UNIVERSIDAD DE EXTREMADURA

TESIS DOCTORAL

APLICACIÓN CLÍNICA DE LAS CELULAS MADRE MESENQUIMALES ALOGÉNICAS DE ORIGEN ADIPOSO A PACIENTES CANINOS CON ENTEROPATÍA INFLAMATORIA CRÓNICA

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"Cuando la gratitud es absoluta, las palabras sobran"

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Índice

Resumen

Resumen

La enteropatía inflamatoria crónica es un proceso idiopático consecuencia de una respuesta inapropiada del sistema inmune a antígenos de la dieta o la microbiota en pacientes predispuestos genéticamente. Los signos gastrointestinales son crónicos, persistentes o recurrentes e impactan enormemente en la calidad de vida del paciente y de su cuidador. El diagnóstico es dificultoso ya que conlleva la realización de múltiples pruebas para descartar patologías que provocan una sintomatología similar. El abordaje terapéutico también es complejo, implicando el paso sucesivo por cambios en la dieta, administración de antibióticos y después de inmunosupresores a medida que no responden a la terapia anterior. Los pacientes requerirán tratamiento de por vida, con los efectos secundarios que esto ocasiona, especialmente en el caso de los inmunosupresores, y un porcentaje de estos no responden a ninguna estrategia terapéutica. Si a estos factores se añade el aumento de su prevalencia en los últimos años, se concluye que la enfermedad constituye un reto para el veterinario. Por todo ello, la búsqueda de biomarcadores que faciliten el diagnóstico y el pronóstico y de nuevas perspectivas terapéuticas para aquellos pacientes refractarios al tratamiento, se hace indispensable.

El presente estudio se centra en el tratamiento de la enfermedad con células madre alogénicas mesenquimales de origen adiposo y en la investigación de nuevos marcadores de la enfermedad. Para ello, el trabajo consta de cuatro artículos de investigación, que comparan la efectividad clínica de la terapia celular con o sin prednisona concomitante, y que estudian los cambios en el proceso inflamatorio y en el estrés oxidativo tras la aplicación de ambas opciones terapéuticas y el valor de la cobalamina como marcador de las lesiones del íleon en animales con enteropatía inflamatoria crónica.

Los resultados revelan que el tratamiento de la enteropatía inflamatoria crónica con células madre es igual de efectivo asociado o no a prednisona, observándose en ambos casos una disminución de los índices clínicos y un aumento de albúmina y cobalamina en los controles efectuados hasta un año tras el tratamiento. Además, la infusión de células madre a pacientes en tratamiento con prednisona, permite disminuir su dosis hasta anularla en algunos casos.

Como marcadores de inflamación, las ratios neutrófilos/linfocitos, linfocitos/plaquetas y el índice de inmunidad-inflamación sistémica se encontraron aumentados en perros enfermos antes del tratamiento en comparación con perros sanos, observándose su disminución progresiva en los controles realizados entre un mes y un año

postratamiento, que a su vez se correlacionó con un descenso en los valores de los índices clínicos.

Por otra parte, en un grupo de perros afectados por la enteropatía inflamatoria crónica se observó que la afectación endoscópica e histopatológica del íleon era totalmente diferente cuando la cobalamina era inferior a los 200 ng/l o superior a 350 ng/l y que la concentración de cobalamina se correlaciona con la severidad de la lesión macro y microscópica del íleon.

Por último, el malondialdehido, el glutation reducido y la albúmina como marcadores de estrés oxidativo se analizaron en pacientes con y sin enfermedad inflamatoria, encontrándose una disminución significativa en la albúmina en los perros afectados con respecto a los sanos. Exceptuando la concentración de albúmina, que aumentó con el tiempo, no se observó ningún cambio en ninguno de estos parámetros tras la administración del tratamiento de células madre con o sin prednisona.

Por tanto, se puede concluir que la aplicación de las células madre como tratamiento de la enteropatía inflamatoria crónica canina proporciona un alivio de los síntomas clínicos que se acompaña de una mejoría del proceso inflamatorio, pudiendo aplicarse de forma única o en combinación con el tratamiento inmunosupresor preexistente. Sin embargo, ambas opciones terapéuticas no parecen tener efecto sobre el estrés oxidativo de los pacientes. Aun así, la albúmina ha demostrado ser un marcador de enfermedad prometedor, mientras que la cobalamina puede proporcionar información sobre el grado de afectación del íleon sin necesidad de su exploración.

Abstract

Abstract

Chronic inflammatory enteropathy is an idiopathic process resulting from an inappropriate response of the immune system to dietary or microbiota antigens in genetically predisposed patients. Gastrointestinal signs are chronic, persistent, or recurrent and greatly impact the patient's and his owner's quality of life. Diagnosis is difficult as it involves multiple tests to rule out pathologies that cause similar symptomatology. The therapeutic approach is also complex, involving successive changes in diet, administration of antibiotics, and then immunosuppressants as they do not respond to previous therapy. Patients will require lifelong treatment, enduring the side effects of the treatment, especially the immunosuppressants, and a percentage of these do not respond to any therapeutic strategy. Adding these factors to the increase in its prevalence in recent years, we can conclude that the disease constitutes a challenge for the veterinarian. For all these reasons, searching for biomarkers that facilitate diagnosis and prognosis and for new therapeutic perspectives for refractory patients is indispensable.

The present study focuses on the treatment of the disease with adipose-derived allogeneic mesenchymal stem cells and on the research of new markers of the disease. The complete study comprises five research articles, comparing the clinical effectiveness of cell therapy with or without concomitant prednisone, and studying the changes in the inflammatory process and oxidative stress after the application of both therapeutic options and the value of cobalamin as a marker of ileum lesions in animals with chronic inflammatory enteropathy.

The results reveal that the treatment of chronic inflammatory enteropathy with stem cells is equally effective associated or not with prednisone, observing in both cases a decrease in clinical indices and an increase in albumin and cobalamin in every checkpoint over one year after treatment. In addition, the infusion of stem cells to patients under treatment with prednisone allows the reduction of the steroid dosage and even its cancellation.

The markers of inflammation neutrophil/lymphocyte, lymphocyte/platelet ratios, and the systemic immunity-inflammation index were found to be increased in dogs with inflammatory enteropathy before treatment compared to healthy dogs. A progressive decrease in the markers was observed in the post-treatment follow-up over a year, which correlated with a decrease in the values of the clinical indexes.

It was observed that the endoscopic and histopathological involvement of the ileum was different when cobalamin was lower than 200 ng/l or higher than 350 ng/l in dogs affected by chronic inflammatory enteropathy and that the cobalamin concentration correlated with the severity of the macro and microscopic lesion of the ileum.

Finally, Malondialdehyde, reduced glutathione, and albumin as markers of oxidative stress were analyzed in patients with and without inflammatory disease. A significant decrease in albumin in affected dogs compared with healthy dogs was found. Except for albumin concentration, which increased over time, no change in any of these parameters was observed after the stem cell treatment with or without prednisone.

Therefore, it can be concluded that the treatment with adipose-derived allogeneic mesenchymal stem cells for canine chronic inflammatory enteropathy provides relief of clinical symptoms accompanied by an improvement of the inflammatory process and can be applied alone or in combination with pre-existing immunosuppressive treatment. However, both therapeutic options do not influence oxidative stress in patients. Still, albumin have proven to be promising disease marker, while cobalamin may provide information on the degree of ileum involvement when it is not endoscopically explored.

Introducción

1. DEFINICIÓN, FISIOPATOLOGÍA Y ETIOPATOGENIA DE LA ENTEROPATÍA INFLAMATORIA CRÓNICA

La enfermedad inflamatoria intestinal crónica canina o, recientemente denominada enteropatía inflamatoria crónica (EIC) canina, es una enfermedad que afecta al tracto gastrointestinal y se caracterizada por la presencia de signos digestivos crónicos. Para llegar al diagnóstico de la EIC, el paciente debe presentar signos clínicos digestivos durante más de 3 semanas de evolución, siendo necesario descartar otras causas que provoquen esta sintomatología (enfermedades infecciosas, metabólicas y neoplásicas) y en el análisis histopatológico del tracto gastrointestinal debe existir un infiltrado inflamatorio (Allenspach K. et al. 2007; Simpson K.W.A & Jergens A.E. 2011).

Esta patología es una causa frecuente de presentación en la clínica veterinaria, sin embargo, no se sabe con exactitud la incidencia debido a la gran variabilidad de resultados que se han obtenido en los diferentes estudios llevados a cabo en centros de referencia, oscilando su prevalencia entre 1 y 17.8% (Dandrieux J.R.S & Mansfield C.S. 2019).

La enfermedad se divide en diferentes subtipos en función de la respuesta de los pacientes a los tratamientos: enteropatía con respuesta a la dieta (ERD o FRE, del inglés Food-Responsive Enteropathy), enteropatía sensible a antibióticos (ERA o ARE, del inglés Antibiotic-Responsive Enteropathy), enteropatía que responde a inmunosupresores (ERI o IRE, del inglés Immunosuppressant-Responsive Enteropathy) y enteropatía que no responde (ENR o NRE, del inglés Non-Responsive Enteropathy). En medicina veterinaria, el término enfermedad inflamatoria intestinal o del inglés inflammatory bowel disease (IBD), se corresponde con el subtipo IRE. Estos pacientes no responden a los cambios de dieta ni a la pauta con antibióticos, además, presentan un infiltrado inflamatorio en el análisis histopatológico de las muestras y esta forma de enfermedad es la que se corresponde con la Enfermedad de Crohn (EC) en medicina humana (Dandrieux J.R. 2016; Isidori M. et al. 2022).

La etiología de esta enfermedad, al igual que ocurre en medicina humana, aún es desconocida. Se cree que es multifactorial, con un origen inmunomediado en el que influyen factores medioambientales, genéticos e inmunológicos.

Los factores medioambientales incluyen los antígenos dietéticos y derivados del microbioma que juegan un papel importante en la patogénesis de la enfermedad (German A.J. et al. 2003; Simpson K.W.A & Jergens A.E. 2011). Los desequilibrios que se producen en el microbioma de estos pacientes se deben a cambios en la composición de los microbios, disminuyendo la diversidad y la proporción de las especies microbianas en comparación con perros sanos. Todo ello da lugar a una disbiosis intestinal, que genera de forma secundaria una inflamación en la mucosa (Xenoulis P.G. et al. 2008).

Con respecto al factor genético de la enfermedad, se han realizado diferentes estudios en los que se ha demostrado una predisposición racial en los perros de raza pastor alemán, weimaraner, rottweiler, bóxer y border collie (German A.J. et al. 2000; Kathrani A. et al. 2011). En medicina humana se han identificado numerosos alelos de riesgo en pacientes con EC y Colitis Ulcerosa (CU), que involucran a genes importantes en la relación del huésped con el microbioma, apoyando el componente genético de la enfermedad. Aunque en medicina veterinaria se haya demostrado esta predisposición racial, aún no se han demostrado estas alteraciones en el genoma (McGovern D.P. et al. 2015).

En la inmunopatología de la EIC se produce una interacción compleja entre la inmunidad innata y la adaptativa. Fisiológicamente, en esta enfermedad tienen una implicación importante las células T helper 1 (Th1) y Th2, más concretamente, en la EC y la CU respectivamente y las células Th17. Por otro lado, las T CD4 de la mucosa son las mediadoras de la respuesta inmunitaria. En esta patología, además, tienen gran importancia los macrófagos que producen el factor de necrosis tumoral alfa (FNT-α) y el desequilibrio que se produce en algunas citoquinas, como las interleuquinas (IL)-1β, IL-6, IL-8, IL-10, IL-12, IL-17, IL-23 y el factor de crecimiento transformante beta-1 (TGF-β) (German A.J. et al. 2003; Sáez A. et al. 2023) (Figura 1).

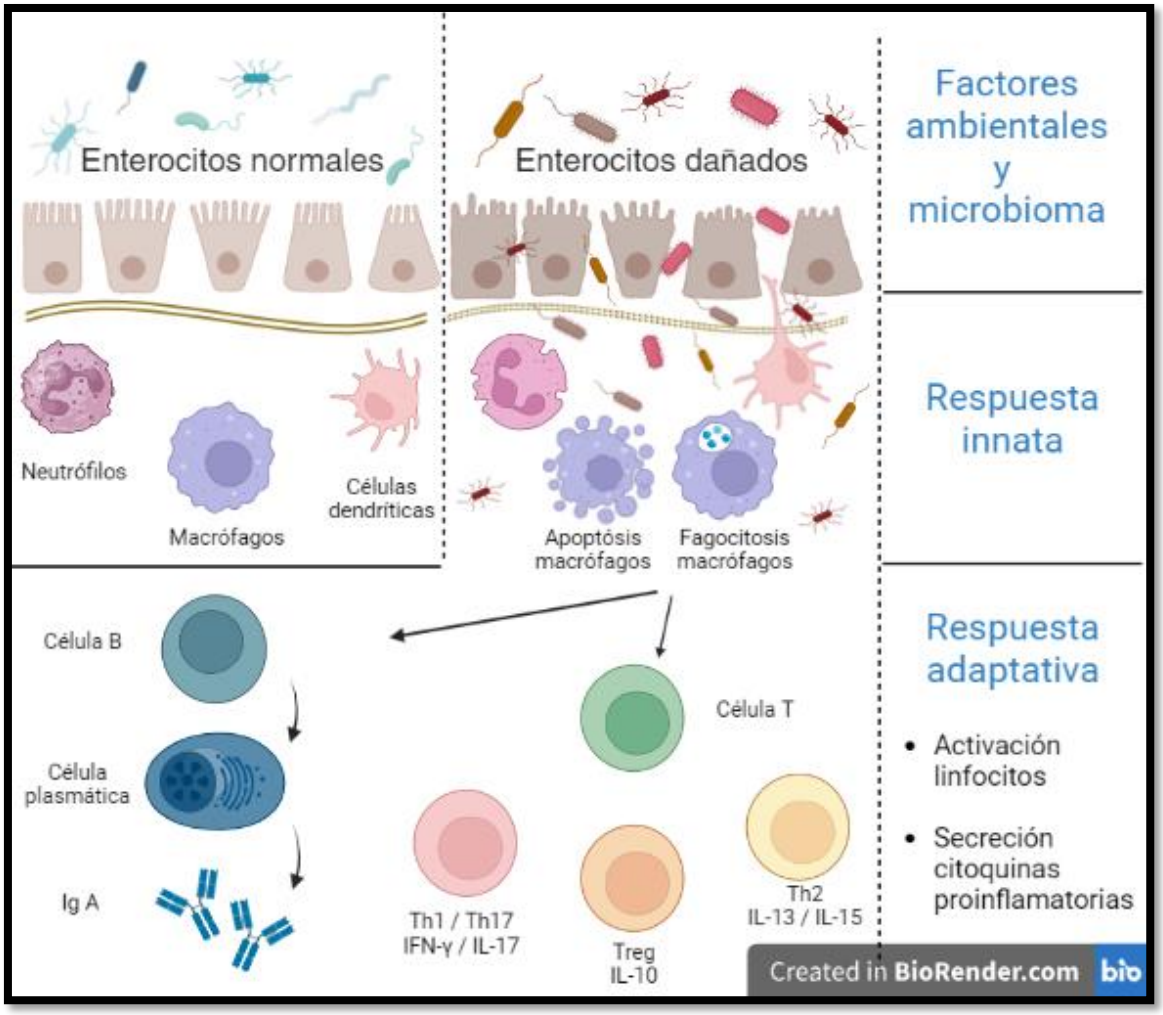

Figura 1. Inmunopatología de la EIC. Población celular clave y mediadores de la homeostasis en el intestino normal y el intestino afectado por la enteropatía inflamatoria crónica.

1.1. DIAGNÓSTICO DE LA ENTEROPATÍA INFLAMATORIA CRÓNICA

El diagnóstico inicial de esta patología se realiza principalmente excluyendo otras causas que puedan estar provocando la sintomatología digestiva crónica. Entre estas causas, destacan las siguientes:

- Enfermedades extragastrointestinales como la insuficiencia renal, hepatopatías, enfermedades pancreáticas, hiperadrenocorticismo, hipoadrenocorticismo e hipocalcemia.
- Enfermedades infecciosas o parasitarias.

- Patologías intestinales de otras etiologías como, por ejemplo, intususcepciones, neoplasias o cuerpos extraños (Simpson K.W.A. & Jergens A.E. 2011).

Para poder descartar todas estas patologías y llegar al diagnóstico definitivo de la EIC es necesario realizar numerosas pruebas diagnósticas. En el procedimiento diagnóstico ideal se incluye una correcta anamnesis y exploración física, una hematología completa y bioquímica sanguínea, incluyendo la medición de albúmina, ácido fólico, cobalamina y TLI (del inglés trypsin-like immunoreactivity), un análisis de orina y análisis coprológico de las heces (incluyendo un kit comercial para la detección de antígenos fecales de *Giardia duodenalis*). Además, se deben realizar radiografías de abdomen y ecografía abdominal completa. Para finalizar el protocolo diagnóstico, se realiza una endoscopia digestiva completa con la consecuente toma de muestras de biopsias de estómago, duodeno, íleon y colon, para el posterior estudio histopatológico de dichas muestras (Simpson K.W.A. & Jergens A.E. 2011).

Una vez descartadas todas las causas extragastrointestinales, la diferenciación de los tipos de EIC se realizará en base a la respuesta que tengan los pacientes a diferentes terapias. Por ello, el paciente presentará FRE si responde a cambios dietéticos utilizando dietas de proteína novel o dietas con las proteínas hidrolizadas, ARE si mejora con la pauta de antibioterapia, siendo el metronidazol y la tilosina los antibióticos más utilizados, IRE (o IBD) si responde al añadir fármacos inmunosupresores (prednisona, ciclosporina, azatioprina, etc.) y, por último, si no responde a cambios dietéticos ni farmacológicos, se llegará a la conclusión de que se trata de NRE (Dandrieux J.R. 2016).

1.2. SIGNOS CLÍNICOS Y ALTERACIONES LABORATORIALES

El curso clínico de la enfermedad, tal y como hemos comentado, suele ser cíclico, con periodos en los que mejora el paciente seguidos de otros en los que empeora la sintomatología. Los pacientes presentan signos gastrointestinales crónicos, sobre todo, vómitos y diarreas, asociados a una pérdida del apetito, borborigmos, dolor abdominal y disminución de peso (Dandrieux J.R. 2016). Otros signos clínicos que también pueden aparecer, aunque con menor frecuencia es la hematoquecia, mucosidad en las heces, tenesmo, melena, hematemesis y polifagia (Craven M. et al. 2004).

En la hematología de estos pacientes se puede observar anemia debido a la pérdida de sangre en el aparato digestivo o por la inflamación crónica. Puede haber un incremento o disminución en el recuento leucocitario secundario a la inflamación que se produce y a los procesos erosivos y ulcerativos de la mucosa. Sin embargo, la aparición de trombocitopenia o trombocitosis es una alteración poco frecuente en estos pacientes (Craven M. et al. 2004).

Por otro lado, las alteraciones que pueden observarse en la bioquímica sanguínea son las siguientes: disminución de proteínas totales, albumina, globulinas y calcio, también incremento de urea, creatinina, ALT (alanino aminotransferasa), fosfatasa alcalina y lipasa pancreática específica canina. Además, se puede observar un aumento o disminución del folato, en función de si existe una disbiosis intestinal o un problema de malabsorción y una disminución de cobalamina sérica. De todas estas alteraciones, las que mayor repercusión presentan en esta enfermedad son la hipoalbuminemia y la hipocobalaminemia (Craven M. et al. 2004).

La hipoalbuminemia se ha encontrado con mayor frecuencia en pacientes que eran refractarios a los tratamientos de la EIC (Craven M. et al. 2004). Sobre esta proteína hablaremos más adelante (apartado 3.4), ya que además de analizar su variación desde el punto de vista del estado nutricional de los pacientes enfermos, se ha estudiado atendiendo a una de las propiedades que se le atribuyen, su capacidad antioxidante (Allenspach, K et al. 2007).

La cobalamina, también llamada vitamina B12, se ingiere con alimentos, principalmente de origen animal, aunque también la puede producir la microbiota intestinal (Watanabe F. 2007). Se ha postulado que, entre un 19 y un 38% de los animales que sufren EIC, presentan hipocobalaminemia (Kather S. et al. 2020).

La EIC afecta a la absorción de esta vitamina ya que la lesión de la mucosa del íleon disminuye la función del receptor cubam, el cual se encarga de absorber la cobalamina, aunque no hay estudios en los que se establezcan los niveles del receptor de cobalamina en pacientes con esta patología (Kather S. et al. 2020). Los bajos niveles de cobalamina se asocian a un aumento en el número de linfocitos intraepiteliales en la mucosa del íleon, demostrándose así que los cambios inflamatorios en este tramo intestinal van asociados a la disminución de esta vitamina en pacientes con EIC (Procoli F. et al. 2013). Esta vitamina

también puede estar disminuida en pacientes con EIC debido a la disbiosis que se genera en el intestino delgado (Berghoff N. & Steiner J.M. 2011).

Por último, se ha demostrado que la disminución del folato y cobalamina son indicadores de problemas de malabsorción intestinal y pueden localizar mejor la enfermedad, ya que el folato se absorbe a nivel proximal (duodeno y yeyuno proximal) y la cobalamina a nivel distal (íleon). Se ha determinado en un estudio reciente que el folato es un biomarcador menos sensible de la EIC en comparación con la cobalamina (Ullal T.V. et al. 2023).

1.3. OTROS MÉTODOS DIAGNÓSTICOS

Tras la realización de los análisis laboratoriales, los métodos de diagnóstico por imagen son esenciales en el protocolo diagnóstico inicial en los pacientes con EIC. Las radiografías simples o de contraste pueden servir para detectar la presencia de masas y cuerpos extraños que estén provocando signos digestivos crónicos. Sin embargo, es más sensible el uso de la ecografía abdominal, esta se utiliza para detectar anomalías que puedan existir en cualquier órgano de la cavidad abdominal, pero, sobre todo, para valorar el espesor de la pared de estómago y asas intestinales, la presencia de líquido intraluminal, la linfadenopatía, los cambios de ecogenicidad de las capas y la dilatación y fruncimiento intestinal (Gaschen L. et al. 2008; Simpson K.W.A. and Jergens A.E. 2011).

Posteriormente, si el paciente no responde a otros tratamientos y se han realizado todas las pruebas anteriormente mencionadas, debe realizarse una endoscopia digestiva completa. Esta prueba es esencial para evaluar de forma directa, rápida y poco invasiva todas las alteraciones que se producen en estos pacientes con EIC, además de descartar otras causas que puedan estar provocando la sintomatología. La endoscopia es imprescindible también para llevar a cabo una correcta toma de muestras de biopsias de cara al análisis histopatológico posterior (Jergens AE. et al. 2016). Es útil para diagnosticar alteraciones infiltrativas, erosivas e inflamatorias, además de cambios anatómicos como linfangiectasia intestinal o linfoma de bajo grado (Jergens A.E. et al. 2022).

Mediante la endoscopia flexible se pueden tomar muestras de tejido de diferentes partes del sistema digestivo (estómago, duodeno, íleon y colon), obteniéndose en algunos

casos incluso del yeyuno a nivel proximal, pero no del resto de yeyuno. Además, los riesgos de perforación digestiva mediante esta técnica son mínimos (Jergens A.E. & Heilmann R.M. et al. 2022).

El grupo de estandarización gastrointestinal de la asociación mundial de veterinarios de pequeños animales (WSAVA del inglés World Small Animal Veterinary Association) publicó en el año 2008 unas plantillas estándar sobre la endoscopia y el análisis histopatológico de gastroenterología en las especies canina y felina. En ellas se detallan los cambios endoscópicos e histopatológicos que se pueden producir durante la EIC en el estómago, duodeno y colon de estas especies con el fin de facilitar y estandarizar su exploración y la recogida de datos durante la misma (Day M.J. et al. 2008).

Sin embargo, en estas guías no se incluían los hallazgos histopatológicos del íleon, posiblemente porque habitualmente, en la endoscopia digestiva completa se suelen tomar muestras solo de estómago, duodeno y colon. Sin embargo, diversos estudios han atribuido gran importancia al estudio del íleon al demostrar que existen numerosas diferencias histopatológicas entre las muestras obtenidas mediante endoscopia del duodeno y del íleon (Procoli F. et al. 2013; Casamian-Sorrosal D. et al. 2010; Jergens A.E. et al. 2016).

En el análisis histopatológico de las biopsias obtenidas se estudia el grado de inflamación que se observa en la lámina propia, el cuál puede ser leve, moderado o grave y el tipo de población celular que se encuentra mayoritariamente, según la cual la EIC puede ser linfoplasmocítica, eosinofílica o granulomatosa. Por último, en este análisis histopatológico se describen los cambios que existen en la arquitectura de la mucosa, es decir, lesiones epiteliales, fibrosis, morfología de las vellosidades, cambios en la criptas y dilatación linfática (Jergens A.E. and Heilmann R.M. et al. 2022).

Recientemente, en medicina humana, se ha utilizado la cápsula endoscópica para evaluar el tracto digestivo de los pacientes con EIC, demostrando un mayor rendimiento con respecto a otras pruebas diagnósticas (como la ecografía, radiografía y endoscopia). Además, es muy útil en la valoración de la respuesta a los tratamientos antiinflamatorios y monitorización de los pacientes, ya que se puede detectar la recaída de algunos pacientes ya tratados (Limpias Kamiya K.J.L. et al. 2021). En medicina veterinaria únicamente se han

llevado a cabo estudios en pacientes con sangrado digestivo (Mabry K. et al. 2019; Stiller J. et al. 2021)

Por otra parte, en los últimos años se han investigado diferentes biomarcadores de inflamación que se podrían utilizar en el momento del diagnóstico, ayudando a determinar la gravedad de la enfermedad y valorar la respuesta a los tratamientos. Entre ellos destacan la proteína C reactiva (PCR) y la calprotectina fecal (Jergens A.E. & Heilmann R.M. et al. 2022).

La proteína C reactiva es una proteína de fase aguda que se eleva en respuesta a inflamación, infección o cáncer (Rhodes B. et al. 2011). Tiene una variación entre individuos alta, por lo que su uso en la EIC es muy limitado (Carney P.C. et al. 2011). Sin embargo, se ha demostrado que una concentración superior a 9,1 mg/L es altamente específica para determinar los pacientes que requieren tratamiento con inmunosupresores en perros con EIC (Heilmann R.M. et al. 2018).

Por último, la calprotectina fecal, que desempeña un papel fundamental en la EIC ya que se acumula en los sitios de inflamación (Heilmann R.M. et al. 2019), ha demostrado ser un biomarcador útil de inflamación intestinal en perros (Heilmann R.M. et al. 2018; Grellet A. et al. 2013). La medición de este parámetro, sin embargo, no está disponible de forma rutinaria en los laboratorios de medicina veterinaria.

2. INFLAMACIÓN EN LA ENTEROPATÍA INFLAMATORIA CRÓNICA

Una de las principales características de la EIC es la presencia de inflamación en la mucosa intestinal. La pérdida de tolerancia inmunológica de la mucosa frente a los antígenos que entran en el sistema digestivo da lugar a una respuesta inmunitaria exacerbada, causando una inflamación crónica no controlada. Además, la presencia de signos digestivos de forma crónica, favorecen el desarrollo de este estado inflamatorio (Dandrieux J.R. 2016; Eissa N. et al. 2019).

Esta inflamación se caracteriza por un aumento de moléculas proinflamatorias (Receptor de tipo Toll [TLRs], FNT-α, IL-1β e Interferón gamma [INF-γ]), que genera un aumento de la proliferación y disminución de la apoptosis de células T proinflamatorias

(células dendríticas, macrófagos T helper tipo 1 y M1), mientras que las células T reguladoras (Treg) están reguladas a la baja. Las células T helper (Th) 17 produce IL-17, la cual contribuye al daño tisular e inflamación. Esta IL presenta un papel esencial en el reclutamiento y activación de granulocitos y, además, en la producción de mediadores proinflamatorios de los macrófagos, generando así una inflamación desproporcionada. El fallo en la capacidad reguladora de los Treg empeora aún más la inflamación. Es importante también el aumento de la polarización de los macrófagos hacia el fenotipo M1 proinflamatorio activado, produciendo un exceso de ROS (del inglés Reactive Oxygen Species) y RNS (del inglés Reactive Nitrogen Species) y citoquinas proinflamatorias. Todo ello empeora aún más el estado inflamatorio provocando así un daño grave de los tejidos (Eiro N. et al. 2022).

En la última década se han investigado numerosos parámetros indicadores de inflamación en la EIC para poder utilizarlos tanto en el diagnóstico inicial como en la monitorización de la enfermedad. Estos biomarcadores son la PCR, la calprotectina fecal, la proteína de unión al calcio S100A12, la N-metilhistamina, la 3-bromotirosina y el receptor soluble de los productos finales de glicación avanzada (Jergens A.E. & Heilmann R.M. et al. 2022).

2.1. MARCADORES INFLAMATORIOS DERIVADOS DE LA HEMATOLOGÍA

Ante la necesidad existente tanto en medicina humana como veterinaria de buscar nuevos marcadores pronósticos en pacientes con EIC, se están estudiando en la actualidad biomarcadores que evalúan el estado inflamatorio del paciente, como son la ratio neutrófilos/linfocitos (NLR), la ratio plaquetas/linfocitos (PLR) y el índice de inmunidad-inflamación sistémica (SII). La utilidad de NLR, PLR y SII como biomarcadores de inflamación sistémica ha sido demostrada. En medicina humana estas ratios se han desarrollado como predictores de morbilidad y mortalidad en multitud de patologías, sobre todo, en pacientes críticos, en pacientes con diferentes tipos de tumores y con problemas cardiacos. Además, se han investigado de igual manera en enfermedades infecciosas como la COVID-19 (Buonacera A. et al. 2022; Simadibrata D.M. et al. 2022; Ye Z. et al. 2022).

Estos novedosos biomarcadores tienen las características de ser muy asequibles en los laboratorios y económicos, ya que se obtienen a través de las hematologías realizadas en la sangre entera que se extrae de forma rutinaria a pacientes con EIC. A pesar del alto número de estudios en los que se han utilizado estas ratios, aún no se ha definido un valor de corte definitivo para ellos (Langley B.O. et al. 2021; Simadibrata D.M. et al. 2022).

2.2. RATIO NEUTRÓFILOS/LINFOCITOS

La ratio neutrófilos-linfocitos se calcula utilizando el cociente entre el recuento de neutrófilos y los linfocitos en un análisis de sangre periférica. Este marcador aúna las dos partes del sistema inmunitario, la respuesta inmune innata gracias a los neutrófilos y la respuesta inmune adaptativa gracias a los linfocitos (Faria S.S. et al. 2016).

Los neutrófilos se liberan como primera línea de defensa del organismo frente a los patógenos gracias a múltiples mecanismos, además, al ser los reguladores de la respuesta inmune innata, reclutan, activan y programan otras células del sistema inmune secretando sustancias proinflamatorias e inmunomoduladoras. Por otro lado, los linfocitos proporcionan una respuesta específica frente a los antígenos regulada por los complejos de histocompatibilidad de clase 1, siendo, por tanto, los responsables de la inmunidad adaptativa (Larosa D.F. & Orange J.S. 2008; Mortaz E. et al. 2018).

En una revisión sistemática realizada sobre la determinación de NLR en pacientes humanos con IBD, la ratio ha demostrado ser un biomarcador muy prometedor, pudiéndose utilizar para la diferenciación clínica y endoscópica de la actividad propia de la enfermedad, además de predecir la respuesta a tratamientos, como, por ejemplo, al infliximab (Langley B.O. et al. 2021).

En medicina veterinaria se ha demostrado que la NLR puede ser un marcador pronóstico prometedor en la EIC canina, ya que se ha demostrado que está significativamente elevada en perros con EIC comparado con perros sanos (Benvenuti E. et al. 2020). La ratio presenta una correlación negativa con las proteínas totales, la albúmina y el colesterol, y una correlación positiva con el CCECAI (del inglés Canine Chronic Enteropathy Clinical Activity Index). También se ha demostrado que la ratio puede determinar la severidad de la enfermedad, ya que los pacientes que no responden a

los tratamientos pautados presentan una NLR más elevada que los que sí responden (Becher A. et al. 2021; Benvenuti E. et al. 2020).

2.3. RATIO PLAQUETAS/LINFOCITOS

La ratio plaquetas/linfocitos se obtiene tras la división del número de plaquetas entre linfocitos, valores obtenidos del análisis hematológico de sangre periférica.

Las plaquetas liberan partículas inflamatorias bioactivas y expresan numerosos receptores inflamatorios en condiciones inflamatorias crónicas, como es el caso de la EIC, por lo que su incremento es indicativo de una inflamación activa. Se ha descrito que en pacientes con EIC se produce una alteración en el número, forma y función de las plaquetas, dando lugar a un estado de activación plaquetaria (Voudoukis E. et al. 2014).

Por otro lado, en pacientes con esta patología, se ha observado un aumento de la apoptosis de linfocitos en la sangre periférica, disminuyendo por tanto el contaje total de este tipo de células. Además, el nivel de leucocitos depende en gran medida del estrés fisiológico y está inversamente relacionado con la inflamación (El-Hodhod M.A. et al. 2013).

La PLR se ha propuesto como un biomarcador no invasivo de la EIC, al encontrarse más aumentada en pacientes con CU que en pacientes en remisión de la enfermedad (Feng W. et al. 2022). Además, se ha demostrado que esta ratio es muy precisa a la hora de identificar pacientes en remisión endoscópica, pudiendo valorar así la gravedad de la enfermedad y la respuesta terapéutica (Chen R. et al. 2020).

