Food and Chemical Toxicology 125 (2019) 78–84

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Skim milk powder with high content of Maillard reaction products affect weight gain, organ development and intestinal inflammation in early life in rats

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ARTICLE INFO

Keywords:
Maillard reactions
Intestinal inflammation
Immune tolerance
Inflammatory cytokines
Autoimmune diseases

ABSTRACT

Background: The intestinal tract is important for development of immune tolerance and disturbances are suggested to trigger autoimmune disorders. The aim of this study was to explore the effect of Maillard products in skim milk powder obtained after long storage, compared to fresh skim milk powder.

Methods: Young rats were weaned onto a diet based on skim milk powder with high concentration of Maillard products (HM-SM, n = 18) or low (C-SM, n = 18) for one week or four weeks. Weekly body weight and feed consumption were noted. At the end, organ weights, intestinal histology, permeability and inflammatory cytokines were evaluated.

Results: Rats fed with HM-SM had after one week, 15% less weight gain than controls, despite equal feed intake. After one week thymus and spleen were smaller, intestinal mucosa thickness was increased and acute inflammatory cytokines (IL-17, IL-1β, MCP-1) were elevated. After four weeks, cytokines associated with chronic intestinal inflammation (fractalkine, IP-10, leptin, LIX, MIP-2, RANTES and VEGF) were increased in rats fed with HM-SM compared to C-SM.

Conclusion: High content of Maillard products in stored milk powder caused an intestinal inflammation. Whether this is relevant for tolerance development and future autoimmune diseases remains to be explored.

1. Introduction

Hypersensitivity and autoimmune disorders like allergy, celiac disease and type 1 diabetes are increasing among children in developed countries (Manna et al., 2016). The reason for this rapid increase is unknown. Genes play a role in the etiology (Todd et al., 1987; Törn et al., 2006) but seem to have a permissive role and yet unknown environmental factors have a precipitating role. The reported rapid increase in incidence suggests a change in the environment leading to different gene-environment interactions. Thus, one or several environmental triggering factors are needed to start the immune mediated process.

Breast milk feeding versus cow milk based formula has been in focus in discussions of etiology for allergy and other autoimmune diseases, but studies have however been inconclusive. In an attempt to decrease the stimulation of specific antigens, infant formula based on hydrolysed proteins has been recommended. The rationale behind this treatment is to make more inert proteins. A recent Cochrane analysis could not confirmed a protective effect on allergy of hydrolysed formula and the topics needs further investigation (Osborn and Sinn, 2006). A very early introduction of hydrolysed formula was instead recently shown to increase islet autoimmunity in children with high risk of type 1 diabetes (Hummel et al., 2017).

Our theory is that the chemical modifications of cow's milk during the processing of skim milk powder, rather than the milk itself, could be the trigger. Non-enzymatic reactions between amino acids and reduced sugars that occur during high temperature, are able to proceed a long time after the reaction initiated. The effect of these Maillard reactions

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https://doi.org/10.1016/j.fct.2018.12.015
Received 1 October 2018; Received in revised form 4 December 2018; Accepted 12 December 2018
Available online 13 December 2018

List of abbreviations

BSA	Bovin Serum Albumin
CML	Carboxymethyl Lysine
C-SM	Control – Skim Milk powder
ELISA	Enzyme-Linked ImmunoSorbent Assay
FITC-D	Fluorescein Isothiocyanate – Dextran
G-CSF	Granulocyte Colony-Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating factor
GRO/KC	Growth Regulated Oncogene - Keratinocyte chemoattractant (also known as CXCL1)
HM-SM	High Maillard - Skim Milk powder
IFNγ	Interferon gamma
IL	Interleukin
IP	Interferon gamma-induced protein 10 (also known as CXCL10)
LIX	Lipopolysaccharide-induced CXC chemokine
MCP-1	Monocyte Chemoattractant Protein 1 (also known as CCL2)
MIP-2	Macrophage Inflammatory Protein-2
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted (also known as CCL5)
SM	Skim Milk powder
TNFα	Tumor Necrosis Factor alpha
VEGF	Vascular Endothelial Growth Factor

