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An infant formula toxicity and toxicokinetic feeding study on carrageenan in preweaning piglets with special attention to the immune system and gastrointestinal tract ☆

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ABSTRACT

A toxicity/toxicokinetic swine-adapted infant formula feeding study was conducted in Domestic Yorkshire Crossbred Swine from lactation day 3 for 28 consecutive days during the preweaning period at carrageenan concentrations of 0, 300, 1000 and 2250 ppm under GLP guidelines. This study extends the observations in newborn baboons (McGill et al., 1977) to piglets and evaluates additional parameters: organ weights, clinical chemistry, special gastrointestinal tract stains (toluidine blue, Periodic Acid–Schiff), plasma levels of carrageenan; and evaluation of potential immune system effects. Using validated methods, immunophenotyping of blood cell types (lymphocytes, monocytes, B cells, helper T cells, cytotoxic T cells, mature T cells), sandwich immunoassays for blood cytokine evaluations (IL-6, IL-8, IL1β, TNF-α), and immunohistochemical staining of the gut for IL-8 and TNF-α were conducted. No treatment-related adverse effects at any carrageenan concentration were found on any parameter. Glucosuria in a few animals was not considered treatment-related. The high dose in this study, equivalent to ~430 mg/kg/day, provides an adequate margin of exposure for human infants, as affirmed by JECFA and supports the safe use of carrageenan for infants ages 0–12 weeks and older and infants with special medical needs.

Abbreviations: ADI, acceptable daily intake; ANOVA, one-way analysis of variance; BW, body weight; CGN, carrageenan; cPs, centipoise; EMEA, European Medicines Agency; FAO, Food and Agriculture Organization of the United Nations; FDA, Food and Drug Administration; GIT, gastrointestinal tract; GLP, Good Laboratory Practice; H&E, hematoxylin and eosin; ICH, International Conference on Harmonisation; IL, interleukin cytokines; IM, intramuscular; IPCS, International Programme on Chemical Safety; JECFA, Joint FAO/WHO Expert Committee on Food Additives; kDa, kiloDaltons; K_3EDTA, potassium ethylene diamine tetraacetic acid; LD, lactation day; LMT, low molecular weight tail; MSE, mean square error; Mw, weight-average molecular weight; PAS, Periodic Acid–Schiff; PGN, poligeenan; NBF, neutral buffered formalin; SD, standard deviation; TNF-α, tumor necrosis factor alpha; USA, United States of America; WHO, World Health Organization.

☆The work included in this paper was partially presented at the 2014 Society of Toxicology Meeting in Phoenix, AZ in the following abstracts: Weiner M.L., et al. (2014) Safety of Carrageenan in Infant Formula: A 4-Week Toxicity Study in Preweaning Piglets. *Toxicol. Sci.* 138 (1) 341 (Abstract); Zeigler B., et al. (2014) Safety of Carrageenan in Infant Formula: A 4-Week Study of the Potential Immune System Effects. *Toxicol. Sci.* 138 (1), 342 (Abstract); Blakemore, W.R., et al. (2014) Safety of Carrageenan in Infant Formula: A 4-Week Toxicokinetic Evaluation in Preweaning Piglets. *Toxicol. Sci.* 138 (1) 343 (Abstract).

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1. Introduction

Carrageenan (CGN) is a high molecular weight polymer derived from certain *Rhodophyceae* (red seaweeds). Food-grade CGN is primarily used to bind water, promote gel formation, improve palatability, thicken and stabilize structure for food products by binding with protein. Chemically, CGN has a molecular backbone of repeating galactose units that may have sulfate groups attached with a weight average molecular weight (Mw) of 200–800 kDa. Regulatory agencies specify a viscosity of ≥5 cPs (centipoise), for food grade CGN.[1] This viscosity is equivalent to ~100–170 kDa Mw.

CGN has been widely used as a food additive for decades in numerous foods, including infant formula, and its safety is based on a large database of studies (Weiner, 2014). In Canada, CGN is approved at a maximum level of 0.05% in infant formula as a suspension agent for calcium salts in lactose-free infant formula, based on milk protein, and at a level of 0.1% in formula based on isolated amino acids and/or protein hydrolysates (Canada Gazette, 2004). This latter formula is used for infants with special medical needs, such as allergies to cow's milk, prematurity and health concerns.

CGN was reviewed by the United Nations' World Health Organization (WHO) Food and Agriculture Organization's (FAO) Joint Expert Committee on Food Additives (JECFA) in 2008. JECFA (2008) reaffirmed the Acceptable Daily Intake (ADI) for CGN as "Not Specified". However, JECFA raised[2] concerns regarding the safety of CGN for use in infant formula for infants under the age of 12 weeks. This concern was based on lack of data on potential effects of CGN ingestion on the neonatal immune system and gastrointestinal tract (GIT). Injection of CGN (intravenous or intraperitoneal) is known to cause inflammatory immune responses in animal models (see Weiner, 2014). This has raised questions whether orally administered carrageenan affects the immune system.