Sin embargo, en medicina veterinaria únicamente existe un estudio desarrollado por Pierini A. y colaboradores en el año 2021, en el que se comparó la PLR entre los perros con EIC que respondían y los que no respondían a tratamientos inmunosupresores. No observaron diferencias entre ambos grupos, sin embargo, se correlacionó positivamente con las proteínas totales, la albúmina y el CCECAI, demostrando que podría ser un marcador de la actividad clínica en este tipo de pacientes (Pierini A. et al. 2021).

2.4. ÍNDICE DE INMUNIDAD-INFLAMACIÓN SISTÉMICA

El índice de inmunidad-inflamación sistémica se obtiene gracias al recuento de neutrófilos, linfocitos y plaquetas. En este caso se calcula mediante la fórmula:

$$SII = Nº\ plaquetas\ x \frac{Nº\ neutrófilos}{Nº\ linfocitos}$$

El SII proporciona una relación entre la inflamación que se genera en el organismo y el estado inmunitario del mismo. Su incremento es indicativo de una elevación en los neutrófilos y plaquetas y una disminución en los linfocitos, por lo que refleja una mayor respuesta inflamatoria y una inmunidad del individuo más débil (Hu B. et al. 2014; Zhang M. et al. 2021).

Este biomarcador, al igual que los anteriores, se ha encontrado más aumentado en pacientes con CU activa que los pacientes en remisión, proponiéndose de igual manera como un marcador prometedor de la EIC (Pakoz Z.B. et al. 2022; Xie Y. et al. 2021). En otro estudio en pacientes con CU se ha demostrado que el SII se puede utilizar para monitorizar la gravedad de la enfermedad y para ajustar el tratamiento en aquellos pacientes en los que aún no se haya realizado una colonoscopia (Zhang M. et al. 2021).

Sin embargo, a pesar de haber sido estudiado en numerosas investigaciones en medicina humana, aún no se ha estudiado ampliamente en la especie canina, siendo analizado en pacientes con melanoma oral tratados con inmunoterapia y quimioterapia metronómica (García J.S. et al. 2022). También se ha utilizado para comparar el grado de inflamación que se puede producir tras la realización de una ovariectomía laparoscópica y convencional (Espadas-González L. et al. 2023). Sin embargo, este biomarcador no se ha estudiado aún en perros con EIC.

3. ESTRÉS OXIDATIVO EN LA ENTEROPATÍA INFLAMATORIA CRÓNICA

3.1. DEFINICIÓN

Se ha demostrado en la actualidad que el estrés oxidativo juega un papel importante en la aparición de la EIC y en su desarrollo (Guan G. & Lan S. 2018). El estrés oxidativo se produce debido a la producción excesiva de radicales libres oxidativos o debido a la falta de moléculas antioxidantes, es decir, es un desajuste en el balance redox, lo cual da lugar al envejecimiento celular (Halliwell B. 1997). La producción de radicales libres (RL) se produce de forma endógena a partir de reacciones metabólicas normales, o bien, de forma exógena secundario a la exposición a contaminantes, fármacos, radiaciones, etc. Este daño producido por los RL da lugar al desarrollo de múltiples enfermedades crónicas (Dröge W. 2002).

En la actualidad se está investigando su implicación en numerosas enfermedades, sobre todo en aquellas que presentan un componente inflamatorio, como es el caso de la EIC (Guan G & Lan S. 2018).

En esta patología, tal y como se ha comentado anteriormente, se produce una respuesta inmune exacerbada, la cual da lugar a un estado inflamatorio de larga duración y a un daño en la mucosa digestiva, causando a su vez una mala perfusión en los tejidos. Este conjunto de alteraciones da lugar a una producción excesiva de especies reactivas de oxígeno y nitrógeno (ROS/RNS). El aumento de ROS produce cambios en las estructuras de los lípidos, proteínas, carbohidratos y ácidos desoxirribonucleicos en los tejidos, generando así una lesión celular, provocando, en definitiva, un daño oxidativo. La generación de este estado de estrés oxidativo, en definitiva, contribuye al inicio y a la progresión de la enfermedad (Yuksel M. et al. 2017).

Debido a la constante formación de ROS, los organismos generan sustancias antioxidantes para contrarrestar los efectos negativos del daño oxidativo. Estas sustancias antioxidantes protegen contra la producción excesiva de radicales libres e impiden las reacciones perjudiciales contra las estructuras biológicas. Además, también pueden transportar o reparar los constituyentes de las células que se han dañado debido al exceso de radicales libres (Dröge W. 2002).

Por todas estas características, se han evaluado determinados antioxidantes y compuestos oxidantes como posibles biomarcadores de la EIC tanto en medicina humana como en medicina veterinaria. En un estudio reciente en medicina veterinaria se han evaluado la capacidad antioxidante reductora cúprica (CUPRAC, del inglés Cupric Reducing Antioxidant Capacity), la capacidad antioxidante equivalente a Trolox (TEAC, del inglés Trolox Equivalent Antioxidant Capacity) y la paraoxonasa 1 (PON1) como marcadores antioxidantes. Por otro lado, se han determinado como biomarcadores favorecedores del estado oxidativo, FOX (ferrous oxidation-xylenol organe), TBARS (Thiobarbituric Acid Reactive Substantes) y ROS. En dicha investigación se observó en los perros con EIC una disminución de los marcadores antioxidantes y un aumento de los oxidantes. Estos resultados apoyan la hipótesis de la implicación del estrés oxidativo en la patogénesis de la EIC (Rubio C.P. et al. 2017).

En otro estudio en el que evaluaron la microbiota fecal y el perfil de metabolitos séricos en perros con EIC comparándolos con perros sanos, sugirieron la presencia de mayor estrés oxidativo en perros con esta patología. En el análisis determinaron un aumento en la actividad de la ruta de las pentosas fosfato en perros con EIC, que es una vía importante para la reducción del glutatión, por lo que juega un papel importante en la protección frente al estrés oxidativo (Minamoto Y. et al. 2015).

Además de los marcadores descritos anteriormente, se han estudiado otros parámetros de estrés oxidativo en tejidos y fluidos en medicina humana, como el malondialdehído (MDA), el glutatión reducido (GSH) y la albúmina (Rubio C.P. et al. 2017; Anderson M.E. 1998; Sitar M.E. 2013).

3.2. MALONDIALDEHÍDO

El MDA es un producto clave en la peroxidación de los lípidos que, al unirse a macromoléculas, altera y elimina su función. Se ha demostrado en las últimas décadas que la peroxidación lipídica juega un papel muy importante en la biología celular y salud humana, siendo, por tanto, muy importante en el daño celular y el estrés oxidativo (Ayala A. et al. 2014).

Este agente es altamente citotóxico y mutagénico, de ahí su utilidad como marcador de estrés oxidativo en múltiples patologías de medicina humana como el carcinoma, pacientes con periodontitis, etc. (Cherian D.A. et al. 2019; Maurya R.P. et al. 2021). También se ha evaluado en humanos con EC, encontrándose aumentado con respecto a pacientes sanos (Alzoghaibi M.A. et al. 2007; Boehm D. et al. 2012). Se ha demostrado además un aumento de este biomarcador tanto en el suero como en la saliva de pacientes que presentan la EC activa en comparación con aquellos que presentan la enfermedad inactiva y con pacientes sanos. Además, se correlacionó de forma positiva con el índice de actividad clínica e inflamación producida en la enfermedad de Crohn (Szczeklik K. et al. 2018).

En medicina veterinaria, el MDA ha sido estudiado en perros con cataratas asociadas a la edad, con dermatitis atópica, con varios tipos de cáncer (como carcinomas mamarios, mastocitomas y osteosarcomas, entre otros), en pacientes con erhlichiosis, parvovirosis y en perros con fallo cardiaco congestivo. En la especie felina se ha analizado en pacientes con enfermedad renal crónica y en gatos infectados con calicivirus y coronavirus felino (Kayar A. et al. 2015; Valle E. et al. 2019; Tabrizi B.A. et al. 2021). Todos los pacientes con estas patologías presentaban los valores de MDA incrementados con respecto a perros y gatos sanos, demostrando así la importancia del estrés oxidativo en diversos estados patológicos (Madany J. 2016; Macotpet A. et al. 2013; Kapun A.P. et al. 2012; Gaykwad C. et al. 2018; Çiftci G. et al. 2021; Nemec Svete A. et al. 2021). Sin embargo, este parámetro no ha sido estudiado anteriormente en perros con EIC.

3.3. GLUTATION REDUCIDO

El GSH actúa como un antioxidante endógeno, participa en procesos importantes como la homeostasis celular y cumple funciones esenciales actuando frente al daño oxidativo que se produce en el organismo. La principal función antioxidante del GSH es la reacción frente a los intermediarios reactivos del nitrógeno, actuando así frente al estrés oxidativo al que se enfrentan las células a niveles fisiológicos. Tiene efectos detoxificadores contra endobióticos malignos y xenobióticos. Los efectos antioxidantes del GSH desempeñan un papel esencial en la regulación de la proliferación y muerte celular a través de la principal vía de señalización reguladora redox en la célula. Por tanto, la

concentración de GSH es un buen indicador de funcionalidad y viabilidad celular y se ha asociado a múltiples procesos patológicos (Denzoin Vulcano, L.A. et al. 2013).

Este marcador ha sido utilizado en medicina humana en múltiples patologías, entre las que cabe destacar el cáncer, la diabetes, artritis reumatoide, cataratas y enfermedad de Párkinson, entre otras (Pastore A. et al. 2003). Además, se ha observado una disminución de los valores de GSH en el suero y la saliva de pacientes con EC activa, comparándolo con aquellos que presentan la enfermedad inactiva y con pacientes sanos (Szczeklik K. et al. 2018).

En medicina veterinaria se ha observado también una disminución en gatos con inmunodeficiencia felina, enfermedad renal crónica y enfermedad hepática y en perros con enfermedad renal, neoplasias, enfermedades cardiovasculares, trastornos endocrinos, neurológicos y micosis sistémica (Denzoin Vulcano, L. A. et al. 2013).

3.4. ALBÚMINA

La albúmina, proteína de fase aguda y utilizada rutinariamente como indicador de las reservas proteicas, en general, del estado nutricional, se considera además una importante molécula antioxidante extracelular (de Castro L.L. et al. 2014). Es la proteína más abundante en el cuerpo humano, representando entre el 50 y 60% de las proteínas plasmáticas totales del organismo. Además, la concentración de albumina es mayor que la de otras moléculas antioxidantes, por lo que es la proteína principal que mayor capacidad antioxidante presenta (Bonifazi M. et al. 2021).

Las propiedades antioxidantes de la albúmina se deben a su estructura bioquímica única. Entre estas propiedades se encuentran una unión fuerte al cobre y débil al hierro, eliminación de radicales libres y la provisión del grupo tiol (Sitar M.E. et al. 2013).

En pacientes con enfermedades hepáticas, nefropatía diabética, sepsis o COVID-19, se ha demostrado que, a medida que se oxidan las moléculas de albúmina por los radicales libres, van disminuyendo sus capacidades antioxidantes. Por tanto, multitud de investigadores actualmente están demostrando que la albúmina oxidada puede ser un buen biomarcador de estrés oxidativo y, además, un factor agravante en multitud de enfermedades (Tabata F. et al. 2021).

En medicina humana se ha estudiado junto con otros biomarcadores antioxidantes en pacientes con EC (Su Q. et al. 2019). En medicina veterinaria, de igual manera, se ha investigado la alteración de la concentración de albúmina en pacientes con EIC (Allenspach K. et al. 2007), tal y como se describe en el capítulo 1.2.

4. MONITORIZACIÓN Y PRONÓSTICO

Uno de los métodos utilizados para monitorizar la enfermedad y determinar su pronóstico es la utilización de dos sistemas de puntuación mediante los cuales se evalúa de forma cuantitativa la gravedad de la EIC. Estos dos sistemas de puntuación son el índice clínico CIBDAI (del inglés Canine Inflammatory Bowel Disease Activity Index) y el índice clínico CCECAI. El primero de ellos evalúa las variables clínicas "actitud y actividad", "vómitos", "consistencia de las heces", "frecuencia de las heces" y "pérdida de peso". El segundo de ellos, además de las que ya se han mencionado, también evalúa "concentración de albúmina", "presencia de ascitis y/o edemas periféricos" y "prurito". Cada una de las variables se puntúa de 0 a 3 y en base a la puntuación final, la enfermedad se clasifica de la siguiente forma: clínicamente insignificante (de 0 a 3), leve (de 4 a 5), moderada (de 6 a 8) y grave (superior a 9). Si el CCECAI es superior a 12, se clasifica como muy grave (Jergens A.E. et al. 2003; Allenspach K. et al. 2007).

El pronóstico de los pacientes con EIC de forma general es pobre. Se ha descrito que hasta un 50 - 60% de los animales presentan FRE, suelen ser jóvenes y tener síntomas clínicos más leves, por lo que presentan un mejor pronóstico. Por el contrario, entre un 15 y 43% de los casos no responden a cambios de dieta ni a tratamientos farmacológicos, por lo que son clasificados como NRE, presentando así un peor pronóstico a largo plazo y, por tanto, una alta tasa de eutanasia (Craven M. et al. 2004; Allenspach K. et al. 2007).

Actualmente, cada vez se realizan más estudios en los que se valoran diferentes marcadores para poder monitorizar la enfermedad y predecir mejor un pronóstico. Los principales parámetros laboratoriales que evalúan la gravedad de los pacientes con EIC son la albúmina, la vitamina B12 (cobalamina), la vitamina D, la vitamina B9 (folato) y el colesterol (Allenspach K. et al. 2007; Titmarsh H. et al. 2015; Wennogle S.A. et al. 2019; Ullal T.V. et al. 2023). Se ha descrito que la cobalamina y la albúmina presentan una correlación muy fuerte, siendo los bajos niveles de ambos parámetros indicativos de un

pronóstico negativo, por lo que sería ideal su determinación conjunta en todos los perros con EIC (Allenspach K. et al. 2007).

Se ha llegado a la conclusión que un alto índice clínico (CCECAI), niveles bajos de cobalamina y albúmina, niveles altos de proteína C reactiva, lipasa pancreática específica canina y ratio neutrófilos/linfocitos, se asocian a pobres respuestas a los tratamientos y, por tanto, a un peor pronóstico. Estos parámetros pueden utilizarse, por tanto, para monitorizar la gravedad de la enfermedad y la respuesta a los diferentes tratamientos utilizados (Allenspach, K et al. 2007; Benvenuti E. et al. 2020).

Otro parámetro que se ha usado para determinar la progresión de la enfermedad y la respuesta a los tratamientos es la PCR, aunque su concentración debe variar hasta 2,7 veces para considerar que se trata de un cambio relevante (Carney P.C. et al. 2011). Por último, la calprotectina fecal también predice la respuesta a los tratamientos en perros con EIC, diferenciando aquellos pacientes que responden o no responden de los perros con IRE (Heilmann R.M. et al. 2018).

Debido a la sintomatología prolongada y recurrente que presentan los perros con EIC, y a la dificultad que existe en el diagnóstico y tratamiento, la calidad de vida de los perros se ve afectada en gran medida. Recientemente se ha demostrado que esta enfermedad afecta al comportamiento del perro, a su calidad de vida y a la relación que existe entre propietario y animal. Debido al estado general del paciente existe un aumento del apego entre el dueño y el perro, además, estos necesitan salir un mayor número de veces de paseo, requieren más cuidados y, al cambiar el comportamiento, los perros suelen buscar más a los propietarios (Marchetti V. et al. 2021). Estos autores han evaluado la calidad de vida de los pacientes atendiendo a los aspectos positivos y negativos de la vida del animal, siendo, por tanto, un aspecto relevante a la hora de optar por la eutanasia o por añadir otros tratamientos adicionales. Por todo ello, la búsqueda de nuevas terapias en perros con esta patología es importante para los investigadores, ya que en los casos que no responden a las sucesivas terapias y se ve afectada la calidad de vida del animal, finalmente los propietarios optan por la eutanasia humanitaria.

5. TRATAMIENTO CONVENCIONAL DE LA ENTEROPATÍA INFLAMATORIA CRÓNICA

El tratamiento de la EIC se basa en una combinación de cambios dietéticos y terapias farmacológicas, además de un suplemento de ácido fólico y cobalamina en el caso de que estos parámetros se encontrasen disminuidos (Simpson K.W.A. & Jergens A.E. 2011).

En las fases iniciales se realizan cambios dietéticos mediante la utilización de proteína hidrolizada o con dietas de eliminación (nuevas fuentes de proteínas o carbohidratos), a los cuales responden la mayoría de los pacientes dentro de las 2 a 4 semanas tras el inicio del cambio de dieta. Es ideal realizar como mínimo dos cambios de dietas diferentes. El siguiente paso es la utilización de antibióticos (como el metronidazol y la tilosina) e inmunosupresores (como la prednisona, azatioprina, ciclosporina o budesonida). Sin embargo, no se ha establecido un protocolo concreto a la hora de la utilización de estos fármacos y hay poca información con respecto a la respuesta a largo plazo a esta terapia farmacológica (Allenspach K. et al. 2016). Además, se ha demostrado que los antibióticos provocan un efecto negativo en la microbiota intestinal, dando lugar a una disbiosis intestinal.

En una reciente revisión llevaba a cabo por expertos en gastroenterología veterinaria, determinan el uso de antibióticos únicamente en aquellos pacientes que presenten una infección documentada en las muestras obtenidas a través de las biopsias endoscópicas. Estos pacientes previamente no han respondido a los cambios de dieta ni a la pauta de tratamiento con probióticos, prebióticos y fármacos inmunomoduladores (Cerquetella et al. 2020).

Asimismo, están descritos los abundantes efectos negativos que tienen los inmunosupresores sobre el organismo en general. Por todo ello, se están estudiando en la actualidad nuevos protocolos y tratamientos alternativos más eficaces y seguros para estabilizar a los pacientes con EIC (Isidori M. et al. 2022).

Uno de estos tratamientos alternativos, es la utilización de probióticos como terapia de apoyo en este tipo de pacientes. En numerosas investigaciones se han obtenido resultados muy controvertidos. Todo ello debido a la diferente población objeto de

estudio, el entorno experimental y las diferentes cepas bacterianas utilizadas. En todo caso, se ha demostrado que esta terapia no presenta efectos perjudiciales en los pacientes tratados (Isidori M. et al. 2022).

Otro de los tratamientos estudiados en la actualidad es el trasplante de microbiota fecal (TMF). En medicina humana se ha investigado en enfermos de Crohn y colitis ulcerosa con el fin de tratar la disbiosis intestinal que contribuye a la patogénesis de esta patología (Paramsothy S et al. 2017). En medicina veterinaria se han desarrollado estudios clínicos, observando una mejoría clínica y una normalización del microbioma tras el TMF, siendo necesarios más estudios para definir la metodología y el protocolo de administración (Murphy T. et al. 2014; Berlanda M. et al. 2021). En otro de los estudios se ha observado igualmente una mejoría clínica, sin embargo, no existían diferencias con respecto al grupo placebo utilizado en el ensayo clínico (Collier A.J. et al. 2021). Recientemente se ha llevado a cabo un ensayo clínico en 41 perros con EIC, en el cuál se ha demostrado que mejora la disbiosis intestinal en perros con esta patología (Toresson L. et al. 2023).

Una de las terapias más actuales y prometedoras es el uso de las células madre mesenquimales (MSCs del inglés mesenchymal stem cells). El uso de esta terapia en trastornos gastrointestinales que presentan un componente inmunitario se ha estudiado tanto en medicina humana (Tsuchiya A. et al. 2017; Zhang X. et al. 2020), como en medicina veterinaria (Webb T.L. & Webb B.C. 2015; Pérez-Merino E.M. et al. 2015(a); Pérez-Merino E.M. et al. 2015(b); Webb T.L. & Webb B.C. 2022), demostrando su eficacia y seguridad tras la aplicación en los pacientes. Sin embargo, aún queda mucho por investigar sobre esta terapia, ya que, aunque en medicina humana el número de estudios es más elevado, son escasos en medicina veterinaria.

6. APLICACIÓN DE CÉLULAS MADRE MESENQUIMALES EN LA ENTEROPATÍA INFLAMATORIA CRÓNICA

7.1. CARACTERÍSTICAS DE LAS CÉLULAS MADRE

Las células madre mesenquimales (CMM o MSCs del inglés Mesenchymal Stem Cells) o también llamadas células estromales mesenquimales son células progenitoras

multipotentes que se pueden encontrar en casi todos los tejidos corporales, aunque las principales fuentes de obtención son el tejido adiposo, la médula ósea y los tejidos asociados al nacimiento (cordón umbilical, placenta y amnios) (Hass R. et al. 2011). Las MSCs se pueden diferenciar en múltiples líneas celulares como son los adipocitos, osteoblastos, hepatocitos, fibroblastos, miocardiocitos y condrocitos (Pittenger M.F. et al. 1999).

Inicialmente se utilizaban las MSCs debido a sus propiedades regenerativas, sin embargo, el concepto de la utilidad de esta terapia está cambiando gracias al conocimiento de sus propiedades antiinflamatorias y, más recientemente aún, a sus propiedades angiogénicas, antifibróticas, antitumorales, antimicrobianas y antioxidantes (Eiro N. et al. 2022) (Figura 2).

Tal y como hemos comentado, este tipo de células presentan propiedades inmunomoduladoras, inhibiendo la proliferación de las células B y las células Th1 y Th17, suprimen la función de las células Natural Killer (NK), la maduración y activación de las células dendríticas (CD) y promueven la diferenciación de los Treg. Mediante estos mecanismos regulan la secreción de citoquinas y las respuestas inmunitarias. Además, tienen como propiedades la localización en tejidos dañados e inflamados (Bernardo M.E. & Fibbe W.E. 2013; Tavakoli S. et al. 2020).

También presentan efectos regenerativos gracias a la secreción de moléculas biológicas como citoquinas, factores de crecimiento y lípidos, capaces de estimular la supervivencia y la recuperación de tejidos y células dañadas. Se ha sugerido incluso que promueven la angiogénesis, ya que, para poder recuperar tejidos dañados, es necesario formar nuevos vasos sanguíneos para poder suministrar nutrientes y oxígeno (Park S.R. et al. 2018).

Se ha descrito de igual manera que las MSCs y el secretoma de las MSCs presentan efectos antifibróticos, lo cual es importante y muy beneficioso, ya que la mayoría de las enfermedades crónicas presentan un estado profibrótico, debido a la presencia de estrés oxidativo e inflamación (Choi A. et al. 2020).

Los efectos antiapoptóticos y antitumorales de las MSCs son de gran interés en los estudios que se realizan recientemente. Sin embargo, es necesario evaluar de forma más

profunda la capacidad oncogénica de las MSCs para mejorar su aplicación clínica. El efecto pro o antitumoral de esta terapia depende en gran medida del origen de las MSCs y del tipo del tumor frente al que se utilizan (Lin W. et al. 2019; Fernández-Francos S. et al. 2021).

Se ha demostrado igualmente que el tratamiento con MSCs presenta unos efectos beneficiosos frente al estrés oxidativo, siendo este un mecanismo fisiopatológico de gran importancia en numerosas patologías, incluida la EIC. Todos los estudios sobre el efecto de las MSCs en el estrés oxidativo sugieren que estas generan una inmunomodulación para prevenir el daño oxidativo. Sobre este aspecto se hablará más adelante (Apartado 7.4) (Stavely R. et al. 2020).

Por último, también se han descrito efectos antimicrobianos de las MSCs. Este tipo de células pueden liberar péptidos antimicrobianos (PAM), los cuáles causan la destrucción celular de los microorganismos. Esto ocurre gracias a la acción de estos PAM contra la integridad de la membrana celular, contra algunos componentes intracelulares específicos y, también, inhibiendo la síntesis de ADN y ARN (Alcayaga-Miranda. et al. 2017).

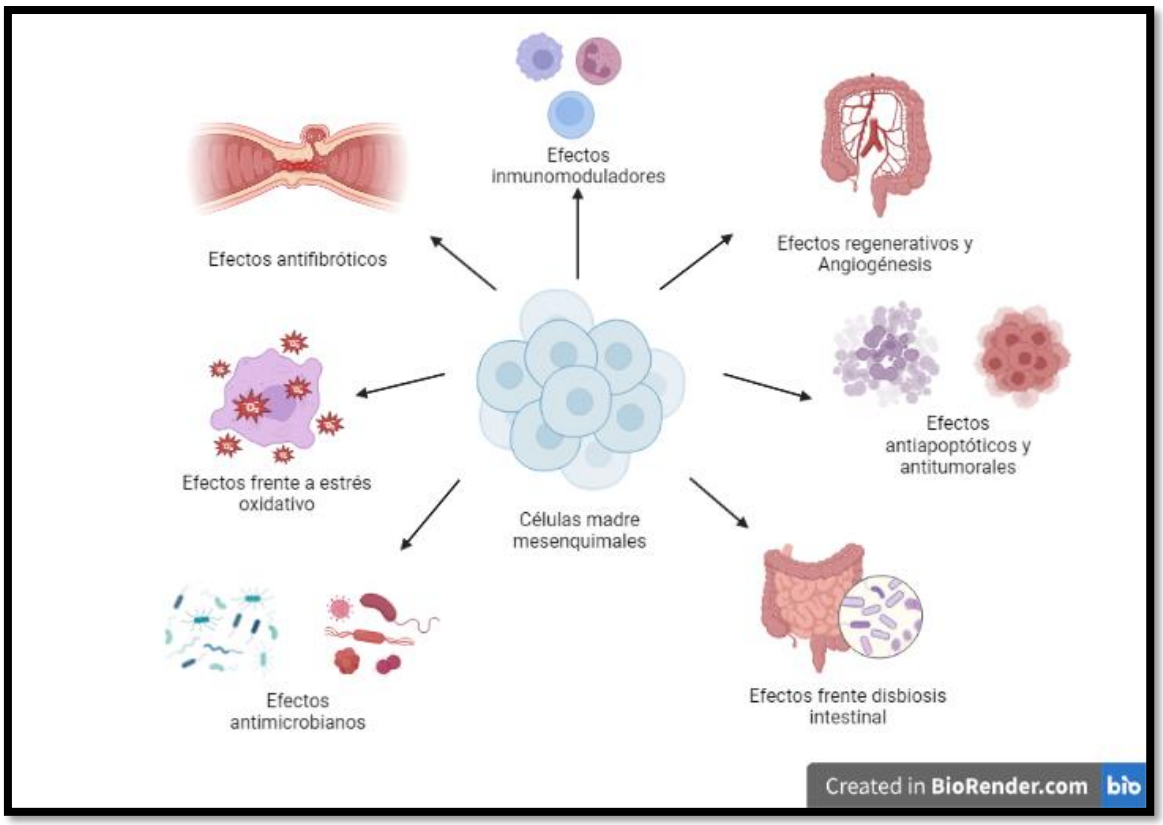

Figura 2. Propiedades de las células madre mesenquimales.

Además, el tratamiento con MSCs está ganando importancia de igual manera debido a los efectos de restauración de la microbiota intestinal. La disbiosis, caracterizada por una composición bacteriana desequilibrada y una biodiversidad restringida, es uno de los factores desencadenantes que da lugar a la EIC, por lo que actuar frente a ello es de vital importancia en estos pacientes (Ocansey D.K.W. et al. 2019). Se ha demostrado que las MSCs promueven la normalización de la microbiota en un estudio con ratones (He R. et al. 2021).

Debido a sus capacidades regenerativas e inmunomoduladoras, a su capacidad de evadir el sistema inmune el huésped, y a su abundancia en el tejido adiposo y médula ósea de los adultos, esta terapia se ha utilizado en medicina humana para tratar multitud de enfermedades. Se han realizado estudios valorando su aplicación en la diabetes, en enfermedades hepáticas, pulmonares, cardiovasculares, renales, musculoesqueléticas y gastrointestinales. También destacan algunas de origen inmunomediado, como lo son el IBD, la artritis reumatoide, el lupus eritematoso sistémico y la esclerosis sistémica (Tyndall A. & van Laar J.M. 2016; Squillaro T. et al. 2016).

6.2. TERAPIA CON CÉLULAS MADRE EN LA ENTEROPATÍA INFLAMATORIA INTESTINAL

La aplicación de las MSCs en medicina humana y medicina veterinaria se ha llevado a cabo en multitud de patologías, utilizándose cada vez con mayor frecuencia en numerosas de enfermedades. Además, en la mayoría de las investigaciones realizadas no se han observado efectos secundarios que perjudiquen a los pacientes a los que se les ha administrado esta terapia (Hoffman A.M & Dow SW. 2016; Dias, I.E. et al. 2019; Fernández-Francos S. et al. 2021).

En medicina humana se han publicado múltiples estudios en pacientes con EC fistulizante (perianal), en la EC luminal y en la CU utilizando la administración local de MSCs en la forma fistulizante y la inyección intravenosa en la forma intraluminal de la EC y en la CU (Adak S. et al. 2017; Zhang X. et al. 2020). En ellos, se han utilizado más frecuentemente células alogénicas (o autólogas), ya que tienen la ventaja de expandirse rápidamente y en masa para alcanzar una alta concentración antes de su administración, derivadas del tejido adiposo, médula ósea, endometrio, cordón umbilical, amnios y

amígdala, administrándose de forma intravenosa, intraperitoneal, mesentérica o de forma local. En la mayoría de las ocasiones las MSCs se están empleando en aquellos pacientes que no responden a otros tratamientos, es decir, aquellos que presentan una enfermedad refractaria (Eiro N. et al. 2022).

La mayoría de estos estudios reportan resultados prometedores, observando la mejora en la sintomatología en pacientes con EC luminal y promueve la curación de las fistulas perianales en la EC fistulizante (Adak S. et al. 2017; Zhang X. et al. 2020). Todos ellos han demostrado que esta terapia celular mejora la consistencia de las heces, el sangrado, el peso corporal, la tasa de mortalidad, la inflamación del colon y la expresión de citoquinas, entre otras características.

También se ha observado que los factores bioactivos de las MSCs que se administran de forma exógena suprimen la activación excesiva del sistema inmune que se produce en pacientes con EIC y, además, estimulan a las células madre resistentes específicas del tejido del paciente construyendo nuevos tejidos (Caplan A.I. 2017).

En lo referente a la medicina veterinaria, un estudio centrado en gatos con IBD, en los que se aplicó la terapia con MSCs de forma intravenosa, observó una mejoría significativa en 5 de los 7 gatos tratados, sin efectos adversos en ninguno de los animales (Webb T.L. & Webb C.B. 2015). Estos mismos investigadores han realizado un estudio reciente en el que administraron las MSCs a 12 gatos con EIC. De forma aleatoria seis de ellos recibieron MSCs alogénicas y los otros seis únicamente la terapia convencional con prednisolona, observándose unos resultados similares con ambos tratamientos, sin presentar efectos secundarios ninguno de los animales a los que se les administró MSCs (Webb T.L. & Webb C.B. 2022).

El primer estudio en la especie canina se llevó a cabo en 11 perros con IBD, mostrando una mejoría clínica y laboratorial medida a través de los índices CIBDAI y CCECAI y las concentraciones de albúmina, folato y cobalamina. Dicha mejoría se prolongó hasta 42 días después de la infusión intravenosa de MSCs en 9 de los 11 animales tratados (Pérez-Merino E.M. et al. 2015(a); Pérez-Merino E.M. et al. 2015(b)). En ambas investigaciones los autores concluyeron que la infusión intravenosa de células madre mesenquimales alogénicas obtenidas de tejido adiposo (Ad-MSCs) eran bien aceptadas y eran eficaces en el tratamiento de perros y gatos con IBD.

Estos trabajos en pequeños animales han demostrado la eficacia y seguridad de esta terapia, sin embargo, en los pacientes caninos del estudio realizado por Pérez-Merino y colaboradores en el año 2015, eliminaron otros tratamientos concomitantes para que no afectara al desarrollo de los ensayos clínicos. Por el contrario, en medicina humana, una vez demostrada la eficacia y seguridad de la aplicación de MSCs, se han realizado estudios con fármacos inmunosupresores de forma concomitante, pudiendo modificar la dosis de estos tras la administración de la terapia celular (Liang J. et al. 2012; Zhang J. et al. 2018).

6.3. CÉLULAS MADRE E INFLAMACIÓN

Las MSCs presentan un efecto antiinflamatorio sobre numerosas células del sistema inmune. Como hemos comentado anteriormente, en esta enfermedad se ven implicadas las principales citoquinas inflamatorias relacionadas con las células Th1 y Th17, siendo las más importantes la IL-12, IL-17, IFN-γ y TNF-α, entre otras. Las MSCs dan lugar a una disminución de las respuestas Th1 y Th17, inhibiendo así las citoquinas específicas de estas células. Además, da lugar a un aumento de las respuestas Th2 y Treg, que a su vez producen mediadores inmunomediados. Todo ello, por tanto, mejora la inflamación que se produce en los órganos del sistema digestivo. Estas MSCs, además, generan IL-10, una citoquina inmunoreguladora que contribuye a mejorar la homeostasis intestinal. Además, las MSCs expresan NOD2 (dominio de oligomerización por unión de nucleótidos que contiene la proteína 2), dando lugar a la secreción de factores antiinflamatorios como PEG2 e IL-10, estimulando la producción de Treg (Eiro N. et al. 2022). Esta terapia celular también modifica la respuesta inmunológica mediante la maduración de las células dendríticas y regula la inflamación gracias a la inducción del cambio de fenotipo de los macrófagos M1 a M2 (los macrófagos M1 dan lugar a citoquinas inmunoestimuladoras y los M2 tienen propiedades inmunosupresoras) (Figura 3).

