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Effectiveness of Celastrol in Reducing Inflammatory Cell Infiltration via H&E in the Liver of High-Fat Diet-Fed ApoE-Knockout Mice

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Abstract

High-fat diet (HFD) promotes liver inflammation by increasing infiltration of inflammatory cells. This study analyzed the effect of celastrol on hepatic inflammation in ApoE-knockout mice on HFD. Treatment with celastrol (1.5, 2, or 2.5 mg/kg/day) for 4 weeks, followed by liver tissue staining with H&E. Quantification of infiltration areas was obtained with NDP.view 2 software and analyzed with the Kruskal-Wallis test. At the maximum dose (2.5 mg/kg), the inflammatory cell infiltration of the liver was significantly decreased in comparison with untreated HFD controls. These results justify the potential use of celastrol as an anti-inflammatory in diet-induced liver inflammation.

Keywords: Celastrol, Inflammatory cell, H&E staining, ApoE-knockout mice

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1.0 Introduction

Inflammation is an immune process that occurs when multiple stimuli cause an inflammatory response, which may result in tissue damage or pathology. Free radicals, damaged cells, oxidative stress, or foreign pathogens are all triggering factors. Under the domain of morbidity and mortality related to inflammation, it was documented that 3 out of 5 individuals die in the chronic stage of inflammatory disease (Pahwa et al., 2018). It has therefore been stated by the World Health Organization (WHO) that chronic diseases are the most significant threat to human health, because of the high prevalence rates, as regards inflammation, and mounting incidence rates each year. The inflammatory cell infiltration is a helpful indicator of inflammation because it is naturally associated. It involves the recruitment

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and migration of immune cells to the site of injury to facilitate healing (Medzhitov, 2008). Pathologically, cellular infiltration of inflammatory cells includes influx of neutrophils, macrophages, lymphocytes, eosinophils, and mast cells, which have definite roles in the inflammatory response. Nonetheless, excess infiltration of inflammatory cells indicates an excessive level of inflammatory response that is higher than normal. Accordingly, the overactive and lengthy inflammatory reaction transforms an acute response into a chronic one. With time, the condition might eventually lead to tissue damage because it cannot be repaired (Chen et al., 2017). The widespread consumption of HFD in modern society has incorporated unhealthy intake of saturated and trans fats, which are among the predominant factors promoting inflammation, especially in the liver (Duan et al., 2018). Celastrol is a quinone methide triterpene found naturally in the *Tripterygium wilfordii* herbal plant, which is commonly cultivated in China, Taiwan, and Japan. It has been described as a potent antioxidant and anti-inflammatory agent, underscoring its role in modulating the effect of inflammation (Allison et al., 2001). The ApoE knockout mouse model is a well-established model for developing the inflammatory disease when fed with HFD (Western-type diet). Multiple studies have found that an HFD in rodents led to inflammation of various organs, including the liver. Thus, celastrol is believed to hold therapeutic potential not only for restoring physiological balance but also for improving health-related behaviors and quality of life by modulating inflammatory infiltrate. The Hematoxylin and Eosin (H&E) stain is a standard, broadly applicable stain among the many stains available in frozen sections to explore the presence of inflammatory cell infiltrates, either by grading the severity of inflammation or by quantifying the inflammatory area (Liu et al., 2023; Bergwik et al., 2024). Hence, the paper aims to utilise H&E staining to examine the effect of celastrol in reducing inflammatory cell infiltration within the liver tissue site of ApoE knockout mice fed an HFD.

2.0 Literature Review

In the study by Schierwagen et al. (2015), using ApoE knockout mice fed a high-fat western diet for seven weeks, it was established that they develop metabolic syndrome, which manifested in stronger proliferation of hepatic inflammatory cells with intense fibrous expression of profibrotic markers. Liver inflammation is also noted via elevated proinflammatory cytokines, that is, TNF-α, IL-6, and IFN-α, with the same animal model of ApoE knockout mice and the exact duration receiving a high-fat western diet (Camargo et al., 2022). The prevalence of high-fat, Western-style diets has played a significant role in increasing the occurrence of inflammation-related health issues, resulting in dependency on care and treatment services. The trend poses a significant threat to public health and underscores the dire need for preventive measures, especially efficient nutritional ones. Celastrol has been of growing interest due to its powerful anti-inflammatory effects. Over the last few years, it has gained wider application in preclinical research, and there has been growing interest in its role in treating inflammatory diseases. Ongoing research supports its therapeutic promise, paving the way for future clinical trials.

