is needed to prevent antibiotics from becoming obsolete and, where appropriate, alternatives to antibiotics should be considered (Carson et al., 2006).

Although only a third of the patients use ozone therapy for patient analgesia, it was possible to statistically verify that the longer professionals use ozone therapy, the more they recommend it for this effect. This fact may be due to the positive effects for this purpose that professionals observe as the time of therapy use increases. Some studies describe the analgesic effect of medicinal ozone in veterinary medicine (Teixeira et al., 2013; Viebahn-Haensler and León Fernández, 2021). The decision to use it for analgesia is possibly associated with the use of this therapy in joint diseases, as already described in humans (de Sire et al., 2021; Manoto et al., 2018) and in experimental models (Xu et al., 2020) or even in the relief of skin burns (Viebahn-Haensler and León Fernández, 2021). After skin diseases, the most commonly treated with medical ozone were discopathies and arthritis (Figure 1). Most musculoskeletal disorders share these pathophysiological processes, and the therapy appears to reduce pain and improve functionality in osteoarthritis (de Sire et al., 2021). The topical use of oxygen-ozone gas for pain relief can be associated with the acupuncture technique, in what is called application in 'acupoints' (Teixeira et al., 2013).

Interestingly, half of the professionals claim to have good results with the use of medicinal ozone, and another large group defines it as excellent. It was statistically verified that as the years of experience with ozone therapy increase, the use of it increases; that is, it seems

277 to be a reaffirmation of the proven beneficial effects in the clinical routine of patients, even
278 though we do not have a large number of baseline studies to date. However, the time using
279 ozone therapy was not able to statistically change the opinion about the results of this, or be a
280 factor that increases or decreases the indication for antibacterial treatments. The training time
281 also did not influence the fact of being more or less adept at integrative therapies, that is,
282 professionals do not tend to increase or decrease the use of therapies used over time. Also, the
283 training time was not related to the use of ozone therapy more or less, nor the number of diseases
284 treated in the clinical routine.

285 The state was also not associated with the number of integrative therapies recommended
286 by professionals, as well as the level of knowledge about ozone therapy and the time of use.
287 Therefore, it can be suggested that the knowledge of the therapy is widespread in the southern
288 states of Brazil.

289 The legislation that regulated the use of ozone therapy in veterinary practice in Brazil
290 (CRMV, 2020a) defined its use as isolated, adjuvant, or complementary. It is prudent that the
291 use of therapy, as stated by the vast majority of veterinarians in this study, be associated with
292 conventional treatment, despite the use alone (as seen by reports of efficacy) being credited. A
293 portion of professionals said they still use it only when the conventional treatment is
294 unsuccessful, which may be relevant, after all, at that moment the professional chooses the 'plan
295 B' that can be good for the patient, not giving up on treating him. The decision of most
296 professionals not to recommend/apply it in a prophylactic way seems quite plausible since there
297 are practically no studies proving benefits in this situation.

298 The lack of proof of efficacy was the main reason why professionals ended up not
299 recommending/applying ozone therapy (which was statistically found to be the main cause of
300 professionals in RS) followed by the lack of technical knowledge for this (the main cause

alleged by professionals from PR and SC). This can be seen as an appeal by professionals to scientific studies in the area. There is a vast field of studies to be considered by teaching and research institutions, seeking evidence of effectiveness, as professionals seek treatments and evidence that ensure their technical conduct, is supported by science. Another need expressed by professionals is the lack of serious courses in the area, for the safe and effective use of the therapy. Once again, the existence of this is reserved to happen after many baseline studies are published, on mechanisms of action and effects obtained from randomized studies.

According to the Resolution that approves the use of ozone therapy in Veterinary Medicine, this clinical activity is exclusive to the veterinarian and must have technical support that indicates safety and efficacy for the treatment of the specific disease or condition (CFMV, 2020a). The fact that the professional has to count on technical support is contradictory, since until now, studies with medicinal ozone are extremely scarce, even more for 'specific diseases or aggravations'. It is seen that integrative and alternative therapies require a lot of research, and often there is no interest in their promotion, especially when it does not add profits to large pharmaceuticals. Thus, studies to provide technical support in the area of ozone therapy are restricted to non-profit research groups interested in the area, which further delays discoveries and research in the area.

The belief on the part of almost all professionals that there will be a strong expansion of the use of medicinal ozone in the coming years is most likely linked to the positive reports in clinical use, which despite the few experiments, are quite optimistic. It is noteworthy that almost 100% of professionals reported recommending at least one integrative therapy (most recommend several). Data from a 1997 survey indicated 42% of pet owners used alternative therapies, and states that as clients investigate and use alternative therapies, veterinary medical professionals tend to want to know these modalities to instruct and be able to apply such

therapies also in pets of these and another tutors (AVMA, 2000). Added to this, the very ineffectiveness of conventional treatments may be motivating the use of these therapies (Memon et al., 2016; Shmalberg and Memon, 2015).

The authors of this work believe that the knowledge regarding integrative therapies and also about ozone therapy does not come from disciplines during graduation. A survey sent to the deans of institutions with a veterinary medicine course revealed that only 1/3 offered the course during the course (rehabilitation and acupuncture topics were predominantly offered), however, more than 2/3 provided clinical care with integrative practices in university veterinary hospitals (Memon, 2021). That is, a certain controversy is perceived by educational institutions, which, on the one hand, value the administration solely of 'evidence-based medicine' (Memon et al., 2016), on the other hand, in clinical practice, they promote their use in the care of animals in the most varied modalities within university veterinary hospitals. The use of these therapies for hundreds or thousands of years, even if they often do not have solid scientific bases, added to the success stories of colleagues, makes it difficult to fully refute their therapeutic potential. The protection of the professional obtained by the authorization of the tutor through informed consent is of paramount importance.

Finally, the fact that there was a predominance of women's participation in this study may be related to the dominance of gender in the contingent of working professionals, according to the last census of the Profession Council of this country (CFMV, 2020a). We found that women indicate more integrative therapies and ozone therapy than men; regardless of whether they were in greater numbers in the study, there was an association between these factors.

Conclusion

The potential for expanding the use of ozone therapy in veterinary medicine is wide. Only in the last two years has its use been regulated in Brazil, and even before that, veterinarians had already been recommending and verifying positive results. It is admirable that this therapy is being one of the most used among the existing integrative, revealing the affection of the professionals with its result. In addition, we can say that one of the main uses, if its effectiveness and mechanisms of action are demonstrated by serious scientific studies, will be antimicrobial treatments, especially when there is the bacterial resistance. For the time being, empirically, its use in clinical routine seems to benefit patients who need analgesia. It is worth noting that the use of medical ozone requires qualification, even required by the class council, since little is known about the adverse effects of its use, and it can even be harmful when used by unprepared professionals without knowledge of concentrations, doses, and routes. The fact that women professionals use more integrative therapies and ozone therapy than men demonstrate the profile of professionals, as well as the greater openness of this group to innovative techniques, however, the user must be done with caution, seeking, whenever existent, foundation scientific to do so.

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Conflict of interests

The authors declare that there are no conflicts of interest regarding this article's research, authorship, and/or publication.

Ethical approval

This study was previously approved by the Ethics Committee for Human Beings, registered under protocol number 43992921.1.0000.5346.

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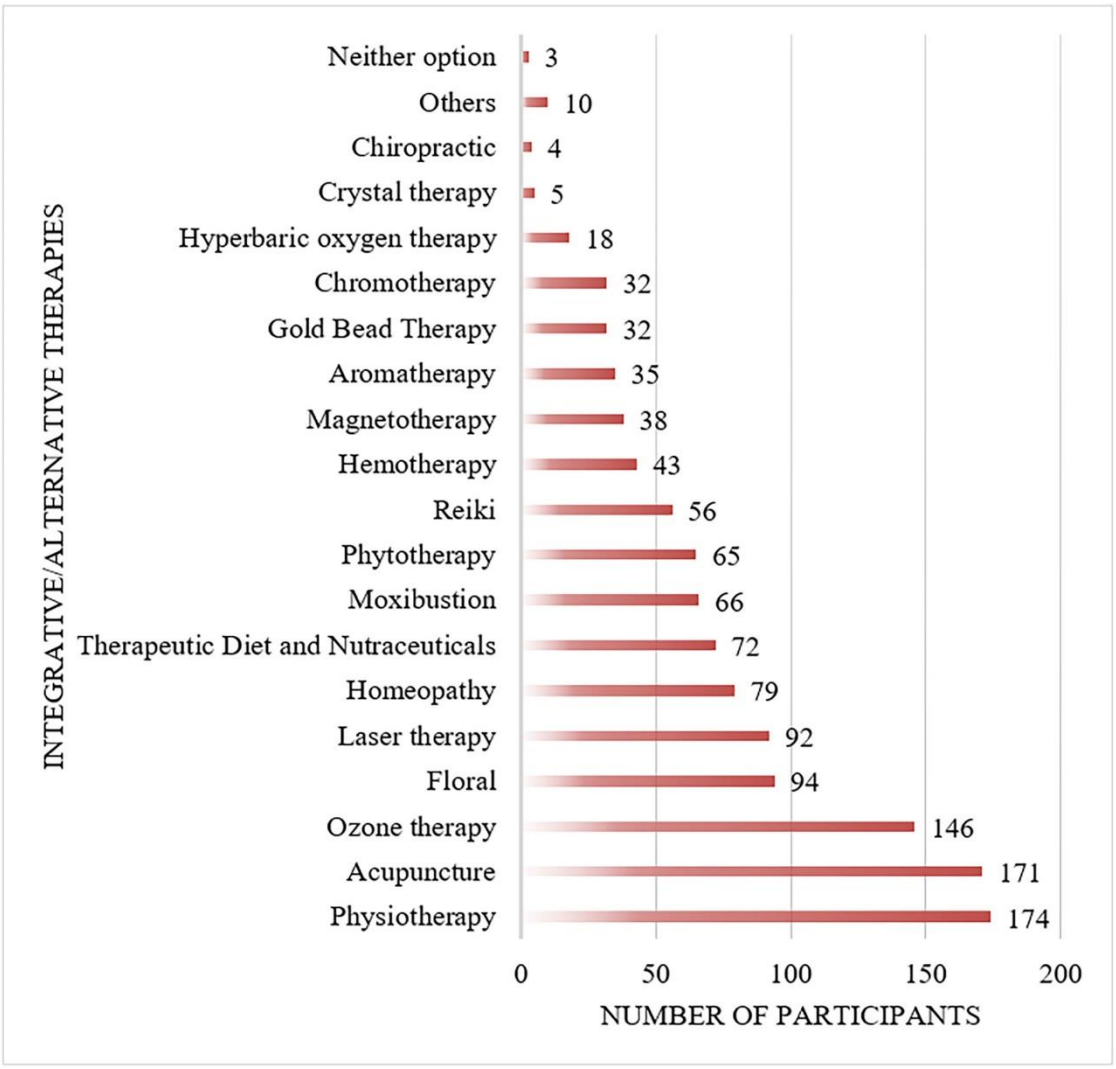
518

519 **Figure 1.** Integrative therapies most applied/recommended by veterinarians in the states of
520 Paraná, Santa Catarina and Rio Grande do Sul in an online questionnaire applied in
521 April/May 2021, among 214 participants.

522



523

524 **Table 1.** Main diseases of small animals (dogs and cats) treated with medicinal ozone by
525 veterinarians in the southern states of Brazil (Paraná, Santa Catarina and Rio Grande do Sul) in
526 a questionnaire applied in April and May 2021.

Diseases	**Nº of participants who indicate or apply**	**% used for a specific purpose**
Healing of open wounds	133	90,5
Dermatitis	93	63,3
Asepsis of contaminated wounds	68	46,3
Post-surgical healing	58	39,5
Arthritis	57	38,8
Discopathies	56	38,1
Otitis	54	36,7
Chronic Kidney Disease	48	32,7
Cystitis	41	27,9
Ophthalmopathies	27	18,4
Epilepsy/myoclonus	25	17,0
Diarrhea	23	15,6
Pyometra	11	7,5
Others	10	6,8

527 The percentage % refers to the 146 participants who apply or recommend ozone therapy. Each
528 participant responded to use for at least 1 disease, and at most 13 diseases. *Others: Neoplasms
529 (4), inflammatory bowel disease (1), oral procedures (3), autoimmune diseases (1), diabetes (1).

