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UNIVERSITAT POLITÈCNICA DE VALÈNCIA

School of Agricultural Engineering and Environment

A model of colitis from the perspective of biophysics as a contribution to the understanding of inflammatory bowel disease

End of Degree Project

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Title: A model of ulcerative colitis from the perspective of biophysics as a contribution to the understanding of inflammatory bowel disease.

Abstract:

The ulcerative colitis is a condition characterized by chronic inflammation of the gastrointestinal tract, which is comprised inside the group of inflammatory bowel diseases (IBD). It leads principally to diarrhoea, weight loss and stool bleeding. Its causes are still unknown, although it is believed that different genetic, immunological, and environmental factors play an important role in its development. The treatments for this pathology, nowadays are not completely successful in all cases. Then, the objective of our project was to detect the chronic ulcerative colitis disease before the appearance of its characteristic symptoms, and to contribute to its understanding from a biophysical point of view.

The outcome of the chronic ulcerative colitis disease was studied in detail by employing one year old C57BL/6 mice. During six weeks, the experimental group was administered with a weekly alternation of a solution of 2% dextran sodium sulphate (DSS), a chemical widely used to induce mouse model of colitis, as it produces symptoms very similar to human ulcerative colitis, and tap water, while the control group was provided only with tap water. All mice were weighed, and their stools were collected and scored three times per week. In addition, mice underwent bioluminescence analysis to visualize the progression of inflammation over time, using the IVIS (In Vivo Imaging System) machine, as well as a colonoscopy. And the blood presence in their stools was detected also by using the IVIS. Finally, with the collected data obtained from a biophysical study, it was monitored the progression of the ulcerative colitis disease and concluded that it can be detected by the *in vivo* bioluminescent study of the mice and its stools at the same time than the onset of the symptoms, being the tissue inflammation detected from the first week of experimentation, being especially appreciable on day 7, and recording the highest radiance values at the beginning of the water weeks, in general.

Key words: Inflammatory bowel disease; Ulcerative colitis; Colonoscopy; Bioluminescence.

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Título: Un modelo de colitis ulcerosa desde la perspectiva de la biofísica como contribución a la comprensión de la enfermedad inflamatoria intestinal.

Resumen:

La colitis ulcerosa es una condición caracterizada por la inflamación crónica del tracto gastrointestinal, la cual se engloba dentro del grupo de las enfermedades inflamatorias intestinales (EII). Provoca principalmente diarrea, pérdida de peso y sangrado en las heces. Sus causas aún se desconocen, aunque se cree que diferentes factores genéticos, inmunológicos y ambientales juegan un papel importante en su desarrollo. Los tratamientos para esta patología hoy en día no son del todo exitosos en todos los casos. Por lo tanto, el objetivo de nuestro proyecto fue detectar esta enfermedad antes de la aparición de sus síntomas característicos, y contribuir a su comprensión desde un punto de vista biofísico.

El desarrollo de la colitis ulcerosa crónica se estudió en detalle empleando ratones C57BL/6 de un año. Durante seis semanas, al grupo experimental se le administró, con alternancia semanal, una solución de sulfato de dextrano sódico (DSS) al 2%, un químico ampliamente utilizado para inducir el modelo de colitis en ratones ya que produce unos síntomas muy similares a la colitis ulcerosa humana, y agua del grifo, mientras que al grupo de control solo se le proporcionó agua del grifo. Se pesaron todos los ratones, y se puntuaron y recogieron sus heces tres veces por semana. Además, los ratones se sometieron semanalmente a análisis de bioluminiscencia para visualizar la progresión de la inflamación a lo largo del tiempo, utilizando la máquina IVIS (In Vivo Imaging System), así como una colonoscopia. La presencia de sangre en sus heces también se detectó utilizando IVIS. Finalmente, con los datos recopilados, obtenidos mediante un estudio biofísico, se monitoreó la progresión de la enfermedad de colitis ulcerosa y se concluyó que se puede detectar mediante el estudio bioluminiscente de los ratones y sus heces al mismo tiempo que la aparición de los síntomas, siendo la inflamación tisular detectable desde la primera semana de experimentación, la cual resultó especialmente apreciable el día 7, y registrándose, en general, los valores más altos de bioluminiscencia al inicio de las semanas de administración de agua.

Palabras clave: Enfermedad inflamatoria intestinal; Colitis ulcerosa; Colonoscopia; Bioluminiscencia.

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Títol: Un model de colitis ulcerosa des de la perspectiva de la biofísica com a contribució a la comprensió de la malaltia inflamatòria intestinal.

Resum:

La colitis ulcerosa és una condició caracteritzada per la inflamació crònica del tracte gastrointestinal, la qual s'engloba dins del grup de les malalties inflamatòries intestinals. Provoca principalment diarrea, pèrdua de pes i sagnat en la femta. Les seues causes són desconegudes, encara que es creu que diferents factors genètics, immunològics i ambientals juguen un paper important en el seu desenvolupament. Els tractaments per a aquesta patologia a hores d´ara no són del tot eficaços en tots els casos. Per tant, l'objectiu del nostre projecte va ser detectar aquesta malaltia abans de l'aparició dels seus símptomes característics, i contribuir a la seua comprensió des d'un punt de vista biofísic.

El desenvolupament de la colitis ulcerosa crònica es va estudiar detalladament emprant ratolins C57BL/6 d'un any. Durant sis setmanes, al grup experimental se li va administrar, amb alternança setmanal, una solució de sulfat de dextrano sòdic al 2%, un químic àmpliament utilitzat per a induir el model de colitis en ratolins ja que produeix uns símptomes molt similars a la colitis ulcerosa humana, i aigua de l'aixeta, mentre que al grup de control només se li va proporcionar aigua de l'aixeta. Es van pesar tots els ratolins, i es van puntuar i recollir la seua femta tres vegades per setmana. A més, els ratolins es van sotmetre setmanalment a anàlisis de bioluminescència per a visualitzar la progressió de la inflamació al llarg del temps, utilitzant la màquina IVIS (In vivo Imaging System), així com una colonoscòpia. La presència de sang en la seua femta també es va detectar utilitzant IVIS. Finalment, amb les dades recopilades, obtingudes mitjançant un estudi biofísic, es va monitorar la progressió de la malaltia de colitis ulcerosa i es va concloure que es pot detectar mitjançant l'estudi bioluminiscent dels ratolins i la seua femta al mateix temps que l'aparició dels símptomes sent la inflamació tissular detectable des de la primera setmana d'experimentació, la qual va resultar especialment apreciable el dia 7, i registrant-se, en general, els valors més alts de bioluminescència a l'inici de les setmanes d'administració d'aigua.

Paraules clau: Malaltia inflamatòria intestinal; Colitis ulcerosa; Colonoscòpia; Bioluminescència.

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LIST OF ACRONYMS AND ABBREVIATIONS

IBD	Inflammatory Bowel Disease
UC	Ulcerative Colitis
CD	Crohn's disease
DSS	Dextran Sodium Sulphate
TNBS	Trinitrobenzene Sulfonic Acid
PRR	Pattern Recognition Receptor
NLR	NOD-like Receptor
TLR	Toll-like Receptor
ROS	Reactive Oxygen Species
NE	Neutrophil Elastase
MPO	Myeloperoxidase
NET	Neutrophil Extracellular Traps
Ctrl	Control
IVIS	In Vivo Imaging System
ROI	Region of Interest

1. INTRODUCTION

1.1. Causes of inflammatory bowel disease

In the present century, an increase in the number of patients suffering from inflammatory bowel disease (IBD) has been registered worldwide, affecting adults and children (Siew C Ng, et al. 2018). IBD mainly comprises two complex and multifactorial conditions, Crohn's disease and Ulcerative Colitis, which are characterized by recurrent and long-lasting inflammation of intestinal epithelium, associated with blood in stool, pain, diarrhoea and weight loss. Even though, the IBD pathogenesis is not completely understood (Antoniou et al., 2016; Ho et al., 2020; Kobayashi et al., 2020; Maronek et al., 2021; Seyedian et al., 2019; Y. Z. Zhang & Li, 2014) it is furtherly known that its symptoms can be from mild to severe or even life threatening (Seyedian et al., 2019).

Crohn's disease (CD) affects any part of the gastrointestinal tract in a non-continuous way, acting on entire layers of the intestine and contrasting with ulcerative colitis (UC), which is found only in the colon and represented by mucosal inflammation (Y. Z. Zhang & Li, 2014). UC is considered an episodic disease, as it comprises periods of acute exacerbation, during which the signs and symptoms of this condition can be easily detected, such us inflammation or tissue injury, and intervals of remission while, despite the damage is still present, the patient can be asymptomatic (Sedghi et al., 1993). This condition may result in patients suffering from iron deficiencies, osteoporosis, and colon cancer if its duration is long enough (8-10 years)(Seyedian et al., 2019). Despite the fact that UC has been lengthy studied, its causes are still unclear, even though it has been commonly related with genetics, deregulations of the immune system, microbiome alterations, and environment.

