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Reducing Pain in Experimental Models of Intestinal Inflammation Affects the Immune Response

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The incidence of inflammatory bowel disease with its two main manifestations, colitis ulcerosa and Crohn's disease, is rising globally year after year. There is still a tremendous need to study the underlying pathomechanisms and a well-established tool in order to better understand the disease are colitis models in rodents. Since the concept of the 3Rs was proposed by Russell and Burch, this would include pain medication in animal models of intestinal inflammation as a reduction of suffering. This review argues against pain medication because the administration of pain medication in its current form has an impact on the inflammatory process and the immune response, thus falsifying the results and the reproducibility and therefore leading to misconceptions.

Lay Summary

Colitis models are a good tool to study underlying pathomechanisms of inflammatory bowel disease. Pain medication to fulfill the concept of the 3Rs has to be considered carefully. This review discusses influences of pain medication on the immune system, functional structures, and inflammatory processes.

Key Words: colitis, inflammation, inflammatory bowel disease, opioids, pain treatment

Introduction

The main forms of inflammatory bowel disease (IBD) are ulcerative colitis (UC) and Crohn's disease (CD), and they differ in manifestations and pathologies. UC mainly affects the colon and is limited to the (sub)mucosa. The inflammation continuously spreads from the rectum to the proximal colon.[1,2] Whereas in CD, inflammation can occur in the whole gastrointestinal tract from mouth to anus. The inflammation is discontinuous (skip lesions) and involves all layers of the intestinal wall (transmural). In line with this, the symptoms, though not all, differ in both entities.[3,4] IBD develops predominantly in the first 3 decades of life. Patients experience diarrhea, weight loss, and fatigue as well as abdominal pain. CD mainly manifests in the terminal ileum (terminal ileitis in 40% of the cases).[5] Expressed pointedly, a CD patient is a 20-year-old woman with pain in the right-sided lower abdomen. The pain usually occurs 1-2 hours after meals. She has diarrhea but no bloody stools. Whereas the typical UC patient, also pointedly expressed, is a 20-year-old man, who quit smoking 3 months ago and is experiencing painful defecation. This patient further experiences diarrhea with bloody stools.

For the treatment of clinical symptoms, various substances are available. Their application and the treatment regimen depend on disease course and severity. The disease course in IBD is intermittent with episodes of inflammation (flare) and remission. Nevertheless, there is no curative therapy available yet. This is due to the still incomplete understanding of the pathogenesis of the disease. Biomedical research leads to new therapies, improved diagnostics, and prevention. Translational research transports research findings into the clinics (from bench to bedside). In translational research, animal studies play an important if not irreplaceable role. Animal models of intestinal inflammation are numerous and helped immensely to understand IBD as a multifactorial disease. The advantages of animal studies include the opportunity to study the onset of a disease. While patients present with clinical symptoms before coming to the clinic, animals allow for studying the trigger as well as the development and factors of chronicity of a disease. Additionally, animal models allow for knockout or knockin of certain factors enabling to study their specific role in disease progression. Whereas in humans, certain procedures and the resulting knowledge would be impossible to gain. This also holds true for intervention studies in order to test for efficacy and safety. When using animals in studying human diseases, this has to be done in a humanly manner. Already in 1959, Russell and Burch[6] published the principles of the 3Rs (replacement, reduction, and refinement) as "The Principles of Humane Experimental Technique." They stated, "it is widely recognized that the humanest possible treatment of experimental animals, far from being an obstacle, is actually a prerequisite for successful animal experiments."[6] This

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would include pain medication in animal models of intestinal inflammation as a reduction of suffering. This review argues against pain medication in animal models of intestinal inflammation because administration of pain medication in its current form has an impact on the inflammatory process and the immune response, thus falsifying the results and therefore leading to misconceptions. With regard to IBD in humans, the following groups of active substances are used for the treatment of clinical symptoms and the suppression of the underlying immune reaction[7,8]:

1) Cortisone compounds like prednisolone or budesonide act anti-inflammatory. These compounds can be applied orally, locally, or intravenously. The application method and dosage influence efficacy and side effects. Oral or intravenous application results in a systemic anti-inflammatory effect, while local application is associated with a more compatible side-effect profile. Hence, cortisone compounds should be applied during the active phase of inflammation and not during remission.[9-11]
2) Aminosalicylates (sulfasalazine and mesalazine) act anti-inflammatory and are applied orally or locally. Sulfasalazine and mesalazine are prodrugs and are cleaved into their active forms in the large bowel. Sulfasalazine and mesalazine are used for treating active disease but also for remission control.[12]
3) Immunosuppressants like thiopurines (eg, 6-mercaptopurine or its prodrug azathioprine) or methotrexate suppress immune reactions and are applied not only in CD and UC, but also in other autoimmune-mediated diseases or organ transplantation.[13,14] Application is highly followed by side effects that include dose-dependent and dose-independent effects.[15] Thiopurine treatment of IBD maintains remission, decreases the need for surgery, and lowers the risk of developing colorectal cancer. Additionally, thiopurine acts synergistic with biologicals (eg, infliximab).[16]
4) Biologicals include anti-tumor necrosis factor α (anti-TNFα) (eg, infliximab, adalimumab, golimumab) binding to and inactivating soluble as well as membrane-bound TNFα.[17] Other biologicals include anti-α4β7 integrin and anti-interleukin (IL)-12/IL-23 therapies.[18,19] These therapies target specific factors involved in IBD progression and chronicity.[20] TNFα is a proinflammatory cytokine produced in high amounts in active IBD. The integrin α4β7 mediates leukocyte homing to the gut and blocking this route reduces leukocyte trafficking, counteracting one pathological factor in IBD. IL-12 and IL-23 belong to the IL-12 cytokine family, which is part of the IL-6 superfamily. IL-12 and IL-23 are immunoregulatory cytokines. They further have the ability to target innate lymphoid cells and $CD4^+$ T cells to produce proinflammatory cytokines.
5) Small molecules including Janus kinase (Jak) inhibitors. Tofacitinib has been approved for UC and inhibits predominantly Jak1 and Jak3 and thus prevents the induction of intracellular proinflammatory pathways. More specific Jak inhibitors are in clinical studies as well as a number of additional small molecules.

In order to reduce pain, all the previously mentioned treatments to ease inflammatory symptoms can be supplemented with antidepressants[21,22] as well as with changes in the patient's diet (eg, by eating smaller portions and avoiding flatulent food).[23,24] However, we emphasize the point that none of these substances and treatment regimens include pain medication. On the other hand, pain medication and management become more important in therapy, as patients can develop a pain memory. Even during episodes of remission with no active inflammation, they still feel pain. Analgesics, like nonselective nonsteroidal anti-inflammatory drugs (NSAIDs) or cyclooxygenase-2 (COX-2)–inhibiting drugs, can accompany the conventional treatment for a short period of time, yet their usage is still controversial.[25,26] There are several studies showing that long-term treatment with NSAIDs or COX-2 inhibitors can lead to an aggravation of intestinal inflammation.[27-29] Besides analgesics, also cannabis is described to relief IBD-related symptoms, but its usage is also controversially discussed because it is associated with a higher risk of surgery in CD patients.[30] Besides pain medication, transcranial direct current stimulation, a noninvasive technique that includes brain stimulation to modulate pain, is described to be useful to relieve abdominal pain.[31] This technique might be a powerful tool to reduce pain medication in IBD patients but is in need of further investigation.

Using animal models of intestinal inflammation for elucidating IBD should be as close to the human situation as possible. In humans, a prophylactic or permanent pain medication is not part of the therapy, and the course of inflammation should be studied without interfering with the immune system. Hence, it is of great importance that experiments done in rodents, investigating the development and chronicity of intestinal inflammation, are carried out without pain medication.