2.1 HFD Effect on Liver Inflammation

Prolonged liver inflammation induced by the HFD is the key factor in liver damage and fibrosis. Recurrent neutrophil and macrophage infiltrate causes hepatocellular damage through proinflammatory cytokines, reactive oxygen species, and proteases. Chronic maintenance of this immune reaction stimulates hepatic stellate cells (HSCs) to secrete extracellular components like collagen that are central to forming fibrosis. According to Hammerich and Tacke (2023), these internal hepatic inflammations are necessary to trigger the activity of stellate cells and promote fibrosis. Therefore, liver inflammation triggered by HFD in the ApoE knockout model results in chronic damage and fibrosis remodeling. In this regard, it is important to quantify infiltrating inflammatory cells and markers of fibrosis by histological techniques in order to determine the effectiveness of anti-inflammatory therapies.

2.2 Therapeutic Effect of Celastrol in Liver Inflammation

Celastrol has shown promising therapeutic effects on the reduction of liver inflammation and metabolic dysfunction caused by HFD. When celastrol at 200 mg/ kg was administered intraperitoneally to wild-type C57BL/6J mice biweekly over four weeks, hepatic metabolic imbalances linked to HFD intake were notably reduced (Zhang et al., 2017). The activation of the Sirt1 pathway facilitated this, inhibiting the expression of Srebp-1c, a major lipid synthesis regulator, thus reducing hepatic steatosis. Moreover, celastrol increased the anti-inflammatory status by suppressing the proinflammatory cytokines, IL-6 and TNF-alpha, but also led to an improvement of the antioxidant status within the liver.

Adding evidence to its anti-inflammatory effect, Zhu et al. (2021) also demonstrated that celastrol inhibited the macrophage-initiated hepatic inflammation in ApoE-knockout mice fed with HFD. This was mediated by down-regulation of cAMP-PKA-NF, and it acts on adenylyl cyclase-associated protein 1(CAP1), which led to diminished expression of pro-inflammatory cytokines. Collectively, these studies suggest that celastrol most likely achieves its hepatoprotection effect through a combination of metabolic and immunomodulatory mechanisms, suggesting its prospects as a treatment option in HFD-induced liver inflammation.

With these findings, several gaps in the literature remain. The existing literature varies in dose or length of time and animal model, thus making it too different to compare. Particularly, the effect of celastrol on the histology of inflammatory cell infiltration in the liver, which is a central characteristic of chronic inflammation and liver fibrosis, has not been thoroughly examined to a great extent using H&E staining. In addition, more studies need to be conducted to determine the optimum dose of celastrol in different models. Hence, additional preclinical experiments to determine the effectiveness of celastrol in ApoE knockout mice fed an HFD-induced liver inflammation are necessary. Our study fills this gap by looking at the protective activity of celastrol towards hepatic inflammatory-cell-infiltration in the ApoE-knockout mice under high-fat-diet feeding, in a quantitative measure of cell-infiltration using H&E staining.

2.3 Preclinical Histopathology Staining Using H&E

H&E staining is one of the most commonly used histological examination methods in biomedical and preclinical studies. As a standard process of staining, H&E provides the opportunity to observe the tissue architecture and cellular morphology of numerous organs and models of diseases. Hematoxylin is a basic stain used to stain acidic structures, mostly nuclei, usually in blue or purple because of the high concentration of DNA and RNA. Eosin, a carboxylic dye, stains the components of the cytoplasm and the extracellular matrix in multiple pink to red shades, which helps to distinguish the tissue elements clearly (Fischer et al., 2008).

H&E staining is essential in preclinical studies using experimental animal models to define structural changes like inflammation, fibrosis, and necrosis. The method facilitates distinct visualization of hepatocellular damage and immune-mediated activities at different pathological statuses. As an example, H&E illustrates the presence of focal inflammation in the liver surrounded by inflammatory infiltrates during murine cytomegalovirus infection (Sjolin et al., 2002), and detects the presence of fibrosis and inflammatory alterations of the liver through H&E staining in a rat model with a chemically induced liver injury (Elrazik et al., 2022). When feeding high-fat diets or genetically engineered mice, like the ApoE-knockout model, H&E staining is a valid tool for determining the degree of liver damage and focal inflammation. It can be compared between groups to determine the effectiveness of various treatments in treating the inflammatory status.

