Review Article

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Gastrointestinal Tract Digestion and Carrageenan:
How Misconceptions have influenced the
Understanding of Carrageenan SafetyMcKim JM^{1*}, Willoughby JA¹, Blakemore WR² and Weiner ML³¹IONTOX, LLC, Kalamazoo, MI, USA²Celtic Colloids Inc., Topsham, ME, USA³TOXpertise, LLC, Princeton, NJ, USA***Correspondence:** James M McKim, IONTOX, LLC, Kalamazoo, MI, USA, E-mail: jmckim@iontox.com

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Abstract

Carrageenan (CGN) is a naturally occurring fiber isolated from various species of red seaweeds (*class Rhodophyceae*). It has been safely consumed for hundreds of years and today is approved for use in the food industry as a food additive by regulatory agencies around the world. Unfortunately, some researchers have used misleading and incorrect interpretations of early studies to suggest that food-grade CGN (*Mw* = 200,000 to 800,000 Da.) is not safe for human consumption. These researchers reference studies conducted with the acid-hydrolysis products of CGN, which include degraded carrageenan (d-CGN; *Mw* = 20,000 to 40,000 Da.) and poligeenan (PGN; *Mw* = 10,000 to 20,000 Da.), as evidence of the potential adverse health effects of high *Mw* CGN. While PGN and d-CGN have been shown to have adverse effects *in vivo*, the same is not true for CGN. Both PGN and d-CGN are made in the laboratory under harsh conditions of low *pH* (< 2.0) and high temperature (80°C), and have distinctly different physical, chemical and toxicological profiles than CGN. Studies have shown that d-CGN and PGN are not formed *in vivo* after ingesting CGN, nor are d-CGN and PGN used as food additives. Yet these differences between d-CGN/PGN and CGN are either not understood or are ignored by many authors in the published literature and the adverse effects observed with d-CGN and PGN are being used to question the safety of CGN. This has caused significant confusion in the literature and with regulators. Here we review the physical, chemical and toxicological properties of CGN, d-CGN and PGN. We then review the ingestion of CGN, how the formation of d-CGN and PGN does not occur *in vivo*. Finally, we discuss recent review providing a prime example of how some publications use misinformation to suggest CGN is unsafe for ingestion.

Keywords: Carrageenan, Poligeenan, Degraded carrageenan, Inflammation, Digestion**Abbreviations:** CGN: Carrageenan; d-CGN: degraded Carrageenan; PGN: Poligeenan; *Mw*: Average Molecular Weight of a Polydisperse sample; *Mn*: Number Average Molecular Weight (total weight of the sample divided by the number of molecules in the sample)

Introduction

Carrageenan (CGN) is a naturally occurring high molecular weight sulfated polysaccharide. It is a linear molecule consisting of galactose sugars, linked together with alternating α -1,3 and β -1,4-glycosidic linkages, with

varying degrees of sulfate groups attached. There are three major forms of carrageenan; lambda- (λ), kappa- (κ) and iota- (ι) CGN. The primary differences between these three forms are the conformation of the galactose sugars and the level and location of sulfate groups; properties which impart different physical characteristics

when mixed with water or food protein [1]. There are no significant differences among the three forms of CGN in terms of toxicological effects and human safety in foods [2]. Food-grade CGN has an average molecular weight (*Mw*) of 200,000 to 800,000 Daltons (Da) and is made up of primarily κ -CGN and λ -CGN though it can also contain ι -CGN as well, depending on the desired gelling or stabilizing effects [3,4]. This review will focus primarily on food-grade CGN and the long-standing confusion with nomenclature. This confusion results in researchers applying adverse toxicological effects only observed with lower molecular weight materials (d-CGN and PGN) to food-grade CGN, where no such adverse effects have been observed.

Food-grade CGN is safe for human consumption and is widely used as a stabilizer, emulsifier, gelling agent, and thickener that also improve texture and mouth-feel in numerous dairy products like ice cream, non-dairy products such as soy milk, lunch meats, and even infant formula. Recently the Joint FAO/WHO Expert Committee on Food Additives (JECFA) deemed food-grade CGN safe for use in infant formulas, stating that “the use of carrageenan in infant formula or formula for special medical purposes at concentrations up to 1000 mg/L is not of concern” as well as mentioning that they “took account of the previous toxicological database on carrageenan, which did not indicate other toxicological concerns” [5]. JECFA added that infant formula containing 300 mg/L CGN (standard infant formula) results in a total exposure of 37-67 mg/kg/day, assuming 100% of caloric intake is CGN containing formula [5]. Though 1000 mg/L formula has a higher total exposure, these levels of CGN in infant formula are only used in special medical formulations where the infant would already be under the care of a doctor [5]. Regulatory agencies around the world, including those in the United States, Europe, China, Japan and Brazil, have found CGN safe for use as a food additive. The US Food and Drug Administration (FDA) has food-grade CGN listed as “generally recognized as safe,” or GRAS [6] and the WHO JECFA has placed carrageenan in the best, safest possible category for a food additive with an acceptable daily intake (ADI) of “not specified” [7]. Additionally, the European Food Safety Authority (EFSA) put an ADI of 75 mg/kg while the International Agency for Research on Cancer (IARC) has stated that carrageenan is non-carcinogenic [8-10]. Recently, EFSA reviewed CGN as a food additive and maintained the existing ADI of 75 mg/kg [11]. This is the most stringent ADI put forth by any regulatory body. To put this in perspective, an average sized male weighing 70 kg would have to ingest 5.25