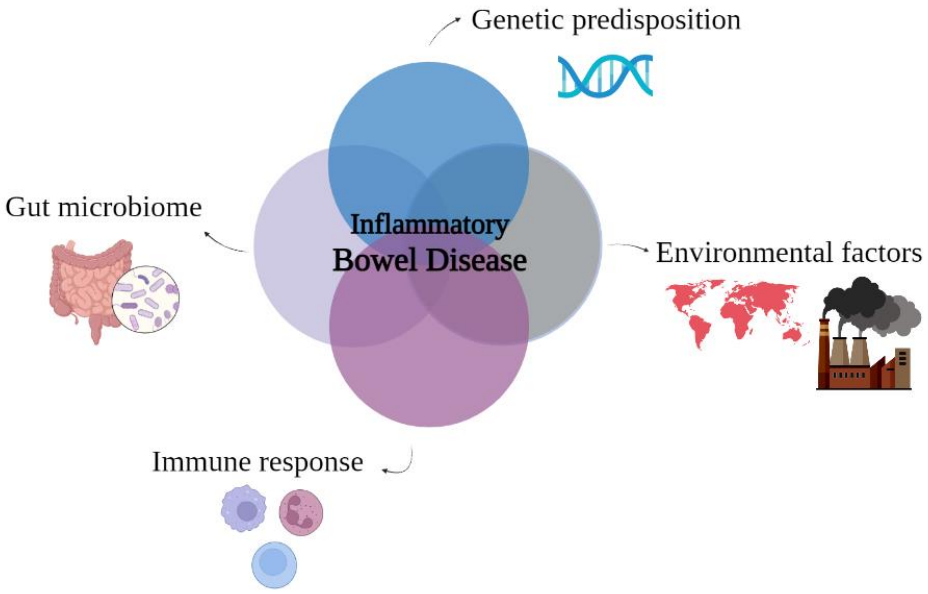

***Figure 1**: Factors that play important roles in the pathogenesis of inflammatory bowel diseases. Figure created with Biorender (BioRender, n.d.)*

a) GENETICS

Recent advances in genetics, especially in genome-wide association studies have shown the existence of certain genes and single nucleotide polymorphisms linked to the development of IBD in patients genetically predisposed to suffer from this disease. Even though it was found that the heritability of those genes is the cause of only the 20%-25% of the registered cases, it can be considered that the genetic component strongly influences the susceptibility of IBD (Mentella et al., 2020; Y. Z. Zhang & Li, 2014). Actually, some evidence supported the hypothesis that there is a higher percentage of Jewish people suffering from CD and UC than non-Jewish people (Seyedian et al., 2019).

There have been found 260 susceptible loci related with the IBD, from which, as was mentioned by Ho et al., 2020, the 67% of them are associated with UC and CD. Among all the identified genes related with the IBD, CARD15, also known as NOD2 is, as defends Eichele & Kharbanda, 2017, the most studied gene because of its function recognising bacteria in the lumen. NOD2 binding to the bacterial peptidoglycan causes the activation of NFκB a proteic complex that plays a key role in the regulation of the immune response, secreting pro-inflammatory mediators that guarantee the immune response against the detected antigen (Atreya et al., 2008; Lu & Zhao, 2020). However, it was observed that the NOD2 polymorphism confers higher risk of suffering from IBD, being from 1.75 till 4 times higher the risk if it is found in heterozygosity, while in homozygosity it increases between 11 and 27 times the possibilities of suffering from UC or CD (Eichele & Kharbanda, 2017).

The NOD2 gene belongs to the family of NOD-like receptors (NLRs), found in the cytoplasm, which together with the toll-like receptors (TLRs), located on the cell surface, form the pattern recognition receptors (PRRs), a family of proteins which are able to identify conserved microbial motifs, such as carbohydrates, nucleic acids or peptides, activating the innate immune response (Sahoo, 2020).

b) IMMUNE RESPONSE

The immune system is composed by the innate immune system, which constitutes the first line of defence, and the adaptative immune system, which is slower than the innate immune response but highly specific. The defect in any of these two pathways has been shown to be related with the development of intestinal inflammation in patients with IBD (Geremia A, 2014).

Innate immunity is non-specific but very fast and is composed by neutrophils, macrophages, dendritic cells, and natural killers. Some studies have shown that these immune cells, as well as the PRR are importantly modified in individuals diagnosed with IBD. The interaction of the PRRs with a pathogen triggers the signalling cascade leading to the activation of NFκB, as has been previously mentioned. Indeed, the literature stands out the NFκB pathway as one of the most influential paths in the development of the UC. However, evidence shows that the activation of NFκB has a dual role, being able to be positive or negative, as its hyperactivation would lead to tissue damage. Because of this, the inhibition of NFκB pathway is being studied as a possible treatment of the IBD (Atreya et al., 2008; Lu & Zhao, 2020; Yan et al., 2018; Y. Z. Zhang & Li, 2014).

In contrast, adaptative immune response takes several days but is very specific and confers "memory". The adaptative pathway is composed by the B and T cells, whose abnormal function results in the IBD signs and symptoms. It has been observed in some studies that the anomalous response of Th1 cells could be related with the intestinal inflammation in CD, while UC is thought to be caused by the Th2 and Th17 cells abnormalities (Eichele & Kharbanda, 2017; Y. Z. Zhang & Li, 2014). Furthermore, it was proved that during the active phase of UC, the number of macrophages in the intestinal lamina propria increase, as a consequence of the autophagy failure, disturbing the intestinal homeostasis (Ho et al., 2020). This accumulation of macrophages conducts to the excessive production of proinflammatory factors, such as IL-1, IL-6 or TNF-α; as well as the stimulation of neutrophils, enhancing the intestinal inflammation and resulting in intestinal epithelial tissue damage. Also, it was observed that the severity of UC disease in humans and mice is directly related with the degree in which the neutrophils penetrate the lamina propria and epithelium (Eichele & Kharbanda, 2017; Ho et al., 2020). Therefore, there have been proposed some new potential therapeutic targets for the improvement of the UC, which consists in the suppression of the proliferation of macrophages, the molecules that modulate them and neutrophils in the lamina propria (Luzentales-Simpson et al., 2021; Ren et al., 2021; Yan et al., 2018).

i) Mechanism of NETosis

Among all the immune cells, we will focus on neutrophils, the most abundant innate immune effector cells in humans. They are phagocytes whose main functions are the pathogen clearance by phagocytosis, degranulation of antimicrobial substances, production of reactive oxygen species (ROS) and the release of neutrophil extracellular traps (NETs), by a process known as NETosis. The NETs formation, composed by granular proteins and chromatin, plays a key role in the neutralization, and kill of viruses, bacteria, parasites and fungi, preventing its propagation (Kobayashi et al., 2020; Papayannopoulos, 2018). NETs can sustain the inflammatory response (Ho et al., 2020). However, it has been demonstrated that an excessive NET production participates in the destruction of the intestinal barrier during the inflammation process, as a consequence of the induction of the epithelial cells' apoptosis by NETs (Lin et al., 2020). Thus, it can be said that NET formation is involved in the protection against infection, but it is also related with the pathology of diverse conditions, as for example the IBD, rheumatoid arthritis or liver disease (Maronek et al., 2021; Wéra et al., 2016). Indeed, it was observed that the severity of the UC was proportional to the neutrophil infiltration rate, postulating that the downregulation of neutrophils and some of its functions as the chemotaxis, ROS or myeloperoxidase formation contribute to the development of UC (Kobayashi et al., 2020; Wéra et al., 2016).

Several proteins that participate in the composition of NETs have been identified, such as histones, neutrophil elastase (NE) or myeloperoxidase (MPO), implying that the constitution of NETs may change according to the stimulus, and these components give the pro-inflammatory and pro-coagulant properties to NETs. When the neutrophils arrive to the infection site, the NETosis process takes place and NETs expand into the extracellular space. The NETs formation can be induced by pathogens, endogenous stimuli and activated platelets, preventing the pathogens to spread. With the neutrophil

activation, there is a release of ROS, which induce the MPO pathway causing the breaking of its azurophilic granules, which at the same time leads to the oxidative activation of NE, needed to block the phagocytosis (Papayannopoulos, 2018). The azurophilic granules produce hydrogen peroxide (H_2O_2) and hypochlorous acid, which can be detected *in vivo* by luminol (5-amino-2,3-dihydro-1,4-pthalazine-1,4-dione), a chemiluminescent substrate. Luminol is a useful biomarker which allows the detection of activated neutrophils by bioluminescence. It has been already used *in vivo* to track the UC, cancer or arthritis progress (Davis et al., 2019; N. Zhang et al., 2013), and the aim of this project was to employ this tool, and the knowledge that the proteins associated with activated neutrophils and NETs are higher in the UC induced animals than in healthy controls, to try to detect the condition before the onset of the disease.

c) ENVIRONMENT

The suggestion of a possible relationship between the environment and the development of the IBD dates from the beginning of the last century, when a significant grow in the number of people suffering from UC in newly industrialised countries was detected, followed by a rising incidence in CD. The rapid industrialization of some areas supposed a pollution increase, together with some changes in diet, and a new lifestyle; stricter hygiene, less infections, and higher access to a wide variety of antibiotics. All these changes, commonly known as "Westernisation" are considered as risk factors for the development of the IBD (Mentella et al., 2020), a condition that nowadays is suffered by 10 million people worldwide, according to the European Federation of Crohn´s and Ulcerative Colitis Associations. From the 20th century until now, the rise in the number of patients having this pathology has been constant, registering an increase of 14.9% of cases each year, what suggests that in 2025 the prevalence will affect more than 30 million people (Ho et al., 2020). Also, the geographical location, ethnic and racial characteristics have been considered as factors that contribute to the differential prevalence of the disease among people. As describes Seyedian et al., 2019, it was observed that the prevalence of IBD was lower in Africa and Asia than in England and North America, highlighting the role of the environmental factors.