Moreover, the evolution of digital pathology tools improved the accuracy and reproducibility of H&E-based analyses. The stained sections can be measured quantitatively using whole-slide scanning and programs like NDP.view or ImageJ, enabling objective aspects of histopathological assessment. Due to its reliability, simplicity, and relevance to diagnosis, H&E staining is still considered the basis of histologic analysis. It has been used as a reference technique in conjunction with immunohistochemical or molecular evaluations in preclinical studies.

3.0 Methodology

For animal work, male ApoE knockout mice from the C57BL/6 strain at 8 weeks old were acclimatized for 1 week and randomized to one of five groups (n = 6 per group): normal-diet control, HFD control, and three HFD groups treated with celastrol intraperitoneally. Celastrol group divides into 3 dose subgroups: 1.5, 2.0, and 2.5 mg/kg body weight (BW) of celastrol dissolved in 2 dimethyl sulfoxide (DMSO). Celastrol groups received the respective doses daily during the last four of the 12 HFD dietary weeks. Animals were sacrificed at 20 weeks of age. As for sample collection, liver tissues were quickly harvested, washed in cold phosphate-buffered saline (PBS), mounted in optimal cutting temperature (OCT) compound, snap-frozen, pre-cooled with dry ice, and immersed in liquid nitrogen. The tissues were then stored at -80 °C and cryosectioned on a cryostat at a 7 µm thickness. Prior to the H&E staining method, sections were air-dried and stained using an optimized commercial H&E stain kit (ab245880, Abcam, UK) as per the manufacturer's instructions.

For H&E staining analysis, the stained slides were scanned under a 40x magnification in a Hamamatsu NanoZoomer S60, and their analysis was performed in NDP.view 2. Based on a freehand ROI tool, two independent researchers traced the outlines of the inflammatory area, and a histopathologist verified the results. The area was then expressed as the inflammatory area (mm^2) divided by the total tissue area (Bergwik et al., 2024). The comparison of data between groups (mean + SEM) was conducted using an analysis of Kruskal-Wallis and a post hoc test (Dunn) in GraphPad Prism 8, where $p < 0.05$ was considered significant. All experiments were in accordance with the institutional and national rules of animal care and were approved by the appropriate IACUC (UiTM CARE: 426/2023).

4.0 Findings

H&E staining in this study visualizes the inflammatory cellular changes in organs where hematoxylin stains cell nuclei purple, and eosin colors cell cytoplasm pink. Inflammatory cell infiltration can be identified as the dark cells gathering in clusters or bands in the tissues or around the vessels with the help of a microscopic view. In groups with 2.5 mg/kg celastrol in liver tissues, a significant and remarkable decrease in inflammatory cell infiltration was observed, whereas the value was about two times lower than that of the HFD group at 0.30 mm^2 of the tissue area. Moreover, a remarkable decrease was seen in the mice receiving a control chow diet, 0.21 mm^2 of the positive tissue area containing an inflammatory cell infiltrate, which was observed compared to the HFD group (Figures 1 and 2).

The inflammatory area was weakly decreased in the 1.5 mg/kg celastrol group compared to the HFD group. However, the inflammatory cell infiltration was still relatively high, and the two groups had no significant difference. This indicates that within this dose, although some anti-inflammatory actions were probably triggered at this dose, there was still no potent histological reaction, suggesting that there is still some anti-inflammatory activity despite the lack of significant histological action. However, the 2.0 mg/kg celastrol-treated group showed a more evident decrease in the inflammatory area, as illustrated by the appearance of fewer infiltrating clusters of immune cells under the microscope. Nevertheless, this trend remained statistically insignificant compared to the HFD group, where the effect on inflammation is merely partial, with a sub-threshold value.