grams of CGN to meet this ADI. This is unlikely given the average ingestion of CGN in a Western diet is 250 mg/day [12]. Some individuals may ingest as much as 2-4 grams of CGN per day [13]. However, this is still below the stringent regulatory ADI set forth by EFSA of 75 mg/kg for the average adult.

The acid hydrolysis of CGN results in molecules with smaller *Mw*, known as degraded-CGN (d-CGN; *Mw* = 20,000 to 40,000 Da.) and poligeenan (PGN; *Mw* = 10,000 to 20,000 Da.). This process is performed in a laboratory setting under extreme conditions, with temperatures at or greater than 80°C in acid with a *pH* of 1-2 [4].

Food-grade CGN does have a lower molecular weight fraction that is referred to as the “low molecular weight tail” or LMT. The molecular weights of the molecules in the LMT are between 20,000-50,000 Da., typically with less than 5% of the entire CGN sample being lower than 50,000 Da [14,15]. Though the presence of this LMT is unavoidable, as it is part of the incomplete natural biological synthesis of CGN by seaweed during the normal life cycle prior to harvest, researchers for the European Commission’s Scientific Committee on Food (SCF) estimated that the LMT could be present at levels up to 5%, though none was specifically detected at that time [16]. To put this in perspective, food-grade CGN is typically used as a food additive at concentrations of 0.1 - 2%. If we assume every item ingested in a day contains the maximum amount of CGN as food additive (2%) and that the LMT portion of all the CGN ingested is 5%, then the LMT ingested comes to 0.1% of the total food ingested each day. As it is unlikely that every part of every meal consumed contains CGN, the percentage of LMT ingested is likely far less than 0.1% of the total ingested food each day. This is important to note as the known adverse toxicological effects observed in effects due to d-CGN and PGN *in vivo* are in response to much higher concentrations in the range of 0.5% - 5% and ingested continually for weeks, even months, and usually provided in drinking water, not bound to food protein [17-22]. These levels of d-CGN and PGN (0.5% - 5%) are orders of magnitude above the maximal amounts expected in the human diet. Thus, the low level of the LMT fraction of CGN is not of toxicological significance.

Human consumption of food-grade CGN as a food additive is completely safe with no demonstrated adverse effects. PGN and d-CGN, on the other hand, have long been shown to cause inflammation and lesions in the GI tract in animal studies, and to cause adverse changes in

the gut microbiome [23]. However, PGN and d-CGN are not used in the food industry, nor have they ever been used in the food industry. The digestion, or lack thereof, of CGN as a food additive, from exposure in food to nearly complete excretion in feces, will be discussed and it will be shown that CGN is not absorbed or degraded in the gastrointestinal tract. Finally, the discussion will show that when high *Mw* CGN was used in well-designed safety studies, no adverse effects were recorded and these findings are in agreement with the regulatory agencies around the world in stating that food-grade CGN is safe for human consumption.

Confusion in CGN safety

Despite the findings by regulatory bodies across the globe, almost unanimously supporting the safety of food-grade CGN, there are researchers that seem to be confused in regards to the published results showing that CGN is safe for human consumption (reviewed in McKim et al., 2018). These researchers falsely suggest that food-grade CGN can cause intestinal inflammation and lesions similar to those observed with Crohn's disease, ulcerative colitis and irritable bowel disease. One such report propagating this false information is a recent review by Martino et al. (2017) [24]. The authors of this review conclude that "Carrageenan administered in animal models consistently results in intestinal ulcerations with histopathological features similar to human IBD." However, this conclusion is untrue and appears to be based on confusion surrounding CGN nomenclature and chemistry. Martino et al. (2017) [24] cited 23 total references in the section of the review titled "The Role of Carrageenan in Intestinal Inflammation in Animal Models" (Table 1). Nine of the referenced studies used d-CGN, not food-grade CGN (Table 1) while another nine of the referenced studies have no mention of either CGN or d-CGN anywhere in the study (Table 1). Only five of the 23 cited studies that Martino et al. (2017) [24] used to suggest CGN induces intestinal inflammation actually used CGN as a test material (Table 1). One study by Onderdonk [25] (ref 26 in the Martino et al. review) stated that CGN was used, but it did not identify the type (λ , κ etc.) nor whether it was food-grade or not. It is also important to note that in the four other publications by Onderdonk cited in Martino et al. review (refs 21, 26, 27 and 30 in the Martino et al. review) [26-29] and in other publications by Onderdonk's group [30,31] the authors stated they were using CGN but when reviewing the Methods, they in fact used d-CGN. It is quite likely that in the single study that stated they used CGN, [25] they in fact used d-CGN and just did not appropriately mention it

