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N^{ε}-carboxymethyllysine in nutritional milk formulas for infants

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ABSTRACT

Production of infant formulas involves high temperature processing for microbiological safety. However, heat processes generate Advanced Glycation End-products (AGEs), including N^{ε}-carboxymethyllysine (CML) formed between lysine and lactose. Formulas manufactured from cow or goat milk, with or without whey adjustment, or hydrolysates of cow whey proteins, were tested for CML levels using a commercially available ELISA kit. CML concentrations ranged from 2 to 210 μg/g protein in formulas containing intact proteins. Median CML concentrations were up to 3-fold greater in formulas containing 60% whey protein compared with 20% whey protein, for both cow and goat formulas. Goat milk formulas contained 7 to 12-fold less CML than cow milk formulas. Formulas made from intact proteins contained lower CML compared to formulas using whey hydrolysates. Western immunoblotting techniques detected higher CML levels in whey proteins compared with casein. This study showed whey addition to infant formula significantly contributes to CML levels.

1. Introduction

Thermal processing of foods is necessary to ensure microbiological safety. However, these processing techniques can also introduce undesirable structural modifications in proteins. A common modification is a non-enzymatic reaction between reducing sugars and free amino groups, known as the Maillard reaction. Maillard reactions can reduce sensory and nutritional quality of products if not carefully managed (Mehta & Deeth, 2016) and result in formation of Advanced Glycation End-products (AGEs) in foods (Nguyen, Van der Fels-Klerx, & Van Boekel, 2014).

Consumption of AGEs from foods has been linked to inflammation, oxidative stress and insulin resistance (Clarke, Dordevic, Tan, Ryan, & Coughlan, 2016; Kellow & Savige, 2013; Uribarri et al., 2015; Van Puyvelde, Mets, Njemini, Beyer, & Bautmans, 2014). AGEs are also being investigated for their association with food allergy (Smith, Masilamani, Li, & Sampson, 2017). Thus, during thermal processing of foods there is a need to monitor and reduce the production of AGEs.

N^{ε}-carboxymethyllysine (CML), formed between reducing sugars and lysine, is often used as a marker of AGE formulation (Nguyen et al., 2014). Several studies have reported that CML levels can vary 10-fold, or more, between different types of milk-based formulations for infants (Birlouez-Aragon et al., 2004; Delatour et al., 2009; Dittrich et al., 2006; Meyer, Al-Diab, Vollmer, & Pischetsrieder, 2011; Šebeková, et al., 2008). Formulas based on hydrolysed proteins contain significantly higher amounts of CML than formulas with intact proteins (Delatour, et al., 2009; Dittrich, et al., 2006; Mericq et al., 2010; Šebeková, et al., 2008).

Bovine milk is used as a base for most infant formulas; goat milk is also considered a suitable source of protein in infant and follow on formulas (EFSA, 2012; Zhou et al., 2014). Originally, infant formulas were based on unmodified cow milk protein and, therefore, had a protein profile comprising 80% casein and 20% whey proteins (Fomon, 2001; Rudloff & Kunz, 1997). Whey-dominant infant formulas were then developed to more closely resemble the content of human milk with 40% casein and 60% whey protein and its amino acid profile (De Wit, 1998; Lönnerdal & Chen, 1990; Rudloff & Kunz, 1997). These formulas subsequently became the industry standard (Fomon, 2001). The present study investigated the impact on CML concentrations in formulations for infants when goat milk was used as an alternative protein source. In addition, the study investigated the impact on CML concentrations in formulas when whey, both intact and hydrolysed, was used in formulations.

2. Materials and methods

2.1. Samples for CML analysis

Powdered formulas used in the study were commercially available internationally. Three formula types containing goat milk proteins with

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20% whey, goat milk proteins with 60% whey or cow milk proteins with 60% whey were obtained from the Dairy Goat Co-operative (N.Z.) Ltd (Hamilton, New Zealand). Formulas containing cow milk proteins with 20% whey or hydrolysed whey cow milk proteins, manufactured by various companies, were purchased from supermarkets within New Zealand. All formulas contained added vegetable oils, lactose, vitamins and minerals to comply with international regulations for feeding 0–12 month old infants. According to the listed ingredients, the main carbohydrate in all formulas was lactose, typically at a ratio of 5–6 g lactose/g protein.

To assess the impact of long-term stability of CML levels in formulas, selected individual cans of goat milk-based formula were kept at ambient temperature for 2–80 months or exposed to cycling temperatures (10 °C to 50 °C, 24 h cycle) for 4 months before assay.

For analysis, formula samples were made up according to manufacturer's instructions, generally being 13 g powder to 90 ml water.

To assess formation of CML in fresh goat and cow milk, samples were sourced from milk-tankers supplying formula manufacturers in New Zealand. These samples consisted of milk pools collected from several farms. Milks were heated in 5 ml aliquots to 95 °C in an oil bath for 0, 1, 3, 10, 30, and 90 min. They were then cooled rapidly in an ice bath and 1 ml aliquots frozen for subsequent analysis.

2.2. CML analysis by ELISA

CML was measured using a commercial CML ELISA kit sourced from Echelon Biosciences (AGE:CML Competitive ELISA II, Cat# K3900s; Salt Lake City, Utah, USA) and used according to their instructions.