has been discussed in terms of loss of nutritional quality (Plakas et al., 1985), diabetes (Knecht et al., 1991) carcinogenesis (Tareke et al., 2000) or even their potential health promoting role as antioxidants (Oh et al., 2013). The composition of skim milk powder with a high content of lactose and proteins makes it an ideal media for Maillard reactions to take place already during processing and later on the reactions continue during storage. To monitor and understand the different Maillard reactions in processed food including powdered milk is a challenge of great importance and vital survey (Ottum and Mistry, 2015; Poulsen et al., 2013). Since the surface area of powders is much greater than of fresh food, skim milk powder has a significantly increased amount of reactive Maillard derivatives in comparison.

We have previously showed an increasing concentration of Maillard reaction products over time in stored milk powders, where storage conditions have great impact on this process (Aalaei et al., 2016a, 2016b). A high humidity and high temperature accelerates this process (Thomsen et al., 2005). When lysine in the milk powder reacts with lactose, lysine will be blocked and consequently not nutritionally available as an essential amino acid in metabolism. Milk powder has an expiry time most often based on colour, physical properties like solubility and smell. In this study we have tested stored milk powder with a high content of the Maillard reactant carboxymethyl lysine (CML), which could be readily measured, and compared with fresh milk powder.

The probability for the gut to be exposed for products like Maillard compounds is high. Milk powder is inexpensive and widely used, especially in prepared foods and instant products. It is often used to enrich protein to the food and to give better texture. The content of CML is crucial for the toxicological effects. New milk powder is therefore different from powder stored in humid atmospheres.

The aim of this study was to investigate if skim milk powder with higher content of Maillard reaction products (HM-SM), simulating longer storage, was more prone to induce intestinal inflammation compared to fresh milk powder in control-food containing lower content of Maillard reaction products (C-SM). This was performed in a rat model where the pups were weaned onto these diets.

2. Material and methods

2.1. Milk powder preparation

Pasteurized (+73 °C) and low heat spray dried cow skim milk powder (SM) produced by Norrmejerier, Umeå, Sweden were purchased to be used in the rat feed. Two kinds of SM were used for the rat feed pellets, one in original condition and a second after controlled storage at 45 °C and 52% relative humidity for 28 days. The storage was carried out using a climate chamber (Vötsch Industrietechnik GmbH, Balingen, Germany). The two milk powders are named control milk powder (C-SM) and high Maillard skim milk powder (HM-SM). These two powders were used as the overall protein source in the animal feed (Table 1).

2.2. Determination of available lysine and CML

The available lysine content of skim milk powders were determined using a dye-binding method previously described and validated (Aalaei et al., 2016b). The method was applied successfully on a variety of skim milk powders manufactured and stored differently (Aalaei et al., 2016a). Measurement of CML content was carried out using a method previously described and validated (Tareke et al., 2013).

2.3. Feed preparation

The experimental diets were based on a laboratory rodent chow (R36, Labfor, Table 1) where, instead of soy and potato proteins, the high Maillard skim milk powder (HM-SM) or control milk powder (C-SM) had been incorporated as the main protein source. The diets were then pelleted and used in the in vivo studies. The analysis of the feed pellets showed that the CML content was almost 10 times higher in the HM-SM then in the C-SM (Table 2).

2.4. Animals

The experiment was approved by the local Malmö-Lund Ethical Review Committee for Animal Experiments and conducted in accordance with the European Community regulation concerning the protection of experimental animals (2010/63/EU and M169/14).The studies were carried out on rats (*Rattus norvegicus*) of the Sprague-Dawley strain (SPRD Han; Taconic M&B, Denmark). The rats were bred and kept under specific pathogen-free conditions in the animal facility at the department of Biology at Lund University (20 ± 1 °C, 50 ± 10% RH, 12:12 h light-dark cycle). At 2–3 days after birth the rat pups were culled to 12 per litter and kept with their dam in separate cages (polycarbonate) on aspen wood bedding (Beekay B & K Universal AB, Sweden), enriched with paper-nesting material (Sizzle-pet, Lillicobiotech). The rats had free access to water and a laboratory rodent chow (R36, Lactamin) placed on the cages lid, however, the cage height was increased using a 7 cm wall extender to hinder the access of

Table 1
Characteristics of skim milk powders and rat feed pellets.