In order to reduce the burden, stress level evaluation of the animal is irreplaceable. A method to quantitate unambiguously if the animal is sensing pain or nociception during intestinal inflammation is unfortunately not yet validated.[32] The stress level and welfare of the animal is scored by different characteristics (eg, reduced self-care, weight loss, stool consistency, or activity). Moreover, the legislation on protection of animals is evaluating the burden level of animals in an experiment not only on the basis of significant stressors in the experimental setup but additionally by the mere duration of the experiment. As an example, transfer colitis is induced in immunodeficient animals by the transfer of T cells from wild-type littermates. The experiment of transfer colitis starts with the transfer of T cells (day 0). During the course of 14-21 days, these transferred cells home to the gut and induce colitis.[33] During this phase of migration and homing, the animals do not experience severe signs of inflammation or pain; clinical symptoms start to show by week 3. Providing pain medication in the model of transfer colitis at this stage would constitute prophylactic treatment and therefore interfere with colitis development.

Besides transfer colitis, there are around 90 other mouse models[34] to investigate colitis. Among these are chemically induced models in which acute or chronic colitis is induced by dextran sodium sulfate[35] or by TNBS (2,4,6-trinitrobenzene sulfonic acid) (described by Morris et al 1989[36]). Furthermore, various models in transgenic knockout mice that spontaneously develop colitis symptoms like IL-10–deficient mice[37] and bacterial-induced colitis models (eg, by *Salmonella typhimurium*)[38] are used to investigate colitis. Because there is no score that is available and validated that categorizes the pain or nociception of the

animals during the period of disease, the researcher needs to carefully balance the gain of knowledge vs the suffering of the animal. Nevertheless, a proposed measure for refinement of IBD models is analgesic treatment, which however can interfere with the research.

Why would pain medication affect the immune response? A graduated scheme (pain ladder) for pharmacologic treatment of pain was established 1996 by the World Health Organization. This scheme suggests the use of analgetic substances for pain medication starting with nonopioid analgesics to strong opioids (Table 1).

First-step pain medication includes, for example, paracetamol or NSAIDs, with their antiphlogistic properties. There are many studies showing numerous effects on the immune system (eg, stimulation of T cells or impairment of CD4 T-cell immunity).[39-41] They can also have immunomodulatory functions on macrophages by modulating cytokine response and cytokine release.[42]

Further, other analgesic substances, especially opioids, have various side effects as well as modulatory effects on the immune system. Opioids like fentanyl and morphine restrict the function of macrophages, natural killer (NK) cells, and T cells.[43] The immune modulation may change depending on pain processing and the immune system. Immune cells like granulocytes, macrophages, and T cells as well as the nervous system are interrelated, as these cells secrete endogenous and exogenous opioid peptides that act on peripheral opioid receptors[43] expressed on various cell types. Interfering with this tightly regulated system in order to manage inflammation and pain will affect inflammatory responses in models of IBD.

Opioids act on cells via binding to opioid receptors, which are expressed on cells throughout the nervous system and immune cells. Opioid receptors are classified as μ, μ1, μ2, and κ type. They play an important role in physiological and pathophysiological processes. Binding or release within the nervous system modulates the peripheral immunity.[44-47] Opioids can additionally bind to toll-like receptors (TLRs) (eg, TLR4 in mice).[48] TLR4 is a pattern recognition receptor recognizing, for example, bacterial components like lipopolysaccharide (LPS). This binding leads to TLR4 activation and results in activation of the innate immune system. In active IBD, the μ opioid receptor (MOR) is overexpressed in the small intestine and colon. This suggests that opioid signaling itself is playing a role in the inflammatory process in IBD.[49] The same group showed that in mice with chemically-induced colitis, the treatment with MOR agonists (like morphine) has an anti-inflammatory effect and could reduce colitis.[50] Decades ago, Wybran et al[51] published their data on the influence of opioids on the immune system stating that various opioids dampen the activation state of T cells. The described immunomodulatory or immunosuppressive functions on the immune system are unwanted effects and will lead to a misinterpretation in experimental inflammation.

