SILVÂNIA MÓL PELINSARI

HOW THE OZONE THERAPY CAN INFLUENCE THE REDOX METABOLISM AND THE INFLAMMATORY PROCESS OF HEPATOCYTES IN MURINE MODELS?
A SYSTEMATIC REVIEW

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção do título de *Magister Scientiae.*

Orientador: Reggiani Vilela Gonçalves

Coorientador: Emerson Ferreira Vilela

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BIOGRAFIA

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Em março de 2021, iniciou o Programa de Pós-Graduação em Biologia Celular e Estrutural, em nível de mestrado, na Universidade Federal de Viçosa.

RESUMO

PELINSARI, Silvânia Mól, M.Sc., Universidade Federal de Viçosa, julho de 2022. **Como a ozonioterapia pode influenciar o metabolismo redox e o processo inflamatório de hepatócitos em modelos murinos? Uma revisão sistemática.** Orientadora: Reggiani Vilela Gonçalves. Coorientador: Emerson Ferreira Vilela.

As doenças hepáticas são responsáveis por cerca de 2 milhões de mortes por ano em todo o mundo e, entre as diferentes patogêneses podemos destacar o desequilíbrio de marcadores oxidativos. O hepatócito desempenha um papel importante no balanço oxidativo por sua ativação direta ou pela ativação indireta de outras células do tecido hepático, como células de kupfer e neutrófilos. Todas essas características tornam as células hepáticas uma importante ferramenta para entender os principais mecanismos envolvidos no processo de estresse oxidativo tecidual. Nesse sentido, a ozonioterapia é uma ferramenta promissora para o tratamento de danos hepáticos, uma vez que reconhecidamente controla a liberação de radicais livres e aumenta a expressão de enzimas antioxidantes. Assim, este trabalho teve como objetivo investigar as principais vias intra-celulares ativadas após a exposição à ozonoterapia, levando em consideração a mensuração de enzimas antioxidantes e marcadores de estresse oxidativo, secundário a ação de radicais livres. Esta revisão sistemática foi realizada com base nas diretrizes PRISMA e utilizando uma busca estruturada no MEDLINE (PubMed), Scopus e Web of Science. Os estudos incluídos são limitados àqueles que utilizaram a terapia com ozônio para controlar o estresse oxidativo tecidual no tecido hepático em modelos murinos. As principais vias celulares ativadas, dose e concentração de ozônio e a relação entre marcadores oxidativos e inflamatórios foram extraídas e comparadas quando possível. A análise de viés e as avaliações de qualidade metodológica foram examinadas por meio da ferramenta Risk of Bias do SYRCLE. Dezenove estudos foram selecionados. Nossos resultados mostraram que a exposição à ozonioterapia possui efeito protetor no tecido hepático, pois diminuiu a inflamação tecidual e consequentemente o estresse oxidativo do tecido. Em relação ao controle da inflamação marcadores inflamatórios foram os TNF-α IL1-β e as alterações teciduais causadas pela ação de radicais livres, foram degeneração hepatocelular, esteatose, inflamação periportal, apoptose e necrose. O tratamento com ozônio promoveu redução de marcadores oxidativos como malondialdeído (MDA), proteína carbonilada (CP), peróxido de hidrogênio (H_2O_2) , 4-HDA (hidroxinonental), conjugado dieno e enzimas pró-oxidantes como mieloperoxidase (MPO), xantina oxidase(XOD), NADPH oxidase (NOX), além de promover um aumento das enzimas antioxidantes Superóxido

dismutase (SOD), Catalase (CAT), e Glutationa (GST). As consequências morfológicas do controle destas vias intracelulares foi a diminuição do processo inflamatório tecidual e consequentemente a diminuição de degenerações e de áreas de necrose após o tratamento com ozonioterapia. Acreditamos que a ozonioterapia é uma terapia eficaz para controlar o estresse oxidativo e a inflamação tecidual , estimulando o equilíbrio redox nas células hepáticas. Considerando uma avaliação detalhada de relatórios e qualidade metodológica, a evidência pré-clínica atual apresenta um alto risco de viés em relação aos modelos animais, dosagem e concentração da terapia com ozônio. Estes resultados mostram que muito ainda precisa ser estudado nesta área para que os resultados encontrados em modelos pré-clinicos possam ser transladados para o contexto clínico. No entanto, esperamos que nossa análise crítica seja útil para mitigar o risco de viés em estudos futuros. Este estudo está registrado na plataforma PROSPERO (CRD42021264362).

Palavras-chave: Estresse oxidativo. Enzimas antioxidantes. Fígado. Inflamação.

ABSTRACT

PELINSARI, Silvânia Mól, M.Sc., Universidade Federal de Viçosa, July, 2022. **How the ozone therapy can influence the redox metabolism and the Inflammatory process of hepatocytes in murine models? A systematic review.** Advisor: Reggiani Vilela Gonçalves. Co-advisor: Emerson Ferreira Vilela

Liver diseases are responsible for 2 million deaths per year worldwide, and among the different pathogenesis, we can highlight the oxidative markers imbalance. The hepatocyte plays an important role in the oxidative balance by its direct activation or by the indirect activation of other cells in the liver tissue, such as Kupfer cells and neutrophils. All these characteristics make liver cells an important factor to understand the main mechanisms involved in the tissue oxidative stress process. In this sense, ozone therapy is a promising tool for the treatment of liver damage, since it is known to control the release of free radicals and increase the expression of antioxidant enzymes. Thus, this work aimed to investigate the main intracellular pathways activated after exposure to ozone therapy, taking into account the measurement of antioxidant enzymes and markers of oxidative stress, secondary to the action of free radicals. This systematic review was performed based on the PRISMA guidelines and using a structured search in MEDLINE (PubMed), Scopus, and Web of Science. The included studies are limited to those that used ozone therapy to control tissue oxidative stress in liver tissue in murine models. The main activated cellular pathways, ozone dose, concentration, and the relationship between oxidative and inflammatory markers were extracted and compared when possible. Bias analysis and methodological quality assessments were examined using the SYRCLE Risk of Bias tool. Nineteen studies were selected. Our results showed that exposure to ozone therapy has a protective effect on liver tissue, promoting a decrease in inflammatory tissue, and consequently decreases oxidative stress in hepatic tissue. Regarding the control of inflammation, the main markers analyzed were TNF-α and IL1-β. Morphological changes caused in the tissue by the action of free radicals were hepatocellular degeneration, steatosis, periportal inflammation, apoptosis, and necrosis. Our results showed that Ozone treatment promoted the reduction of oxidative markers such as malondialdehyde (MDA), carbonyl protein (CP), hydrogen peroxide (H_2O_2), 4-HDA (hydroxynonental) conjugated diene and pro-oxidant enzymes such as myeloperoxidase (MPO), xanthine oxidase (XOD), and NADPH oxidase (Nox). In addition, ozone therapy promoted an increase in the antioxidant enzymes Superoxide dismutase (SOD), Catalase (CAT), and Glutathione (GST). The morphological consequences of the control of these intracellular pathways were the

reduction of the tissue inflammatory process, and consequently, the reduction of degenerations and necrosis areas after treatment with ozone therapy. We believe that ozone therapy is an effective therapy to control oxidative stress and tissue inflammation by stimulating redox balance in liver cells. Considering a detailed assessment of reports and methodological quality, the current preclinical evidence presents a high risk of bias concerning animal models, dosage, and concentration of ozone therapy. These results show that much still needs to be studied in this area so that the results found in pre-clinical models can be translated into the clinical context. However, we hope that our critical analysis will be useful in mitigating the risk of bias in future studies. This study is registered on the PROSPERO platform (CRD42021264362).

Keywords: Ozone therapy. Oxidative stress. Antioxidant enzymes. Liver. Inflammation

LISTA DE SIGLAS E ABREVIATURAS

AST -aspartate aminotransferase

ALT -alanine aminotransferase

CAT Catalase

GSH-Px Glutathione peroxidase

GSH -Glutathione

GST- Glutathione S-transferase

H_2O_2 -hydrogen peroxide

IL-1- interleucina 1

TNF-α – fator de necrose tumoral

MDA-malondialdehyde

MPO -Myeloperoxidase

NOX -NADPH-oxidase

Nrf2 Erythroid 2-related factor 2

CP-carbonylated protein

ROS- Reactive oxygen species (ROS)

SOD- Superoxide dismutases

TBARS-Thiobarbituric acid reactive substances

SUMÁRIO

1- REVISÃO DA LITERATURA

1.1 Estresse oxidativo no fígado

O fígado é um órgão central de desintoxicação e metabolismo de nutrientes, sendo mais susceptível ao estresse oxidativo e à inflamação produzida a partir de toxinas e metabólitos no corpo (LI et al., 2016). As células parenquimatosas, células de Kupffer, células estreladas hepáticas e células endoteliais são mais expostas ao estresse oxidativo sendo as mitocôndrias e peroxissomos e retículo endoplasmático liso as principais organelas das células parenquimatosas envolvidas neste processo (LI et al., 2015).

As Espécies Reativas de Oxigênio ou de Nitrogênio (EROs e ERNs), são radicais livres cujo elétron desemparelhado encontra-se nos átomos de oxigênio ou nitrogênio, e sua produção ocorre naturalmente no organismo nas mitocôndrias e no retículo endoplasmático liso de todas as células, no entanto, este processo é mais acentuado em células responsáveis pela detoxificacão do organismo como por exemplo os hepatócitos através das enzimas do citocromo P450 (CICHOŻ-LACH & MICHALAK, 2014).

O estresse oxidativo causa danos hepáticos provocando alterações severas de lipídios, proteínas e conteúdo de DNA, modulando vias biológicas que regulam a transcrição de genes, expressão de proteínas, apoptose celular e ativação de células estreladas hepáticas (LI et al.,2016). Portanto, o estresse oxidativo e a inflamação estão interligados e associados ao agravamento de doenças hepáticas (LI et al., 2015). Nesse contexto o estresse oxidativo causa modulação da expressão proteíca por meio da ativação de fatores de transcrição redox-sensíveis, como fator nuclear κB (NF-κB), proteína ativadora-1 (AP-1), proteína 1 de resposta ao crescimento precoce (EGR-1) e proteínas G (CICHOŻ-LACH & MICHALAK, 2014) promovendo a expressão de citocinas pró-inflamatórias aumentando a expressão de TNF-α, IL-6, IL-12 (IDDIR et al., 2020). Essses achados demonstram que a inflamação contribui para o estresse oxidativo, uma vez que na lesão hepática, o estresse oxidativo ativa NF-kB, Egr-1 e AP-1, levando a uma resposta inflamatória, além de ativar vias de morte celular nos hepatócitos (ARROYAVE-OSPINA et al., 2021).

O comprometimento crônico do metabolismo lipídico está relacionado com o desequilíbrio oxidante/antioxidante, que afetam as organelas relacionadas ao metabolismo, levando à lipotoxicidade celular, peroxidação lipídica, estresse crônico do retículo endoplasmático (RE) e disfunção mitocondrial e desencadeia vias de estresse dos hepatócitos, levando à inflamação e fibrogênese (ARROYAVE-OSPINA et al., 2021). Assim, o

desequilíbrio entre agentes oxidante e antioxidantes em favor dos oxidantes leva ao processo de estresse oxidativo celular (SIES, 2020). Neste contexto, existe dentro das células um sistema antioxidante eficaz que controla o metabolismo redox. Entre as principais proteínas que exercem esta função podemos destacar a superóxido dismutase (SOD) que desempenha um papel fundamental na remoção de radicais livres de ânion superóxido e gera peróxido de hidrogênio (H_2O_2); a catalase (CAT) responsável por eliminar H_2O_2 gerado pela SOD e converte-lo em água e oxigênio e a glutationa peroxidase (GPX) que catalisa a redução de H_2O_2 em água (LIU et al., 2022).

