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Intestinal anti-inflammatory activity of calcium pyruvate in the TNBS model of rat colitis: Comparison with ethyl pyruvate

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ABSTRACT

Pyruvate is a key intermediate of the carbohydrate metabolism with endogenous scavenger properties. However, it cannot be used in clinics due to its instability. Ethyl pyruvate (EP) has shown better stability as well as an antioxidant and anti-inflammatory activity. Calcium pyruvate monohydrate (CPM) is another stable pyruvate derivative that could also provide the benefits from calcium, fundamental for bone health. Considering everything, we propose CPM as a therapeutic strategy to treat diseases with an immune component in which there is also a significant dysregulation of the skeletal homeostasis. This could be applicable to inflammatory bowel disease, which is characterized by over-production of pro-inflammatory mediators, including cytokines and reactive oxygen and nitrogen metabolites that induces intestinal mucosal damage and chronic inflammation, and extra-intestinal symptoms like osteopenia and osteoporosis.

The effects of CPM and EP (20, 40 and 100 mg/kg) were evaluated on the trinitrobenzenesulfonic acid (TNBS) model of colitis in rats, after a 7-day oral treatment, with main focus on colonic histology and inflammatory mediators.

Both pyruvates showed intestinal anti-inflammatory effects in the TNBS-induced colitis. They were evident both histologically, with a recovery of the mucosal cytoarchitecture and a reduction of the neutrophil infiltration, and through the profile of inflammatory mediators (IL-1, IL-6, IL-17, IL-23, iNOS). However, CPM appeared to be more effective than ethyl pyruvate. In conclusion, CPM exerts intestinal anti-inflammatory effect on the TNBS-induced colitis in rats, although further experiments are needed to explore its beneficial effects on bone health and osteoporosis.

1. Introduction

Pyruvate, the anionic form of pyruvic acid (2-oxo-propanoic acid), is a pivotal biochemical intermediate of the carbohydrate metabolism. Almost all carbohydrates are metabolized through pyruvic acid, so a few hundreds of grams are produced by the human body every day. In addition to being an important energy-bearing metabolite, it most probably works as an endogenous scavenger of reactive oxygen species (ROS). Furthermore, many evidences support its pharmacological effect improving the cardiac function after coronary ischemia and reperfusion and critical medical conditions, like severe sepsis, acute respiratory distress syndrome, burn injury, acute pancreatitis and stroke [15]. However and despite its properties, pyruvate cannot be used in clinical practice due to its instability in solution [15].

The ethyl ester of pyruvic acid, ethyl pyruvate (EP), has shown much better stability in aqueous solutions, and to be a pharmacologically active molecule in different models of redox- and inflammation-mediated cellular or tissue injury [15], including ischemia/reperfusion [39], pancreatitis [9], sepsis [40] and intestinal

Abbreviations: CPM, calcium pyruvate monohydrate; EP, ethyl pyruvate; GSH, glutathione; IBD, inflammatory bowel disease; ICAM-1, intercellular adhesion molecule-1; IFNγ, interferon γ; IL, interleukin; iNOS, inducible nitric oxide synthase; LTB_4, leukotriene B_4; MCP-1, monocyte chemoattractant protein-1; MPO, myeloperoxidase; ROS, reactive oxygen species; SAZ, sulphasalazine; TNBS, trinitrobenzenesulfonic acid.

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inflammation [10]. The mechanisms involved include the scavenging of ROS and the inhibition of NF-κB activation by scavenging ROS and also other pathways not well described [17]. It has been reported, both in vitro and in vivo, that pyruvates are not only able to inhibit the pro-inflammatory interleukin (IL)-6 and tumour necrosis factor (TNF)-α, but to increase the production of the anti-inflammatory cytokine IL-10 [42]. Furthermore, EP may also serve as a metabolic substrate that could reduce ATP depletion and mitochondrial damage.

Other pyruvate derivatives have been developed like calcium pyruvate monohydrate (CPM) that has been synthesized avoiding destabilizing reaction conditions [32]. This molecule has been used as anti-obesity or slimming aid [37] due to the fact that pyruvate is one of the smallest carbohydrate molecules existing, which does not lead to an insulin release once ingested. But it also provides the benefits from calcium, which is well known for its pivotal impact in bone health as well as in osteoporosis prevention, and beyond this, it is discussed to play a role in obesity control [12] and in lowering the risk of hypertension and colon cancer [44].

Taken together the effects described for pyruvate as a scavenger of ROS and anti-inflammatory, and the beneficial effects exerted by calcium in osteoporosis prevention, it could be interesting to think on calcium pyruvate as a therapeutic strategy to treat diseases with an immune component in which there is also a significant dysregulation of the skeletal homeostasis. This is the case of inflammatory bowel disease (IBD), in which there is an abnormal synthesis of proinflammatory mediators including cytokines and reactive oxygen and nitrogen metabolites that leads to intestinal mucosal damage and chronic tissue inflammation [38]; together with extra-intestinal manifestations [41] like osteopenia and osteoporosis in many cases [24]. Furthermore, IBD treatment is suboptimal nowadays. The therapies usually involve the use of aminosalicylates, glucocorticosteroids or immunosuppressants, including biologicals like TNF-α monoclonal antibodies [11]; however, these drugs may display limited beneficial actions and/or serious complications and side-effects, thus limiting their chronic administration in these patients [13].

Thus, the aim of the present study is to evaluate the potential use of the stable and pure CPM in the treatment of IBD, and compare it with ethyl pyruvate, which has previously shown anti-inflammatory effects in acute and chronic murine colitis [10]. CPM has been also tried as a form of calcium supplementation for the treatment of osteoporosis in postmenopausal women showing a good bioavailability and tolerability [35]. We have evaluated the intestinal anti-inflammatory properties of both compounds in the trinitrobenzenesulfonic acid (TNBS) model of colitis in rats, a well established model of intestinal inflammation that mimics many histopathological and immune characteristics of human IBD.

2. Methods

2.1. Chemicals and reagents

Calcium pyruvate monohydrate (CPM) was provided by Phyto-Lab GmbH & Co. KG (Vestenbergsgreuth, Germany). All other chemicals, including ethyl pyruvate, were obtained from Sigma–Aldrich Quimica (Madrid, Spain), unless otherwise stated.

