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Maillard Reaction Products from highly heated food prevent mast cell number increase and inflammation in a mouse model of colitis

Issam Al Amir PharmD, PhD^a, David Dubayle PhD^b, Anne Héron PhD^{c,d},
Carine Delayre-Orthez PhD^{a*} and Pauline M. Anton PhD^{aC*}

^a *Transformations et Agroressources, Institut Polytechnique UniLaSalle, Beauvais, France*

^b *CNRS UMR 8119, Biomedical center, Paris Descartes University, France*

^c *Department of Human Physiology, Paris Descartes University, France*

^d *Clinical Research Unit URC28, Victor Jousselin Hospital, Dreux, France*

*The authors contributed equally to the work

Issam Al Amir: issamalamir@gmail.com / 19 rue Pierre Waguet F-60000 Beauvais, France

David Dubayle: david.dubayle@parisdescartes.fr / 45 rue des Saints-Pères, F-75006 Paris, France

Anne Héron: anne.heron@parisdescartes.fr / 44 avenue du Président J. F. Kennedy, F-28102 Dreux, France

Carine Delayre-Orthez: carine.delayre@unilasalle.fr / 19 rue Pierre Waguet F-60000 Beauvais, France

Pauline M Anton: pauline.anton@unilasalle.fr / 19 rue Pierre Waguet F-60000 Beauvais, France

^C**Corresponding author:** Pauline M Anton – UniLaSalle – 19 rue Pierre Waguet – BP 30313 – 60026 Beauvais cedex 03 France. Phone: +33 (0)344 063 868. Mail: pauline.anton@unilasalle.fr.

List of Abbreviations

AGEs: Advanced Glycation End Products

ATP: Adenosine TriPhosphate

CD: Crohn's Disease

DSS: Dextran Sodium Sulphate

HH: Highly Heated

IBD: Inflammatory Bowel Disease

iNOs: inducible Nitric Oxide Synthase

MC(s): Mast Cell(s)

MDS: Macroscopic Damage Score

MH: Mildly Heated

MPO: MyeloPerOxidase

NH: Non Heated

NO: Nitric Oxide

UC: Ulcerative Colitis

ABSTRACT

Links between food and inflammatory bowel diseases (IBDs) are often suggested but the role of food processing has not been extensively studied. Heat treatment is known to cause the loss of nutrients and the appearance of neoformed compounds such as Maillard reaction products. Their involvement in gut inflammation is equivoqual as some may have pro-inflammatory effects while other seem to be protective. As IBDs are associated with the recruitment of immune cells, including mast cells, we raised the hypothesis that dietary Maillard Reaction Products generated through heat treatment of food may limit the colitic response and its associated recruitment of mast cells. An experimental model of colitis was used in mice submitted to mildly and highly heated rodent food. Adult male mice were divided in 3 groups and received either non heated, or mildly or highly heated chow during 21 days. The last week of the study, each group was split in two sub-groups, submitted or not (controls) to dextran sulphate sodium (DSS) colitis. Weight variations, macroscopic lesions, colonic myeloperoxidase (MPO) activity and mucosal MC number were evaluated at the end of the experiment. Only highly heated chow significantly prevented DSS-induced weight loss, MPO activity and mast cell number increase in the colonic mucosa of DSS colitic mice. We suggest that Maillard Reaction Products from highly heated food may limit the occurrence of inflammatory phases in IBD patients.

Keywords

Maillard Reaction Products; Dextran Sulfate Sodium salt; Intestinal barrier; Mast cell;
Experimental intestinal inflammation; Food heat treatment