530

Table 2. Results of the questionnaire carried out to veterinarians on the use of integrative therapies in small animals, especially the ozone therapy, in southern Brazil (states of Paraná, Santa Catarina and Rio Grande do Sul) in the year 2021 with 213 participants.

Question	Alternative	Responses number	%
Participants' gender (213):	Male	46	21.60%
	Female	167	78.40%
Age groups they fall into (213):	Into 23-29 years	69	32.39%
	Into 30-39 years	100	46.95%
	Into 40-49 years	32	15.02%
	Into 50-59 years	9	4.23%
	Into 60-69 years	3	1.41%
Training time – Medicine Veterinary course (213):	Graduated less than 10 years ago	146	68.54%
	Graduated ago 11-20 years	48	22.54%
	Graduated ago 21-30 years	14	6.57%
	Graduated ago 31-45 years	5	2.35%
About the use of integrative therapies* (213):	Apply/recommend at least one	210	98.59%
	Do not apply/recommend any of them	3	1.41%
Know the ozone therapy or at least having heard about it (213):	Know or heard about it	203	95.31%
	No know or heard about it	10	4.69%
Among the who know or have heard of it (203):	Apply to their patients	28	13.79%
	Do not apply it but recommend a professional who does it	118	58.13%
	Do not apply and do not recommend ozone therapy	57	28.08%
Reasons why the participants do not apply and do not recommend medical ozone therapy (57):	Lack of efficacy evidence	22	38.60%
	Doesn't have technical knowledge	20	35.09%
	Lack of interest in this area	8	14.04%
	There is no good acceptance of the tutors for the practice	5	8.77%
	Doesn't have the necessary equipment	2	3.51%

Question	Alternative	Responses number	%
About conducting a preparatory course among professionals who apply ozone therapy (28):	Took a preparatory course	23	82.14%
	Don't have a course, but I intend to do it	3	10.71%
	Did not take course for that	2	7.14%
Among the who recommend ozone therapy to be performed by another professional (118):	Do not have a trained person in the establishment where they work and recommend an external professional	61	51.69%
	Do not have it in the establishment yet but intend to invest in the specialization of some collaborator to perform the therapy	23	19.49%
	Already have a qualified professional to apply the therapy in the establishment itself	34	28.81%
Species in which ozone therapy is applied/recommended (146):	Use on dogs and cats	118	80.82%
	Use only on dogs	27	18.49%
	Use only on cats	1	0.68%
Results with ozone therapy among the who apply or recommend it (146):	Excellent	57	39.04%
	Good	75	51.37%
	Reasonable	14	9.59%
	Bad	0	0.00%
About the prophylactic/preventive use of ozone therapy (146):	Apply/recommend	26	17.81%
	Do not apply/recommend	93	63.70%
	Sometimes apply/recommend	27	18.49%
About the moment of choice of ozone therapy in the treatment (146):	Use it in combination with conventional treatment	127	86.99%
	Use it as a last option, when the patient does not improve with conventional treatment	17	11.64%
	Use it as a first option	2	1.37%
About the use of medicinal ozone seeking antibacterial/antifungal action instead of conventional treatments (146):	Doesn't recommend the replacing	87	59.59%
	Sometimes recommend the replacing	46	31.51%
	Recommended replacement	13	8.90%
On the recommendation of ozone therapy in cases of resistance to conventional antibacterials (146):	Never recommend	100	68.49%
	Already recommended	46	31.51%
Among the professionals who already indicated it for antimicrobial treatment with resistance to antibacterials (46):	Had good results	34	73.91%
	Sometimes had good results	9	19.57%
	Not had good results	3	6.52%

Question	Alternative	Responses number	%
On the indication of ozone therapy aiming analgesic effect (146):	Do not use it for that	65	44.52%
	Sometimes use for this	27	18.49%
	Use for this	54	36.99%
About the fact that ozone therapy will have a strong expansion in the coming years (203):	Believe that yes	180	88.67%
	Believe that no	23	11.33%

*Integrative therapies represented in Figure 1.

536 **SUPPLEMENTARY MATERIAL**

537 **Table 1.** Chi-Square and Fisher tests results for the associations of the study variables.

	Association	Test	P-value	Conclusion
1	Gender *vs* number of integrative therapies used	Chi square	0.00123	There is association
2	Gender *vs* use ozone therapy	Chi square	0.00811	There is association
3	Age *vs* knowledge about ozone therapy	Fisher	0.54566	There is no association
4	Age *vs* uses ozone therapy in a prophylactic way	Fisher	0.16665	There is no association
5	Age *vs* uses ozone therapy for antimicrobial purposes	Fisher	0.16268	There is no association
6	Age *vs* time using ozone therapy	Fisher	0.00817	There is association
7	Training time *vs* time using ozone therapy	Fisher	0.01230	There is association
8	Training time *vs* number of integrative therapies used	Fisher	0.08561	There is no association
9	Time using ozone therapy *vs* number of diseases treated with ozone therapy	Fisher	0.27585	There is no association
10	Time using ozone therapy *vs* results with ozone therapy	Fisher	0.84223	There is no association
11	Time using ozone therapy *vs* success in antimicrobial treatment	Fisher	0.37911	There is no association
12	Time using ozone therapy *vs* recommendation for analgesic effect	Fisher	0.00704	There is association
13	State that works *vs* number of integrative therapies used	Fisher	0.60669	There is no association
14	State that works *vs* knowledge of ozone therapy	Fisher	0.58360	There is no association
15	State that works *vs* use (application/recommendation) of ozone therapy	Chi square	0.50624	There is no association
16	State that works *vs* reason why they do not recommend/apply ozone therapy	Fisher	0.03822	There is association
17	State that works *vs* time using ozone therapy	Fisher	0.54948	There is no association

538

5. MANUSCRITO 2 – ANTIMICROBIAL EFFECT OF OZONIZED SALINE SOLUTION ON BACTERIA CAUSING CYSTITIS IN DOGS

Manuscrito submetido para publicação no periódico:

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ANTIMICROBIAL EFFECT OF OZONIZED SALINE SOLUTION ON BACTERIA CAUSING CYSTITIS IN DOGS

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ABSTRACT - Bacterial cystitis is a common clinical problem in dogs and is one of the main reasons for the empirically administration of antimicrobials. This can facilitate the selection of bacteria that are multidrug-resistant to antibiotics. In this context, it is urgent to understand and validate therapeutic modalities that complement antimicrobial treatment in cystitis cases. Ozone therapy has been proposed by scientists owing to the various mechanisms of action in a range of pathologies, both in human and animal medicine. Thus, the objective of this study was evaluated the *in vitro* antimicrobial activity of ozonized saline solution over standard strains and resistant or multi-drug resistant isolates commonly associated with cystitis in dogs. The plating method was used to evaluate the antimicrobial activity of the exposure of 1 mL of phosphate buffer solution containing 10^8 colony forming units (CFU) mL^{-1} for 60 s to 4 mL of ozonized saline solution at 78 µg mL^{-1} over the standard strains (*Staphylococcus aureus* and *Escherichia coli*) and resistant or multi-resistant isolates (*Proteus mirabilis*, *Klebsiella* sp. e *Enterococcus* sp.), and the broth microdilution test to determine the minimum inhibitory and bactericidal concentration for *S. aureus*, *E. coli* and *Pseudomonas aeruginosa* strains from the same solution. The *plating* was the only assay in which treatment with ozonized saline resulted in bactericidal activity, except for *P. mirabilis*. These results are promising, as it is urgent to understand and validate therapeutic alternatives to antimicrobial treatment in cystitis cases with limited allopathic therapy due to increased bacterial resistance.

Keywords: NaCl 0.9% ozonized solution; urinary tract infection; ozone therapy; bacterial resistance; integrative therapy.

INTRODUCTION

The urinary tract infection (UTI) of bacterial etiology is a common disease in dogs and is among the main reasons for the use of antimicrobials (WEESE et al., 2019). However, in most cases, these drugs are administered empirically, without performing a urine culture and antibiogram,

tests necessary both for the diagnosis and to guide treatment decisions for this condition (BARTGES, 2004; BALL et al., 2008).

Empirical administration of antimicrobials is of concern as it may result in the selection of multidrug-resistant (MDR) microorganisms or alter the microbiota, predisposing them to further infections (WILCOX, 2009). Also, in selecting resistant bacterial populations, inappropriate use of antimicrobials can cause adverse effects on the patient (WONG et al., 2015), significant morbidity to animals, and become financially costly (JOHNSTONE, 2019). Pressure from tutors to resolve the patient's discomfort and clinical condition has been identified as a possible reason for adherence to empirical treatments in cases of UTI (SØRENSEN et al., 2018).

The rate at which the resistance of microorganisms to conventional antimicrobial therapies is increasing is alarming (FRIERI et al., 2017). Thus, the development of new treatment options and alternative antimicrobial therapies is a major global challenge for the single health scientific community.

Medical ozone (O_3) therapy has been proposed and studied as a potential adjuvant or alternative for cases of MDR bacterial infection in human and veterinary medicine (ROWEN, 2019; SCIORSCI et al., 2020). Ozone is a powerful non-antibiotic biocidal gas, easily dissolved in water (HEMS et al, 2005), widely used as a sterilant for microorganisms present in drinking water (NGWENYA et al., 2013). The bactericidal effect of O_3 is based on the direct attack on microorganisms by oxidation of their biological membranes (THANOMSUB et al., 2002). Once added to aqueous solutions, O_3 rapidly decomposes, generating reactive oxygen species (ROS) - superoxide ($\bullet O_2^-$), hydroperoxide radicals ($HO_2\bullet$) and hydroxyl radicals ($OH\bullet$) (TOMIYSU et al., 2015). These formed ROS attack the bacterial cell surface oxidizing mainly two groups: polyunsaturated fatty acids and amino acids from peptides, enzymes or proteins. Once damaged by oxidation, these free radicals penetrate through the microbial cell membrane, causing damage to DNA or intracellular proteins, which impacts repair and transcription, and can thus result in cell lysis or death (PATIL et al., 2011). Furthermore, unlike antibiotics, bacteria cannot develop resistance to the ozone mode of action (ROWEN, 2019), thus preventing the resistance and the spread of antibiotic-resistant bacteria (SCIORSCI et al., 2020).

Specifically, in cases of cystitis, case reports in both Wistar rats and humans have revealed antibacterial effects of both distilled water and ozonized saline for some cystitis-causing agents in these species (TASDEMIR et al., 2013; VILELA, 2015; LOGAN, 2018). Thus, we hypothesize that treatment with medicinal ozone may be promising in bacterial cystitis in companion animals. Thus, this study aimed to evaluate the *in vitro* antimicrobial activity of ozonized saline solution on standard strains and bacterial isolates with a resistant or multidrug resistance profile commonly associated with cystitis in dogs.

MATERIAL AND METHODS

1.1.Experimental space, materials and supplies

To evaluate the bactericidal activity of the ozonized saline solution, all the antimicrobial tests were carried out at the Bacteriology Laboratory of the Federal University of Santa Maria (LABAC/UFSM). The culture media used in this study (MacConkey Agar, Blood Agar, Mueller-Hinton Agar and Plate Count Agar [PCA]) were purchased commercially (LaborClin®, Pinhais, Brazil) and the ozonized saline solution (NaCl 0.9% - Sanobiol®) was generated in an ozone generator (O_3) device (Ozone and Life, model OeL1.5RM, São José dos Campos, Brazil), coupled to medical oxygen (O_2) cylinder.

1.2.Strains and isolates

To carry out this study, the standard strains *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 25853) were used, and the isolated bacteria *Klebsiella* sp. (SBP 60/21), *Enterococcus* sp. (SBP 65/21) and *Proteus mirabilis* (SBP 65/18). The *Klebsiella* sp. and *Enterococcus* sp. were recovered from the urine of two dogs with cystitis and that of *P. mirabilis* from a case of otitis in a canine.