	Protein content (g per 100 g)	Available Lysine (g per 100 g SMP)	CML (μg per g pellets)
HM-SM powder	37 ± 2[a]	1.82 ± 0.16	not analysed
C-SM powder	37 ± 2[a]	3.26 ± 0.08	not analysed
HM-SM pellets	18.5[b]	not analysed	576.6 ± 25.4
C-SM pellets	18.5[b]	not analysed	66.3 ± 5.7

[a] From suppliers content list.
[b] From Table 2.

Table 2
Nutritional composition of the diet (R36, Breeding feed for mouse and rat, Labfor, Stockholm, Sweden).

1280 kJ/100 g metabolizable energy
18.5% raw protein (based on soy and potato protein)
4.0% raw fat
55.7% Nitrogen-free extract (NFE, carbohydrates)
3.5% Fibre
6.3% Ash
(< 12.0% Water)

the solid chow by the pups.

2.5. Animal experiment 1 - four weeks of exposure

Rats from 3 litters (n = 36) at the age of 21 days (3 weeks) were removed from their dam (weaned) and taken into this experiment. The rats were randomized into two groups and put on a diet containing the experimental HM-SM based pellets (n = 18) or the control C-SM based pellets (n = 18). The ad libitum intake of food was measured per cage. The rats were followed and each week their body weights and the feed consumption (per cage) were measured. After four weeks the rats were sacrificed by inhalation of an overdose of isoflurane. Blood samples were taken by heart puncture and plasma was obtained after centrifugation and stored at −80 °C until analysis. At autopsy, the liver, pancreas, spleen, thymus and intestine were dissected and weighted. Fresh faecal samples (pellets) were collected from the distal colon and stored at −80 °C until analysis. Distal jejunal samples were dissected from 8 HM-SM and 8 C-SM rats for ex vivo studies.

2.6. Animal experiment 2 - one week of exposure

Based on the results we received from experiment one we wanted to repeat the experiment for one week where another set of 36 rats from 3 litters were weaned onto the two diets, the HM-SM based diet (n = 18) or the control C-SM based diet (n = 18). At start of the experiment and at the end after one week all the rats were weighted and then sacrificed. Blood and faecal was collected and organ weights were measured as in the first experiment.

2.7. Intestinal ex vivo permeability

A segment (≈13 cm) from the distal jejunum was excised and immediately immersed in room-tempered, oxygenated modified Krebs–Ringer buffer, pH 7.4 (Nejdfors et al., 2000a).The segment was divided into three parts, opened along the mesenteric border and mounted as tissue sheets in Ussing diffusion chambers (Precision Instrument Design, Los Altos, CA, USA) with an exposed area of 1.78 cm^2 (Grass and Sweetana, 1988; Nejdfors et al., 2000b). The half-chambers on the mucosal and serosal sides were filled with 5 mL of buffer and continuously oxygenated with 95% O_2 in the circulation buffer by gas lift and kept at 37 °C. At the start of the experiments (time = 0) the buffer on the mucosal side was replaced with 5 mL of buffer containing Mannitol, instead of glucose, and containing the different-sized marker molecules, bovine serum albumin (BSA, Fraction V, 66 500 Da, 25 mg/mL), FITC-dextran 4400 (FD, 4400 Da, Sigma–Aldrich Co, 1 mg/mL) and ^{14}C-mannitol (182 Da, Perkin-Elmer, 0.031 mCi/mL). Samples were taken from the serosal receiver side for marker analyses and replaced with an equal volume of buffer each 20 min during the 120 min incubation. Under these conditions, the intestinal samples were considered to be viable during the time period studied (Pantzar et al., 1993). The permeation of the markers from the mucosal to the serosal side in Ussing diffusion chambers is expressed as the apparent permeability coefficient, Papp (mean ± SD x1^{-6} cm/s).