2.2. TNBS model of rat colitis

This study was carried out in accordance with the 'Guide for the Care and Use of Laboratory Animals' as promulgated by the National Institute of Health and the protocols approved by the Ethic Committee of Laboratory Animals of the University of Granada (Spain) (Ref. No. CEEA-2010-286). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals [20,27]. 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 provided with food and tap water *ad libitum*. They were randomly assigned to nine groups ($n = 10$). Three groups received treatment with CPM (20, 40 and 100 mg kg^{-1}); other three received treatment with ethyl pyruvate (20, 40 and 100 mg kg^{-1}); and the remaining was treated with sulphasalazine (30 mg kg^{-1}). All compounds were dissolved in 1 ml of carboxymethylcellulose (0.2%) in water solution, and administered daily by oral gavage. An untreated TNBS control group and a non-colitic group were included for reference, which received the vehicle used to administer the test compounds. Colonic inflammation was induced in control and treated groups as previously described [8]. Briefly, rats were fasted overnight, anesthetized with halothane and given 10 mg of TNBS dissolved in 0.25 ml of 50% ethanol (v v^{-1}) by means of a Teflon cannula inserted 8 cm through the anus. During and after TNBS administration, the rats were kept in a head-down position until they recovered from anaesthesia, and then returned to their cages. Rats from the non-colitic group were administered intracolonically 0.25 ml of phosphate buffered saline instead of TNBS. The treatments were given from the day of the colitis induction until the sacrifice of the rats with an overdose of halothane, seven days later. Animal body weights, occurrence of diarrhoea, and water and food intake were recorded daily throughout all the experiments. Once the animals were sacrificed, the colon was removed aseptically and placed on an ice-cold plate, longitudinally opened and cleaned from their luminal contents with cold saline. Afterwards, it 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 experiment, according to the criteria described before [5].

Colon samples (0.5 cm^2) containing all the layers were taken from a region of the inflamed colon corresponding to the adjacent segment to the gross macroscopic damage and were fixed in 4% buffered formaldehyde for the histological studies. Equivalent colonic segments were also obtained from the non-colitic group. The colon was subsequently minced, aliquoted and kept frozen at −80 °C until biochemical determinations and RNA extraction was performed.

2.3. Histological studies

Cross-sections were selected and embedded in paraffin. Full-thickness sections of 5 μm were obtained at different levels and stained with haematoxylin and eosin. The histological damage was evaluated by a pathologist observer, who was blinded to the experimental groups, according to the criteria previously described [4].

Immunohistochemistry evaluation of the myeloid marker CD11b was performed in colonic tissue sections from the different experimental groups. Briefly, deparaffinised and rehydrated tissue sections were treated in a steamer for 20 min in citrate buffer for antigen retrieval. After blockade of endogenous peroxidase and unspecific protein binding, anti-CD11b antibody (NB110-89474SS; Novus Biologicals, Littleton, CO, USA) was used at 1:500 dilution for one hour. Presence of specific binding was detected by brown precipitate using the DAB detection method following manufacturer instructions (ab80437 EXPOSE Rabbit specific HRP-DAB Detection IHC Kit v2; Abcam, Cambridge, MA, USA), and haematoxylin was used as counterstain.

2.4. Biochemical determinations in colonic tissue

Myeloperoxidase (MPO) activity was measured according to the technique described previously [23]; the results were expressed as MPO units per gram of wet tissue; one unit of MPO activity was defined as that degrading 1 μmol hydrogen peroxide/min at 25 °C. Total glutathione (GSH) content was quantified with the recycling assay described by Anderson [3], and the results were expressed as nmol per g wet tissue. In order to evaluate tissue IL-1β levels, the colonic samples were homogenized (1/5 w v^{-1}) in phosphate buffered saline supplemented with 0.1% sodium dodecyl sulfate (SDS), 0.1% sodium deoxycholate, 1% Triton X-100 and protease and phosphatase inhibitors (aprotinin, leupeptin and phenylmethylsulfonyl fluoride). The cytokine was quantified by enzyme-linked immunosorbent assay (R&D Systems Inc., Minneapolis, MN, USA) and the results were expressed as pg per g wet tissue. Finally, lipid peroxidation was determined spectrophotometrically by measuring the colonic malonyldialdehyde (MDA) levels as previously described [48]. Colonic samples were homogenized (1:2.5 w v^{-1}) in a solution containing 1.15% KCl. A standard curve using 1,1,3,3-tetramethoxypropane as source of MDA was used. Colonic MDA levels were expressed as nmol per g wet tissue.

2.5. Analysis of gene expression in colonic samples by RT-qPCR

Total RNA from colonic samples was isolated using Trizol® (Thermo Fisher Scientific Inc., Waltham, MA USA) following the manufacturer's protocol. All RNA samples were quantified with the Thermo Scientific NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA USA) and 2 μg of RNA were reverse transcribed using oligo(dT) primers (Promega, Southampton, UK). Real time quantitative PCR amplification and detection was performed on optical-grade 48 well plates in a Eco™ Real-Time PCR System (Illumina, CA, USA) with 20 ng of cDNA, the KAPA SYBR® FAST qPCR Master Mix (Illumina, CA, USA) and specific primers at their annealing temperature (Ta) (Table 1). To normalize mRNA expression, the expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured. The mRNA relative quantitation was calculated using the ΔΔCt method.

2.6. Western blot analysis of NF-κB

Cytoplasmic and nuclear fraction extracts from colonic samples were obtained by using the Nuclear Extract Kit, following manufacturer instructions (Active Motif, Carlsbad, CA, USA). Proteins were separated on 10% SDS–PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes. Total cytoplasmic p65 NF-κB and nuclear phospho-NF-κB p65 were detected with the corresponding specific primary antibodies, L8F6 and Ser536, respectively (Cell Signaling Technology, Beverly, MA, USA). Both antibodies were used at a 1:1000 dilution and incubated overnight at 4 °C. The membranes were then incubated with HRP-conjugated secondary antibodies from Santa Cruz Biotechnologies (Santa Cruz, CA, USA) at a 1:2000 dilution for 1 h at room temperature before visualizing by using ECL detection reagents.

2.7. Statistics

All results are expressed as the mean ± SEM. Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA) and post hoc least significance tests. Differences between proportions were analyzed with the chi-squared test. All results are expressed as the mean ± SEM. Differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA) with Tukey post-hoc test. Nonparametric data (macroscopic and microscopic scores) were analyzed by the Kruskal–Wallis test. All statistical analyses were carried out with the GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA, USA), with statistical significance set at $P < 0.05$.

Table 1
Primer sequences used for real-time PCR assays in the colonic tissue.