The antimicrobial susceptibility test was performed by the disc diffusion method (CLSI, 2013), and all isolates were considered resistant or MDR bacteria according to the criteria proposed by MAGIORAKOS et al. (2012). The following antimicrobial susceptibility profiles were observed in the isolates used: *P. mirabilis* (SBP 65/18) - sensitive to amikacin (30 μg), ampicillin (2 μg), ciprofloxacin (5 μg), florfenicol (30 μg), gentamicin (10 μg), marbofloxacin (5 μg), neomycin (30 μg) and sulfamethoxazole/trimethoprim (25 μg), resistant to amoxicillin + clavulanic acid (30 μg), cefadroxil (30 μg), cephalexin (30 μg), cephalothin (30 μg),

doxycycline (30 µg) and enrofloxacin (5 µg); *Klebsiella* sp. (SBP 60/21) – sensitive to amikacin (30 µg), cefepime (30 µg), ciprofloxacin (5 µg), doxycycline (30 µg), gentamicin (10 µg), neomycin (30 µg) and sulfamethoxazole/trimethoprim (25 µg)), resistant to amoxicillin + clavulanic acid (30 µg), azithromycin (15 µg), cefovecin (30 µg), ceftiofur (30 µg), ceftriaxone (30 µg) and enrofloxacin (5 µg); *Enterococcus* sp. (SBP 65/21) – sensitive to norfloxacin (10 µg), intermediate sensitivity to amoxicillin + clavulanic acid (30 µg) and enrofloxacin (5 µg), resistant to amikacin (30 µg), cephalexin (30 µg), cephalothin (30 µg), marbofloxacin (5 µg), nitrofurantoin (300 µg), and sulfamethoxazole/trimethoprim (25 µg).

Strains and isolates were kept lyophilized and stored at -20ºC and before antibacterial assays, the isolates were seeded on Muller-Hinton Agar (MH) and incubated at 37°C for 24 h.

1.3. Antibacterial activity of ozonized saline solution (O_3SS) by the plating method

In the first stage of this study, two standard strains were used: *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) to evaluate the effectiveness of the ozonated saline solution against Gram-positive and negative bacteria. Next, assays were performed with isolates of multidrug-resistant bacteria commonly identified in cases of cystitis in dogs (*Enterococcus* sp., *Klebsiella* sp. and *P. mirabilis*). All strains and isolates were submitted to three different treatments: pure saline solution (SS); saline solution saturated with medical oxygen (O_2SS) and ozonized saline solution (O_3SS).

To obtain the ozonized saline solution (O_3SS), a volume of 500 mL of saline solution was ozonized for 10 min at a concentration of 78 µg mL^{-1} in a continuous flow of ¼ L min^{-1} of medicinal O_2 in a device that generated O_3 (Ozone and Life, model OeL1.5RM, São José dos Campos, Brazil). To generate the saline solution saturated with oxygen (O_2SS), a volume of 500 mL of 0.9% NaCl solution was used, which was kept in contact with oxygen gas for 10 min, at a flow of ¼ L min^{-1} in the device that generates O_3, but with the batcher turned off, so there is only the output of O_2 gas.

For the test, 1.0 mL of the bacterial solution, containing approximately 10^8 colony forming units (CFU) mL^{-1}, was mixed, within 20 min, with 4.0 mL of O_3SS, O_2SS or SS. After the initial incubation, the solution was exposed to vortexing for one minute (60 s) and 0.5 mL of this solution was added to 4.5 mL of phosphate buffer solution – (PBS), as described by Song et al.

(2018). Then, 50 µL of this solution was seeded, in triplicate, in Petri dishes and incubated at 37˚C for 24 h. After this period, the CFU count of each plate was performed and corrected for CFU mL^{-1}.

1.4. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Based on the results of the tests of antimicrobial activity of O_3SS carried out by the plating method, it was decided to carry out the broth microdilution methodology to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of this solution. This assay was performed using two treatments: O_3SS and its respective negative control (SS), according to the M07-A9 microdilution protocol defined by CLSI (CLSI, 2012). Strains of *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 25853) were used. For this test, the saline solution initially ozonized at a concentration of 78 µg mL^{-1} for 10 min was subjected to dilutions from 1:2 to 1:64, thus obtaining an O_3 concentration in this solution that varied between 39 to 1.22 µg mL^{-1}, respectively. The technique was performed within 20 min after ozonation of the saline solution.

For CBM determination, 10 µL of each O_3SS sample corresponding to each strain, equal to or greater than the MIC value, was transferred to Muller-Hinton Agar plates, followed by incubation at 37º C for 24 h. After this period, it was observed if there was a growth of CFU on the plates.

1.5. Statistical analysis

Data were analyzed using the IBM SPSS *Statistics* 26 software. The average of CFU mL^{-1} between the different groups were compared using Analysis of Variance (ANOVA). The Tukey test was subsequently performed to identify the different treatments, adopting a significance level of 5%.

RESULTS

Regarding the antibacterial assays, O_3SS demonstrated bactericidal activity for the standard strains of *E. coli* and *S. aureus*, and the isolates of *Klebsiella* sp. and *Enterococcus* sp., when compared to control solutions (SS and O_2SS), presenting a p value < 0.05 for each genus and/or species of bacteria (Table 1). Regarding the CFU mL^{-1} counts, it is possible to observe that for

the *Klebsiella* sp. and *Enterococcus* sp. O_3SS zeroed this count, while for *E. coli* and *S. aureus* strains, there was a reduction of 99.06% and 99.72%, respectively (Figures 1, 2, 3 and 4). On the other hand, for the *P. mirabilis* isolate, treatment with O_3SS did not show antibacterial activity ($p = 0.949$) compared to control solutions (SS and O_2SS) (Figure 5).

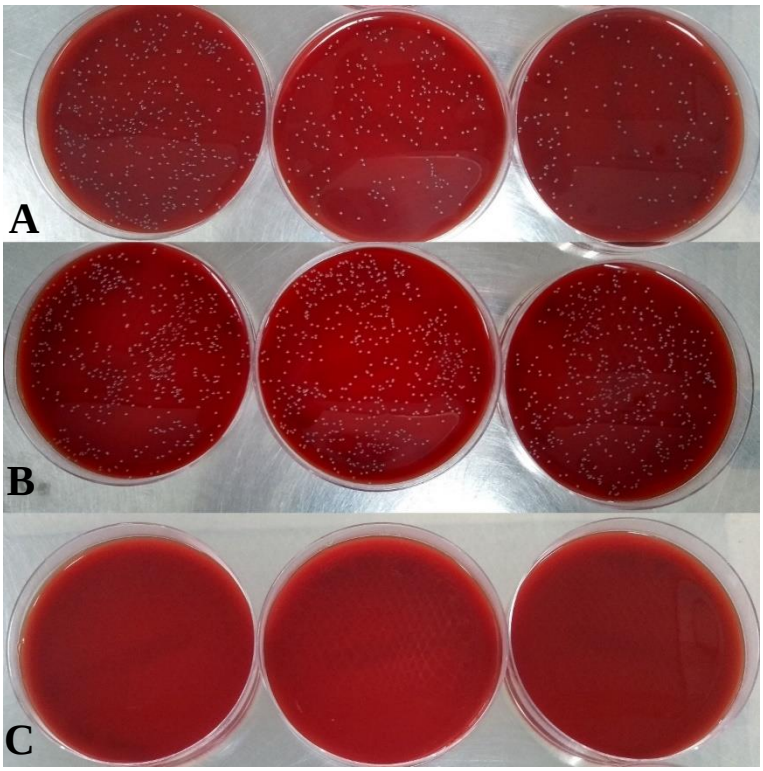
Table 1. The average number of colony forming units (CFU) mL^{-1} after each kind of treatment and for each bacteria gender/specie

Genus/Specie	**SS**		**O_2SS**		**O_3SS**		***p-Value***
	Average	**DP**	**Average**	**DP**	**Average**	**DP**	
S. aureus	2.48×10^{7} **a**	6.55×10^{6}	2.64×10^{7} **a**	8.39×10^{5}	2.33×10^{5} **b**	2.08×10^{5}	0,000
E. coli	9.68×10^{6} **a**	2.82×10^{6}	1.18×10^{7} **a**	4.16×10^{6}	2.67×10^{4} **b**	1.15×10^{5}	0,005
Enterococcus sp.	4.74×10^{6} **b**	2.09×10^{6}	8.28×10^{6} **a**	4.39×10^{6}	0 **c**	0	0,001
Klebsiella sp.	5.2×10^{6} **b**	9.23×10^{5}	6.27×10^{6} **a**	8.93×10^{5}	0 **c**	0	0,000
P. mirabilis	1.44×10^{7} **a**	9.02×10^{5}	1.44×10^{7} **a**	2.41×10^{6}	1.50×10^{7} **a**	3.70×10^{6}	0,949

Data expressed as mean and standard deviation. Values of $p < 0.05$ (ANOVA) on the same line indicate a significant difference between the groups. Different letters superscript on the same line indicates a significant difference between the methodologies according to Tukey's test ($p < 0.05$).

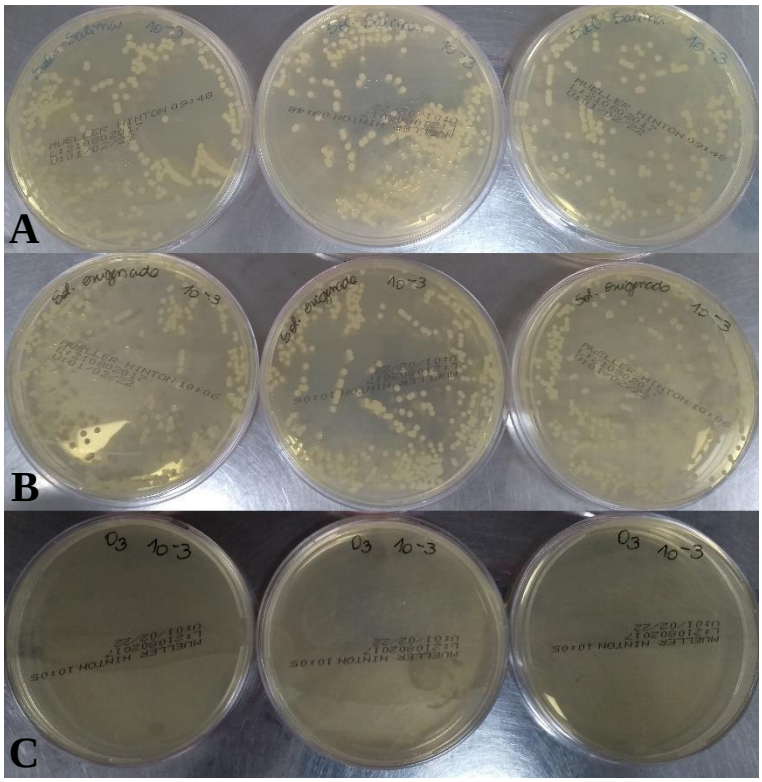
CFU: colony forming units; SD: standard deviation; SS: pure saline solution; O_2SS saline solution saturated with oxygen; O_3SS ozonized saline solution; *S. aureus: Staphylococcus aureus; E. coli: Escherichia coli; P. mirabilis: Proteus mirabilis*

Figure 1. *Enterococcus sp.* colony-forming units (CFU) mL^{-1} count in the different treatment groups at 10^{-3} dilution: A – pure saline solution (SS); B – saline solution saturated with oxygen (O_2SS) and C – saline solution ozonated at 78 µg mL^{-1} (O_3SS). Note that O_3SS zeroed the CFU counts on the three plates tested (C). Culture medium: Blood Agar.



