Studies on Effects of D-Mannitol on Absorption of Calcium and Magnesium in Growing Rats

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Abstract of thesis

In recent years, many indigestible sugars have been actively developed in lab researches and food industry because of the physiological properties that they are beneficial to human and animals' health. D-mannitol is one of indigestible sugars used widely in sweet and low-caloric foods. D-mannitol does not alter glycemic or insulinemic indices after ingestion. D-mannitol is partially absorbed, but it is not metabolized in the small intestine. D-mannitol is utilized by local bacterial in the large intestine. In this research, effects of D-mannitol on the absorption of calcium (Ca) and magnesium (Mg) and the mechanism were investigated

1. Effects of D-mannitol on the absorption and retention of calcium and magnesium

Experiment 1: To estimate the effect of D-mannitol on the absorption and retention of Ca and Mg, four-week-old male Wistar rats were divided into five groups (n=7) and were given the experimental diets containing 0, 2, 4, 6 or 8% D-mannitol for twenty-eight days. The feces of the rats were collected twice from day 5 to 9 and from day 20 to 24 of the feeding trial to determine the apparent absorption of Ca and Mg. In the last 7 days of the feeding trial, a non-absorbable marker Cr-CWC was added to the experimental diets to estimate Ca and Mg absorbability in the intestinal segments. Tibias and femurs of the rats were collected.

Apparent Ca absorption and Ca retention in bone were significantly increased by 6 and 8% D-mannitol diets. Apparent Mg absorption was significantly increased by 4, 6 and 8% D-mannitol diets, while Mg retention in bone was significantly increased by 8% D-mannitol diet. Ca/Cr and Mg/Cr in cecal digesta were similar in all groups. Fecal Ca/Cr was significantly decreased by 6 and 8% D-mannitol diets, and Mg/Cr was significantly decreased by 4, 6 and 8% D-mannitol diets.

Experiment 2: To estimate the effect of D-mannitol on cecal parameters, nine-week-old male Wistar rats were divided into three groups (n=7) and were fed the experimental diets containing 0, 4 or 8% D-mannitol for seven days.

A significant decrease in cecal pH was concomitant with a significant change in cecal organic acid concentrations after D-mannitol consumption. Cecal weight and cecal content weight in the ratswere significantly increased by 4 and 8% D-mannitol diets. Cecal tissue weight of the rats was significantly increased by 8% D-mannitol diet.

The results mentioned above suggest that the absorption and retention of Ca and Mg are promoted by 6 and 8% D-mannitol diets. The increase of the absorption of Ca and Mg occurs in the large intestine, and it may be contributed by the fermentation of D-mannitol in cecum.

2. Role of cecum to the effect of D-mannitol on calcium absorption

Twenty eight eight-week-old growing male Wistar rats were used in this experiment. The half of the rats was cecectomized. Cecectomized rats and non-cecectomized rats were divided into two subgroups (n=7) to be fed two different experimental diets containing 0 or 4% D-mannitol for twenty-eight days. Ca balance tests were carried out from day 8 to 12 and from 22 to 26 of the feeding trial. During Ca blance tests, feces and urine of the rats were collected for 24 hours each day.

Ca absorption and retention were significantly decreased by cecectomy. In the cecectomized rats, Ca absorption and retention of the rats were significantly lowered by D-mannitol diet. In the noncecectomized rats, cecal parameters such as cecal weight, cecal tissue weight and cecal content weight were significantly increased, and cecal pH was significantly lowered by D-mannitol diet. The proportion of short chain fatty acids in cecum was significantly modified by D-mannitol diet. Furthermore, the amount of cecal soluble Ca and the ratio of cecal soluble Ca to cecal total Ca were significantly increased by D-mannitol diet. These results suggest that the stimulatory effect of dietary D-mannitol on the absorption and retention of Ca is markedly decreased by the cecectomy. Cecal fermentation of D-mannitol plays a decisive role in its effect on the intestinal Ca absorption and Ca retention in body.

From the present study, it can be demonstrated that the bioavailability of Ca and Mg is increased by dietary D-mannitol in growing rats at a level of 6 and 8%, and the increase of Ca and Mg absorption probably depends on D-mannitol fermentation in the cecum. These results can provide basic information about the fermentation in the large intestine and the advisable dose for the application of D-mannitol as a food material, especially for the people who tend to suffer the deficiency of Ca and Mg, such as adolescents, the elderly, pregnant women and osteoporosis patients

Chaper 1 General Introduction

1.1 Indigestible oligosaccharide and sugar alcohols

1.1.1 Definition

Over the past two decades, many indigestible oligosaccharides and sugar alcohols were actively developed and utilized not only in lab researches but also in food industry because of their excellent physiological properties: they are both scientifically interesting and beneficial to human and animal health, for they serve as low caloric sweeteners. The major part of the food is digested in the stomach and the small intestine facilitated by a large number of digestive enzymes. Carbohydrates that escape the hydrolysis of the endogenous enzymes are defined as indigestible carbohydrates. They are partially or not digested in the small intestine and become available for the large intestinal metabolism. These indigestible oligosaccharides and sugar alcohols are fermented by intestinal bacteria, forming organic acids (short chain fatty acids and lactic acids) and gases (carbon dioxide, hydrogen, and methane) (Cummings 1984; Cummings and Macfarlane 2002).