As mentioned previously, the inhibition of the inflammatory cell infiltration was most apparent in the group receiving 2.5 mg/kg celastrol, with the measured inflammatory area being significantly decreased compared to the untreated HFD group. This decrease was histologically evident and accompanied by statistical significance of the quantification of inflammatory cell infiltration area, implying that the anti-inflammatory properties of celastrol are more definite at higher dosing. The results show a dose-dependent pattern with higher levels of celastrol resulting in a gradual reduction of liver inflammation. This pattern is justified by the typical H&E-stained images in Figure 2, where inflammatory cell infiltration is represented by black arrows across groups. It is important to note that dense clusters of immune cells can be observed in the group of HFD, whereas the number is significantly decreased in the group of 2.5 mg/kg treatment,

which agrees with the quantified data. Thus, celastrol at a dose of 2.5 mg/kg drives an effective anti-inflammatory effect in this model of HFD-induced liver inflammation in the ApoE knockout mice.

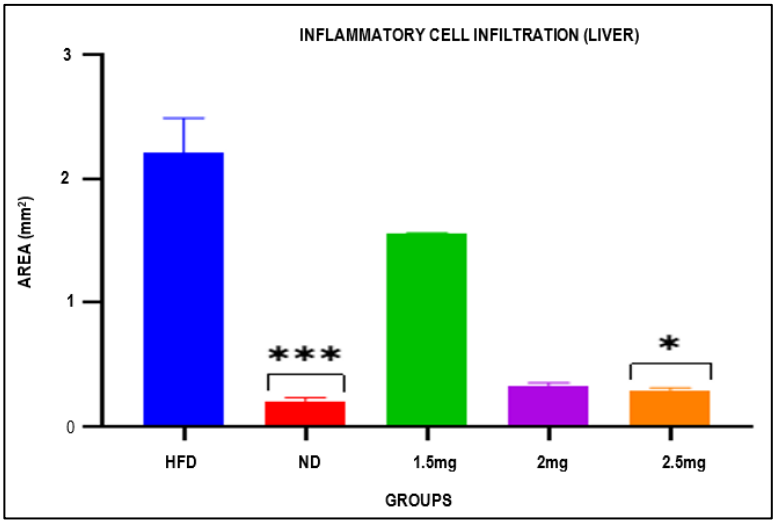

Fig. 1: Quantification of the inflammatory cell infiltration into the liver in HFD, control normal chow diet (ND), and in celastrol treatment groups of 1.5 mg, 2 mg, and 2.5 mg, respectively. The results are reported as mean ±SEM of cell infiltration in the liver area. (*P < 0.05, ***P < 0.001 compared with the HFD group, Kruskal-Wallis test)