1.2 Doenças Hepáticas Crônicas

As doenças hepáticas crônicas são quase sempre caracterizadas por aumento do estresse oxidativo, independentemente da causa do distúrbio hepático (CICHOŻ-LACH & MICHALAK, 2014). Portanto, fatores como álcool, drogas, poluentes ambientais e irradiação podem levar ao estresse oxidativo e contribuir para o desenvolvimento de doenças e lesões hepáticas (LI et al., 2015).

Dados atuais, mostram que cerca de 2 milhões de mortes por ano ocorrem devido a doenças do fígado em todo o mundo (DUTTA et al., 2022), sendo o álcool um dos principais responsáveis (ASRANI et al., 2019). Além disso, com o estilo de vida moderno e urbanizado, mudanças nos hábitos alimentares foram inseridos e a ingestão de dieta com alto teor calórico, tem proporcionado um aumento de doenças hepáticas gordurosas não alcoólica (DHGNA) (WEI et al., 2022). Outra patologia hepática comum, associada ao estresse oxidativo é o carcinoma hepatocelular. Este tipo de câncer afeta aproximadamente uma em cada 1.000 pessoas, sendo mais comum em pessoas com doença hepática crônica (ASRANI et al., 2019) como a infecção pelo vírus da hepatite B (HBV) (WEI et al., 2022).

As doenças hepáticas geram gastos no mundo em torno de US$ 20.673,70 milhões em 2020 e estima-se que atinja US$ 36.455,70 milhões até 2030 , requerendo ações terapêuticas eficientes e acessíveis à população (MALI et al., 2021).

1.3 Mecanismo de ação da Ozonioterapia

A palavra ozônio tem sua origem do grego “ozein” e significa cheiro (YAMAMOTO et al., 2021). O ozônio (O_3) é um gás incolor formado por três átomos de oxigênio sendo sintetizado por meio de geradores específicos, em que ocorre descargas elétricas sobre a

molécula de oxigênio, que se quebra liberando átomos que se ligam em outras moléculas de oxigênio, formando o ozônio, que é altamente reativo sendo instável e oxidante, por isso é ativo em sua ação biológica o que o torna um importante aliado da medicina (REZENDE et al., 2021).

Os tratamentos usando ozônio iniciaram durante a primeira guerra mundial no hospital militar Queen Alexandra em 1916, com a finalidade de cicatrização, sendo destacado três características como curativo: 1) É um forte estimulante e determina um aumento fluxo de sangue para a parte afetada; 2) É um germicida, que destrói todos os microrganismos impedindo o desenvolvimento de infecções; 3) Tem grandes poderes na formação de oxiheomoglobina fornecendo mais nutrientes para o tecido durante o seu reparo (STOKER, 1916). Desde então, diversas frentes de estudos têm investigado a ação do ozônio, bem como sua melhor dose e efeito em diferentes patologias (DAYANI et al., 2019; VIEBAHN-HAENSLER et al., 2021; HIDALGO-TALLÓN et al., 2022).

O mecanismo de ação do ozônio é baseado na produção de espécies reativas de oxigênio de forma controlada e assim é capaz de estimular a produção de agentes antioxidantes (SCASSELLATI, et al., 2020). Neste contexto, o leve estresse oxidativo gerado pelo ozônio promove a ativação do fator transcricional mediador do fator nuclear eritroide 2 relacionado ao fator 2 (Nrf2), um domínio envolvido na transcrição de elementos de resposta antioxidante (ARE). Portanto, o ozonio ativa o Nrf2 e inibe a via NF-κB, apresentando propriedades antioxidantes e anti-inflamatórias, uma vez que o ozonio reduz a expressão de citocinas pró-inflamatorias como TNF-α, IL-1β e IL-6 (CHIRUMBOLO et al., 2021). Assim, a ozonioterapia atua nas vias de sinalização, preservando o equilíbrio redox celular, a função mitocondrial e a regulação dos fatores de transcrição e a modulação do sistema imunológico (MENENDEZ-CEPERO, 2018).

Em fluidos e tecidos humanos o ozônio reage rapidamente com água e ácidos graxos poliinsaturados (PUFA), criando dois grupos: espécies reativas de oxigênio (EROS) e produtos de ozonização lipídica (LOP) (DE SOUZA et al., 2021). A principal EROS produzida é o peróxido de hidrogênio (H_2O_2), porém outras EROs foram identificadas como produtos de reações de ozônio, como o íon superóxido e o radical hidroxila (OH^-) (DE SIRE et al., 2021; DE SOUZA et al., 2021). O H_2O_2 , íon superóxido e o radical hidroxila atuam como mensageiros do ozônio. Esses produtos gerados pelo ozônio podem funcionar como moléculas sinalizadoras demonstrando o efeito imunomodulador do ozônio, regulando a interação entre estresse oxidativo e inflamação (CHIRUMBOLO et al., 2021).

Baseado em toda esta fisiologia, a exposição ao ozônio deve acontecer de forma regulada e segura. A "janela terapêutica" para o ozônio pode variar de 0,21 μmol/ml (10 μg/ml O_3 para cada ml de sangue) a 1,68 μmol/ml (80 μg/ml O_3 para cada ml de sangue) pois nessa faixa de dosagem o sistema antioxidante é capaz de neutralizar o ozônio e mantém seu benefício biológico, enquanto doses mais altas são tóxicas (CHIRUMBOLO et al., 2021). No entanto é necessário mais pesquisas e estudos sobre os mecanismos de ação do ozônio para melhor compreensão das vias celulares e teciduais que são ativadas durante sua exposição. Além disto, é importante entender quais as melhores doses e frequencia de exposição estão associadas aos melhores efeitos terapêuticos em hepatócitos. Por isto visando preencher esta lacuna do conhecimento nos realizamos uma revisão sistemática de estudos pré-clínicos para demonstrar os efeitos terapêuticos da ozonioterapia em hepatócitos.

2- OBJETIVO GERAL

Investigar a influência da terapia com ozônio no estresse oxidativo no tecido hepático em modelos murinos.

2.1 OBJETIVO ESPECÍFICO

- Compreender a relação entre o equilíbrio redox e o processo inflamatório no interior dos hepatócitos após a exposição à terapia com ozônio.

- Mapear as vias de sinalização que podem contribuir para ampliar a compreensão dos esforços envolvidos na ozonoterapia e inflamação no estresse tecidual hepático.

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4- ARTIGO I

4.1 ARTIGO DE REVISÃO

How the ozone therapy can influence the redox metabolism and the inflammatory process of hepatocytes in murine models? A systematic review

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ABSTRACT

Liver diseases are responsible for 2 million deaths per year worldwide, and among the different pathogenesis, we can highlight the oxidative markers imbalance. The liver is a very important organ, responsible for the detoxification of the body, and during this process, there is an increase in the number of mitochondria and P450 cytochromes proteins inside the cells. This process generates ATP, but also free radicals and reactive oxygen species (ROS) that can harm cells and liver tissue. Therefore, the hepatocyte plays an important role in the oxidative balance by its direct activation or by the indirect activation of other cells in the liver tissue, such as Kupfer cells and neutrophils. All these characteristics make liver cells an important factor to understand the main mechanisms involved in the tissue oxidative stress process. Perhaps the activation of oxidative mechanisms during the process of cellular injury, secondary to different aggressive agents, justifies the ineffectiveness of current treatments for liver diseases, making the recovery of this tissue a challenging task. In this sense, ozone therapy is a promising tool for the treatment of liver damage since it is known to control the release of free radicals and increase the expression of antioxidant enzymes. Thus, this work aimed to investigate the main intracellular pathways activated after exposure to ozone

therapy, taking into account the measurement of antioxidant enzymes and markers of oxidative stress, secondary to the action of free radicals. This systematic review was performed based on the PRISMA guidelines and using a structured search in MEDLINE (PubMed), Scopus, and Web of Science. The included studies are limited to those that used ozone therapy to control tissue oxidative stress in liver tissue in murine models. The main activated cellular pathways, ozone dose, concentration, and the relationship between oxidative and inflammatory markers were extracted and compared when possible. Bias analysis and methodological quality assessments were examined using the SYRCLE Risk of Bias tool. Nineteen studies were selected. Our results showed that exposure to ozone therapy has a protective effect on liver tissue, promoting a decrease in inflammatory tissue, and consequently decreases oxidative stress in hepatic tissue. Regarding the control of inflammation, the main markers analyzed were TNF-α and IL1-β. Morphological changes caused in the tissue by the action of free radicals were hepatocellular degeneration, steatosis, periportal inflammation, apoptosis, and necrosis. Our results showed that Ozone treatment promoted the reduction of oxidative markers such as malondialdehyde (MDA), carbonyl protein (CP), hydrogen peroxide (H_2O_2), 4-HDA (hydroxynonental) conjugated diene and pro-oxidant enzymes such as myeloperoxidase (MPO), xanthine oxidase (XOD), and NADPH oxidase (NOX). In addition, ozone therapy promoted an increase in the antioxidant enzymes Superoxide dismutase (SOD), Catalase (CAT), and Glutathione (GST). The morphological consequences of the control of these intracellular pathways were the reduction of the tissue inflammatory process, and consequently, the reduction of degenerations and necrosis areas after treatment with ozone therapy. We believe that ozone therapy is an effective therapy to control oxidative stress and tissue inflammation by stimulating redox balance in liver cells. Considering a detailed assessment of reports and methodological quality, the current preclinical evidence presents a high risk of bias concerning animal models, dosage, and concentration of ozone therapy. These results show that much still needs to be studied in this area so that the results found in pre-clinical models can be translated into the clinical context. However, we hope that our critical analysis will be useful in mitigating the risk of bias in future studies. This study is registered on the PROSPERO platform (CRD42021264362).

Keywords: Ozone therapy, Oxidative stress, Antioxidant enzymes, Liver, Inflammation

1. INTRODUCTION

Liver diseases are responsible for 2 million deaths per year worldwide (DUTTA et al., 2022). Among its causes, we can mention alcohol-associated liver disease (AALD), non-alcoholic fatty liver disease, viral hepatitis, and autoimmune hepatitis, among others (ASRANI et al., 2019). The annual costs for the treatment of liver diseases can estimate to billions of dollars per year due to the complexity of the diseases. Generally, chronic liver diseases are associated with inflammation tissue and increased oxidative stress (CHEN et al., 2020). Oxidative stress affects major cellular components, and is commonly emphasized in the pathogenesis of various degenerative and chronic diseases, which can result in serious damage to the human body (UCHIDA et al., 2020). Therefore, the role of oxidant agents in cells is complex and depends on the balance between oxidant and antioxidant particles (JIANG et al., 2021). When the excessive production of free radicals and ROS occurs, with a decrease in the antioxidants enzymes like superoxide dismutase, Catalase, and Glutathione, it can promote modifications in proteins, lipids, and DNA cells. Consequently promoting degeneration and cell death.