Por un lado, las propiedades inmunológicas y antiinflamatorias de las MSCs tanto in vivo, como in vitro se encuentran ampliamente definidas y, además, se ha demostrado que la interacción entre las MSCs y las células del sistema inmunitario favorece la cicatrización y remodelación de los tejidos orgánicos (Regmi S. et al. 2019).

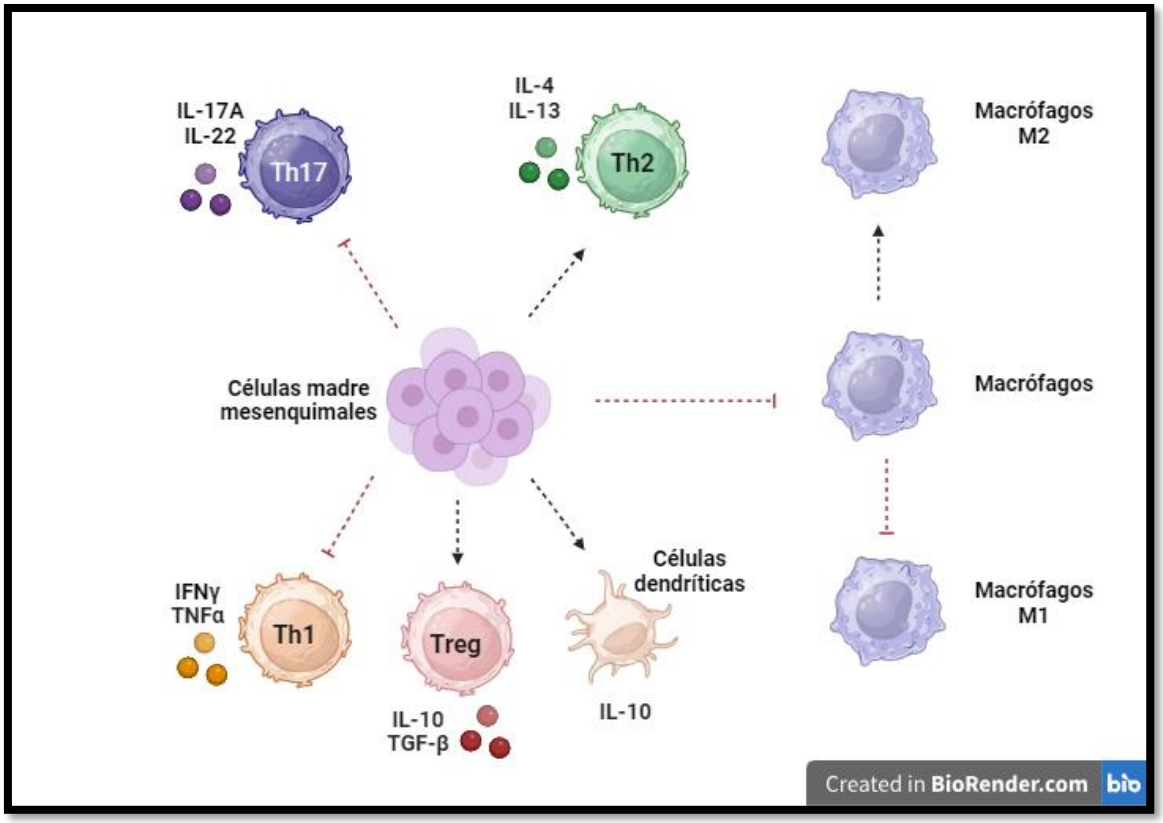

Figura 3. Efectos del tratamiento con células madre mesenquimales sobre las células del sistema inmune

6.4. CÉLULAS MADRE Y ESTRÉS OXIDATIVO

Las propiedades de la terapia con MSCs incluyen efectos antioxidantes directos, como la disminución de los agentes oxidantes eliminando los RL y la donación de mitocondrias sanas a las células dañadas o, efectos indirectos, como el incremento de las defensas antioxidantes en otras células del organismo y la alteración de la bioenergética celular. Además, las propiedades inmunosupresoras de las MSCs impiden la generación de ROS. Todas estas características disminuyen el estrés oxidativo, aportando numerosos beneficios como terapia frente a multitud de patologías (Inan M. et al. 2017; Jung K.J. et al. 2020; Stavely R. et al. 2020; He J. et al. 2021) (Figura 4).

Diferentes estudios llevados a cabo en medicina humana y veterinaria han demostrado que las MSCs presentan propiedades beneficiosas actuando frente al estrés oxidativo en diferentes patologías, como en lesiones isquémicas, lesiones por quimioterapia y radioterapia, lesiones diabéticas, lesiones traumáticas, en el

envejecimiento, trastornos cognitivos, lesiones sépticas y en enfermedades inflamatorias intestinales (Stavely R. et al. 2020).

Sin embargo, aún no está bien definido si los marcadores de estrés oxidativo son útiles para monitorizar la EIC y establecer el pronóstico en estos pacientes. Además, sería conveniente de igual manera, determinar en un futuro si la aplicación terapéutica de antioxidantes se podría utilizar como parte del tratamiento de la enfermedad (Rezaie A. et al. 2007).

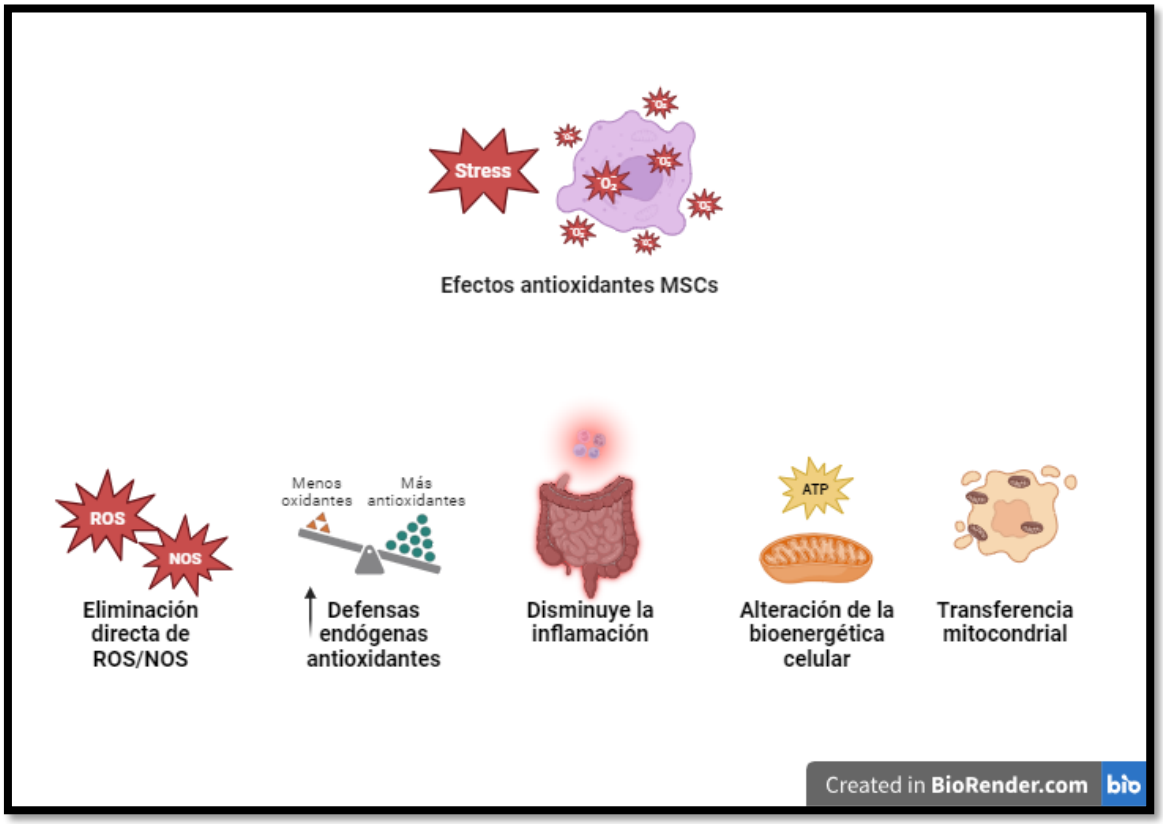

Figura 4. Efectos del tratamiento con células madre mesenquimales sobre el estrés oxidativo.

6.5. LIMITACIONES DEL TRATAMIENTO CON CÉLULAS MADRE

A pesar de los resultados prometedores de esta terapia celular, el equilibrio entre el riesgo y el beneficio de su infusión continúa aun siendo difícil de predecir (Heslop J.A. et al. 2015).

Este tratamiento presenta algunas limitaciones, sobre todo debido al corto periodo de viabilidad de las MSCs tras su administración sistémica, la poca capacidad de diferenciación tras realizar la administración en el paciente y, por último, debido al deterioro que sufren estas células madre con el paso de la edad. Esto hace que se necesiten dosis mayores para alcanzar el efecto terapéutico deseado, lo que conlleva a un mayor riesgo de empeorar las condiciones teratogénicas de esta terapia (Prockop D.J. 2017).

También se han observado algunos efectos tóxicos o rechazo incluso de esta terapia celular. A pesar de existir numerosos estudios satisfactorios en pacientes con EC o CU, también se han publicado algunas investigaciones en las que dicha terapia puede ser perjudicial y empeorar la enfermedad (Markovic B.S. et al. 2018). Se ha descrito que la administración intravenosa puede provocar un atrapamiento en la microvasculatura y en el pulmón, lo que podría desembocar en efectos indeseados como, por ejemplo, tromboembolismos pulmonares e infartos (Jung J.W. et al. 2013).

Todo ello, unido al coste elevado de lo que supone esta terapia celular en lo que respecta al aislamiento, cultivo, diferenciación, expansión y criopreservación, junto con el gran número de células que se requieren para realizar un tratamiento óptimo (Eiro N. et al. 2022).

Por último, otra importante limitación de esta terapia es la escasa estandarización de los protocolos que se utilizan. Esto se debe a que los diferentes estudios sobre la aplicación de las MSCs utilizan distintas pautas referentes a dosis, velocidades de infusión y vías de administración. Además, los resultados también varían en función del tejido del que se extraigan, el/los paciente/s donante/s y los laboratorios que procesan las MSCs. Debido a estos últimos aspectos existe una gran dificultad para la aprobación de fármacos basados en MSCs. Por último, también influyen en ello las grandes diferencias con respecto a la regulación que existen entre países (Grégoire C. et al. 2017; López-Beas J. et al. 2020).

Justificación

La enteropatía inflamatoria crónica es una enfermedad crónica, multifactorial, fruto de complejas interacciones entre el sistema inmune, la dieta y el microbioma intestinal en un huésped genéticamente susceptible. La sintomatología de la enfermedad es principalmente digestiva, con vómitos y diarreas, que presentan un curso crónico, alternando brotes de exacerbación con otros de mejoría (Jergens A.E. and Heilmann R.M. et al. 2022). A largo plazo, el paciente presenta una pérdida de peso, un estado grave de malnutrición y una merma de la calidad de vida que afecta a su comportamiento y también al estado de ánimo de los propietarios (Marchetti V. et al. 2021).

La prevalencia de la enfermedad es desconocida, pero hasta el 20 o 30 % de las visitas al veterinario se relacionan con estos síntomas (Jergens A.E. and Heilmann R.M. et al. 2022). La EIC puede ser una importante causa de morbilidad e incluso de mortalidad en la especie canina (Allenspach K. et al. 2016; Craven M. et al. 2004; Howard B. 2019) y cualquier raza pueda ser afectada (Kathrani A. et al. 2011).

El diagnóstico es largo y complejo, y requiere múltiples pruebas y ensayos terapéuticos, así como la exploración endoscópica del tracto digestivo y del examen histológico de las biopsias adquiridas durante la endoscopia. Los costes y la dificultad del diagnóstico han impulsado la búsqueda de marcadores de la enfermedad, aunque pocos se usan en la práctica clínica diaria (Langlois D.K. et al. 2023). Durante la endoscopia, el íleon suele ser inexplorado por la dificultad para su entubación, sin embargo, se conoce que su afectación conduce a la disminución de la absorción de cobalamina, por lo que su concentración podría servir como marcador del grado de afectación ileal sin necesidad de su exploración.

Si bien hasta el 50 - 60% de los pacientes responden a los cambios de dieta como tratamiento de la enfermedad, el resto requerirán tratamiento de por vida con inmunosupresores como los corticosteroides o la ciclosporina. Además de los efectos secundarios que este tratamiento origina en los pacientes, hasta un 15 - 43% no responden de forma adecuada a estos fármacos, siendo los pacientes que presentan peor pronóstico y tasa de eutanasia más alta (Jergens A.E. and Heilmann R.M. et al. 2022).

Por todo ello, investigar en la fisiopatología, marcadores de diagnóstico, pronostico y tratamiento de la EIC es una necesidad justificada. Estudios previos en pacientes humanos con enfermedad de Crohn (Duijyestein M et al. 2010; Forbes G.M et al. 2014) y

en animales de experimentación en los que se provocaban formas de colitis (Hayashi Y. et al. 2008; Tanaka, F. et al. 2008; Zhang J. et al. 2008; Zhang Q et al. 2009) demostraron la eficacia prometedora del uso de las células madre para la enfermedad inflamatoria intestinal y pavimentaron el camino hacia la primera investigación clínica sobre aplicación de las células madre como tratamiento de la EIC en el perro (Pérez-Merino E.M. et al. 2015(a); Pérez-Merino E.M. et al. 2015(b)). Estos trabajos se centraron en la evolución clínica y laboratorial de los pacientes y en los cambios endoscópicos e histológicos tras el tratamiento. Si bien los resultados clínicos fueron alentadores, los cambios histológicos y endoscópicos no fueron manifiestos y además el seguimiento solo se extendió a los tres meses post administración de las células (Pérez-Merino E.M. et al. 2015(a); Pérez-Merino E.M. et al. 2015(b)). Por todo ello, es patente la necesidad de confirmar la efectividad clínica aumentando el número de pacientes tratados, y el plazo de seguimiento y dar respuesta a otras cuestiones como la posibilidad de administrarlas conjuntamente con el tratamiento convencional con inmunosupresores.

Además, los animales de compañía suponen un modelo más realista para determinar la utilidad de esta terapia celular, ya que las enfermedades que desarrollan de forma natural se pueden asemejar mucho a las condiciones que se dan en los humanos (Arzi B. et al. 2021).

Las células madre han demostrado capacidad de migrar a los lugares de inflamación y de ejercer efectos antiinflamatorios a través de la secreción de ciertos factores (Kang J.W et al. 2008; Matthay M.A. et al. 2010; Song J.I. et al. 2017; Regmi S. et al. 2019). Para evaluar la inflamación, es tendencia el uso de los marcadores derivados de sangre completa, por su accesibilidad y coste, puesto que solo requieren de una extracción de sangre completa y el recuento de neutrófilos, linfocitos y plaquetas (Sacoor C. et al. 2021; Bertani L. et al. 2020; Xie Y. et al. 2021; Zhang M.H. 2021). Por ello, parece adecuado analizar si el tratamiento con células madre provoca algún cambio en el proceso inflamatorio asociado a la EIC medido a través de estos marcadores.

También se ha demostrado que el estrés oxidativo juega un importante papel en la patogénesis del IBD y, por ello, ciertos componentes oxidantes y antioxidantes han sido evaluados como posibles biomarcadores en medicina veterinaria (Yuksel M. et al. 2017; Guan G, Lan S. 2018). Sin embargo, algunos como el malondialdehido o el glutatión reducido no han sido estudiados en la EIC canina y se desconoce si el tratamiento con

células madre tiene efectos sobre estos marcadores. Además, los tratamientos que se estudian en pacientes con EIC podrían enfocarse a eliminar los radicales libres, disminuir las concentraciones de citoquinas y enzimas oxidantes, además de mejorar la capacidad antioxidante celular. Es necesario, por tanto, caracterizar mejor la implicación del estrés oxidativo en la patogénesis y progresión de la EIC de cara al desarrollo de nuevas terapias (Tian T. et al. 2017).

Objetivos

Por todo lo expuesto anteriormente, los objetivos de la presente Tesis Doctoral son los siguientes:

- Evaluar la coadministración de células madre mesenquimales derivadas de tejido adiposo (Ad-MSCs) y prednisona en pacientes diagnosticados de EIC con el fin de reducir la dosis de corticosteroides.

- Comparar los cambios en los marcadores de inflamación derivados del análisis de la hematología en perros diagnosticados de EIC antes y después de la terapia con Ad-MSCs.

- Determinar si la concentración de cobalamina en perros con EIC se relaciona con el grado de afectación endoscópica e histológica del íleon.

- Evaluar la concentración plasmática de los biomarcadores de estrés oxidativo malondialdehido, glutation reducido y albúmina, en perros con EIC tratados con Ad-MSCs (con o sin prednisona concomitante).

Trabajos publicados

TRABAJO 1

Effects of Allogeneic Mesenchymal Stem Cell Transplantation in Dogs with Inflammatory Bowel Disease Treated with and without Corticosteroids

José Ignacio Cristóbal, Francisco Javier Duque, Jesús María Usón-Casaús, Patricia Ruiz, Esther López, Eva María Pérez-Merino.

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Article

Effects of Allogeneic Mesenchymal Stem Cell Transplantation in Dogs with Inflammatory Bowel Disease Treated with and without Corticosteroids

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Simple Summary: The conventional treatment of canine inflammatory bowel disease (IBD) includes corticosteroids, but they cannot contain the disease effectively in a percentage of patients. Still, their suppression can lead to a worsening. Moreover, the application of mesenchymal stem cells (MSCs) as an alternative has yielded promising results. However, they have been always infused after a washout period of any other immunosuppressants. Therefore, the feasibility and effects of the combination of stem cells and prednisone in IBD-dogs will be evaluated for the first time in this study. A single infusion of MSCs were administered to a group of IBD-dogs without any treatment and to another having prednisone treatment with poor response. The changes in two clinical indices, albumin and cobalamin concentration were assessed after one, three, six and 12 months. In both groups, an alleviation of the disease severity and an increase in albumin and cobalamin concentrations were observed at each visit. In parallel, the steroid dosage was gradually reduced until it was suppressed in all patients a year after the stem cell infusion. Therefore, the benefits of stem cell transplantation in dogs with inflammatory bowel disease receiving or not prednisone are significant and lasting.

Abstract: Mesenchymal stem cells have proven to be a promising alternative to conventional steroids to treat canine inflammatory bowel disease (IBD). However, their administration requires a washout period of immunosuppressive drugs that can lead to an exacerbation of the symptoms. Therefore, the feasibility and effects of the combined application of stem cells and prednisone in IBD-dogs without adequate response to corticosteroids was evaluated for the first time in this study over a long-term follow up. Two groups of dogs with IBD, one without treatment and another with prednisone treatment, received a single infusion of stem cells. The clinical indices, albumin and cobalamin were determined prior to the infusion and after one, three, six and 12 months. In both groups, all parameters significantly improved at each time point. In parallel, the steroid dosage was gradually reduced until it was suppressed in all patients a year after the cell therapy. Therefore, cell therapy can significantly and safely improve the disease condition in dogs with IBD receiving or not receiving prednisone. Furthermore, the steroid dosage can be significantly reduced or cancelled after the stem cell infusion. Their beneficial effects are stable over time and are long lasting.

Keywords: inflammatory bowel disease; chronic enteropathy; stem cells; corticosteroids

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1. Introduction

Inflammatory bowel disease (IBD) is the collective term for a group of chronic enteropathies characterized by persistent or recurrent gastrointestinal (GI) signs and inflammation of the GI tract [1]. Even though the pathogenesis of this disease has not yet been

clarified, trials in humans and mice have determined that it is a combination of genetic causes, environmental factors and the host's own immune system [2,3].

To treat this condition, immunosuppressants are commonly administered to patients that fail to respond to either dietary changes or antibiotics. The most widely used drug is prednisone, although studies have also been conducted with other immunosuppressive drugs, including cyclosporine, azathioprine and budesonide [4]. However, there is still a percentage of animals that are non-responsive to this conventional treatment. Additionally, immunomodulators often induce numerous side effects. Thus, new strategies and safe treatments for IBD are urgently needed to improve the control of the disease, such as emerging and promising therapies with mesenchymal stem cells (MSC).

The application of MSC to treat immune-related gastrointestinal disorders have been investigated both in human [5,6] and in small animals [7–9], demonstrating efficacy and safety in all the species. However, while the number of clinical trials in human medicine is high, the number of trials in veterinary medicine is scarce, and several questions still remain unanswered.

Clinical trials in small animals have mostly been designed to establish the efficacy and safety of the new therapy, so, a washout period before the treatment and the absence of any other medical treatment during the trial was required. On the contrary, in most human clinical trials conducted on Crohn's patients, once efficacy and safety issues were solved, concomitant immunosuppressive agents were allowed, with their dosage being modified after the cell administration [10–12]. The design of those studies better reflects a common situation that occurs in human and veterinary medicine, in which a long suppression of conventional treatment is not possible in many patients because it could exacerbate the symptoms, putting them at risk. However, stem cells to treat canine IBD have never been applied jointly with concomitant conventional therapy, and it would be of great interest to know whether the MSC infusion during the conventional treatment could change the course of the disease.

To be precise, the goal of this study is to establish if the co-administration of adipose derived mesenchymal stem cells (Ad-MSC) and prednisone would allow the prednisone dose to be subsequently reduced. Furthermore, we investigate whether the effects of combined Ad-MSC and corticosteroids are greater or lesser than the administration of Ad-MSC alone. Additionally, despite good results regarding the use of MSC in dogs and cats with IBD, their long-term effectiveness has not yet been evaluated and the number of cases in both studies has been very small. Due to this, the secondary aim of this work is to expand the number of dogs with IBD treated with stem cells and their clinical and laboratory follow-up in the medium and long term.

1. Materials and Methods

1.1. Animals

Dogs diagnosed with IBD and treated in the Internal Medicine consultation of the Veterinary Teaching Hospital of the University of Extremadura (VTH-UEx) were included in the study, which was approved by the UEx Animal Care and Use Committee and by the Government of Extremadura (File nº 20160822, approved August 2016). Prior information was provided, and all owners signed the written consent.

1.2. Groups

The animals in this study were divided in two groups according to the received treatment:

- MSC group: Animals without any treatment at least 21 days before MSC administration.
- P-MSC group: Animals treated with prednisone at the time of MSC administration (all other immunosuppressant drugs except prednisone were withdrawn at least 21 days before the MSC administration). The starting dose of prednisone in dogs in this group was between 0.75 and 2 mg/kg per day. In addition, the prednisone dosage was assessed

and reduced at the different time points post-treatment if CIBDAI and CCECAI scores dropped more than 30% of the previous value. If not, the dosage was maintained.

All owners were advised to feed their dogs with a hypoallergenic or novel protein diet to minimize diet interaction and variability.

1.1. Initial Tests

The dogs in this study, prior to inclusion, had a history of chronic gastrointestinal signs (at least three weeks) and failed to respond to symptomatic therapies. IBD diagnosis was confirmed on the basis of routine diagnostic tests performed at the VTH-UEx. Referred animals were re-evaluated and those initial tests were repeated at the hospital. The diagnostic procedure included a detailed anamnesis, a complete physical examination and a complete blood count and biochemistry, including the measurement of albumin, folate, cobalamin and trypsin-like immunoreactivity (TLI). Blood hematology was carried out using an automatic analyzer (Spincell 5 Compact®, Spinreact, Barcelona, Spain), blood chemistry was performed on the Saturno 100 VetCrony® (Crony Instruments, Rome, Italy) automatic analyzer and, finally, the folate, cobalamin and TLI values were analyzed in the external laboratory Laboklin (Madrid, Spain).

Subsequently, abdominal radiographs and a full abdominal ultrasound were performed. The radiographs were performed using the X-ray equipment Siemens Axiom MultiX MT® (Siemens AG, Muenchen, Germany), and the abdominal ultrasounds using the Philips® HD 11XEDS Ultrasound System (Philips, Eindhoven, The Netherlands). A urinalysis and stool analysis were performed on three consecutive days together with a Giardia test. Once extra-intestinal pathologies and parasitic and infectious diseases that could cause chronic digestive signs were ruled out, a digestive endoscopy (gastroduodenoscopy and ileocolonoscopy) was performed. Finally, mucosal samples endoscopically obtained were histopathologically analyzed, thus confirming the presence of inflammation. Food and antibiotic responsive enteropathies were excluded in all dogs according to the recommendations of the World Small Animal Veterinary Association Gastrointestinal Standardization.

The criteria for patient inclusion were adult dogs (≥1 year of age) with histologically confirmed IBD and without adequate response to immunosuppressants.

To establish the severity of each dog illness, the Clinical Inflammatory Bowel Disease Activity Index (CIBDAI) [13] and the Canine Chronic Enteropathy Clinical Activity Index (CCECAI) [14] were calculated. These indices evaluate different variables: attitude and activity, appetite, vomiting, stool consistency, stool frequency and weight loss for CIBDAI. The same variables apply to CCECAI but also include serum albumin concentration and the presence of ascites and pruritus. Each variable is scored from 0 to 3, and the disease is finally classified as follows: 0 to 3 is clinically insignificant, 4 to 5 is mild IBD, 6 to 8 is moderate IBD and 9 or more is severe IBD. If CCECAI is over 12 points, the classification is very severe IBD.

1.2. Isolation and In Vitro Expansion of Adipose-Derived Mesenchymal Stem Cells

Isolation, cell culture and characterization of adipose-derived mesenchymal stem cells were performed by ICTS Nanbiosis (Unit 14 at CCMIJU).

Ad-MSC were obtained from subcutaneous adipose tissue from the falciform ligament of healthy donors (n = 4) during conventional ovariectomy. The cells were washed twice with phosphate buffer saline (PBS) and digested at 37 °C for 30 min with 1.5% of collagenase type V (Sigma, St. Louis, MO, USA) at 37 °C with agitation. The digested samples were washed twice with Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and filtered through a 40 µm nylon mesh. Cells were seeded onto tissue culture flasks and expanded at 37 °C and 5% CO^2. Following 48 h in culture, the non-adherent cells were removed. Adhered cells were passaged at 80–90% confluence by trypsinization (0.25% trypsin solution) and seeded to a new culture at a density of 5000–6000 cells/cm^2. Culture medium was changed every 4–7 days.

Cells were expanded over three passages and then cryopreserved in fetal calf serum (FCS) with 10% of dimethyl sulfoxide (DMSO). The MSC phenotype of the adherent cells was verified according to the International Society for Cellular Therapy guidelines [15]. Flow cytometry analyses demonstrated a positive expression of CD44, CD29, CD105 and MHC class I molecules, as well as a negative expression for MHC class II molecules. In vitro differentiation assays towards osteogenesis, adipogenesis and chondrogenesis were performed at the Minimally Invasive Surgery Centre-Unit 14 from Nanbiosis (https://www.nanbiosis.es, Accessed on 9 February 2021) as previously described [16].

A second expansion was performed to obtain the final product for in vivo administration. The day of the treatment, cells were carefully thawed at 37 °C, FCS and DMSO were removed by centrifugation of the cells, and the pellet was resuspended in 50 mL of vehicle (physiological saline solution). Additionally, a mycoplasma test was performed in all cell lines.

1.1. Adipose-Derived Mesenchymal Stem Cell Administration

Patients received an intravenous single dose of 4×10^6 cells/kg body weight. For the administration of Ad-MSC, thawed cells were resuspended and diluted in physiological saline (SSF) to a final volume of 100 to 250 mL (volume was established according to animal weight). The infusion was administered through a peripheral IV cannula over an average of 30 min. All animals were monitored before, during and 1 h after administration.

1.2. Study Procedure

Just prior to the administration of the Ad-MSC (T0), clinical exam, hematology, serum biochemistry and abdominal ultrasound were performed, and CIBDAI and CCECAI scores, cobalamin (hypocobalaminaemia < 200 ng/L) and albumin (hypoalbuminemia < 2.5 g/dL) serum concentrations, and prednisone dosage for the P-MSC group were obtained.

Patients were followed up at 30 (T1), 90 (T3), 180 (T6) and 365 (T12) days after cell infusion, and the same parameters were assessed at each of the time points and compared with the previous values.

Clinical remission was defined as a decrease of over 75% in the CIBDAI and CCECAI T12 scores. Indeed, animals showing CIBDAI or CCECAI scores lower than 3 are considered to be in remission of the disease in this study.

1.3. Statistical Study

Statistical analysis was performed using the Sigma Plot 12.0 Extract Graphs and Data Analysis software (USA/Canada). First, a Shapiro-Wilk test was performed to analyze the normality of the data distribution. All variables analyzed (clinical indices, albumin and cobalamin concentrations and prednisone dosage) had a non-Gaussian distribution. Data are presented as mean values standard deviation.

For each group, differences in data before and after treatment at each control (T0, T1, T3, T6 and T12) were compared using a Tukey test.

Finally, to determine the differences between values from both groups for each studied parameter at each visit, a Mann-Whitney U test was carried out.

Statistical significance was set at $p < 0.05$.

2. Results

2.1. Animals and Groups

Thirty-two dogs were included in the study, 19 of them in the MSC group and 13 in the P-MSC group. A majority of them were mixed breed (18.75%), followed by German Shepherd (12.5%), Yorkshire Terrier (12.5%), French Bulldog (9.4%) and Boxer (6.3%). The rest of the breeds, representing 3.12% each, were Beagle, West Highland White Terrier, American Staffordshire Terrier, Bichon Maltese, Pitbull Terrier, Poodle, Siberian Husky, Spanish Mastiff, Golden Retriever, Greyhound, Pomeranian, Cocker Spaniel and Spanish Water Dog. There were no statistically significant differences in age ($p = 0.056$) or weight ($p = 0.673$) between both groups (Table 1).

Table 1. Number of animals, average age and range (years), sex and average weight and range (kilograms) of the patients in the cell therapy (MSC) group and the combined therapy (P-MSC) group.

	MSC	P-MSC
Animals	19	13
Age	4.18 (1–14)	5.84 (1–11)
Sex (Male/Female)	12/7	7/6
Weight	13.3 (3–41)	13.8 (2.2–26.2)

1.1. Symptomatology

Prior to the administration of the Ad-MSC, all animals had gastrointestinal symptoms for a period greater than 3 weeks (mean time of 14 months). Clinical signs showed, from highest to lowest frequency, were watery diarrhea, pasty stools, decreased appetite/anorexia, weight loss, vomiting, increased frequency of defecation, apathy, hematochezia, melena, ascites and hematemesis. During the initial evaluation (T0), each patient showed 4 symptoms on average. After Ad-MSC administration, the number of symptoms decreased in most patients from both groups, presenting an average of 2 symptoms at T1, 1 at T3 and T6 and no symptoms at T12.

1.2. Characterization of Adipose-Derived Mesenchymal Stem Cells

Adipose-derived mesenchymal stem cells were isolated and in vitro expanded as previously described [16]. The cells were firstly characterized by flow cytometry, being positive for CD44 CD29 and negative for MHC class II (data not shown). Additionally, the adipogenic, chondrogenic and osteogenic differentiation potential was evaluated according to standard protocols as previously described [16]. Our results revealed the multipotency of cells using dexamethasone, and Alizarin Red, Alcian Blue and Oil Red O allowed us to visualize each differentiation (Figure 1). Briefly, Figure 1A shows the morphology of in vitro cultured cells under standard conditions. The Figure 1B–D shows the microscopic images of adipogenic, chondrogenic and osteogenic differentiations respectively.

1.3. Results of the Parameters Studied

1.3.1. Clinical Activity Indices

According to the clinical indices, the study group at T0 included 1 animal with medium, 12 with moderate, 10 with severe and 9 with very severe IBD. Baseline CIBDAI and CCECAI population mean scores were 8.75±3.37 (moderate IBD) and 9.80±3.42 (severe IBD), respectively.

Table 2 shows pre- and post-MSC infusion values of the CIBDAI and CCECAI scores in both groups. There were no statistically significant differences between the two groups in entry CIBDAI and CCECAI scores (Table 2).

For CIBDAI, a drop of 7.36 points for the MSC group and 8.53 for the P-MSC group were recorded a year after the cell infusion (Figure 2). In the cell therapy group, a statistically significant reduction in T1, T3, T6 and T12 CIBDAI scores was observed when compared to T0 ($p < 0.05$). In the prednisone and cells joint treatment group, that reduction was only significant for the values obtained at T3, T6 and T12 when compared to T0 ($p < 0.05$) (Table S1).