in the Methods, particularly as it appears to be a follow-up study to a previous manuscript using d-CGN [28]. Therefore, only 4 of the 23 studies cited by Martino et al. [24] in the section titled "The Role of Carrageenan in Intestinal Inflammation in Animal Models" most certainly used CGN. Of these four, three show no adverse effects with CGN administration alone (refs 34, 35 and 36 in the Martino et al. review) [32-34]. The papers by Wei et al. [32] and Wu et al. [33] only show that in rodents, continual exposure to high levels of κ -CGN may exacerbate the ulcerations induced by other mechanisms. In the remaining four articles cited that actually use CGN, only one, Weiner et al. [34] used food-grade CGN. In fact, one study cited by Martino et al. (2017) [24] that used CGN, not PGN or d-CGN, actually shows some health benefits and the anti-inflammatory potential of λ -CGN (ref 33 in the Martino et al. review) [35]. Therefore, the authors are using studies that showed adverse effects observed with PGN and d-CGN, and claiming those results also apply to CGN. In addition, only a single study cited by Martino et al. (2017) [24] used food-grade CGN, and that study showed no adverse effects [34]. As PGN and d-CGN are very different molecules than CGN, both in *Mw* as well as toxicological effects, it is important not to confuse them.

This misinformation in the Martino et al. review [24] and other studies may be primarily due to problems in nomenclature in the early work with CGN published in the mid-20th century. Authors often referred to d-CGN as CGN throughout these early publications and the only way to discern whether the authors used CGN or d-CGN in a study is by reviewing the materials and methods used to produce the test sample and to discern if the CGN was hydrolyzed to low *Mw* forms or not. Another area of confusion is the route of administration. As a food additive, food-grade CGN provides excellent gelling, thickening and emulsifying properties, and these effects are due to the ability of food-grade CGN to tightly bind to food proteins. Therefore, the human GI tract rarely sees CGN in its native unbound or free form when used as a food additive. However, many *in vivo* studies on food-grade CGN are performed with CGN provided either in drinking water or in a pill form, not tightly bound to food protein. Any adverse effects observed in these studies cannot be properly correlated to human ingestion of CGN bound to food protein.

Digestion in the oral cavity

Digestion and breakdown of food begins in the oral cavity. Chewing (mastication) of the food physically breaks down the food into smaller, more manageable pieces in

order to be swallowed, while saliva moistens the food to make it easier to swallow and starts the digestive process. In addition to moistening the food, saliva also contains a number of proteins and enzymes. The primary and the most well-known of these enzymes is alpha-amylase. Alpha-amylase breaks down amylose and amylopectin polysaccharides (starches), as well as glycogen, in food. These starches are broken down into smaller and less complex sugars [36]. Alpha-amylase acts on the α -1,4 glycosidic linkages in polysaccharides, hydrolyzing these bonds. As CGN is comprised of α -1,3 and β -1,4-glycosidic linkages, and does not contain α -1,4 glycosidic linkages, this enzyme is incapable of breaking down CGN into d-CGN or PGN.

In addition to alpha-amylase, saliva contains a number of other enzymes, including lysozyme, salivary lipase, lactoferrin, salivary peroxidase, phosphatases, peptidases, and salivary kallikrein [37]. Most of these enzymes aid in maintenance of oral health by providing innate immunity; killing bacteria, fungi and/or viruses and/or preventing their propagation [38]. However, salivary peptidases aid in the digestion of large proteins found in food into smaller peptides that are easier for the peptidases found in the stomach to digest. These enzymes cleave the peptide bonds between amino acids using either water or another amino acid residue to perform a nucleophilic attack. Given that CGN does not contain amino acids, or peptide bonds, these peptidases do not digest CGN into smaller molecules. Salivary lipase, also called lingual lipase, hydrolyzes long-chain fatty triglycerides into partial glycerides and free fatty acids [39]. In fact, up to one third of fatty acid digestion is performed by lingual lipase [40,41]. However, this digestion does not occur in the oral cavity, as the lingual lipase only hydrolyzes lipids in an acidic environment (pH 3.0 - 6.0) which is not found in the oral cavity [39,42,43]. As CGN is not a fatty acid, lingual lipase will not hydrolyze CGN into d-CGN. CGN hydrolysis does not occur in the oral cavity as the pH is not low enough and there are no enzymes present in saliva that will hydrolyze the glycosidic linkages of CGN, as just discussed above.