In brief, the following method was used. Eight standards were prepared from the stock 10 μg/ml standard provided in the kit (standard range 0.004 to 10.0 μg/ml). An aliquot (50 μl) of standard or sample was added to the CML detection plate, followed by 50 μl of CML detector. The plate was then incubated for 1 h at room temperature (RT) with gentle shaking. Following plate washing, a secondary detector solution was added (100 μl) and incubated for 0.5 h at RT with shaking. Following further washing, a substrate solution was added and the coloured end-product detected photometrically. The colour intensity was inversely proportional to the concentration of CML in the sample. A standard curve was produced by plotting standard concentration against optical density (OD) values. Concentration of the unknown sample was determined from the standard curve. Levels of CML in samples were expressed per g of total protein.

2.3. CML detection using immunoblotting with anti-CML antibody

Western blot techniques were used to identify which proteins in milk and formula samples had CML modifications, based on known molecular weight and migration patterns of the major milk proteins (Meyer et al., 2011).

Samples were separated by gel electrophoresis using 12% Bis-Tris Criterion XT pre-cast polyacrylamide gels (Cat# 345-0117, Bio-Rad Laboratories, Auckland, NZ) and XT MOPS running buffer (Cat# 161-0788, Bio-Rad). Samples were prepared to 3 mg/ml protein in XT sample buffer (Cat# 161-0324, Bio-Rad) with XT reducing agent (Cat # 161-0792, Bio-Rad) and boiled for 5 min. Samples (equivalent to 30 μg protein) and a molecular weight marker (Kaleidoscope Cat# 161-0324, Bio-Rad) were loaded into individual wells. The electrophoresis was conducted at 100 V (constant) for 140 min.

The gel was then equilibated for 10 min at RT in transfer buffer (Towbin buffer, Bio-Rad). Proteins were then electro-eluted to a Bio-Trace PVDF membrane (Pall Life Sciences, Pensacola, FL, USA) using a Criterion blotting system (Bio-Rad). The membrane was stained using Ponceau S and an image captured. The membrane was destained in MQ water then blocked for 30 min with rocking at RT, using 0.01 M Tris buffered saline containing 0.05% Tween-20, and 1% BSA (TBS-T-BSA). The membrane was then probed using rabbit anti-CML (1:3,000 in TBS-T-BSA, Cat# ab27684, Abcam, Melbourne, VIC, Australia) and incubated overnight with rocking at 4 °C. The membrane was washed with TBS-T. CML-specific bands were visualised following incubation for 1 h at RT with goat anti-rabbit IgG conjugated to horse radish peroxidase (1:15000 in TBS-T-BSA, Cat# P0448, Dako, DK-2600, Glostrup, Denmark) using a chemiluminescence detection system (ImageQuant LAS 4000, GE). Density data were analysed using Quantity One (Bio-Rad).

2.4. Statistical analysis

Results were expressed as median and range. A Kruskal-Wallis test was used to check for significance between formula types. *P* value was set at 5%. Mann–Whitney *U* test was used to test differences between selected formulas.

3. Results

3.1. CML levels in different formulas

CML concentrations in all formulas, as detemined by ELISA analysis, are shown in Table 1 with formulas grouped according to protein source and whey percentages. The inter assay coefficient of variation for the CML ELISA was 4.2% and 14.5% for two samples measured over three different assays with mean values of 3.6 and 59.2, respectively. In formulas containing intact proteins, CML concentrations ranged from 2 to 210 μg/g protein. CML concentrations for formulas manufactured using goat milk were consistently lower than formulas manufactured using cow milk ($P < 0.05$). CML in formulas using hydrolysates of cow whey protein were elevated compared to formula manufactured with intact proteins ($P < 0.05$). Furthermore, CML was elevated in formulas with 60% whey protein compared to formulas with 20% whey protein ($P < 0.05$), irrespective of whether these formulas contained goat or cow milk proteins.

3.2. Impact of storage on CML levels in goat milk formulas

Goat milk formulas were stored at ambient temperature for up to 80 months, with temperatures ranging between 17 and 27 °C over that time. Levels of CML in formulas were variable, ranging from 3.1 to 5.8 μg/g protein, with an overall average of 3.4 μg/g protein (Fig. 1). Over this storage period there was no trend for increased CML levels. In goat milk formula that had been subjected to the fluctuations of temperature cycling of 10–50 °C over 24 h for a period of 4 months, average CML levels were 13.4 μg/g protein. These levels were approximately 4-fold higher than long term ambient temperature storage.

3.3. Formation of CML in heated fresh milks

Table 2 shows the concentration of CML in fresh goat and cow milk samples that were subjected to 95 °C heating over 1–90 min. As expected, unheated fresh milk had low levels of CML. After 30 min heating at 95 °C, concentrations of CML in fresh goat milk were elevated 2-fold above time zero, compared to a 6-fold elevation for cow milk.

Table 1
Concentration of CML per g protein in formulas, as determined by ELISA analysis. Data are expressed as median and range.

Protein source	% whey	Number tested	CMLμg/g of protein	Range
Cow	60	18	95	51–211
Cow	20	7	49	17–121
Goat	60	11	13	10–30
Goat	20	12	4	2–12
Cow whey hydrolysate	100	4	233	42–900