Accompanying the environmental factors that contribute to the appearance of the IBD, it should be mentioned the discovery of Leslie et al., n.d. , who detected that the vitamin D deficiency could be related with the development of CD and UC. Research provides the conclusion that murine models, which had deficiencies in vitamin D were more susceptible to the treatment with DSS to induce colitis (Y. Z. Zhang & Li, 2014). Also, it has been proposed that high levels of stress, depression and anxiety may play a key role in the worsening of IBD pathogenesis (Y. Z. Zhang & Li, 2014).

On the other hand, some protective agents have also been identified, as for example smoking and the appendectomy, which have been pointed out by many authors as shielding against the UC induction. Nevertheless, due to the multiple well-known drawbacks of tobacco, and the complications that an appendectomy entails, its real benefits are controversial (Ho et al., 2020; Kobayashi et al., 2020; Y. Z. Zhang & Li, 2014).

d) MICROBIOTA

It is estimated that the human gut microbiota is composed by more than 1000 bacterial, fungi and virus species, whose diversity usually remains stable over time. The microbiome comprises one hundred times more genes than the human genome, and it acts in symbiosis with the host, protecting him/her against pathogenic infections, producing vitamins and facilitating the digestion, among other functions. Furthermore, it has been identified a dependency relationship between the gut microbiota and the immune system, developed during the first years of life (Ho et al., 2020; Seyedian et al., 2019). This association is the responsible of keeping a homeostatic state between the host and its microbiome, which is maintained under normal conditions.

Recent studies have shown that the homeostasis can be disturbed by diseases, antibiotic intake, or important changes in diet. However, the treatment of UC with antibiotics, in some cases, has been successful, as well as the implementation of gluten free diets, which improve the UC-like symptoms, or Mediterranean diets, which have been related with the decrease of the inflammatory markers. Also, a significant reduction in the biodiversity of faecal microbiome in people diagnosed with IBD was noticed, in comparison with healthy people, pointing out the correlation between the IBD and the dysbiosis (imbalance in the number or type of microbial colonies that have colonized the human digestive tract). Focusing on the UC pathogenesis, it was observed a decrease in the variety of protective microbes that compose the human microbiota, at the same time that an enrichment of inflammatory organisms was notified (Eichele & Kharbanda, 2017; Kobayashi et al., 2020; Mentella et al., 2020; Y. Z. Zhang & Li, 2014).

1.2. Mouse model

With the persistent aim to study, characterise, understand and treat the IBD, many animal models have been employed, being mice the preferred laboratory animal for the last century. Then, mice are very well known by the scientific community (Hickman et al., 2017).

Mice present many advantages that have make them the mainly used animals in the study of UC, as its small size and low space and food requirements, being very economical. Also, its short lifespan, fast reproduction and easy handle and transport, and the fact that they are biologically very similar to humans and get many of the same diseases, for the same genetic reasons. Furthermore, mice can be genetically manipulated to mimic any human disease or condition. Especially, mouse models are useful for comparative studies, due to many mice can be treated and evaluated easily at the same time, what makes them an indispensable tool to study and discover the IBD pathogenesis as well as to evaluate potential therapeutics (Eichele & Kharbanda, 2017; Hickman et al., 2017; Wang et al., 2019). Concretely, the C57BL/6 inbred mouse strain is extensively employed in biomedical, immunological and nutritional research, as well as for many human diseases, like obesity, cancer or DSS-induced IBD. This mouse strain was proven to be generally more suitable for the study of chronic UC than others, such as BALB/c. However, any important differences were observed between the several sub-strains of C57BL/6 strain for the IBD study (Kim et al., 2021).

The UC can be induced in mice by different ways. It can be chemically induced, by the employment of dextran sulphate sodium (DSS), trinitrobenzene sulfonic acid (TNBS) or oxazolone. Among them, the most widely used mouse model of colitis, and the one that was employed in the present thesis is DSS, an anticoagulant chemical which induced IBD in an easy, fast, controllable and reproducible way. TNBS is a heptaning agent that induces the immunologic response of the colonic proteins in the host, while oxazolone is also a heptaning agent, but it induces colonic inflammation in a different way than TNBS, causing acute superficial inflammation. Besides, this condition is commonly produced by the employment of cell transfer, or IL-10 knockout, furtherly studied by other researchers (Almero, 2007; Antoniou et al., 2016; Chassaing et al., 2014; Kiesler et al., 2015; Kotla et al., 2022).

1.3. Detection and diagnosis

The establishment of an optimal treatment for the IBD requires a previous accurate diagnosis. Nowadays, the diagnostic criteria employed on medicine to detect UC is mainly based on clinical symptoms, endoscopic and histological analysis, however, no definitive diagnostic method has been found so far (Kobayashi et al., 2020).

Initially, the detection of the characteristic symptoms (bloody and/or mucosal stool, frequent bowel movement, pain…) by the patient is of great importance to diagnose correctly the IBD. After this indispensable step, many other methods can be employed, such as an endoscopic evaluation of the extent and severity of the disease, but it can produce a perforation in patients suffering severe colitis, then it is not recommended in some cases. Also, a histopathological study, performing biopsies from each segment of the bowel, or some biochemical tests could be useful for the interpretation of the condition. When the endoscopy is contraindicated, further analytic methods are implemented, such as the x-rays analysis or the white blood cell scan which determine how much inflammation is present, the MRI, bowel ultrasonography or computed tomography (Kobayashi et al., 2020).

Despite all the technical advances achieved, no method has been already applied in the hospitals for the detection of the UC before the onset of the characteristic symptoms of this condition. Furthermore, most of the methods currently utilized require a previous preparation; as for example the computerized tomography, which takes many x-rays images at the same time. Before it, the patient is asked to drink a contrast agent, which may cause diarrhoea (*DIAGNOSING CROHN'S DISEASE AND ULCERATIVE COLITIS*, 2010; Minesh Khatri, 2020). Then, finding a way to detect the disease prior the first symptoms appear would be the first step to understand better the IBD, being able to treat it before there is an irreversible damage in the intestine, thus improving the patient quality life and the chances of recovery.

1.4. Treatment

The two conditions that involve the IBD are difficult to cure, being in some cases unresolved and causing hight morbidity (Kim et al., 2021; Luzentales-Simpson et al., 2021). Historically, the treatments employed in patients suffering from this condition tried to eliminate or reduce the symptomatology, or in some cases, to keep the disease at a fixed stage, nevertheless no cure exists, and lifelong treatment is required (Mentella et al., 2020). Usually, the therapy of the IBD comprises jointly self-care and medical treatment, reducing the associated symptoms by the adoption of good lifestyle habits, such us avoiding alcohol and coffee intake, fatty foods, or daily products, and learning how to manage the stress. However, not a universal formula to improve the symptoms has been found, being the response of each patient to the same therapy different.

Among the medical treatments, commonly aminosalicylates, corticosteroids, antibiotics and immunosuppressive drugs are employed, however, any of them have shown totally satisfying results on patients. In contrast, a prolonged administration of nonsteroidal anti-inflammatory drugs, has been proven to be related with higher risk of CD and UC (Lu & Zhao, 2020; Mentella et al., 2020; Seyedian et al., 2019; Y. Z. Zhang & Li, 2014). Also, it has been proposed the strategy of blocking the activation of NFκB to prevent the mucosal inflammation in IBD patients (Atreya et al., 2008; Yan et al., 2018), and the removal of the affected region by surgery has been employed in some people, however all of them present side effects and have not resulted totally successful (Luzentales-Simpson et al., 2021), then further study is needed in this area to find a useful and safe treatment for the UC disease.

2. OBJECTIVES

2.1. General Objectives

The objective of the present project was to detect the chronic ulcerative colitis disease before the appearance of the characteristic symptoms of it, employing an *in vivo* bioluminescent analysis. With this investigation we tried to contribute to the understanding of the IBD, from a biophysical point of view, through the in-depth study of the evolution of the disease. To achieve it, the chronic UC was induced in mice with DSS, whose observable symptoms, and the progression of the inflammation in their colon were monitored.

2.2. Specific Objectives

1. Handle, mark, immobilize, shave and inject alive mice in a secure and suitable way.
2. Sacrifice mice according to the retro-orbital bleeding technique and extraction of its organs.
3. Detection of symptoms related to UC.
4. Preparation of DSS and luminol solutions for the induction of UC and observation of bioluminescence in mice respectively.
5. Obtention of images by using a colonoscope and evaluation of the state of the colon in mice.
6. Employment of the In Vivo Imaging System (IVIS) for the observation of bioluminescent images in mice, and their stools, and extraction of data from them.
7. Analysis of statistical data from a biophysical point of view.
8. Introduction to the GraphPad software.

3. MATHERIALS AND METHODS

3.1. Animals

In this experiment, one year old C57BL/6 mice (Charles River, Wilmington, MA, USA) were employed. All the animals were kept under standard conditions, at a temperature of 22-24°C, with a 50% of humidity, and with cycles of 12 hours of light and 12 hours of darkness. Mice were fed with standard chow and tap water *ad libitum*. Prior the initiation of the project, the animals were habituated to these conditions. The experiment was approved by the State Veterinary and Food Administration of the Slovak Republic (decision 3041/17-221/3) and by the Ethics committee of the Comenius University in Bratislava, Slovak Republic.