1 - Introduction

Inflammatory bowel diseases (IBDs) are complex multifactorial human diseases including Ulcerative Colitis and Crohn's Disease. In genetically predisposed patients, these pathologies involve inflammation of the gastro-intestinal tract [1] and mobilize innate and adaptative immune cells. Mast cells (MCs) are immune cells produced by the bone marrow. They have been suspected to be implicated in these chronic diseases [2,3]. MCs use the blood vessels to penetrate the gastrointestinal tract. In particular, they infiltrate in the *lamina propria* of the intestinal mucosa and participate in the immune response. In physiological conditions, the number of MCs in the intestinal mucosa is low. MCs exert regulatory functions at the gastrointestinal barrier. They regulate not only epithelial secretion and barrier functions, but also blood flow, vascular permeability, peristalsis and recruitment of other immune cells. Like other leukocytes (neutrophils, lymphocytes and macrophages), MCs prevent the invasion of pathogens in parenchyma, thus limiting the risk of sepsis or anaphylaxis. For instance, in response to exogenous bacteria, viruses, parasites or allergens, infiltrated MCs act by degranulation and release various substances involved in inflammation: enzymes (tryptase and chymase), cytokines (interleukins, interferon and transforming growth factor), lipid metabolites (leukotrienes and prostaglandins), proteoglycans (heparin), neuropeptides (vasoactive intestinal peptide), ATP (adenosine triphosphate) and nitric oxide through different pathways among which Ca^{2+} upregulation [4]. In pathological conditions, the number of MCs greatly increases in the intestinal mucosa and their inflammatory effects result in appearance of diarrhea, impairment of epithelial and endothelial barrier and chronic inflammation [5]. Finally, MCs release biogenic amines (histamine and serotonin) that act on sensory nerve endings, causing abdominal pain [6]. Thereby, food matrix might modulate mast cell degranulation that will impact the course of the

inflammatory reaction [7]. Indeed, it was shown that ingestion of some species of wild edible fungi that activate MCs and cause inflammation is prevented by black cumin, an anti-inflammatory condiment [8]. Moreover, several *in vitro* and *in vivo* studies have suggested that the inhibition of mast cell activation and degranulation by natural substances [9] or biophysical therapies [10,11] would have effective anti-inflammatory, analgesic and regenerative effects.

Among the numerous factors contributing to the etiology of IBD and thus the activation of mast cells, diet plays a crucial role through the numerous nutrients (amino acids, vitamins), xenobiotics (pesticides ...) or neoformed compounds (polycyclic aromatic hydrocarbons) present in the food matrix. Among neoformed compounds, and especially in heated food, we may find Maillard Reaction Products (MRPs) that are a mixture of several compounds. This large family of molecules comes from the glycation of amino-acids (mainly Lysine Asparagine) with reduced sugars under specific temperature, pressure and humidity dependent conditions. They include Amadori Products, advanced glycation end products (AGEs), premelanoidins and melanoidins. The mix of MRPs will then be dependent of the food matrix and of the physical conditions to which the food will be submitted. Due to differences in molecular weight and variability of their presence in the food matrix, they differ in terms of gastrointestinal bioavailability and absorption [12,13]. Their effect on gut homeostasis is equivocal and seems largely to depend upon the form of MRPs considered, and needs further investigation. In this study, we investigated the recruitment of MCs in an IBD mice model: the DSS (Dextran Sulfate Sodium)-induced colitis, a model known and used for a long time in mice [14–16]. The DSS colitis model mimics bloody diarrhea, weight loss, mucosal ulceration and neutrophil infiltration into the colon of mice [17]. Due to the clinical signs, DSS colitis mimics most of the

inflammatory responses observed in ulcerative colitis in human. In a previous work, we demonstrated that repeated exposure to highly heated food rich in Maillard Reaction Products (MRPs) in mice limited colonic inflammation [18].

Then, since mast cell degranulation is observed during IBD, and as consumption of MRPs is limiting an experimental colitis in rodents, we hypothesized that dietary MRPs could, as well as other natural substances, limit mast cell activation and degranulation by exerting anti-inflammatory effects. This study was aimed at determining if highly heated food could limit mast cell infiltration, contributing to the reduction of inflammatory reaction in DSS-colitic mice. To test this hypothesis, macroscopic lesions, intestinal mast cell number and myeloperoxidase activity have been measured following 3 weeks of standard, mildly and highly heated food in healthy and colitic mice.

2 – Methods and materials

2.1 – Diet preparation

Standard A04 rodent maintenance chow was used (SAFE diets- Augy – France). Mild heat treatment consisted of A04 pellets rehydration in 1/3 (w/w) of deionised water before proceeding to oven-heat treatment (Memmert UFE400, France) at 150°C for 90 min. Highly heated pellets were rehydrated in 2/3 (w/w) of deionized water and oven-heated at 150°C for 240 min according to previously described [18].