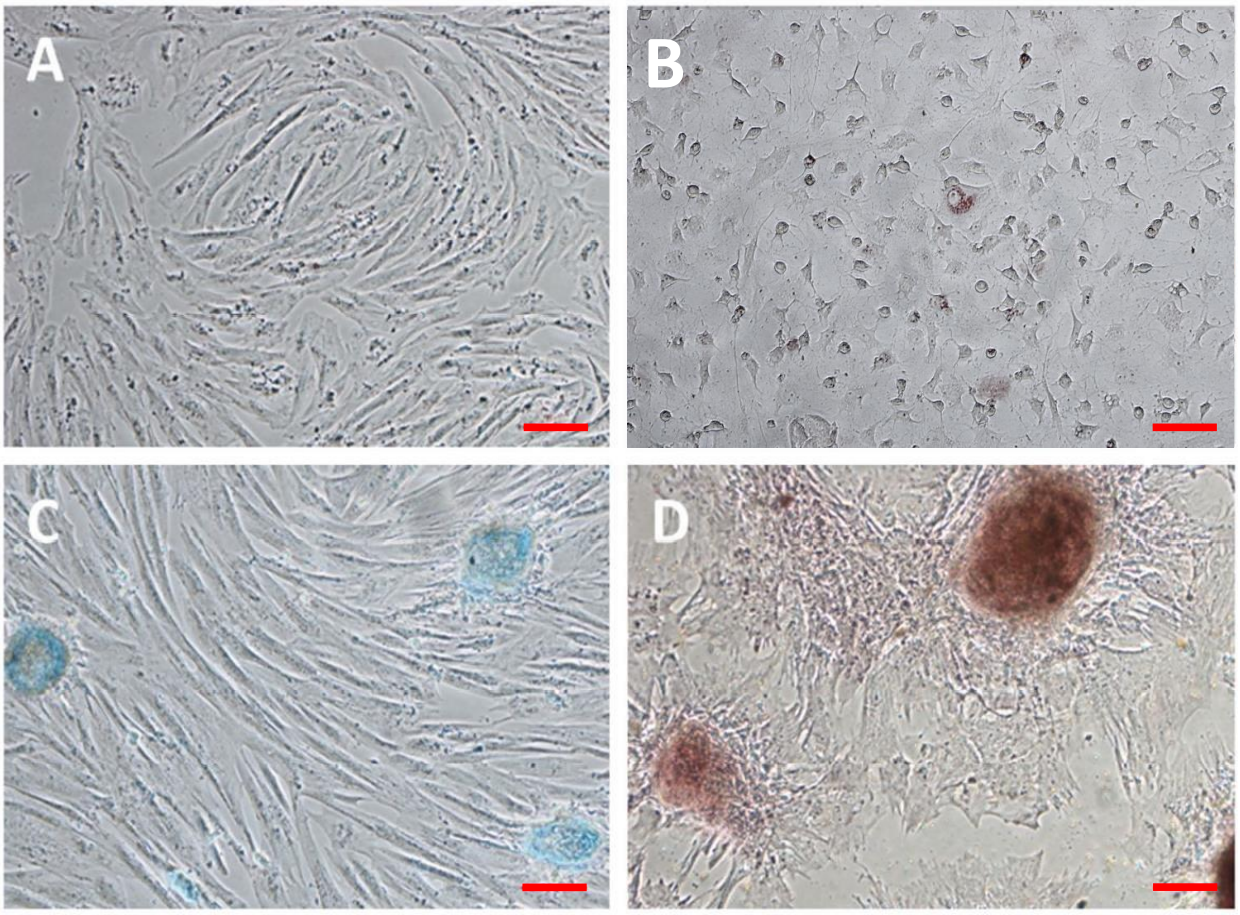


Figure 1. Adipose-derived mesenchymal stem cells (**A**) and their adipogenic (**B**), chondrogenic (**C**) and osteogenic (**D**) differentiations. The differentiations were induced as described in material and methods section. Microscopy images of in vitro differentiations at 10× magnification. Scale red bars detail 100 µm.

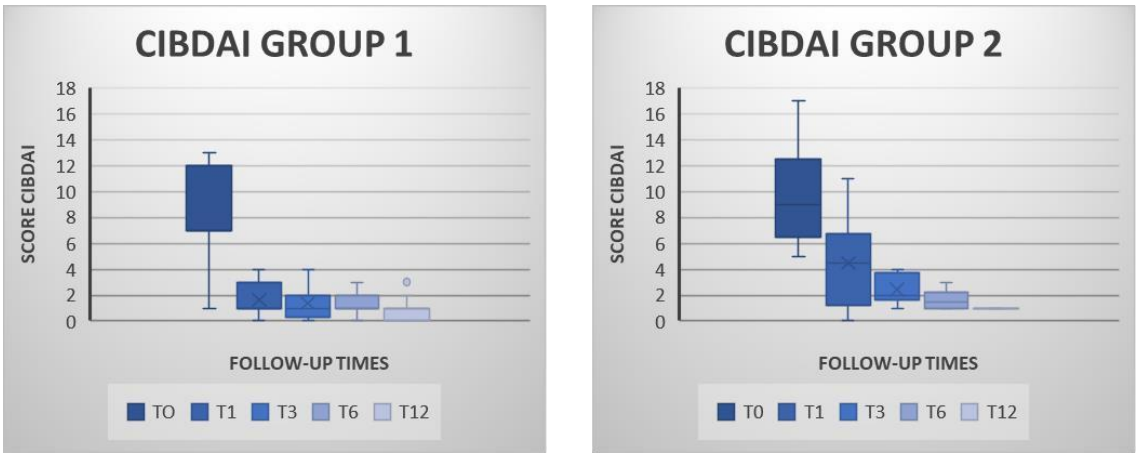
Table 2. Mean and standard deviation of the clinical indices CIBDAI and CCECAI, albumin, cobalamin and the prednisone dose (mg/kg/day) in the MSC and P-MSC groups. Significance level (*p*-value) between the MSC and P-MSC groups.

	MSC GROUP	P-MSC GROUP	*p*-Value
CIBDAI			
T0	8.21 ± 2.97	9.54 ± 3.87	$p = 0.280$
T1	1.63 ± 1.44	4.46 ± 3.24	$p = 0.006$
T3	1.38 ± 1.31	2.44 ± 1.12	$p = 0.035$
T6	1.38 ± 0.86	1.67 ± 0.82	$p = 0.542$
T12	0.85 ± 0.89	1.00 ± 0.00	$p = 0.426$
CCECAI			
T0	8.90 ± 2.90	11.12 ± 3.79	$p = 0.142$
T1	1.77 ± 1.78	5.13 ± 3.92	$p = 0.007$
T3	1.18 ± 1.33	2.94 ± 1.57	$p = 0.017$
T6	1.56 ± 1.33	1.83 ± 0.75	$p = 0.540$
T12	0.83 ± 0.94	1.20 ± 0.45	$p = 0.246$

Table 2. *Cont*

	MSC GROUP	P-MSC GROUP	*p*-Value
ALBUMIN (g/dL)			
T0	3,03 ± 0,81	2,29 ± 0,62	$p = 0,010$
T1	3,21 ± 0,41	2,49 ± 0,45	$p < 0,001$
T3	3,42 ± 0,43	2,81 ± 0,51	$p = 0,008$
T6	3,51 ± 0,27	2,98 ± 0,61	$p = 0,024$
T12	3,60 ± 0,20	3,10 ± 0,27	$p = 0,007$
COBALAMIN (pg/mL)			
T0	271,46 ± 136,72	322,86 ± 250,83	$p = 0,954$
T1	308,76 ± 153,57	350,93 ± 250,51	$p = 1,000$
T3	365,26 ± 83,33	424,00 ± 253,56	$p = 0,832$
T6	446,10 ± 132,42	396,68 ± 191,78	$p = 0,286$
T12	587,84 ± 149,42	420,78 ± 163,54	$p = 0,033$
PREDNISONE DOSAGE (MG/KG/DAY)			
T0		1,60 ± 0,54	
T1		1,19 ± 0,60	
T3		0,46 ± 0,59	
T6		0,15 ± 0,31	
T12		0,00 ± 0,00	

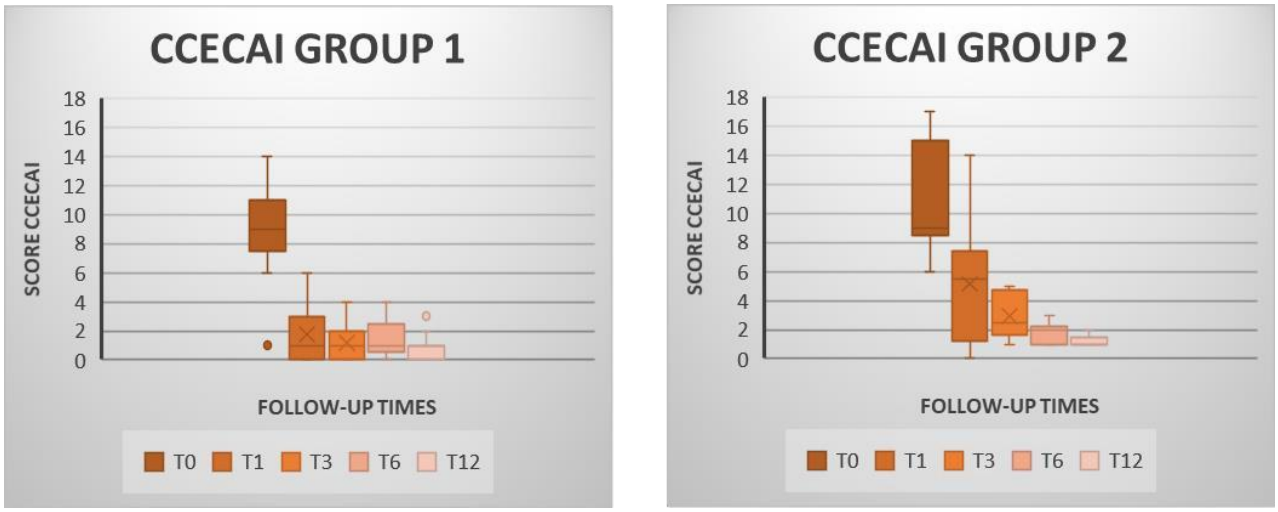
CIBDAI (Clinical Inflammatory Bowel Disease Activity Index), CCECAI (Canine Chronic Enteropathy Clinical Activity Index), MSC (Mesenchymal Stem Cells), P-MSC (Prednisone-Mesenchymal Stem Cells).



(a) (**b**)

Figure 2. Values of the Clinical Inflammatory Bowel Disease Activity Index (CIBDAI) for the cell therapy group (MSC group; figure (**a**)) and for the combined prednisone and stem cells group (P-MSC group; figure (**b**)) at each check point. In both groups, it can be observed how the CIBDAI decreases progressively over the successive control points, highlighting the decrease between T0 and T1. Box and Whisker Plot: The graphs show the median (line inside the box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers).

CCECAI scores fell 8.06 points in the MSC group and 9.91 in the P-MSC group a year after (Figure 3). In both groups, a statistically significant reduction in T1, T3, T6 and T12 scores was observed when compared to T0 ($p < 0.05$) (Table S1).



(**a**) (**b**)

Figure 3. Values of the Canine Chronic Enteropathy Clinical Activity Index (CCECAI) for the cell therapy group (MSC group; figure (**a**)) and for the combined prednisone and stem cells group (P-MSC group; figure (**b**)) at each review. In both groups, it is observed how the CCECAI decreases gradually over the time, highlighting the decrease between T0 and T1. Box and Whisker Plot: The graphs show the median (line inside the box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers).

There were no significant differences among the values obtained from the post-cell treatment follow-up controls in any index or group (Table S1).

Indices reduction from baseline were significantly greater in the MSC group at T1 and T3 than in the combined therapy group (Table 2). All animals in both groups showed clinically insignificant IBD at the annual revision.

Clinical remission was achieved by 57% of the animals (11/19) in the MSC group and by 30% (4/13) in the P-MSC group at T1. At T3, 68% (13/19) of the MSC-patients and 61% (8/13) of the P-MSC patients achieved clinical remission. Six months after the administration of the MSC (T6), remission was observed in 84% (16/19) of the animals in the MSC group and also in 84% (11/13) of the animals in the P-MSC group. Finally, in the annual check-up (T12), 100% of the animals achieved remission in both groups.

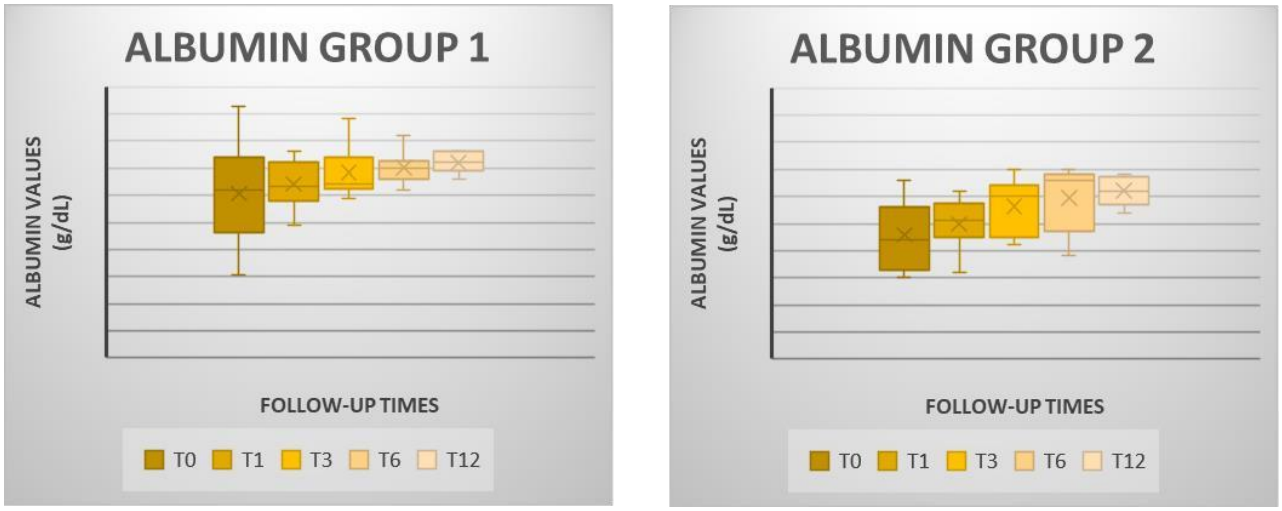
1.1.1. Albumin

At the time of diagnosis, 13 of the 32 patients had hypoalbuminemia (40.6%), 5 of them in the MSC group and 8 in the P-MSC group. The baseline mean albumin value of the study population was 2.67 ± 0.75 g/dL. The T0 albumin concentration was significantly lower in P-MSC animals than in MSC animals (Table 2).

After cell administration, the albumin concentration improved gradually in both groups, with only one dog being hypoalbuminemic at T1 and none at the following visits in the MSC group. In the prednisone group, the five animals remained hypoalbuminemic at T1, but decreased to 3 at T3, 2 at T6 and none at T12.

A year later, the albumin concentration had increased by 0.57 g/dL in the MSC group and 0.81 g/dL in the P-MSC group (Figure 4). The increase in albumin concentration at each follow up was not significant at any control when compared to T0 in the MSC group. However, in the P-MSC group, a significant increase in albumin was observed at T6 and T12 reviews compared to T0. No differences between the values obtained from the

other controls were noticed (Table S1). Albumin levels remained significantly lower in the prednisone group than in the MSC group at each checkpoint (Table 2).



(a) (**b**)

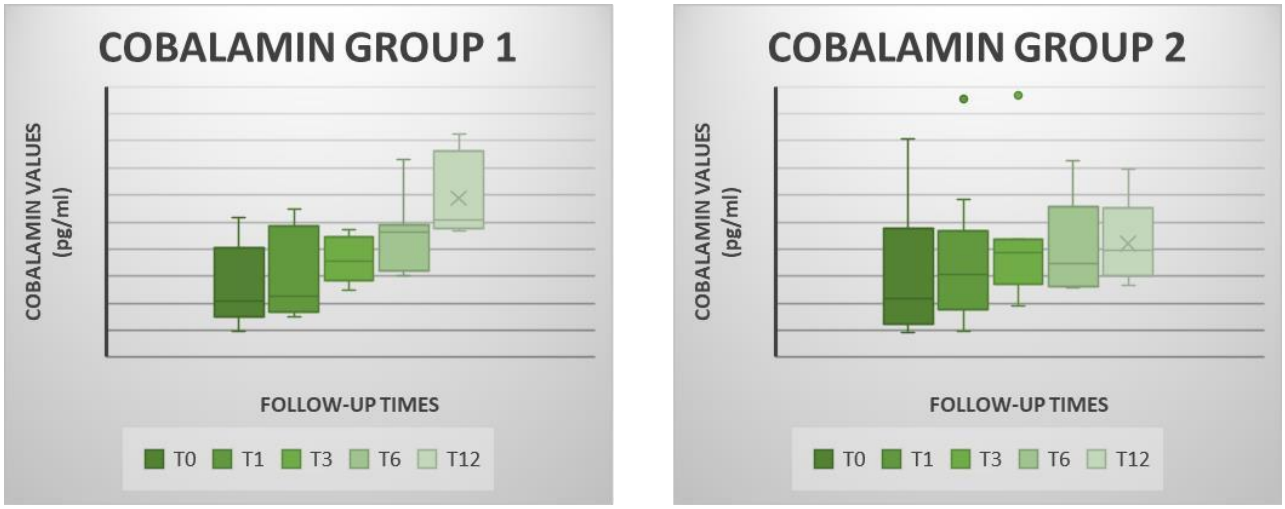
Figure 4. Albumin concentration values for the cell therapy group (MSC group; figure (**a**)) and for the combined prednisone and stem cells group (P-MSC group; figure (**b**)) at each review. In both groups, it is observed how the albumin value increases every checkpoint, presenting higher values in MSC group (group 1). Statistically significant differences in albumin concentration at each checkpoint were observed between both groups ($p < 0.05$). Box and Whisker Diagram: the graphs show the median (line inside the box), 25th percentiles and 75 (box) and minimum and maximum values (whiskers).

1.1.2. Cobalamin

Prior to cell treatment, 19 of the 32 patients in the study (59%) had hypocobalaminemia, 11 in the MSC group and 8 in the P-MSC group. Mean cobalamin concentration at T0 for the study group was 292.34 ± 189.38 pg/mL. Even though mean values of cobalamin in the MSC group were lower than in the prednisone group at T0, the difference was not significant (Table 2).

After a month, 7 patients from the MSC group and 5 from the P-MSC group remained hypocobalaminemic. Three months later, they decreased to 3 and 2 in the MSC and P-MSC group, respectively. At T6 and T12, the cobalamin concentration was normal in all the dogs of the cell group. In the prednisone group, hypocobalaminemia persisted in 2 dogs at T6 and in 1 dog at T12.

A year after MSC infusion, cobalamin had increased by an average of 316.38 pg/mL in the MSC group and only by 97.92 pg/mL, on average, in the P-MSC group (Figure 5). In the MSC group, a significant increase in cobalamin was observed at T6 visit when compared to T0, and in T12 when compared to T0, T1 and T3. No significant differences were detected among the different cobalamin concentrations over time in the P-MSC group (Table S1). The rise in cobalamin was significantly higher only at T12 in the MSC group than in the combined prednisone and cell group (Table 2).



(**a**) (**b**)

Figure 5. Cobalamin concentration values observed at each control point for the cell therapy group (MSC group; figure (**a**)) and for the combined prednisone and stem cells group (P-MSC group; figure (**b**)). Statistically significant differences were noted between both groups at T12 ($p < 0.05$). In both groups, cobalamin increases over the periodic reviews, showing the MSC-group (group 1) the steepest increase. Box and Whisker Diagram: the graphs show the median (line inside the box), 25th and 75th percentiles (box) and minimum and maximum values (whiskers).

1.1.1. Prednisone Dosage

After the administration of MSC, the prednisone dose was progressively reduced over the successive controls (Figure 6). Compared to the T0 dose, the decrease in prednisone was significant at each follow-up control (T1, T3, T6 and T12). In addition, a significant decrease in the dose was observed at T6 and T12 when compared with the dose administered at T1 (Table 2).

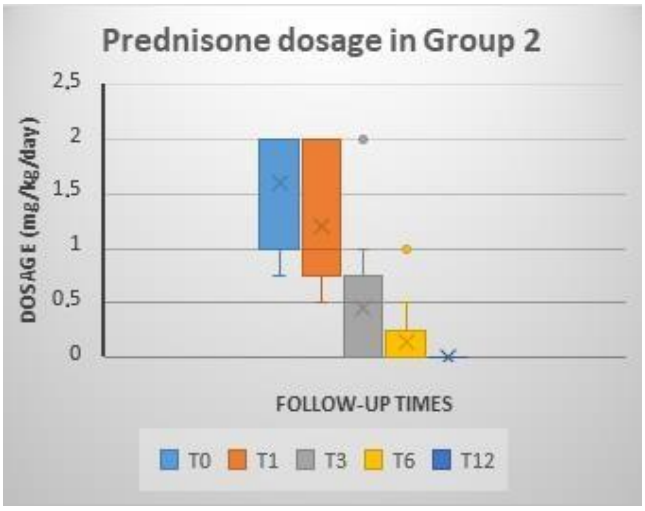


Figure 6. Values of the prednisone dosage (mg/kg/day) in the P-MSC group at each of the reviews.

2. Discussion

MSC have been used in veterinary medicine in immune-mediated diseases due to the important role they play in regulating the immune system. Studies have been conducted using this cell therapy in patients with canine atopic dermatitis, keratoconjunctivitis sicca,

granulomatous meningoencephalitis, feline chronic gingivostomatitis, feline asthma and canine and feline chronic IBD [7,17].

Previous preliminary studies on feline and canine IBD offered promising results, reporting a significant improvement in clinical signs [8,9]. Thus, MSC seem to be a suitable alternative therapy for dogs and cats with IBD. However, those data were obtained from IBD patients after a washout period of corticosteroids. Therefore, the results could not be generalized to refractory patients that are concurrently receiving conventional treatment.

Canine IBD is routinely treated with steroids, cyclosporine or azathioprine, separately or combined [1]. In some of our patients, prior to inclusion in the study, an attempt to suppress that treatment was unsuccessful. In particular, the suppression of steroids led to a rapid worsening of the patients, but even keeping them, the CIBDAI score corresponded to a severe IBD. The recurrent nature of the disease, the length of the treatment or individual features might explain why steroids washout could be possible for some dogs but notfor others before the cell infusion. Therefore, it was imperative to study the interaction between MSC and the drug most commonly used in conventional therapy.

In human medicine, numerous studies in which MSC of different origins were administered to IBD patients in combination with immunosuppressant therapy have been carried out, concluding that their co-administration was safe, the steroid dosage significantly decreased and the patients' conditions also improved significantly [10–12]. Likewise, in this study, the use of MSC and prednisone concomitantly in dogs was shown to be safe. Furthermore, it seems that the MSC therapy had a distinctive role since patients who had poorly responded to steroids presented an improvement in their physical and laboratorial condition while reducing the prednisone dosage to the point of its suppression. This indicates that MSC can attenuate immune malfunction in IBD dogs.

Different investigations have shown that the co-administration of MSC and other IBD treatments leads to synergy, can repair the mucosa and can improve intestinal inflammation [6]. Schneider et al. evaluated human chorion-derived MSC for cell viability, cell polarity, nuclear morphometry, F-actin and focal adhesion kinase distribution and cell migratory properties in the presence of the immunosuppressive drugs azathioprine (AZA) or dexamethasone at concentrations similar to those used in clinical treatments. They found that the stemness, cell viability and nuclear morphometry of MSC were not affected by these two drugs [18].

On the other hand, this study also shows that both cell therapy modalities (alone or combined) seem to be equally effective with some minor differences. Even though the clinical indices decreased considerably after the MSC administration, their fall was higher in the combined therapy group, even from a worse starting situation [18]. As some authors described, it could be attributed to the joint action of MSC and prednisone, which reduces inflammation in the gastrointestinal tract of patients and inhibits the exacerbated response of the immune system that occurs in IBD [4,19]. The pace of clinical improvement also seems similar for both therapies but is slightly slower for the combined therapy.

Hypoalbuminemia affected a higher number of dogs in the combined therapy group. In fact, it was the only group that started off from an average albumin concentration below the normal level. This condition has been described more frequently in patients with advanced disease who require the administration of immunosuppressants [20]. Furthermore, it was shown that human patients with hypoalbuminemia due to ulcerative colitis required corticosteroid cycles in an earlier period compared to those patients who presented albumin within the reference range [21]. Therefore, since the dependence of corticosteroids in these patients is strong, hypoalbuminemic dogs might be the target for the combined therapy. Hypoalbuminemia has been described as a negative prognostic factor [14,22]. However, in this study, MSC infusion both alone and combined with steroids was able to reverse the situation in all hypoalbuminemic animals, reaching albumin values within the reference range. That return to normality required more time in prednisone-dependent dogs, and they never attained the maximum values obtained in patients who did not require the co-administration of corticosteroids.

According to various studies, the prevalence of hypocobalaminemia in IBD patients varies between 19% and 38% [23]. In this study, however, 59% of the patients showed a cobalamin concentration below the reference range at the beginning of the study. A low cobalamin concentration is particularly common in patients with severe enteropathy and steroids treatment needs. Cobalamin deficiency is associated with small intestinal dysbiosis and impaired absorption at the distal small intestine and has been considered a negative prognostic marker [14,22]. To increase its concentration in IBD dogs, cobalamin supplementation is usually required, and different protocols have been described for that purpose [24]. However, in this study, normocobalaminemia was restored in all dogs without the external administration of this vitamin. This can be attributed to MSC stabilization of cell homeostasis by regulating and preserving the cells of the intestinal epithelium [25]. Furthermore, the increase was greater in the prednisone-free group. The explanation is not clear, and a greater number of studies is needed.

MSC have been reported to secrete a variety of immunosuppressive molecules, thereby reducing inflammation in a generalized manner. They also promote wound healing and tissue regeneration by secreting the so-called transforming growth factor β (TGF-β) and fibroblast growth factor. Furthermore, these cells can differentiate into fibroblasts and endothelial cells to form granulation tissues. Therefore, they have regenerative and anti-inflammatory qualities, which are beneficial for IBD [26].

However, the short duration of earlier studies (42 days, 2 and 3 months) [8,9] left the issue of the length of the observed positive effects unanswered until now. At present, this study clearly shows that the improvement in clinical signs remains stable over a year after the cell infusion. Even though, studies with longer term follow up (over 3 years) have proven that conventional immunosuppressant therapy (either with a single drug or with a combination of several drugs) turns out to be less promising over time [27]. Therefore, continued monitoring of the patients is mandatory to detect if that also happens with cell therapy.

The main limitation of the study is the small number of recruited patients, since most dogs with chronic enteropathies responded to special diets or antibiotics. Another weakness might be the lack of a prednisone-treated control group. However, this study is not intended to assess the efficacy of MSC when compared to steroids, but to prove the feasibility of the combination of both and to restate the utility of cell therapy for canineIBD by increasing the number of MSC-treated dogs and the follow-up period.

The origin and type (autologous or allogeneic) of MSC, their laboratory preparation and the method of administration (route, dosage, schedule, pretreatment conditioning) can also affect the final outcome of the treatment [28]. Therefore, the information obtained from this study is only a small contribution to solve the long list of issues about cell therapy. Thus, there is a need for more prospective studies to standardize the methodology.

1. Conclusions

Ad-MSC therapy can significantly and safely improve the disease condition in dogs with IBD both without treatment and receiving a stable steroid dose. Furthermore, the steroid dosage can be significantly reduced or cancelled after Ad-MSC infusion. Their beneficial effects are stable over time and are long lasting.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ani11072061/s1. Table S1. Significance level (*p*-value) between the values of each studied parameter (CIBDAI, CCECAI, albumin and cobalamin concentration) obtained at each review (T0, T1, T3, T6 and T12) in the MSC and P-MSC groups.

Author Contributions: Conceptualization, J.I.C., F.J.D. and E.M.P.-M.; methodology, J.I.C.; J.M.U.- C.; E.L.N. and P.R.; data curation, J.I.C.; writing—original draft preparation, J.I.C. and E.M.P.-M.; writing—review and editing, F.J.D. and J.M.U.-C.; supervision, E.M.P.-M.; project administration, J.M.U.-C.; E.M.P.-M.; funding acquisition, E.M.P.-M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The protocol was approved by the UEx Animal Care and Use Committee and by the Government of Extremadura. (File nº 20160822, approved August 2016).

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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TRABAJO 2

Complete Blood Count-Derived Inflammatory Markers Changes in Dogs with Chronic Inflammatory Enteropathy Treated with Adipose-Derived Mesenchymal Stem Cells.

José Ignacio Cristóbal, Francisco Javier Duque, Jesús María Usón-Casaús, Rafael Barrera, Esther López, Eva María Pérez-Merino.

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Article

Complete Blood Count-Derived Inflammatory Markers Changes in Dogs with Chronic Inflammatory Enteropathy Treated with Adipose-Derived Mesenchymal Stem Cells

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Simple Summary: Mesenchymal stem cells exhibit anti-inflammatory properties, and their administration to dogs with chronic inflammatory enteropathy has been shown to be safe and effective. Neutrophil/lymphocyte ratio, platelet/lymphocyte ratio, and the systemic immuno-inflammation index are considered novel biomarkers to assess the inflammatory status of patients. The present study aimed to compare the clinical evolution and the changes in these inflammatory biomarkers in dogs with chronic enteropathy before and after cell therapy. The values of the three inflammatory biomarkers were higher in dogs with chronic enteropathy before the treatment compared to the values obtained from healthy dogs. After treatment, those values decreased significantly over time, and nine months later, no difference was observed between healthy dogs and dogs with chronic enteropathy in two of the three studied markers. A relationship between the amelioration of the clinical signs and the decrease in the blood inflammatory markers was also established. These results demonstrate that the clinical improvement of patients with chronic enteropathy treated with stem cells is accompanied by a normalization of the inflammatory biomarkers studied.

Abstract: Adipose-derived mesenchymal stem cells (Ad-MSCs) exhibit anti-inflammatory and immunomodulatory activities. The neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and systemic immune-inflammation index (SII) have been reported as novel biomarkers of the inflammatory state; however, they have never been examined in dogs with chronic inflammatory enteropathy (CIE) treated with Ad-MSCs. This study aimed to compare the clinical evolution and the changes in the NLR, PLR, and SII in dogs with CIE before and after cell therapy. Sixteen dogs with CIE were administered a single intravenous dose of Ad-MSCs. The canine chronic enteropathy clinical activity index (CCECAI), NLR, PLR, and SII were assessed before treatment (T0) and at 2 (T2) and 9 (T9) months post-treatment and compared over time and with the reference values obtained from a group of healthy dogs. NLR, PLR, and SII were significantly increased at T0 compared to the reference values, decreasing significantly over time. At T9, the NLR and SII did not differ from the reference values, but PLR remained above the reference values. A correlation was observed between CCECAI and the three markers. These findings show that the clinical improvement of dogs with CIE treated with Ad-MSCs is accompanied by a normalization of the inflammatory status.

Keywords: cell therapy; chronic inflammatory enteropathy; neutrophil-to-lymphocyte ratio; platelet- to-lymphocyte ratio; systemic immune-inflammation index

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1. Introduction

Chronic inflammatory enteropathy (CIE) describes a group of disorders of the canine enteral tract characterized by persistent gastrointestinal signs whose diagnosis is based on histologically confirmed inflammation in the intestinal mucosa after excluding other identifiable digestive and extra-digestive causes of vomiting and diarrhea [1].

Based on the patient's response to treatment, the enteropathy can be considered food-responsive (FRE), antibiotic-responsive (ARE), immunosuppressant-responsive (IRE), or nonresponsive (NRE) [2]. The terms IBD and IRE are considered synonymous for dogs in which the histology has confirmed the presence of intestinal inflammation. Canine IRE is comparable to Crohn's disease (CD) in humans [1].

Corticosteroids and other immunosuppressants are commonly used to treat CIE; however, nonresponsiveness and adverse reactions limit their usage [3–5]. Adipose-derived mesenchymal stem cells (Ad-MSCs) are a promising alternative therapy to treat chronic enteropathies in dogs and cats, which has been shown to be safe and effective [2,6–8]. After administration, Ad-MSCs not only migrate directly to the site of inflammation but also exert indirect anti-inflammatory effects through secretory factors [9–12].

Moreover, novel biomarkers related to the inflammatory status of patients have been developed [13]. The neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and systemic immune-inflammation index (SII) have been considered new markers for the assessment of the severity of ulcerative colitis in humans [14–16]. In dogs, the NLR and PLR also provide additional information regarding the severity of CIE [17–19]. However, the SII has not been investigated in veterinary patients.

Despite the immunomodulatory and anti-inflammatory properties of the MSCs [11,12,20] and the usefulness of NLR, PLR, and SII as indices of systemic inflammation [17,21,22], these markers have never been examined in patients treated with Ad-MSCs.

Therefore, the present study aimed to compare the clinical evolution and the changes in the NLR, PLR, and SII in dogs with CIE before and after cell therapy.

1. Materials and Methods

1.1. Animals

The study included dogs diagnosed with CIE at the Internal Medicine Unit of the Veterinary Teaching Hospital of the UEx (VTH-UEx). The Animal Care and Use Committee of the UEX and the Regional Government approved the study's design, and all the clients signed an informed consent form.

The diagnosis of CIE was confirmed by excluding other causes of chronic diarrhea based on routine diagnostic tests. All the referred patients were examined again at the VTH-UEx, and the tests were repeated. The diagnostic procedure included a complete blood count, chemistry profile, urinalysis, serum trypsin-like immunoreactivity test, serum canine pancreatic lipase immunoreactivity test, serum cobalamin and folate concentrations, stool examination, abdominal radiography, and ultrasound examination, as well as a histopathological review of mucosal biopsy specimens obtained via both gastroduodenoscopy and ileocolonoscopy. Gastrointestinal biopsies were assessed according to the criteria proposed by the World Small Animal Veterinary Association Gastrointestinal Standardization Group for diagnosing gastrointestinal inflammation in dogs and cats [23,24]. None of the dogs underwent immunosuppressant therapy two weeks prior to endoscopy.

The CCECAI [25] is a standard index used to evaluate the severity of the disease in the dogs of this study by assessing attitude and activity, appetite, vomiting, stool consistency, stool frequency, weight loss, serum albumin concentration, and presence/absence of ascites and pruritus and scored from 0 to 3. After adding the scores from each item, a total score is obtained, according to which the disease will be considered insignificant, 0–3; mild, 4–5; moderate, 6–8; severe, 9–11; and very severe, ≥12.

Food (with hydrolyzed or novel protein diets) and antibiotic (with metronidazole or tylosin) trials were completed before administering immunosuppressive drugs. The diet and antibiotic trials were repeated at the VTH-XXX in the referred dogs. Immunomodulatory therapy was administered using prednisolone, budesonide, cyclosporine, or chlorambucil, either alone or in combinations at the clinician's discretion.