A total number of twelve male and twelve female mice were chosen for this experiment. The animals were divided in two groups each sex, the control group (CTRL), which contained four mice, and the experimental group (DSS), with eight mice. All mice were weighed to ensure that the average group weight was balanced between both groups from the same sex, being for females 25.45 g the CTRL, and 24.25 g the DSS in average, while for males was 31,38 g the control group and 30,05 g the DSS group. Furthermore, each animal was labelled individually with a mark in the tail, and a personal ID number was given to them to have an individual tracing of them.

3.2. Induction of Colitis

The murine model of ulcerative colitis was induced with a solution of DSS 2% in tap water (molecular weight = 40,000, AppliChem, Darmstadt, Germany), by mixing 6 g of DSS powder with 300 mL of tap water, following the procedure generally showed in Figure 2. This solution was administrated only to mice from the experimental group, while mice in the CTRL group received tap water during the entire experiment. As this trial aimed to study the chronic ulcerative colitis disease, the experiment lasted for 6 week (42 days) during which there were alternated one week of DSS administration with a wash out week, in which all mice drank only tap water. Each bottle of DSS water solution and tap water were emptied and refilled at least once per week, changing it more often if any growth or turbidity was observed, and recording each time the weight of the bottle. The last day of experimentation (day 42), after performing the required measurements, all mice were sacrificed by using the retro-orbital bleeding procedure, under the anaesthesia effects. All mice blood was collected, correctly labelled, and stored for future scientific projects. Also, the liver, spleen, intestine, and colon of each mouse were extracted, weighed and measured for a possible future use.

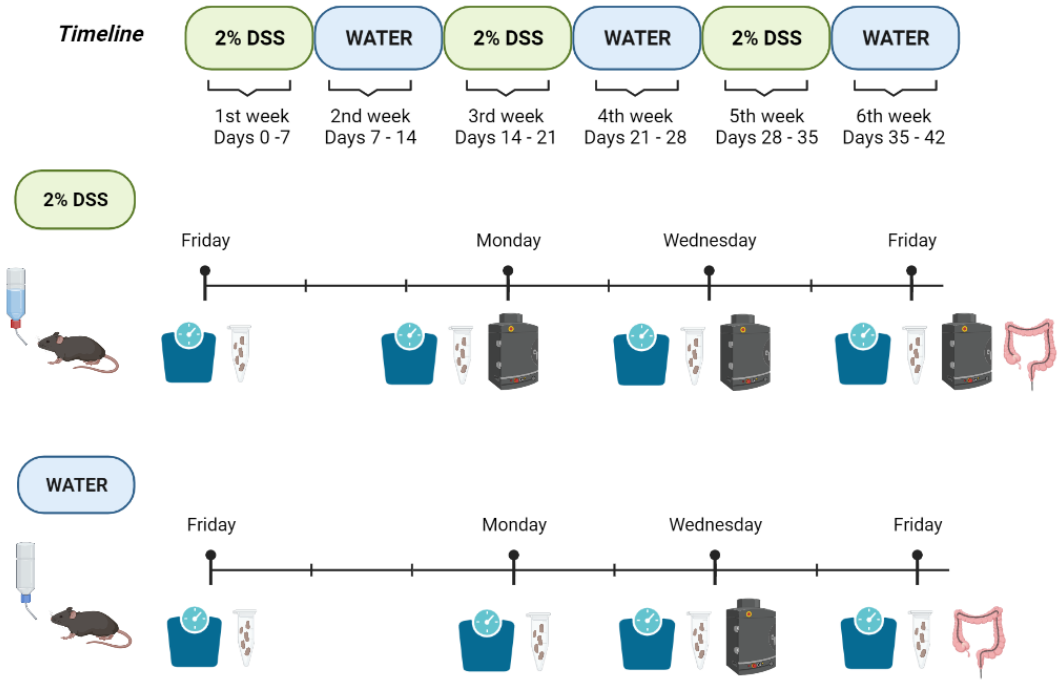

Figure 2: *Schematic representation of the procedures carried on to evaluate the progression of the ulcerative colitis disease in DSS induced mice, according to the experimental week and the day of the week. Figure created with Biorender (BioRender, n.d.)*

3.3. Evaluation of Disease

3.3.1. Assessment of Colitis Severity

To evaluate the progression and severity of the induced ulcerative colitis, mice body weight, stool consistency, rectal bleeding and water intake were checked three times per week (Mondays, Wednesdays and Fridays) during the 6 weeks of experimentation, as showed in Figure 2.

Stool consistency was scored, together with rectal bleeding, according to the classification described by Kim et al., 2021; Maronek et al., 2021, on a scale from 0 to 3, representing as follows: Thick, formed stool without blood: 0; Soft stool, blood not present: 1; Watery stool, blood not present: 2; and soft or watery stool with the presence of blood: 3. All the stool samples were collected separately in labelled Eppendorf tubes and stored frozen at -20°C. Also, the behavioural alterations in mice were macroscopically evaluated.

3.3.2. Animal Endoscopy

animal endoscopy was used as a complementary tool for the evaluation of the chronic UC progression. The endoscopic study was performed every week on Friday, except on the last week. In total five times during the whole experimental process (days 14, 21, 28, 35 and 42) to all mice. This procedure was carried out having anaesthetized previously all mice by the inhalation of isoflurane (Isoflorano, Cristália Pharmaceutical

Chemicals, São Paulo, SP, Brazil) mixed with oxygen. After the procedure, mice were supervised until they woke up, during this time any alterations in animal behaviour were detected, mice remained active and did not show any sign of pain.

The rectum and part of the colon visualization was performed with the portable veterinary device Tele Pack Vet X LED RP100 (Karl Storz, Tuttlingen, Germany). This instrument is composed by an endoscope of 1.9 mm in diameter, light source, camera, monitor and insufflation pump. The endoscope was inserted into the anus approximately 8 cm having removed, by a ventral massage the present stools, before the introduction of the rigid endoscope. Epithelium of the colon was analysed according to a validated endoscopic scoring system to evaluate the severity of colitis, focusing in 4 categories described by (Maronek et al., 2021): Vascular translucency, presence of fibrin deposits, bleeding and reddening of the colon mucosa, according to the scores in Table 1.

Table 1: *Endoscopy scoring system. The table describes score values of each examined category. Table took from (Maronek et al., 2021).*

Category	Score	Description
Translucency	0	Vascularization fully visible
	1	Vascularization partially visible
	2	Vascularization not visible
Fibrin	0	No fibrin is present in the mucosa
	1	Small fibrin deposits in the mucosa
	2	Large fibrin deposits in the mucosa
Bleeding	0	No bleeding
	1	Several sites of mucosal bleeding
	2	Many sites of mucosal bleeding, may obstruct camera of the endoscope, bleeding may start spontaneously or as a reaction to contact with endoscope, blood may directly flow out of the anus
	3	Profound mucosal bleeding, usually obstructs camera of the endoscope, bleeding often starts spontaneously and blood usually flows out of the anus
Reddening	0	No reddening visible
	1	Several sites of mucosal reddening
	2	Many sites of mucosal reddening

3.3.3. In Vivo Ulcerative Colitis Bioluminescence Study in Mice

The bioluminescent study was performed three times per week (Monday, Wednesday and Friday) during the DSS weeks and one time per week (Wednesday) during the wash out weeks. Bioluminescent images were captured using the In Vivo Imaging System (IVIS) Spectrum (Perkin Elmer, Santa Clara, CA, USA) and analysed using IVIS image software (Living Image). To acquire the image, some steps were followed as are described:

3.3.3.1. Chemiluminescent solution

Firstly, a solution of 75 mg of luminol (Luminol sodium salt, Biosynth Carbosynth, UK) in 1,5 mL of sterile PBS was prepared (enough for the administration of all mice, to avoid variations in concentration between preparations). This solution was hidden from the light during its preparation, storage, and use, due to luminol is a light sensitive chemical compound, and its

exposition to the light could alter the results obtained in the bioluminescent study (Hyde & Skare, 2018).

3.3.3.2. Animal preparation

Before performing the bioluminescent analysis, all mice ventral side was shaved, from between the legs up to the bottom of the rib cage using an electric hair cutter. This procedure was performed having immobilised the mice and being careful of not damaging their skin. Also, it was stablished the order and position in which each mouse would be introduced in the IVIS machine.

3.3.3.3. Machinery setting up

Firstly, the XGI-8 anaesthesia system was prepared, according with the manufacturer instructions and as it is described by (Hyde & Skare, 2018) but carrying out some small variations, briefly:

a. Weigh F-air canisters, filled with activated carbon filters, prior to each use of the XGI-8 anaesthesia system. Filters must be changed after the initial weight increases 50 g.

b. Verify that the isoflurane level is enough according to the imaging time, it cannot be added once the machine is working.

c. Turn on the evacuation pump and the valve of the oxygen tank. After this, turn on the oxygen handle of the XGI-8 unit.

d. Turn dial of the isoflurane vaporizer to 2.5-3.5, according to the experimental requirements, considering the isoflurane resistance developed by the mice after few weeks.

e. Toggles for the IVIS flow and animal isolation chamber should be set at 0.25 lpm and 1.5 lpm, respectively, and will be turned on and off throughout the experiment as needed.