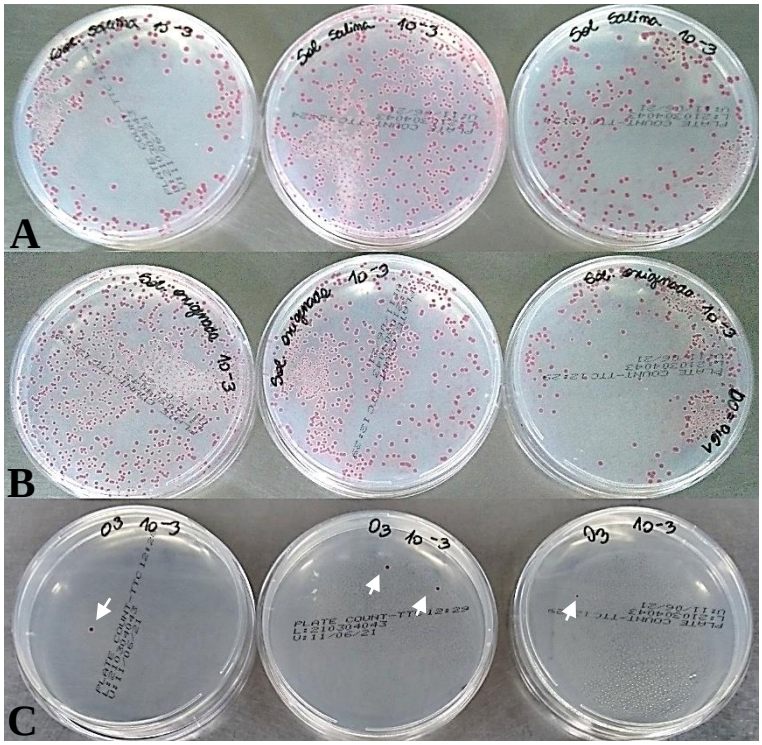
Source: author's collection.

Figure 2. *Klebsiella* sp. colony-forming units (CFU) mL^{-1} count in the different treatment groups at 10^{-3} dilution: A – pure saline solution (SS); B – saline solution saturated with oxygen (O_2SS) and C – saline solution ozonated at 78 µg mL^{-1} (O_3SS). Note that O_3SS zeroed the CFU counts on the three plates tested (C). Culture medium: Mueller-Hinton Agar.



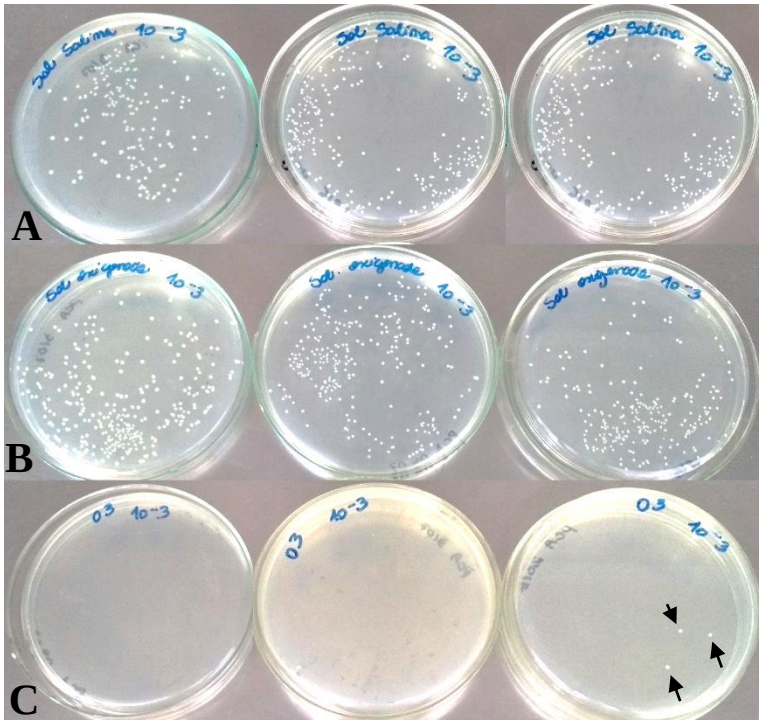
Source: author's collection.

Figure 3. *Escherichia coli* colony-forming units (CFU) mL^{-1} count in the different treatment groups at 10^{-3} dilution: A – pure saline solution (SS); B – saline solution saturated with oxygen (O_2SS) and C – saline solution ozonated at 78 µg mL^{-1} (O_3SS). Note the marked difference in the number of CFUs (arrows) between O_3SS and the others (O_2SS and SS). Culture medium: Plate Count Agar.



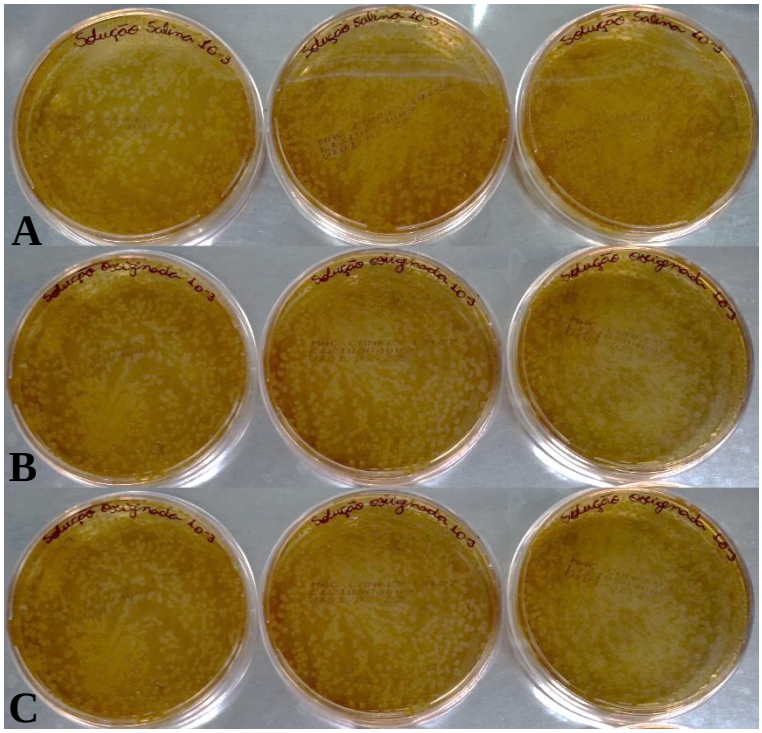
Source: author's collection.

Figure 4. *Staphylococcus aureus* colony-forming units (CFU) mL^{-1} count in the different treatment groups at 10^{-3} dilution: A – pure saline solution (SS); B – saline solution saturated with oxygen (O_2SS) and C – saline solution ozonated at 78 µg mL^{-1} (O_3SS). Note the marked difference in the number of CFUs (arrows) between O_3SS and the others (O_2SS and SS). Culture medium: Plate Count Agar.



Source: author's collection.

Figure 5. *Proteus mirabilis* colony-forming units (CFU) mL^{-1} count in the different treatment groups at 10^{-3} dilution: A –pure saline solution (SS); B – saline solution saturated with oxygen (O_2SS) and C – saline solution ozonated at 78 µg mL^{-1} (O_3SS). Observe the absence of O_3SS antibacterial activity compared to the others (SS and O_2SS). Cultivation medium: MacConkey Agar.



Source: author's collection.

When comparing the antibacterial activity between the control solutions, there was no statistical difference between the treatments with SS and O_2SS for *E. coli*, *S. aureus*, *Klebsiella* sp. and *P. mirabilis*. For the isolate *Enterococcus* sp., there was a greater proliferation in the treatment with O_2SS, on average 74.44% higher than that of SS.

In the MIC and CBM assays, no inhibitory and/or bactericidal activity was observed in any of the dilutions against the standard strains of *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 25853), both for the SS solution (negative control), and for the O_3SS.

DISCUSSION

In this study we evaluated the *in vitro* antibacterial activity of ozonized saline on standard strains and on MDR clinical isolates commonly identified as etiologic agents of cystitis in dogs.

Ozone therapy, despite having been useful since the time of the First World War for the treatment of septic gangrene in German soldiers (BOCCI et al., 2009), was replaced by the use of antibiotic therapy due to the practicality of application and potency, in which just one or two doses were enough to resolve the infectious process.

However, with the emergence of bacterial resistance to antimicrobials, both in human and veterinary medicine, the treatment options for UTIs have become increasingly limited, especially in those individuals who have coexisting conditions that compromise the storage and voiding function of the lower urinary tract (RAFATPANAH et al., 2017). Studies in both guinea pigs and humans have shown promising results from the application of O_3 dissolved in water as an alternative to antimicrobial treatment due to its high reactivity and potent oxidizing effect (TASDEMIR et al., 2013; VILELA, 2015; LOGAN, 2018).

Specifically, concerning ozonated water, *in vitro* studies in medicine and dentistry have shown that relatively short exposure times to this solution (around 60 s) can reduce the count of Gram-positive and Gram-negative bacteria, as well as *Candida albicans* (HUTH et al., 2009; JOHANSSON et al., 2009; SADATULLAH et al., 2012; SONG et al., 2018; TONON et al., 2021). In this work, we demonstrated that 60 s of exposure to O_3SS was able to reduce more than 99% the CFU mL^{-1} count of *S. aureus* and *E. coli*, and 100% of the counts of *Klebsiella* sp. and from *Enterococcus* sp. Similar results were also observed in other *in vitro* studies that used ozonized water for sanitizing dental instruments for *S. aureus, E. coli* and *Enterococcus* sp., for antisepsis of root canals and skin wounds, but some with a time of exposure to ozonated solution greater than 60 s (NAGAYOSHI et al., 2004; CÉSAR et al., 2012; SONG et al., 2018).

The choice of ozone concentration in the ozonized solution (78 µg mL^{-1}) was based on the highest concentration that the generator is capable of producing, to achieve maximum performance, considering that the greater the dissociation of oxygen, the greater the formation of ozone (HEMS et al., 2005). In addition, the bubbling time of SS to the oxygen-ozone (O_2/O_3) gas mixture was established based on the study carried out by SANTOS DE PAULA et al. (2021), in which the volume of 500 mL of this solution reaches the maximum saturation concentration with O_3 after 10 min of exposure to the O_2/O_3 mixture. The maximum time of 20 min for adding the bacteria solution to O_3SS was established based on previous studies that

demonstrated that O_3 dissolved in water maintains its effect for approximately 30 min (BIALOSZEWSKI et al., 2011; BURKE et al., 2012).

Despite using the maximum concentration that the generator is capable of producing and respecting the 20 min limit of the bactericidal action of the ozonated saline solution, ozone did not demonstrate effective activity against all types of bacteria tested in this study. The lack of efficacy of O_3SS on the *P. mirabilis* isolates observed in this research suggests that the antibacterial activity of this solution may be linked to the profile of the infecting strain or also to the exposure time of the microorganism to the solution, which was 60 s. To date, based on the literature, no study describes the antibacterial activity of O_3SS against this species of bacteria, much less with the multidrug resistance profile presented by the tested isolate.

The absence of inhibitory and/or bactericidal activity of O_3SS observed in the MIC and CBM assays were also reported in another study. However, the concentration of ozone injected in the saline solution was 8 µg mL^{-1} (BORGES et al., 2017), significantly lower than that used in our experiment (78 µg mL^{-1}). The fact that O_3SS is subjected to different dilutions and, therefore, to lower concentrations, could be one of the factors that impair or even prevent the action of dissolved O_3 in the solution and the ROS generated by it on the plasma membrane of bacterial cells.

Considering that bacterial resistance to antimicrobials is an emerging public health problem and that the transmission of resistance genes between pathogens originating from pets and humans has already been documented (DAMBORG et al., 2016; PITOUT; DEVINNEY, 2017), the results of this study are promising in terms of public health. Our results may encourage *in vivo* research that evaluates the effectiveness and protocols of bladder lavage with O_3SS in cases of bacterial cystitis in companion animals, until obtaining the results of bacterial culture and antibiogram, or even be an alternative treatment depending on the type of bacteria involved.

CONCLUSION

The present study demonstrated that O_3SS (78 µg mL^{-1}) can be considered a promising antibacterial agent for cases of cystitis and even skin infections in dogs caused by *E. coli*, *S. aureus*, *Klebsiella* sp. and *Enterococcus* sp. In this study, we did not find the antibacterial activity of this solution against the isolate MDR *P. mirabilis*, which suggests that the action of

O_3SS may be related to the genus/species of bacteria, as well as the exposure time of the microorganism to it. Therefore, based on our results, further studies that test different times of exposure to O_3SS are necessary to evaluate the antibacterial activity of this solution on these and other genera/species of bacteria.

ACKNOWLEDGMENTS

The authors thank the Federal University of Santa Maria for the opportunity to develop this research, the Coordination for the Improvement of Higher Education Personnel (CAPES – financial code 001) and the National Council for Scientific and Technological Development (CNPq - funding code 870589/1997-0) for its funding.