Digestion in the stomach

Once a bolus of food has been swallowed, it travels down the esophagus and into the stomach. It has been assumed that if acid hydrolysis of CGN was possible, and then the stomach would be the most likely location for this to occur due to the high acidity and digestive enzymes present in the stomach. In fact, there have been a few studies that have demonstrated that CGN degradation

happens, at least to some degree, in the GI tract after ingestion. An early study by Pittman et al. [44] analyzed distribution of various hydrolyzed CGN preparations of differing Mws ranging from $M_n = 5000 Da$ to $M_n = 314,000 Da$, including fecal samples, from monkeys, guinea pigs and rats that were fed various preparations of this partially hydrolyzed CGN in drinking water. The authors then used gel electrophoresis to measure both the standard sample (prior to administration to animals) as well as the d-CGN isolated from fecal samples. They noted that in most instances, the electrophoretic mobility (relative to a dye used) was greater in the fecal samples than it was in the standard samples. This result was used by Pittman et al. [44] to suggest that some sort of degradation occurred in the GI tract. However, the authors also noted that only the highest M_n samples were observed to have a change in the dosed sample vs. the fecal samples, and it is unlikely that only the highest M_n samples underwent some form of digestion or enzymatic degradation while the other lower M_n samples did not. This is not a surprising result and does not suggest hydrolysis or digestion of CGN occurred. The free CGN in feces would easily interact with any residual protein present in the feces. The higher the Mw of both the protein and the CGN, the stronger the interaction. This would make it harder to quantitatively isolate the CGN [45,46]. Therefore, it would not be surprising for the Mw of the CGN extracted from feces to be slightly lower than the original test material. In addition, Pittman et al. [44] admitted to substantial run to run variations in the fecal analysis. These issues with run to run variation in attempting to accurately assess the size and Polydisperse nature of CGN and d-CGN, particularly, still exist today. It is also important to note that all samples fed to animals had undergone some form of acid hydrolysis prior to administration, thus the results observed in this study do not necessarily correlate well to the ingestion of unhydrolyzed food-grade CGN.

In another pair of studies, food-grade CGN was dissolved in simulated gastric fluid (pH 1.0 - 1.9) for up to 6 hours at $37^\circ C$ [47,48]. It is important to note that these studies by Ekstrom et al. assessed pure food-grade CGN dissolved in water, not bound to food proteins as is the case for the majority of dietary CGN that is ingested [47,48]. That being said, in the first study, κ -CGN and ι -CGN were incubated at pH 1.0 for 6 hours at $37^\circ C$, which resulted in substantial degradation of the samples to smaller Mw fragments, with κ -CGN being much more susceptible to hydrolysis than ι -CGN, with most of the κ -CGN having a $Mw < 25,000 Da$ [47]. In a follow-up study, a 2 hour incubation of food-grade κ -CGN in simulated gastric fluid (pH 1.2) resulted in ~90% of the CGN in the

sample having a mass of $<100,000$ Da and 25% of the sample having a mass of $\sim 20,000$ Da, while at pH 1.9, the rate of degradation was lower (65% of the carrageenan had a mass of $\sim 100,000$ Da and 10% had a mass of $<20,000$ Da after 2 hours) [48]. These results initially appear to suggest that food-grade CGN could easily be converted at physiological temperatures in the human gut, particularly when it is well known the pH of the human stomach is quite low due to the presence of hydrochloric acid (HCl). However, additional work later conducted Capron et al. found drastically different results than Ekstrom et al. [49]. Using a simulated gastric fluid, Capron et al. showed that only 10% of κ -CGN is hydrolyzed to a mass $<100,000$ Da, while ι -CGN is even more stable than κ -CGN [49].