Gene	Sequence (5′–3′)
IL-1β	FW: GATCTTTGAAGAAGAGCCCG RV: AACTATGTCCCGACCATTGC
IL-6	FW: CTTCCAGCCAGTTGCCTTCTTG RV: TGGTCTGTTGTGGGTGGTATCC
IL-12	FW: ACGCTACCTCCTCTTCTTG RV: ATGTCGTCCGTGGTCTTC
IL-17	FW: TGGACTCTGAGCCGCATTGA RV: GACGCATGGCGGACAATAGA
IL-23	FW: ATCCAGTGTGGTGATGGTTGTG RV: TGTCCGAGTCCAGCAGGTG
CINC-1	FW: CCGAAGTCATAGCCACACTCAAG RV: TCACCAGACAGACGCCATCG
MCP-1	FW: TCTTCCTCCACCACTATGC RV: TCTCCAGCCGACTCFATTG
ICAM-1	FW: GTGAACTGCTCTTCCTCTTG RV: AGTGGTCTGCTGTCTTCC
MUC-2	FW: ACCACCATTACCACCACCTCAG RV: CGATCACCACCATTGCCACTG
TFF-3	FW: ATGGAGACCAGAGCCTTCTG RV: ACAGCCTTGTGCTGACTGTA
iNOS	FW: AAGAGACGCACAGGCAGAGG RV: AGCAGGCACACGCAATGATG
GADPH	FW: CCATCACCATCTTCCAGGAG RV: CCTGCTTCACCACCTTCTTG

3. Results

3.1. Macroscopic and microscopic effect of CPM and EP on TNBS rat colitis

The administration of TNBS to rats induced an intestinal inflammatory process that was evidenced, in the course of the experiment, by anorexia and the presence of diarrhoea in most of the animals from control group when compared to normal rats (data not shown). Correspondingly, these colitic animals showed a reduced body weight gain with time in contrast with the higher weight gain observed in non colitic rats (data not shown). Once the rats were sacrificed, the colonic tissue from control colitic rats showed an inflamed and necrosed area, typically affecting 5–6 cm of the large intestine, thus obtaining a score value of 7.9 ± 0.2 (Table 2). In addition, bowel wall thickening, as an index of oedematous tissue, together with a shortening in the colonic length, was clearly evidenced, thus resulting in an almost 2.5-fold increase in the colonic weight/length ratio in rats from the untreated colitic group when compared with non-colitic rats (Table 2).

Although none of the treatments showed a beneficial impact on the body weight evolution in colitic rats during the seven days following TNBS instillation, the administration of the different doses of CPM (40 and 100 mg kg^{-1}), ethyl pyruvate (40 and 100 mg kg^{-1}) or sulphasalazine (30 mg kg^{-1}) resulted in a significant reduction in the extent of colonic damage when compared with untreated control colitic group (Table 2). However, no beneficial effect was obtained with any of the different treatments on the colonic weight/length ratio (Table 2).

The histological assessment of the colonic samples confirmed the intestinal anti-inflammatory effect of either calcium pyruvate

Table 2
Effects of several doses of calcium pyruvate monohydrate (CPM), ethyl pyruvate (EP) and sulphasalazine (SAZ) on colonic macroscopic damage score, weight/length ratio, myeloperoxidase (MPO) activity, malonyldialdehyde (MDA) and glutathione (GSH) content in TNBS experimental rat colitis.

Group (n = 10)	Damage score (0–10)	Weight/length (mg cm^{-1})	MPO (mU per g tissue)	MDA (nmol per g tissue)	GSH (nmol per g tissue)
Non-colitic	0	80.3 ± 4.0	2.0 ± 0.3	94.1 ± 4.1	1488 ± 141
TNBS control	7.9 ± 0.2	201.8 ± 15.2	83.8 ± 7.7	132.0 ± 12.6	403 ± 58
CPM (20 mg/kg)	7.1 ± 0.4	186.0 ± 22.8	48.2 ± 7.2*	105.6 ± 7.2	389 ± 55
CPM (40 mg/kg)	5.7 ± 0.4*	184.6 ± 25.1	48.8 ± 8.2*	85.3 ± 13.7*	675 ± 67*
CPM (100 mg/kg)	6.6 ± 0.6*	198.9 ± 15.0	57.7 ± 11.5*	78.2 ± 9.3*	768 ± 100*
EP (20 mg/kg)	6.9 ± 0.6	185.4 ± 31.1	58.1 ± 11.9	108.2 ± 7.3	560 ± 74
EP (40 mg/kg)	6.1 ± 0.4*	172.0 ± 15.8	61.5 ± 9.8	90.5 ± 12.3*	715 ± 88*
EP (100 mg/kg)	6.0 ± 0.5*	186.4 ± 13.2	54.7 ± 10.8*	89.6 ± 10.2*	870 ± 124*
SAZ (30 mg/kg)	6.7 ± 0.4*	180.4 ± 16.9	82.8 ± 8.2	95.7 ± 4.6*	900 ± 118*

Data are expressed as mean ± SEM.
All colitic groups statistically differ from non-colitic group ($P < 0.05$), except for MDA, in which there were only differences between non-colitic and TNBS control groups.
* $P < 0.05$ vs. TNBS control group.

or ethyl pyruvate, since both treatments facilitated the recovery of the colonic histology, as evidenced by a significant reduction in the microscopic scores in comparison with the untreated TNBS colitic control group (Fig. 1). In this group, the inflammatory process was characterized by an intense epithelial ulceration and necrosis affecting more than 75% of the surface in most of the animals. The colon showed a severe transmural inflammation, with massive infiltration of granulocyte cells, predominately neutrophils, in the lamina propria, and a mixture of granulocytes and mononuclear cells (macrophages, lymphocytes and plasmatic cells) in the submucosa, muscularis and serosa layers. The presence of oedema was evidenced in the majority of the specimens. The grade of lesion was considered as severe or very severe, giving a mean score value of 34.4 ± 3.3 (Fig. 1). The treatment with CPM promoted the recovery of the intestinal cytoarchitecture in all colonic layers compared with the control group, being the reduction of the microscopic score statistically significant at the doses of 40 and 100 mg kg^{-1}. Thus, the recovery of the mucosa was evident, and the ulceration affected less than 50% of the mucosa surface in all samples, showing complete restoration in half of the samples. In addition, goblet cells appeared replenished with their mucin content. Although the leukocyte inflammatory infiltrate occurred, this was considered as slight to moderate in most of the samples, being neutrophils again the predominant cell type. In these groups, the grade of lesions could be considered as moderate, with mean score values of 17.2 ± 4.4 and 12.2 ± 1.7, at doses of 40 and 100 mg kg^{-1} of CPM, respectively ($P < 0.05$ vs. TNBS control group) (Fig. 1). Similarly, the administration of ethyl pyruvate (40 and 100 mg kg^{-1}) to colitic rats was associated with an improvement of the altered colonic histology in comparison with untreated TNBS control group. However, there was no dose-dependent effect in these groups of rats, since the higher efficacy was achieved at doses of 40 mg kg^{-1}, which showed similar characteristics to those reported for both active doses of CPM. In this group, most of the samples (8 out of 10) showed a clear recovery of the mucosa, since the ulceration of the mucosa typically affected less than 50% of the surface in all samples, and only one sample showed ulceration affecting more than 75% of the mucosal surface. Also, the crypts appeared almost completely recovered in most of the samples and the goblet cells replenished with their mucin content. In this case, the inflammatory infiltrate was also evident, mainly composed by granulocytes, being moderate in the majority of the samples; however, on three of the samples this inflammatory infiltrate was considered as moderate to severe, affecting even the muscular layer. The lesions in the groups of colitic rats treated with ethyl pyruvate were assigned mean score values of 16.7 ± 2.9 and 22.4 ± 2.6, at doses of 40 and 100 mg kg^{-1}, respectively ($P < 0.05$ vs. TNBS control group) (Fig. 1). Finally, the effect observed with sulphasalazine (30 mg kg^{-1}) on colonic histology was similar to that obtained with the lowest dose of CPM or ethyl pyruvate (20 mg kg^{-1}), without showing statistical differences with the score value obtained in the colitic control group (Fig. 1). The immunohistochemical study of the tissue has confirmed an intense myeloid infiltrate in the colon in the TNBS control group. The treatment with the active doses of wither ethyl and calcium pyruvate clearly decreased this infiltrate (Fig. 2).

