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A new therapeutic association to manage relapsing experimental colitis: Doxycycline plus *Saccharomyces boulardii*

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ABSTRACT

Immunomodulatory antibiotics have been proposed for the treatment of multifactorial conditions such as inflammatory bowel disease. Probiotics are able to attenuate intestinal inflammation, being considered as safe when chronically administered. The aim of the study was to evaluate the anti-inflammatory effects of doxycycline, a tetracycline with immunomodulatory properties, alone and in association with the probiotic *Saccharomyces boulardii* CNCMI-745. Doxycycline was assayed both in vitro (Caco-2 epithelial cells and RAW 264.7 macrophages) and in vivo, in the trinitrobenzenesulfonic acid (TNBS) model of rat colitis and the dextran sodium sulfate (DSS) model of mouse colitis. In addition, the anti-inflammatory effect of the association of doxycycline and the probiotic was evaluated in vitro and in vivo in a DSS model of reactivated colitis in mice. Doxycycline displayed immunomodulatory activity in vitro, reducing IL-8 production by intestinal epithelial cells and nitric oxide by macrophages. Doxycycline administration to TNBS-colitic rats (5, 10 and 25 mg/kg) ameliorated the intestinal inflammatory process, being its efficacy comparable to that previously showed by minocycline. Doxycycline treatment was also effective in reducing acute intestinal inflammation in the DSS model of mouse colitis. The association of doxycycline and *S. boulardii* helped managing colitis in a reactivated model of colitis, by reducing intestinal inflammation and accelerating the recovery and attenuating the relapse. This was evidenced by a reduced disease activity index, colonic tissue damage and expression of inflammatory mediators. This study confirms the intestinal anti-inflammatory activity of doxycycline and supports the potential use of its therapeutic association with *S. boulardii* for the treatment of inflammatory bowel diseases, in which doxycycline is used to induce remission and long term probiotic administration helps to prevent the relapses.

1. Introduction

Different antibiotics have been described to comprise within their therapeutic effects the modulation of the immune response [1]. This is of increasing interest in the pharmacological treatment of diseases where infectious and inflammatory factors converge [2–5]. However, the potential of immunomodulatory antibiotics in managing intestinal conditions, such as inflammatory bowel disease (IBD) [6–8], is poorly documented.

IBD pathogenesis is characterized by a genetic predisposition and an aberrant intestinal immune response to environmental factors [9]. Among the latter, an imbalance on the intestinal microbiota (dysbiosis) seems to play a key role [8,10–16]. Despite this, IBD therapy has been mainly focused on suppressing the immune system rather than on restoring the composition of the altered microbiota, which can be achieved by the administration of probiotics, prebiotics or antibiotics [8]. Since no causative pathogen has been specifically identified, broad-spectrum antibiotics have shown the best results [17].

Semi-synthetic second generation tetracyclines, such as doxycycline and minocycline, have been reported to exert immunomodulatory activities in addition to their antimicrobial properties

Abbreviations: CFU, colony forming units; DAI, disease activity index; DSS, dextran sulfate sodium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, glutathione; IBD, inflammatory bowel disease; LPS, lipopolysaccharide; MPO, myeloperoxidase; TNBS, trinitrobenzene sulfonic acid.

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[2,18]. Both compounds have been shown to possess anti-apoptotic, immunosuppressive and anti-inflammatory properties in different inflammatory diseases [19]. The mechanisms behind these effects comprise inhibition of different enzymes involved in inflammatory processes, such as nitric oxide synthase, phospholipase A2 and matrix metalloproteinases; scavenging of oxygen free radicals, and regulation of immune cell activation and proliferation [20]. Considering all this, second generation tetracyclines seem a promising approach for the treatment of IBD. In fact, minocycline's intestinal anti-inflammatory effects have been previously well described in different models of experimental colitis [21–23]; however, doxycycline has not been fully tested in these intestinal conditions [24].

The aim of this study was to determine if the intestinal anti-inflammatory activity ascribed to minocycline is a property shared by doxycycline, thus broadening the therapeutic choice for the treatment of IBD. For this purpose, we first compared the immunomodulatory properties of doxycycline to those displayed by minocycline in vitro. Likewise, the in vivo intestinal anti-inflammatory effect of doxycycline was tested in comparison with minocycline in the TNBS model or rat colitis [25] and confirmed in the DSS model in mice [26].

Despite the usefulness of antibiotics for the treatment of IBD [27], several studies have reported that discontinuation of antibiotic therapy results in a high relapse rate, and long-term antibiotic therapy is associated to increased risk of drug side effects and antibiotic resistance [28,29]. In this case, remission could be maintained by the administration of probiotics, which have been previously reported to exert beneficial effects in IBD due to their immunomodulatory properties [30,31]. Yeasts are commonly used in combination with doxycycline, remaining their growth, phenotype or functions unaffected by the antibiotic [32]. In particular, the probiotic *Saccharomyces boulardii* CNCMI-745 has been shown to modulate the gut immune response, inducing intestinal homeostasis, which supports its effectiveness in intestinal inflammatory states [33,34]. For this reason, we evaluated the association of doxycycline and *S. bourlardii* both in vitro and in vivo, in a model of reactivated colitis in mice, as a therapeutic strategy aimed at inducing remission and preventing the onset of symptoms after antibiotic discontinuation [23].