2.8. Intestinal morphometry

Samples from the proximal and distal jejunum were dissected and immediately fixed in 10% neutral formalin solution. Then the samples were paraffin-embedded, routinely processed and cut and then stained with haematoxylin and eosin. Microscopic intestinal morphology was studied in regard to villi height, crypt depth and thickness of mucosa and muscle layers.

2.9. Cytokine multiplex assay

Cytokines were analysed with a premixed 27-plex magnetic bead multiplex assay (RECYMAG65K27PMX, Millipore, France) including EGF, Eotaxin, Fractalkine, G-CSF, GM-CSF, GRO/KC, IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17, IL-18, IP-10, Leptin, LIX, MCP-1, MIP-2, RANTES, TNFα and VEGF, according to the

Table 3
Weekly mean body weight for rats on High Maillard Skim milk powder diet (HM-SM) and rats on control skim milk powder (C-SM). Results are expressed as mean ± standard deviation if a normally distributed data otherwise as median and interquartile range in brackets [Q3-Q1]. Comparison between groups were done with Student's t-test or Mann-Whitney *U* test, respectively.

	HM-SM 1w (n = 18)	C-SM 1w (n = 18)	p-value	HM-SM 4w (n = 18)	C-SM 4w (n = 18)	p-value
Start (age 3 weeks)						
Weight (g)	46.8 ± 4.4	47.2 ± 5.7	ns	50.9 ± 3.4	50.1 ± 3.8	ns
Weight gain (g)	N.A	N.A		N.A	N.A	
Food intake (g)	N.A	N.A		N.A	N.A	
After 1 week (age 4 weeks)						
Weight (g)	76.0 ± 6.89	81.2 ± 7.1	0.01	81.2 ± 5.7	85.7 ± 7.0	0.04
Weight gain (g)	29.1 ± 3.4	34.8 ± 5.3	< 0.001	31.5 [33–39]	36.5 [28–32]	< 0.001
Food intake (g)	66.0 ± 4.5	62.7 ± 6.6	ns	68.9 ± 2.2	64.9 ± 2.7	ns
After 2 weeks (age 5 weeks)						
Weight (g)	N.A	N.A		123.0 ± 11.0	128.1 ± 12.5	ns
Weight gain (g)	N.A	N.A		74 [62–77]	76 [70–86]	ns
Food intake (g)	N.A	N.A		103.6 ± 5.2	99.2 ± 4.8	ns
After 3 weeks (age 6 weeks)						
Weight (g)	N.A	N.A		166.8 ± 17.6	171.2 ± 24.8	ns
Weight gain (g)	N.A	N.A		111 [102–126]	112 [101–144]	ns
Food intake ()	N.A	N.A		122.8 ± 12.0	123.6 ± 10.5	ns
After 4 weeks (age 7 weeks)						
Weight (g)	N.A	N.A		207.9 ± 37.5	211.1 ± 39.8	ns
Weight gain (g)	N.A	N.A		146 [126–192]	145 [131–199]	ns
Food intake (g)	N.A	N.A		130.2 ± 21.8	131.7 ± 18.9	ns

manufacturer's technical guidelines. Blood plasma samples were diluted 1:2 in assay buffer prior to analysis and measured in duplicates in the 4-week-experiment but not in the one week experiment since many of the samples were below detection limit in the four week experiment. We included 36 animals in the four-week experiment that left only 24 reaction spots available for the one week experiment. Thus 12 animals from each group were randomized for cytokine analysis in the one week experiment. A Bio-plex 200 instrument was used to record the median fluorescent intensity (MFI) data and sample concentrations were estimated using a 5-parametric logistic regression curve. The reported intra-assay coefficient of variation was between 2.2 and 6.3%.

2.10. Calprotectin in faeces

The faecal samples were thoroughly mixed with extraction buffer (1:50, w/v), centrifuged at 3000 × *g* for 10 min and calprotectin was analysed in the supernatant using a commercial ELISA kit according to manufacturer's instructions (Immundiagnostik AG, Bensheim, Germany). The detection limit was reported as 0.076 ng/mL.