3.2. Effects of CPM and ethyl pyruvate on biochemical parameters in TNBS rat colitis

The beneficial effects observed histologically were confirmed biochemically. In control colitic rats, the TNBS-induced colonic damage was associated with an increased MPO activity in comparison with non-colitic rats, as an index of the intense neutrophil infiltration into the inflamed tissue. All the assayed doses of CPM significantly reduced this enzyme activity in colitic rats, thus suggesting a lower leukocyte infiltration in the colonic tissue, in accordance with the observations from the histological studies (Table 2). However, when ethyl pyruvate was considered, only the highest dose (100 mg kg^{-1}) achieved statistical significance in reducing this enzyme activity in comparison with untreated colitic group (Table 2). Consistently, colitic rats evidenced a colonic glutathione content depletion together with increased MDA production, consequence of the colonic oxidative stress that takes place in the inflamed colonic tissue. However, the modifications in glutathione content and MDA production were significantly ameliorated by either CPM or ethyl pyruvate (at doses of 40 and 100 mg kg^{-1}), as well as by sulphasalazine, thus revealing an improvement in the altered oxidative status that characterizes this experimental model of colitis (Table 2).

3.3. Effects of CPM and ethyl pyruvate on gene and protein expression in TNBS rat colitis

The colonic inflammatory status in TNBS-induced colitic rats was also characterized by an altered expression of the different colonic markers assayed in the present study. Although both CPM and ethyl pyruvate exerted an intestinal anti-inflammatory effect in this experimental model of colitis, they do not display the same profile when all these markers are considered. Thus, CPM treatment reduced the expression of IL-1β, both at the mRNA and protein levels, whereas ethyl pyruvate was not able to significantly affect the expression/production of this proinflammatory cytokine (Fig. 3). Similarly, only CPM (at the dose of 40 mg kg^{-1}) significantly reduced the expression of IL-6 and IL-12 in the colonic tissue from colitic rats (Fig. 3). Sulphasalazine significantly reduced the colonic production of IL-1β as well as the expression of IL-6. Additionally, the colonic expression of the Th-17 related cytokines,