2.2 - Animals and Study design

Male Balb/c mice (n=36) aged 8 weeks (21-25g) were randomly divided into three groups (n=12 per group) and housed in stainless steel cages under controlled temperature (21 ±1°C) and a 12 h light-dark cycle, with free access to food and water. Weight and food intake were monitored throughout the study. The last week of treatment, each group was split into two sub-groups (n=6 per sub-group): one received dextran sulfate sodium (DSS) salt and the other one had no treatment. DSS (36-42000 Da - 3% w/v) was purchased from MP Biomedical France and added in the drinking water for 6 days. Bottles were changed every other day. Two days before the end of the treatment, DSS was removed from water. Experiments were conducted at the Animal House Unit of LaSalle Beauvais after receiving the prior approval from both the animal protocol review committee and the Picardie Council of veterinary office in France (C-60-200-01). Mice have received standard chow (non heated, NH, n=12) mildly heated chow (MH, n=12) or highly heated chow (HH, n=12) daily for three weeks, as described in the section 2.1 diet preparation.

2.3 - Assessment of DSS-induced colitis

2.3.1 - Macroscopic lesions:

At the end of the experiment before collecting the samples, animals were sacrificed and a midline laparotomy was performed to determine the score of colitis-induced macroscopic lesions, according to the literature [17]. DSS lesion severity was evaluated based on weight loss (from 0-no loss to 4-more than 20% loss), stool consistency (from 0-consistent to 4-diarrhea), occult or gross bleeding (from 0-none to 4-gross bleeding) and gross blood content (from 0-no blood to 3-blood in more than 2/3 of the colon). In the most severe case, a necrotic colon would be assigned a macroscopic damage score (MDS) of 15 in arbitrary unit (AU). Then, the proximal colon pieces were immediately removed to perform various experimental analyses. The first half (3cm long from ceco-colonic junction) was used for MPO activity assay and the second half for MC quantification.

2.3.2 – Mast Cell quantification:

MCs are unequivocally identified by their proteoglycan content, such as heparin and chondroitin sulfate. In the presence of toluidine blue in an acidic medium, their cytoplasmic granules containing proteoglycans present the classical metachromatic reaction and appear purplish-red [19,20]. Colonic tissues were collected and immersed in 4% formaldehyde to assess microscopic tissue infiltration of MCs. Fixed colonic tissues were dehydrated in successive baths of increasing alcohol concentration solutions, xylene-treated and embedded in paraffin using standard procedures. Serial transverse sections (thickness 4 μm) were mounted on SuperFrost

microscope slides, deparaffinized in xylene, hydrated in decreasing alcohol concentration solutions and stained for 90s in 0.05% aqueous solution of toluidine blue (Fluka 89640) acidified with HCl to pH 2.3. Then, sections were rinsed for 15s in acidified distilled water at the same pH, and differentiated and dehydrated in increasingly alcohol solutions (70% alcohol for 60s and 95% alcohol for 120s). Finally, sections were air-dried, xylene-treated and coverslipped with Eukitt [19]. Observation of MCs inside the colonic tissue was performed using a conventional photonic microscope (Olympus BH-2) connected to a color video camera (JVC KY-F50). MC identification was performed at magnification x200 with a blue filter.

2.3.3 - MPO activity:

The inflammatory reaction is a process associated with immune cell activation and neutrophil infiltration. Myeloperoxidase (MPO) is almost exclusively found in neutrophils cytoplasmic granules and is considered to be a good marker for quantifying the level of inflammatory reaction. As previously described [21], tissue (1st half of the colon) were lysed in a phosphate buffer (50mM - pH=6) containing hexadecyl trimethyl ammonium bromide (0.5% m/v) with a polytron (PT 1200E, Kinematica AF, Switzerland). Homogenates were then submitted to 3 cycles of freezing and thawing (-80°C - 10 min/37°C - 15 min), and then sonicated (Bioblock scientific, France) before centrifugation (6000 g - 4°C - 15 min). Supernatants were collected to measure MPO activity and protein concentration. MPO activity was assessed in supernatants diluted in a reaction buffer containing O-dianisidine dihydro-chloride (1mg/mL) and hydrogen peroxide (5 x 10⁻⁴% v/v); pure MPO from human leucocytes (Sigma Aldrich, France) was used as a standard. Absorbance was measured at 450 nm after 15 min of incubation. Supernatants' total protein

content was assessed based on Lowry method following the manufacturer's recommendations (Bio Rad DC Protein Assay, France).