The criteria for patient inclusion were adult dogs (≥1 year of age) with histopathologic confirmation of inflammation in intestinal biopsies and an inadequate response to immunosuppressants, defined as a reduction of <30% in the CCECAI scores after immuno-

suppressive therapy compared with their corresponding pretreatment values. Patients with pregnancy, sepsis, extreme physical impairment, and concomitant diseases were excluded.

A group of healthy dogs that were routinely examined at the VTH-XXX served to establish the reference values for each study parameter. The inclusion criteria for this group were healthy adult dogs (>1 year) whose clinical history, physical examination, blood hematology and biochemistry, urinalysis, and fecal examination did not reveal any abnormality. Dogs with digestive signs or signs of any other transient disease that were reported during the last month were excluded.

1.1. Isolation and In Vitro Expansion of Adipose-Derived Mesenchymal Stem Cells (Ad-MSCs)

Ad-MSCs were isolated from subcutaneous adipose tissue in a conventional surgery of female dog castration, as previously described [8]. Briefly, adipose tissue was digested using collagenase type V, washed, and filtered. The cells obtained were cultured and expanded in Dulbecco's Modified Eagle Medium (DMEM) culture medium with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37 °C and 5% CO_2. Adherent cells were phenotypically characterized by flow cytometry following the specifications of the International Society for Cellular Therapy guidelines [26] and in vitro differentiated towards osteogenic, adipogenic, and chondrogenic lineages. Expanded cells were cryopreserved until the day of administration, when the cells were thawed and resuspended in 50 mL of physiological saline solution. All the procedures described above were performed at the Minimally Invasive Surgery Centre-Unit 14 from Nanbiosis (https://www.nanbiosis.es, accessed on 5 February 2022).

1.2. Study Protocol

A 3-week washout period for immunosuppressive drugs was mandated before the administration of MSCs. Dogs that fulfilled the inclusion criteria received a single dose of Ad-MSCs. The thawed cells were resuspended and diluted in physiological saline to a final volume of 100–250 mL (according to animal weight). The infusion was administered over 30 min through a peripheral intravenous cannula at a target dose of 4× 106 cells/kg body weight. The dogs were monitored during the infusion and for 60 min prior to discharge.

A physical examination was performed, and blood samples were collected just before the treatment (T0) and during subsequent visits at 2 and 9 months (T2 and T9) after treatment.

Whole blood was used for routine hematology with differential white blood cell counts, which was performed at the Clinical Pathology Laboratory of VTH-XXX using an automatic analyzer (Spincell 5 Compact, Spinreact®, Barcelona, Spain). An automatic leukocyte count was performed to obtain the absolute value of each white blood cell type. Serum chemistry profile was performed using a Saturno 100 VetCrony® automatic analyzer (Crony Instruments, Rome, Italy). White blood cell, absolute platelet, neutrophilic, and lymphocytic counts were recorded, and the CCECAI, NLR, PLR, and SII were calculated for each time point. The NLR and PLR were calculated from the differential count by dividing the absolute neutrophil and platelet counts, respectively, by the absolute lymphocyte count. The SII values were calculated using the formula: platelet × neutrophil/lymphocyte counts.

A single blood sample was collected from each recruited healthy dog to obtain the normal values for neutrophil, platelet, and lymphocyte counts, NLR, PLR, and SII. The differences between these reference values and those obtained from the dogs with CIE at each time point (T0, T2, and T9) were analyzed.

1.3. Statistical Study

Data were analyzed using SPSS v22.0 (IBM Corp., Armonk, NY, USA). The Shapiro–Wilk test was used to determine the normality of the quantitative variables. Since most of the data were not normally distributed, their descriptive statistics were reported in terms of median and range (min-max).

Changes over time in the neutrophil, lymphocyte, and platelet counts, and NLR, PLR, and SII values in the treated dogs were compared using the Friedman rank test with post hoc Wilcoxon comparisons. The Mann–Whitney U test was used to compare the values of the treated dogs with their respective reference values. The effect size was calculated using Cohen's d statistic when significant differences were detected.

Because the values were not normally distributed, correlation analysis between the inflammatory ratios and the CCECAI was carried out using Spearman's rho. Statistical significance was set at $p < 0.05$.

1. Results

1.1. Population

A total of 16 dogs with CIE were enrolled in this study: three Yorkshire Terrier, two mixed breeds, and one each of Boxer, American Staffordshire Terrier, Bichon Maltese, American Pitbull Terrier, French Bulldog, Pomeranian, Spanish Mastiff, Spanish Water Dog, Greyhound, German Shepherd, and Poodle. There were 11 males (two neutered) and five females (two spayed). The median age and body weight was 4.3 years (range, 1–14 years) and 13.7 kg (range, 3–41 kg).

Histological evaluation showed the presence of lymphocytic-plasmacytic inflammatory infiltrates in all the dogs.

The reference value for each parameter was obtained from a group of 20 healthy dogs comprising 11 males and 9 females. Their ages ranged from 1 to 9 years (median, 3.6 years). The breeds included five mixed breeds, five Labrador Retrievers, three Border Collies, two Patterdale Terriers, and one each of Dalmatian, English Setter, German Shepherd, Spanish Mastiff, and Jack Russel Terrier. Age and sex did not differ between the healthy and treated dogs.

The descriptive statistics of the clinical indices, absolute neutrophil counts, lymphocyte, and platelet counts, NLR, PLR, and SII from the dogs with CIE and the reference values are reported in Table 1.

Table 1. Descriptive statistics of the studied variables.

	Reference value	T0	T2	T9	P
CCECAI		8.5 (1 – 14)[a]	1.75 (0 – 6)[b]	1 (0 – 4)[c]	<.0001
Effect size (95% CI)	-		2.83 (1.79; 3.73)	3.42 (2.26; 4.40)	
Neutrophils (x10³/µl)	5.78 (2.65 – 10.24)	**7.8 (3 – 16.9)[a]**	6.25 (3.53 – 7.9)[b]	5.88 (4.26 – 7.12)[b]	.003
Effect size (95% CI)			0.85 (0.11; 1.55)	0.96 (0.21; 1.67)	
Lymphocites (x10³/µl)	3.05 (1.40 – 5.52)	**1.49 (0.95 – 3.18)[a]**	**2 (0.96 – 4.83)[a]**	2.38 (1.46 – 3.71)[b]	.039
Effect size (95% CI)			-0.76 (-1.46; -0.03)	-1.25 (-1.97; -0.46)	
Platelets (x10³/µl)	141 (67 – 501)	**243.5 (119 – 611)[a]**	221 (116 – 324)[a]	214 (112 – 341)[b]	.019
Effect size (95% CI)			0.60 (-0.12; 1.29)	0.76 (0.02; 1.46)	
NLR	2.11 (0.74 – 5.62)	**4.58 (1.62 – 17.24)[a]**	**3.24 (1.68 – 5.76)[b]**	2.35 (1.47 – 4.39)[c]	.002
Effect size (95% CI)			0.96 (0.20; 1.66)	1.21 (0.43; 1.93)	
PLR	56.41 (15.34 – 198.02)	**181.42 (102.35 – 364.29)[a]**	**107.54 (45.53 – 179.73)[b]**	**89.37 (50.80 – 145.40)[c]**	
Effect size (95% CI)			1.28 (0.49; 2.01)	1.72 (0.87; 2.48)	
SII (x103)	360.98 (52.93 – 1503)	**1071.76 (475.94 – 6156.43)[a]**	**662.13 (273.18 – 1227.55)[b]**	547.66 (308.08 – 878.23)[c]	
Effect size (95% CI)			0.99 (0.23; 1.69)	1.16 (0.39; 1.88)	

Abbreviations: CCECAI, Canine Chronic Enteropathy Clinical Activity Index; CG, control group; CIE, chronic inflammatory enteropathy; NLR, neutrophil–lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; SII systemic immune-inflammation index. T0, T2, and T9, values of CIE dogs prior to treatment, 2 and 9 months after the treatment. Data are expressed as median and range (min-max). *p* values correspond to Friedman rank test. Within a row, data without a common superscript (a, b, c) differ ($p < 0.05$) according to the Wilcoxon post hoc test. Effect sizes (Cohen's d) between T0 and the stated timepoint are given for each variable. Text in bold indicates a statistically significant difference ($p < 0.05$) with the respective normal value.

1.1. Clinical Indices

The CIE group included one dog with an insignificant disease, seven dogs with moderate disease, and eight dogs with severe disease. Compared with the baseline scores, post-treatment CCECAI scores showed a significant decrease at each time point. This reduction was also significant between T2 and T9 (Table 1, Figure 1).

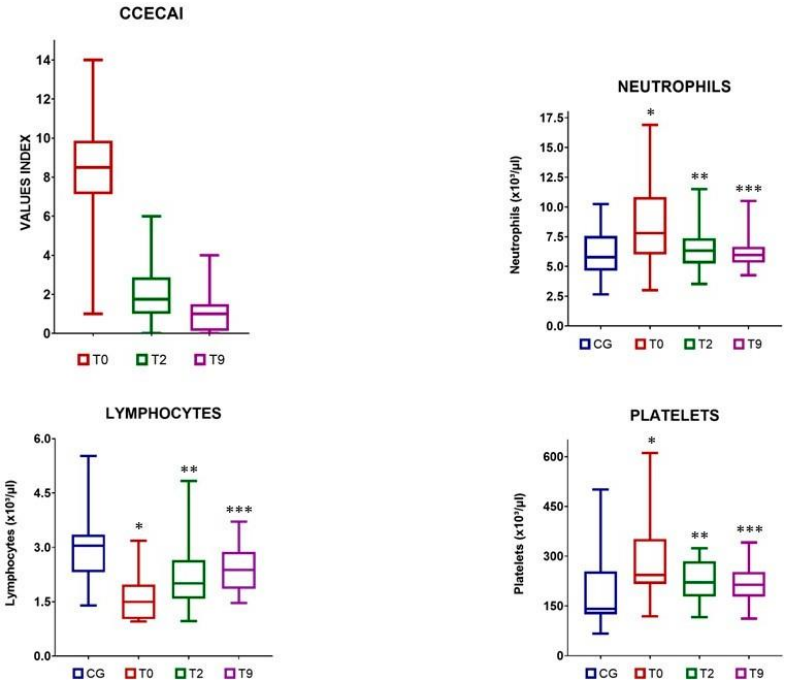

Figure 1. Canine Chronic Enteropathy Clinical Activity Index (CCECAI), neutrophil, lymphocyte, and platelet values in the control group (CG) and in dogs with chronic inflammatory enteropathy (CIE) treated with stem cells at different time points: before administration (T0), at 2 months (T2), and at 9 (T9) months after therapy. Neutrophils: * $p = 0.03$; effect size (95% CI): 0.78 (0.08; 1.45); ** $p = 0.644$; effect size (95% CI): −0.04 (−0.70; 0.61); *** $p = 0.962$; effect size (95% CI): −0.19 (−0.84; 0.47). Lymphocytes: * $p < 0.0001$; effect size (95% CI): −1.62 (−2.34; −0.83); ** $p = 0.02$; effect size (95% CI): −0.78 (−1.44; −0.08); *** $p = 0.05$; effect size (95% CI): −0.71 (−1.37; −0.02). Platelets: * $p = 0.01$; effect size (95% CI): 0.77 (0.07; 1.44); ** $p = 0.063$; effect size (95% CI): 0.36 (−0.31; 1.01); *** $p = 0.171$; effect size (95% CI): 0.20 (−0.46; 0.86).

1.1. Changes in the Neutrophil, Lymphocyte, and Platelet Counts

The dogs with CIE had a significantly higher neutrophil count at T0 than the healthy dogs. After MSCs infusion, the number of neutrophils decreased significantly at T2 and T9 compared to that at T0. No differences were observed between T2 and T9 (Table 1, Figure 1). There were no significant differences between the reference values and the number of neutrophils in the dogs with CIE, both at T2 and T9 (Figure 1).

The lymphocyte number at T0 was significantly lower in the dogs with CIE than in the healthy dogs. Although the lymphocyte numbers increased after treatment, significant differences were observed only between T0 and T9 and not between T0 and T2 in the CIE dogs (Table 1, Figure 1). The lymphocyte counts in the CIE group were significantly lower than the reference values at T2; however, no such differences were observed at T9 (Figure 1).

Compared to the reference values, platelets were significantly elevated in the CIE group prior to MSCs administration. However, a significant decrease in platelet counts was observed in these dogs at T9 (Table 1, Figure 1). Furthermore, the post-treatment platelet counts were not significantly different from the reference values (Figure 1).

1.2. Blood Inflammatory Indices

Pretreatment NLR, PLR, and SII values of the dogs with CIE were significantly higher than those of healthy dogs. After infusion, a significant decrease was observed at T2 and T9 for all three ratios compared with the T0 pretreatment values. In addition, the difference in the drop between T2 and T9 was significant. (Table 1, Figure 2). Significant differences were

observed between NLR, PLR, and SII values at T2 and the corresponding reference values. However, the NLR and SII values at T9 did not differ significantly from the reference values. The PLR of treated dogs remained significantly above the reference value at T9, but the effect size was smaller than that between T0 and the reference value.

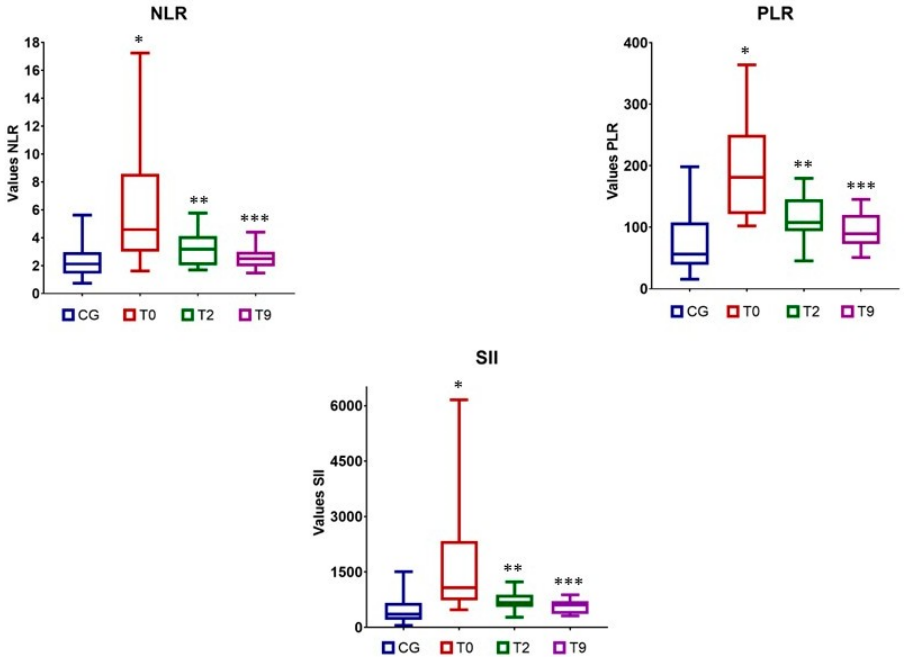

Figure 2. Neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and systemic immune-inflammation index (SII) in the control group (CG) and in dogs with CIE treated with mesenchymal stem cells at the different time points: before treatment (T0), at 2 months (T2), and at 9 (T9) months after therapy. NLR: * $p < 0.0001$, effect size (95% CI): 1.29 (0.54; 1.98); ** $p = 0.02$; effect size (95% CI): 0.66 (−0.03; 1.32); *** $p = 0.298$, effect size (95% CI): 0.14 (−0.52; 0.79). PLR: * $p < 0.0001$, effect size (95% CI): 1.94 (1.11; 2.68); ** $p = 0.005$; effect size (95% CI): 0.96 (0.24; 1.63); *** $p = 0.04$; effect size (95% CI):0.52 (−0.16; 1.18). SII: * $p < 0.0001$, effect size (95% CI): 1.28 (0.54; 1.97); ** $p = 0.008$; effect size (95% CI): 0.76 (0.06; 1.42); *** $p = 0.131$, effect size (95% CI): 0.27 (−0.39; 0.93).

1.1. Association between Inflammatory Indices and the Parameters Analyzed

A positive correlation was found between the NLR, PLR, and SII. All three blood markers were significantly associated with CCECAI and negatively correlated with lymphocyte count. Furthermore, the NLR, PLR, and SII were positively correlated with neutrophil, platelet, and platelet and neutrophil numbers, respectively (Table 2, Figures 3 and 4).

Table 2. Correlation study between the blood inflammatory markers and rest of parameters analyzed.

	NLR		**PLR**		**SII**	
	Spearman ϱ	**p value**	**Spearman ϱ**	**p value**	**Spearman ϱ**	**p value**
Neutrophils	0.71	<0.0001	0.38	0.01	0.73	<0.0001
Lymphocites	-0.82	<0.0001	-0.67	<0.0001	-0.59	<0.0001
Platelets	0.003	0.99	0.54	<0.0001	0.54	<0.0001
NLR	-	-	0.62	<0.0001	0.77	<0.0001
PLR	0.63	<0.0001	-	-	0.87	<0.0001
SII	0.77	<0.0001	0.87	<0.0001	-	-
CCECAI	0.52	<0.0001	0.53	<0.0001	0.495	<0.0001

Abbreviations: CCECAI, Canine Chronic Enteropathy Clinical Activity Index; NLR, neutrophil–lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; SII systemic immune-inflammation index.

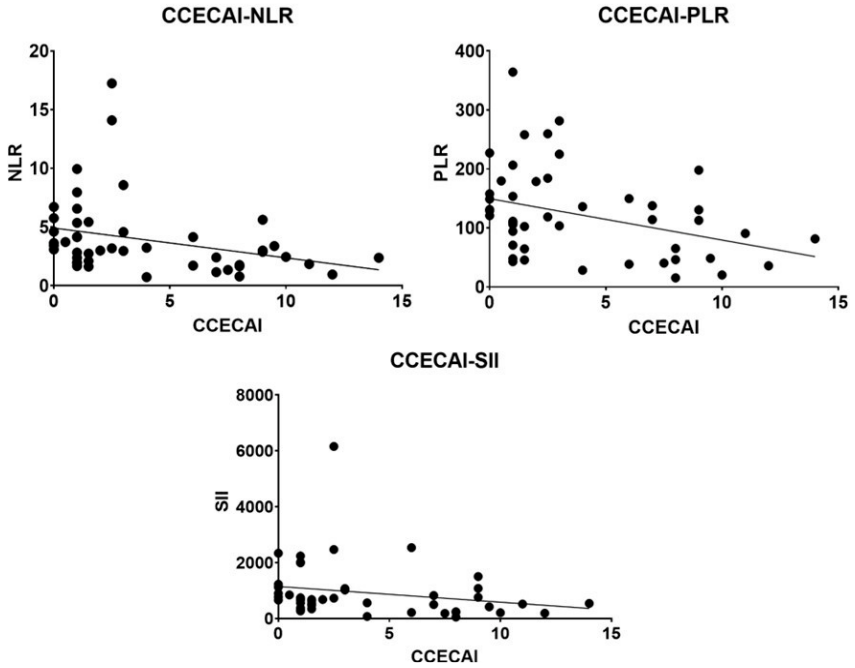

Figure 3. Correlation between CCECAI and each white blood cell-based inflammatory marker. CCECAI, Canine chronic enteropathy clinical activity index; NLR, neutrophil–lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; SII systemic immune-inflammation index.

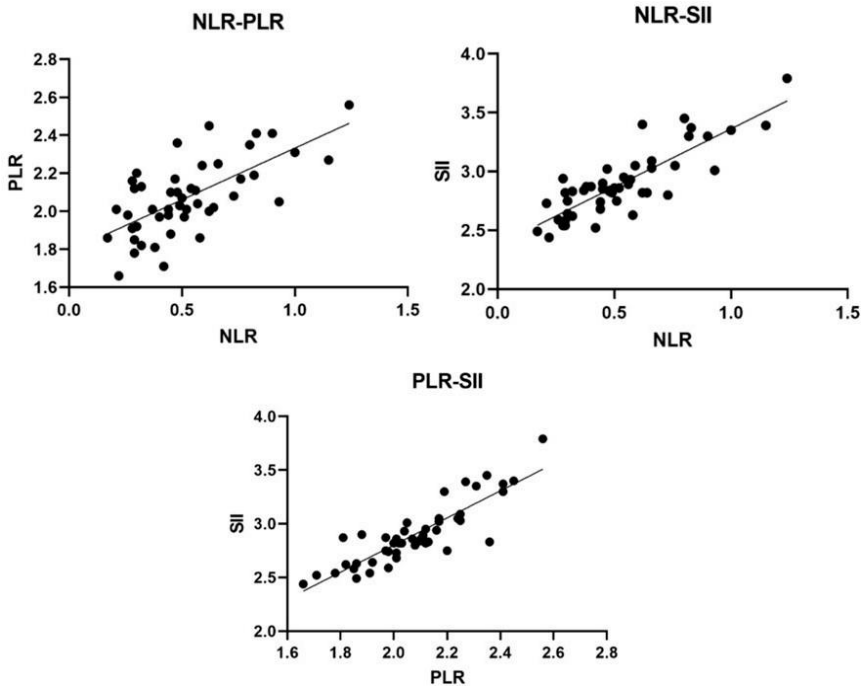

Figure 4. Correlation among the three inflammatory markers. NLR, neutrophil–lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; SII, systemic immune-inflammation index.

1. Discussion

To the best of our knowledge, this is the first study to describe the values of SII in dogs and report the changes in white blood inflammatory markers over time in dogs with CIE treated with MSCs.

Our results reinforce those of earlier studies reporting that the clinical signs of dogs with CIE ameliorated after cell therapy [6,8]. The clinical improvement was accompanied by a decrease in the NLR, PLR, and SII values after treatment with Ad-MSCs.

In this study, dogs with CIE had higher neutrophil and platelet counts and lower lymphocyte counts than healthy dogs. Similar modifications associated with inflammatory processes have been described in other studies [17,27]. Reactive thrombocytosis was also reported in 6–32% of dogs with chronic enteropathy [28].

After cell therapy, we observed a decline in neutrophil and platelet counts and an increase in lymphocyte count in dogs with CIE, with these values returning to normal. A study analyzing the peripheral immune changes in patients with CD treated with autologous hematopoietic stem cell transplantation reported a transient increase in the percentage of circulating Treg cells during the first six months [29]. Systemic administration of MSCs was shown to attenuate subsequent polymorphonuclear neutrophil-predominant inflammatory responses linked to experimental ventilator-induced lung injury in rats [30].

Alterations in NLR, PLR, and SII are often considered in human CD studies. However, these biomarkers have been poorly studied in dogs with CIE.

In the present study, the NLR reference value was in the range reported by other authors [17,18,31], and our group of dogs with CIE showed an NLR value consistent with that reported for dogs with similar CCECAI [18]. However, our PLR reference value was below that described in a previous study on PLR in dogs [31], and the median PLR values of the dogs with CIE were slightly higher than the only value previously described for the same disease [19]. These discrepancies might be due to the fact that normal ranges are unknown, and researchers estimated cutoff points within their sample population. The differences in the study's design and sample sizes might result in an inconsistent range of cutoff points [32–34].

The NLR was significantly higher in dogs with CIE than in healthy dogs in the present study, as previously reported in humans [35,36] and dogs [17]. PLR and SII between dogs with CIE and healthy dogs have never been compared; however, it has been demonstrated that both markers are higher in human patients with ulcerative colitis than in controls [14–16,37].

In the present study, NLR, PLR, and SII decreased after MSCs infusion. In dogs with CIE, the frequency of thrombocytosis was shown to reduce after immunosuppressant treatment (from 32% to 23%) [28]. In humans, the NLR of patients with active Behçet's disease showed a significant reduction after colchicine-corticosteroid treatment; however, it remained higher than that of inactive patients or healthy controls [38]. In contrast, in this study, only the PLR values remained higher in the treated dogs than in healthy dogs.

In a study on patients with ulcerative colitis who received antitumor necrosis factor drugs, a decrease in baseline NLR and PLR was observed at week 8; this decrease was maintained till week 54 of treatment [14]. Similarly, in this study, a decrease in the inflammatory indices was observed at two months after infusion, as well as at the 9-month follow-up, where the levels remained low and within the normal range.

Moreover, this study confirms the positive correlation between NLR [17,18] and PLR [19] and disease severity, as observed by previous authors, as well as the correlation between SII and disease severity. In humans with ulcerative colitis, the decrease in NLR and PLR was correlated with clinical remission after treatment with antitumor necrosis factor drugs [14].

Our results reinforce the conclusions of earlier studies, providing further evidence that NLR can be used to determine the severity of canine CIE, suggesting that it could be used as a routine test for CIE activity and in patient monitoring [17,18], similar to that of humans.

Changes in other inflammatory markers after cell therapy have also been analyzed in dogs. Topical administration of MSCs has been shown to significantly decrease CD4, IL-1, IL-6, and tumor necrosis factor-alpha (TNF-α) levels in dogs affected by keratoconjunctivitis sicca [39].

Similarly, a decrease in CD8+ T lymphocytes, serum, and synovial C-reactive protein, was reported in dogs with ruptures of the cranial cruciate ligament at 4 and 8 weeks after intravenous and intra-articular injection of autologous canine bone marrow MSCs [40].

The mechanisms that control the dysregulated immune response following MSCs administration are still not entirely clear. The response of the MSCs to immune cells is highly complex and depends on the inflammatory signals in the microenvironment. MSCs can exert both pro- and anti-inflammatory effects; they adopt an anti-inflammatory phenotype during inflammatory conditions but maintain a proinflammatory phenotype in the absence of inflammation [12,41,42].

In tissues with a low concentration of TNF-α, interferon-gamma (IFN-γ), and other inflammatory cytokines, MSCs acquire an inflammatory phenotype after their engraftment, endorsing the host defense against infections [41,42]. Conversely, MSCs develop an anti-inflammatory phenotype when engrafted in tissues with high concentrations of the before mentioned inflammatory cytokines [42]. The increase in cytokines produced by the inflammatory immune cells benefits the generation of an immunoregulatory phenotype in MSCs and activates the further secretion of MSC-derived immunosuppressive soluble factors. Consequently, there are suppression effects of the MSCs on the immune response and inflammation boost [43,44].

The main limitation of this single-center study is the small cohort of client-owned dogs. Another limitation might be the lack of a comparison group of dogs with CIE treated with corticosteroids as standard treatment. However, the effect of corticosteroids and other immunosuppressants on white blood cells could hamper the correct interpretation of the results. It has been widely demonstrated that corticosteroids can decrease lymphocyte counts and increase peripheral granulocytes [45,46].

Furthermore, different results might be obtained according to the MSCs employed since the immunosuppressive ability of Ad-MSCs is both dose- and cell-passage-dependent [47]. Moreover, the donor age, sex, and tissue source of the MSCs might lead to significant variations in the chemical–physical characteristics of their secretome, thus affecting their immunomodulatory capacity [6–8,10–12,29].

1. Conclusions

This study describes the changes in white blood-cell-based inflammatory markers in dogs with CIE receiving an intravenous infusion of Ad-MSCs. Our results demonstrated that NLR, PLR, and SII decreased significantly at two months after MSCs administration, and NLR and SII reached normal levels at 9 months post-treatment. These changes in the blood inflammatory markers were accompanied by significant clinical improvement.

Author Contributions: Conceptualization, J.I.C., F.J.D. and E.M.P.-M.; methodology, J.I.C., J.U.-C., E.L., E.M.P.-M., F.J.D. and R.B.; data curation, J.I.C.; writing—original draft preparation, J.I.C. and E.M.P.-M.; writing—review and editing, F.J.D., J.U.-C.; supervision, E.M.P.-M.; project administration, J.U.-C. and E.M.P.-M.; funding acquisition, E.M.P.-M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The protocol was approved by the UEx Animal Care and Use Committee (File nº 20160822, approved 22 August 2018).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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TRABAJO 3

Relationship between serum cobalamin concentration and endoscopic ileal appearance and histology in dogs with chronic inflammatory enteropathy

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Relationship between serum cobalamin concentration and endoscopic ileal appearance and histology in dogs with chronic inflammatory enteropathy

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Abstract

Background: It has not been determined whether ileal appearance differs among dogs with chronic inflammatory enteropathy (CIE) and different serum concentrations of cobalamin.

Objective: To compare endoscopic and histologic ileal findings in dogs with CIE and different serum cobalamin concentrations and then evaluate the correlation of ileal changes to cobalamin serum concentration using updated scoring systems to assess the ileum.

Animals: Sixty-eight dogs with CIE.

Methods: Retrospective study. Frequency of ileal features and ileal histologic and endoscopic scores (IHS and IES) were obtained and compared among CIE dogs with severe hypocobalaminemia (SHC; <200 ng/L), hypocobalaminemia (HC; 200-350 ng/L), or normocobalaminemia (NC; >350 ng/L). The correlation of IHS and IES with cobalamin was evaluated.

Results: Friability, villus atrophy, crypt dilatation, epithelial injury, and intraepithelial lymphocytes were more frequent in SHC than in NC dogs (all $P \leq .01$). Median SHC-IES (2; range, 0-4) was higher than NC-IES (1; range, 0-5; $P = .004$). Median SHC-IHS (6; range, 3-9) was higher than HC-IHS (4; range, 1-7; $P < .001$) and NC-IHS (3; range, 1-8; $P < .001$). Cobalamin concentration correlated negatively with IES ($\rho = -.34$, $P = .005$) and IHS ($\rho = -.58$, $P < .001$).

Conclusions and Clinical Importance: Ileal features and involvement degree markedly differed when cobalamin was <200 or >350 ng/L in CIE dogs. With updated scales to assess the mucosa, greater ileal damage was associated with lower serum cobalamin concentration.

KEYWORDS
canine, endoscopy, histopathology, small bowel disease

Abbreviations: CCECAI, canine chronic enteropathy clinical activity index; CIBDAI, clinical inflammatory bowel disease activity index; CIE, chronic inflammatory enteropathy; HC, hypocobalaminemia; IES, ileal endoscopic score; IEL, intraepithelial lymphocytes; IHS, ileal histologic score; NC, normocobalaminemia; PLE, protein-losing enteropathy; SHC, severe hypocobalaminemia; VTH-UEx, Veterinary Teaching Hospital of the University of Extremadura; WSAVA, World Small Animal Veterinary Association.

1 | INTRODUCTION

Chronic inflammatory enteropathy (CIE) in dogs is a gastrointestinal disorder characterized by histological evidence of inflammation in the small intestine.[1] Endoscopy and biopsy sampling of the digestive tract is a crucial tool in the diagnosis of CIE. During the examination, ileoscopy is recommended due to the extreme disparity between ileal, duodenal, or colonic histopathologic findings.[2,3] Nevertheless, the tendency is to explore and biopsy only the stomach, duodenum, and colon due to the difficulty of ileal intubation.[4,5] In those cases, possible injuries to the ileum might have been overlooked.

It has been postulated that chronic mucosal disease affecting the ileum reduces the epithelial expression or function of the cubam receptor, leading to reduced mucosal uptake of cobalamin.[6] On this basis, serum cobalamin concentration might be expected to be a marker of ileal mucosa damage. However, a single earlier study failed to correlate hypocobalaminemia with increased ileal histologic scores obtained according to the World Small Animal Veterinary Association (WSAVA) criteria.[4] Nevertheless, that system showed a high subjectivity and did not include a specific evaluation of ileal biopsy specimens.[7,8]

A new simplified histological scale was developed that found associations not identified by using the WSAVA scheme.[9] However, the relationship between cobalamin and ileum injury has never been reassessed using this histological scale. An added difficulty is that hypocobalaminemia has been defined by different thresholds.[4,10-14] Similarly, a validated endoscopic activity scale for inflammatory bowel disease in dogs was described a few years ago.[15] Nevertheless, there is no description of the endoscopic appearance of the ileum in hypo- or normocobalaminemic dogs with CIE or of the correlation between ileal endoscopic and histologic findings.

Our hypotheses were that dogs with CIE will have different ileal features according to different serum cobalamin concentrations, and ileum damage assessed using updated scales will correlate with cobalamin concentration. If so, serum cobalamin concentration could provide clinicians with information about the severity of ileal damage in CIE dogs if the ileum is not explored and sampled.

Therefore, there were 3 goals of this study. First, to describe and compare histologic and endoscopic findings in the ileum of dogs with CIE with normal and low concentrations of serum cobalamin assessed using updated and validated scales. Second, to determine whether the association between serum cobalamin concentration and ileal mucosa damage can be detected with those scales different from the traditional WSAVA system. Third, to evaluate the relationship between ileal endoscopic activity, histologic changes, and clinical severity in light of those scoring systems.

2 | MATERIAL AND METHODS

2.1 | Study design and selection criteria

The electronic clinical database of the Veterinary Teaching Hospital of the University of Extremadura (VTH-UEx) was searched from January 2016 to December 2020 to identify retrospective cases of CIE in dogs. Only dogs with CIE (diagnosed based on the histologic finding of inflammatory infiltrates within the lamina propria) and in which full gastrointestinal endoscopy had been performed, ileum endoscopic biopsies had been obtained, and serum cobalamin concentration had been determined, were included in the study.