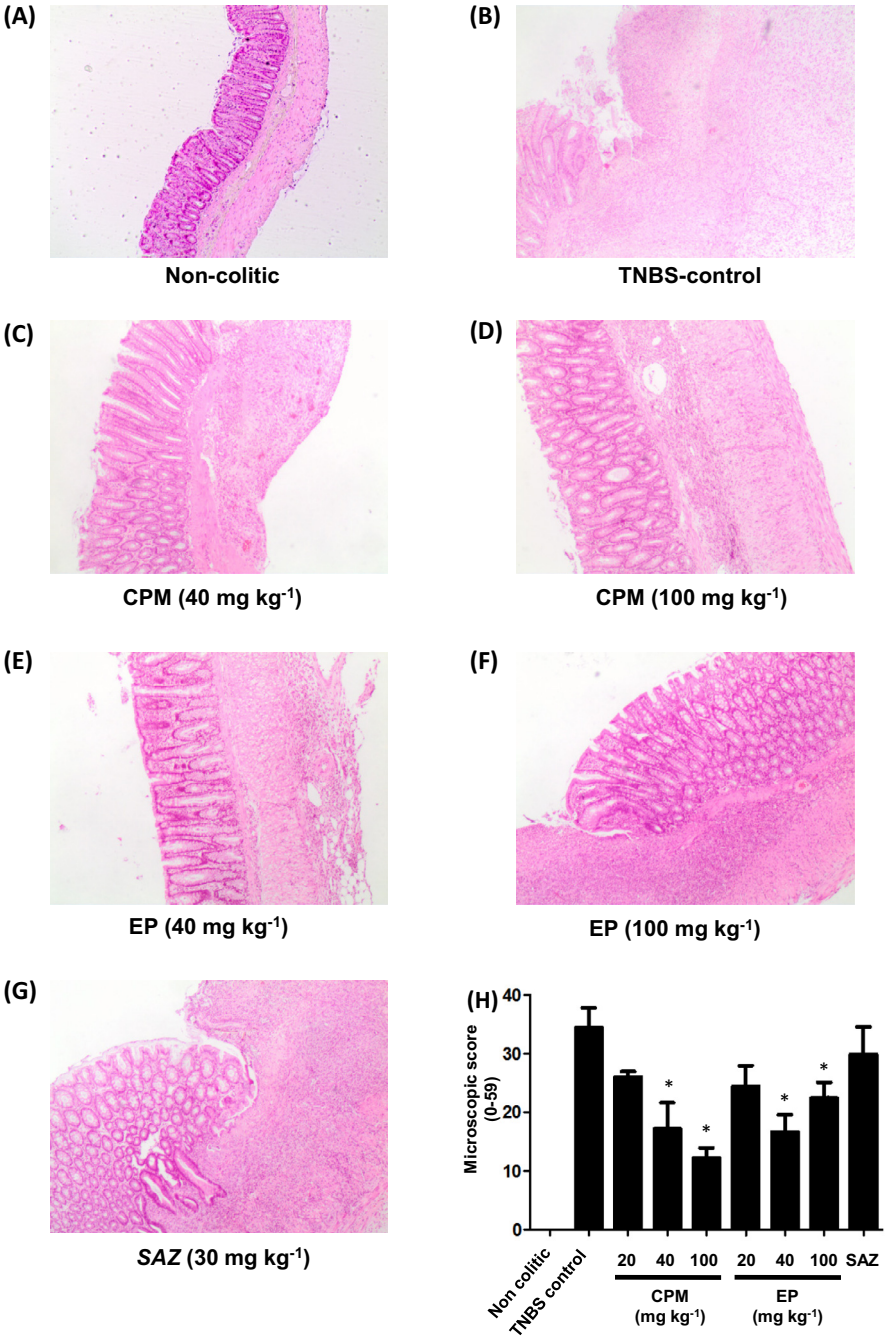

Fig. 1. Histological sections of colonic tissue stained with haematoxylin and eosin showing the effects of different doses of calcium pyruvate monohydrate (CPM), ethyl pyruvate (EP) and sulphasalazine (SAZ) in TNBS rat colitis: (A) non-colitic; (B) TNBS-control; (C) CPM (40 mg kg^{-1}); (D) CPM (100 mg kg^{-1}); (E) EP (40 mg kg^{-1}); (F) EP (100 mg kg^{-1}); (G) SAZ (30 mg kg^{-1}); and (H) microscopic score assigned according the criteria previously described [4]. Data are expressed as means ± SEM; $^{*}P < 0.05$ vs. TNBS control group.

IL-17 and IL-23, was also increased by the intestinal inflammation induced by the TNBS, but significantly down-regulated in colitic rats treated with either CPM or ethyl pyruvate, showing the former this beneficial effect at doses of 20 and 40 mg kg^{-1} in both cytokines, whereas only one of the doses of ethyl pyruvate assayed significantly reduced the expression of IL-17 (20 mg kg^{-1}) or IL-23 (40 mg kg^{-1}) (Fig. 4). Sulphasalazine only significantly decreased the expression of IL-23 in colitic rats (Fig. 4).

The expression of the inducible protein iNOS was also increased in the untreated colitic control group in comparison with non colitic rats. Both pyruvate treatments were able to significantly reduce this protein expression, but whereas CPM exerted this effect with all the doses studied, ethyl pyruvate only showed a significant effect at the dose of 40 mg kg^{-1} (Fig. 4). Sulphasalazine was devoid of any significant effect when compared with the colitic control group (Fig. 4).

Other three mediators involved in the intestinal inflammatory response, and related to the chemotaxis and/or infiltration of leukocytes, cytokine-induced neutrophil chemoattractant (CINC)-1, monocyte chemoattractant protein (MCP)-1 and intercellular adhesion molecule (ICAM)-1, were also evaluated by qPCR. The results showed that colonic inflammation significantly increased

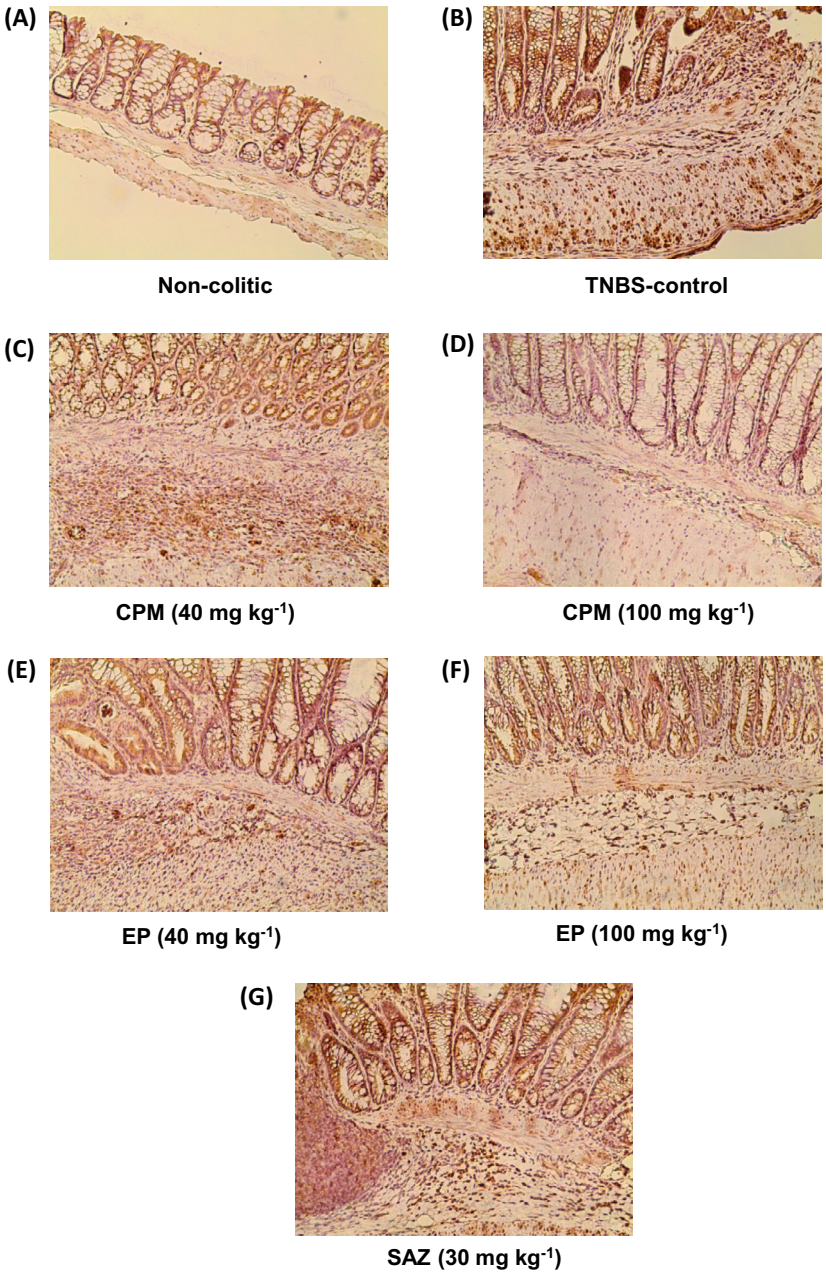

Fig. 2. Immunostaining of the myeloid marker CD11b performed in histological sections of colonic tissue from TNBS rats treated with different doses of calcium pyruvate monohydrate (CPM), ethyl pyruvate (EP) and sulphasalazine (SAZ): (A) non-colitic; (B) TNBS-control; (C) CPM (40 mg kg^{-1}); (D) CPM (100 mg kg^{-1}); (E) EP (40 mg kg^{-1}); (F) EP (100 mg kg^{-1}); (G) SAZ (30 mg kg^{-1}).

the expression of these markers in TNBS colitic rats in comparison with non-colitic (Fig. 5). The treatment with CPM (at any of the doses assayed) was associated with a significant inhibition in the expression of the three proteins, whereas ethyl pyruvate (also in all the doses studied) significantly reduced the expression of MCP-1 and ICAM-1; however, sulphasalazine only reduced the expression of ICAM-1 when compared with untreated colitic rats (Fig. 5).