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6. ARTIGO 1 – EFFECTIVENESS OF OZONIZED SALINE SOLUTION IN THE TREATMENT OF *PROTEUS* SPP BACTERIAL CYSTITIS

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Effectiveness of ozonized saline solution in the treatment of *Proteus* spp. bacterial cystitis

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ABSTRACT

Bacterial cystitis is a common clinical problem among cats and dogs and is one of the main reasons for the administration of antimicrobials. This can cause serious damage to public and animal health, as this practice facilitates the selection of bacteria that are multidrug-resistant to antibiotics. In this context, it is urgent to understand and validate therapeutic modalities that complement antimicrobial treatment in cystitis cases. Ozone therapy has been proposed by scientists owing to the various mechanisms of action in a range of pathologies, both in human and animal medicine. This paper describes the bactericidal action of two different protocols of bladder irrigation with ozonized saline solution (59 µg/mL) in a paraplegic canine with recurrent bacterial cystitis caused by *Proteus* spp. In the first protocol, the bladder instillations were applied once a day for three consecutive days while in the second, successive lavages were performed throughout the day until a significant reduction in the presence of bacteria in the urine sediment. In this study, we were able to demonstrate that repeated bladder instillation within 24 hours was the most effective treatment for *Proteus* compared to a single instillation on successive days.

Key words: bactericidal effect**;** lower urinary tract infection; medicinal ozone; ozone therapy

INTRODUCTION

Bacterial urinary tract infection (UTI) is a common clinical problem among cats and dogs and is the main reason for antimicrobial administration.[1,2] Approximately 14% of all dogs present with at

least one episode of UTI during their lifetime.[3] In dogs, UTI occurs more often in females, elderly animals, and those with certain concomitant illnesses.[4-6] Spinal cord injury is a coexisting condition that increases UTI risk in both human beings and animals; this is because of an impairment of storage and voiding function in the lower urinary tract.[7]

With the emergence of antimicrobial resistance, treatment options for UTI and other infections are becoming increasingly limited. Unconventional treatments such as oral immunostimulants, mannose, acupuncture, vaginal and oral estrogen, and, more recently, ozone therapy, have been proposed and studied.[8-15]

There have been medical reports of the resolution of refractory bacterial cystitis when treated with medicinal ozone, rather than antimicrobials.[13,15] An experimental study demonstrated the antimicrobial effect of bladder irrigation with ozonized saline solution (O_3SS) as a treatment for *Escherichia coli*-induced cystitis.[16] Given these promising results of ozone therapy in the treatment of bacterial cystitis and the lack of such studies in dogs, this study reported the effectiveness of two different protocols of bladder irrigation with O_3SS in a paraplegic canine with recurrent bacterial cystitis caused by *Proteus* spp.

CASE REPORT

A 14-year-old female Dachshund, paraplegic, weighing 10.2 kg, was referred to the ozone therapy sector of the Veterinary Hospital of the Federal University of Santa Maria, Santa Maria, Brazil, because of recurrent bacterial cystitis caused by *Proteus* spp. Two months after treatment with enrofloxacin, amoxicillin, and potassium clavulanate, the patient developed adverse effects such as pharmacodermia and corneal ulcers.

Alternative treatments were discussed in our team and ozone therapy was selected, which consisted of bladder instillations of O_3SS once a day for 3 consecutive days. To obtain O_3SS, 1 L of 0.9% NaCl solution was ozonized for 10 minutes at a concentration of 59 μg/mL in an O_3 generator apparatus (model OeL1.5RM, Ozone and Life, São José dos Campos, Brazil), with an O_2 flux of 0.25 L/min. After antisepsis of the external genitalia with 1% chlorhexidine gluconate, a bladder catheterization was performed using a sterile 8-Fr urethral catheter (Mark Med, Bragança, São Paulo, Brazil) lubricated with lidocaine hydrochloride jelly (20 mg/g). First, the urinary bladder was drained through the urethral catheter aided by syringes and 10 mL of urine was separated for urinalysis, culture, and antibiogram. Next, within a 20-minute interval, the bladder was washed with 1 L of O_3SS at a concentration of 59 μg/mL.

Table 1 shows the results of the urine analysis (urinalysis, culture, and antibiogram) before the treatment (D0), and after the first (D1), second (D2), and third (D3) treatments. As the urinary bacterial load did not reduce after D3, we started a conventional treatment with amoxicillin and potassium clavulanate (12.5 mg/kg, twice a day; 10 days), which effectively resolved cystitis.

Table 1: Urinalysis, urine culture, and antibiogram profile of the treatment protocol performed once a day for 3 consecutive days in a female Dachshund canine with bacterial cystitis before the treatment (D0), after the first treatment (D1), after the second treatment (D2), and finally after the third treatment (D3).

	D0	D1	D2	D3
Urinalysis				
Color	Yellow	Yellow	Yellow	Yellow
Odor	Sui generis	Malodorous	Malodorous	Sui generis
Turbidity	Cloudy	Cloudy	Cloudy	Cloudy
Specific gravity	1036	1032	1036	1030
pH[a]	8.5	8	8	8
Protein[a]	1+	2+	2+	1+
Transitional epithelial cells (/400 x magnification field)[b]	1	2	1	0
Red blood cell (/400 x magnification field)[b]	6.8	3.6	1.2	2.4
White blood cell (/400 x magnification field)[b]	>50	24.2	30.8	10.2
Bacteria[c]	Many[d]	Many[d]	Many[d]	Many[d]
Struvite[c]	Many	Few	Few	Moderate
Uroculture (colony-forming unit/mL)				
Proteus spp.	1.2×10^7	6.5×10^7	5.9×10^7	2.5×10^7
Antibiogram				
Gentamicin	Susceptible	Susceptible	Susceptible	Susceptible
Marbofloxacin	Susceptible	Susceptible	Susceptible	Susceptible
Neomycin	Susceptible	Susceptible	Susceptible	Susceptible
Nitrofurantoin	Resistant	Resistant	Resistant	Resistant
Amoxicillin + clavulanic acid	Susceptible	Susceptible	Susceptible	Susceptible
Enrofloxacin	Intermediate	Intermediate	Intermediate	Intermediate
Cephalotin	Susceptible	Susceptible	Susceptible	Susceptible
Trimethoprim + sulfadiazine	Susceptible	Susceptible	Susceptible	Susceptible

Note: Superscript a was measured with a urine reagent strip. Superscript b indicates the data were the average between 10 slide fields. Superscript c was determined by microscopic examination. Superscript d indicates the presence of cocci and bacilli-type bacteria.

Because the animal had paraplegia, the tutor was instructed to perform daily bladder compressions to induce urine drainage. However, 6 months after the first treatment, the tutor noted color and odor alterations in the animal's urine. The patient underwent another physical examination, but no clinical alterations were observed. However, on ultrasound examination, the bladder presented medium repletion filled with suspended anechoic and echogenic material (sediment) and small hyperechogenic, punctiform, and linear structures suggestive of crystals or small clots. The bladder wall had thickened, with an irregular mucosal layer and evident stratification of the layers (diameter, approximately 0.82 cm; **Figure 1A**).

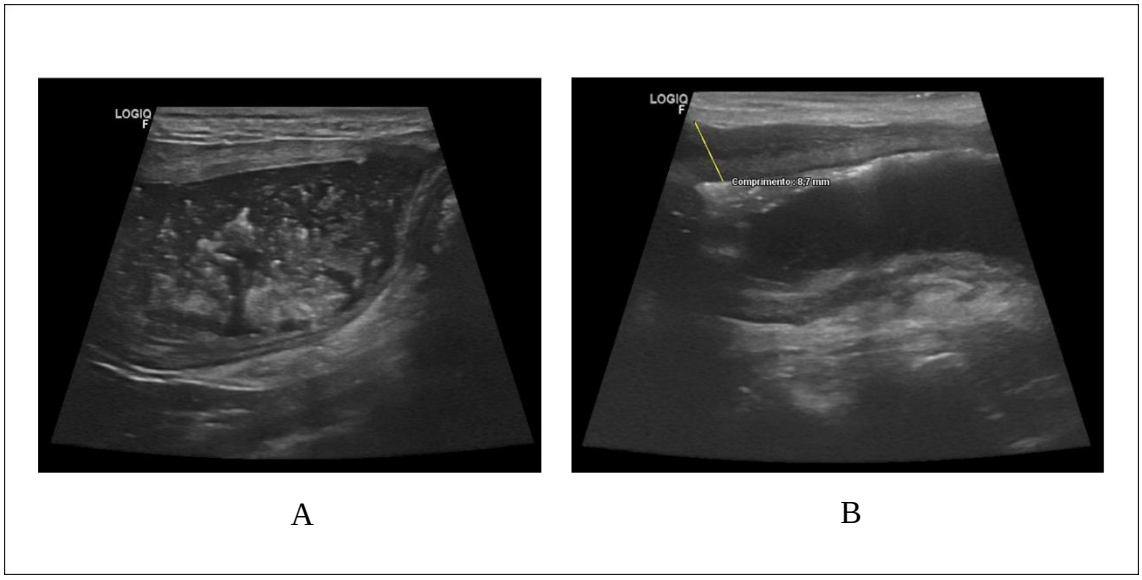

Figure 1: Ultrasound of the bladder of a female Dachshund canine with bacterial cystitis at 6 months after the first treatment.

Note: (A) The evident and accentuated stratification of the urinary bladder wall layers, in addition to the presence of anechoic and echogenic content in suspension (sediment) and small hyperechogenic, punctiform, and linear structures suggestive of crystals or small clots before the beginning of treatment. (B) The marked reduction in suspended echogenic content (sediment) and small hyperechogenic, punctate, and linear structures suggestive of crystals or small clots.

A bladder catheterization was performed following the aforementioned antisepsis procedure for urine collection and initiation of therapy. Due to the previous therapeutic failure (3 consecutive days of bladder irrigation), we adopted a new protocol in which successive lavages were performed throughout the day until a significant reduction in the presence of bacteria in the urine sediment. This was achieved after the third bladder irrigation, performed every two hours. For each irrigation, 1 L of O_3SS for 10 minutes at a concentration of 59 µg/mL in an O_3 generator device, with an O_2 flux of 0.25 L/min.

Table 2 shows the results of the urinalysis, which was performed on the same day before treatment (T0), and after the first (T1), second T2, and third (T3) lavages.

To report this case, we had the consent of the guardian of the animal and the veterinarians who participated in the case.

Table 2: Urinalysis, urine culture, and antibiogram profile of the treatment protocol performed on the same day of a female Dachshund canine with bacterial cystitis, before treatment (T0), after the first bladder irrigation (T1), after the second bladder irrigation (T2) and, finally, after the third bladder irrigation (T3).

	T0	**T1**	**T2**	**T3**
Urinalysis				
Color	Brown	#	#	Red
Odor	Malodorous	#	#	*Sui generis*
Turbidity	Cloudy	#	#	Cloudy
Specific gravity	1040	#	#	1028
pH[a]	8.5	#	#	8
Protein[a]	3+*	#	#	3+*
Transitional epithelial cell (/400 x magnification field)[b]	6*	#	#	1
Squamous epithelial cell(/400 x magnification field)[b]	2	#	#	1
Red blood cell (/400 x magnification field)[b]	>100*	#	#	>500*
White blood cell (/400 x magnification field)[b]	>200*	#	#	18*
Bacteria[c]	Many[d]	#	#	Few*
Struvite[c]	Moderate	#	#	0*
Uroculture (colony-forming unit/mL)				
Proteus spp.	7.9×10^{7}*	9.0×10^{6}*	6.9×10^{2}*	5.6×10^{2}*
Antibiogram				
Gentamicin	Susceptible	Susceptible	Susceptible	Susceptible
Marbofloxacin	Susceptible	Susceptible	Susceptible	Susceptible
Neomycin	Intermediate*	Intermediate*	Intermediate*	Susceptible
Nitrofurantoin	Resistant	Resistant	Resistant	Resistant
Amoxicillin + clavulanic acid	Susceptible	Resistant*	Intermediate*	Intermediate*
Enrofloxacin	Susceptible*	Susceptible*	Susceptible*	Susceptible*
Cephalotin	Intermediate*	Intermediate*	Intermediate*	Susceptible
Trimethoprim + sulfadiazine	Resistant*	Intermediate*	Intermediate*	Intermediate*

Note: Superscript a was measured with a urine reagent strip. Superscript b indicates the data were the average between 10 slide fields. Superscript c was determined by microscopic examination. Superscript d indicates the presence of cocci and bacilli-type bacteria. Superscript * indicates the difference in the observation time points between Table 1 and Table 2.