For each case, other causes of persistent signs of gastrointestinal disease (>3 weeks) were ruled out by routine hematologic and serum biochemical findings, fecal parasitology, abdominal ultrasonography, measurement of serum canine trypsin-like immunoreactivity concentration, and histopathological review of endoscopically obtained mucosal biopsy specimens. Proper food (hydrolyzed or selected protein diets not previously prescribed as Hill's z/d, and Royal Canin Hypoallergenic or Royal Canin GI Low Fat and Hill's i/d Low Fat in cases of protein-losing enteropathy [PLE] for a minimum of 4 weeks) and antibiotic trials (metronidazole 10 mg/kg, PO, q 12 hours for a minimum of 3 weeks) were completed before recommending endoscopic examination. Dogs with clinically relevant concurrent extragastrointestinal disease (pancreatitis, hepatic dysfunction, kidney disease, leishmaniasis, and ehrlichiosis), with causes of gastrointestinal disease other than inflammatory enteritis or for which a complete medical record could not be obtained, were excluded. Dogs were excluded if they had received corticosteroids or other anti-inflammatory or immunosuppressant medication within 2 weeks before undergoing endoscopy and sampling.

Recorded data included age, breed, sex, and the clinical severity of disease for each dog according to 2 previously published scoring systems: the Clinical Inflammatory Bowel Disease Activity Index (CIBDAI)[16] and the Canine Chronic Enteropathy Clinical Activity Index (CCECAI).[17] For each case, the presence of hypoalbuminemia (serum albumin concentration <3 g/dL) was also recorded.

Cobalamin values were analyzed by an external laboratory (Laboklin, Madrid, Spain). Dogs were placed in either the CIE with normal serum cobalamin concentration (>350 ng/L) group (NC), the CIE with hypocobalaminemia (cobalamin concentration 200-350 ng/L) group (HC), or the CIE with severe hypocobalaminemia (cobalamin concentration <200 ng/L) group (SHC). The lower cutoff of 200 ng/L was chosen because it has been proven to be significantly associated with disease outcome[17] and was used in a referential study[4] whose results will be compared to ours. The upper cutoff of 350 ng/L was chosen based on the increased likelihood of clinically important cobalamin deficiency below this concentration; in veterinary practice, cobalamin supplementation is commonly initiated whenever serum cobalamin concentration is <350 ng/L.[10,12,13]

2.2 | Ileum assessment

The ileal endoscopic examination was carried out using a Fuji 2200 or a Storz PV-SG28-140 flexible video endoscope. The endoscopy video recordings of each case were reviewed, assessed, and scored independently by 2 experienced endoscopists using a validated quantitative endoscopic activity score for inflammatory bowel disease in dogs.[15]

This scale grades ileum granularity, friability, erosions, and lymphatic dilatation from 0 to 2 (0, absent; 1, moderate; 2, severe) with a maximum lesion score of 8. Each dog was assigned an ileal endoscopic score (IES; the mean of the scores of the 2 endoscopists).

Flexible endoscopic biopsy forceps with 2.5-mm smooth-edged oval cups were used to collect 8 to 10 mucous membrane specimens from the ileum for histopathological evaluation. All ileal histologic preparations were re-examined and reviewed by a single board-certified pathologist blinded to the endoscopy exam and clinical information for each case. To obtain the ileal histologic score (IHS) for each dog, different morphologic/inflammatory features (villus stunting, crypt dilatation, lacteal dilatation, surface epithelial injury, and lamina propria infiltrates: lymphocytes, eosinophils, and neutrophils, or neutrophils) were scored 0 (absent), 1 (mild), 2 (moderate), or 3 (severe), according to a simplified histopathologic scoring system.[9] The maximum possible score was 21. The presence of intraepithelial lymphocytes (IEL) was independently scored in the same way.

1.1 | Statistical analysis

Data were collected and imported into SPSS Statistics for Windows, version 26 (IBM Corp, Armonk, New York). Categorical data were described using frequencies and proportions. Normality was assessed using a Shapiro-Wilk test. Most data were not normally distributed, so their descriptive statistics were reported as median and range (min-max).

Kruskal-Wallis and Mann-Whitney post hoc tests were used for between-group comparisons of quantitative variables (albumin and cobalamin concentrations, IHS, IES, CIBDAI, and CCECAI). Categorical variables (histologic and endoscopic features) were compared by the chi-squared or Fisher's exact test. For such analyses, ordinal categorical variables were converted into binary variables where its absence was scored as 0 points and its presence as 1 point. The correlations between variables were evaluated by Spearman's correlation analysis. Statistical significance was set at $P < .05$.

2 | RESULTS

2.1 | Study sample

Sixty-eight dogs were included in this study. There were 20 mixed-breed dogs, 4 German Shepherd dogs, 3 each of Cocker Spaniel, Labrador Retriever, and West Highland White Terrier, 2 each of American Staffordshire Terrier, English Beagle, English Setter, Boxer, Shar Pei, Siberian Husky, Medium Poodle, Spanish Hound, French Bulldog, Epagneul Breton, Pyrenean Mastiff, and Weimaraner and 1 each of Argentine Dogo, Bernese Mountain Dog, Dalmatian, Border Collie, Greyhound, Belgian Shepherd Malinois, Giant Schnauzer, Chow Chow, Portuguese Water Dog, Staffordshire Bull Terrier, and Standard Schnauzer. Forty-one dogs were male (4 castrated), and 27 were female (10 spayed). The study sample median age was 5 years (range, 1-14 years), and there was no significant difference in age between groups ($P = .53$).

Twenty-nine dogs (29/68, 43%) were normocobalaminemic (NC group). Thirty-nine (39/68, 57%) showed low concentrations of cobalamin, among which 19/68 (28%) were assigned to the HC group and the remaining 20/68 (29%) to the SHC group.

2.2 | Clinical indices and laboratory findings

Significant differences were found in CIBDAI among the 3 groups, and CCECAI was significantly different between the SHC and the other 2 groups. However, CCECAI scores were similar between the HC and NC groups (Table 1). As anticipated by group definitions, differences in cobalamin concentration among the 3 groups were statistically significant (Table 1). In the NC group, 7/29 (24%) dogs showed hypoalbuminemia (median albumin concentration, 3.2 g/dL; range, 1.70-4.20 g/dL). In groups HC (median albumin concentration, 3.2 g/dL; range, 1.40-3.80 g/dL) and SHC (median albumin concentration, 2.79 g/dL; range, 1.7-3.7 g/dL), 5/19 (26%) and 13/20 (65%) dogs, respectively, showed low concentrations of cobalamin and albumin simultaneously. Albumin concentration did not differ significantly among the 3 groups ($P = .09$).

TABLE 1 Data of the indices analyzed in the three groups of dogs with CIE according to the cobalamin serum concentration

	SHC	HC	NC	*P*
Cobalamin concentration (ng/L)	145 (95-198)[a]	288 (200-343)[b]	415 (354-804.40)[c]	<.0001
CIBDAI	10 (6-14)[a]	7 (1-8)[b]	5 (2-11)[c]	<.0001
CCECAI	8 (8-15)[a]	7 (2-10)[b]	5 (2-14)[b]	<.0001
IHS	6 (3-9)[a]	4 (1-7)[b]	3 (1-8)[b]	<.0001
IES	2 (0-4)[a]	1 (0-4)[ab]	1 (0-5)[b]	.009

Note: Data are represented by median (min-max range). Reported *P* values are for the Kruskal-Wallis test. Within a row, data without a common superscript differ ($P < .05$).

Abbreviations: CCECAI, canine chronic enteropathy clinical activity index[17]; CIBDAI, clinical inflammatory bowel disease activity index[16]; CIE, chronic inflammatory enteropathy; HC, hypocobalaminemia; IES, ileal endoscopic score[15]; IHS, ileal histologic score[9]; NC, normocobalaminemia; SHC, severe hypocobalaminemia.

TABLE 2 Comparative of the endoscopic findings in the ileum of dogs with CIE between the different cobalamin groups

	SHC	HC	NC	*P*
Friability	8/20 (40%)[a]	3/19 (15.8%)[ab]	3/29 (10.3%)[b]	.03
Granularity	13/20 (65%)[ab]	14/19 (73.7%)[a]	12/29 (41.4%)[b]	.03
Erosions	3/20 (15%)[a]	0/19 (0%)[a]	3/29 (10.3%)[a]	.23
Lymphangiectasia	8/20 (40%)[a]	6/19 (31.6%)[a]	5/29 (17.2%)[a]	.42

Note: *P* values correspond to chi-squared test. Within a row, data without a common superscript differ ($P < .05$).
Abbreviations: CIE, chronic inflammatory enteropathy; HC, hypocobalaminemia; NC, normocobalaminemia; SHC, severe hypocobalaminemia.

TABLE 3 Comparative of the histopathologic findings in the ileum of dogs with CIE between the different cobalamin groups

	SHC	HC	NC	*P*
Villus atrophy	18/20 (90%)[a]	12/19 (63.2%)[ab]	11/29 (37.9%)[b]	<.0001
Epithelial injury	8/20 (40%)[a]	6/19 (31.6%)[ab]	3/29 (10.3%)[b]	.04
Crypt dilatation	16/20 (80%)[a]	13/19 (68.4%)[a]	8/29 (27.6%)[b]	.002
Lacteal dilatation	11/20 (55%)[a]	6/19 (31.6%)[a]	8/29 (27.6%)[a]	.11
LP lymphocites	20/20 (100%)[a]	19/19 (100%)[a]	29/29 (100%)[a]	1
LP eosinophils	4/20 (20%)[a]	3/19 (15.8%)[a]	4/29 (13.8%)[a]	.84
LP neutrophils	8/20 (40%)[a]	3/19 (15.8%)[a]	7/29 (24.1%)[a]	.39
IEL	14/20 (70%)[a]	3/19 (15.7%)[b]	3/29 (10.3%)[b]	<.0001

Note: *P* values correspond to chi-squared test. Within a row, data without a common superscript differ ($P < .05$).
Abbreviations: CIE, chronic inflammatory enteropathy; HC, hypocobalaminemia; IEL, intraepithelial lymphocites; LP, lamina propria; NC, normocobalaminemia; SHC, severe hypocobalaminemia.

1.1 | Endoscopic findings

Endoscopic intubation and exploration of the ileum were achieved in all dogs. The most common endoscopic abnormality recorded, present in 39/68 (57%) cases, was the increase in mucosal texture (granularity), mostly to a mild degree, followed by lymphangiectasia in 19/68 (28%) cases. Friability was noted in 14/68 (20%) dogs, and 6/68 (9%) dogs exhibited mucosal erosions.

The frequency of each endoscopic lesion according to severity degree and group is reported in Table S1. Friability, erosions, and lymphangiectasia were most frequent in the SHC group, and granularity was most common in the HC group. However, the only significant differences were in the proportion of friability between SHC and NC dogs and the proportion of granularity between HC and NC dogs (Table 2). Furthermore, the SHC group showed the highest frequency for every severity category of each endoscopic lesion, except for the mild increase in granularity, which was found most often in the HC group.

Finally, concerning IES, the maximum score obtained was 5 (out of a possible 8), and 59/68 (87%) dogs obtained scores between 0 and 3. The IES was significantly higher in the SHC group than in the NC group. There were no other differences among the groups (Table 1).

1.2 | Histopathologic findings

Ileal biopsy samples were obtained by endoscopy in all cases. All dogs had primary lymphoplasmacytic inflammation and showed some alteration in the studied ileal histologic features, to varying extents. An increased number of lamina propria lymphocytes was observed in all dogs, to a moderate degree in 37/68 (54%) cases. The second and third most frequent features present in the ileal biopsies were villous atrophy, found in 41/68 (60%) dogs, and crypt dilatation, found in 37/68 (54%) dogs, followed by lacteal dilatation in 25/68 (37%) dogs and an increase in IEL in 20/68 (29%) dogs. Infiltration of neutrophils in the lamina propria (18/68, 26%), epithelial injury (17/68, 25%), and infiltration of eosinophils in the lamina propria (11/68, 16%) were the less common abnormalities.

The SHC group had a significantly greater proportion of dogs with villus atrophy and epithelial injury than the NC group and a greater proportion of cases with increased IEL than both the NC and HC groups. The occurrence of crypt dilatation was significantly higher in the SHC and HC groups than in the NC group. Epithelial injury, lacteal dilatation, and lamina propria infiltration were more frequently observed in the SHC group, but the differences among the groups for all 3 features were not significant (Table 3).

The frequency of each histopathologic feature according to severity degree and group is presented in Table S2. Moderate and severe degrees of histologic features were observed most frequently in the SHC group. The highest IHS obtained was 9 (out of a maximum possible of 21), and 48/68 (65%) dogs had scores ≤5. Among the 3 groups, IHS was significantly higher in the SHC group. No differences in IHS were found between the HC and NC groups (Table 1).

TABLE 4 Spearman correlation coefficients between the different variables analyzed in CIE dogs

		IES	IHS	IEL	CIBDAI	CCECAI	ALB
Cobalamin		ρ = −.34 *P* = .005	ρ = −.58 *P* < .001	ρ = −.46 *P* < .001	ρ = −.58 *P* < .001	ρ = −.51 *P* < .001	ρ = .21 *P* = .08
All population	IES		ρ = .49 *P* < .001	ρ = .11 *P* = .33	ρ = .25 *P* = .03	ρ = .31 *P* = .009	
	IHS			ρ = .46 *P* < .001	ρ = .67 *P* < .001	ρ = .70 *P* < .001	
NC	IES		ρ = .50 *P* = .006		ρ = .24 *P* = .90	ρ = .03 *P* = .85	
	IHS				ρ = .25 *P* = .18	ρ = .27 *P* = .15	
HC	IES		ρ = .18 *P* = .44		ρ = .43 *P* = .06	ρ = .41 *P* = .77	
	IHS				ρ = .52 *P* = .01	ρ = .67 *P* = .002	
SHC	IES		ρ = .08 *P* = .73		ρ = .24 *P* = .30	ρ = .09 *P* = .68	
	IHS				ρ = .56 *P* = .009	ρ = .55 *P* = .01	

Abbreviations: ALB, albumin; CCECAI, canine chronic enteropathy clinical activity index[17]; CIBDAI, clinical inflammatory bowel disease activity index[16]; CIE, chronic inflammatory enteropathy; HC, hypocobalaminemia; IEL, intraepithelial lymphocites; IES, ileal endoscopic score[15]; IHS, ileal histologic score[9]; NC, normocobalaminemia; SHC, severe hypocobalaminemia.

1.1 | Correlation study

According to Spearman's test, cobalamin concentration showed a weak negative association with IES and a moderate negative correlation with IHS. An increased presence of IEL was also moderately correlated with lower serum cobalamin. Similarly, a moderate negative correlation of cobalamin with CCECAI and CIBDAI was observed. No correlation was found between cobalamin and albumin concentrations. Endoscopic and histologic scores were somewhat correlated. IEL showed a moderate positive correlation with the histologic score but not with the endoscopic score. CCECAI and CIBDAI were directly and moderately correlated with IHS but poorly with IES (Table 4).

The within-group correlations showed no correlation between the clinical indices and histological or endoscopic indices in the NC group. However, IHS and IES were significantly positively correlated. On the contrary, a significant positive correlation was detected between the clinical indices and IHS in the HC and SHC groups, but not with IES nor between histologic and endoscopic indices (Table 4).

2 | DISCUSSION

This study compares the endoscopic and histologic abnormalities in the ileum of CIE dogs with different serum cobalamin concentrations. The only 3 studies that previously addressed ileal findings in dogs with chronic enteropathy focused on potential associations with the duodenal mucosa histology[2-4] or possible correlations between histological changes and clinical activity.[9] However, none provided any description of ileal features and their frequency in dogs with enteropathy, and only 1 secondarily addressed the cobalamin impact.[4]

The gross endoscopic appearance of the ileum has been traditionally assessed using the endoscopic guidelines developed by the WSAVA Gastrointestinal Standardization Group.[8] During endoscopy, the report forms provided are extremely useful in helping to ensure that examinations are complete. However, the endoscopic activity score used in this study is the only validated scale in veterinary medicine to assess endoscopy activity in dogs with inflammatory bowel disease.[15] It was designed to exclude the more infrequent or useless parameters and select those features with the best interobserver agreement and reproducibility among experienced endoscopists. However, neither the WSAVA guidelines nor the scale used in this study provided any representative image of the peculiarities of the ileal mucosa in dogs with CIE.

In our study, the most frequent findings were increased mucosal texture (granularity) and multifocal white granular foci present in the ileal mucosa. Granularity has never been reported as a feature of the ileum affected by CIE, but ileal lymphangiectasia has been described previously in 3 dogs from a small sample of 10 with gastrointestinal disorders.[18]

We showed that endoscopic lesions, such as erosions, lymphangiectasia, and particularly mucosal friability, were more likely, and gross ileal injury was significantly greater in dogs with CIE when the cobalamin concentration was <200 ng/L than when it was >350 ng/L. In addition, intermediate concentrations provided little useful information, and the endoscopic ileum involvement could resemble that of 1 or other extreme. However, because cobalamin and endoscopic activity demonstrated a weak inverse correlation in

our study, it might be expected that as cobalamin approaches the upper margin, the characteristics of the ileum resemble more closely those of normocobalaminemic dogs and vice versa.

According to a recent study, ileoscopy can be challenging and involve certain risks. Iatrogenic ileocecocolic perforation is a rare but serious complication that might occur, mainly when lower endoscopy is performed by a novice endoscopist.[19] This might be a deterrent to performing routine ileoscopy and could be a possible reason for the lack of knowledge on ileum appearance.

It has been stated that low serum cobalamin concentration suggests a focal or diffuse mucosal disorder affecting absorption in the distal (ileum) small intestine and is an indication for endoscopy.[5] The WSAVA International Gastrointestinal Standardization Group recommended obtaining ileal biopsies in animals whenever gastroduodenoscopy or colonoscopy seems indicated because ileal biopsy is recognized as providing valuable information not always found in duodenal or colonic biopsies.[8] However, the decision to perform ileoscopy is not usually determined by the cobalamin concentration. In fact, cobalamin concentration is often unknown at the time of the endoscopy, as the results of the gastrointestinal panel might be pending when the procedure is performed based on the client appointment and the hospital schedule. Then, the final decision is dependent mainly on the endoscopist's experience, clinical signs, or even on anesthetic considerations because of the increased exploration time.[4,5] Our study demonstrated that cobalamin might be a suitable indicator of ileal involvement in CIE dogs without ileoscopy. Nevertheless, the decision to perform ileoscopy should be based on the diagnostic value of the ileum exploration and sampling. An early study in dogs found that the concordance of histologic diagnosis between duodenal and ileal sites was high for lymphoplasmacytic enteritis (73%) but low for eosinophilic enteritis (17%).[3] The same study also confirmed the discordance between upper and lower gastrointestinal biopsies for small-cell lymphoma in dogs.[3] In our opinion, these are compelling reasons to perform ileoscopy. Nevertheless, in those cases in which CIE was the diagnosis and endoscopy did not include ileoscopy, this study and cobalamin concentration might provide the clinician with insight into the ileum's condition.

The simplified histopathologic model[9] used in this study was designed and later refined as an extension of the original WSAVA criteria[7,8] to reduce interobserver variability and improve consistency in the diagnostic interpretation of gastrointestinal inflammation between pathologists. Furthermore, the WSAVA system does not include ileal templates for histopathologic evaluation of ileal biopsies, although there is a general agreement on the need to explore and sample the ileum.[7-9] Consequently, the ileum has usually been assessed and scored as the duodenum. Our study now offers a description of the specific characteristics of the ileal mucosa of dogs with CIE.

All dogs in this study showed some histopathological abnormality. In a previous study, microscopic lesions were found in 80% of ileal samples of dogs with enteropathy.[2] Accordingly to 1 study, the likelihood of obtaining adequate mucosal samples and identifying pathology is better in the ileum than in the duodenum due to its relatively thinner mucosa.[5]

As noted macroscopically, histopathological changes in the ileal mucosa are more frequent and severe when the cobalamin serum concentration is <200 ng/L, and significantly more scarce and milder abnormal features can be observed when concentrations are >350 ng/L. However, our study detected an increased presence of crypt dilatation in the intermediate range when the cobalamin fell <350 ng/L.

The presence of IEL in the small intestine was not included as a parameter in the simplified histopathologic scoring system.[9] However, because it has been correlated with hypocobalaminemia,[4] we decided to analyze it separately. This was valuable as it confirmed the relationship between lower cobalamin concentration and the rise of IEL. Moreover, the proportion of increased IEL proved to be the only parameter that differed between the 2º of hypocobalaminemia in our study. Furthermore, dogs with SHC had a greater proportion of villous stunting, epithelial injury, crypt dilatation, and IEL and to a more severe degree than NC dogs.

In our study, morphologic features, except for lacteal dilatation, vary significantly between cobalamin concentrations, whereas inflammatory infiltrates, except for IEL, were not different between groups. This phenomenon was also observed in the small intestine when histopathologic features were compared between hypo- vs normoalbuminemic dogs diagnosed with CIE.[20] Another study argued that morphological features appeared to be more important than the intensity of duodenal inflammation in the assessment of lymphoplasmacytic enteropathy in dogs.[21]

Our data shows that ileal involvement can be found more frequently when cobalamin is <200 ng/L, with features distinct from those observed when the concentration is >350 ng/L. Furthermore, the severity of the histologic injury is significantly greater when the cobalamin concentration is <200 ng/L than >200 ng/L. These findings support previous studies in which dogs with chronic enteropathy and initial serum cobalamin concentrations below the cutoff value of 200 ng/L had a markedly higher chance of a negative outcome.[17] Thus, 200 ng/L is the value below which hypocobalaminemia should be considered in studies on CIE in dogs.

Our study confirms the correlation between hypocobalaminemia and the clinical severity of disease (CCECAI) reported previously.[4,22] Contrarily, however, while the presence of hypocobalaminemia could not be related to the ileal histologic WSAVA scores,[4] we found that cobalamin concentration was inversely associated not only with the ileal histopathologic damage but also with endoscopy activity in our study. The assessment scales used in our study have likely contributed to achieving these results.

Previously, it was claimed that of the 3 mechanisms that reduce cobalamin availability from the small intestine (congenital disorders of receptor function, excess competition from the intestinal microbiome or decreased mucosal absorptive capacity) the latter was likely to be the most important in clinical veterinary medicine.[23] Cobalamin is essential for many cell functions and mucosal regeneration, and a deficiency can contribute to mucosal inflammatory infiltration and villous atrophy.[24,25] On this basis, and the correlation between hypocobalaminemia and the increased number of IEL and lacteal

dilatation in the ileal mucosa, an association between ileal mucosal inflammatory changes and hypocobalaminemia in dogs with chronic enteropathy has been expected.[6,21] Our results confirmed the relationship and showed that cobalamin might be considered an indicator of ileal mucosa involvement in dogs with CIE. This exploratory research should be complemented by an evaluation of the expression of cobalamin-receptors in dogs with CIE.

Our third aim was to review the relationships of clinical severity with endoscopic and histologic ileal activities using updated scales rather than the WSAVA's, which could not establish any association between clinical activity and ileal histology.[4] More recently, a nearly significant positive correlation between the CCECAI and the IHS was found using the simplified scoring system.[9] The present study goes a step further, definitively establishing a moderate positive correlation between the IHS and clinical activity. The reasons for this improved correlation might lie in the larger sample of this study and the study sample cobalamin serum concentrations. We noted that clinical and histologic ileal indices directly correlated in the dogs with cobalamin concentrations <350 ng/L but not in the NC group. Thus, the correlation between ileal histology and clinical activity improves at lower cobalamin concentrations. Conversely, the study referred to earlier[9] did not provide data concerning cobalamin concentration.

A few studies have attempted to associated endoscopic lesions with clinical severity and histopathologic features.[16,26-28] Each employed a different endoscopic scale, but all agreed on the lack of a relationship among them. The absence of a validated scale and the differences in operator experience have been suggested to explain these discordant results. With the use of a validated endoscopic scale and a simplified histologic scale, a slight positive correlation between endoscopic and histopathologic involvement of the ileum was found in our study. Nevertheless, we did not observe a relationship between clinical and endoscopy activity.

The within-group correlations showed how histology and endoscopy are only associated in dogs with normal cobalamin concentrations, probably due to the scarcity of lesions in the ileum at both the microscopic level and the macroscopic level. On the contrary, as cobalamin decreases, the microscopic changes occur with greater intensity than the macroscopic ones, thus resulting in a lack of correlation between the endoscopic and histological indices.

In this study, the more severe ileal damage correlated not only with lower cobalamin concentration but also with higher clinical indices. Low cobalamin concentration and high clinical activity indices have been previously associated with poor prognosis.[17] Thus, it could be inferred that high endoscopic and histologic ileal scores together with high clinical indices and low cobalamin concentration could worsen the prognosis of dogs with CIE. Future research that includes the outcome of dogs with CIE is required to determine the effect of the endoscopic and histologic ileal damage on the prognosis of the disease.

In this study, hypoalbuminemia and hypocobalaminemia coexisted more frequently in animals with higher clinical activity and more severe and extensive intestinal involvement, as noted previously.[17,21,29] Although some studies associated hypocobalaminemia with hypoalbuminemia in dogs with chronic enteropathy,[17,22,30] no correlation between the trend of cobalamin and albumin concentrations was found in the present study. Despite this, it is indisputable that the proportion of hypoalbuminemic individuals was highest in the group of dogs with SHC. The prevalence of hypocobalaminemia ranges from 19% to 54% in dogs with chronic enteropathies[14] but reportedly increases to 43%-75% in dogs with nonneoplastic, noninfectious causes of PLE.[6] However, a review showed that low serum cobalamin was described in only 8 of 23 studies on PLE dogs.[31] Despite this, a clear limitation of our study is the lack of a group containing dogs with PLE. Consequently, future research to investigate and compare the ileal changes in hypo- and normocobalaminemic PLE and non-PLE dogs is recommended.

This study has other limitations. The first is the low quality of biopsied samples obtained endoscopically because of the potential to miss lesions deep within the submucosal and muscularis layers.[32] As mentioned above, biopsy sample quality is influenced by operator experience, the endoscopic technique of sample collection, the number of specimens collected, and the biopsy specimen handling. Superior endoscopic specimen quality is obtained by using an endoscope that allows the largest forceps possible (2.8 better than 2.2). Fenestrated forceps cause fewer crush artifacts and yield larger biopsy specimens than nonfenestrated models.[5] Six adequate or 10 to 15 marginal samples should be collected from the canine duodenum, depending on the lesion being sought. The exact number of recommended endoscopic specimens from the ileum is unknown, although 3 to 5 adequate biopsies seem to be enough.[33] To be considered adequate, a biopsy sample should contain the full thickness of the mucosa and be wide enough to have at least 3 to 4 intact and preferably contiguous villi. Fewer biopsy samples are needed to establish a diagnosis as the quality of the tissue increases. However, it is typically easy to obtain high-quality biopsies from the ileum because ileal mucosa is relatively thin, allowing for full-thickness specimens with minimal effort.[5] In general, skilled endoscopists must take fewer samples than less-skilled endoscopists to achieve the same number of adequate samples.[8] Tissue samples should be carefully removed from the biopsy forceps and placed directly into 10% formalin or collected in a foam-lined tissue cassette before fixation.[5,8] In an effort to maximize the quality of the histopathologic analysis and to fulfill the recommendations mentioned, 2.5-mm smooth-edged fenestrated oval-cupped biopsy forceps were used, and a minimum of 8 to 10 good-quality biopsies were collected by experienced endoscopists in this study. Samples were submitted in 2 forms: free-floating in formalin; in commercial cassettes with sponges.

Another limitation of this study is that mainly medium- and large-sized dogs, which allowed ileal intubation with an endoscope of 8 to 9 mm in diameter and a 2.8-mm channel, were included. Thus, breeds highly predisposed to PLE, like the Yorkshire Terrier, are underrepresented in this study. No purebred Yorkshire Terrier, Bichon Frise, or Maltese was included but only crosses of those and similar breeds in which ileoscopy could be performed with the endoscope described.

Finally, a breed predisposition for hypocobalaminemia has been identified.[11] Predisposed dogs, like German Shepherd dogs (n = 4),

Labrador Retrievers (n = 3), Shar Peis (n = 2), Greyhounds (n = 1), and Border Collies (n = 1), represented 16% (11/68) of the study sample in our study. Although we cannot rule out potential selection bias, other studies on CIE described cohorts with a similar proportion of those breeds.[3,4,10,17,21,22,25,29,34-37] In those in which cobalamin concentration was also detailed, the percentages of hypocobalaminemic dogs[4,10,21,22] were similar to that of our study. Only 2 studies reported percentages of dogs with cobalamin <200 ng/L higher (42%)[3] or lower (19%)[17] than that we have observed (30%).

Therefore, this study sample can be considered representative of what could be found in routine clinical practice.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

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TRABAJO 4

Oxidative Stress in Dogs with Chronic Inflammatory Enteropathy Treated with Allogeneic Mesenchymal Stem Cells.

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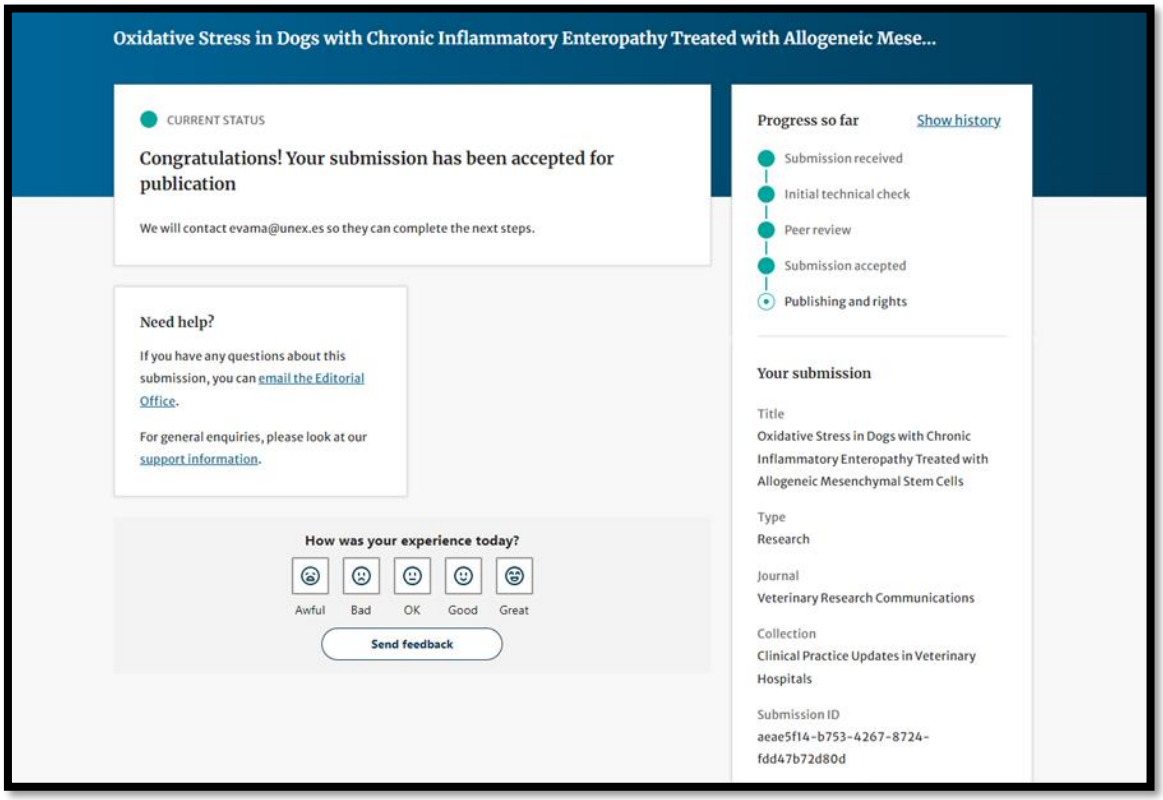

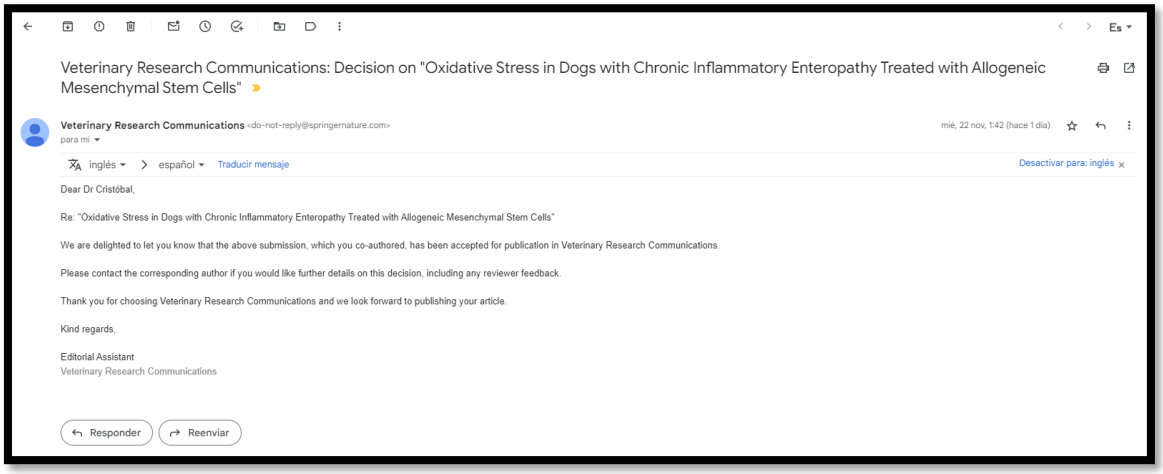

Oxidative Stress in Dogs with Chronic Inflammatory Enteropathy Treated with Allogeneic Mesenchymal Stem Cells

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Abstract

The search for new biomarkers in patients with chronic inflammatory enteropathy (CIE) is ongoing in the human and veterinary medicine fields. Oxidative stress biomarkers (malondialdehyde [MDA], reduced glutathione [GSH], and albumin) have been studied in humans with chronic enteropathies, but among them, only albumin has been studied in dogs with CIE. Moreover, the effect of mesenchymal stem cell (MSCs) treatment with or without prednisone on these parameters has never been studied in dogs with CIE. These parameters were compared between healthy dogs (n = 12) and dogs with CIE, and before and 1, 3, 6, and 12 months after the treatment with MSCs alone (n = 9) or together with prednisone (n = 11). The relationship between the Canine Inflammatory Bowel Disease Activity Index (CIBDAI) and oxidative stress was evaluated. Albumin was the only parameter that significantly differed between dogs with CIE and healthy dogs (p = 0,037). Differences were observed only in albumin values after combined treatment with MSCs and prednisone. No differences were observed in MDA and GSH after treatment with MSCs with or without prednisone. Albumin could help stage canine CIE, as well as its prognosis, as has already been demonstrated, although it is essential to evaluate this parameter for its antioxidant capacity, and therefore it could be a good biomarker of oxidative stress in this pathology. However, the treatment with MSCs seems unable to modify any of the analyzed oxidative stress parameters.