Finally, the expression of proteins involved in colonic epithelial integrity, mucin MUC-2 and the trefoil factor (TFF)-3, was evaluated. TNBS-induced inflammation significantly reduced their expression while both CPM and ethyl pyruvate restored their expression to normal values. However, whereas CPM did it at all the doses studied, ethyl pyruvate was only effective at the two highest doses (40 and 100 mg kg^{-1}) (Fig. 6). These effects correlated with the improvement observed in the mucosal layer when the histological studies were performed. In this study, sulphasalazine was also able to significantly increase the expression of MUC-2 and TFF-3 in the colonic tissue, in comparison with the corresponding colitic control group (Fig. 6).

3.4. Effects of CPM and ethyl pyruvate on the expression of phospho-NF-kB in the colon in TNBS rat colitis

When trying to investigate the mechanisms of action of the CPM, we focused on the activity of the NF-kB, since it is one of the key regulators of the inflammatory response. The TNBS instillation induced an increased of the phosphorylation of the p65 subunit of the NF-kB complex in the nuclear extracts of the colonic tissue. The treatments with both pyruvates manage to

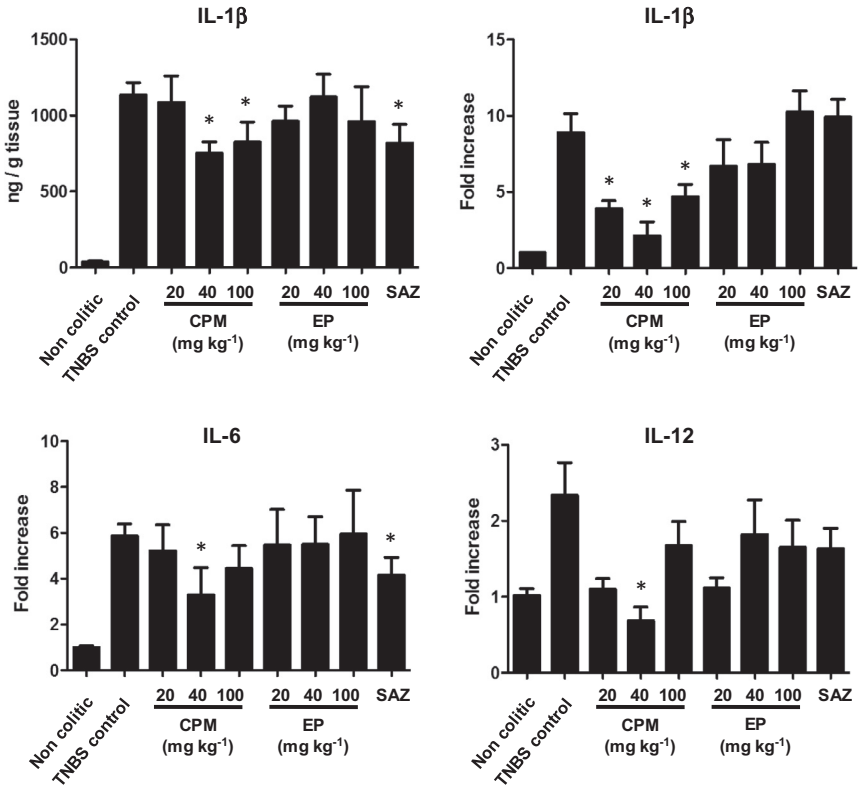

Fig. 3. Effects of different doses of calcium pyruvate monohydrate (CPM), ethyl pyruvate (EP) and sulphasalazine (SAZ) in TNBS rat colitis on colonic IL-1β measured by ELISA and expressed as ng/g of tissue, and the mRNA expression of IL-1β, IL-6 and IL-12 quantified by real-time PCR and shown as fold increases. Data are expressed as means ± SEM (n = 10); $^{*}P < 0.05$ vs. TNBS control group.

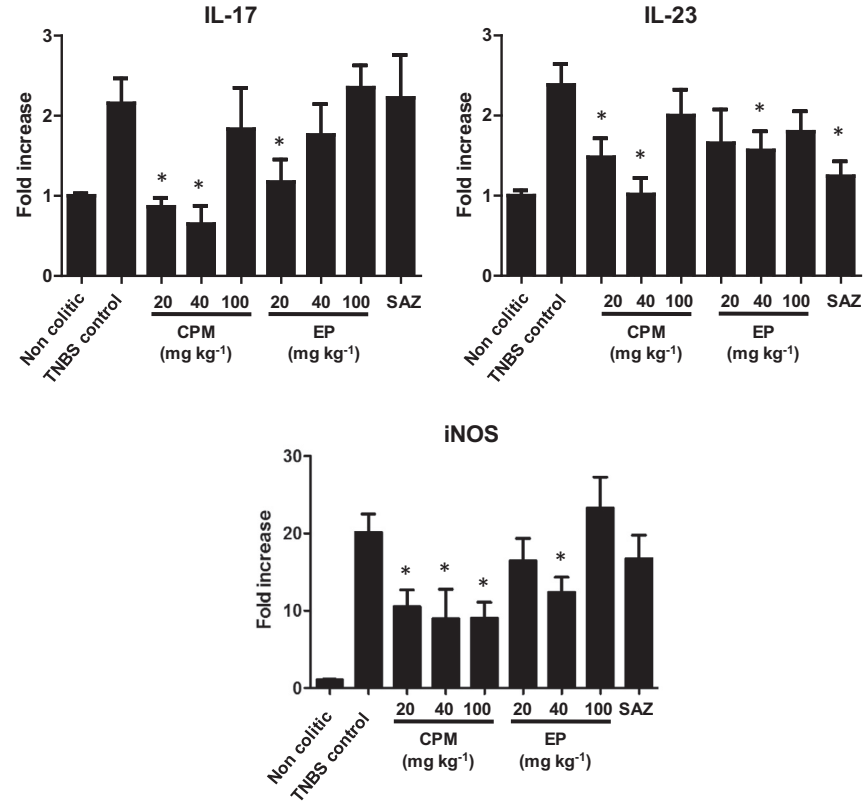

Fig. 4. Effects of different doses of calcium pyruvate monohydrate (CPM), ethyl pyruvate (EP) and sulphasalazine (SAZ) in TNBS rat colitis on mRNA expression of IL-17, IL-23 and iNOS quantified by real-time PCR and shown as fold increases. Data are expressed as means ± SEM (n = 10); $^{*}P < 0.05$ vs. TNBS control group.