Earlier work by McGill et al. (1977) had shown CGN to be safe to infant baboons fed infant formula with up to 1220 ppm CGN (~432 mg/kg/day) from birth for 112 days. There were no effects on health, growth, blood, fecal or urine parameters or any histopathological effects on the GIT tissues (McGill et al., 1977). McGill et al. (1977) did not specifically look at immune system effects or absorption of CGN. JECFA (2008) considered McGill et al. (1977) to lack a full evaluation of the GIT because no special stains, such as toluidine blue, were used to visualize mucosal mast cells, indicators of immune system responses, and because potential CGN absorption was not evaluated.

The present study in preweaning piglets sought to evaluate (1) the potential absorption of CGN in the GIT, (2) the presence of CGN in serum following ingestion of swine-adapted infant formula containing CGN via toxicokinetic analysis and (3) to assess the impact of CGN on the developing immune system. No regulatory study guidelines for the evaluation of constituents in infant formula exist (International Programme on Chemical Safety (IPCS), 2009). The preweaning piglet chosen for this study is considered a good model to evaluate nutritional status, growth and development and basic physiology, including the GIT and immune system, which have been shown to resemble humans (Barrow, 2012; Guilloteau et al., 2010; Helm et al., 2007; Odel et al., 2014; Penninks et al., 2012). The dosing period (0–28 days) was chosen because it corresponds to the preweaning period and is equivalent to about the first 23 months of life for a human (Barrow, 2012; Buelke-Sam, 2002). Toluidine blue was used to visualize mucosal mast cells and Periodic Acid–Schiff (PAS) reagent was used to stain the goblet cells of the jejunum for evaluation of possible toxicological effects. A detailed immune system evaluation, including immunophenotyping, cytokine evaluation and immunohistochemistry of the GIT, was conducted. This study is the first time that CGN has been evaluated for safety in infant formula using new technologies for immune system parameters; detailed GIT staining and measurement of CGN plasma absorption in an established animal model for the preweaning period. This study provides a thorough evaluation of this major food additive for its use in infant formula under Good Laboratory Practice (GLP) guidelines and also demonstrates the value of the preweaning piglet model for safety evaluation of food additives used in infant formula.

Table 1
Study design for the 28-day study.

Group number[a]	Dose volume: mL/kg bw/day	CGN dose concentration: ppm	Number of males per group	Number of females per group
Main study				
1	500	0	6	6
2	500	300	6	6
3	500	1000	6	6
4	500	2250	6	6
Toxicokinetic study				
5	500	0	3	3
6	500	300	3	3
7	500	1000	3	3
8	500	2250	3	3

[a] Corresponding main study and toxicokinetic groups were combined to facilitate data interpretation. All animals were dosed from Lactation Day 2 (Study Day 1) for 28 consecutive days.

2. Materials and methods

The study was conducted by MPI Research, Mattawan, MI, USA in accordance with GLP Regulations[3] and was based on current guidelines and guidance documents for preclinical juvenile studies for drugs (Buelke-Sam, 2002; European Medicines Agency (EMEA), 2008; International Conference on Harmonisation (ICH) Harmonised Tripartite Guidelines, 2010; United Stated Food and Drug Administration (FDA), 2000; United States Food and Drug Administration (FDA) Center for Drug Evaluation and Research (CDER), 2006). Table 1 outlines the study design for this study. Table 2 summarizes the frequency of animal observations (mortality, morbidity, clinical observations, body weight, and feed consumption) and types and frequency of all parameters evaluated, the times of collection and animal groups studied. A separate set of animals was designated for the toxicokinetic phase to avoid excessive handling of any particular animal, since piglets are prone to stress when handled too much.

2.1. Test material characterization

The test material sample of κ/λ-CGN (FMC Lot. 90303011) was fully characterized for molecular weight (Mw), percentage of the Mw below 50 kDa, known as the Low Molecular Weight Tail (LMT) as per European requirements, and met interna-

[1] Commission Regulation. (2012), 2031/2012 of 9 March 2012. Official Journal of the European Union L83 55 L 83/140 – L 83/141; Food and Agriculture Organization of the United Nations. (2007) Combined Compendium of Food Additive Specifications; CGN – 68th session of JECFA, 2007; Food Chemicals Codex. (2013). FCC Monographs, Edition 8, Supplement 2. 219–228; Japan Food Additives Association (2009) Japan's Specifications for Food Additives, Eighth Edition, the Ministry of Health and Welfare, 344.

[2] "The JECFA Secretariat clarified that it was not the existence of data raising any specific concerns, rather the lack of data on the potential impact of carrageenan on the immature gastrointestinal and immune systems which led to the conservative conclusion. As a general principle, JECFA considered that the ADI was not applicable to infants under the age of 12 weeks, in the absence of specific data to demonstrate the safety of these substances for this age group." (Codex Committee on Food Additives, 2008).

[3] United States Food and Drug Administration (FDA) Good Laboratory Practice (GLP). Regulations, 21 CFR Part 58; Organisation for Economic Co-operation and Development (OECD) 2002. Consensus Document "The Application of the Organisation for Economic Co-operation and Development Principles of GLP to the Organisation and Management of Multi-Site Studies" ENV/JM/MONO (2002); Japanese Ministry of Health, Labour and Welfare (MHLW) Good Laboratory Practice Standards Ordinance No. 21, March 26, 1997.