Free radicals and ROS are primarily produced in the mitochondria of the cells via cytochrome P450 enzymes. Due to its metabolic activity, mainly detoxification of the body, the liver constitutes an organ that is particularly susceptible to oxidative stress. When this tissue suffers damage an imbalance of redox metabolism and the activation of the important inflammatory pathways like Egr-1, NF-kappaB, and AP-1, as well as G proteins occurs (CICHOŻ-LACH, et al., 2014). The activation of these pathways promote the expression of inflammatory markers that attract more defense cells, like macrophages and neutrophils, creating a pro-oxidant feedback environment. Within the liver tissue, mitochondria and the endoplasmic reticulum generate ROS, with mitochondria being considered the most relevant generators of ROS (CHEN et al., 2020). This pathological chain reaction exposes the liver to great oxidative stress and can result in the death of hepatocytes by necrosis or apoptosis. However, the mechanisms involved in this process remains poorly understood, and more investigations are necessary to understand the real relationship between the main pathways activated and the interrelation among them.

Considering the crucial role of oxidative stress and subsequently the inflammatory process in liver diseases, antioxidant therapies are considered a great option for the treatment of liver disorders. In this context, the search for alternative therapies that reduce liver damage has grown substantially (GÜVENDI et al., 2020). Therefore, ozone therapy has become a

promising tool because it works by stimulating the immune system locally and systemically, leading to a fast and safe tissue repair process (GÜVENDI et al., 2020). Ozone therapy is a therapy that uses a gas mixture with 5% ozone (O_3) and 95% oxygen (O_2). The ozone (O_3) is a molecule composed of three oxygen atoms, including a stable pair (O_2) and an unstable third atom, which gives ozone its beneficial effects (HERNÁNDEZ et al., 2020). Several studies have indicated that ozone therapy promotes the activation of the Nrf2 pathway. This pathway increases the antioxidants enzyme expression, reduces pro-inflammatory cytokine levels (GALIÈ et al., 2019; SCASSELLATI et al., 2020), and increases cellular adaptation to oxidative stress (SAMPAIO et al., 2018). Therefore, the oxidative preconditioning generated in liver cells by ozone exerts a protective effect by stimulating the endogenous antioxidant system, and consequently by the stimulus, the anti-inflammatory system (GALIÈ et al., 2019). However, the study's outcomes remain inconclusive and controversial, reinforcing the importance of doing a critical analysis of available evidence.

Considering that current evidence is based on fragmented data, a better understanding of the pathways and mechanisms cellularly activated after ozone therapy exposure on oxidative stress in liver tissue is essential. Thus, we used a systematic review framework to integrate the pre-clinical evidence (*in vivo*) to investigate the relevance of ozone therapy in the treatment of liver diseases with a focus on oxidative balance mechanisms and their relationship with the inflammatory process. We believed mapping signaling pathways may contribute to broadening the understanding of the mechanisms involved in ozone therapy that relate to oxidative stress and inflammation in liver tissue damage. The methodological quality of the studies was reviewed and the risk of bias associated with the current evidence was also critically analyzed.

2. MATERIALS AND METHODS

2.1. Focus question

This systematic review was based on the following focus question: What is the influence of ozone therapy on oxidative stress in liver tissue in murine models? What are the main cellular pathways activated after ozone therapy exposure? What is the relationship between oxidative stress and the inflammatory process inside hepatocytes after ozone therapy exposure?

2.2. Search strategy

This systematic review was conducted based on the PRISMA guidelines (preferred reporting items for systematic reviews and meta-analysis) (PAGE et al., 2020). The protocol details for this systematic review were registered in Prospective International Registry of Systematic Reviews (PROSPERO). Details of the Population, Intervention, Comparators, and Outcomes (PICO) can be found in Table S1. An extensive literature search was being carried out using the electronic databases Medline /PubMed (https://www.ncbi.nlm.nih.gov/pubmed), Scopus (https://www.scopus.com/home. URI), and Web of Science (https: //www-periodicos-capes-gov-br.ez35.periodicos.capes.gov.br). For all databases, the search filters were based on three complementary levels: (i) Ozone, (ii) Liver, and (iii) Antioxidant, which were combined by Boolean connectors [AND]. Search filters were initially developed for PubMed. The search algorithms [MeSH Terms] and [TIAB] were applied to identify the indexed records and those recently published in an indexing process, respectively. In addition, a back search (manual search) was performed, in which the reference list of each included study was manually screened for additional eligible studies that were not retrieved by our search. The descriptors created as a search strategy were detailed in the supplementary materials (Table S2).

2.3. Eligibility criteria

After record identification in the three databases, the duplicate studies were removed. Then, an initial selection based on title and abstract was performed. In this initial selection, we included pre-clinical studies in murine models that assessed the effects of ozone therapy on the oxidative balance in hepatic damage. All studies that evaluated the oxidative stress and antioxidant potential of ozone in liver cells from murine models were included in this research. All timings, frequencies, and dosages of ozone exposure were eligible for inclusion. Secondary (literature reviews, letters to the editor, case studies, comments, and editorials) and *in vitro* studies were also excluded. After the initial screening, all relevant studies were recovered in full text and evaluated by the eligibility criteria. We excluded studies that either had no full text available or did not meet the criteria described above.

2.4. Data extraction and management

Two independent investigators (SMP, MMS) selected eligible studies by reviewing their titles and abstracts. The kappa test was done for the selection (kappa = 0.925). Publication data was extracted through standardization information such as (1) publication characteristics and animal models (author, country, ethics committee, statistical analysis, lineage, sex, age, and weight); (2) Cell Oxidative stress (oxidative markers; antioxidant enzymes: SOD, CAT, GST; ROS and free radicals); (3) inflammatory markers, inflammatory cells and liver injury parameters. After, data were compared between reviewers and conflicting information was corrected. The features we collect from the studies and used for their evaluation are presented in Table S3; S4; S5. Any disagreements regarding the extracted data were resolved during discussions with two additional reviewers (RVG and RDN).

2.5.Bias analysis

The quality of the studies was assessed through the risk of bias (RoB), a tool of Systematic Review Centre for Laboratory Animal Experimentation) (SYRCLE), designed specifically for animal studies (Hoojimans et al., 2014). The following methodological domains based on RoB were evaluated considering the following: Q1 and Q2 considers selection bias; Q3 considers performance bias due to knowledge; Q4 considers detection bias due to knowledge of interventions by outcome evaluators; Q5 considers attrition bias (quantity, nature, or processing of incomplete results data); Q6 considers reporting bias due to selective result reporting. In addition, we asked eight additional questions that contributed to the judgment of the studies; Q7 considers that the conditions in which the animals were kept were reported (temperature, humidity, light/dark cycles, water, and food); Q8 Consider whether information about the intervention is complete (dose, time and interval of exposure of the intervention); Q9 considers allocation information (individual, collective, how many per allocation) Q10 considers whether the study was approved by the ethics committee; Q11 considers whether the study reports dropouts and/or exclusions from any group and the reason; Q12 considers whether the methodology used to obtain the results is validated, if it is available, or if it is replicable; Q13 considers whether the statistical methods used were reported; Q14 considers whether the study directly addresses the review issue. The items in the RoB tool were scored with “yes” (low risk of bias); “no” (high risk of bias); or “unclear” (indicating that the item was not adequately reported, and therefore, the risk of bias was unknown). Based on these items we constructed a figure in the Review Manager 5.4 program,

centered on Cochrane Collaboration (RoB 2.0), to demonstrate the risk of bias across all studies included.

3. RESULTS

3.1.Selection of PRISMA-Guided Studies

Our search strategy allowed us to retrieve 931 studies (PubMed: 221; Scopus: 378; Web of Science: 332). After removing 290 duplicates, 624 studies were excluded due to inappropriate topics selected by reading the titles and abstracts. 470 studies were read in full (full text), and 456 were excluded using the eligibility criteria. After reading the bibliographic references of the 14 selected articles, 5 studies were added, totaling 19 studies (Figure 1).

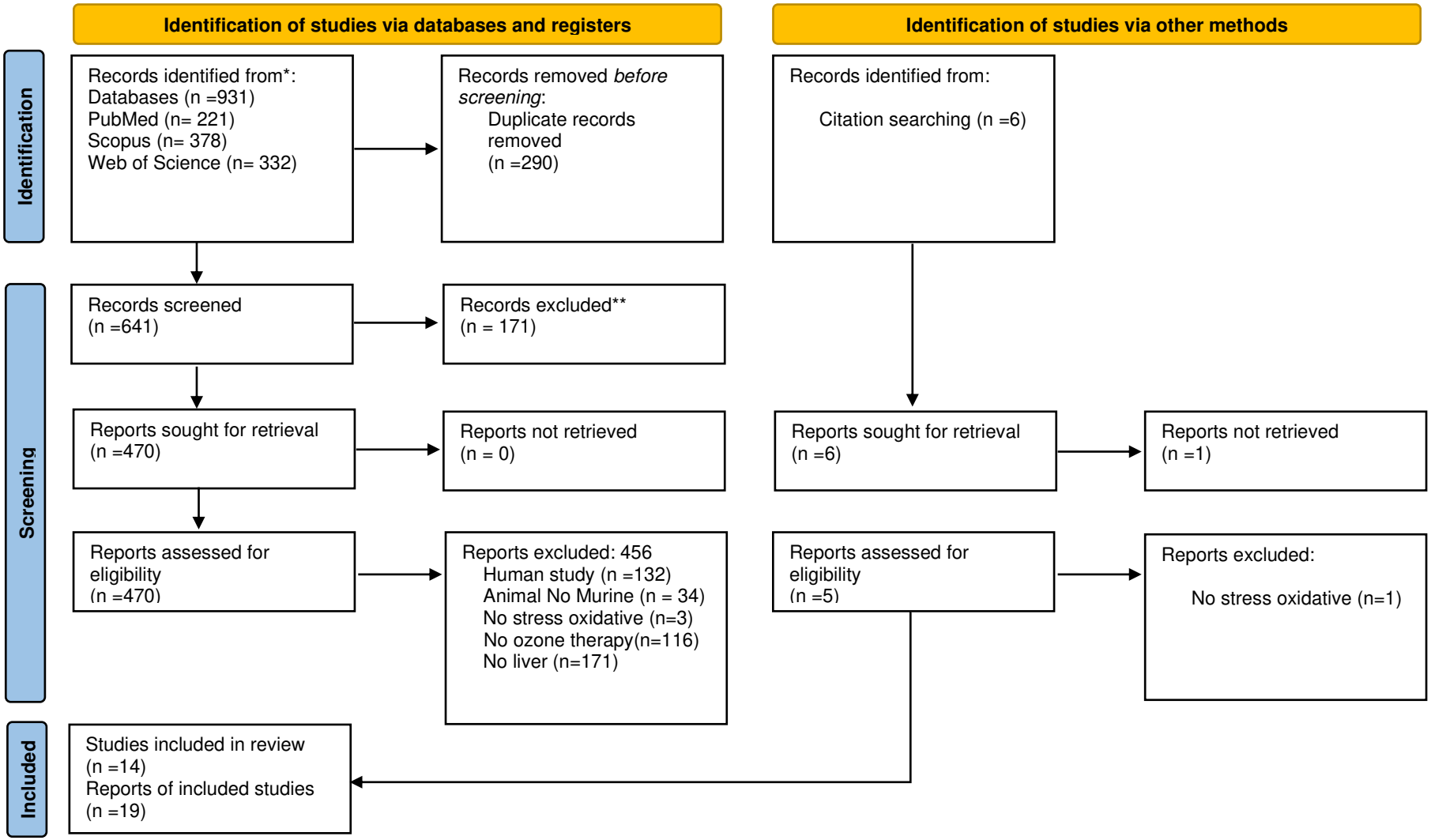

Figure 1- *Consider, if feasible to do so, reporting the number of records identified from each database or register searched (rather than the total number across all databases/registers). **If automation tools were used, indicate how many records were excluded by a human and how many were excluded by automation tools. *From:* Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. DOI: 10.1136/BMJ.n71. For more information, visit: http://www.prisma-statement.org_**Fig 1-PRISMA diagram.** Different phases of the selection of studies for conducting qualitative and quantitative analyses. Flow diagram of the systematic review literature search results. Based on "Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement." http://www.prisma-statement.org.