Keywords: chronic inflammatory enteropathy; mesenchymal stem cells; malondialdehyde; glutathione; albumin.

Introduction

Canine chronic inflammatory enteropathy (CIE) comprises a chronic idiopathic inflammation of the gastrointestinal mucosa tract alternating periods during which the patient is stable with flares of disease activity (Dandrieux 2016). According to whether the patient responds to dietary changes or immunosuppressant therapy the disease is called food-responsive enteropathy or immunosuppressant-responsive enteropathy, also known as IBD (inflammatory bowel disease). Until recently, a third category named antibiotic-responsive diarrhea (ARE) was recognized as one form of canine enteropathy. However, more recent works recommended avoiding the empirical antimicrobial treatment trial in the diagnostic work-up of canine CIE, due to the detrimental effects of the indiscriminate use of antibacterial drugs on the patient and the general population (Cerquetella et al. 2020). In addition, the low number of dogs responsive to antibiotics casts doubts on the true existence of an ARE group (Cerquetella et al. 2020; Jergens and Heilmann 2022).

Patients that do not respond to any of the treatments are included in the non-responsive enteropathies group (Dandrieux 2016; Isidori et al. 2022).

Oxidative stress has been demonstrated to play an important role in the pathogenesis of IBD in humans and dogs (Rezaie et al. 2007; Rubio et al. 2016 a, b; Segarra et al. 2016; Yuksel et al. 2017; Rubio et al. 2017). Oxidative stress occurs due to the excessive production of oxidative radicals or the lack of antioxidant molecules (Strober et al. 2007). In IBD, the excessive immune response that occurs secondary to chronic inflammation and tissue poor perfusion due to mucosal damage leads to the overproduction of reactive oxygen and nitrogen species (ROS/RNS). Structural modification and functional inhibition of cellular lipids, proteins, carbohydrates, and DNA associated with ROS/RNS-derived oxidative damage contribute to the development and progression of inflammation of the intestinal mucosa (Pravda 2005; Circu and Aw 2011).

Because of all these characteristics, certain antioxidants and oxidative compounds such as Trolox equivalent antioxidant capacity (TEAC), cupric reducing antioxidant capacity (CUPRAC), paraoxonase 1 (PON1) ferric reducing ability of plasma (FRAP) and total serum thiol concentrations have been studied to

evaluate the antioxidant response and oxidative damage in dogs with IBD (Rubio et al. 2016 a, b; Segarra et al. 2016; Rubio et al. 2017).

Other oxidative stress parameters, studied mainly in tissues and biological fluids from humans, include malondialdehyde (MDA), reduced glutathione (GSH), and albumin (Anderson 1998; Sitar et al. 2013; Rubio et al. 2017).

MDA is produced during lipid peroxidation, a process involved in the pathogenesis of numerous inflammatory diseases and malignancies. It promotes intramolecular or intermolecular protein/DNA cross-linking, altering the biochemical properties of biomolecules, leading to different pathological states (Ayala et al. 2014). GSH is an endogenous antioxidant that acts against reactive nitrogen intermediates and has detoxifying effects against malignant endobiotics and xenobiotics. It is therefore a good indicator of cell functionality and viability and has been associated with multiple pathological processes (Denzoin Vulcano et al. 2013). Albumin is the most abundant protein in the body and is used as a marker of protein reserves and nutritional status, although it is also an important extracellular antioxidant molecule. Its antioxidant properties include the elimination of free radicals and the provision of the thiol group (Tabata et al. 2021).

In humans, these three parameters have been shown to be biomarkers of oxidative stress in Crohn's disease (CD) (Boehm et al. 2012; Szczeklik et al. 2018; Su et al. 2019). In veterinary patients, albumin has been studied in dogs with CIE from the point of view of nutritional status; however, recently, in multiple investigations, more importance has been given to the redox status of albumin as an aggravating factor in multiple pathologies, which could be important when creating new drugs (Tabata et al. 2021). Furthermore, in patients with CIE it has been described as an important marker of poor prognosis (Volkman et al. 2017).

In veterinary medicine, the oxidative role of MDA has been studied in dogs with atopic dermatitis (Kapun et al. 2012), congestive heart failure (Nemec Svete et al. 2021), different types of cancer (Macotpet et al. 2013), dogs infected by Babesia (Crnogaj et al. 2010) and cats infected by coronavirus (Kayar et al. 2015). GSH has been studied in dogs and cats with hepatopathies (Center et al. 2002), cardiovascular diseases, numerous tumors (Viviano et al. 2009), and hemolytic and non-hemolytic anemia (Woolcock et al. 2020). However, they have never been investigated in patients with CIE.

Moreover, the safe administration and the anti-inflammatory capacity of allogeneic adipose-derived mesenchymal stem cells (MSCs) in the treatment of CIE in dogs has been described in previous research (Pérez-Merino et al. 2015; Cristóbal et al. 2021; Cristóbal et al. 2022). Although the antioxidant properties of the MSCs, exerted either by decreasing the activity of oxidizing agents or promoting the antioxidant defenses, have been demonstrated in different diseases, such as gastrointestinal inflammation, and ischemic injuries (Eiro et al. 2022), the variation in oxidative stress after the application of this novel cell therapy in dogs with CIE remains unexplored.

Considering the above, the present study has two aims. The first is to evaluate the plasma concentration of the oxidative stress biomarkers MDA, GSH, and albumin in healthy dogs and dogs with CIE. The second aim is to investigate possible changes in these parameters in dogs with CIE treated with MSCs with or without concomitant prednisone.

Materials and Methods

This research was developed at the Veterinary Teaching Hospital of the University of Extremadura. The Animal Care and Use Committee of the UEx and the Government of Extremadura approved this study (File No. 20160822, approved 22 August 2018). The owners of the animals included in the study were informed in detail and signed an informed consent.

Groups

A control group of clinically healthy dogs over one year old, with no digestive signs or other pathologies and no treatments in the last year, attending the internal medicine clinic of the Veterinary Teaching Hospital of the University of Extremadura (VTH-UEx) for routine check-ups, was formed to compare oxidative stress biomarkers between dogs with CIE and healthy dogs. The absence of alterations in routine blood tests or abdominal ultrasound was also an inclusion criterion for this group.

A group of dogs with CIE was formed including dogs over one year of age, with digestive signs of more than three weeks of evolution that did not respond to diet, antibiotic, and immunosuppressant-based treatments previously established according to standard guidelines (Jergens and Heilmann 2022). Complete anamnesis, physical examination, complete blood count and blood biochemistry (including measurement

of albumin, folic acid, cobalamin, and trypsin-like immunoreactivity [TLI]), urinalysis, coprological analysis with Giardia test and abdominal ultrasound were performed for the diagnosis of CIE. Those in which inflammation was confirmed by histopathological analysis of endoscopic biopsies were included. Dogs with other pathologies, sepsis, physical damage, and pregnant bitches were excluded.

The group of dogs with CIE was subdivided into two treatment groups to compare the effect of treatment on oxidative stress biomarkers in dogs with CIE:

- MSCs group: untreated dogs at least 21 days prior to MSCs administration.

- MSCs+Prednisone (P) group: dogs treated with prednisone (doses between 0.75 and 2 mg/kg) due to worsening symptomatology during the washout period of 21 days prior to MSCs infusion. After treatment, if the Canine Inflammatory Bowel Disease Activity Index (CIBDAI), described by Jergens and colleagues (Jergens et al. 2003), improved (with a decrease of more than 30% from the previous value), the prednisone dose was reduced. If not, the prednisone dose was not changed.

Study design

Blood was collected from all dogs in both groups prior to any treatment and plasma was frozen at -85 °C to analyze and compare MDA, GSH, and albumin levels between the Control Group and CIE group. The resulting CIBDAI of the CIE dogs was related to the three oxidative stress biomarkers.

A single dose of adipose tissue-derived allogeneic MSCs was administered to patients with CIE from the MSCs and MSCs+P groups. Intravenously, 4×10^6 cells/kg body weight diluted in physiological saline (100 - 250 ml depending on the weight of the animal) was infused over 30 minutes. Blood samples were collected to determine MDA, GSH, albumin levels, and CIBDAI scores were obtained at one (T1), three (T3), six (T6), and 12 (T12) months after MSCs administration (T0). In each group, the difference between all screening times before and after treatment was assessed by relating CIBDAI to oxidative stress biomarkers.

MSCs culture

Subcutaneous adipose tissue was obtained from a female dog undergoing conventional spay surgery. MSCs were extracted from this tissue, which was digested with collagenase type V, washed, and filtered. MSCs were cultured and expanded in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine

serum (FBS) and penicillin/streptomycin at 37°C and 5% CO_2. Using flow cytometry, following the guidelines of the International Society for Cell Therapy (Dominici et al. 2006), adherent cells were phenotypically characterized, in addition to differentiating in vitro to chondrogenic, osteogenic and adipogenic lineages. The expanded MSCs were cryopreserved until use, at which time they were thawed and resuspended in 50 ml of physiological saline.

Determination of Oxidative Stress Biomarkers

MDA was estimated according to the spectrophotometric method of Ohkawa et al. adapted for microplate assays (Ohkawa et al. 1979). Plasma extracts were incubated at 95 °C for 1 h in a mixture of 20% acetic acid (adjusted to pH 3.5 using NaOH), 8.1% SDS (with 0.05% butylated hydroxytoluene), and 0.8% solution of thiobarbituric acid. Then, a solution of n-butanol:pyridine (15:1, v/v) was added to the reaction mixture, shaken (5 min), and centrifuged (10,000 ×g at 4 °C for 5 min). The upper organic layer (pink-colored) was removed and read at 532 nm. MDA formation was expressed as micromoles of MDA equivalents per milligram of total protein using a calibration curve of 1,1,3,3-tetramethoxypropane (6.25-100 nmol/mL).

GSH was determined according to the fluorometric method described by Hissin and Hilf (Hissin and Hilf 1976). Samples were deproteinized by the addition of 50% cold trichloroacetic acid and then centrifuged (10,000 ×g at 4 °C for 10 min). Afterward, samples (50 µL) were incubated with 1 mg/mL of the fluorescent reagent o-phthalaldehyde in 0.1 M sodium phosphate (pH 8.0) containing 5 mM EDTA. The reaction mixture was incubated at 20 °C for 45 min, followed by fluorimetric measurement (the excitation and emission wavelengths were set at 420 and 350 nm, respectively). The calibration curve of GSH (3.20–320 µmol/mL) as an external standard was used for quantification.

Albumin analysis was performed using an automatic blood biochemistry analyzer (Saturno 100 Vetcrony® Instruments, Rome, Italy) and a Spinreact® commercial laboratory kit (Spinreact SA, Girona, Spain).

Statistical Analysis

Graphpad Prims software (version 8) was used to analyze the data. Concentrations of oxidative stress markers (MDA, GSH, and albumin) and CIBDAI index were compared between healthy dogs and CIE dogs. In addition, the values of these parameters were compared before and after treatment with MSCs at different times. A study of the normality of each component was performed using the Shapiro-Wilk test. The results were not normally distributed, so they were presented as the median and interquartile range (IQR).

Comparisons between the parameters of the control group and the CIE group were established using the Mann-Whitney U test. For analysis of changes over time, the Kruskal-Wallis test was performed. Dunn's test was used as a post hoc test. Spearman's test was used to study the correlations between MDA, GSH, and Albumin and the CIBDAI scores. The degree of significance was considered if $p < 0.05$.

Results

Study Population

The control group consisted of 12 healthy dogs of different breeds: Border Collie (n = 3), mixed breed (n = 3), Spanish Water Dog (n = 2), Jack Russel (n = 1), Samoyedo (n = 1), Poodle (n = 1), and English Setter (n = 1) of which five were females and seven were males. The median and range (minimum-maximum) age of the dogs was 2.75 (1.5 - 8.5) years, and the median and range (minimum-maximum) weight was 16.7 (2.60 to 21.5) kg.

The group of dogs with CIE included 20 dogs subdivided into 9 dogs in the MSCs group and 11 in the MSCs + P group. The breeds included were German Shepherd (n = 4), mixed-breed (n = 4), French Bulldog (n = 2), Yorkshire terrier (n = 2), Poodle (n = 1), Mastiff (n = 1), Greyhound (n = 1), Boxer (n = 1), Pomeranian (n = 1), Spanish Water Dog (n = 1), Husky (n = 1), Golden Retriever (n = 1). The median and range (minimum-maximum) age of the dogs was 4 (1 - 14) years, and the median and range (minimum-maximum) weight was 15.9 (2 to 26) kg. There were 13 males and 7 females. Dogs with CIE showed a median and range (minimum-maximum) of the CIBDAI value of 7.5 (5 – 14). According to this index, 8 dogs had severe disease (CIBDAI ≥ 9), 10 moderate disease (CIBDAI 6-8), and 2 mild disease (CIBDAI 4-5).

Albumin was significantly lower in the CIE group before the treatment than in the control group (p = 0.037). No statistical differences were observed for the MDA and GSH between groups (p = 0.58 and p = 0.27, respectively). (Figure 1, Table 1).

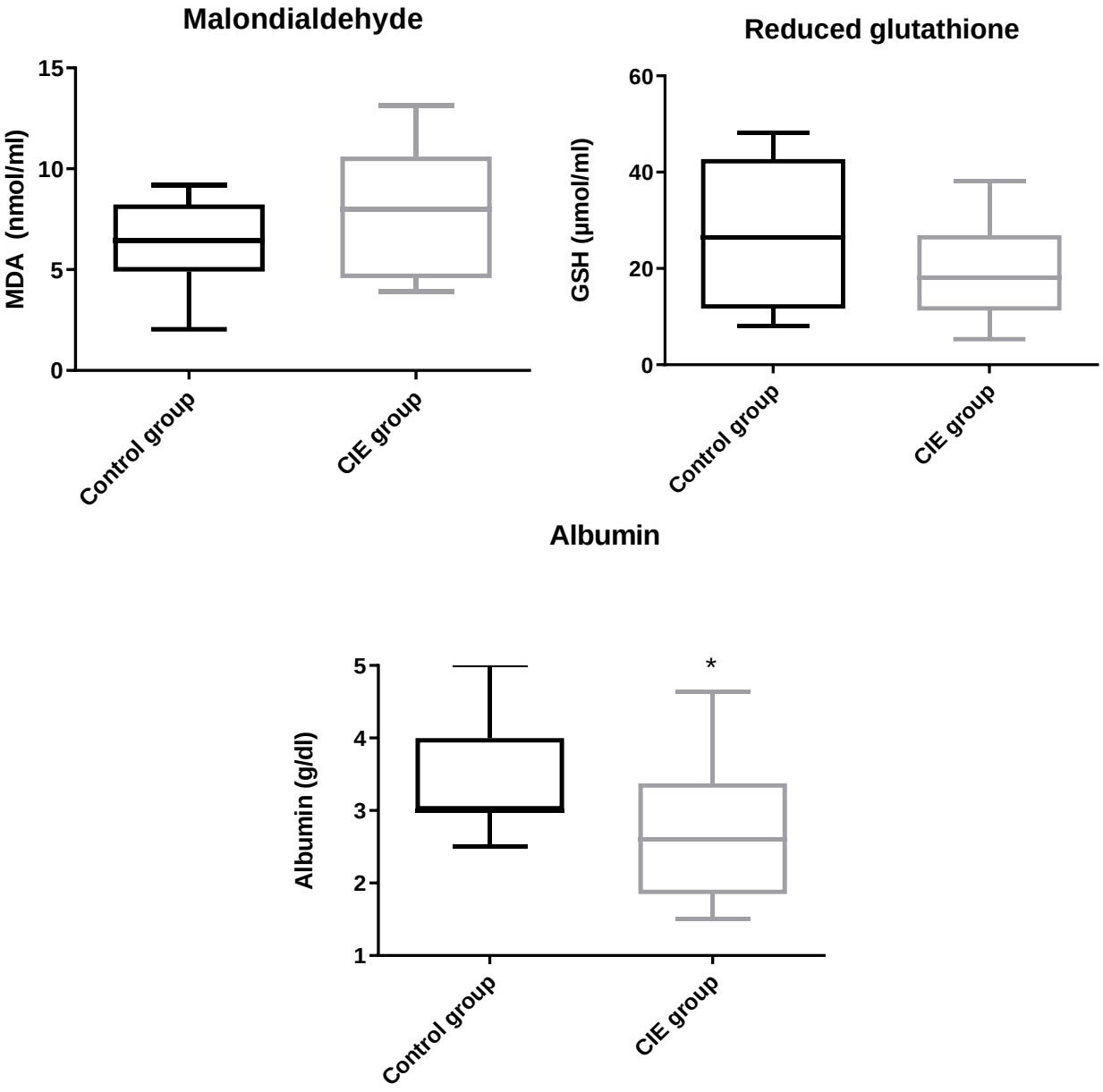

Fig 1. Comparison of malondialdehyde (MDA), reduced glutathione (GSH), and albumin between the control and chronic inflammatory enteropathy (CIE) groups. Asterisks indicate significant differences with the other group.

Table 1.

The median and interquartile range of MDA, GSH and albumin in control dogs and dogs with CIE plasma.

	MDA	GSH	Albumin
CONTROL GROUP	6.46 (3.33)	26.39 (31.06)	3.00 (0.63)
CIE GROUP	7.98 (6.14)	18.00 (15.67)	2.60 (0.98)*

*Significant differences with the other group ($p < 0.05$).

Changes in CIBDAI index and oxidative stress biomarkers after treatment with MSCs

CIBDAI decrease was more pronounced at T1. It stabilized at T3 with a slight increase at 6 and 12 months. All post-treatment times present significant differences with respect to T0 ($p = 0.005$) (Figure 2, Table 2).

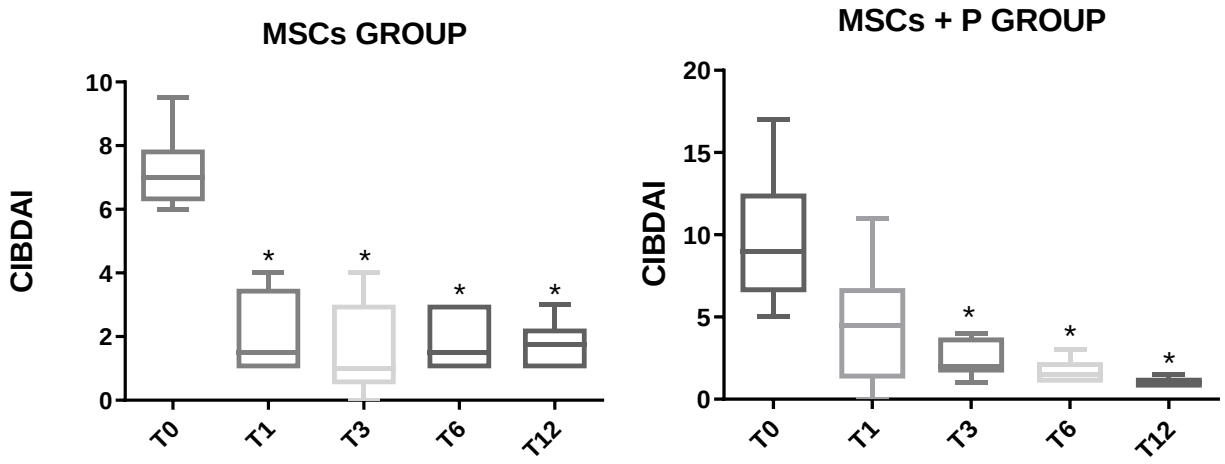

Fig 2. Canine Inflammatory Bowel Disease Activity Index (CIBDAI) in dogs with chronic inflammatory enteropathy (CIE) before treatment (T0) with mesenchymal stem cells (MSCs) or with MSCs and prednisone (MSCs+P) and at different review times: one (T1), three (T3), six (T6), and 12 months (T12). Asterisks indicate significant differences with T0.

Table 2.

The median and interquartile range of MDA, GSH, albumin and CIBDAI scores pretreatment (T0) and one (T1), three (T3), six (T6), and 12 months (T12) after treatment in dogs with CIE treated with mesenchymal stem cells (MSCs group) alone o combined with prednisone (MSCs+P group).

	MSCs group				MSCs+P group			
	MDA	GSH	ALBUMIN	CIBDAI	MDA	GSH	ALBUMIN	CIBDAI
T0	4.15 (2.29)	28.06 (9.39)	3.20 (1.53)	7.00 (1.63)	5.90 (7.17)	10.36 (3.02)	2.20 (1.15)	9.00 (6.00)
T1	3.35 (3.75)	27.15 (7.01)	3.10 (1.20)	1.50 (2.50)**	5.05 (4.16)	13.61 (6.02)	2.60 (0.65)	4.50 (5.50)
T3	2.54 (1.83)	26.20 (5.92)	3.20 (0.08)	1.00 (2.50)**	5.26 (4.31)	11.18 (3.15)	3.00 (1.10)	2.00 (2.13)*
T6	6.11 (7.69)	25.99 (8.53)	3.40 (0.30)	1.50 (2.00)**	4.50 (5.09)	11.58 (3.94)	3.30 (1.13)**	1.50 (1.25)*
T12	2.80 (1.12)	25.28 (6.62)	3.65 (0.45)	1.75 (1.25)**	6.28 (4.76)	12.37 (2.83)	3.30 (0.35)**	1.00 (0)*

*Differences with T0 for that parameter ($p < 0.05$)

**Differences with T0 for that parameter ($p < 0.01$)

No differences were observed in MDA, GSH, and albumin at any time point before and after the treatment ($p = 0.30$, $p = 0.89$, and $p = 0.15$, respectively) (Figure 3, Table 2).

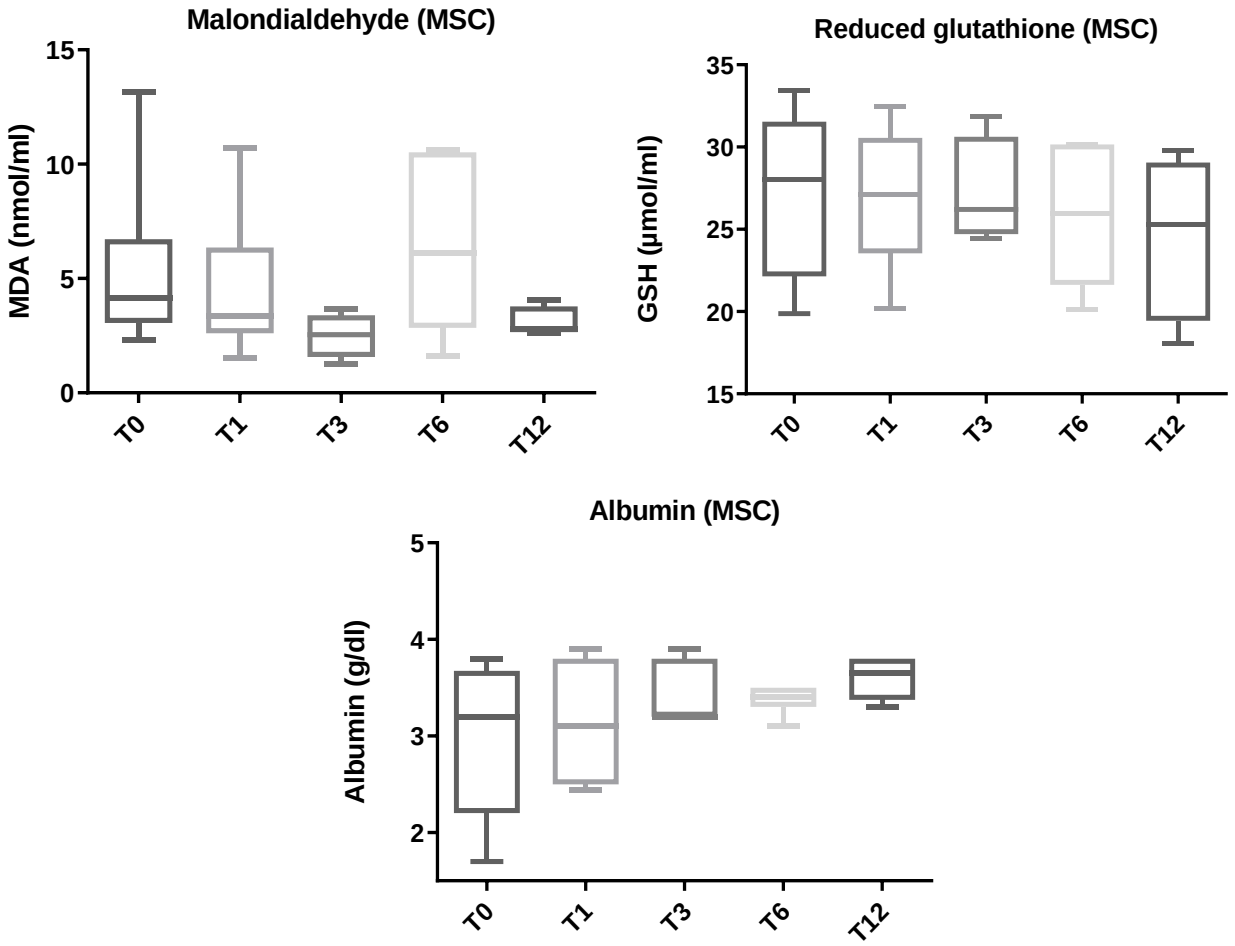

Fig 3. Malondialdehyde (MDA), reduced glutathione (GSH), and albumin in dogs with chronic inflammatory enteropathy (CIE) before treatment (T0) with mesenchymal stem cell (MSCs group) and at different review times: one (T1), three (T3), six (T6), and 12 months (T12).

Changes in CIBDAI index and oxidative stress biomarkers after treatment with MSCs and prednisone

CIBDAI showed a progressive decrease after treatment with MSCs together with corticosteroids. CIBDAI values at T3, T6, and T12 differed significantly from T0 ($p = 0.02$) (Figure 2, Table 2).

No difference was found in MDA and GSH in dogs with CIE before and at any time point after the treatment with MSCs and prednisone ($p = 0.84$ and $p = 0.47$, respectively). Significant differences were observed in albumin between T0 and T6 ($p = 0.008$) and between T0 and T12 ($p = 0.006$) (Figure 4, Table 2).

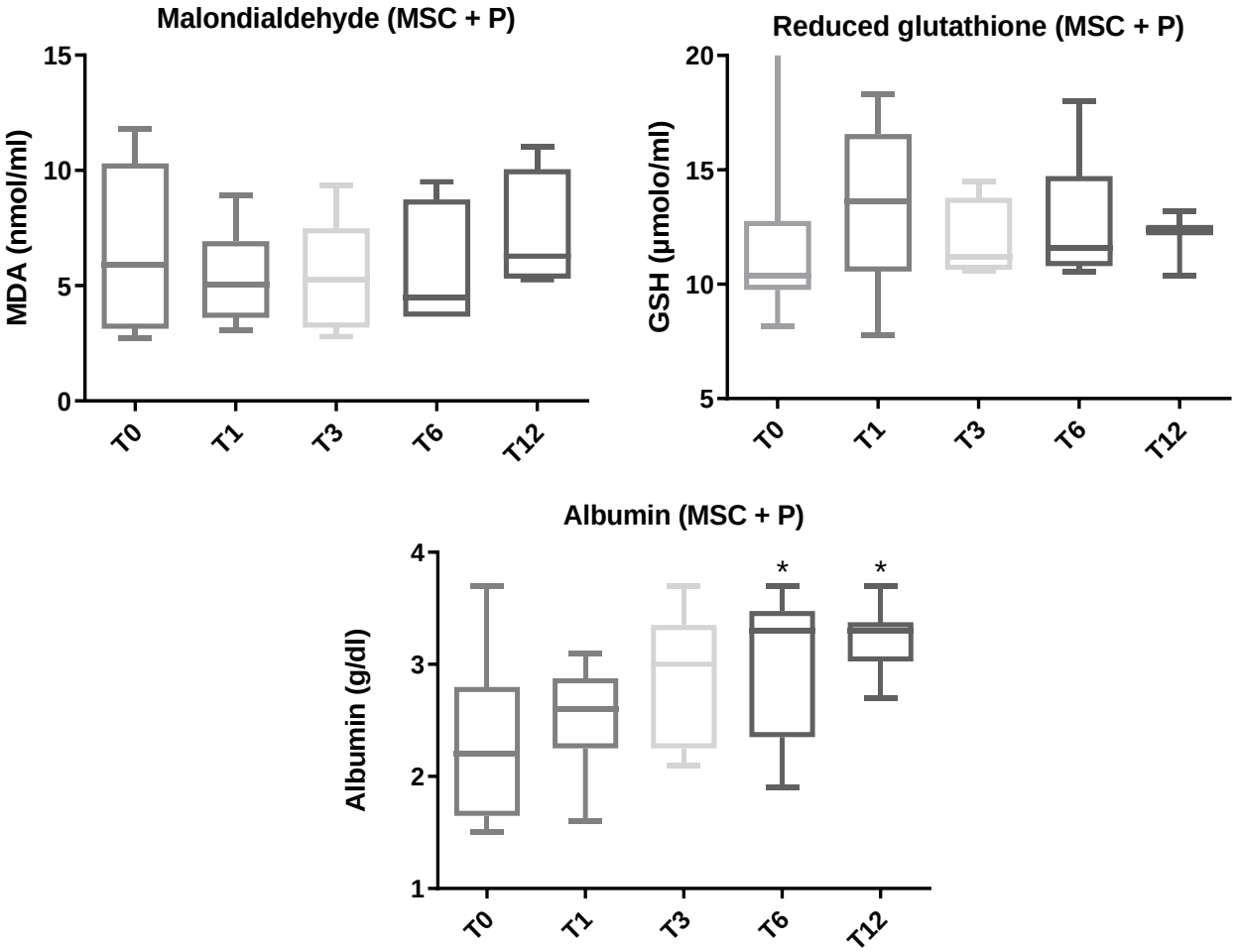

Fig 4. Malondialdehyde (MDA), reduced glutathione (GSH) and albumin in dogs with chronic inflammatory enteropathy (CIE) before combination treatment (T0) with mesenchymal stem cell and prednisone (MSCs+P group) and at different review times: one (T1), three (T3), six (T6), and 12 months (T12). Asterisks indicate significant differences with T0.

Correlation study

A significant positive correlation was found between the changes in the CIBDAI scores and the MDA ($\rho = 0.62$; $p = 0.05$) and GSH ($\rho = 0.43$; $p = 0.05$) values after the treatment in the MSCs+P group. No other significant correlation was observed.

Discussion

Due to the strong implication of oxidative stress in the pathophysiology of CIE, different oxidative and antioxidant biomarkers are currently being increasingly investigated. In the present study, an analysis of biochemical parameters related to oxidative stress in dogs with CIE was carried out compared with healthy animals. In turn, changes in these biomarkers after administration of MSCs alone or in combination with prednisone were evaluated.

In dogs with CIE, antioxidant serum biomarkers TEAC, CUPRAC, and PON1, have been shown to be decreased with respect to healthy dogs, but not FRAP (Rubio et al. 2016a; Rubio et al. 2016b; Segarra et al. 2016; Rubio et al. 2017). In a recent study, dogs exhibited an increase in determinable reactive oxygen metabolites (dROMs), and Oxidative Stress index (OSi), whereas no difference was found in Serum Antioxidant Capacity (SAC), compared to healthy dogs (Candellone et al. 2022).

Nevertheless, there was no data in the veterinary literature relating to MDA in canine CIE. In humans, the results of the changes in MDA during Crohn's disease are not consistent. Some studies described an increase in MDA values in CD patients, thus supporting an increase in free radicals in these patients and demonstrating an important role of oxidative stress in this disease (Alzoghaibi et al. 2007; Boehm et al. 2012; Achitei et al. 2013) and even the association of the increase of MDA in saliva and serum with the Crohn's disease activity index (Szczeklik K. et al. 2018). However, as happened in the present study, MDA has been reported to be unchanged in the colon of patients with ulcerative colitis or Crohn's disease (Bhaskar et al. 1995; Koch et al. 2000; Koch et al. 2002; Tüzün et al. 2002). The differences in these results have been attributed to the different origins of the samples examined (human plasma, breath alkanes, or mucosal biopsies) and to the different biochemical techniques utilized to estimate free radical production (Karp and Koch 2006).

In dogs and cats, higher MDA values were found in dogs with age-related cataracts (Madany 2016), cancer-bearing dogs (Macotpet et al. 2013), Ehrlichia canis (Çiftci et al. 2021) and parvo-infected dogs (Gaykwad et al. 2018), and dogs with atopic dermatitis (Kapun et al. 2012), but the present study has failed to show that increase in dogs with CIE.