2.11. Statistical analyses

Normal distribution of data was analysed with D'Agostino-Pearson´s test. If data deviated from a normal distribution, a log-transformation was applied or non-parametric tests were used. Comparison between groups were analysed using Student's t-test or Mann-Whitney *U* test. Results are expressed as mean ± standard deviation if a normal distribution of data apply, median followed by interquartile range in brackets [Q3-Q1] if data deviates from a normal distribution despite log-transformation. A p-value less than 0.05 was considered as statistically significant. Statistical analyses were performed using MedCalc for Windows v17.4.4 (MedCalc Software, Ostend, Belgium).

3. Results

3.1. Body weight and food intake

Weaning rat pups on the skim milk based diets resulted in a body growth of about 30 g after one week, the weight gain being statistically significantly less, about 15%, in the HM-SM group as compared to the C-SM group ($p < 0.001$) (Table 3). This despite no significant difference in feed consumption between the two groups. The difference in weight gain disappeared after one week and no differences in body weight gain or feed consumption were found between the dietary groups between 2 and 4 weeks. To confirm that the reduced weight gain observed after one week was not by chance the experiment was repeated for one week with the same outcome in weight gain ($p < 0.001$) (Table 3).

3.2. Organ weights

The HM-SM diet affected the lymphoid organs, since both the thymus and spleen organ weights were significantly lower as compared to the rats fed the C-SM diet, at one week after weaning (Table 4). In contrast, the weight of the small intestines, relative to body weight, was higher in the HM-SM exposed rats as compared to the controls. However, after four weeks with the different diets no difference in organ weights between groups could be observed.

3.3. Inflammatory cytokines

Even though the multiplex assay was designed to analyse 27 different inflammatory markers, some were not possible to evaluate due to concentrations below the detection limit, possibly due to matrix-effects of multiplexing. However, several early proinflammatory mediators differed between the groups after four weeks exposure of HM-SM diet (Table 5). The chemokines fractalkine, the macrophage inflammatory protein 2 alpha (MIP-2α), interferon-gamma-inducible protein 10 (IP-10) and RANTES as well as lipopolysaccharide induced CXC chemokine (LIX) were significantly higher in HM-SM exposed rats compared to the controls. Similarly, levels of growth factors like vascular epidermal growth factor (VEGF) as well as leptin was also higher in the HM-SM exposed rats after four weeks. The other cytokines available in the assay either failed to reach the detection limit or did not differ between the groups in the four weeks experiment.

Levels of early inflammatory cytokines IL-1β, IL-17 and monocyte chemoattractant protein (MCP-1) were significantly increased after one-week exposure (Table 5). The other cytokines included in the assay were generally higher in the HS-SM group but did not reach statistical significance even if IL-10 and IL-18 showed some strong tendency.

Calprotectin levels in faeces did not differ in any of the groups.

3.4. Intestinal morphology and permeability

The morphometric analysis of the small intestines one week after weaning showed generally higher values for the mucosa thickness, villi height, crypt depth in the HM-SM rats compared to the controls, however, this was only significant for the mucosa thickness in the distal small intestine (Table 6). After four weeks of dietary exposure no differences in the intestinal morphology could be found (Table 6).

The intestinal in vitro permeability of the different-sized marker molecules, mannitol, FITC-D4 and BSA, was tested after the four weeks experiment, but no significant differences in the marker permeability were found between the feeding groups of rats.

Table 4
Organ weights (mg) and organ weights relative to body weight (mg/g body weight) after 1-week of dietary exposure to high Maillard skim milk powder diet (HM-SM) and control skim milk powder (C-SM) respectively. Results are expressed as mean ± standard deviation. Comparison between groups were done with Student's t-test. NA = not analysed.