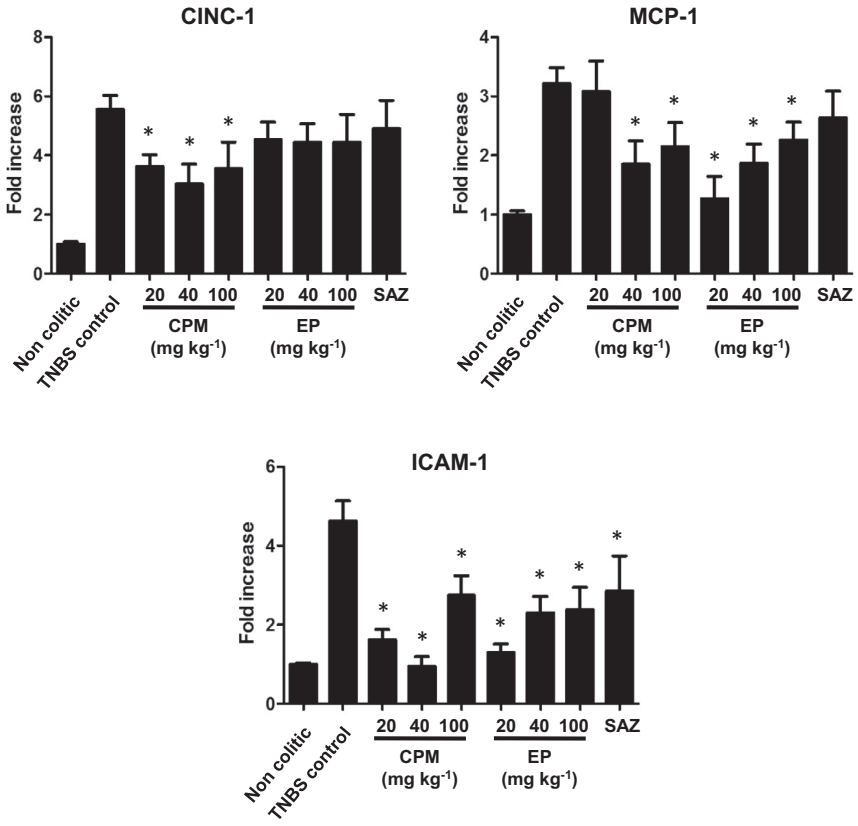

Fig. 5. Effects of different doses of calcium pyruvate monohydrate (CPM), ethyl pyruvate (EP) and sulphasalazine (SAZ) in TNBS rat colitis on mRNA expression of CINC-1, IL-23 and iNOS quantified by real-time PCR and shown as fold increases. Data are expressed as means ± SEM ($n = 10$); $^{*}P < 0.05$ vs. TNBS control group.

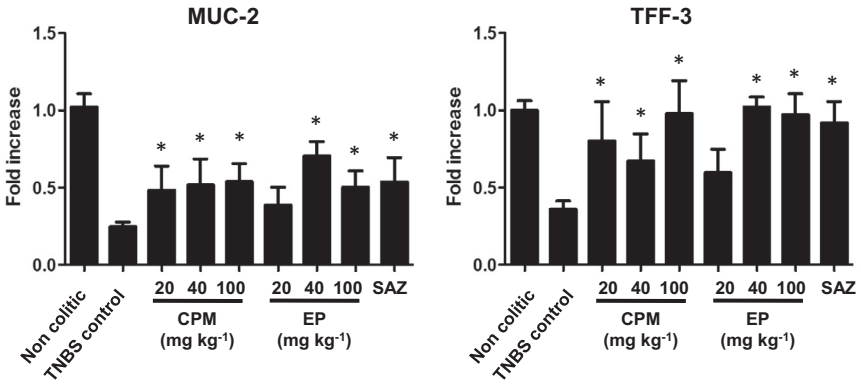

Fig. 6. Effects of different doses of calcium pyruvate monohydrate (CPM), ethyl pyruvate (EP) and sulphasalazine (SAZ) in TNBS rat colitis on mRNA expression of MUC-2 and TFF-3 quantified by real-time PCR and shown as fold increases. Data are expressed as means ± SEM ($n = 10$); $^{*}P < 0.05$ vs. TNBS control group.

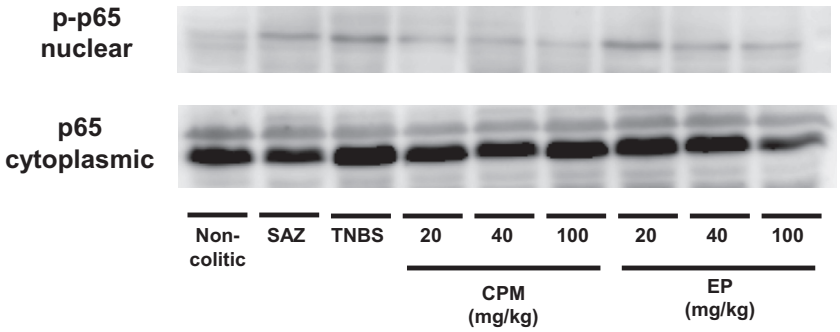

Fig. 7. Effect of calcium pyruvate monohydrate (CPM) (20, 40 and 100 mg kg^{-1}), ethyl pyruvate (EP) (20, 40 and 100 mg kg^{-1}) and sulphasalazine (SAZ) (30 mg kg^{-1}) on colonic phospho-p65 NF-κB subunit nuclear levels. Western blots with colonic protein samples from cytoplasmic and nuclear cell fractions were probed for total p65 and phospho-p65 antibodies, respectively.

reduce the phosphorylation of the p65 subunit, as shown in the Fig. 7, in comparison with the non-treated colitic group.