3.1.1. Animal Model Characteristics

The general characteristics of the selected studies and experimental models are shown in Table S3. The studies were published between 1996 and 2020 and were carried out in several countries, mainly Cuba followed by Turkey, Spain, Poland, and Egypt (Figure 2). Rats were the main animal model used in the studies (n = 17; 89.47%), followed by mice (n = 2; 10.53%). Among strains of rats, most studies used Wistar rats (n = 14; 73,68%) and Sprague-Dawley (n = 3; 15.79%) (Figure 2). Among mice, there was Balb/c (n = 2; 10.53%). The experimental animals analyzed were male (n = 14) and female (n = 5) with variations in weight and age (Figure 2). All rat studies reported the weight and age of the animals, which ranged from 172 to 300 g, aged 3 to 6 months, respectively. In mice, the weight of the animals ranged from 18 to 20 g, and these studies did not report age.

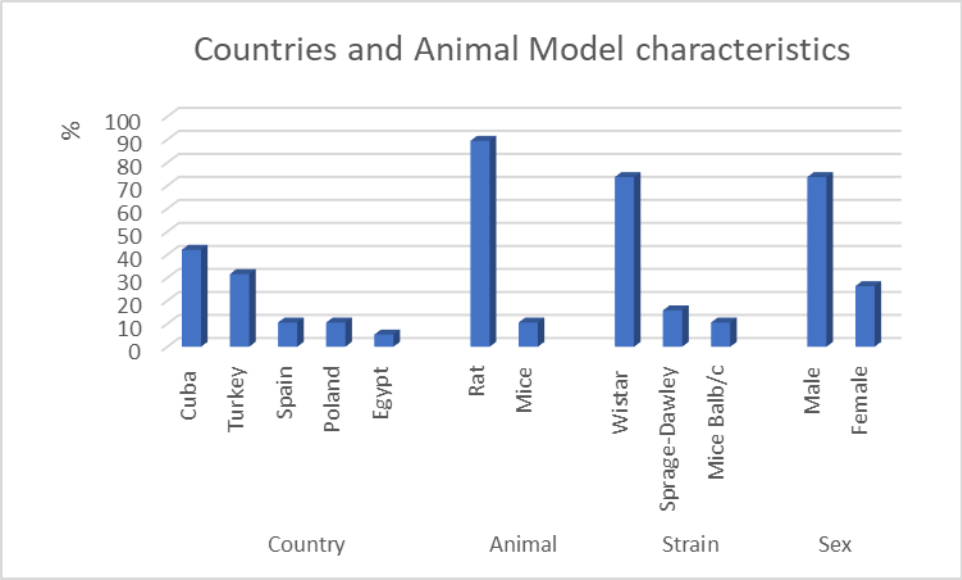

Figure 2- Countries and Animal Model characteristics od the studies included in this review

3.1.2 Methods used to cause liver injury

Different methods were used to promote liver injury. In most studies, the Ischemia/Reperfusion technique was used, corresponding to 26.32% (n = 5), followed by LPS (lipopolysaccharide) 15.79% (n = 3), CCl4 (carbon tetrachloride) 10.53% (n = 2), and Cd (Cadmium). In addition to, fecal material, acetaminophen, Methotrexate, alcohol, iron dextran, aging, ionizing radiation corresponded to 5.26% (n = 1, each), and mandibular

defect. Mandibular bone defect increases oxidative stress as bone damage increases free radical production, contributing to oxidative damage in the liver (ERDEMELI et al., 2019).

3.1.3. Ozone Characteristics

The doses of ozone applied ranged from 0.2 mg/kg to 1.2 mg/kg. Ozone concentrations ranged from 3.8 to 67 µg/mL (Table S4). Ozone administration routes were performed intraperitoneal, corresponding to 57.89% (n = 11) and 42.11% rectal (n = 8). In 89% (n = 17) of the studies, the application of ozone occurred daily during the period of the experiment. The duration of ozone treatment ranged from 1 to 450 days, with most of the studies using between 4 and 6 days. The duration of treatments can be divided into three time intervals: 1 to 10 days (n = 10; 48%), 15 to 27 days (n = 8; 38%) and 450 days (n = 3; 14%) (Table S5).

3.2.Main outcomes

3.2.1. Ozone therapy and Metabolism redox

Among oxidative markers, the most cited in the studies are: Malondialdeido (52.6% n = 10), followed by 4HDA (15.78% n = 3), H_2O_2 (10.53%; n = 2), Diene conjugate (5.26%; n = 1), and protein carbonyls (5.26%; n = 1).

Our results showed that about 70% of the doses applied were between 0.5 to 1 mg/kg. One of the first events that occur in oxidative stress is lipid peroxidation. The primary products formed are hydroperoxides and conjugated dienes, both have been used as a marker for oxidative stress. Unstable compounds produced in cells, either enzymatically or as byproducts of oxygen metabolism, are difficult to quantify. In our review, the studies that quantify these compounds showed that there was a reduction after ozone therapy exposure (15.8% n = 3). The next compound formed in this oxidation chain is MDA (TBARS) byproducts, this compound is more stable than hydroperoxides, but still considered very unstable. In all studies that analyzed MDA after liver injury, showed there was a reduction in MDA levels (52.6% n = 10), revealing that ozone treatment prevented oxidative damage in liver tissue, strengthening the antioxidant defense system. At the end of the peroxidation chain is 4HAD, making this compound is more stable. In our review (15.8% n = 3), the

studies that analyzed this marker showed that after ozone therapy exposure there was a reduction in this compound.

Carbonyl protein is another important oxidation product within cells. The higher the carbonyl content in the cells, the more protein oxidation occurs by reactions with aldehydes. Our review showed that ozone therapy reduces the number of carbonyl proteins within cells. Our result also showed that endogenous ROS, such as hydrogen peroxide (H_2O_2), was reduced inside the cells after ozone therapy exposure (10.5% n = 2). H_2O_2 is recognized as a destructive molecule and is considered the most common ROS produced inside the cells. This has shown us that ozone therapy can act in the superoxide chain inhibiting tissue oxidation.

In our review, some studies analyzed the pro-oxidant and anti-oxidant enzymes capacity. It was observed that the enzyme-like Myeloperoxidase (MPO) (5.26% n = 1), NOX (NADPH oxidase) (10.5% n = 2), and xanthine oxidase (XOD) (10.5% n = 2) play a key role in the production of ROS, and so is an important biomarker of oxidative damage reduced after ozone therapy exposure. An important pro-oxidant enzyme described in the studies, and included in this review, was xanthine oxidase (XOD). These enzymes are formed from XDH (xanthine dehydrogenase), and are responsible for converting hypoxanthine into xanthine leading to excessive production of ROS. Our results showed that after ozone therapy exposure there was a reduction in the XOD (10.5% n = 2) expression during liver damage reducing the ROS quantity generation. Following the same idea, the MPO (myeloperoxidase) enzyme secreted by neutrophils, macrophages, and responsible for inducing oxidative stress in the tissue, was reduced after ozone therapy exposure (5.26% n = 1).

Some studies (68% n = 13) also showed that after ozone exposure there was an increase in the total antioxidant enzymes. One antioxidant enzyme evaluated in this study was: SOD (superoxide dismutase) (57.89% n = 11). SOD isoforms were also analyzed in which Cu-Zn-SOD (5.26% n = 1) and Mn-SOD (5.26% n = 1). Other antioxidant enzymes evaluated were: CAT (Catalase) (47.37% n = 9), GST (glutathione-S-transferase) (15.79% n = 3), GSH (36.84% n = 7), GSSG (10.53% n = 2), GPx (31.57% n = 6). In 9 studies there was an increase in SOD (82%), and in 2 studies there was a reduction in SOD after ozone treatment. In the group treated with ozone therapy, there was an increase in endogenous SODs returning to the normal state. This implies cellular protection by reducing the availability of superoxide anion and reducing liver damage. In cases where liver damage increased SOD, ozone preconditioning reverted SOD levels to normal levels indicating that ozone establishes redox equilibrium.

Another important enzyme that participates in the antioxidant defense system is Catalase. In three studies (15.8%) there was no effect of ozone on the CAT content. In two studies (10.5%) there was an increase, and in four studies (21%) there was a reduction in the CAT. Concerning Glutathiones, in two studies (10.5%) GST increased, and in one study (5.26%) there was no effect of ozone on GST content. In seven studies there was an increase in GSH (36.8%), in one study (5.26%) the GPX was not changed, in one studies (5.26%) the GPX decreased, and in four studies the GPX increased (21%). In two studies (10.5%) the GSSG was reduced. Due to ozone treatment, it is observed there is an adaptation of the tissues to oxidative stress by inducing enzymes or activating the metabolic pathways. Thus, maintaining a redox balance with an increase in glutathione levels, and a decrease in lipid peroxidation regulating the cell's thiol-redox status.

3.2.2. Ozone therapy and inflammation

The most frequent inflammatory parameters reported were cellular markers, cytokines, morphological changes and biochemical markers. Our review revealed that ozone therapy was efficient in controlling the inflammatory process; it promoted a decrease in the total leukocytes number, especially macrophages (Kupfer cells) and neutrophils (21%). Consequently, important pro-inflammatory markers produced by these cells also presented a decreased expression, like TNF-α (15.8% n = 3) and IL-1β (5.26% n = 1).

Our results showed that the main morphological changes present in the tissue associated with the inflammatory process were: periportal inflammation (21% n = 4), vascular congestion (10.5% n = 2) and cell death as necrosis (10.5% n = 2); with ozone therapy being efficient to reduce this damage, possibly due to its anti-inflammatory and antioxidant capacity.

In addition, chemotactic markers in inflammatory cells were also identified. Immunity-associated neopterin is an immunity-associated biochemical in cells produced in monocytes/macrophages that allows monitoring of the progression of inflammatory markers (OZGER et al., 2021; DHILLON et al., 2021). In our study, ozone treatment reduces macrophages in liver tissue, decreasing neoptyrin levels and consequently reducing inflammation. In addition, Phospholipase A was reduced (5.26% n = 1) after ozone treatment. Thus, reducing the hydrolysis of phospholipids and consequently the processes associated with inflammation.