Organ weight	HM-SM 1w (n = 18)	C-SM 1w (n = 18)	p-value	HM-SM 4w (n = 18)	C-SM 4w (n = 18)	
Liver (mg)	3525 ± 482	3740 ± 384	ns	10538 ± 2928	10054 ± 2293	ns
(mg/g body weight)	46.3 ± 3.7	45.6 ± 2.3	ns	50.1 ± 4.1	47.1 ± 5.8	ns
Pancreas (mg)	410.8 ± 36.8	447.4 ± 35.5	0.005 ns	1014 ± 117	1021 ± 98	ns
(mg/g body weight)	5.4 ± 0.4	5.5 ± 0.6		4.9 ± 0.9	5.0 ± 0.8	ns
Spleen (mg)	245 ± 35	304.5 ± 39.7	< 0.001	610 ± 153	555 ± 109	ns
(mg/g body weight	3.2 ± 0.3	3.7 ± 0.4	0.002	2.8 ± 0.4	2.8 ± 0.2	ns
Thymus (mg)	315 ± 55	384.7 ± 45.9	< 0.001	600 ± 95	583 ± 105	ns
(mg/g body weight)	4.1 ± 0.5	4.7 ± 0.6	0.007	2.8 ± 0.5	3.0 ± 0.5	ns
Prox. small intestine (mg)	1764 ± 396	1657 ± 174	ns	N.A	N.A	
(mg/g body weight)	22.4 ± 1.6	20.2 ± 1.2	0.005			
Distal small intestine (mg)	1522 ± 201	1465 ± 176	ns	N.A	N.A	
(mg/g body weight)	20.0 ± 1.6	17.9 ± 1.3	< 0.001			

Table 5
Proinflammatory mediators that were higher in plasma after one and 4 weeks exposure in rats exposed to high Maillard reaction products in spray dried milk (HS-SM) diet compared to the control group (C-SM). Results are expressed as median and interquartile range in brackets [Q3-Q1]. Comparison between groups were done using Mann-Whitney *U* test.

After 1 week	HM-SM (n = 12)	C-SM (n = 12)	p-value	Functions
IL-1β	148 [78–257]	63 [51–87]	0.009	Early mediator of intestinal inflammation and increased intestinal permeability
IL-17	52 [35–75]	31 [14–40]	0.032	Promote neutrophil recruitment. Upregulated in intestinal inflammation and barrier function.
MCP-1	1.2 [1.0–1.3]	1.0 [0.9–1.1]	0.026	Produced by intra-epithelial cells during IL-1β stimulation. Upregulated in intestinal inflammation
IL-18	225 [143–293]	143 [107–213]	ns	IL-1 family cytokine involved in controlling barrier function in colitis.
After 4 weeks	HM-SM (n = 18)	C-SM (n = 18)	p-value	
Fractalkine (pg/ml)	275 [235–346]	204 [165–251]	0.010	Lymphocyte recruitment in the intestinal tract and mediator of intestinal inflammation
MIP-2α (ng/ml)	1.3 [1.2–1.4]	1.2 [1.1–1.3]	0.006	Neutrophil and lymphocyte recruitment in the intestinal tract
LIX (ng/ml)	2.5 [1.9–3.4]	1.4 [1.2–2.4]	0.045	Involved in intestinal inflammation. Neutrophil recruitment,.
IP-10 (pg/ml)	666 [561–812]	551 [433–644]	0.048	Neutrophil recruitment, Inducer of IFNγ and Th1 cell mediated immunity
RANTES (pg/ml)	1130 [824–1366]	830 [642–1176]	0.027	Monocyte and lymphocyte recruitment.
VEGF (pg/ml)	161 [144–204]	144 [106–165]	0.043	Angiogenesis, mediator of intestinal inflammation
Leptin (ng/ml)	73.2 [52.2–86.9]	49.6 [37.2–56.1]	0.009	Appetite hormone, T cell mediated intestinal autoimmunity, growth of inner organs such as pancreas, thymus and small intestine.

4. Discussion

In this study we have demonstrated an increased inflammatory response in young rats when exposed after weaning to skim milk powder containing high concentrations of Maillard reaction products. This occurred with a reduced weight gain after one week exposure to milk powder diet formula containing high levels of Maillard reaction products (HM-SM). This observation was replicated when the study was repeated in an additional experiment. The difference in weight gain and body weight disappeared when the exposure to HM-SM diet was extended up to four weeks but signs of intestinal inflammation was still observed.