GSH decrease has been reported in ill dogs affected by several diseases including a small number of them affected by gastrointestinal diseases compared to healthy animals (Viviano et al. 2009). In our study, however, that difference between dogs with CIE and healthy dogs was not observed . In humans, a study showed a decrease in the GSH in both the healthy and inflamed ileum of patients with CD compared to the ileum of healthy control patients (Iantomasi et al. 1994) and others in the colon (Holmes et al. 1998; Sido et al. 1998; Miralles-Barrachina et al. 1999). However, a study measuring GSH in the saliva of patients with CD has shown that GSH concentrations are decreased, but only in active CD (Szczeklik et al. 2018) and another demonstrated that there were no differences in colonic total antioxidant capacity comparing control colon to inactive and active ulcerative colitis in spite of the decreased tissue levels of GSH. This last study suggested that the depletion of glutathione in ulcerative colitis may be a specific disorder rather than a secondary defect attributable to global oxidative stress. It remains unclear whether glutathione depletion is related to increased consumption or to malnutrition that alters glutathione biosynthesis (Koch et al. 2000; Karp and Koch 2006).

Our results showed that the albumin value was decreased in CIE dogs relative to the control group as observed in human patients together with the depletion of other antioxidant markers such as FRAP, GSH, bilirubin, and uric acid (Szczeklik K et al. 2018; Su et al. 2019). This alteration has been consistently reported in many studies on canine CIE, and it has been considered a parameter of poor prognosis (Craven et al. 2004; Allenspach et al. 2007; Cristóbal et al. 2021). However, those studies do not approach the study of albumin from the point of view of its antioxidant qualities. Serum albumin is involved in redox homeostasis in the circulation. The most abundant thiol in plasma is the Cys34 residue, which is found in albumin. It exerts antioxidant activity, prevents systemic oxidative stress, and eliminates ROS and RNA. Furthermore, it has been shown that, as albumin molecules are oxidized by free radicals, the antioxidant capacities of this protein decrease. Therefore, researchers are currently demonstrating that oxidized albumin is a good biomarker of oxidative stress, as well as being an aggravating factor in multiple pathologies (Tabata et al. 2021).

Moreover, several features of the MSC therapy have been demonstrated in relation to oxidative stress, including direct antioxidant effects, such as the scavenging of free radicals and the donation of healthy mitochondria to damaged cells, and indirect effects, such as the enhancement of antioxidant defenses in

other cells of the body and the alteration of cellular bioenergetics. In addition, the immunosuppressive effects of MSCs prevent the generation of ROS. All these characteristics lead to a decrease in oxidative stress, generating beneficial effects against multiple pathologies (Inan et al. 2017; Jung et al. 2020; Stavely and Nurgali 2020; He et al. 2021).

It has been shown that MSCs could repair damaged brain tissue and decrease oxidative stress levels in humans by inhibiting the production of ROS/RNS (Calió et al. 2014). Another study showed the therapeutic effect of MSCs on damaged small intestine tissue from humans with intestinal ischemia/reperfusion was associated with increased antioxidant capacity and decreased oxidative stress, as indicated by a lower level of MDA and increased activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (Gpx) (Inan et al. 2017). Similar findings were reported in mice in which treatment with MSCs was shown to decrease oxidative stress by reducing ROS production, decreasing the MDA level, and increasing the activity of antioxidant enzymes SOD, CAT, and Gpx (Jung et al. 2020).

Furthermore, other treatments seemed able to modify the biomarkers in dogs such as N-acetylcysteine treatment that proved to improve glutation S-transferase activity and decrease MDA concentrations in parvo-infected dogs (Gaywad et al. 2018). However, in the present study, only albumin concentration improved but no changes in MDA and GSH levels were observed after the application of MSCs alone or in combination with prednisone in dogs with CIE.

One of the main limitations of this study is the low number of patients that we were able to include in each of the groups studied. The variability that may exist between the different breeds and sizes of the dogs included in the study is another limitation, as it may influence the values obtained for oxidative stress biomarkers. In addition, the limited literature on the determination of oxidative stress biomarkers in canine species makes it difficult to interpret the results obtained in our study.

In conclusion, when investigating the three biomarkers of oxidative stress in dogs with CIE, only differences in albumin values were observed, with no differences in MDA and GSH parameters. The treatment with MSCs, either alone or in combination with corticosteroids, was shown to be effective in CIE; however, only the albumin value varied (increasing) after the MSCs treatment, whereas the rest of the

oxidative stress parameters analyzed were not significantly modified by the treatment. It would be of interest to consider albumin as a biomarker of oxidative stress, as well as a nutritional marker, due to its antioxidant qualities.

Ethics approval

The protocol was approved by the UEx Animal Care and Use Committee (File nº 20160822, approved 22 August 2018). Informed consent was obtained from all owners of the animals participating in the study.

Author contribution

All authors contributed to the study's conception and design. Material preparation, data collection and analysis were performed by José Ignacio Cristóbal, Francisco Javier Duque, Jesús Usón-Casaús, María Salomé Martínez, María Prado Míguez and Eva María Pérez-Merino. The first draft of the manuscript was written by José Ignacio Cristóbal and Eva María Pérez-Merino and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflict of Interest Statement

The authors declare no conflict of interest.

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Resultados y discusión

En el primer trabajo de esta tesis, se evaluaron los principales cambios clínicos y laboratoriales que se produjeron en los perros con EIC tratados con MSCs como terapia única o acompañada de prednisona. Tras la administración de las MSCs a perros diagnosticados de IBD, el CIBDAI disminuyó de forma significativa al mes, 3, 6 y 12 meses en el grupo de perros tratados únicamente con MSCs, y a los 3, 6 y 12 meses en el grupo de perros tratados con MSCs junto con prednisona con respecto al inicio del tratamiento. El CCECAI, por otro lado, se redujo de forma significativa al mes, 3, 6 y 12 meses con respecto a las puntuaciones iniciales en ambos grupos. La remisión clínica de los animales tras el mes de tratamiento se alcanzó en el 57% de ellos en el grupo tratado con MSCs y en el 30% en el grupo tratado con MSCs y prednisona, alcanzándose el 100% de los perros a los 12 meses postratamiento.

Con respecto al análisis de albúmina, en el grupo tratado con MSCs el número de perros con hipoalbuminemia pasa de 5 a 1 después de un mes de la administración de células madre, y ninguno de ellos lo es en las siguientes visitas. El grupo tratado con prednisona y MSCs pasó de 8 animales hipoalbuminémicos a 5 tras 1 mes de tratamiento, alcanzando la normalización de todos los pacientes en la revisión anual. Sin embargo, el aumento de los valores de albúmina únicamente fue significativo a los 6 y 12 meses en el grupo tratado con MSCs y prednisona.

El número de perros con hipocobalaminemia fue reduciéndose progresivamente tras los tratamientos, pasando de 11 a 7 al mes de la infusión en el grupo tratado con MSCs y de 8 a 5 en el grupo tratado con MSCs y prednisona. Del primer grupo todos los pacientes alcanzaron la normalidad a los 6 meses, mientras que en el segundo grupo únicamente un paciente permaneció hipocobalaminémico en la revisión anual. El incremento de cobalamina fue significativo únicamente en el grupo tratado solo con células madre, siendo significativo a los 6 y 12 meses con respecto a los valores iniciales. Además, hubo diferencia a los 12 meses con los valores obtenidos al mes y tres meses postratamiento.

Por último, en todos los pacientes del estudio incluidos en el grupo P-MSCs la dosis de prednisona pudo ir reduciéndose de forma progresiva y significativa en cada control realizado.

Los resultados obtenidos en previos estudios sobre la administración de MSCs a perros y gatos con IBD, hacían referencia a pacientes sin tratamiento concomitante, en los

que se estableció un tiempo para eliminar otros inmunosupresores antes de la infusión (Webb T.L. & Webb C.B., 2015; Pérez-Merino EM et al. 2015). Este trabajo describe por primera vez la posibilidad de combinar las MSCs con el tratamiento convencional y, si mejora la sintomatología, incluso poder disminuir la dosis de prednisona, lo cual es clínicamente beneficioso, ya que en muchos casos la supresión de los inmunosupresores da lugar a un empeoramiento de la clínica de estos pacientes.

En este estudio se demuestra que ambas modalidades de terapia celular (sola o combinada con prednisona) parecen ser igual de efectivas con algunas diferencias menores. En medicina humana, la combinación de inmunosupresores con MSCs ya se ha llevado a cabo de forma segura, observándose una mejoría de los pacientes, pudiendo disminuir las dosis de los inmunosupresores, tal y como sucede en este estudio (Duijyestein M et al. 2010; Zhang J et al. 2018; Liang J et al. 2012). Numerosas investigaciones demuestran que la coadministración de MSCs y otros inmunosupresores en pacientes con IBD pueden mejorar la inflamación intestinal y reparar la mucosa dañada (Zhang X et al. 2020). También se ha demostrado que la troncalidad, la viabilidad celular y la morfometría nuclear de las MSCs no se ven afectadas por la azatioprina o la dexametasona, fármacos utilizados en el tratamiento del IBD (Schneider N. et al. 2015).

En lo referente al análisis de albúmina, su disminución afectó mayormente al grupo de perros con terapia combinada, ya que esta hipoalbuminemia se ha descrito con mayor frecuencia en aquellos pacientes que necesitan la administración de inmunosupresores (Dandrieux J.R.S. 2016). En este estudio la infusión de MSCs sola o combinada con corticosteroides pudo revertir la hipoalbuminemia en todos los animales, alcanzándose valores dentro del rango de referencia, aunque tardaron más tiempo en alcanzar esta normalidad el grupo de perros que tomaban la prednisona.

Por otro lado, la hipocobalaminemia va asociada a una disbiosis de intestino delgado y a la alteración en la absorción intestinal, sobre todo en la parte distal (íleon), considerándose un marcador pronóstico negativo (Craven M. et al. 2004; Allenspach K. et al. 2007). Para poder aumentar esta vitamina es necesario su administración exógena, habiendo diferentes protocolos para ello (Toresson L. et al. 2016). Sin embargo, en este estudio, la normalización de la cobalamina se alcanzó sin la suplementación externa. Esto puede atribuirse al efecto de las MSCs, las cuales mejoran la estabilización de la

homeostasis celular y la preservación de las células del epitelio intestinal (Sémont A. et al. 2010).

Dado que los anteriores ensayos clínicos llevaban a cabo un corto periodo de seguimiento (42 días, 2 y 3 meses) (Webb T.L. & Webb C.B., 2015; Pérez-Merino E.M. et al. 2015(a); Pérez-Merino E.M. et al. 2015(b)) no se había determinado el efecto de la administración de MSC a largo plazo. Este estudio demuestra que los efectos positivos de esta terapia y la mejoría clínica se mantiene estable incluso hasta 1 año tras la administración de las MSCs. Sin embargo, en lo que respecta a la terapia inmunosupresora, se ha demostrado que estos fármacos a más largo plazo (más de 3 años) resultan ser menos prometedores (ya sea un solo fármaco o la combinación de varios fármacos) (Allenspach K. et al. 2016). Por lo que es imprescindible la monitorización de los pacientes tratados con la terapia celular para determinar su efecto a un plazo más largo aún.

En el segundo trabajo de nuestra investigación, se evaluaron los cambios inflamatorios y del sistema inmune que se produce en perros con EIC tras la administración de las MSCs a través de la determinación de los biomarcadores NLR, PLR y SII. Se compararon los valores de estas ratios entre perros con EIC y perros sanos y, además, en los pacientes con EIC se evaluaron estos parámetros previos al tratamiento y tras la infusión de las MSCs, a los 2 y 9 meses.

Estas ratios no han sido evaluadas tras la administración de MSCs. Además, el SII no había sido descrito previamente en medicina veterinaria hasta la publicación de este estudio clínico.

En los resultados de esta segunda parte de nuestra investigación, tanto los neutrófilos como las plaquetas se encontraban aumentados y los linfocitos disminuidos en los perros con EIC con respecto a los perros sanos. Tras la administración de las MSCs los neutrófilos disminuyeron de forma significativa a los 2 meses con respecto a los valores previos al tratamiento. A los 9 meses, tanto la disminución de las plaquetas y los neutrófilos, como el aumento de los linfocitos fueron significativos.

Las tres ratios analizadas (NLR, PLR y SII) se encontraban significativamente más elevadas en el grupo de perros con EIC con respecto a los pacientes sanos, disminuyendo de forma significativa a los 2 y 9 meses tras la infusión de las MSCs con respecto al valor obtenido previo al tratamiento. Entre el grupo de perros sanos y enfermos se observaron

diferencias en las tres ratios a los dos meses del tratamiento, sin embargo, no hubo ninguna a los 9 meses postratamiento.

Por otra parte, se encontró una correlación positiva entre las tres ratios y entre las tres ratios y el índice clínico CCECAI, y negativa entre las tres ratios y el número de linfocitos. La ratio NLR se correlacionó positivamente con los neutrófilos, la ratio PLR con las plaquetas y la ratio SII con los neutrófilos y plaquetas.

En base a los resultados obtenidos, se demuestra que la mejoría clínica de los pacientes con EIC a los que se les administró MSCs va acompañada de una disminución de las ratios NLR, PLR y SII.

La elevación de neutrófilos y plaquetas, junto con la disminución de linfocitos se ha descrito de igual manera en pacientes afectados por procesos inflamatorios (Blackwood L. et al. 2016; Benvenuti E. et al. 2020). La trombocitosis, además, afecta entre un 6 y un 32% de los perros con EIC (Marchetti V. et al. 2010). Tras la administración de las MSCs disminuyeron los neutrófilos y plaquetas y aumentaron los linfocitos. En un estudio en humanos con EC tratados con células madre autólogas de origen hematopoyético, se demostró un aumento en el porcentaje de células T reguladoras circulantes (Clericii M. et al. 2011). En otra investigación se ha demostrado que las MSCs atenúan la respuesta inflamatoria (con predominio de los neutrófilos polimorfonucleares) en ratas con lesiones pulmonares inducidas por ventilación (Lai T.S. et al. 2015).

En este estudio, el valor medio de referencia de la ratio NLR y el de los perros con EIC es similar al descrito en otros estudios (Benvenuti E. et al. 2020; Becher A. et al. 2021; Neumann S. 2021), aunque la media obtenida de PLR en perros sanos es inferior a la descrita en otros estudios (Neumann S. et al. 2021) y la de los perros con EIC se encuentra por encima de la media descrita en el único estudio existente (Pierini A. et al. 2021).

En el estudio se observa como la ratio NLR es superior perros con EIC que en perros sanos, al igual que se describe en otros estudios en medicina humana y veterinaria (Okba A.M. et al. 2019; Jeong Y. et al. 2021). También las ratios PLR y SII están aumentadas en nuestros pacientes con EIC, pero no hay datos de otros estudios al no haber sido anteriormente comparados entre perros sanos y con EIC. Sin embargo, en personas con

CU sí se ha observado un incremento de estas dos ratios en comparación con individuos sanos (Bertani L. et al. 2020; Xie Y. et al. 2021).

En lo referente al efecto de las MSCs, las tres ratios disminuyeron tras su administración. Se ha demostrado que NLR disminuye en personas con enfermedad de Behçet tras la administración de corticosteroides y colchicina (Djaballah-Ider F. and Touil-Koukoffa C. 2020). Por otro lado, en personas con CU, se ha demostrado que las ratios NLR y PLR disminuyeron a las 8 semanas de la terapia con FNT-α (Bertani L. et al. 2020). En otro ensayo clínico desarrollado en perros con EIC se ha descrito que la trombocitosis disminuye tras el tratamiento con inmunosupresores (Marchetti V. et al. 2010).

El mecanismo de control de la respuesta inmune tras la administración de las MSCs aún no está del todo esclarecido. Esta respuesta depende de señales inflamatorias en el microentorno ya que las MSCs pueden presentar efectos pro- y antiinflamatorios (Bernardo M.E. & Fibbe W.E. 2013).

Esta investigación además confirma la correlación positiva entre las ratios NLR y PLR y la gravedad de la enfermedad, coincidiendo con otros estudios (Benvenuti E. et al. 2020; Becher A. et al. 2021; Pierini A et al. 2021). Además, se confirmó la correlación entre el SII y la gravedad de la enfermedad. De forma similar, en personas con CU, la disminución de NLR y PLR se ha correlacionado con la remisión clínica en pacientes tratados con FNT- α (Bertani L. et al. 2020).

Nuestros resultados corroboran la utilidad del uso de NLR para determinar la gravedad de la EIC en perros, pudiendo utilizarse como un marcador de rutina a la hora del diagnóstico y monitorización de la enfermedad, similar a lo descrito en medicina humana.

El objetivo del tercer trabajo fue relacionar los niveles de cobalamina con el grado de afectación endoscópica e histológica del íleon en los pacientes con EIC.

De los 68 perros incluidos en esta investigación, el 43% eran normocobalaminémicos (NC, concentraciones superiores a los 330 ng/L), el 28% se asignaron al grupo con hipocobalaminemia (HC, cobalamina entre 200 y 300 ng/L) y el

29% al grupo con hipocobalaminemia severa (SHC, cobalamina eran inferiores a 200 ng/L).

Se observaron diferencias significativas en el índice CIBDAI entre los 3 grupos y sólo entre el grupo SHC y los otros dos grupos en el índice CCECAI.

En lo referente a los hallazgos endoscópicos, la anormalidad más frecuente fue la granularidad (57% de los casos), seguido de linfangiectasia (28%), friabilidad (20%) y erosiones en la mucosa (9%). Sólo se observaron diferencias en la friabilidad entre los grupos SHC y NC y en la granularidad entre los grupos HC y NC. La afectación endoscópica fue significativamente más grave en el grupo SHC que en el NC, sin embargo, no se observó ninguna otra diferencia entre los grupos.

Con respecto a los hallazgos histopatológicos, se observó principalmente un incremento de linfocitos en la lámina propia (54 % de los casos) seguido de una atrofia de las vellosidades (60%) y dilatación de las criptas (54%). El grupo SHC mostró significativamente más atrofia de las vellosidades y daño epitelial que el grupo NC y, además, aumento significativo de IES (puntuación endoscópica ileal) con respecto a los grupos NC y HC. Los grados moderados y graves de hallazgos histológicos fueron observados con mayor frecuencia en el grupo SHC. El grado de afectación histológica fue significativamente superior en el grupo SHC que en los otros dos, pero no se encontraron diferencias entre los perros HC y NC.

La concentración de cobalamina mostró una correlación negativa débil con la afectación endoscópica, moderada con la afectación histológica, con la presencia de linfocitos intraepiteliales (IEL) y con los índices CCECAI y CIBDAI. En el grupo de perros normocobalaminémicos, se observó una correlación entre el grado de afectación histológica y endoscópica del íleon, mientras que en los grupos HC y SHC se observó una correlación positiva entre los índices clínicos y el grado de afectación histológica.

Este estudio demuestra que la concentración de la cobalamina podría ayudar al clínico a conocer el estado del íleon sin necesidad de su exploración endoscópica, la cual suele resultar dificultosa para endoscopistas más inexpertos y conlleva una prolongación del tiempo anestésico (Woolhead, V. L. et al. 2020). Por ser normalmente inexplorado, el íleon se ha puntuado y evaluado generalmente como el duodeno (Washabau, R. J. et al.

2010), sin embargo, nuestro estudio ofrece por primera vez una descripción de las características específicas de la mucosa ileal en los pacientes con EIC.

En los animales de este estudio se ha descrito un aumento de la granularidad y la linfangiectasia en la mucosa ileal. La primera de las características nunca se ha descrito en el íleon de pacientes con EIC, pero si la segunda en un estudio con un pequeño número de perros (Malancus R.N. et al. 2017).

Este estudio demuestra que las erosiones, linfangiectasia y friabilidad se observan con mayor frecuencia en los perros con concentraciones de cobalamina inferiores a 200 ng/L que en aquellos con más de 350 ng/L. Sin embargo, las concentraciones intermedias proporcionan información escasa sobre el posible aspecto del íleon. Aun así, dada la correlación inversa entre la cobalamina y la actividad endoscópica encontrada en este estudio, se podría esperar que, a medida que aumenta la cobalamina, las características del íleon se asemejarán a la de los perros normocobalaminémicos y viceversa.

Todos los perros de este estudio mostraron anormalidades histopatológicas frente al 80% de un estudio anterior (Casamian-Sorrosal D. et al. 2010). Estos cambios en la mucosa ileal son más frecuentes y más graves cuando la cobalamina es inferior a 200 ng/L, sin embargo, las anormalidades son leves con concentraciones superiores a 350 ng/L. Esto respalda la cifra umbral de 200 ng/L, concentración por debajo de la cual los animales presentan peor pronóstico (Allenspach K. et al. 2007). La correlación entre la presencia de IEL y la cobalamina ha sido observada en este estudio como en otros anteriores (Procoli F. et al. 2013) pero además la presencia de IEL es el único parámetro que diferencia entre los dos grados de hipocobalaminemia de nuestro estudio (<200 o 200-350 ng/L).

Esta investigación confirma la correlación entre la hipocobalaminemia y la gravedad clínica de la enfermedad (CCECAI) descrito previamente en otros estudios (Procoli F. et al. 2013; Volkmann M. et al. 2017). Sin embargo, mientras estudios anteriores no pudieron relacionar el daño histológico del íleon con la concentración de cobalamina (Procoli F. et al. 2013), este estudio ha demostrado la asociación de la concentración de cobalamina con el daño histológico y endoscópico del íleon.

También ha demostrado la relación entre la severidad de la enfermedad y el grado de afectación histológica del íleon que tradicionalmente no se había establecido (Procoli

F. et al. 2013), si bien un artículo previo ya había conseguido una correlación casi significativa entre el CCECAI y la afectación histológica ileal (Allenspach, K. A et al. 2019).

La correlación entre las lesiones endoscópicas e histológicas del íleon observada en este estudio fue débil, aunque estudios anteriores no encontraron ninguna (Roth, L. et al. 1990; Jergens, A. E. et al. 2003; García-Sancho, M. et al. 2007; Schreiner, N. M. et al. 2008). Pensamos que el uso de una escala validadas y simplificadas para la evaluación del íleon ha contribuido a hallar esa relación en la población. Analizando por grupos, se observa que la correlación endoscopia-histología solo se da en el grupo de animales normocobalaminémicos, pero no en el de hipocobalaminémicos. Creemos que, en el primero, la escasez y levedad de las lesiones a ambos niveles permiten esa relación, sin embargo, a medida que la cobalamina disminuye, los cambios microscópicos aparecen con mayor intensidad que los macroscópicos, perdiéndose la correlación.

Por último, en este ensayo clínico la hipoalbuminemia e hipocobalaminemia coexistieron en aquellos animales con índices clínicos altos y daño intestinal grave y extenso. Aunque muchos estudios han relacionado la hipocobalaminemia con la hipoalbuminemia (Allenspach K. et al. 2007; Moser K. et al. 2018; Craven M. et al. 2004), en este estudio no se observó dicha correlación, sin embargo, sí se ha descrito que la mayoría de los individuos hipoalbuminémicos se encontraban en el grupo SHC.

En el último trabajo, se investigó el papel del estrés oxidativo en perros diagnosticados de EIC, cuya importancia fisiopatológica ya se ha demostrado anteriormente tanto en medicina humana como en veterinaria (Yuksel M. et al. 2017; Rubio C.P. et al. 2017), sin embargo, en esta ocasión, se utilizaron marcadores de estrés oxidativo diferentes a los utilizados por otros autores como son el agente oxidante malondialdehido (MDA) y dos antioxidantes, el glutation reducido (GSH) y la albúmina, comparándolos entre los perros sanos y perros con EIC. Además, se determinaron los cambios que se pueden producir en estos biomarcadores de estrés oxidativos tras la administración de las células madre como tratamiento único o junto con la administración de prednisona.

Los resultados de este estudio demostraron que los pacientes con EIC presentan una disminución significativa de la concentración de albúmina con respecto al grupo control, pero no existen diferencias en los valores de MDA y GSH.

Tras la administración de MSCs como terapia única o combinada con prednisona, se observó una mejoría clínica, ya que disminuyó progresivamente el CIBDAI, siendo esta bajada significativa al mes, 3, 6 y 12 meses en el primer caso y a los 3, 6 y 12 meses en el segundo con respecto a los valores pretratamiento.

Sin embargo, no se observaron diferencias en las determinaciones de MDA y GSH a lo largo del tiempo y solo los valores de albúmina disminuyeron significativamente a los 6 y 12 meses postratamiento en el grupo tratado con MSCs y prednisona

Por último, hubo una correlación significativa entre los valores del CIBDAI y los biomarcadores MDA y GSH tras el tratamiento con MSCs y prednisona.

En este estudio no se observaron diferencias en el MDA entre los perros sanos y pacientes con EIC. En medicina humana, sin embargo, los cambios descritos en el MDA durante la EC no son consistentes. Por un lado, algunos estudios sí encuentran diferencias en personas con EC con respecto a individuos sanos, corroborando, por tanto, un aumento en los radicales libres en estos pacientes, demostrando así el papel del estrés oxidativo en la EC (Boehm D. et al. 2012; Achitei D. et al. 2013). De igual forma se ha descrito un aumento de MDA en el suero y saliva, asociándose a la gravedad de la EC y a la inflamación que produce esta patología, además, este biomarcador se correlacionó con el índice de actividad clínica y la inflamación generada en la EC (Szczeklik K. et al. 2018). Sin embargo, otros estudios no observan modificaciones en el MDA en el colon de pacientes con CU o EC (Bhaskar et al. 1995; Koch et al. 2000; Koch et al. 2002; Tüzün et al. 2002) con respecto a personas sanas, como ha sucedido en el presente estudio. Esta variabilidad en los resultados se ha atribuido a los distintos orígenes de las muestras examinadas (plasma humano, alcanos del aliento o biopsias de mucosa intestinal) y a las diferentes técnicas utilizadas para estimar la producción de radicales libres (Karp y Koch 2006).

Con respecto al GSH, tampoco se apreciaron diferencias entre perros con EIC y perros sanos, sin embargo, se ha descrito su disminución en perros afectados por numerosas enfermedades, en comparación con animales sanos (Viviano et al. 2009). En investigaciones llevadas a cabo en medicina humana se ha observado una disminución en este biomarcador en el íleon sano y enfermo de pacientes con EC comparándolo con el íleon de pacientes sanos (Iantomasi T. et al. 1994) y en el colon de pacientes con CU y EC

(Holmes et al. 1998; Sido et al. 1998; Miralles-Barrachina et al. 1999). En otro estudio se demostró una disminución en las concentraciones de GSH en pacientes con la EC activa (Szczeklik K. et al. 2018).

Por último, el valor de la albúmina se encontró disminuido en los pacientes con EIC con respecto a los valores del grupo control. Esto mismo se ha demostrado en anteriores investigaciones desarrolladas en perros con EIC, y, además, se ha considerado como un parámetro de mal pronóstico (Craven M. et al. 2004; Allenspach K. et al. 2007). Sin embargo, la albúmina siempre se ha estudiado desde el punto de vista de sus características como proteína de reserva y estatus nutricional. Por lo que en esta investigación se ha dado mayor relevancia a las importantes funciones de esta molécula como antioxidante extracelular en este tipo de pacientes.

Se ha descrito que, según se van oxidando las moléculas de albúmina, la capacidad de esta proteína va disminuyendo. Por lo tanto, los investigadores actualmente están demostrando que la albúmina oxidada es un buen biomarcador de estrés oxidativo, además de ser un factor agravante en multitud de patologías (Tabata F. et al. 2021). En medicina humana, esta proteína se ha estudiado junto a otros marcadores antioxidantes, como la bilirrubina, ácido úrico, GSH y la capacidad de reducción férrica del plasma (FRAP del inglés Ferric Reducing Antioxidant Power), observándose en concentraciones más bajas en personas con EC que en pacientes sanos (Szczeklik K. et al. 2018; Su Q. et al. 2019).

En medicina humana se ha demostrado que las MSCs pueden reparar el tejido dañado cerebral y disminuir el nivel de estrés oxidativo mediante la inhibición de la producción de ROS y RNS (Calió M.L. et al. 2014). Otro estudio comprobó las mismas propiedades de esta terapia en pacientes con daño intestinal que presentan isquemia/reperfusión intestinal mediante la disminución del estrés oxidativo y el aumento de las capacidades antioxidantes. Todo ello demostrado gracias a la disminución de MDA y un incremento de superóxido dismutasa (SOD), catalasa (CAT) y GPx (Inan M. et al. 2017). Sin embargo, en lo referente al efecto de las MSCs sobre el estatus oxidativo, en esta investigación no se observaron cambios en los niveles de MDA y GSH tras la administración de MSCs solas o combinadas con prednisona. Sin embargo, se observó una mejoría de la albúmina en ambos grupos de tratamiento.

Conclusiones

1. La terapia con Ad-MSCs puede mejorar la clínica de los pacientes con EIC de forma segura y significativa tanto como terapia única, como acompañada de corticosteroides. Además, la dosis de corticosteroides puede reducirse, e incluso suprimirse, tras el tratamiento con Ad-MSCs. Sus beneficios son duraderos y estables en el tiempo.

2. Los marcadores inflamatorios basados en el contaje de leucocitos y plaquetas (NLR, PLR y SII), disminuyen significativamente a medio y largo plazo tras la infusión de Ad-MSCs, alcanzando niveles normales a los 9 meses. Esta evolución en los biomarcadores inflamatorios va asociada con mejoría clínica de los pacientes.

3. Con la utilización de las escalas actualizadas y validadas para evaluar la mucosa del íleon se consigue establecer una asociación entre la concentración de cobalamina y el grado de lesión macroscópica y microscópica, por lo tanto, la concentración de cobalamina puede ser útil para predecir el grado de afectación del íleon sin su exploración.

4. La albúmina es el único marcador de estrés oxidativo afectado en perros diagnosticados de EIC y la única que varía tras la infusión de MSCs mientras que el resto de los parámetros de estrés oxidativo no se modificaron (MDA y GSH). Es necesario, por tanto, darle mayor importancia a la albúmina como biomarcador de estrés oxidativo, gracias a sus propiedades antiinflamatorias.

Conclusions

1. Ad-MSCs therapy can safely and significantly improve the clinical outcome of patients with CIE both as monotherapy and in combination with corticosteroids. In addition, the corticosteroid dose can be reduced or even abolished after treatment with Ad-MSCs. The benefits are long-lasting and stable over time.

2. Inflammatory markers based on leukocyte and platelet counts (NLR, PLR and SII) decrease significantly in the medium and long term after Ad-MSCs infusion, reaching normal levels at 9 months. This evolution in inflammatory biomarkers is associated with clinical improvement in patients.

3. Using the updated and validated scales to assess the ileal mucosa, an association between cobalamin concentration and the degree of macroscopic and microscopic injury can be established, therefore, cobalamin concentration can be useful to predict the degree of ileal involvement without examination of the ileum.

4. Albumin is the only oxidative stress marker affected in dogs diagnosed with CIE and the only one that varies after MSC infusion, while all other oxidative stress parameters remain unchanged (MDA and GSH). It is therefore necessary to give greater importance to albumin as a biomarker of oxidative stress due to its anti-inflammatory properties.

Listado de abreviaturas

Ad-MSCs: Células madre mesenquimales alogénicas obtenidas de tejido adiposo

ALT: Alanino aminotransferasa

ARE: del inglés Antibiotic-Responsive Enteropathy

CAT: Catalasa

CCECAI: del inglés Canine Chronic Enteropathy Clinical Activity Index

CD: Células dendríticas

CU: Colitis ulcerosa

CUPRAC: del inglés Cupric Reducing Antioxidant Capacity

CIBDAI: del inglés Canine Inflammatory Bowel Disease Activity Index

CMM: Células madre mesenquimales

EC: Enfermedad de Crohn.

EIC: Enteropatía inflamatoria crónica

ENR: Enteropatía no responsiva

ERA: Enteropatía responsiva a antibióticos

ERD: Enteropatía responsiva a la dieta

ERI: Enteropatía responsiva a inmunosupresores

FNT-α: Factor de necrosis tumoral alfa

FRAP: del inglés Ferric Reducing Antioxidant Power

FRE: del inglés Food-Responsive Enteropathy

FOX: del inglés Ferrous Oxidation-Xylenol Organe

GPx: Glutation peroxidasa

GSH: Glutation reducido

HC: Hipocobalaminemia

IBD: del inglés Inflammatory Bowel Disease

IEL: Linfocitos intraepiteliales

IES: Puntuación endoscópica ileal

IL: Interleuquina

INF: Interferon

IRE: del inglés Immunosuppressant-Responsive Enteropathy

MDA: Malondialdehído

MSCs: del inglés Mesenchymal stem cells

NC: Normocobalaminemia

NK: Células Natural Killer

NLR: del inglés Neutrophil to Lymphocite ratio

NOD2: dominio de oligomerización por unión de nucleótidos que contiene la proteína 2

NRE: del inglés Non-Responsive Enteropathy

PAM: Péptidos antimicrobianos

PCR: Proteína C reactiva

PLR: del inglés Platelet to Lymphocite ratio

PON1: Paraoxonasa 1

RL: Radicales libres

RNS: del inglés Reactive Nitrogen Species

ROS: del inglés Reactive Oxygen Species

SAC: Capacidad antioxidante sérica

SHC: Hipocobalaminemia grave

SII: del inglés systemic immune-inflammation index

SOD: Superóxido Dismutasa

TBARS: del inglés Thiobarbituric Acid Reactive Substantes

TEAC: del inglés Trolox Equivalent Antioxidant Capacity

TGF-β: del inglés Transforming growth factor-beta

Th: Células T helper

TLI: del inglés trypsin-like immunoreactivity

TLR: Receptores tipo Toll (del inglés Toll-like receptor)

TMF: Trasplante de Microbiota Fecal

Treg: Células T reguladoras

WSAVA: del inglés World Small Animal Veterinary Association

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