After this, Living Image programme was stablished according to the user guide of the programme (*Living Image® Software User's Manual*, 2002) and following the indications described by (Hyde & Skare, 2018), introducing some variations, briefly:

a. Open the Living Image (64-bit) software and select the initialization button in the acquisition control panel. Wait the green light to open the door of the IVIS machine, which will appear once the initialization is completed, and the needed temperature is achieved (-80ºC).

b. Let the luminescent, photographic imaging mode, overlay boxes, F/stop, that measures the amount of light that is let into your lens opening through a hole (Foreground Inc, 2020), and emission filter,

which allows the desirable fluorescence from the sample to reach the detector, while blocking unwanted traces of excitation light (Chroma Technology Corporation, 2020), as are stayed by default, as well as other parameter such us subject height, or temperature. The default temperature of the platform is 37 °C, the average corporal temperature of mice.

c. Choose the measurement option, time and series that better suits to the experiment. In our case, it was chosen the bioluminescence imaging, and using the manual settings they were stablished 2 series of study, one at 5 minutes and another one at 10 minutes.

d. Prepare the IVIS platform. Insert the required number for nose cones. Black shields should be placed between the nose cones to block signal from the neighbouring mouse allowing quantification of an individual specimen without overlapping bioluminescence. Adjust the field of view in the software control panel to include all mice.

e. Create a folder to save the acquired images.

Later, the bioluminescent images were obtained. For the *in vivo* bioluminescent analysis, animals were anaesthetized by inhalation of isoflurane (Isoflorano, Cristália Pharmaceutical Chemicals, São Paulo, SP, Brazil) inside the isolation chamber, as it has been mentioned before, according to the order previously seated for its measurements. After waiting for them to fall asleep (7-10 minutes), mice were injected subcutaneously with the chemiluminescent solution previously prepared (point 3.3.3.1). Each mice received an injection of 0,1 mL of this solution, using a 0,3x12 mm needle. Just after being injected, they were located into the IVIS platform for imaging, placing each mouse in a nose cone in an upside-down position, facing the ventral side with the camera located at the top of the machine, and the flow of isoflurane to the IVIS was turned on. In all measurements, the control mice were located in the left positions, while the DSS mice were in the right positions, in this way, it was easier later to compare images from different mice.

Once the two images had been taken (5 and 10 minutes) and saved in the preselected folder, mice were returned to their cages positioned over a heating blanket, to keep warm the animals, and were monitored during its waking up. This procedure was repeated as many times as was required to obtain pictures from all mice. Finally, the flow of isoflurane to the IVIS, as well as to the isolation chamber were turned off, and the XGI-8 system was shouted down, according to the manufacturer instructions.

3.3.4. Detection of Blood in the Stool of colitis mice by IVIS

3.3.3.4. Stool preparation

Throughout the experiment, to study the progression of the intestinal inflammation related with the DSS-induced ulcerative colitis, stools of all mice were collected and placed in a separate 1.5mL Eppendorf, appropriately labelled. The samples were stored in a freezer at -20°C.

Before the measurement, the stored samples were defrosted and 500µL of distilled water were added to each sample. Subsequently, the samples were completely homogenized by using the vortex (Vortex V-1plus, Biosan, Riga, Latvia). The obtained mixture was centrifugated for 2 minutes at a relative centrifugal force of 8000g (Centrifuge 5804R, Eppendorf, Hamburg, Germany). After this, 200µL of the resulting supernatant were introduced into clean and labelled 2mL Eppendorf tubes and they were frozen until the next use.

3.3.3.5. Luminol Solution preparation for Blood detection in Stool and Image Acquisition

For the induction of bioluminescence in the prepared samples, a solution of 32mg of luminol (SERVA, Heidelberg, Germany) were mixed with 2.4g of KOH (Sigma-Aldrich, Saint-Louis, Missouri, USA) and dissolved in 40mL of distilled water. This solution was stored at 4°C, without access to light. Before the analysis of the samples, the solution was mixed with H2O2 in a ratio of 1:1. Then, 300µL of luminol solution were added to the 200µL of sample previously stored, and immediately placed in the IVIS system and started the measurement. The samples were organized by days, introducing all the samples of the same day together in the IVIS machine, and one picture was obtained from each group of samples.

3.4. Data Analysis

3.4.1. Quantification of IVIS Bioluminescence

IVIS data was obtained from the Living Image 4.5.2 (64-bit) programme, following the manufacturer information (Living Image® Software User's Manual, 2002)and the protocol described by Hyde & Skare, 2018, but introducing some variations one more time.

For the measurements, the first bioluminescent picture obtained from the present experiment was opened. It was checked that the maximum count was between 600 and 60000, due to images below 600 or over 60000 counts cannot be used for ROI quantification. After this, the units were changed to photons and the binning was stablished at 4, where better resolution could be seen without losing too much sensitivity. Also, the region of interest (ROI) for measuring the bioluminescence was selected in the control mice drawing a circle, using the circle tool, and creating a ROI that generously covered all the abdominal area of the mouse, inside which the bioluminescent signal was found. This same ROI was kept constant in area and positioning within all experiments, displacing it to be located in the ventral region of

each mouse. Finally, it was pressed “Measure ROI” to obtain all the information of each ROI in the picture, which includes the total flux (photons/sec (p/s)), average radiance (photons/s/cm2/sr), standard deviation, minimum and maximum radiance. This data was exported to an Excel file, where useful side information was added, such as the mouse ID, group (CRLT/DSS), the day of experimentations in which this picture was obtained, and the time (5/10 minutes). The same procedure was carried out with each picture obtained during the experimentation days, copying, and pasting the same ROI, to be able to compare the pictures.

According to the stool samples, the procedure was similar. Firstly, it was ensured that the maximum count was between 600 and 60000, later, the units were changed to photons and the binning was stablished at 4. The region of interest (ROI) for measuring the bioluminescence was selected in the first sample, drawing a ROI that covered the whole Eppendorf, and this ROI was copied and pasted in all the other samples keeping constant the area and position of it. Then, the ROI was measured, and the average radiance data was obtained.

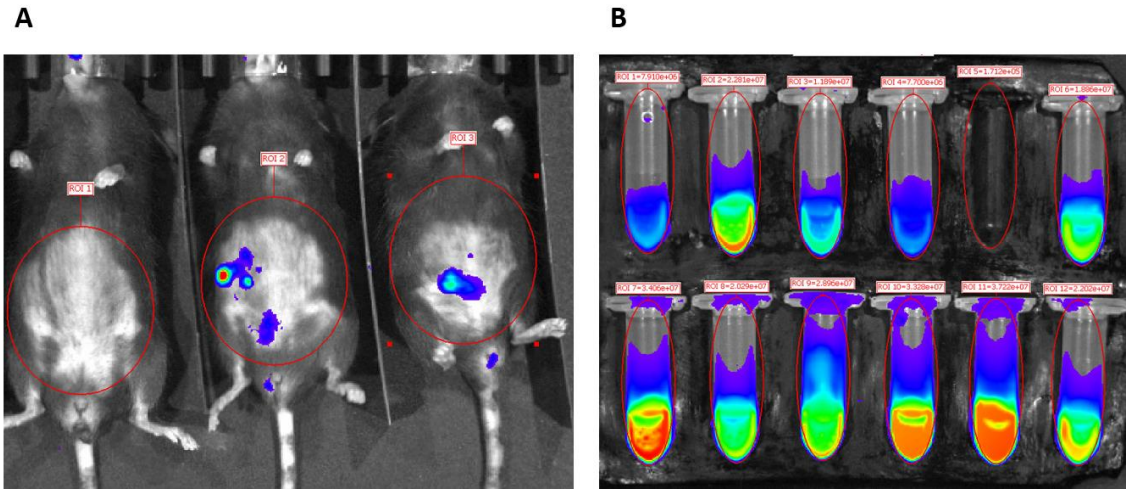

Figure 3: *Representation of the ROI drawing for the bioluminescent measurement in (A) mice, and (B) stool samples.*

3.4.2. Statistical Analysis

All the recorded data from the weight and stool consistency variations in mice, as well as the data obtained from the IVIS pictures were introduced in GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). Normality of the data was studied by applying the Normality and Lognormality Tests, also the normal QQ plot was obtained. Afterwards, the outliers were identified. Finally, a multiple test analysis was performed. Data were analysed using either Mann-Whitney test, a non-parametric version of the T-test, when comparing two groups, or two-way ANOVA when comparing more than 2 groups. Data are presented as mean $\pm$ standard deviation. P-values less than 0.05 were considered statistically significant.

4. RESULTS

With the main objective of characterising the ulcerative colitis from a biophysical point of view and trying to detect it before the onset of the disease, the UC was induced on mice by the administration of DSS, whose symptoms were monitored often, comparing them with the data obtained from the bioluminescent study.