After weaning the rats are in a rapidly growing stage and weaning is a vulnerable transient phase of the rat's life adapting to the solid food with profound maturation of the digestive, barrier and immune functions of the gut. The lower weight gain after one week in the rats fed with HM-SM is considered to be clinically significant for the rat and for the development of the gut. The mechanism behind the insufficient weight gain is unknown. The intake of feed and thus calories was not significantly different, and even numerically higher in the HM-SM group despite the lower weight gain. Anorexia, nausea or aversion against the food containing high levels of Maillard products was not indicated. Further, there was no indication of increased metabolism or catabolism due to fever or any concomitant disease. The high Maillard milk powder diet contains less lysine than the control diet. Lysine deficiency could retard the growth but in that case the effect would be expected to continue for several weeks. The most plausible explanation is therefore an inferior uptake of nutrients through the gut, maybe mediated by a proinflammatory response. Reduction in body weight in rats fed with high concentration of Maillard reaction products has been observed in previous studies (Delgado-Andrade et al., 2013; Seiquer et al., 2010). Reduced food intake due to Maillard products in the fed was proposed as the most likely explanation. In our study we have tracked ingested food and have no evidence for lower food intake in animals fed with HM-SM.

The whole small intestine, proximal jejunum and distal small intestine, weighted significantly more in relation to total body weight, while spleen and thymus weighted less in proportion to total body weight after one week in rats fed with HM.SM (Table 4). Microscopic investigation of the intestines showed that the mucosa thickness was increased in the HM-SM rats, together with an indication of increased villi length and crypt depth (Table 6). The increased weight of the intestine could be due to increased amount of interstitial fluid caused by an inflammatory process. In this case it could explain a defect uptake of nutrients thought the gut epithelium.

The weight of the liver was similar in both groups, indicating that HM-SM diet did not influence the nutritional state of the animals. The weight of the thymus and spleen were however reduced in the Maillard exposed animals. This of interest since these organs are of great importance for development of immunological tolerance.

After four weeks, a compensation of the delayed growth was observed and the body weight in the groups become equal. A careful investigation of the gut after four weeks could not reveal any different trait regarding histology or function of the gut and the permeability of

Table 6
Morphometric analysis of intestinal mucosa from the proximal and distal small intestine of rats after 1 week and 4 weeks of dietary exposure to a high Maillard skim milk powder diet (HM-SM) and control skim milk powder diet (C-SM). Results are expressed as mean ± standard deviation. Comparison between groups were done with Student's t-test.

After 1 week		Mucosa thickness (μm)	Villi height (μm)	Crypt depth (μm)	Muscularis thickness (μm)
Proximal jejunum	C-SM	369 ± 39	288 ± 32	78 ± 16	30 ± 12
	HM-SM	376 ± 34	293 ± 26	104 ± 83	38 ± 36
	P-value	ns	ns	ns	ns
Distal jejunum	C-SM	231 ± 57	178 ± 34	78 ± 16	26 ± 3
	HM-SM	268 ± 39	188 ± 26	82 ± 9	27 ± 4
	P-value	0.03	ns	ns	ns
After 4 weeks					
Proximal jejunum	C-SM	637 ± 49	512 ± 42	122 ± 13	31 ± 3
	HM-SM	638 ± 60	507 ± 55	124 ± 10	33 ± 6
	P-value	ns	ns	ns	ns
Distal jejunum	C-SM	421 ± 39	296 ± 27	127 ± 13	40 ± 5
	HM-SM	452 ± 83	317 ± 52	136 ± 30	49 ± 23
	P-value	ns	ns	ns	ns