2.4 - Statistical analyses

Data are expressed as means \pm standard error of the mean (SEM). Statistical analyses were performed using Graph Pad Prism software. A Shapiro-Wilk test and a Bartlett's test were used to test all variables for normality distribution and homogeneity of variances respectively. A one-way ANOVA followed by a post hoc test of Tukey, whenever significant differences among means were found, was used to compare weight variations and MPO activities. The macroscopic lesions and mast cell number were analysed by a Kruskal-Wallis test followed by a test of Dunn whenever significance among medians were observed. The statistical significance was $P < .05$.

3 - Results

3.1 - Highly heated chow limits weight loss

As expected, DSS induced a significant ($P < .05$) weight loss in mice fed with standard non heated (DSS-NH) chow (Table 1). However, feeding animals with mildly heated (DSS-MH) and highly heated (DSS-HH) chow resulted in less weight loss (but not significantly) or no weight loss respectively (Table 1). Follow up of food intake confirmed that thermic treatment of food did not induce any food intake variation (data not shown).

3.2 – Colitis is limited by highly heated chow consumption.

3.2.1 - Macroscopic lesions:

In control animals, no macroscopic lesions were observed (Table 1). In DSS-colitic mice receiving standard chow (DSS-NH), a significant increase of MDSs expressed in Arbitrary Units (AU) (9.4 ± 0.7 AU vs 0 in CTL-NH, $P < .001$) was observed (Table 1). The increase of MDSs was also significant in DSS-colitic mice under mildly heated chow (7.1 ± 0.7 AU vs 0 in CTL-MH, $P < .01$). By contrast, in the DSS-HH group, MDSs were not significantly elevated in comparison to MDSs of respective control (5.7 ± 0.7 AU vs 0 in CTL-HH, $P < .05$) (Table 1). High Heat treatment limited DSS-induced macroscopic lesions (Table 1).

3.2.2 – Mast Cells identification:

In histological sections of colonic mucosa, MCs were revealed by toluidine blue staining. In control conditions, no or rare MCs were found in the mucosa (Figure 1A). As illustrated in Figure 1B, MCs infiltrated the intestinal parenchyma of DSS-colitic mice. MCs were particularly localized in the *lamina propria* of the intestinal mucosa. At higher magnification, proteoglycans granules were observed inside and outside the MCs illustrating a fine degranulation process (Figure 1C and 1D, black arrows).

3.2.3 – Mast Cells number quantification

Heat treatment of chow did not change the number of MCs quantified in the *lamina propria* of the colonic mucosa of control mice (CTL-NH, CTL-MH, CTL-HH) (Figure 2). In contrast, in DSS-colitic mice, MC number was significantly increased by 14 times in DSS-NH ($P < .01$) and by 11 times in DSS-MH ($P < .05$) groups, but this increase was not significant ($P < .05$) in the DSS-HH group (Figure 2). Heat treatment limited the presence of MCs in the *lamina propria* of colitic mice (Figure 2).

3.2.4 – Myeloperoxidase activity (Figure 3):

Ingestion of NH, MH or HH chow did not have any influence on MPO activity in control mice (CTL) (Figure 3). In contrast, in the NH treated chow group, we observed a significant increase (+70%) of MPO activity in DSS colitis mice (169.2 ± 18.8 vs 99.7 ± 5.1 MPO U/mg protein; $P < .001$) (Figure 3). A lower but significant increase (+50%) of MPO activity was observed in mice that received MH chow (151.3 ± 12.4 vs 96.1 ± 6.1 MPO U/mg protein; $P < .05$) (Figure 3). In DSS-colitic mice that ingested HH chow no significant increase (+30%) of MPO activity was observed (131 ± 8.4 vs 93.8 ± 6.8 MPO U/mg protein) (Figure 3).