In a fasted state, the average pH of the human stomach is approximately $2.16 \pm$ for men and $2.79 \pm$ for women, [50] though it has also been reported to be a slightly higher ($pH = 2.9 \pm 0.33$) [51] and a slightly lower ($pH = 1.3 - 1.7$) [52,53]. Regardless of the fasting pH of the stomach, immediately after ingesting a meal, the pH in the human stomach increases to 4.5 - 6.7 range [52-54]. The pH then slowly begins to drop over a time period of 2-4 hours down to the fasted pH levels, with the rate of return being considerably slower (in the 2-hour range), in younger and healthier individuals [52-54]. Though the data varies depending on meal size and methodologies used to assess GI emptying times, it is generally accepted that the half-emptying time for most people is 2.5- 3 hours and that the full-emptying time is 4-5 hours [55-57]. These studies show that even if CGN is ingested, it immediately encounters an environment in the pH range of 4.5 - 6.7. CGN has not been shown to be significantly degraded in this range of pH . Though the pH of the stomach eventually returns to ~ 2.0 after 2-4 hours post meal, the half emptying times are $\sim 2.5 - 3$ hours, and full emptying times are $\sim 4-5$ hours. Therefore, most of the ingested CGN will not encounter the pH ranges or extended times that have been reported to hydrolyze CGN in vitro or under the conditions used to manufacture d-CGN in the laboratory. This is of particular relevance to the rodent studies as well, since the pH of stomachs in the rat and mouse when fasted are around pH 4.0 and does not change much after feeding [58].

Though acid hydrolysis of CGN in human stomach does not occur, nor has it ever been definitively proven to occur, there are a number of digestive enzymes in the stomach so the potential to cleave higher molecular weight CGN into lower molecular weight d-CGN or PGN should be addressed. The primary digestive enzymes

present in the stomach after a meal are pepsin, salivary lipase (present in saliva coating the food) and the gastric lipase. Both gastric and salivary lipase have a pH optimum range of 3.5-6.43, [59] and hydrolyze ingested fats into partial glycerides and free fatty acids [39,59]. As stated earlier, CGN does not contain any long-chain fatty triglycerides, thus, neither the gastric lipase nor the salivary lipase present in the stomach will act to hydrolyze CGN to d-CGN.

Salivary amylase is another enzyme present in the stomach after a meal that acts to break down carbohydrates. However, as CGN does not contain the α -1,4 glycosidic linkages, salivary amylase will not hydrolyze CGN to d-CGN in the oral cavity or stomach. Finally, pepsin is one of the prominent digestive enzymes in the stomach [60,61]. It digests proteins into smaller peptides and amino acids, with an optimal activity pH range of 2.0 - 4.5 [60,61]. However, as pepsin only acts on peptide bonds, not the α -1,3 and β -1,4-glycosidic linkages found in CGN, it is incapable of hydrolyzing CGN [60,61].

Digestion in the small intestine

After the digestive process is completed in the stomach, the resulting slurry, known as chyme, then passes through the pylorus and on to the duodenum (start of the small intestine) via peristaltic waves [62]. The small intestine is made up of three regions, the duodenum, the jejunum, and the ileum. The remaining digestive processes occur in the duodenum, where the chyme mixes with bile from the liver/gall bladder and pancreatic juice. The digestion is completed via several enzymes, and the resulting nutrients are absorbed through the wall of the jejunum and ileum. Though the pH of the duodenum, jejunum, ileum and large intestine are important for proper functions, it never exists at the levels needed for hydrolysis of CGN ($pH < 2.0$). The median pH in the duodenum in the fasted state of healthy human subjects is 6.1 (range of 5.8 - 6.7) while the media pH increases slightly after a meal to 6.3 (range of 6.0 - 6.7) [52,53]. The pH in the lumen of the small intestines gradually increases from pH 6 in the proximal small intestine to about pH 7.4 in the terminal ileum. The pH in the caecum drops to about 5.7, but again gradually increases to about pH 6.7 in the rectum [63]. This means there is no chance for acid hydrolysis of CGN to occur in the small intestine. The only way conversion of CGN to d-CGN would occur in the small or large intestines is for enzymatic hydrolysis to occur.

There are a number of enzymes present in the duodenum that function to complete the digestive

process. The digestive enzymes present in the duodenum are secreted by the pancreas, which releases trypsin, chymotrypsin, pancreatic lipase, pancreatic amylase, and nucleosidases (RNase and DNase) into the duodenum via the pancreatic duct. Though trypsin and chymotrypsin only act on peptide bonds, and, therefore, do not act to hydrolyze CGN to d-CGN, they likely result in separation of the food-grade CGN from the protein to which it was tightly bound. Pancreatic lipase and pancreatic amylase function very similarly to salivary lipase and salivary amylase, respectively. Pancreatic lipase digests fatty while pancreatic amylase digests carbohydrates, primarily starches, which were not digested by salivary amylase. However, since pancreatic lipase hydrolyzes long-chain fatty triglycerides into partial glycerides and free fatty acids, and CGN does not contain fatty acids, lipase will not hydrolyze CGN. The same is true for pancreatic amylase, which cleaves α -1,4-glycosidic linkages and not the α -1,3 and β -1,4-glycosidic linkages found in CGN. Therefore, pancreatic amylase will not hydrolyze CGN to d-CGN. The same is true for the RNases and DNases, which hydrolyze phosphodiester bonds. As CGN has no phosphodiester bonds, these enzymes will not hydrolyze CGN. Once the digested meal has passed through the duodenum, it moves into the jejunum and then into the ileum, where the absorption of the digested nutrients occurs. Studies have shown that as the *Mw* of ingested CGN or d-CGN increases, the absorption decreases [44]. Therefore, virtually all the food-grade CGN (*Mw* = 200,000 to 800,000 Da.) that is ingested during any given meal will remain intact (unhydrolyzed) and will pass through the GI tract unaltered [14,45,46].