Effect on Granulocytes

During the colitis development, neutrophils are the first cells recruited to the site of inflammation with the purpose to limit invasion of bacteria through the leaky mucosal barrier.[52,53] In numerous studies, it was shown that part of the anti-inflammatory effect of NSAIDs is mediated by regulating neutrophil adhesion and migration by downregulation of L-selectin.[54,55] It was shown that the administration of opioids inhibits the migration and phagocytic potential of neutrophils.[45] A study of our lab has shown that blockade of neutrophil migration results in increased mortality and aggravation of colitis in mice and rats.[56] Further, the animals present with extraintestinal manifestations of the eyes.[56] Moreover, the activity of CXCR2, which plays an important role in neutrophil migration from the bone marrow, is reduced.[57] Additional to inhibited migratory capacity, functional processes are significantly influenced. For example, the secretion of reactive oxygen species by neutrophils is blocked when treated with opioids.[58] Welters et al[59] described an immunosuppressive effect of morphine by inhibition of nuclear binding of nuclear factor κB (NFκB) in neutrophils and monocytes. The role of mast cells in intestinal inflammation is still controversially discussed. Besides their proinflammatory function, they can also influence fibrosis and wound healing.[60,61] In addition, it has been described that mast cells drive innate and adaptive type 2 immunity in helminth infections.[62] Ennis et al[63] provided evidence indicating that a variety of opioids change

TABLE 1. Analgesic Substances for Pain Relief According to Pain Ladder

Pain Ladder	Substance	Examples
Step 1	Nonopioid analgesics	Salicylates (acetylsalicylic acid) Phenylacetic acid derivates (like diclofenac and indomethacin) 2-Phenylpropionic acid derivates (like ibuprofen, ketoprofen, and naproxen) 4-Aminophenole derivates (like paracetamol) Pyrazolone (like metamizole or dipyrone and phenazon) Selective COX-2 inhibitors (like celecoxib and parecoxib)
Step 2	Mild opioids	Tramadol Tilidine (plus naloxone) Dihydrocodeine
Step 3	Strong opioids	Buprenorphine Fentanyl Hydromorphone Morphine Oxycodone

Abbreviation: COX-2, cyclooxygenase-2.

the secretion of histamine by mast cells and inhibit mast cell function. Moreover, the LPS-induced TNFα production of intraperitoneal mast cells is inhibited by fentanyl.[64] Long-term administration of fentanyl has a strong immunosuppressive effect on mast cells.[65]

Influence on Monocytes and Macrophages

Macrophages are key players in preservation of gut homeostasis and the resolution of inflammation.[62] By influencing processes like the recruitment of neutrophils to the intestine and the differentiation and expansion of pathogenic Th17 cells, they play a major role in IBD.[66] In some models of intestinal inflammation (eg, in IL-10–deficient mice), a treatment with the NSAID sulindac leads to a strong infiltration of macrophages within 4 weeks.[67] In the same model, the application of piroxicam accelerates the onset of colitis symptoms and is even used to induce colitis.[68] Various studies show that the administration of opioids influences critical immune functions of macrophages. Tramadol, among others, relieves pain through activating μ opioid receptors. In-vitro studies using human blood showed that tramadol does not change the number of macrophages, but rather shifts their polarity. Tramadol inhibits inflammatory macrophages, which produce proinflammatory cytokines like IL-6 and TNFα, while promoting alternatively-activated macrophages, which are instrumental for wound healing and tissue repair.[69,70] The same effect was reported for buprenorphine.[71] The effects of morphine on macrophages show dose-dependent differences. While low-dose morphine affects phagocytosis, high-dose morphine induces apoptosis.[46,72] Moreover, there is a dose-dependent influence on LPS-induced secretion of the cytokines IL-6 and TNFα.[73] Roy et al[73] indicated that this effect is mediated by a modulation of the NFκB activity by LPS. While low-dose morphine increases the activity of NFκB, high-dose morphine is decreasing the activity. Besides morphine, also NSAIDs influence NFκB activity. There are several investigations showing that part of their anti-inflammatory effect is due to NFκB inhibition.[74,75]

Long-term treatment with morphine leads to a reduced number of macrophages and influences their proliferative capacity. Besides this, long-term administration of morphine leads to a significantly delayed recruitment of macrophages and neutrophils to sites of inflammation.[76] Moreover, it is discussed that the bactericidal activity of macrophages could be reduced due to a significant reduction of LPS-induced production of nitric oxygen.[77] Additionally, cell interactions like antigen presentation by macrophages is reduced by opioids, resulting in a lower antibody production.[78]