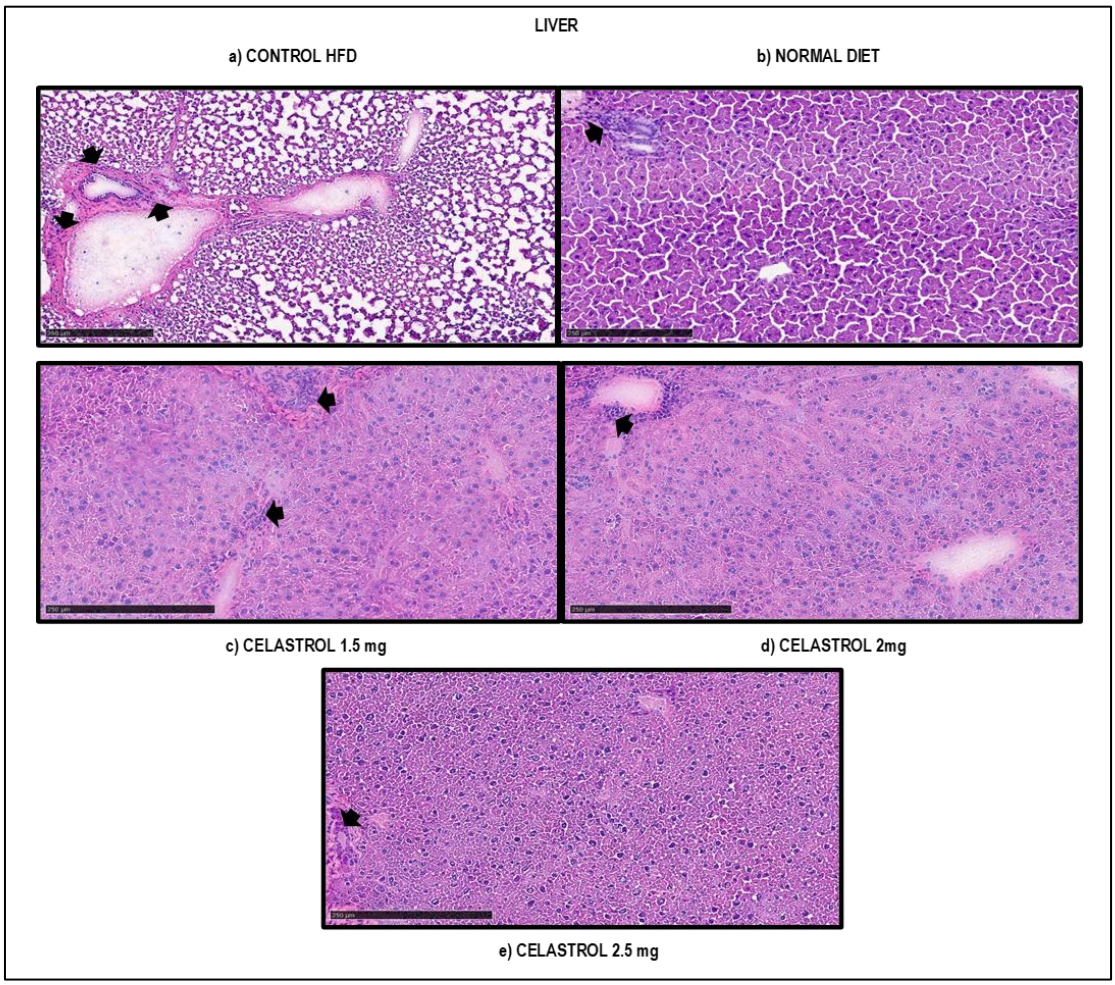

Fig. 2: The representative H&E-stained liver sections depicting the inflammatory cell infiltrations in all animal groups, i.e., a) control HFD, b) control normal chow diet (ND), and the celastrol treatment groups of c) 1.5 mg, d) 2 mg, and e) 2.5 mg. The nuclei are violet (blue-purple hematoxylin), and the cytoplasm is light pink (eosin). Black arrows represent a cluster of inflammatory cells.

5.0 Discussion

H&E staining histological analysis is a gold-standard method to demonstrate focal inflammatory foci in the liver since hepatocyte cytoplasm has a contrasted eosinophilic pink color compared to the basophilic infiltrating leukocytic nuclei. This method is particularly informative with models of HFD steatohepatitis, in which portal tract-centred clusters of neutrophils and mononuclear cells are frequently seen around portal tracts and in the sinusoid. Quantitative H&E analysis in our ApoE knockout mouse cohort revealed that the HFD induced an increase in inflammatory cell infiltration, thereby demonstrating that the genetic-dietary pair model in this study, i.e, HFD induced inflammation in ApoE knockout mice, consequently reproduces the pro-inflammatory hepatic environment as described in prior HFD studies. Positive tissue areas containing inflammatory cell infiltrate were observed compared to the HFD group (Figures 1 and 2).

A definite downward pattern was observed in the celastrol dose-response. A smaller decrease in the total inflammatory area was only observed with the 1.5 mg/kg dose, indicating that only a fraction of the celastrol molecular targets are engaged at this dose concentration. Increasing the dose to 2.0 mg/kg produced a more significant histological improvement. Sinusoids were less congested, periportal aggregates were less dense, but wide dispersion retained the group mean above the statistical significance level. Markedly, 2.5 mg kg/kg exhibited the most extreme outcome, which reduced the HFD inflammatory burden by half, and was found significant in the Kruskal-Wallis post-hoc test ($p < 0.05$). Morphologically, the portal triad radius became smaller, sinusoids opened, and their architecture achieved a more normal-diet-like appearance. These results reveal a dose-dependent hepatic protection where 2.5 mg/kg was identified as the most optimum dose effective in the model.