4.1. Effects of DSS on Body Weight of mice

In the female group, Figure 4 (A), a time dependent body weight loss was recorded since the beginning of the experiment till day ten, where the average weight of the experimental mice decreased around a 12% its initial value. In the control group just a 4% decrease on day ten was detected. During the second experimental week, the average body weight of all mice increased, reaching the maximum on control mice at day fourteen. The experimental group, this same day arrived in average to at 95.89% of its initial body weight. At the beginning of the third week, a 96.3% of the relative body weight was achieved by both groups, due to a body weight decrease in the control group. This value was kept by the control group until the end of the experiment. Nevertheless, in the DSS group a continuous body weight decrease was recorded from the day seventeen till the day twenty-four, achieving a minimum of 82.4%. In the fourth week, the body weight of the DSS group increased until a 96.8% on day thirty-one, decreasing again during the next DSS week, followed by an improvement kept till the end of the experiment, finishing the sixth week with a mean of 92% in the DSS group.

Regarding to the relative body weight variations in male mice, Figure 4 (B), both groups suffered an increase in their body weight on day three, being greater the increase registered in the control group than in the experimental group. This value was maintained in the control group till the day seven. The DSS group went into time dependent body weight loss, suffering a 12.3% decrease between days five and seven, what forced the removal of males from the study at the end of the first week.

Comparing the relative body weight variations in male and female mice, as it is shown in Figure 4 (C), stronger effects were detected on male. More than a 10% of the average body weight decrease was recorded on experimental male mice group at day seven, while at the same time, less than a 5% of decrease was registered in the female experimental group.

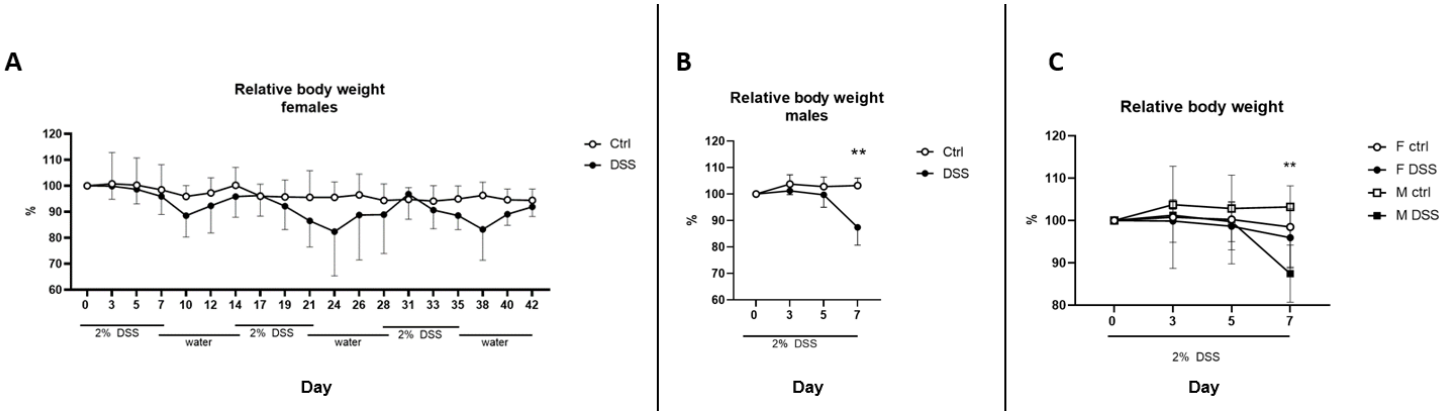

Figure 4: *Changes in the relative body weight of C57BL/6 mice in the experimental (DSS) and control (Ctrl) group. (A) Female mice during 42 days, alternating a week of 2% dextran sulphate sodium (DSS) supplementation with a week of water intake. (B) Male mice during a week of 2% DSS administration. (C) Comparison according to the sex during 7 days of 2% DSS intake. Each value is represented as the mean ± SD for four mice in the control (Ctrl) group of each sex, and the alive mice at the moment of the measurement for the experimental (DSS) group of each sex. T-test, *P<0.05, **P<0.01, ***P<0.001.*

4.2. Evaluation of Colitis Progression according to the Stool

In experimental female mice group, Figure 5 (A), the stool consistency values where between 1 and 2. The recorded scores followed a decreasing tendency during the water intake (second, fourth and sixth weeks), being the minimum average value obtained on day fourteen, while the increase was related with the DSS solution intake (first, third and fifth weeks). The highest values were obtained the last day of each DSS week, being the maximum achieved on day twenty-one. However, in the control group, the scores of stool consistency and rectal bleeding were kept in average between 0 and 1 without following any detected patron.

In experimental male mice, Figure 5 (B), it was recorded a continuous increase since the first day of experimentation, achieving the maximum score of 3 on day seven. In contrast, on the control group, the stool consistency and rectal bleeding scores were kept at an average value between 0 and 1 from day three until day five, and a minimum of 0 was achieved on day seven. No more values were obtained for male mice because they were excluded from the experimentation.

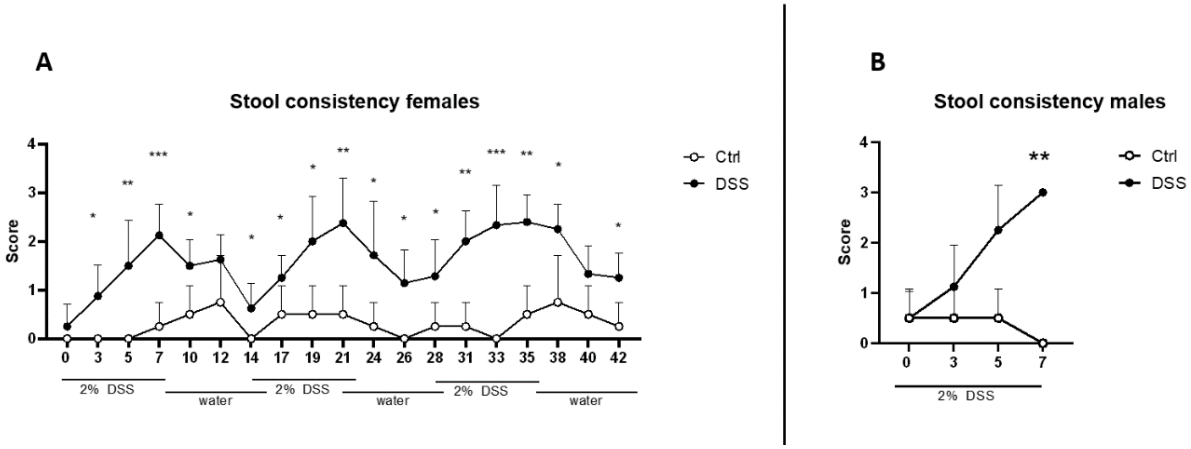

Figure 5: *Symptoms were scored according to the stool consistency and rectal bleeding in one year old C57BL/6 mice, administrated with 2% DSS solution (DSS), compared to the control group (Ctrl), administrated only with tap water. (A) Female mice during 6 experimentation weeks, alternating in the experimental group a week of 2%DSS administration with a week of tap water intake. (B) Male mice during 1 week of experimentation. The scores indicate the severity of abnormal signs. Multiple T-test, *P<0.05, **P<0.01, ***P<0.001, data is significant.*

4.3. Endoscopic Results

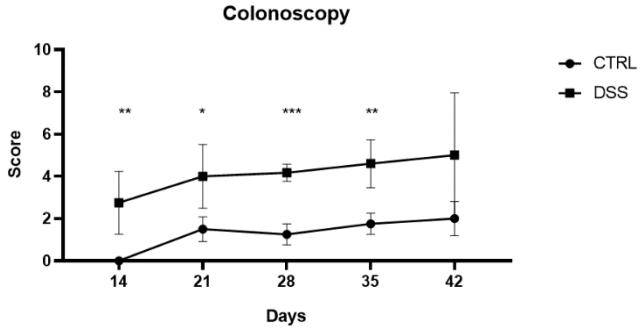

Figure 6: *Endoscopic average total score of translucency, fibrin, bleeding and reddening in one year old C57BL/6 female mice intestine. Multiple unpaired T-tests, *P<0.05, **P<0.01, ***P<0.001, data is significant.*

As it can be seen in Figure 6, the scores registered in the experimental (DSS) group are higher than in the control (ctrl) group. In the DSS group, a constant increase was detected from the beginning, mean of 2.75, till the end of the experimentation, when a score of 5 was obtained. Similarly, in the ctrl group it was appreciated a score increase, from the first endoscopic analysis, where a value of 0 was registered till the last day,

with a mean of 2. However, contrasting with the DSS group, in the ctrl it was observed a general improvement on day twenty-eight, in relationship with the previous analysed day, while in the DSS group any improvement was registered from a general point of view.

Going deeper, vascularization was partially visible in all mice of the control group, not fibrin, bleeding or reddening were detected during the whole project (Figure 7 (A and C)). In the experimental group, during the first two weeks, vascularization was not appreciated, while just few sites of mucosal reddening were detected. Any fibrin or bleeding was recorded (Figure 7 (B)). In the third week, several sites of mucosal reddening were found, but not further changes were appreciated in the other evaluated categories (Figure 7 (D)). Mucosal bleeding was detected sporadically in few mice from the fourth week in advance, also reddening became more notorious in all of them (data not shown).