2. Materials and methods

2.1. Reagents

All studies were carried out in accordance with the "Guide for the Care and Use of Laboratory Animals" as promulgated by the National Institute of Health. All chemicals, including the antibiotics, were obtained from Sigma–Aldrich (Madrid, Spain), unless otherwise stated. The doses of antibiotics used in the animal models were chosen according to previous results of our group and were equivalent to the therapeutic dose in humans (calculated as described elsewhere [35]). *Saccharomyces bourlardii* CNCMI-745 was provided by Biocodex (Beauvais, France).

2.2. In vitro studies

Caco-2 cells (human colon adenocarcinoma cells) and RAW 264.7 cells (mouse macrophages) were obtained from the Cell Culture Unit of the University of Granada (Granada, Spain) and cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% FBS and 2 mM L-glutamine, in a humidified 5% CO_2 atmosphere at 37 °C. Caco-2 cells were seeded onto 24-well plates at a density of 5×10^5 cells per well and grown until formation of a monolayer. Then, they were pre-treated for 24 h with minocycline (MNC) (50 μM) or different concentrations of doxycycline ranging from 1 to 50 μM. To study the effects of *S. boulardii*, cells were pre-incubated for 2 h with the probiotic at a concentration of 10^8 UFC/ml, and they were washed three times afterwards. Probiotic conditioned medium was obtained by incubating the probiotic in medium at 10^8 UFC/ml for 2 h, followed by centrifugation and collection of the supernatant. Cells were incubated with the conditioned medium for 2 h. In the association studies, cells were incubated with doxycycline (25 μM) for 24 h and *S. boulardii.* or its conditioned medium were added during the last 2 h. Following the pre-treatments, the cells were stimulated were stimulated with IL-1β (1 ng/ml) for 20 h. Untreated unstimulated cells and untreated cells were used as negative and positive controls. Then the supernatants were collected, centrifuged at 10,000 × *g* for 5 min and stored at −80 °C until IL-8 determination by ELISA (Biosource, Invitrogen™) was performed.

RAW264.7 cells were seeded onto 24-well plates at a density of 5×10^5 cells per well and grown until confluence. They were cultured with antibiotics and/or *S. boulardii* or conditioned medium as described above and then stimulated with LPS (100 ng/ml) for 20 h; similarly, positive and negative controls were included. Supernatants were collected and centrifuged at 10,000 × *g* for 5 min, and nitrite levels were measured using the Griess assay [36]. Cell viability was examined by the MTT-test as described elsewhere [37].

2.3. Trinitrobenzenesulfonic acid (TNBS) model of rat colitis

Female Wistar rats (180–200 g) obtained from Janvier (St Berthevin Cedex, France) were housed in makrolon cages, maintained in an air-conditioned atmosphere with a 12 h light–dark cycle, and they were provided with free access to tap water and food. They were randomly assigned to 6 groups ($n = 10$). Four of them received antibiotic treatment: doxycycline (5, 10 or 25 mg/kg) and minocycline (40 mg/kg). The antibiotics were dissolved in 2 ml of distilled water and administered daily by oral gavage. An untreated TNBS control group and a non-colitic group were also included for reference, which received the vehicle.

Colonic inflammation was induced in control and treated groups as previously described [38] by the administration of 10 mg of TNBS dissolved in 0.25 ml of 50% ethanol (v/v) by means of a Teflon cannula inserted 8 cm through the anus. The treatment with antibiotics started the day of the colitis induction, to avoid their possible preventive effect, as it has been widely described [39], and examine their curative effect. It continued for 7 days until the death of the rats with an overdose of halothane.

Animal body weights, occurrence of diarrhea and water and food intake were recorded daily throughout all the experiment. Once the animals were sacrificed, the colon was removed aseptically and placed on an ice-cold plate and longitudinally opened. Afterwards, the colonic segment was weighed and its length measured under a constant load (2 g). Each colon was scored for macroscopically visible damage on a 0–10 scale by two observers unaware of the treatment, according to the criteria previously described [38]. The colon samples were subsequently sectioned in different fragments to be used for histological study and biochemical determinations.

2.4. Dextran sodium sulfate (DSS) model of mouse colitis

Female C57BL/6J mice (7–9 weeks old; approximately 20 g) obtained from Janvier (St Berthevin Cedex, France) were assigned to a non-colitic ($n = 8$) and DSS colitic group ($n = 32$). The colitis was induced by adding DSS (3%, w/v) (36–50 KDa, MP Biomedicals, Ontario, USA) in the drinking water for a period of 7 days [26]. Colitic mice were randomly divided in four groups of 8 animals each: control mice, which were given distilled water (200 μl) and three groups treated with doxycycline at different doses, 7.5, 15