Discussion

Food-grade CGN has been widely used for decades and is safe for human consumption. CGN is commonly found in dairy products, dairy substitutes (such as almond milk and soy milk), deli meats, nutritional supplements, beverages, and infant formula. The three primary forms of CGN used in food-grade CGN (λ , κ and ι) exhibit no major differences among them in terms of toxicological effects and human safety in foods [2]. However, despite clear evidence regarding the safety of food-grade CGN, there has been significant confusion in the scientific literature and the public realm between the high molecular weight food additive CGN (*Mw* = 200,000 - 800,000 Da.) and the CGN acid hydrolysis products d-CGN (*Mw* = 20,000 to 40,000 Da.) and PGN (*Mw* = 10,000-20,000 Da.). PGN and d-CGN clearly have adverse toxicological effects when administered orally to Guinea pigs, monkeys, rats, and mice [17-22].

In early works PGN was often incorrectly called “degraded carrageenan” (d-CGN) and some early studies often referred to d-CGN as CGN, adding to the confusion. This confusion has resulted in some innocent, but incorrect conclusions in research papers and review, therefore proper study design is of utmost importance [64,65]. Over the past decade or more several researchers have attempted to set the record straight regarding issues of size and nomenclature [23,64-69]. Despite these publications, the confusion regarding nomenclature has fueled misinterpretations of toxicological data by researchers [24,70-72] and by consumer groups [73,74]. In fact, the mini-review by Martino et al. (2017) [24] is an example of misinformation resulting in incorrect conclusions by the authors despite the fact that the regulatory authorities have stated that CGN used as a food additive is completely safe for the general population [5-10,75].

The first sentence under the subheading the Role of Carrageenan in Intestinal Inflammation in Animal Models states “It has been demonstrated that when guinea pigs are supplied with degraded carrageenan in their drinking water, ulcerations develop in 100% of the animals in their large intestine by the end of a 30-day period (16)” [24]. The authors admit that the study they are referencing used d-CGN, but blamed CGN for the adverse effects observed in the references of the study (reference #16 in the Martino et al. review is reference # 17 in this review) [17]. The authors go on to state that the “... lesions induced by carrageenan in the guinea pigs’ large bowel resemble features of human UC (10). Carrageenan has also produced ulcerative lesions in rabbits, mice, and rats that were associated with weight loss, anemia, diarrhea, visible or occult blood, and sometimes mucus in the feces (17)” [24]. Both of the references cited used degraded carrageenan, not food-grade CGN or undegraded CGN (reference #10 and #17 in Martino et al. references #76 and #77 in this review) [76,77]. This point is important because, the authors are misleading readers by incorrectly referring to d-CGN or PGN as food-grade CGN (Table 1).

Many of the recent studies that do purport to show adverse effects of CGN were performed in vitro systems. In fact, it has been suggested that CGN induces inflammation in the colon of animals and humans by Toll-like receptor 4 (TLR4) inductions of the BCL10 and NF-kappa B signal transduction pathways resulting in an up regulation of interleukin 8 (IL-8) secretion [78-80]. However, this data cannot be reproduced. For example, CGN does not bind TLR4, and it does not induce inflammatory signaling in

Table 1: This table contains all of the [23] references from the section of Martino et al. (2017) [1] titled “The Role of Carrageenan in Intestinal Inflammation in Animal Models.” Acid-hydrolyzed degraded carrageenan is denoted as d-CGN. Lambda-carrageenan is denoted as λ -CGN. Kappa-carrageenan is denoted as κ -CGN. Food Grade Carrageenan is denoted as FG-CGN. Studies that utilized neither d-CGN, λ -CGN, κ -CGN nor FG-CGN are denoted with an X.