3.2.3. Secondary outcomes

Regarding secondary outcomes, we observed the labeling of different enzymes, intracellular activators and polysaccharides. In this context, that ozone therapy promoted an increase in Ca^{2+}ATPase (5.26% n = 1) and reduced Ca^{2+} levels (5.26% n = 1), markers that are altered during liver damage. Another enzyme that was reduced by ozone therapy was Calpain, decreasing oxidative stress and damage inside cells (5.26% n = 1). In addition, ozone treatment increased ATP and ADP (5.26% n = 1), providing more energy to cells to maintain high metabolism during the inflammatory process. Some markers that show an overload in liver tissue were also described in our review, with emphasis on AST (aspartate aminotransferase) and ALT (alanine aminotransferase); these markers being reduced after exposure to ozone therapy.

In addition, ozone therapy proved to be effective in maintaining hepatic glycogen content, indicating that ozone offers protection against glycogen reduction (5.26% n = 1%), preventing its degradation into lactate. Thus, decreasing intracellular acidosis associated with anaerobic glycolysis.

Lipofuscin is an important marker of aging within cells (LI et al., 2021). It constantly accumulates and is intensely related to the excessive production of reactive oxygen species (ILIE et al., 2020). Our results showed that there was a reduction of this marker after exposure to ozone therapy. Most of the studies included in this review attributed these results to the antioxidant capacity of ozone therapy.

The results described above are shown in Table 1b, while Figure 3 shows the effect of ozone on the protection of liver cell damage in a murine model.

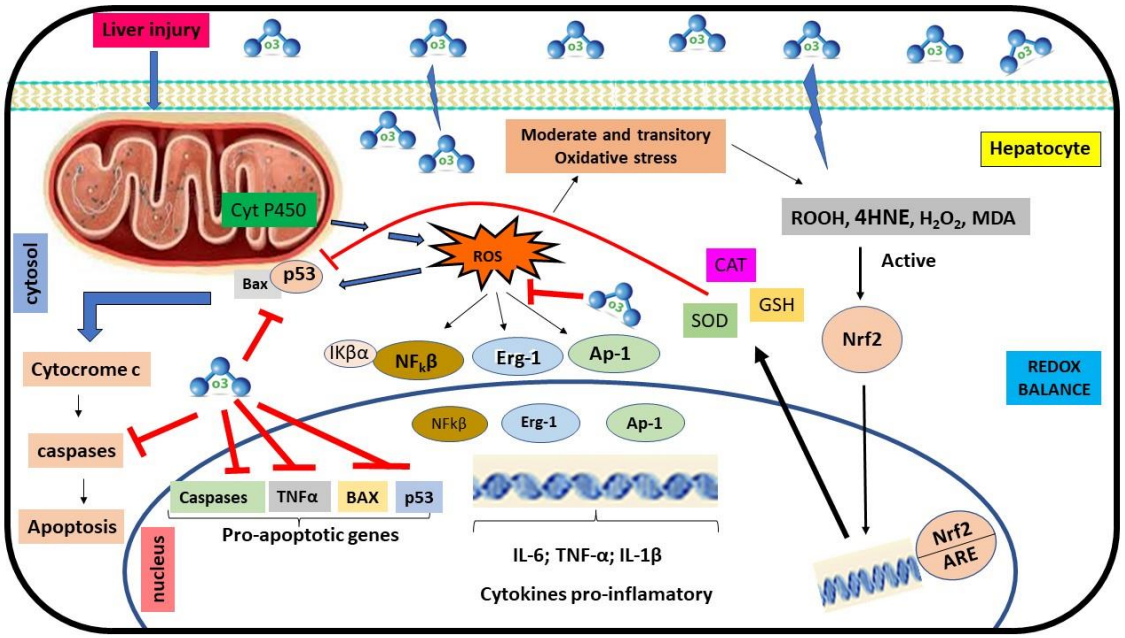

Figure 3- ROS overproduction causing oxidative stress and the effect of ozone on the protection of liver cell damage in a murine model.

Reactive oxygen species (ROS) can directly activate p53, play a role as a transcription factor and activate the expression of pro-apoptotic genes. Among these, Bak (Bcl-2 antagonist/homologous killer) and Bax (Bcl-2-associated protein X) can stimulate the activation of cytochrome C in the mitochondrial membrane, and in turn activates caspase. O3 administration decreases the expression of tumor necrosis factor-α (TNF-α), Bax and p53, inflammation, and cell death. Enzymes such as SOD (Superoxide dismutase), CAT (catalase), and GSH-Px (glutathione peroxidase) can regulate p53, Bax, and Bcl-2. Ozone increases ATP synthesis in mitochondria and does not depend on the presence of glucose, as occurs in the Krebs Cycle due to an increase in cellular metabolism. The therapeutic action of ozone occurs through the process characterized by the formation of ROS and LOPs (Lipid Oxidation Products), and both act in different phases. While ROS acts immediately and is neutralized by antioxidant systems, LOPs are distributed throughout the tissues and have the function of reducing potential toxicities. The production of LOPs occurs after the oxidation of polyunsaturated fatty acids in the cell membrane. ROS are produced in mitochondria through cytochrome P450 and can cause damage to proteins, lipids, and DNA. They activate transcription factors such as Egr-1, NF-kappaB, and AP-1, but ozone inhibits these inflammatory proteins, reducing liver damage. Ozone can activate Nrf2, which regulates gene expression through the antioxidant response element (ARE). The protein (keap1)/Nrf2 ARE signaling pathway primarily regulates anti-inflammatory gene expression and inhibits the progression of inflammation. Under moderate oxidative stress induced by ozone, Nrf2 translocates to the nucleus, where it binds to ARE genes. This leads to the inhibition of the NF-κB pathway, reducing the expression of pro-inflammatory cytokines. Therefore, ozone decreases the levels of pro-inflammatory markers IL-6, TNF-α, and IL-1β.

Table 1a. Main results of the action of ozone therapy on oxidative stress in liver tissue

	Oxidative markers					Pro-oxidant enzymes			Antioxidant markers								Infla. cells		Infla. mark		Morphologic parameters				Other analyzes																
	MDA	H_2O_2	PC	4-HDA	CD (Conjugado dienos)	NOX(NADPH OXIDASE)	XOD	MPO	SOD	Cu, Zn-SOD	Mn-SOD	CAT	GSH	GST	GSSG	GPX	Kupfer cell	Neutrofilos	TNF-α	IL-1β	Necrosis	degeneration	congestion	periportal inflammation	Ca-ATPase	Ca^{2+}	Calpain	Phospholipase A	Lipofuscin	Lactate	ATP + ADP	Ácido urico	Glicogênio	Neopttirna	AST	ALT
Laszczyca, et al., 1996												=		=		=																								
León, et al., 1998	-								+			=	+												+			-							-				
Peralta, et al., 1999		-							+				+					-			-									-					-	-			
Peralta, et al., 2000		-					-																								+				-	-			
Jalil et al., 2001	-								+			=																		-		-	+							
Ajamieh et al., 2002				-			-										-									-	-								-	-			
Ajamieh et al., 2004				-		-			+			-	+		-																				-	-			
Ajamieh et al., 2005				-					+	-	+	-	+		-							-													-	-			
Zamora et al., 2005	-											-		+		+			-																				
Madej et al., 2007									-			-																												
Guanche et al., 2010	-				-				+			+				+																								
Rodríguez et al., 2011	-								+					+		+																								
Gul et al., 2012	-					-			+							+					-														-	-	-		
Gultekin et al., 2012	-								+								-		-			-	-	-												-	-			
Safwat et al., 2014	-		-										+			-													-										
Aslaner et al., 2015	-							-					+				-	-	-	-		-		-											-	-			
Erdemli et al., 2018	-								-			+	+																										
Adali et al., 2019																						-	-	-											-	-			
Guvendi et al., 2020																								-															

Obs: PC=protein carbonylate; Infla marks= inflammatory markers ; Infla. cells = Inflammatory cells

+	Increased
-	Reduced
=	Not effect

3.3 Risk of Bias and Methodological Quality Assessments

Detailed results for the bias analysis are shown in Figure 4 and Figure 5. No study met all the methodological criteria analyzed. Regarding selection bias, the sequence generation process presented a high risk of bias in 73.68% of the studies (n = 14). In terms of allocation concealment, 26.32% (n = 5) presented a high risk of bias, while 73.68% (n = 14) presented an unclear risk. None of the articles reported a random accommodation or blinding of caregivers (Blinding of participants and personnel, blinding of outcome assessment, respectively), and the outcome was assessed as presenting a high risk of bias. Incomplete outcome data was adequately addressed in 78.95% of the studies (n = 15); all studies were free from selective reporting (n = 19) and clear data on the conditions of the animals (n = 19). Regarding intervention, 100% of the studies (n = 19) presented clear data. In terms of the unit of allocation, 57.89% of the studies (n = 11) presented unclear data, 26.32% of the studies (n = 5) presented low risk, and 15.79% of the studies (n = 3) presented a high risk of bias. Regarding ethical approval, 47.37% of the studies (n = 9) presented unclear data, 31.58% of the studies (n = 6) presented low risk, and 21.05% (n = 4) presented a high risk of bias. All withdrawals and exclusions studies presented a low risk of bias. In addition, three studies (15.79%) presented unclear data, 78.95% (n = 15) presented a low risk of bias, and 5.26% (n = 1) had a high risk of bias for Tool validated. In 89.47% of the studies (n = 17) information was not clear concerning statistical methods, while 10.53% (n = 2) had a high risk of bias. In the applicability item, 100% of the low risk of bias was obtained. In 100% of the studies (n = 19), they presented an unclear other bias.

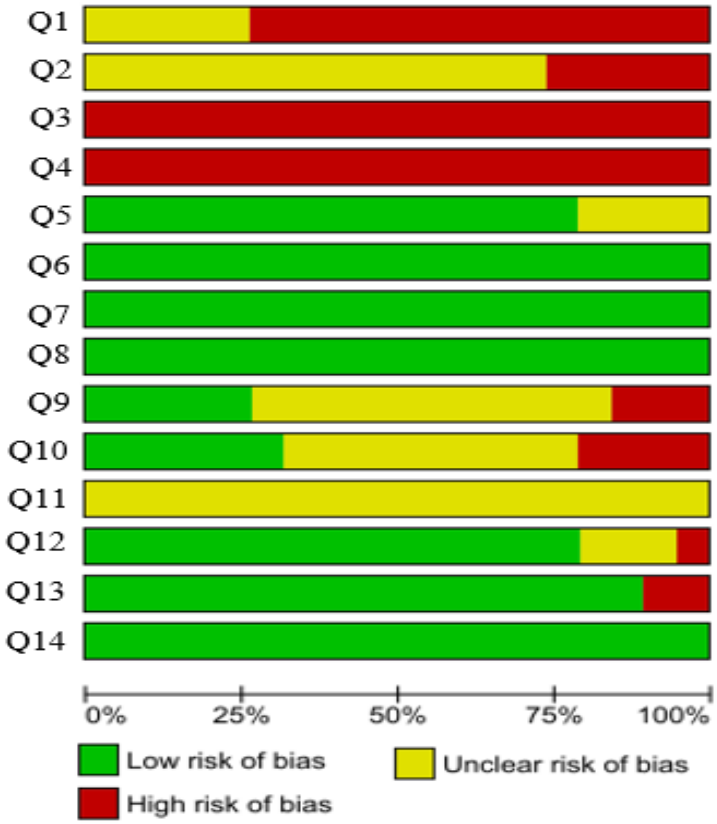

Figure 4- Results for the risk of bias and methodological quality indicators for all studies included in this systematic review that evaluated the effect of ozone therapy on oxidative stress in liver tissue. The items in the Systematic Review Center for Laboratory animal Experimentation (SYRCLE) Risk of Bias assessment were scored with "yes" indicating low risk of bias, "no" indicating high risk of bias, or "unclear" indicating that the item was not reported, resulting in an unknown risk of bias. Q1 and Q2 consider selection bias; Q3 considers performance bias due to knowledge; Q4 considers detection bias due to knowledge of interventions by outcome evaluators; Q5 considers attrition bias (quantity, nature, or processing of incomplete results data); Q6 considers reporting bias due to selective result reporting. In addition, we added seven additional questions that contributed to the judgment of the studies; Q7 considers that the conditions in which the animals were kept were reported (temperature, humidity, light/dark cycles, water, and food); Q8 considers whether information about the intervention is complete (dose, time and interval of exposure of the intervention); Q9 considers allocation information (individual, collective, how many per allocation) Q10 considers whether the study was approved by the ethics committee; Q11 considers whether the study reports dropouts and/or exclusions from any group and the reason; Q12 considers whether the methodology used to obtain the results is validated, available, or replicable; Q13 considers whether the statistical methods used were reported; Q14 considers whether the study directly addresses the review issue.