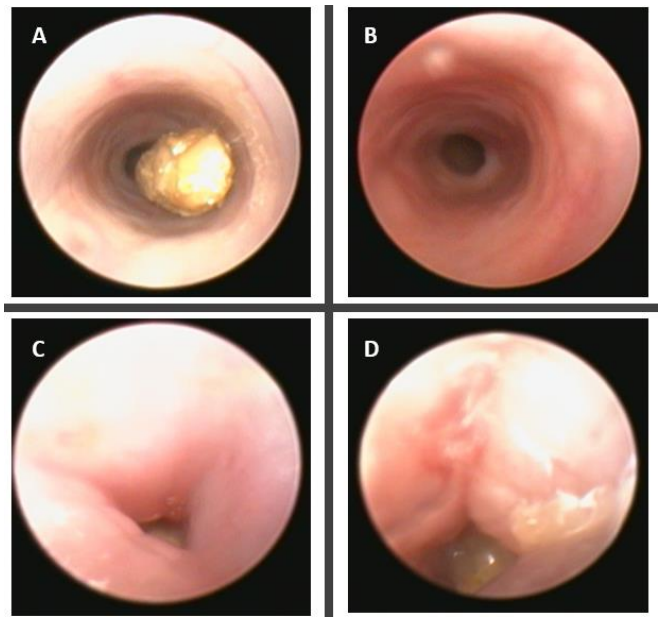

Figure 7: *Endoscopic visualization of rectum on (A) control mice at day 14; (B) experimental (DSS) mice at day 14; (C) control mice at day 42; (D) DSS mice at day 42.*

4.4. In Vivo Bioluminescence Study

4.4.1. IVIS in Mice

On time 5 minutes, Figure 8 (A), the maximum radiance was achieved by the DSS group on day twenty-six, recording an increase of more than 1800 [p/s/cm²/sr] compared to the previous measurement, while any change was detected on the control group these days. On days twelve and thirty-one the signal was also especially high in the experimental group. The highest increase in the DSS category was captured between

days seven (mean of 626.125 [p/s/cm²/sr]) and twelve (mean of 2511.14 [p/s/cm²/sr]), while on day thirty-one the maximum in bioluminescence in the control group was registered. In contrast to the DSS, the ctrl group did not suffer from big changes in bioluminescence during the whole experiment. The minimum in both groups was recorded on day seven. Finally, a constant decrease from day thirty-one till the end of the experiment was detected in both groups.

On time 10 minutes, Figure 8 (B), the bioluminescent values acquired followed a similar tendency than those obtained at 5 minutes. Nevertheless, in the DSS group, the maximum radiance was registered on day twelve coinciding with the maximum in the control group, while at 5 minutes it was achieved on day twenty-six. Furthermore, at 10 minutes, in the DSS group, the bioluminescent value was higher on day thirty-one than twenty-six, decreasing progressively from day thirty-one till the end of the experiment, as well as the control group. Currently, the control group did not suffer big variations in bioluminescence, being the radiance values obtained at 10 minutes generally lower than the ones obtained at 5 minutes. The minimum value of both groups was registered on day seven, while the maximum value in the control group was obtained on day three.

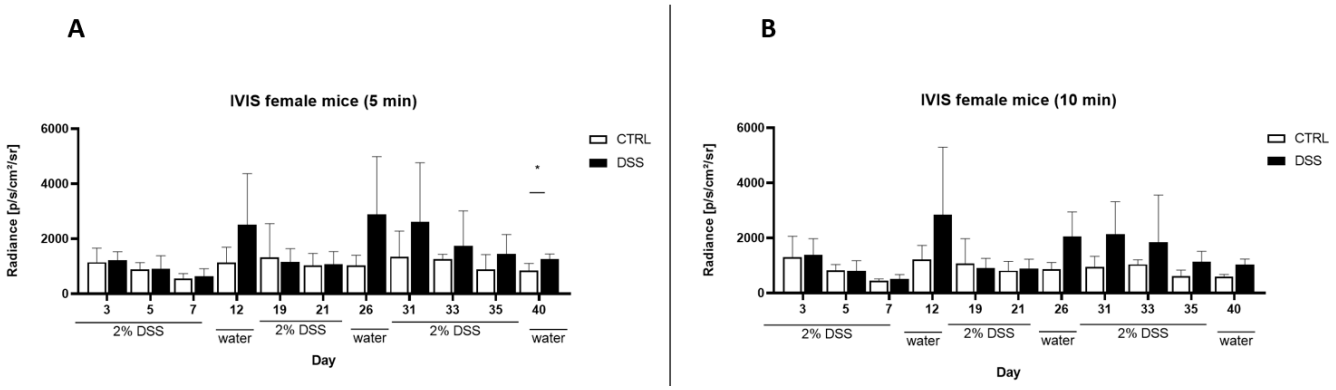

Figure 8: *Luminol bioluminiscence detection of inflammation in intestine by IVIS. Total bioluminiscence during six weeks in DSS induced colitis mice (DSS) vs control mice (CTRL). The bioluminescence was measured at 5 minutes (A) and at 10 minutes (B). Multiple T- test, *P<0.05, **P<0.01, ***P<0.001, data is significant.*

4.4.2. IVIS of Stool

Maximum radiance values were obtained in the third and fifth weeks in both groups, Figure 9, which are DSS weeks. According to the experimental group, the highest radiance values in average correspond with days nineteen, twenty-one and thirty-five, however, the maximum radiance in the ctrl group was obtained on day thirty-three. In contrast, the lowest values in the DSS group were acquired during the second week, which was the first wash out. Concretely on day fourteen the lowest radiance values were registered in both groups. Comparing the fluctuations of the DSS and ctrl groups, both groups increase and decrease together, being the values from the control group lower in all cases.

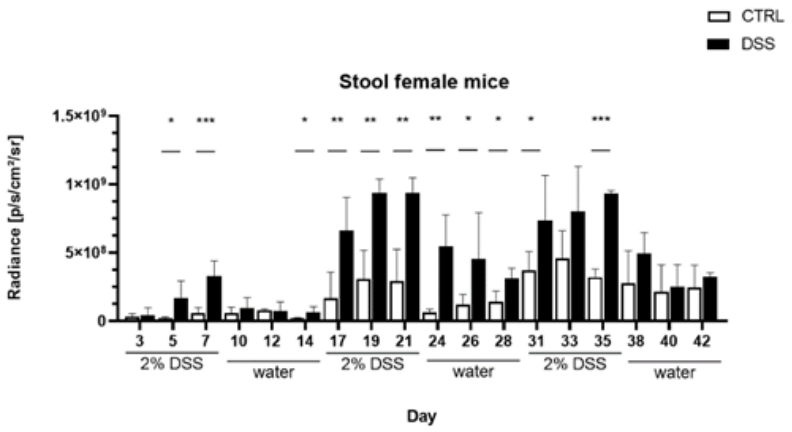

Figure 9*: Detection of blood in stool by bioluminiscence during six weeks, alternating a week of 2% DSS administration with a week of water intake in one year old C57BL/6 female mice. Some of the differences between the average bioluminiscence of DSS mice and control mice were significant. Multiple t-test *P<0.05, **P<0.01, ***P<0.001, **** P<0.0001.*

5. DISCUSSION

The characteristic signs and symptoms of the UC disease were monitored in each mouse, as well as the immunological response, measuring the neutrophilic activation by the detection of their chemiluminescent signal, based on its interaction with luminol, a useful biomarker already employed in medicine for the *in vivo* evaluation of many conditions, such as cancer or arthritis (Davis et al., 2019). Apart from it, also the amount of blood in stool was recorded, employing the quality of the luminol to emit a chemiluminescent light when it reacts with the iron of the haemoglobin (Harris, 2002).

In this study it has been proved that the DSS administration to one year old C57BL/6 mice induces a colonic epithelial damage, and symptomatology (body weight loss, decrease in stool consistency and rectal bleeding) comparable with the symptoms of human ulcerative colitis disease. These results coincide with previous works (Eichele & Kharbanda, 2017; Chassaing et al., 2014; Kim et al., 2021; Maronek et al., 2021; Wang et al., 2019).

Generally, DSS is a highly water-soluble sulphate polysaccharide, which has a wide range of molecular weights (from 5 to 1400 kDa). The potential of DSS for inducing colitis is dependant of its molecular weight. For the murine model in mice, usually a molecular weight between 36 and 50 kDa is employed (Kim et al., 2021; Chassaing et al., 2014). But the severity of the damages related with the administration of this chemical, capable of penetrate the mucosal membrane of the intestine, also depends on other factors, such as the concentration (usually between 1% and 5%), the duration and frequency of administration, the animal strain, age, weight, and environmental factors, among others (Chassaing et al., 2014; Eichele & Kharbanda, 2017). For this reason, with the aim to characterise a chronic model, it was decided to alternate a week of 2% DSS intake with a water week, to reduce the severity of the symptoms in short term. Furthermore, the employment of C57BL/6 mice breed was not arbitrary, it has been shown by many researchers that this mouse type is suitable for the study of DSS-induced UC (Hickman et al., 2017; Kim et al., 2021).

With reference to the results of the body weight variations and the stool scores obtained in this investigation, it has been demonstrated that the efficacy of the DSS water solution intake varies according to the animal gender too, previously observed by other authors (Chassaing et al., 2014). This sex differences, that lead us to remove the males from our experiment at the end of the first week, while the females lasted from the six weeks previously agreed, could be caused by the normally lower circulating levels of estrogens in males than females (Goodman et al., 2018), due to as was discussed by Bábíčková et al., 2015, estrogens participate in the reduction of colonic motility, they contribute to the retention of water by regulating the Cl^- reabsorption and modify the intestinal epithelial barrier. Probably, this explains the dramatic weight decrease observed in experimental male mice at the end of the first DSS week. Nevertheless, the sex differences were not further studied in our project, but we could demonstrate that the administration of 2% DSS solution, alternating weekly with water, works well for one year old C57BL/6 female mice for the study of chronic ulcerative colitis, whose body weight reduction, as well as the appearance of the symptoms was

progressive and slow. In contrast, to carry out the same study with male mice, another strain or DSS concentration should be employed.