Reference Number in Martino et al. (2017) [1]	Reference Number in this Article	Material Used or Discussed	Result
[10]	[76]	d-CGN	2-5% d-CGN administered to guinea pigs in drinking water for 25-40 days resulted in intestinal lesions in almost all animals. Lesions were similar to ulcerative colitis in humans.
[11]	[84]	d-CGN	Sprague-Dawley administered 2% d-CGN in drinking water for up to 8 weeks. Intestinal lesions (similar to ulcerative colitis in humans) observed. Splenocyte proliferation was diminished in d-CGN rats, but was restored when cultured in the presence of a nitric oxide synthase inhibitor.
[16]	[18]	d-CGN	5% d-CGN administered to guinea pigs in drinking water resulted in caecum, colon and rectum in 100% of animals by Day 30. Lesions were similar to ulcerative colitis in humans.
[17]	[77]	d-CGN	Review of d-CGN induction of lesions in both guinea pigs and rabbits.
[21]	[26]	d-CGN	5% d-CGN co-administered with antibiotics (gentamicin and clindamycin) to guinea pigs in drinking water for 14 days. Lesions were reduced in the presence of clindamycin, but not gentamicin, suggesting the makeup of the intestinal microflora plays a significant role in ulceration.
[22]	[27]	d-CGN	5% d-CGN co-administered with antibiotics (metronidazole, gentamicin and sulfamethoxazole-trimethoprim) to guinea pigs in drinking water for 21 days. With d-CGN alone, ulcerations formed with a concurrent increase in coliform counts. Gentamicin and sulfamethoxazole-trimethoprim reduced coliform counts, but had no effect on the rate or severity of lesions. Metronidazole protected most animals from ulcerations and reduced anaerobe levels but not coliform levels.
[27]	[28]	d-CGN	Guinea pigs were either non-immunized or immunized with <i>B. vulgatus</i> prior to administration of 5% d-CGN in drinking water. The guinea pigs were also fed either standard feed or feed with viable <i>B. vulgatus</i> . All animals receiving d-CGN resulted in ulcerations, however, the immune group developed more severe lesions, with the most severe lesions detected in <i>B. Vulgatus</i> immune animals fed both d-CGN and daily doses of viable <i>B. vulgatus</i> .
[30]	[29]	d-CGN	Guinea pigs, immunized with numerous different <i>B. vulgatus</i> antigens, were then administered 5% d-CGN drinking water as well as fed viable <i>B. Vulgatus</i> . Though lesions developed in all animals, the severity was dependent on the strain of <i>B. vulgatus</i> and the antigen used for immunizations.
[31]	[85]	d-CGN	Rabbits were administered 1% d-CGN in drinking water for up to 9 weeks. Lesions were rapidly induced and were characterized by a marked reduction in the proportion of the caecal epithelial glycoprotein sialic acids that were neuraminidase-resistant and/or substituted in the side chain. This suggests d-CGN may reduce and/or alter constituents of the mucin layer that coats and protects the gastro-intestinal epithelium.
[18]	[86]	X	No discussion of d-CGN or CGN

[19]	[87]	X	No discussion of d-CGN or CGN
[20]	[88]	X	No discussion of d-CGN or CGN
[23]	[89]	X	No discussion of d-CGN or CGN
[24]	[90]	X	No discussion of d-CGN or CGN
[25]	[91]	X	No discussion of d-CGN or CGN
[28]	[92]	X	No discussion of d-CGN or CGN
[29]	[93]	X	No discussion of d-CGN or CGN
[32]	[94]	X	No discussion of d-CGN or CGN
[26]	[25]	CGN*	Guinea pigs were either non-immunized or immunized with <i>B. vulgatus</i> prior to administration of 5% CGN (though likely used d-CGN) in drinking water. The guinea pigs were also fed either standard feed or feed with viable <i>B. vulgatus</i> . All animals receiving CGN/d-CGN resulted in ulcerations, however, the immune group developed more severe lesions, with the most severe lesions detected in <i>B. Vulgatus</i> immune animals fed both d-CGN and daily doses of viable <i>B. vulgatus</i> .
[33]	[35]	λ-CGN	Lambda CGN administered intra peritoneally (40 µL of 2 mg/mL solution) and orally (0.001 - 0.02% in drinking water) to mice 6 days a week for up to 5 weeks. Macrophages and splenocytes were isolated and compared to controls. CGN appeared to be a ligand for TLR4/MyD88, can induce Th1-cytokines, and reduce histamine release <i>in vivo</i> . However, abrogation of oral tolerance does not follow, so CGN may be an effective dietary supplement for the prevention/improvement of allergy-related diseases.
[34]	[32]	κ-CGN	Mice were administered κ-CGN for 14 days prior to TNBS (a known inducer of intestinal inflammation). The κ-CGN + TNBS mice exhibited increased loss of body weight, mortality, colonic inflammation, expression of IL-2, TNF-α and IL-6, and decreased expression of IL-10, compared to TNBS alone.
[35]	[33]	κ-CGN	Mice were administered kappa-CGN (1.7 mg/kg, 8.3 mg/kg, and 41.7 mg/kg) for 7 days prior to oral gavage of <i>C. freundii</i> DBS100. When <i>C. freundii</i> DBS100 was provided after 1 week of κ-CGN administration, the mice showed increased loss of body weight, increased mortality and ulcerations compared to mice administered <i>C. freundii</i> DBS100 alone. Authors did note that all three groups of mice receiving κ-CGN alone showed no adverse effects in this study.
[36]	[34]	FG-CGN	Prewaning piglets were fed 0, 300, 1000 and 2250 ppm FG-CGN daily for 28 days, under GLP conditions. The high dose in this study was equivalent to 430 mg/kg/day. No adverse effects observed in the immune system or the GI tract.