DISCUSSION

Herein, we document the care provided to a paraplegic canine patient with recurrent cystitis caused by a multidrug-resistant strain of *Proteus* spp. None of the drug approaches − different combinations of antibiotics for different periods− could resolve the infection. Therefore, the patient was referred to our hospital for ozone therapy.

The ineffectiveness of conventional treatments due to microbial resistance to antibiotics has been extensively reported, and both veterinarians and guardians have been searching for alternatives to cure or improve the living conditions of companion animals. The literature has demonstrated the antimicrobial effects of ozone in dogs in preventing dental plaque formation and its prophylactic use to reduce bacterial colonization in the conjunctival sac and periocular skin.[17,18]

In our first therapeutic approach, we performed the conventional protocol of bladder irrigation, based on the results obtained in an experimental study.[16] On the 1st day, a urine sample was sent for culture and an antibiogram. Despite successive days of bladder irrigation, the infection did not respond to treatment− urinalysis showed the persistence of bacteria. At the end of the lavages, the urine culture results of the D0 sample revealed bacterial susceptibility to amoxicillin-clavulanate; therefore, these drugs were prescribed. At this time, the infecting strain was identified as *Proteus* spp., which is characterized by invasive growth and rapid multiplication: the bacterial population doubles every 3.5 hours at 37°C.[19] Therefore, bacteria that were not eliminated through bladder irrigation could recolonize the bladder mucosa within 24 hours. The colony-forming unit (CFU) count increased from one day to the next as it did between D0 and D1 (**Table 1**). We hypothesized that the inability of ozone therapy to kill the residual bacteria might cause bacterial recolonization. In agreement with our hypothesis, another study observed the same effect following treatment with ozonized distilled water of root canals experimentally infected with *Enterococcus faecalis* and *Candida albicans*.[20] After 10 days of antibiotic treatment, a complete remission of the infection was observed.

In the second therapeutic moment, 6 months later, in a new recurrence in the same patient, due to the previous failure, we performed successive irrigations on the same day. At T3 (after 3 washes), the bacterial count dropped drastically, following which we stopped the washes. This result was confirmed by the urine culture, which showed a decrease in the bacterial population (from almost 80 million CFU/mL to 560 CFU/mL). According to the literature, to consider that bacteriuria is consistent with cystitis, a bacterial count of > 10,000 CFU/mL is needed for samples obtained by catheterization; any value < 1000 CFU/mL should be considered as contamination.[21] Therefore, we achieved the resolution of the clinical condition with the isolated use of bladder irrigation with O_3SS.

During the procedure, urinalysis showed an increase in the red blood cell count. We considered

this event to be related to the removal of the biofilm that covered the bladder mucosa, visibly altered by a chronic inflammatory process (**Figure 1**), which facilitated the diapedesis of these cells into the bladder lumen. Persistent bleeding, probably due to the antioxidant and anti-inflammatory actions of ozone on biological membranes,[22,23] was not reported. After washing, the bladder environment changed considerably. This change was visible on the ultrasound image, characterized by a marked reduction in the suspended echogenic content (sediment) and small hyperechogenic, punctiform, and linear structures suggestive of crystals or small clots (**Figure 1B**). Furthermore, the moderately distended urinary vesicle was approximately 0.14 cm thick.[24] In this case, the thickness was significantly increased (0.82 cm), probably in response to the chronic inflammatory process.

O_3SS selected a population with intermediate susceptibility to amoxicillin-clavulanate. Conversely, it seems to have changed the pattern of susceptibility to cephalothin and neomycin, which could expand the therapeutic options. We did not find any publications that strongly linked this action to ozone therapy. Therefore, in a controlled experiment with a sufficient sample size, which is a limitation in this report, we may be able to statistically perceive this correlation.

This report demonstrates that the effective bactericidal action of O_3SS in bacterial cystitis depends on the infecting agent. Therefore, it is important to know the characteristics of the pathogenic bacterial growth to determine the most effective protocol. Repeated bladder instillation within 24 hours was the most effective treatment for *Proteus* compared to a single instillation on successive days.

Author contributions

Study conception and design, and data collection and interpreting: AME and AB. Urine analysis and cooperation among the authors: NVB. Carrying out and interpreting the urine culture and antibiogram: CM and DC. Manuscript revision: JFC and CMA. All authors approved the final version of the manuscript.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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Ethical approval

Not applicable. To report this case, we had the consent of the guardian of the animal and the veterinarians who participated in the case.

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7 DISCUSSÃO

A medicina veterinária busca constantemente alternativas com embasamento científico capazes de auxiliar na resolução das enfermidades animais (MEMON et al., 2016). A ineficácia de tratamentos convencionais, bem como, o alto índice de efeitos colaterais e a resistência microbiana aos antibióticos, faz com que os profissionais e tutores busquem a medicina integrativa para a cura ou melhoria da condição de vida dos animais de companhia (MEMON et al., 2016). A OZT vem ganhando explanação por cientistas devido aos vários mecanismos de ação em uma gama de enfermidades, tanto na medicina humana como animal (SCASSELLATI et al., 2020; SCIORSCI et al., 2020). O custo reduzido também é um fator atrativo e que contribui na expansão do uso clínico (SCASSELLATI et al., 2020).

Apesar de ser antiga, inclusive tendo sido utilizada com sucesso no tratamento antimicrobiano e na cicatrização de feridas de soldados durante a Primeira Guerra Mundial (BOCCI, 2005), a terapia com ozônio medicinal ficou por muito tempo esquecida. Prova da escassez de estudos randomizados, bem como do seu esquecimento, são as pesquisas em bases de dados: quando inserido na base ‘Pubmed’ o termo ‘ozone therapy’ são encontrados atualmente (2023) 4363 textos completos de artigos publicados, destes, apenas 223 são testes controlados (randomizados) e 319 são ensaios clínicos; o restante trata-se de meta-análises, revisões e livros. A maior quantia de publicação surgiu nos últimos três anos: em 2020 foram 297 textos completos publicados, em 2021, 308 e em 2022, 267. Já com o termo ‘ozone therapy veterinary’ aparecem apenas 113 textos completos, sendo o auge nos últimos dois anos (2021 e 2022) com 16 e 15 publicações respectivamente, demonstrando a grande lacuna de conhecimento na área veterinária, e ao mesmo tempo, o seu potencial de expansão. Nesse sentido, nosso grupo de pesquisa tem tentado elucidar as principais prescrições de ozônio medicinal recomendadas aos pacientes por médicos veterinários e verificar, por meio de um estudo *in vitro* e outro *in vivo*, a ação antimicrobiana da solução salina ozonizada (SSO_3) sobre cepas padrão e sobre isolados bacterianos resistentes e com perfil de multirresistência comumente associados a cistite em cães, resultando em dois manuscritos e um relato de caso.

O objetivo da investigação junto aos profissionais veterinários de pequenos animais, o qual se deu por meio de um questionário e resultou em um dos manuscritos, foi verificar as principais prescrições de tratamentos com ozônio medicinal na clínica de pequenos animais. Admirou-nos o fato de quase 100% dos participantes utilizarem alguma ou várias terapias integrativas na sua rotina, e o fato de a OZT já estar entre as três mais utilizadas no espaço

geográfico do nosso estudo, considerando que o seu uso foi liberado em nosso país em outubro de 2020 (CFMV, 2020). As principais prescrições da OZT são interessantes de serem levantadas, uma vez que pouco são os estudos científicos experimentais que comprovam os efeitos, bem como, raros os estudos sobre os mecanismos de ação do ozônio *in vivo*, havendo um vasto potencial a ser explorado.

Foi possível verificar que, os profissionais que utilizam a terapia relatam vários resultados promissores, em especial quando usada para a cicatrização de feridas abertas, para o tratamento de dermatites, para a assepsia de feridas contaminadas e a cicatrização pós-cirúrgica. Vários estudos e relatos demonstraram a eficácia do ozônio medicinal na cura de transtornos de pele (SMITH et al., 2017; XIAO et al., 2017; ANZOLIN et al., 2020). Xiao et al. (2017) demonstraram em seu estudo que a aplicação de óleo ozonizado reduziu significativamente a área de lesões de pele e acelerou a cicatrização, por meio do aumento da migração de fibroblastos e diminuição da inflamação. Além de acelerar a cicatrização, os efeitos se estendem a redução da infecção microbiana, efeito de desbridamento, modulação da fase inflamatória, estímulo à angiogênese, bem como reações biológicas e enzimáticas que favorecem o metabolismo do oxigênio, melhorando a cicatrização da ferida (ANZOLIN et al., 2020). É sabido que as dermatopatias apresentam grande prevalência na clínica de pequenos animais, sendo o motivo mais frequente para os animais serem levados ao médico veterinário (SCOTT et al., 1996), o que pode também colaborar para o uso predominante nessas situações, além dos resultados positivos bem documentados para as patologias e feridas de pele.

A partir deste questionário, pudemos observar que o uso do ozônio medicinal no tratamento de infecções resistentes aos antimicrobianos sem dúvida é um dos pontos altos da sua utilização, uma vez que a resistência aos antimicrobianos é uma ameaça a saúde pública global devido ao esgotamento da pipeline de antibacterianos e o rápido desenvolvimento de mecanismos de resistência. No presente estudo, um terço dos profissionais que recomendam/aplicam a OZT declararam ter tido bons resultados quando aplicaram a terapia nos casos de resistência a antimicrobianos; destes, a maioria relatou eficácia no tratamento, o que parece creditar o efeito antibacteriano do ozônio.

A terapia com ozônio foi validada como tratamento alternativo antisséptico contra a maioria dos microrganismos envolvidos nas infecções cutâneas e feridas contaminadas (BORGES et al., 2019; ROTH et al., 2020). Marchegiani et al. (2019) demonstraram que a limpeza da pele periocular com óleo ozonizado e a instilação de colírio ozonizado no saco conjuntival foi capaz de reduzir a flora bacteriana pré-operatória dessas regiões com efeito

equivalente à solução de iodopovidine 5%, evidenciando a atividade microbicida do ozônio. Ao contrário do que ocorre com os antibióticos, as bactérias não têm a capacidade de desenvolver resistência ao modo de ação do ozônio (ROWEN, 2018), evitando assim a formação de genes de resistência e a disseminação de bactérias resistentes a antibióticos (SONG et al., 2018; SCIORSCI et al., 2020).

Apesar de não ser uma das principais prescrições do nosso levantamento junto aos veterinários, o uso do ozônio medicinal na cistite bacteriana despertou interesse no grupo de pesquisa, pois trata-se de uma afecção que acomete frequentemente cães e está entre os principais motivos para o uso significativo de antimicrobianos (LING, 1984; WEESE, 2019). A urocultura combinada ao teste de suscetibilidade a antibióticos (antibiograma) constitui a base laboratorial para o diagnóstico de infecção do trato urinário (ITU), sendo o melhor instrumento para orientar as decisões de tratamento (BALL et al., 2008; BARTGES et al., 2004). No entanto, comumente os clínicos iniciam o tratamento antimicrobiano empírico com base apenas no diagnóstico presuntivo, antes mesmo de obter resultado de cultura e antibiograma (BALL et al., 2008). Este uso inapropriado de antimicrobianos pode provocar uma variedade de prejuízos não somente para a saúde do paciente, mas também para o tutor e para a saúde pública, uma vez que, facilita e aumenta a seleção de bactérias resistentes a antibióticos, dificultando e prolongando o tratamento desses animais (JOHNSTONE, 2020; WILCOX, 2009; WONG et al., 2015). Além disso, a constatação da transmissão de bactérias multirresistentes entre espécies torna essa questão ainda mais preocupante (DAMBORG et al., 2016; PITOUT; DEVINNEY, 2017).