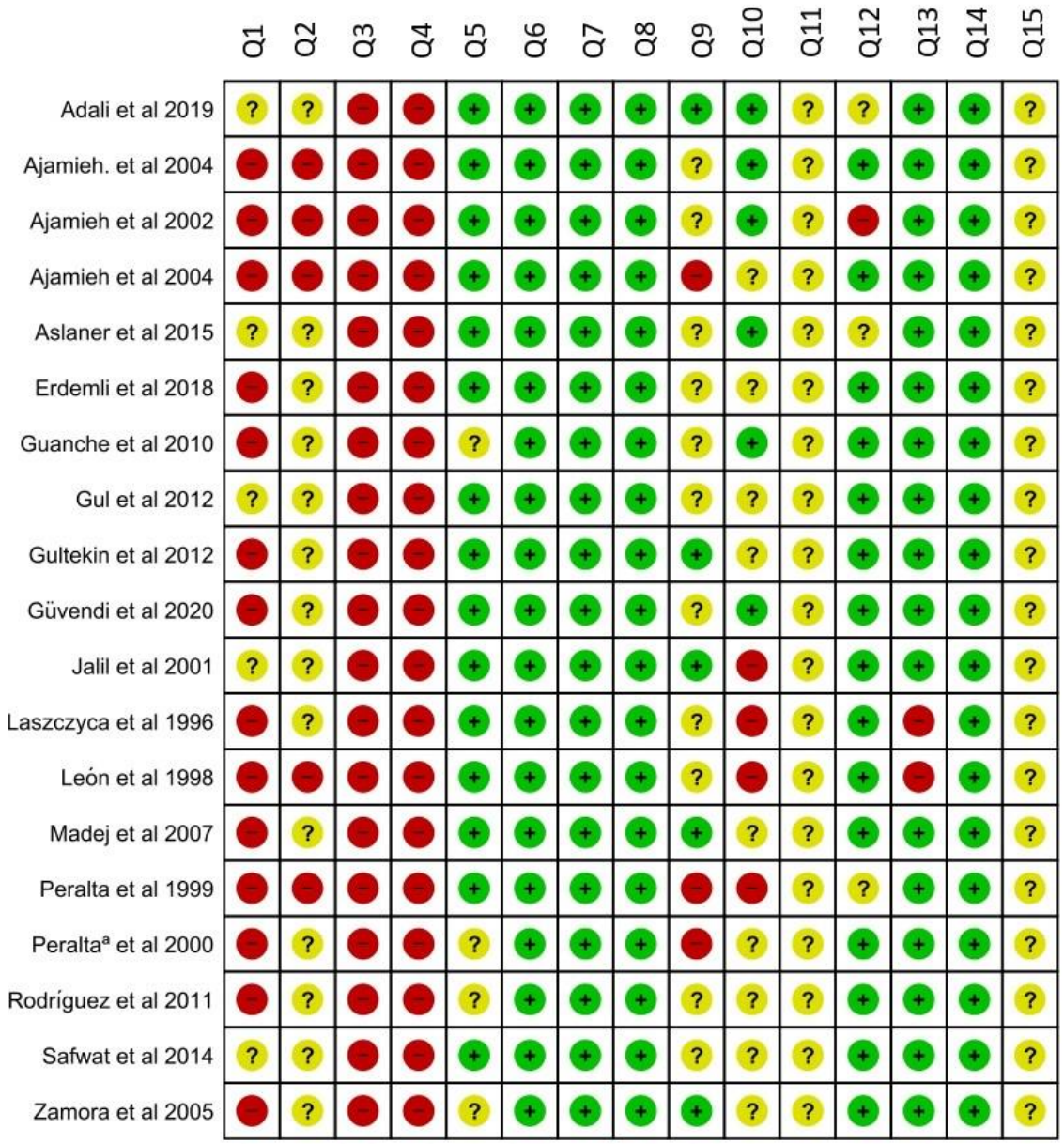

Figure 5: Risk of bias summary- review authors' judgments about the risk of bias items for each included study. Green: low risk of bias; Yellow: unclear risk of bias; and Red: high risk of bias.

4. DISCUSSION

The knowledge about redox metabolism and the role of oxidative stress in liver diseases has indicated that there is a direct relationship between redox imbalance and the inflammatory process. It's known that the inflammatory process and oxidative events are connected processes, and potentially mediated by pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1 and IL-6. Cytokines which stimulate intense reactive species production in liver tissue. Currently, the search for alternative therapies that reduce liver damage have grown increasingly, indicating a promising market (GÜVENDI et al., 2020). Ozone therapy has become a promising therapy, as it acts by stimulating the immune system locally or systemically, leading to a fast and safe tissue repair process (GÜVENDI et al., 2020). However, there is a knowledge gap regarding the understanding of the entire liver recovery process, especially concerning the effect of these alternative therapies on oxidative stress and the inflammatory process in liver diseases. Therefore in our study, we performed a systematic review to investigate the use of ozone therapy to treat hepatic damages, with a focus on the redox metabolism and inflammatory process in murine models.

4.1. Characteristics of the study and the animal model

Despite being investigated for some decades (SCASSELLATI et al., 2017), only in a few countries is ozone regulated as a therapy in medical practice, such as in Italy, France, Greece, Turkey, Cuba, Russia, China, Portugal, Japan, Spain and the United States of America (HERNÁNDEZ et al., 2021). This fact may justify our findings by the predominance of studies in countries such as Cuba, followed by Turkey and Spain. In our review, most of the studies that analyzed ozone therapy in liver tissue were done in Cuba and Turkey. Possibly because it was in these countries that the regulation for the use of ozone therapy occurred earlier in relation to other countries. In Cuba, there are 39 clinical centers for ozone therapy regularly attending to the population within its largest hospitals, incorporating ozone therapy into their care routines since 2009 (ISCO3, 2020). Between 2000 and 2020, there was an increase in studies because of the regularization of ozone therapy in the health system, beginning in the year 2000 in most countries that already have this therapy implemented (ISCO3, 2020). Another interesting element identified in the studies was that the majority used male rats as experimental models, probably because males present less hormonal fluctuations and therefore less behavioral changes than females (ZUCKER & BEERY,

2010). One of the main advantages of using a murine model for liver injury is the ability to obtain samples to do oxidative stress analyses and inflammation markers quantification, and even easier for histopathological and biochemical follow-up of liver injury (BEAL et al., 2018). In addition, they are affordable, widely available, easy to maintain and handle, and a large number of animals can be used for experiments generating a greater degree of reliability in the results (NOGUEIRA et al., 2020). Therefore, the most used animals were Wistar rats, weighing between 180-300 g. Possibly because these animals have greater weight, are easier to manipulate, and may obtain more tissue to analyze. Thus, for the induction of liver damage in murine models, the main methodology used was the induction of ischemia. Hepatic injury by ischemia and reperfusion (I/R) can occur in different clinical situations, but mainly in complex liver surgeries, such as liver transplantation (AN & KANG, 2021). The second most used method was the induction of inflammation by Lipopolysaccharide (LPS). Liposaccharides are endotoxins of low acquisition cost and are highly effective in inducing inflammation, which makes the LPS model an alternative in studies that require modulation of the immune system (BASAURI, 2021). The absence of information, such as the age of the animals, was also neglected by most studies. Age is an important feature to assess oxidative stress in liver injuries, and the evolution of the inflammatory process, so this is considered a critical reporting bias (IZZO et al., 2021).

4.1.1. Intervention characteristics

Most studies presented intraperitoneal administration of ozone therapy, which is a simple application technique with reduced gas loss (MADEJ, 2007). Usually when different drugs are tested in the preclinical study, the intraperitoneal route is the most used, mainly due to the difficulty of access to animals, being considered an experimental method (ISCO3, 2020). Another route used in our studies is rectal insufflation. Rectal insufflation of ozone is easy, painless, and less invasive. Several studies have demonstrated the effectiveness of this application, thus being the most common route in different types of treatments (DAVYDOVA et al., 2022; TOMAN et al., 2019). Rectal ozone is a safe, effective, low-cost, and simple option; making the results found in the studies that used this route easier to translate for the human context because this practice has been adopted in clinical trials around the world (YOUSEFI et al., 2022). As a general rule, ozone is administered in cycles that vary between 15 and 20 sessions, and by rectal insufflation, can be performed daily or three times a week (ISCO3, 2020).

Another important feature of the reported intervention was the dose. The most utilized ozone dose in the studies was 1 mg/kg (concentration of 50 μg/mL), because it represents a concentration of 50 μg/mL, and a concentration greater than 60 or 80 has a toxic effect (ISCO3, 2020); so, it used the highest dose of ozone that does not cause intoxication in the animal. Our findings showed that a dose lower than 0.14 mg/kg (concentration of 40 μg/mL) did not affect treatments, demonstrating that the hepatic effect is dose-dependent. Concentrations ranging from 10 μg/mL to 50 μg/mL are safe and effective (ISCO3, 2020). Thus, at low concentrations and doses, ozone acts as a bioregulator of redox balance, improving antioxidant capacity and activating important anti-inflammatory pathways like NrF2 (COSTANZO et al., 2015; VIEBAHN-HAENSLER et al., 2021); protecting cells from oxidation and suppressing inflammatory responses.

Associated with information on doses, another important feature of the intervention is frequency. Most studies reported that ozone treatment was performed once a day. Possibly because the frequency is related to the duration of the treatment, and the durations of the experiments were very short, highlighting between 5, 10, and 15 days. The number of treatment sessions and the dose of ozone administered depends on several factors, such as the patient's general condition, age, and disease (ISCO3, 2020). However, a therapeutic protocol adapted to each patient is necessary as it depends on the clinical evaluation, and if the protocol is validated and recognized by the international scientific community of ozone therapy (LACERDA et al., 2022).