A significant decrease in the number of inflammatory cells, as well as distortion of the sinusoids, was detected in the livers of celastrol-treated animals using H&E staining, indicative of celastrol's ability to maintain liver tissue architecture (Wang et al., 2020). Celastrol has been found to stimulate AMP-activated protein kinase (AMPK), which subsequently up-regulates SIRT3; this AMPK-SIRT3 axis has been associated with decreased NF-kB activity and subsequent decreased liver inflammation and improved cellular homeostasis (Wang et al., 2020).

The significance of mitigating liver inflammation can be supported by past research that revealed that NLRP3-inflammasome activation, along with IL-17 and TNF-alpha signaling, contributes to hepatocellular damage and fibrosis in mouse models (Wree et al., 2018). With long-term high-fat intake, inflammatory conditions change in the adipose tissue to inflammation in the liver, resulting in simple steatosis to steatohepatitis with inflammation and hepatocellular ballooning (Stanton et al., 2011). Histologic testing, specifically by H&E staining, has demonstrated that chronic exposure to metabolic stress or environmental toxins leads to the development of classical non-alcoholic fatty liver disease (NAFLD). As an example, according to a study conducted by Schneider et al. (2023), liver sections stained with H&E of C57BL/6 mice, which had become exposed to a high-fat diet as well as vehicle emissions, displayed extreme macrovesicular steatosis, hepatocyte ballooning, and discrete zones of inflammatory cell infiltration, indicators characteristic of steatohepatitis. These results propose the significance of the H&E stain in the localization and visualization of hepatic injury and inflammation.

A similar histological method of H&E staining in our study showed a decrease in leukocyte inflammatory infiltration and the enhancement of sinusoidal architecture, especially in the 2.5 mg/kg celastrol-treated group. The above observations indicate that celastrol can reverse inflammatory progression by normalizing hepatic microstructures and constraining immune cell influx. Collectively, the histopathological similarities of our results with those of previous models of NAFLD support the therapeutic potential of celastrol in treating liver inflammation and slowing down fibrotic liver progression in a model of diet-induced liver injury.

Even though our findings enhance the evidence of celastrol as a therapeutic for liver inflammation, several limitations should be considered. Celastrol has a narrow therapeutic window, and therefore, the dose used to treat the liver could still be in the range of causing systemic-toxicity effects, and further study needs to be conducted to ensure the range of toxicity dose. Furthermore, the abnormal lipoproteins of ApoE knockout animals may change the distribution and pharmacodynamics of celastrol. Thus, the efficacy of celastrol should also be validated in humanized lipid models. Importantly, the H&E stain has helped demonstrate overall inflammatory infiltration but fails to identify particular populations of immune cells involved. Including immunohistochemistry with markers like F4/80 recognizing macrophages and Ly6G recognizing neutrophils, would detail the specific cell subsets most responsive to celastrol and define whether celastrol acts primarily upon innate immunity.

6.0 Conclusion

Conclusively, celastrol reduced inflammatory cell infiltration within the liver tissue of ApoE knockout mice fed with HFD, most effectively at the dose of 2.5 mg/kg. This dose demonstrated both statistical and morphological positivity, indicating its potential as an anti-inflammatory therapy in metabolic liver disease. Anti-inflammatory effects were observed and measured using H&E stain to demonstrate celastrol's ability to decrease the buildup of immune cells and maintain hepatic structure. However, a few limitations were addressed, such as focusing exclusively on the animal liver tissue, which may limit the generalizability of the findings to other inflamed organs treated by celastrol. This study used H&E staining, which provides a detailed general overview of cellular morphology rather than direct quantification of specific inflammatory cell types. Although this study mainly examined the histopathological effects of celastrol on inflammation, the results may also have broader behavioral and physiological impacts, including cognition. By mitigating inflammatory cell infiltration within key organs, celastrol may indirectly emphasize the significance of inflammation control under diverse stimuli. For the following research directions, future research should focus on how celastrol affects individual subsets of immune cells and cytokines. Furthermore, the effectiveness of dose-reduction measures, in combination therapy, and their clinical applicability through investigation in human-derived liver tissues. Moreover, the study of the exact cellular mechanism underlying the anti-inflammatory effects of celastrol in hepatic tissue should be performed in the future.

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Paper Contribution to Related Field of Study

The article contributes to histopathology, specifically using H&E staining to quantify inflammatory cell infiltration. It highlights the potential of celastrol as an emerging therapeutic for treating inflammation in the liver, providing valuable insights for future research.

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