*Materials and Methods state that CGN was used, but the type kappa, lambda, food grade etc. was not mentioned. In addition, every published article we could identify with AB Onderdonk as an author used d-CGN. In addition, this appears to be a follow-up of a previous study that used d-CGN, so it is likely that this study used d-CGN as well.

either intestinal or hepatic cell lines [68]. In addition, JEFCA agrees that the design of many of the studies was not up to scientific standards and stated "The Committee agreed with the problems that have been pointed out by others in some of the methodological aspects of the *in vitro* studies ... the Committee noted that the *in vitro* studies with carrageenan were not validated by assessment of responses to a positive control, such as a known inflammatory substance ... there are also

difficulties in extrapolating findings from *in vitro* studies on human intestinal cell cultures to draw conclusions on risk assessment for humans *in vivo*. This aspect is particularly relevant given that *in vitro* systems reflect only one component of the *in vivo* processes for prevention of gut inflammation, which are known to be complex [5]".

In contrast to PGN or d-CGN administration, it was observed that guinea pigs exposed to high concentrations

of CGN in the water or diet (1-5%, respectively) for long periods of time exhibited no adverse effects on growth or behavior, and an evaluation of the GI tracts showed no differences from control animals [21] These results were also reported in other species including monkeys (concentration and route of administration not provided), rats (5% CGN in diet) and Guinea pigs (1% CGN in drinking water) [21] Interestingly, there is even some evidence that the ingestion of food-grade CGN has some health benefits. Some research has suggested that there may be a link between human consumption of CGN and decreased cholesterol and low-density lipoproteins (LDL, the “bad” cholesterol), as well as, increased immune status parameters and a decrease in inflammation biomarkers in human volunteers [81-83] These studies provide evidence that is contrary to the hypothesis posited by Martino et al., [24] however reference to these studies was omitted.

The only way for ingested CGN to cause the adverse health effects suggested by Martino et al. [24] and other researchers is if CGN is hydrolyzed into d-CGN or PGN in the human GI tract. As this review shows, there are no enzymes in the human GI tract that will hydrolyze CGN into d-CGN or PGN [15,45,46]. In addition, there is only one place in the human body with the potential conditions for acid hydrolysis of CGN at $pH < 2.0$: the stomach. However, as the pH of the stomach increases rapidly upon drinking or ingestion of a meal, the conditions needed for acid hydrolysis of CGN do not occur in the human gut. Though the pH of the stomach tends to return to ~ 2.0 around two to three hours after a meal, most of the ingested CGN has left the stomach by that time and has passed through the pyloric sphincter and has entered the duodenum. Even if there is a small amount of hydrolysis of CGN into smaller Mw molecules during the digestion of a meal, the exposure of the gut under these circumstances would be well below levels of d-CGN or PGN that have been shown to have adverse effects in animals or humans.

Conclusions

Food-grade CGN has been safely used for decades as a gelling, thickening and stabilizing agent. The safety of food-grade CGN has been evaluated by numerous governmental agencies around the world and unanimously found to be safe for human consumption. Though CGN is safe, the artificially-produced hydrolysis products, d-CGN and PGN, have been demonstrated to have adverse effects, mimicking Chron's disease or ulcerative colitis, in animals [17-22]. Some researchers have relied on misinformation to suggest that CGN

may not be safe (reviewed in McKim et al., 2018). These researchers use the data collected from d-CGN and PGN exposure studies and suggest that CGN causes the same adverse effects. The only way this correlation could be accurate is if much of the ingested CGN was hydrolyzed to d-CGN or PGN inside the human GI tract. As this review shows, the conditions required to hydrolyze a substantial quantity of the ingested CGN to either d-CGN or PGN do not exist in the human body. Therefore, CGN should continue to be recognized as a safe food additive. Care should be exercised by researchers and peer reviewers in the use of correct nomenclature and the use of published literature used to support their hypotheses.

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Competing interests statement

Author James M. McKim is the owner of IONTOX, LLC, an in vitro toxicology contract research organization and consulting company. Author Jamin A. Willoughby Sr. is employed by IONTOX, LLC. Author William R. Blakemore is the sole employee and owner of Celtic Colloids Inc., a consulting company. Author Myra L. Weiner is the sole employee and owner of TOXpertise, LLC, a consulting company.

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