No contexto do aumento da incidência de cistites resistentes a antimicrobianos, a terapia com ozônio medicinal tem obtido destaque na medicina humana, devido a suas propriedades antimicrobiana, anti-inflamatória, imuno-estimulante, analgésica e antioxidante (BOCCI, 2005; SCIORSCI et al., 2020). Na medicina humana há relatos de resolução de cistites refratárias ao tratamento com antimicrobianos, quando tratadas com OZT (LOGAN, 2018; VILELA, 2015). TASDEMIR et al. (2013) demonstraram experimentalmente o papel protetor do ozônio contra os danos causados pelo estresse oxidativo e nitrosativo que ocorre na vesícula urinária durante um quadro de cistite induzida por *E. coli* em ratos Wistar. Foi possível determinar também o efeito antimicrobiano do ozônio, sendo que dos oito animais infectados experimentalmente e que foram submetidos à lavagem intravesical com solução salina ozonizada, apenas um manteve cultura positiva após o término do tratamento.

Vilela (2015) relata a melhoria laboratorial e clínica de uma mulher com infecção bacteriana crônica do trato urinário refratária a múltiplos antimicrobianos após três instilações de água destilada ozonizada realizadas diariamente, com um tempo de permanência da solução de 15 minutos na vesícula urinária. Logan (2018) também reportou que o tratamento com duas instilações intravesicais de água destilada ozonizada por semana, durante um período de 12 semanas, associadas a auto-hemoterapia maior foi efetivo no caso de uma mulher com cistite crônica causada por *E. coli* produtora de β-lactamases de espectro estendido (EC-ESBL).

Desta forma, buscamos verificar a atividade antimicrobiana da SSO_3 sobre as principais bactérias implicadas como agentes causadoras de cistite em cães. Por meio de um estudo *in vitro* (Manuscrito 2), foi possível verificar que a exposição de um minuto à SSO_3 em concentração de 78 µg mL^{-1} demonstrou atividade bactericida para as cepas padrão de *E. coli* e *S. aureus*, e para os isolados de *Klebsiella* sp. e *Enterococcus* sp., quando comparadas às soluções controle (solução salina pura e solução salina saturada com oxigênio). Por outro lado, para o isolado de *P. mirabilis*, o tratamento com a SSO_3 não apresentou atividade bactericida em comparação com as soluções controle, o que sugere que a ação da SSO_3 pode estar relacionada ao gênero/espécie de bactéria, bem como, ao tempo de exposição do microrganismo a mesma. Esta hipótese é reforçada pelo artigo científico, no qual documentamos dois diferentes protocolos de lavagem vesical com SSO_3, em dois momentos diferentes, em um canino paraplégico com cistite bacteriana recorrente causada por *Proteus* spp. Neste, a primeira abordagem terapêutica utilizada, a qual consistiu em repetidas instilações de 60 mL de SSO_3 a uma concentração de 59 µg mL^{-1}, uma vez ao dia, por três dias consecutivos, não foi efetiva. Já, o segundo protocolo utilizado, que consistiu de sucessivas lavagens em um único dia, respeitando-se um intervalo de 2 horas entre as mesmas, resultou em uma redução significativa da presença de bactérias na sedimentoscopia da urina e na urocultura.

Acreditamos que o insucesso do primeiro protocolo de lavagem vesical ocorreu porque as bactérias remanescentes puderam recolonizar a mucosa vesical, uma vez que o *Proteus* spp. tem por característica crescimento invasivo e rápida multiplicação, cuja população de bactérias duplica a cada 3,5 horas a 37 °C (RAUPRICH et al., 1996), e que o ozônio não possui efeito residual (CARDOSO et al., 2008).

A inatividade da SSO_3 sobre a cepa *Proteus mirabilis* no estudo *in vitro* e a ação bactericida sobre a cepa *Proteus* spp. no estudo *in vivo*, parece estar relacionada ao perfil de susceptibilidade da cepa infectante, uma vez que a do estudo *in vitro* era multirresistente, enquanto que a do estudo *in vivo*, era apenas resistente a dois antimicrobianos (nitrofurantoína

e a trimetoprima + sulfadiazina). Além disso, o tempo de exposição da solução de bactérias no estudo *in vitro,* e a própria ação mecânica exercida pelas consecutivas instilações e drenagens da SSO_3 até que o volume de 1 L fosse utilizado, podem ser considerados como fatores adicionais que podem ter contribuído para a ação bactericida da SSO_3 observada no estudo *in vivo,* uma vez que a concentração de O_3 utilizada neste estudo foi inferior à utilizada no estudo *in vitro.*

Além de reduzir a concentração bacteriana nos casos de cistite, há estudos em mulheres que descrevem o efeito analgésico e anti-inflamatório do O_3 medicinal (MUZI; TATI, 2017; NEIMARK et al., 2014). De acordo com Muzi e Tati (2017), todas as mulheres que participaram do estudo relataram diminuição da sensação de disúria após a primeira aplicação intravesical de 100 mL de gás O_3 a uma concentração de 30 µg mL^{-1}. Já Neimark e colaboradores (2014) demonstraram em seu estudo que houve uma regressão significativa do edema e hiperemia da mucosa vesical após a insuflação intravesical de SSO_3 a uma concentração de 2-4 µg mL^{-1}. Os autores acreditam que os sinais de linfangiogênese e neoangiogênese observados nas lâminas das biópsias vesicais das mulheres tratadas com o O_3 melhoraram a drenagem da mucosa vesical, o que, por sua vez, contribuiu para a regressão do processo inflamatório. De acordo com Sorensen e colaboradores (2018), a necessidade de iniciar o tratamento para resolver o desconforto do paciente e a própria pressão dos tutores são apontados como as principais razões para a adesão aos tratamentos empíricos em casos de ITU em animais de companhia. Dessa forma, apesar de não haver estudos que comprovem esse efeito em casos de cistite em cães, poderíamos sugerir o uso intravesical da SSO_3 na rotina como um "protocolo emergencial", com o intuito de aliviar o desconforto do paciente até a obtenção do resultado da urocultura e antibiograma. Essa tese pode ser afirmada com mais convicção neste caso, uma vez que, essa ideia também é sugerida no nosso estudo das prescrições por médicos veterinários (Manuscrito 1), os quais utilizam a OZT visando a analgesia dos pacientes. Incrementa esse dado o fato de termos associação positiva entre o uso da OZT para essa finalidade e o tempo de atuação dos profissionais, o que significa que quanto mais tempo os profissionais utilizam essa modalidade terapêutica, mais a recomendam visando esse efeito, uma vez que obtiveram bons resultados.

Assim, a partir dos dados obtidos com esta tese foi possível verificar que a OZT está entre as terapias integrativas mais recomendadas pelos profissionais, apesar de recentemente regulamentada (2020) e que a SSO_3 pode ser um agente antibacteriano promissor para os casos de cistite e até mesmo infecções cutâneas em cães causadas por *E. coli*, *S. aureus*, *Klebsiella*

sp. e *Enterococcus* sp. No entanto, são necessários, estudos randomizados que avaliem diferentes tempos de exposição da SSO_3 a fim de se avaliar a atividade antibacteriana dessa solução sobre estes e outros gêneros/espécies de bactérias. Porém, na medicina veterinária, a dificuldade para a realização de estudos experimentais randomizados e controlados com o uso de animais de companhia é uma realidade, uma vez que, as instituições de ensino superior não dispõem de animais de pesquisa *pets*, devido aos conflitos com os comitês de ética, pois teríamos que provocar a cistite bacteriana nos animais, e com a própria sociedade, que muitas vezes não entende o intuito e a necessidade desses.

Além disso, no caso de estudos experimentais com *pets*, além de termos de inocular uma determinada cepa bacteriana para provocar a cistite, seriam necessários um grande número de animais e os mesmos teriam de ser homogêneos. Neste caso, somente sobram os estudos com cobaias, o que é complicado no caso da cistite devido à dificuldade de sondagem destes animais, e também os estudos *in vitro*, os quais apresentam uma lacuna grande quando são transpostos para os tratamentos *in vivo*.

8. CONCLUSÃO

Com os resultados obtidos no conjunto de estudos que compõe essa tese foi possível demonstrar que o potencial de expansão do uso da OZT na medicina veterinária é amplo e que, mesmo antes de sua regulamentação no Conselho Federal de Medicina Veterinária, os veterinários já vinham recomendando e verificando resultados positivos. É admirável o fato desta terapia estar sendo uma das mais usadas entre as integrativas existentes devido ao curto tempo de regulamentação, sendo utilizada principalmente para o tratamento de feridas contaminadas e dermatites. Desta forma acreditamos que um dos principais usos serão os tratamentos antimicrobianos, principalmente quando da resistência bacteriana aos antimicrobianos.

O presente estudo demonstrou que a atividade bactericida da SSO_3 depende do agente infeccioso envolvido, do perfil de resistência do mesmo e também do protocolo terapêutico aplicado e do tempo de exposição do agente bacteriano à mesma. No entanto, de forma geral, acreditamos que a SSO_3 pode ser um potencial agente antibacteriano para os casos de cistite e até mesmo infecções cutâneas em cães causadas por *E. coli*, *S. aureus*, *Klebsiella* sp. e *Enterococcus* sp. Portanto, baseado em nossos resultados, estudos mais aprofundados que testem diferentes protocolos e tempos de exposição à SSO_3, principalmente estudos *in vivo* randomizado com grupos controle não tratado e grupos controle tratado com uma terapia convencional consagrada, utilizando-se uma amostragem significativa de animais, são necessários a fim de se avaliar a atividade antibacteriana dessa solução sobre cistites e até mesmo infecções cutâneas causadas por estes e outros gêneros/espécies de bactérias avaliados neste estudo.

De forma geral, os dados deste estudo forneceram um leque de usos potenciais da OZT na clínica médica de pequenos animais que poderá ser explorado principalmente pela comunidade científica para traçar estudos experimentais futuros com uso de cobaias e estudos clínicos com animais de estimação, buscando a consolidação do conhecimento acerca do ozônio medicinal na medicina veterinária.

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ANEXO A

CASE REPORT

Effectiveness of ozonized saline solution in the treatment of *Proteus* spp. bacterial cystitis

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Abstract

Bacterial cystitis is a common clinical problem among cats and dogs and is one of the main reasons for the administration of antimicrobials. This can cause serious damage to public and animal health, as this practice facilitates the selection of bacteria that are multidrug-resistant to antibiotics. In this context, it is urgent to understand and validate therapeutic modalities that complement antimicrobial treatment in cystitis cases. Ozone therapy has been proposed by scientists owing to the various mechanisms of action in a range of pathologies, both in human and animal medicine. This paper describes the bactericidal action of two different protocols of bladder irrigation with ozonized saline solution (59 µg/mL) in a paraplegic canine with recurrent bacterial cystitis caused by *Proteus* spp. In the first protocol, the bladder instillations were applied once a day for three consecutive days while in the second, successive lavages were performed throughout the day until a significant reduction in the presence of bacteria in the urine sediment. In this study, we were able to demonstrate that repeated bladder instillation within 24 hours was the most effective treatment for *Proteus* compared to a single instillation on successive days.

Key words: bactericidal effect; lower urinary tract infection; medicinal ozone; ozone therapy

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Introduction

Bacterial urinary tract infection (UTI) is a common clinical problem among cats and dogs and is the main reason for antimicrobial administration.[1,2] Approximately 14% of all dogs present with at least one episode of UTI during their lifetime.[3] In dogs, UTI occurs more often in females, elderly animals, and those with certain concomitant illnesses.[4-6] Spinal cord injury is a coexisting condition that increases UTI risk in both human beings and animals; this is because of an impairment of storage and voiding function in the lower urinary tract.[7]

With the emergence of antimicrobial resistance, treatment options for UTI and other infections are becoming increasingly limited. Unconventional treatments, such as oral immunostimulants, mannose, acupuncture, vaginal and oral estrogen, and, more recently, ozone therapy, have been proposed and studied.[8-15]

There have been medical reports of the resolution of refractory bacterial cystitis when treated with medicinal ozone, rather than antimicrobials.[13,15] An experimental study demonstrated the antimicrobial effect of bladder irrigation with ozonized saline solution (O_3SS) as a treatment for *Escherichia coli*-induced cystitis.[16] Given these promising results of ozone therapy in the treatment of bacterial cystitis and the lack of such studies in dogs, this study reported the effectiveness of two different protocols of bladder irrigation with O_3SS in a paraplegic canine with recurrent bacterial cystitis caused by *Proteus* spp.