4 - Discussion

Heat treatment of food matrix is a frequently used technique to improve sanitary status/quality of food generating new organoleptic properties and, in some conditions, different nutritional

quality. Heat treatment modifies functionality of some nutrients (e.g. increased satiating power of food [22]). However, if exposed to severe or long heat treatment, food may lose some nutritional qualities and potentially deleterious substances may be generated including MRPs. Depending on the level of heat treatment many different MRPs may be generated and they may have opposite effects on health. Some substances are associated with inflammatory reactions such as Advanced Glycation End products (AGEs) [23,24] whereas others (e.g. melanoidins) appear to have protective effects on health [25,26]. In a previous work, we have shown that moderate and high treatment of food is associated with increased levels of AGEs and melanoidins that are proportional to the amount of browning [18]. However, we did not investigate the impact of AGEs and melanoidins on the inflammatory reaction when they are generated simultaneously.

In this study, we submitted rodent chow to a heat treatment generating sufficiently different amounts of AGEs and melanoidins in order to compare differences with standard chow (not heated). In a previous work, we demonstrated that mice submitted to heated food for 21 days did not display any alteration of their health status [27]. In the present study, we showed that moderate and high heat treatment of rodent chow had no incidence on food intake or weight gain of animals when submitted to this diet for three weeks (CTL NH/CTL MH/CTL HH). We also showed that heat treatment of food does not have any pathological consequence in the colon of naive adult mice. This is corroborated by the absence of modification of MPO activity, a good indicator of an inflammatory reaction. MPO activity is a marker of neutrophil infiltration and of the activation of mucosal MCs in the colon. Our results suggest that heat treatment will neither impact the nutritional properties of rodent chow nor affect the health status of mice.

Although we did not observe any alteration of the gut mucosa in mice due to ingestion of heated chow, the consequences could be different under gut inflammatory conditions as depicted in IBD. IBDs are chronic gut inflammations of unknown etiology linked to gut homeostasis disruption and regrouping mainly Crohn's Disease (CD) and Ulcerative Colitis (UC). In our IBD model, mice submitted to DSS and receiving standard chow developed an acute experimental colitis, associated with significant weight loss, and reduction of food intake. We also observed ulcerations in the colonic mucosa that are close to those observed in UC in humans [17].

Moreover, DSS colitic mice had an increased MC number in the colonic mucosa in relation with an inflammatory reaction evidenced by increased MPO activity. Mucosal MC activation and increased MPO activity in DSS colitis have already been described in the literature [28,29]. Furthermore the onset and/or the evolution of IBD symptoms are due to several factors including food. However, to our knowledge, there are only few studies that confirm it [30] and most often work is focused on antioxidants because of their importance in the limitation of oxidative stress and its associated inflammatory response [31]. Furthermore, these few studies are focused on food matrix composition but the consequences of food processing is rarely evaluated. The present study points out for the first time the consequences of food processing on the onset of an experimental colitis in rodents. In fact, we observed significant reduction of the response in the DSS-colitic groups in relation with the diet they received. Mice exposed to highly heated chow (HH diet) and then submitted to DSS colitis (HH/DSS group) did not developed any significant inflammatory reaction; this group showed significantly lower MPO activity and no activation of mucosal MCs in comparison to DSS colitic mice under NH diet (NH/DSS group). Similar results were also observed in the MH/DSS group to a lesser extent. However, due to the multiplicity of

molecules generated by Maillard Reaction, further research is needed to determine if these effects are linked to one category of molecules (e.g. melanoidins) or to a mix of them (e.g. melanoidins and AGEs).

Recent literature describes that oral administration of DSS is known to initiate architectural alteration of the colonic mucosa including ulceration, crypt dilation, goblet cells depletion or mixed cell infiltration mainly involving neutrophils and macrophages [32]. Moreover, due to their localization close to the intestinal epithelium, MCs become activated and will, in turn, release mediators enhancing neutrophil influx and perpetuating ongoing inflammation [33,34]. Our present results suggest that ingestion of mildly and highly treated food limit neutrophil infiltration and mast cell activation. In a previous work we had suggested that MRPs may be responsible for a limitation of the inflammatory reaction in DSS colitic mice [27]. This hypothesis is corroborated by the literature since [5-(5,6-dihydro-4H-pyridin-3-ylidenemethyl) furan-2-yl]-methanol (F3-A) is known to inhibit the release of NO, an inflammatory mediator and of its activating enzyme iNOS in Caco2 cells [35]. Thus, by limiting NO release in the intestinal mucosa, we may then limit neutrophil infiltration and MC activation. However, the exact role of NOS and of the other underlying mechanisms leading to the limitation of the immune cells activation remain to be elucidated among which the involvement of iNOS pathway.