the gut membrane was not altered for any of the different sized test markers (Table 6).After one week we found elevated levels of cytokines normally associated with acute intestinal inflammation (Table 5) (Al-Sadi and Ma, 2007; Fujino et al., 2003; Khan et al., 2006) in HM-SM fed rats. IL-1β and IL-18 are well-known mediators in the pathogenesis of innate intestinal inflammation (Ligumsky et al., 1990; Nowarski et al., 2015) and involved in recruitment of IL-17 secreting cells in the intestinal tract (Coccia et al., 2012). IL-17 also promotes inflammation and is mainly expressed by Th17 cells in the intestinal tract and by innate lymphoid cells (ILCs) in the gut. MCP-1 is a chemokine produced by intraepithelial cells during IL-1β stimulation (Waterhouse et al., 2001) and is upregulated in patients with intestinal inflammation (Mazzucchelli et al., 1996; Reinecker et al., 1995). Our findings are in accordance to previous descriptions of intestinal inflammation.

After four weeks, the body weight recovered and the structure of the intestine was normalized. At the same time the profile of cytokines in HM-SM fed rats (Table 5) suggested a chronic gut inflammation. One of these, fractalkine, is a key cytokine found upregulated in patients with Crohn's disease. Also, neutralizing antibodies targeting fractalkine have significant therapeutic effects in animal colitis models (Kobayashi et al., 2007). MIP-2α, IP-10, LIX and RANTES were found elevated in our study (Table 5). They are all expressed by intestinal cells during inflammation and are strong mediators of neutrophil recruitment (Hoermannsperger et al., 2009; Kwon et al., 2005; Ohno et al., 1997; Ohtsuka et al., 2001; Zgraggen et al., 1997; Ajuebor et al., 2001). The higher levels of anti-inflammatory IL-10 in the rats fed with HM-SM could be explained as a possible compensatory mechanism to restore the barrier function during the inflammatory response (Kucharzik et al., 1998; Lee, 2015).

Leptin was significantly increased after four weeks exposure in the HM-SM group compared to the control animals. Although leptin is a hormone mostly known for acting in body weight and energy balance, leptin also has a role in immune regulatory as observed in animal models of multiple sclerosis (Sanna et al., 2003) and in regulation of intestinal autoimmunity in a mouse model (Siegmund et al., 2004). Also, VEGF was significantly higher in HM-SM fed rats compared to controls (Table 5), which is in line with inflammatory bowel disease in man and experimental models of colitis in animals (Ardelean et al., 2014; Scaldaferri et al., 2009).

This inflammatory process recognized both after one week and after four weeks may indicate a disturbed antigen presentation and peripheral tolerance in the intestinal tract. The peripheral tolerance mechanism is important to inactivate autoreactive lymphocytes and maintain immune homeostasis. An intestinal inflammation is probably not the only reason to a later development of autoimmune diseases, but could be a first step to start a progressive cascade of events leading loss of tolerance.

The effects of Maillard products on nutrition and health is insufficiently studied in humans and considering the wide use of food containing milk powder further studies are necessary (Pischetsrieder and Henle, 2012). High content of Maillard products in formula has been described previously and a 35 fold higher CML concentration had been measured in formula compared to breast milk (Dittrich et al., 2006). Formula fed infants became more insulin resistant than breast fed children but the mechanism was not clear (Klenovics et al., 2013). The eventually biological effects of the low content of lysine in skim milk powder is also a question that has to be further investigated. The possible association between skim milk powder and onset of autoimmunity like type 1 diabetes needs to be addressed.

6. Conclusion

In conclusion, we found that hot and humid storage conditions of skim milk powder significantly increased the level of Maillard products. Feeding young rats with skim milk powder containing high content of Maillard products caused an intestinal inflammation, a reduced weight gain and lymphoid organ development. We propose that these Maillard reaction products could alter the immunological barrier function in the gut and trigger the development of autoimmune diseases.

Conflicts of interest

The authors report no conflict of interest.

Acknowledgements

We thank Camilla Björklöv and Agnieszka Czopek for excellent help with the animal experiments and technical assistance and prof Thomas Wiebe for fruitful discussions. The Study was funded by Dr Per Håkanssons foundation to MH and MR, the Swedish diabetes child foundation to MH, the Swedish Medical Research Council to IS, funds from Lund University and Skåne University Hospital to MLO.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2018.12.015.

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