4.1.2. Ozone therapy and redox metabolism

Excessive generation of free radicals promotes an imbalance between oxidant and antioxidant products inside the cells, promoting the oxidative stress process (VELLOSA et al., 2021). Generally, during liver diseases the generation of oxidative stress in the tissue is associated with the decoupling of the electron transport chain (YANG et al., 2022). This process leads to the oxidation of biomolecules with consequent loss of their biological functions and/or homeostatic imbalance, whose manifestation is the potential oxidative damage against cells and tissues (VELLOSA et al., 2021). Thus, identifying alternative therapies involved in the control of redox metabolism in liver diseases may represent a rational and useful strategy to develop an antioxidant therapy to treat hepatic lesions. In this sense, ozone therapy is currently the most promising non-invasive therapy to recover hepatic tissue. In this review, we observed that the methodological analyses carried out focused on

lipidic and protein oxidative markers and pro and antioxidant enzymes, since the free radicals and ROS are very unstable, which makes their quantification difficult.

In our review, there was a predominance of analyses of the lipidic peroxidation and markers like hydroperoxides, MDA, and 4HAD. In all the studies, there was a decrease in the production of these markers after ozone therapy exposure. This shows that this therapy is efficiant to control the lipoperoxidation and subsequently the attack of free radicals and ROS in cellular membranes. The positive effect of the ozone therapy on the redox metabolism may occur due to ozone acting as a pro-oxidant modulator by inducing secondary messengers that are aldehydes and hydroxy hydroperoxides (ozone peroxide). Thus, forming H_2O_2 and a second aldehyde-4-hydroxynonenal (4-HNE), in which develops an adaptive and regulated response in the antioxidant systems controlling oxidative stress by the increase in the expression of the antioxidant enzymes. In addition, there is a direct relationship between the oxidative markers and the inflammation process; Ozone therapy achieves modulation of the Nrf2 and NF-kB pathways (CLAVO et al., 2019). These pathways, especially NrF2 are involved in the initiation of mild oxidative stress, capable of eliciting cell antioxidant expression without causing stress-related injury. Therefore, it is very important to establish a good treatment protocol for ozone therapy exposure, to ensue the production of controlled pro-oxidant molecules without promoting cellular damage. In addition, the administration of doses between 10 μg/NmL to 50 μg/NmL stimulate antioxidant enzymes including superoxide dismutase, glutathione peroxidase, and glutathione transferases, strengthening the antioxidant enzymes system (GALIÈ et al., 2019; CHIRUMBOLO et al., 2021).

Another important oxidative marker described in the studies included in this review was protein carbonyl. This marker is related to oxidation of the proteins that promote a modification of native amino acid side chains in carbonyl proteins, which can lead to a loss of protein function (YANG et al., 2020). These promote the misfolding of proteins and compromise their functions leading to their inactivation (OZTURK et al., 2018). Ozone reduces the number of carbonyl proteins within cells through upregulation of heat shock protein 90 (HSP90), which prevents the insertion of carbonyl groups into the primary structure of proteins, and consequently prevents their misfolding (SAFWAT et al., 2014). Oxidative stress inside the cells is a important factor in promoting unwanted protein misfolding. When this protein accumulates inside the cells and organelles it can produce various disorders (SCASSELLATI et al., 2020). Similarly, Kołota et al., 2020 showed that in alcoholic liver disease there is an intensification of oxidative stress by the consumption of ethanol, generating an increase in the carbonyls protein levels. On the other hand, ozone

therapy exposure reduced the deleterious biochemical and histopathological effects through increased total antioxidant capacity, and decreased oxidative markers decreasing the formation of bad misfolding proteins. In addition, Scassellati et al., 2020 showed that ozone treatment induces moderate oxidative stress by activating Nrf2 to the nucleus where it binds to the ARE elements of genes encoding important antioxidant enzymes. This modulates protein degradation systems, showing that ozone therapy has a high potential to coordinate the production and elimination of the negative misfolding proteins inside the cells.

Our findings showed the use of ozone therapy can reduce pro-oxidant enzymes such as Myeloperoxidase (MPO), Nox (NADPH oxidase), and xanthine oxidase (XOD). These enzymatic pathways are directly linked with the increase in oxidative markers like Hydrogen peroxide and ROS, which are responsible for attacking cellular membranes, proteins, and DNA. This can be explained by the fact that after treatment with ozone there is an increase in the superoxide dismutase and catalase activity levels. These enzymes are responsible for accelerating the passage of the electron and consequently promoting a reduction in the time and quantity of the Superoxide ion, H_2O_2, and ROS. These are harmful molecules produced during the electron transportation in inner mitochondrial membranes (PIECHOWIAK et al., 2020). In this sense, the oxidases like NOX and XOD, play an important role in redox signaling (PRIETO-BERMEJO et al., 2017; SIROKMÁNY et al., 2019). Especially by increasing the production of ROS, anion superoxide, and hydrogen peroxide by the mitochondrial NOX activity and Xanthine oxidase (XOD) activation, respectively. On the other hand, ozone therapy reduces the NOX activation and reduces xanthine accumulation and consequently reduces free radicals and ROS accumulation, in which confirms its antioxidant effect.

The action of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S-transferase (GST) activated by ozone through the positive regulation of Nrf2, neutralizes pro-oxidants linked to inflammation such as xanthine oxidase (CENCI et al., 2022). All of these results showed us that ozone therapy has an important antioxidant function by stimulating oxidative preconditioning or improving adaptation to oxidative stress, and increases the activity of antioxidant enzymes to address ROS-mediated pathophysiological conditions (WEI et al., 2018). The reaction of ozone with the cell membrane and lipoprotein-bound polyunsaturaed fatty acids (PUFAs) H_2O_2 from ozonized lipid products (LOP) products, can function as a cell membrane and mixing stimulants, demonstrating the immunomodulatory effect of ozone through the status of antioxidant lipid generators (LOP) (CHIRUMBOLO et

al., 2021). LOP dissociates Keap1-Nrf2 and activates the Nrf2-ARE pathways, thereby increasing antioxidant enzymes after ozone treatment. In addition, the activation of this pathway is directly related to the inflammatory process. Their activation indicated that the oxidative and inflammatory processes are deeply related during the damaged hepatic, and that ozone therapy is a good therapy to control the oxidative process inside the cells.

In our review the antioxidant role of ozone therapy was also demonstrated by the increase in the antioxidant enzymes expression like SOD and GSH, associated with a reduction in the oxidized glutathione (GSSG). We already know that reduced glutathione (GSH) is considered to be one of the most important scavengers of ROS, and its ratio with oxidized glutathione (GSSG) may be used as a marker of oxidative stress. Within cells, total GSH is responsible for reverting free glutathione from its oxidise form (GSSG), so the ratio of reduced glutathione to oxidized glutathione is often used as a marker of cellular toxicity. These results corroborate our findings of the important antioxidant effect presented by ozone therapy, since there was a increase in GSH and a decrease in GSSG after ozone therapy exposure. In our review, we observed that the administration of ozone in models of ischemia reduces the impact of damage and triggers an antioxidant response (CHIRUMBOLO et al., 2021). Thus, ozone therapy can induce a controlled oxidative stress capable of stimulating an adaptive antioxidant response in healthy tissues (CLAVO, et al., 2019). While moderate oxidative stress will activate the nuclear transcriptional factor-factor 2, inducing the transcription of antioxidant response elements leading to the production of numerous antioxidant enzymes.

4.1.3. Ozone therapy and inflammation

The studies included in this review showed that ozone therapy reduces the inflammatory process by the down regulation of pro-inflammatory markers. These results are directly related to the decrease in the quantity of inflammatory cells, like neutrophils and kupfer cells after ozone exposure. We already know that phagocytes hold an important role in the inflammatory and oxidative processes, and these cells represent a link between both processes involved in the development of different diseases. Macrophages and neutrophils produce free radicals and inflammatory markers triggering oxidative stress and inflammation-promoting lesions in different tissues, especially the liver (IDDIR et al., 2020). Excess ROS can oxidize biomolecules including RNA/DNA, lipids, or proteins, or they can structurally modify proteins and genes to trigger signaling cascades that can lead to the initiation of the

inflammatory response (IDDIR et al., 2020). Inflammatory stimuli can trigger the activation of intracellular signaling pathways involved in the expression of inflammatory mediators causing the release of microbial products and cytokines. This includes interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF), showing that there is a relationship between inflammation and oxidative stress (IDDIR et al., 2020). Ozone is a modulator of the inflammatory process by modulating macrophage phagocytic activity, regulating Nrf2/ARE, and blocking the NF-κB pathway, reducing inflammation (DE SIRE et al., 2022). Activation of the antioxidant signal Nrf2 can attenuate the inflammatory response, moreover, it plays an important role in the intracellular signaling pathways of inflammation (DE SIRE et al., 2022). Ozone may act as a promising therapeutic agent for inflammatory diseases (WEI et al., 2018). In addition, the pro-inflammatory response is associated with the formation of ROS and the consequent generation of oxidative stress (DLUDLA et al., 2019). Ozone treatment reduces the levels of pro-inflammatory cytokines (TNF-α, IL-1β) due to its anti-inflammatory effects, thereby reducing oxidative stress by blocking the NF-κβ pathway and activating the Nrf2 pathway (FERNÁNDEZ-CUADROS et al., 2022). Therefore, in general, ozone therapy is an interesting alternative for the treatment of liver diseases with a focus on oxidative balance mechanisms. The main outcomes of this is ozone treatment proved to be effective in reducing cell degeneration and necrosis. Ozone blocks apoptotic processes by reducing the expression of caspases, Tumor Necrosis Factor-a (TNF-α), Bcl-2-associated protein X (Bax), and p53 genes. Apoptosis can occur through the action of Bax molecules (promotes the activation of cytocrome C), p53, and Caspase molecules (blocking the cell cycle) (SCASSELLATI et al., 2020).

4.1.4. Ozone therapy and other markers

Ozone therapy increases ATP as it stimulates the Krebs cycle in the mitochondria, increasing oxidative carboxylation of pyruvate, and stimulating the production of adenosine triphosphate (ATP) (SCASSELLATI et al., 2020). In addition, ozone therapy increased Ca^{2+} ATPase activity and reduced calcium and calpain levels. Our results showed that ozone therapy promoted a reduction in calcium content. Calcium is a messenger associated with hepatic processes and its dysregulation is related to liver injury (OLIVA-VILARNAU et al., 2018), so the control of intracellular calcium is considered a therapeutic target for liver injury. Ca^{2+} ATPase is a transport protein present in the plasma membrane that transports Ca^{2+} ions out of the cytoplasm vital for regulating the amount of Ca^{2+} within cells (HOSSAIN et al.,

2020). The increase in Ca^{2+} ATPase activity through ozone treatment will reduce the calcium content inside the cell. In addition, ozone promoted the reduction of another protease called calpain. Calpains are calcium-dependent cysteine proteases (SPINOZZI et al., 2021), and the involvement of calpain in liver dysfunction depends on its mediation of oxidative stress and inflammation, which are the most important contributors to the initiation and progression of liver dysfunction (LI & LU, 2018). Calpain plays an essential role in liver disease and its inhibition may protect against liver damage (XU et al., 2021). Therefore, ozone exerted a beneficial effect in reducing calpain.