Case Report

A 14-year-old female Dachshund, paraplegic, weighing 10.2 kg, was referred to the ozone therapy sector of the Veterinary Hospital of the Federal University of Santa Maria, Santa Maria, Brazil, because of recurrent bacterial cystitis caused by *Proteus* spp. Two months after treatment with enrofloxacin, amoxicillin, and potassium clavulanate, the patient developed adverse effects such as pharmacodermia and corneal ulcers.

Alternative treatments were discussed in our team and ozone therapy was selected, which consisted of bladder instillations of O_3SS once a day for 3 consecutive days. To obtain O_3SS, 1 L of 0.9% NaCl solution was ozonized for 10 minutes at a concentration of 59 µg/mL in an O_3 generator apparatus (model OeL1.5RM, Ozone and Life, São José dos Campos, Brazil), with an O_2 flux of 0.25 L/min. After antisepsis of the external genitalia with 1% chlorhexidine gluconate, a bladder catheterization was performed using a sterile 8-Fr urethral catheter (Mark Med, Bragança, São Paulo, Brazil) lubricated with lidocaine hydrochloride jelly (20 mg/g). First, the urinary bladder was drained through the urethral catheter aided by syringes and 10 mL of urine was separated for urinalysis, culture, and antibiogram. Next, within a 20-minute interval, the bladder was washed with 1 L of O_3SS at a concentration of 59 µg/mL.

Table 1 shows the results of the urine analysis (urinalysis, culture, and antibiogram) before the treatment (D0), and after the first (D1), second (D2), and third (D3) treatments. As the urinary bacterial load did not reduce after D3, we started a

Table 1: Urinalysis, urine culture, and antibiogram profile of the treatment protocol performed once a day for 3 consecutive days in a female Dachshund canine with bacterial cystitis before the treatment (D0), after the first treatment (D1), after the second treatment (D2), and finally after the third treatment (D3)

	D0	D1	D2	D3
Urinalysis				
Color	Yellow	Yellow	Yellow	Yellow
Odor	Sui generis	Malodorous	Malodorous	Sui generis
Turbidity	Cloudy	Cloudy	Cloudy	Cloudy
Specific gravity (kg/m³)	1036	1032	1036	1030
pH[a]	8.5	8	8	8
Protein[a]	1+	2+	2+	1+
Transitional epithelial cells (/400× magnification field)[b]	1	2	1	0
Red blood cell (/400× magnification field)[b]	6.8	3.6	1.2	2.4
White blood cell (/400× magnification field)[b]	>50	24.2	30.8	10.2
Bacteria[c]	Many[d]	Many[d]	Many[d]	Many[d]
Struvite[c]	Many	Few	Few	Moderate
Uroculture (colony-forming unit/mL)				
Proteus spp.	1.2×10^7	6.5×10^7	5.9×10^7	2.5×10^7
Antibiogram				
Gentamicin	Susceptible	Susceptible	Susceptible	Susceptible
Marbofloxacin	Susceptible	Susceptible	Susceptible	Susceptible
Neomycin	Susceptible	Susceptible	Susceptible	Susceptible
Nitrofurantoin	Resistant	Resistant	Resistant	Resistant
Amoxicillin + clavulanic acid	Susceptible	Susceptible	Susceptible	Susceptible
Enrofloxacin	Intermediate	Intermediate	Intermediate	Intermediate
Cephalotin	Susceptible	Susceptible	Susceptible	Susceptible
Trimethoprim + sulfadiazine	Susceptible	Susceptible	Susceptible	Susceptible

Note: Superscript a was measured with a urine reagent strip. Superscript b indicates the data were the average between 10 slide fields. Superscript c was determined by microscopic examination. Superscript d indicates the presence of cocci and bacilli-type bacteria.

conventional treatment with amoxicillin and potassium clavulanate (12.5 mg/kg, twice a day, 10 days in total), which effectively resolved cystitis.

Because the animal had paraplegia, the tutor was instructed to perform daily bladder compressions to induce urine drainage. However, 6 months after the first treatment, the tutor noted color and odor alterations in the animal's urine. The patient underwent another physical examination, but no clinical alterations were observed. However, on ultrasound examination, the bladder presented medium repletion filled with suspended anechoic and echogenic material (sediment) and small hyperechogenic, punctiform, and linear structures suggestive of crystals or small clots. The bladder wall had thickened, with an irregular mucosal layer and evident stratification of the layers (diameter, approximately 0.82 cm; **Figure 1A**).

A bladder catheterization was performed following the aforementioned antisepsis procedure for urine collection and initiation of therapy. Due to the previous therapeutic failure (3 consecutive days of bladder irrigation), we adopted a new protocol in which successive lavages were performed throughout the day until a significant reduction in the presence of bacteria in the urine sediment. This was achieved after the third bladder irrigation, performed every two hours. For each irrigation, 1 L of O_3SS for 10 minutes at a concentration of 59 μg/mL in an O_3 generator device, with an O_2 flux of 0.25 L/min.

Table 2 shows the results of the urinalysis, which was performed on the same day before treatment (T0), and after the first (T1), second T2, and third (T3) lavages.

To report this case, we had the consent of the guardian of the animal and the veterinarians who participated in the case.

Discussion

Herein, we document the care provided to a paraplegic canine patient with recurrent cystitis caused by a multidrug-resistant strain of *Proteus* spp. None of the drug approaches - different combinations of antibiotics for different periods - could resolve the infection. Therefore, the patient was referred to our hospital for ozone therapy.

The ineffectiveness of conventional treatments due to microbial resistance to antibiotics has been extensively reported, and both veterinarians and guardians have been searching for alternatives to cure or improve the living conditions of companion animals. The literature has demonstrated the antimicrobial effects of ozone in dogs in preventing dental plaque formation and its prophylactic use to reduce bacterial colonization in the conjunctival sac and periocular skin.[17,18]

In our first therapeutic approach, we performed the conventional protocol of bladder irrigation, based on the results obtained in an experimental study.[16] On the 1st day, a urine sample was sent for culture and an antibiogram. Despite successive days of bladder irrigation, the infection did not respond to treatment - urinalysis showed the persistence of bacteria. At the end of the lavages, the urine culture results of the D0 sample revealed bacterial susceptibility to amoxicillin-

Table 2: Urinalysis, urine culture, and antibiogram profile of the treatment protocol performed on the same day in a female Dachshund canine with bacterial cystitis before treatment (T0), after the first bladder irrigation (T1), after the second bladder irrigation (T2), and finally after the third bladder irrigation (T3)

	T0	T1	T2	T3
Urinalysis				
Color	Brown	#	#	Red
Odor	Malodorous	#	#	Sui generis
Turbidity	Cloudy	#	#	Cloudy
Specific gravity (kg/m³)	1040	#	#	1028
pH[a]	8.5	#	#	8
Protein[a]	3+*	#	#	3+*
Transitional epithelial cell (/400× magnification field)[b]	6*	#	#	1
Squamous epithelial cell(/400× magnification field)[b]	2	#	#	1
Red blood cell (/400× magnification field)[b]	>100*	#	#	>500*
White blood cell (/400× magnification field)[b]	>200*	#	#	18*
Bacteria[c]	Many[d]	#	#	Few*
Struvite[c]	Moderate	#	#	0*
Uroculture (colony-forming unit/mL)				
Proteus spp.	7.9×10^{7}*	9.0×10^{6}*	6.9×10^{3}*	5.6×10^{2}*
Antibiogram				
Gentamicin	Susceptible	Susceptible	Susceptible	Susceptible
Marbofloxacin	Susceptible	Susceptible	Susceptible	Susceptible
Neomycin	Intermediate*	Intermediate*	Intermediate*	Susceptible
Nitrofurantoin	Resistant	Resistant	Resistant	Resistant
Amoxicillin + clavulanic acid	Susceptible	Resistant*	Intermediate*	Intermediate*
Enrofloxacin	Susceptible*	Susceptible*	Susceptible*	Susceptible*
Cephalotin	Intermediate*	Intermediate*	Intermediate*	Susceptible
Trimethoprim + sulfadiazine	Resistant*	Intermediate*	Intermediate*	Intermediate*

Note: Superscript a was measured with a urine reagent strip. Superscript b indicates the data were the average between 10 slide fields. Superscript c was determined by microscopic examination. Superscript d indicates the presence of cocci and bacilli-type bacteria. Superscript * indicates the difference in the observation time points between Tables 1 and 2.

clavulanate; therefore, these drugs were prescribed. At this time, the infecting strain was identified as *Proteus* spp., which is characterized by invasive growth and rapid multiplication: the bacterial population doubles every 3.5 hours at 37°C.[19] Therefore, bacteria that were not eliminated through bladder irrigation could recolonize the bladder mucosa within 24 hours. The colony-forming unit (CFU) count increased from one day to the next as it did between D0 and D1 (**Table 1**). We hypothesized that the inability of ozone therapy to kill the residual bacteria might cause bacterial recolonization. In agreement with our hypothesis, another study observed the same effect following treatment with ozonized distilled water of root canals experimentally infected with *Enterococcus faecalis* and *Candida albicans*.[20] After 10 days of antibiotic treatment, a complete remission of the infection was observed.

In the second therapeutic moment, 6 months later, in a new recurrence in the same patient, due to the previous failure, we performed successive irrigations on the same day. At T3 (after 3 washes), the bacterial count dropped drastically, following which we stopped the washes. This result was confirmed by the urine culture, which showed a decrease in the bacterial population (from almost 80 million CFU/mL to 560 CFU/mL). According to the literature, to consider that bacteriuria is consistent with cystitis, a bacterial count of > 10,000 CFU/mL is needed for samples obtained by catheterization; any value < 1000 CFU/mL should be considered as contamination.[21] Therefore, we achieved the resolution of the clinical condition with the isolated use of bladder irrigation with O_3SS.

During the procedure, urinalysis showed an increase in the red blood cell count. We considered this event to be related to the removal of the biofilm that covered the bladder mucosa, visibly altered by a chronic inflammatory process (**Figure 1**), which facilitated the diapedesis of these cells into the bladder lumen. Persistent bleeding, probably due to the antioxidant and anti-inflammatory actions of ozone on biological membranes,[22,23] was not reported. After washing, the bladder environment changed considerably. This change was visible on the ultrasound image, characterized by a marked reduction in the suspended echogenic content (sediment) and small hyperechogenic, punctiform, and linear structures suggestive of crystals or small clots (**Figure 1B**). Furthermore, the moderately distended urinary vesicle was approximately 0.14 cm thick.[24] In this case, the thickness was significantly increased (0.82 cm), probably in response to the chronic inflammatory process.

O_3SS selected a population with intermediate susceptibility to amoxicillin-clavulanate. Conversely, it seems to have changed the pattern of susceptibility to cephalothin and neomycin, which could expand the therapeutic options. We did not find any publications that strongly linked this action to ozone therapy. Therefore, in a controlled experiment with a sufficient sample size, which is a limitation in this report, we

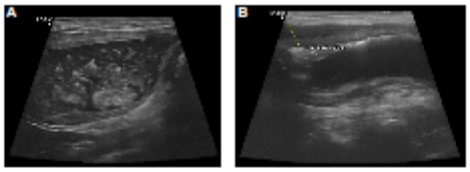

Figure 1: Ultrasound of the bladder of a female Dachshund canine with bacterial cystitis at 6 months after the first treatment.
Note: (A) The evident and accentuated stratification of the urinary bladder wall layers, in addition to the presence of anechoic and echogenic content in suspension (sediment) and small hyperechogenic, punctiform, and linear structures suggestive of crystals or small clots before the beginning of treatment. (B) The marked reduction in suspended echogenic content (sediment) and small hyperechogenic, punctate, and linear structures suggestive of crystals or small clots.

may be able to statistically perceive this correlation.

This report demonstrates that the effective bactericidal action of O_3SS in bacterial cystitis depends on the infecting agent. Therefore, it is important to know the characteristics of the pathogenic bacterial growth to determine the most effective protocol. Repeated bladder instillation within 24 hours was the most effective treatment for *Proteus* compared to a single instillation on successive days.

Author contributions
Study conception and design, and data collection and interpreting: AME and AB. Urine analysis and cooperation among the authors: NVB. Carrying out and interpreting the urine culture and antibiogram: CM and DC. Manuscript revision: JFC and CMA. All authors approved the final version of the manuscript.

Conflicts of interest
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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