Influence on Lymphocytes

Apart from macrophages, opioids like morphine and fentanyl also affect NK cells and T cells.[43] Fentanyl and buprenorphine have suppressive effects on immune responses in the mouse: acute as well as continuous administration affected lymphoproliferation, NK cell activity, and IL-2 and interferon γ production.[79] T cells express three opioid receptor types and the balance of T helper cells shifts depending on the receptor type engaged. Binding of fentanyl induces the production of IL-4, while morphine reduces the production of IL-4.[47] These changes in the cytokine profile can lead to an induction of B-cell differentiation and a change in humoral immunity.[80] A long-term challenge (12-24 months) with morphine increases the expression of the MOR on T cells.[81] Additionally, immune cells responsive to opioids accumulate in inflamed tissues when opioids are applied in the long term.[81] Cornwell et al[82] showed that long-term treatment with morphine leads to an increase of regulatory T cells and Th17 cells. Furthermore, Campana et al[81] showed a reduction in NK cell numbers during long-term treatment. Therefore, long-term opioid treatment might lead to immunotolerance, as shown for fentanyl.[79]

Effect on Intestinal Structures

Especially in the gastrointestinal tract, the application of NSAIDs can cause severe damage such as bleeding, ulceration, and changes of the intestinal barrier integrity.[83-86] Moreover, several studies describe NSAID-induced enteropathy with serious gastrointestinal complications.[87,88] Administration of morphine not only influences cell subsets of the innate and adaptive immune system, but also has some critical effects on components of the extracellular matrix (ECM). Studies on cancer metastasis show that the treatment with opioids changes the production of matrix metalloproteinases, which are important for remodeling and degradation of the ECM.[89] Remodeling of the ECM is one of the early features in the progress of pathogenesis in IBD.[90] In a different context, it is also described that morphine can inhibit cell adhesion and cell migration to ECM components.[91] Both are fundamental immune processes in colitis models. Additionally, morphine and fentanyl weaken the mucosal barrier by changing the organization of tight junction proteins in the gut epithelium.[92,93] The expression of occludin and zonula occludens-1, both responsible for stabilization of the mucosal barrier, is decreased by opioids, leading to complications like a disrupted epithelial barrier, a change in permeability, and a higher bacterial translocation.[92,93] Because a disrupted barrier is one of the diagnosed triggers to induce IBD in humans, using opioids as a pain treatment in colitis studies is fundamentally influencing the disease model. Further, a study from 2018 proved that after only 1 day of opioid treatment, the gut metabolome is changing, bringing along changes of the intestinal microbiota. The bacterial dysbiosis has been associated with a perturbed gut homeostasis in mice.[93,94] Another feature that also plays a role in intestinal inflammatory models is the motility of the intestine.[95] Opioids are known to reduce gut motility and can thereby cause unwanted injuries or perforation of the tissue.[96,97]

Conclusions

There are justifiable reasons against pain treatment in animal models of intestinal inflammation. Analgesics have a diverse spectrum of effects on the immune system. By changing recruitment, proliferation, differentiation, and polarization of various cell subsets, the application of pain medication then further highly influences effector functions like cytokine production, phagocytosis, and cytotoxicity of the immune cells. Changing rudimental processes that are essential for the pathology of disease development prevents production of reproducible data and their right interpretation. Consciously applying pain medication in models of intestinal inflammation means a deliberate falsification of already well-studied models for intestinal inflammation. In a greater sense, it counteracts with the 3Rs. Because in the

long-term, the production of inconclusive data is leading to a higher amount of animal experiments needed to generate decisive results. There is still a lack of alternatives to treat pain in animals. One possibility is to reduce unnecessary stress to comfort the animals (eg, by staffing the cages with hiding places and wood for nibbling). In case a pain medication is absolutely necessary, the influence on the immune system should be taken into account for the interpretation of the results.

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Conflicts of Interest

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