Elevated liver enzymes AST (aspartate aminotransferase) and ALT (alanine aminotransferase) occur due to damage to hepatocytes (SHARMA et al., 2021). Studies point to the effectiveness of alcohol in induced damage (JIANG et al., 2021) by the use of drugs, such as paracetamol (KAGAWA et al., 2018), demonstrating that AST and ALT levels increased regardless of the cause of the injury (SHARMA et al., 2015). In our studies, ozone reduced ALT and AST levels in ischemia-reperfusion-induced liver injury, in the experimental model of radiation damage, damage from alcohol, medication, and CCL4 ingestion. As ALT and AST are produced by hepatocytes and only released in case of damage to these cells, we can highlight in this context, the cytoprotective effect of ozone on hepatocytes reducing the release of ALT and AST. Liver injuries reduce glycogen levels through increased oxidative stress and inflammation (PARK et al., 2020). The maintenance of glycogen is important for proper cellular functioning, as its reduction impacts homeostasis, leading to oxidative stress. (MALINSKA et al., 2020). Ozone preserves the glycogen content, thus reducing liver damage, generating accumulation of cytochrome P450 system enzymes and antioxidant enzymes, and increasing the number of glycogen molecules (SCASSELLATI et al., 2020). Furthermore, the oxidative preconditioning ability of ozone has been reported to preserve liver glycogen content and reduce lactic acidosis (ONAL et al., 2015).

4.2 Methodological quality and risk of bias

The risk of bias analysis was performed individually to ensure the validity of the findings and to assess the methodological quality of the studies, demonstrating that the application of standardized protocols is essential for the reproducibility and synthesis of the results. Bias analysis showed that key features such as blinding of participants (caregivers and outcome assessor) were not reported or unclear in the studies. In addition, some registries provided incomplete result data and insufficient information, which affects the accuracy of the

results. It is important to emphasize that all types of reviews have limitations, and these limitations are clear and more evident in systematic review studies due to the use of specific tools to evaluate the quality of the evidence. In our review, the biggest limitation was the heterogeneity of the studies, which makes the task of comparing them difficult. The lack of information regarding the age of the animals was also neglected by most studies, which may be a reporting bias as it compromises the quality of the report.

We also observed that individual studies only analyzed a few oxidative markers, markers of the antioxidant system, pro-inflammatory cytokines, inflammatory cells, and morphological parameters. In individual studies, each element of methodological bias may be associated with variability in the objectives of different studies. However, it is important to emphasize that all types of reviews have limitations, and these limitations are most evident in systematic review studies, which extract information from primary studies to understand the process in its entirety. Thus, it is worth mentioning that our findings are important for understanding the mechanism of action of ozone and its therapeutic treatments, describing important points of bias, and we hope to contribute to future studies, avoiding those elements of bias that impair the quality of the evidence.

5. CONCLUSION

After the individual studies were analyzed, it was possible to conclude that ozone therapy has a beneficial effect for models of liver injury. Through both the decrease of oxidative stress in tissue and inflammatory markers and consequently decreases pathological processes such as degeneration and necrosis. In addition, ozone regulates the Nrf2/ARE antioxidant pathway and blocks the NF-κB inflammatory pathway. This consequently increases the expression of antioxidant enzymes and reduces the levels of pro-inflammatory cytokines (TNF-α, IL-1β), controlling the oxidative stress and inflammatory process. Thus, acting as a promising therapeutic agent for inflammatory diseases. However, it is worth mentioning that the therapeutic function of ozone is also associated with the generation of moderate oxidative stress promoted by the activation of secondary messengers that form H_2O_2 and aldehyde-4-hydroxynonenal (4-HNE), which stimulate the production of antioxidant enzymes. However, future studies are needed to understand the mechanisms of ozone action, standardization of doses and concentrations, and exposure time for different liver injuries. Therefore, we hope that this review will be used as a guide improving future research on ozone treatment in liver disease.

Supplementary data

Table S1. Complete search strategy with search filters and the number of studies retrieved from databases **PubMed-Medline e Scopus**.

Table S1- Research Question Components following the PICO Strategy

Abbreviations	Parameters (description)	Question Components
P	Population	Murine models
I	Intervention	Ozone therapy
C	Comparison	Animals that did not receive ozone
O	Outcomes	Oxidative stress in liver tissue

Table S2- Full search strategy in PubMed and Scopus, including search terms and filters.

Data base	Descriptors	Items Found	Time	Date
PubMed	#1 Ozone Filter ("ozone"[MeSH Terms] OR Ozone therapy [TIAB])	15,779	07/04/2021	17:23
	#2 Therapeutics Filter ("therapeutics"[MeSH Terms] OR therapy[TIAB] OR "therapeutic use"[Subheading] OR "therapeutic uses"[MeSH Terms] OR therapeutic use[TIAB])	8,934,939	07/04/2021	17:23
	#3 Antioxidant filter ("antioxidants"[MeSH Terms] OR "antioxidants"[Title/Abstract] OR "oxidative stresses"[Title/Abstract] OR "antioxidative stress"[Title/Abstract] OR "oxidative stress injury"[Title/Abstract])	172,003	07/04/2021	17:24
	Total: #1 ABD #2 AND #3	221	07/04/2021	17:25

Data base	Descriptors	Items Found	Time	Date
Scopus	#1 Ozone Filter (TITLE-ABS-KEY ("ozone") OR TITLE-ABS-KEY ("Ozone therapy"))	112,509	07/04/2021	17:33
	#2 Therapeutics Filter (TITLE-ABS-KEY ("therapeutics") OR TITLE-ABS-KEY("therapy") OR TITLE-ABS-KEY("therapeutic use"))	4,851,800	07/04/2021	17:33
	#3 Antioxidant filter (TITLE-ABS-KEY(""antioxidants") OR TITLE-ABS-KEY ("antioxidants") OR TITLE-ABS-KEY ("oxidative stresses") OR TITLE-ABS-KEY ("antioxidative stress") OR TITLE-ABS-KEY ("oxidative stress injury"))	650,154	07/04/2021	17:34
	Total: #1 AND #2 AND #3	379	07/04/2021	17:34

Data base	Descriptors	Items Found	Time	Date
WEB OF SCIENC	#1 Ozone Filter TS=ozone OR TS=Ozone therapy	96.550	07/04/2021	17:33
	#2 Therapeutics Filter TS=therapeutics OR TS=therapy OR TS=therapeutic use OR TS=therapeutic uses OR TS=therapeutic use	2.852.755	07/04/2021	17:33
	#3 Antioxidant filter TS=antioxidants OR TS=antioxidants OR TS=oxidative stresses OR TS=antioxidative stress OR TS=oxidative stress injury	661.260	07/04/2021	17:33
	Total: #1 and #2 and #3	332	07/04/2021	17:33

Table S3 - Animal model Characteristics

Animal Model: Rat	Country	Strain	Sex	Age (months)	Weith (g)	Total number
Laszcyca , et al.1996	Poland	Wistar	M	?	266 - 299	?
León, et al. 1998	Cuba	Sprague -Dawley	F	?	220 - 250	60
Peralta, et al. 1999	Spain	Wistar	M	?	250 - 300	18
Peralta[a], et al. 2000	Spain	Wistar	M	?	250 - 300	56
Jalil, et al. 2001	Cuba	Sprague-Dawley	F	?	200 - 250	40
Ajamieh, et al. 2002	Cuba	Wistar	M	?	250 - 300	32
Ajamieh, et al. 2004	Cuba	Wistar	M	?	250 - 275	50
Ajamieh, et al. 2005	Cuba	Wistar	M	?	250 - 275	60
Madje, et al. 2007	Poland	Wistar	M	4	164 - 180	60
Guanche, et al. 2010	Cuba	Wistar	M	?	?	60
Gul, et al. 2012	Turkey	Sprague-Dawley	M	?	200 - 250	27
Gultekin, et al. 2012	Turkey	Wistar	F	?	200 - 230	30
Safwat, et al. 2014	Egypt	Wistar	M	3	180 - 220	60
Aslaner, et al. 2015	Turkey	Wistar	M	?	250 - 300	18
Erdemli, et al. 2018	Turkey	Wistar	M	3 - 4	200 - 250	36
Adali, et al. 2019	Turkey	Wistar	F	4 - 6	190 - 250	48
Guvendi, et al. 2020	Turkey	Wistar	F	4 - 6	190 - 250	48
Animal Model: Mice	**Country**	**Strain**	**Sex**	**Age**	**Weith**	**Total number**
Reference						
Zamora, et al. 2005	Cuba	Balb /c mice	M	?	18-20	35
Rodriguez, et al. 2011	Cuba	Balb/c mice	M	?	18-20	50

Table S4- Intervention characteristics ozone therapy and liver injury

Reference	Control	Liver injury	Applied dose (mg/kg)	Ozone concentration (µg/mL)	Route	Frequency	Duration (Day)
Laszczyca et al., 1996	Cadmium	Cadmium	0.14 **	40	Intraperitoneal	Once a day	10
León et al., 1998	CCl_4	CCl_4	1	50	Intraperitoneal	Once a day	15
Peralta et al., 1999	Ischemia-reperfusion	Ischemia-reperfusion	1	50	Rectal insufflation	Once a day	10
Peralta et al., 2000	Untreated	hepatic ischemia	1	50	Rectal insufflation	Once a day	10
Jalil et al., 2001	CCl_4	CCl_4	1	50	Rectal insufflation	Once a day	15
Ajamieh et al., 2002	ischaemia/reperfusion	hepatic ischemia	1	50	Rectal insufflation	Once a day	15
Ajamieh et al., 2004	ischaemia/reperfusion	ischaemia/reperfusion	1	50	Rectal insufflation	Once a day	15
Ajamieh et al., 2005	ischemia/reperfusion	hepatic ischemia	1	50	Rectal insufflation	Once a day	15
Zamora et al., 2005	LPS	LPS	0.2; 0.4; 1.2	3,8 ; 7,6; 22,8 **	Intraperitoneal	Once a day	5
Madej et al., 2007	LPS	LPS	0.15	54	Intraperitoneal	Once a day	10
Guanche et al., 2010	Sepsis	Sepsis (fecal material)	?	10, 30; 50	Intraperitoneal	Once a day	5
Rodríguez et al., 2011	LPS	LPS	0.2; 0.4; 1.2	3,8; 7,6; 22,8 **	Intraperitoneal	Once a day	5
Gul et al., 2012	acetaminophen	acetaminophen	0.7	60	Intraperitoneal	Once time	1
Gultekin et al., 2012	Saline solution	ionizing radiation	0.7	60 ***	Intraperitoneal	Once a day	5
Safwat et al., 2014	Untreated	ageing	0.6	67 **	Rectal insufflation	1) twice weekly/90 days once per week/ 450 days 2) three times weekly / 120 days	450
Aslaner et al., 2015	Methotrexate	Methotrexate	0.45 **	25	Intraperitoneal	every day	15
Erdemli et al., 2018	Mandibular defect	mandibular defect	0.6	17	Rectal insufflation	Once a day	15
Adali et al., 2019	Alcoholic	ethyl alcohol	0.5	?	Intraperitoneal	every day	7
Guvendi et al., 2020	Iron overload	iron dextran	0.5	?	Intraperitoneal	Six days a week	27

* Not informed in the text; ** Calculated (Considered average weight); *** 60 mg/mL (change to microgram would be 60000 µg); LPS lipopolysaccharide

Table S5: Frequency and duration of days of ozone treatments

Days	Frequency	Percentage (%)
1	1	5,26
5	4	21,05
7	1	5,26
10	4	21,05
15	6	31,58
20	1	5,26
27	1	5,26
450	1	5,26

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