4. Discussion and conclusions

Nowadays several drugs are available for the treatment of human IBD, including salicylates, glucocorticoids, immunosuppressants as well as biologicals. The main goal of the therapy is to induce and then maintain remission of all the symptoms associated with the intestinal inflammation, and thus provide an enhanced quality of life to these patients. However, and although these drugs usually show efficacy, there is a high risk of side effects that can concern patient compliance and quality of life, thus limiting their use for prolonged periods of time. This has encouraged the scientific community to search for new therapeutic strategies that combine efficacy and safety. It is interesting to note that, in the last decade, the research has mainly focused on the development and clinical application of several compounds that inhibit specific inflammatory signalling pathways, like cytokines, adhesion molecules, chemokines, etc. Unfortunately, and without a clear justification, the majority of trials with such drugs have failed in IBD patients [29]. Since several inflammatory pathways are involved in IBD, it is plausible that targeting concurrently two or more of them may be more beneficial than inhibiting selectively single pathways. This could be the case of pyruvates, which display antioxidant and immunomodulatory properties, responsible for the different beneficial effects previously described in different inflammatory conditions [15]. In fact, ethyl pyruvate has been reported to show intestinal anti-inflammatory activity in experimental colitis in mice [10], which has been confirmed in the present study, showing beneficial effects in the TNBS experimental model of rat colitis, which resembles human IBD [14]. Furthermore, we have demonstrated for the first time that other pyruvate salt, calcium pyruvate monohydrate, also exerts intestinal anti-inflammatory effects in the same model of experimental colitis. It is interesting to note that the results obtained revealed differences between both pyruvate derivatives, showing CPM a better profile than ethyl pyruvate. These differences may in part be due to their different physicochemical characteristics that determine their rate of absorption. Although both pyruvate derivatives are quickly absorbed [35], ethyl pyruvate is a very apolar lipophilic molecule that goes through the intestinal mucosa better that the ionic salt form of calcium pyruvate which is more hydrophilic. Moreover, calcium pyruvate has shown in previous works [31] that in an aqueous solution exists in a sol–gel equilibrium and is not dissociated completely in a wide range of pH so a fraction of it could stay up to colon and also exert a local action. Consequently, we assume that ethyl pyruvate acts systemic due to fast absorption and calcium pyruvate may act both systemic and partly local. Thus, although both compounds showed similar intestinal anti-inflammatory effects in the macroscopic or microscopic colonic analysis, also comparable to sulphasalazine, the evaluation of the different inflammatory mediators revealed some differences. In fact, the increased colonic expressions of IL-1β, IL-6 and IL-12 were only significantly reduced in those colitic rats treated with CPM. Long ago, it was first described an increased production of IL-1β and IL-6 in the intestinal mucosa of patients with IBD [34]. IL-1β signalling has been proposed to play a critical role in gut homeostasis and intestinal inflammation [46]. More recently, it has been shown that, in colonic inflammation, IL-1β is mainly produced by infiltrated neutrophils [43]. In fact, when colonic MPO activity, a reliable marker of neutrophil infiltration in intestinal inflammation, was determined in the colonic tissue from colitic rats, only CPM showed a significant reduction, thus revealing a limited neutrophil infiltration, also confirmed in the histological analysis. The diminished neutrophil infiltration could justify the lower colonic IL-1β expression and production observed in colitic rats treated with calcium pyruvate. Furthermore, it has been shown that neutrophil-derived IL-1β is a critical inducer of IL-6 production by intestine-resident mononuclear phagocytes [43], thus explaining the decreased colonic IL-6 production observed after calcium pyruvate treatment to colitic rats. The lower neutrophil infiltration observed in these treated-colitic rats was associated with a reduced expression of CINC-1, as well as of ICAM-1 and MCP-1, which have been proposed to be proteins involved in initiating inflammation via recruitment and retention of leucocytes and neutrophils [6,36], and in turn responsible for the amplification of the inflammatory response and up-regulated expression of other main pro-inflammatory mediators like IL-1β [33]. Furthermore, the treatment of colitic animals with calcium pyruvate resulted in the reduced expression of colonic IL-17, which plays a key role in the Th17-mediated inflammatory response reported in intestinal inflammation [16]. IL-17 has been shown to be one of the most deleterious mediators that actively participates in tissue inflammation, since it contributes to neutrophil or other immune cells migration to the target tissue and their subsequent activation, enhances dendritic cell maturation and induces inflammatory cytokines, chemokines and matrix metalloproteases, thus facilitating the development of chronic intestinal inflammation in the intestine [1,25]. The inhibitory effect on colonic IL-17 expression observed in calcium pyruvate-treated colitic rats, was correlated with a reduced expression of IL-23. It has been reported that the up-regulation of this cytokine, in the presence of high levels of IL-6 and IL-1β, which resembles a proinflammatory environment like that occurring in intestinal inflammation, seems to be crucial in the polarization of naïve Th cells into Th17 [2,45].

Moreover, it is well known that activation of the transcription factor NF-κB is crucial for intestinal inflammation, both in the initial steps of inflammation as well as in its perpetuation [19]. In fact, in has been reported that this transcription factor is involved in the increased expression and release of several pro-inflammatory mediators, including adhesion molecules (ICAM-1), chemokines (CINC-1), or cytokines (TNFα, IL-1β, IL-6, and IL-12) [30]. Previous studies have shown the ability of ethyl pyruvate to inhibit NF-κB activation both in vivo [47] and in vitro [17,18], probably through inhibition of the nuclear translocation of RelA [28]. In consequence, this inhibitory effect on NF-κB activation could be also ascribed to calcium pyruvate, as demonstrated in the present study, thus resulting in the reduced expression of the different inflammatory mediators in colitic rats.

The ability of CPM to modify the activation of NF-κB can also contribute to its inhibitory effects on colonic iNOS expression. This is an inducible enzyme predominantly expressed at sites of inflammation, whose increased expression has been associated with the activation of NF-κB [7]. This enzyme produces excessive NO, and when combined with other inflammatory mediators like ROS may be detrimental to the integrity of the colon and contribute to the development of the intestinal damage that characterizes the inflammatory reaction [22].

Finally, the beneficial effects showed by both pyruvate derivates were also associated with a recovery of the expression of MUC-2 (the primary constituent of the mucus layer in the colon) and TFF-3 (a bioactive peptide that participates in epithelial protection and repair) [21], thus preserving the mucus-secreting layer that covers the intestinal epithelium and acts as a physical barrier protecting its integrity. This can be relevant in the beneficial effects showed by these compounds, since an impairment of the epithelial barrier function has been proposed to be one of the first events that occur in intestinal inflammation, facilitating the access of antigens from the intestinal lumen and triggering the exacerbated immune response [26].

In conclusion, the present study confirms the beneficial effects previously described for ethyl pyruvate ester in an experimental model of intestinal inflammation. Additionally, another pyruvate, the calcium pyruvate monohydrate salt, has also demonstrated to be effective in the same model of experimental colitis, displaying a better profile when considering the key pro-inflammatory mediator. Further experiments should be considered to also explore the beneficial effects of calcium in the extra-intestinal symptoms like osteopenia or osteoporosis that commonly appear in this pathology.

Author contributions

F.A., A.R.-N., N.G.-M., P.U., J.G.-M., T.V., D.C. and M.E.R.-C. performed the experiments. J.G., I.P. and M.E.R.-C designed the experiments; F.A., A.R.-N., N.G.-M., P.U., J.G.-M., D.C., M.E.R.-C. and J.G. analyzed the data; and J.G., I.P. and M.E.R.-C. wrote the manuscript.

Conflict of interest

I.P. is inventor of patents on pyruvates and was involved in the study design and manuscript write-up, but neither in data acquisition nor in data processing. No conflict to disclose for the rest of the authors.

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