In conclusion, there remain to determine which components of the HH chow have protective effects on colitis, we accept our research hypothesis. Indeed, this study confirmed that HH chow (which we have described as being richer in melanoidins), prevent the induction of the

inflammatory reaction in mice. Moreover, this is in accordance with other studies investigating the health benefits of food rich in melanoidins such as bread crust. These data, together with other ongoing work, could contribute to a better understanding of health risks/benefits of heat treatment. These data are a first step of evidence that heat treatment of food shall be taken into account in the development of nutritional recommendations with potential to reduce the occurrence of inflammatory spikes in IBD patients.

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Legends of figures

Figure 1: MCs identification by their proteoglycan contents

A. Histological section of mouse colonic mucosa after a toluidine blue histochemistry. This mouse received non-heated diet for 21 days (control conditions CTL). No or rare MCs were found in the colonic mucosa under control conditions. **B.** In this section, the mouse received 3% of dextran sodium sulfate (DSS) during the last week of treatment to induce inflammatory reaction. The microphotography illustrates the location of two MCs in the *lamina propria* (lp) of the mucosa (*E: Epithelium* of the mucosa). **C-D:** High magnification of the two MCs observed in **B** microphotography. MCs degranulation was identified by the presence of granules out of the cell body (black arrows).

Figure 2: Prevention of DSS colitis induced mast cell number increase by highly heated food.

Standard (NH), mildly (MH) and highly (HH) treated food containing increasing amounts of MRPs were distributed during 21 days. DSS colitis was induced the last week of the study. The second half of the proximal colon was harvested and fixed. Transverse sections (4 sections/piece of colon) were then mounted before being stained with an acidified solution of toluidine blue.

Mast cell number was evaluated for each slice and a mean count per mouse was determined (mean number/4 sections). Six mice per group were used. Values are expressed as means \pm SEM.

** $P < .01$ vs the corresponding control group (CTL). Abbreviations: CTL, control; DSS, Dextran Sulfate Sodium; HH, Highly heated, MH, Mildly heated; NH, Non Heated; NS, Not Significant;

Figure 3: Prevention of DSS colitis induced increase of myeloperoxidase activity by highly heated food.

Standard (NH), mildly (MH) and highly (HH) treated food containing increasing amounts of MRPs were distributed during 21 days. DSS colitis was induced the last week of the study. The first half of the proximal colon was harvested and snap frozen before proceeding to the tissular myeloperoxidase (MPO) assay. Results are expressed as units of MPO activity (U MPO) per total protein content (mg total protein). Six mice per group were used. Values are expressed as means \pm SEM. *, *** $P < .05, .001$ vs the corresponding control group (CTL). Abbreviations: CTL, control; DSS, Dextran Sulfate Sodium; HH, Highly heated, MH, Mildly heated; MPO, myeloperoxidase; NH, Non Heated; NS, Not Significant;