Considering the fluctuations in the female evaluated symptoms, it is highly appreciable that the average body weight, as well as the stool consistency decreased during the administration of DSS solution to experimental mice, while a recovery was recorded during the wash out weeks. These results were expected, taking into account the epithelial damage that DSS causes in the mucosal layer of the digestive tract. Nevertheless, almost the same fluctuations, but in a lower degree, are appreciated in the control group, which was not supplied with DSS at any time. These changes probably are a consequence of the stress suffered during the DSS weeks, due to during the washout weeks the IVIS measurement was performed in mice just once, however, in the DSS weeks it was performed three times, with all that this entails, anaesthesia, injection, displacement and handling of the animals.

In this study, also a low endoscopy was employed to evaluate the UC progression, a method widely used during the last decade to assess colon and rectal injury, as well as the tumour development. This evaluation method, together with the non-invasive clinical analysis previously mentioned, provide more objective results, which helped us to demonstrate the negative effects that the DSS ingestion causes on the colon and intestine, since the body weight or stool consistency can be biased by many factors, like fluid intake or stress. Furthermore, despite the small diameter of the mice colon, this popular method has been demonstrated to be safe, reproducible and fast (Machado et al., 2020).

According to the pictures obtained from the endoscopies, the average total score of translucency, fibrin, bleeding and reddening was obtained, proving the damaging consequences that the DSS intake has on the mucosal layer of the gut epithelium. In the five measurements performed, the scores obtained in the experimental group are, in average, 2 points over the ctrl group, meaning that the epithelial state was worse in experimental mice during the whole project. The worsening of the mucosal layer is slight and continuous in both groups. In the experimental group it can be explained because the damage induced during the DSS weeks has not time enough to completely recover during the water weeks. Higher worsening is recorded after the DSS weeks, however, during the wash out weeks, the immune system could be still activated, trying to totally get rid of the damaging chemical, and causing a detectable injure on the colon.

On the other hand, focusing on the control group, the worsening could be caused by the anaesthesia administration, as well as the colonoscopy, because it should be taken into account that it is an invasive method, which even has been considered as safe, and any damage was detected on the evaluated mice, it can cause some harm or discomfort on the animals. Then, other brand-new non-invasive method was employed to reveal the mechanism leading to symptoms of UC in mouse models, the *in vivo* bioluminescent imaging.

The early establishment of medical treatment for the IBD is essential to reduce the incidence and severity of the possible complications that this disease entails, such as intestinal stenosis (narrowing of the intestine that difficult the food pass), among others. Therefore, obtaining an early diagnosis is of great importance (EUROPA PRESS, 2022).

That is why, in the present project, following the main objective of detecting the UC before the appearance of the first symptoms, it was employed the IVIS to perform an *in vivo* bioluminescent analysis. However, our experiment is focused just on mice.

The IVIS, at low temperatures can detect the radiance emitted from NETs when they interact with a bioluminescent marker, in our case luminol, and allow us to track the inflammation progress. Concretely, the bioluminescence signal identified by luminol, has been shown to depend on the MPO, one of the proteins that constitute NETs. The MPO is the main component of the neutrophil´s azurophilic granules, and it is also present in the lysosome of monocytes (Gutowski et al., 2017; N. Zhang et al., 2013). Both, neutrophils and monocytes are highly present in inflammation sites. Thus, IVIS offers a sensitive and qualitative analysis in a non-invasive way, what afford the possibility to perform multiple measurements in the same animal lowering the number of animals needed for the study (Magistri et al., 2019). However, its usefulness to detect the UC disease before the onset of symptoms is ambiguous.

Regarding the relative body weight variations in female mice, and the results obtained from the *in vivo* bioluminescence in mice at 5 and 10 minutes, there is a concordance between the results obtained. Regardless there are more days in which the relative body weight was registered than the bioluminescence, it can be appreciated how, generally, the decreases in body weight are represented by an increase in the radiance values. It could be said that the measures taken at 5 minutes represent slightly better the variations in the severity of the inflammation than the bioluminescence measured at 10 minutes, nevertheless, the data obtained in both could help us to recognize the UC disease.

Even though the successful results obtained by IVIS, it did not show the inflammation earlier than the variations in bodyweight or stool consistency, which already aware us about the worsening of the mice health. This could be caused because, as was shown in the histological samples by other authors, the excessive neutrophil recruitment, which is identified by the bioluminescent imaging, leads to the tissue damage and clinical disease activity. Furthermore, it has been proved in other projects that the DSS administration causes inflammation as a consequence of the significant rise in proinflammatory cytokines, as well as MPO accumulation, which leads to the colon injury in mice (Murphy et al., 2010). This data contrasts with the results obtained by Y. L. Chen et al., 2016, in whose experiments were able to detect the autoimmune ulcerative colitis, by the bioluminescent imaging, around a week before registering body weight loss and changes in stool consistency. Nevertheless, in their project they employed T cell transfer model of colitis, instead of DSS, which is the most common model employed to examine the starting and progression of chronic colitis mediated by T cells (Eri et al., 2012).

According to the stools, it can be appreciated that the information got in radiance follows faithfully the recorded scores of stool consistency, what could help us to confirm the existence of this disease. Even the small changes in stool consistency (scores from 0 to 1), which in real life are almost unappreciated, by the *in vivo* bioluminescent imaging they can be detected. In the control group, also the IVIS data and the scores in stool consistency and rectal bleeding show the same line of evolution, nevertheless, the

fidelity in the control group is not as exact as in the experimental group. For example, on day thirty-three, the value obtained in radiance is $8.02x10^8$ [p/s/cm²/sr], what represents a hight value, in comparison with the radiance obtained from other days, while the score of the stool this same day is 0. This could be caused because of the high radiance of the DSS samples, which were spread to the neighbouring samples, modifying the results, something that may not had happen if they had been shotted one by one.

In general, the radiance values obtained in the last 4 weeks in stools are higher than in the first 2 weeks, still having the same stool consistency scores. This outcome confirms the hypothesis that, even though during the water weeks the animals improved their symptoms, the intestinal epithelium had not enough time to come back to the original state, then, always some remaining damage could be identified by the employment of IVIS.

So, even though the IVIS machine has demonstrated in this study to be a useful tool for the UC detection by using a luminol-dependant bioluminescent imaging, as well as was evidenced by other authors with different conditions; like uveitis (Gutowski et al., 2017), dermatitis, arthritis, and tumours (Gross et al., 2009), we could not achieve our main objective of predict this condition before the appearance of the symptoms. The manifestation of the disease was visualized at the same time, or even before than the *in vivo* bioluminescent indicators of this condition.

Moreover, the bioluminescent imaging has some limitations. One of them is that the luminescence produced in tissues deeper than 1 cm is difficult to be detected then, this method of diagnosis is almost restricted to mice. Also, areas with high pigmentation result an impediment for the light emission therefore, for this type of experiments there are recommended the use of white or albino mice, or in cases of using brown mice, as was our case, it raises the necessity to shave the animals to allow the photon transmission (H. Chen & Thorne, 2012). However, as the mice were just shaved in the area of interest, we cannot be completely sure about the appearance of inflammation only in the colon or also in other parts of the body, which were not detected due to the absorption of emitted light by the fur. Especially considering that with the DSS administration, mice can develop a systemic inflammation, being NETs present everywhere around the body, not just in the colon, due to, as it was demonstrated by Metzger et al., 2019, the DSS-induced colitis leads to inflammatory bone changes, therefore, some signal could be lost.

Another issue that must be considered when using the IVIS is that, because of the lack of study in this area, there have not been stablished any radiance values that determine the existence of the UC, then, further research on the prediction or identification in advance of this disease is needed to be able to determine when a mouse is suffering from ulcerative colitis.

6. CONCLUSION

Through this project, it has been evaluated the progression of the UC, helping us to better understand the pathology of this condition, which affects the daily life of many people worldwide.

It has been shown that, even though the bioluminescence can be considered an effective quantifiable test for the monitoring of inflammation in mice colon, our principal objective of detecting the UC disease before the symptomatology appearance, has not been achieved. However, the *in vivo* bioluminescent study with IVIS, can be considered a promising non-invasive method, which has been shown in this study, to offer a clearer and easier to interpret diagnosis than colonoscopy, an invasive method that is currently used routinely in hospitals to diagnose patients with ulcerative colitis. Therefore, a deeper study on the possibilities offered by bioluminescence in the field of detection and diagnosis of UC should be carried out, considering the use of other strains of mice or other methods of inducing the disease.

Finally, it is of great importance to mention that the results obtained from the evaluation of mice stool coincide with those obtained from the evaluation of the abdominal area, which leads us to consider the study of the stool as an encouraging tool for the detection of the UC, even raising the possibility of its introduction into human medicine, however, further evidence is needed.

On the other hand, the specific objectives have been achieved during the progression of the present project, offering us specific skills regarding the methods used for the induction of UC, its monitoring and evaluation of them in mice.

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