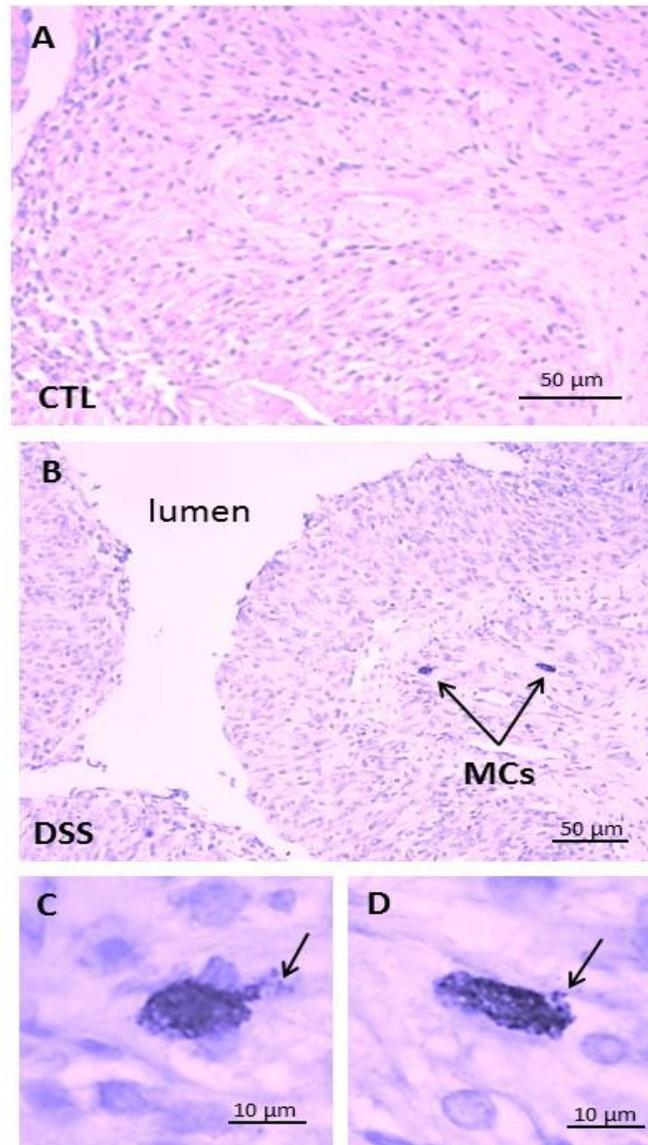


Figure 1

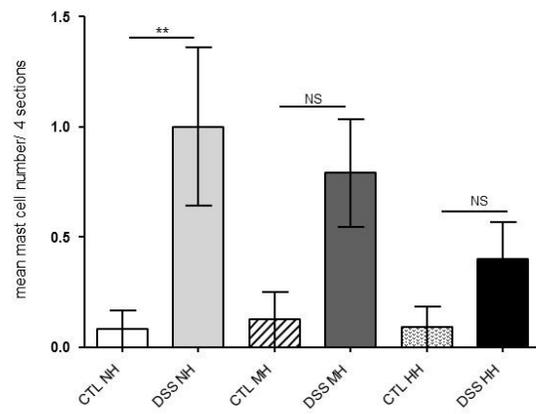


Figure 2

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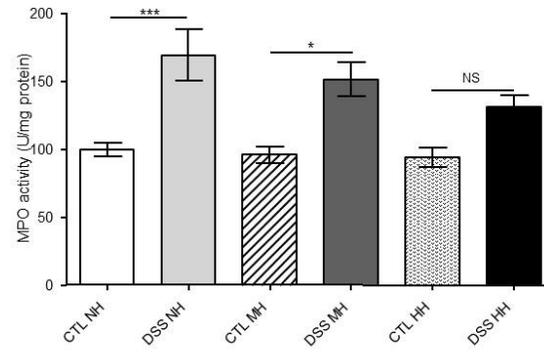


Figure 3

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Table 1 Effect of mild and high heat treatment of food on weight variation and macroscopic lesions in healthy and colitic mice

	Diet and treatment groups					
	NH		MH		HH	
	CTL	DSS	CTL	DSS	CTL	DSS
Measurements						
Weight variation (%)	103.8 ± 1.1	95.4 ± 2 *	102.7 ± 1.2	97.4 ± 2.3	103.6 ± 0.4	101.6 ± 2.2
MDS (AU)	0	9.4 ± 0.7 ***	0	7.1 ± 0.7**	0	5.7 ± 0.7

Macroscopic damage scores (MDS) are expressed in arbitrary unit (AU) and weight variations in percentage (%) of weight variation between the 1st and the last day of the experiment. Three groups of mice received either non heated chow (NH), mildly heated chow (MH) or highly heated chow (HH) during 21 days. The DSS colitis was induced in half of each group of mice the last week of treatment. Values are expressed as means ± SEM, n=6. *, **, ***

P< .05, .01, .001 vs the corresponding control group. Abbreviations: AU, Arbitrary Units; CTL, control; DSS, Dextran Sulfate Sodium; HH, Highly Heated; MDS, Macroscopic Damage Scores; MH, Mildly Heated; NH, Non Heated.