Oligosaccharides are a group of carbohydrates whose molecules contain two to ten monosaccharides connected by glycoside bonds, and are called disaccharides, trisaccharides, and so on in accordance with the number of monosaccharide contained. Indigestible oligosaccharides include fructooligosaccharides (FOS), raffinose, lactulose, galactooligosaccharides, etc.

The defining characteristic of sugar alcohols is with an alcohol group (>CH-OH) replacing the carbonyl group (>C=O) in the aldose and ketose moieties of mono-, di-, oligo- and polysaccharides; hence generally carry the suffix ‘-itol’ in place of the suffix ‘-ose’ according to modern carbohydrate nomenclature (McNaught 1996). Thanks to the desirable properties, the agents of indigestible but fermentable sugar alcohols such as xylitol, maltitol, sorbitol, lactitol and mannitol are exciting research targets.

1.1.2 Properties

1.1.2.1 Low caloric, do not increase blood glucose and insulin secretion

Indigestible oligosaccharides and sugar alcohols are widely used as low caloric or non-caloric food sweeteners (Levin et al. 1995; Livesey 1992). Compared to the traditional food sweetener sucrose, indigestible oligosaccharides and sugar alcohols have equivalent sweetness and lower calorie. Indigestible carbohydrates are characterized by a low glycemic index (Thorburn et al. 1993; Liljeberg et al. 1999). Oral administration of fructooligosaccharide did not change the level of glucose, fructose, and insulin in plasma, indicating that fructooligosaccharide was not absorbed directly into blood (Yamada, 1991). Dietary supplementation of 5% fructooligosaccharide (weight/weight) did not change serum lipid profiles and glucose levels, but lowered 3.6 fold serum insulin concentrations compared to sucrose diet (Kaume, 2011). Galactitol or mannitol caused lower blood glucose. In addition, lower total serum cholesterol and liver ascorbic acid were led by the ingestion of galactitol, mannitol, as well as xylitol (Mäkinen and Hämäläinem 1985). Sorbitol contributed little to the plasma glucose and insulin responses (Ellis and Krantz, 1941), and neither did erythritol (Bornet et al. 1996).

Table 1.1 Relative sweetness and energy of indigestible oligosaccharides and sugar alcohols

Name	Sweetness relative to sucrose	Energy (kcal/g)
Arabitol	0.7	0.2
Erythritol	0.8	0.2
Glycerol	0.6	4.3
Isomalt	0.5	2.0
Lactitol	0.4	2.0
Maltitol	0.9	2.1
Mannitol	0.5	1.6
Sorbitol	0.6	2.6
Xylitol	1.0	2.4
Palatinose	0.4	-
Lactulose	0.6-0.7	-
Fructooligosaccharides	0.3-0.6	-
Xylooligosaccharide	0.5	-
Galactooligosaccharide	0.2-0.4	-

Source: Antonio Zamora and Oku and Nakamura 2002

All those indigestible oligosaccharides and sugar alcohols are low caloric, and do not increase the blood glucose nor induce insulin secretion, because they do not get metabolized in the small intestine to be an energy source. Relative sweetness and energy of several indigestible oligosaccharides and sugar alcohols are shown in Table 1.1.

1.1.2.2 Change intestinal transit time and increase stool mass

Indigestible sugars in diet have been shown to affect gastrointestinal functions such as increase in stool mass and shortened transit time of materials from mouth to anus. Stephen and Cummings (1980) reported that fibers (fibers here refer to dietary fibers, which constitute plant cell wall polysaccharides and lignin, but not to crude fiber) affect large bowel function, increasing stool bulking in human. Increment of stool weight had been explained by water holding properties of indigestible carbohydrates until Eastwood et al found an increase in dry weight in the stools of their subjects who were given bran as dietary fiber supplement. Here the fermentation of indigestible oligosaccharides in thought to result in the production of biomass. The biomass accounts for at least 300 g/kg on a dry weight basis of feces (Roberfroid et al. 1993), which is responsible for the increased fecal bulk and fecal dry matter in human. Diets containing high amount of fiber result in large, soft stools that traverse the intestine rapidly (Burkitta et al. 1972). Fecal bulking is a main characteristics induced by dietary fiber feeding.

The transit time of indigestible sugars is a major factor which is directly related to its effect on stool weight and size. The transit time of the diet in the large intestines (cecum and colon) correlates with the gastrointestinal tract fermentation in the large intestine in non-ruminant animals. The end product of the fermentation such as butyric acid impacts the transit time through the gastrointestinal tract. Higher proportion of butyric acid generally leads a shorter cecal transit time (Mathers and Dawson 1991). Changes in transit time may alter bacterial activity and modify the bacterial pathways; as a consequence, the proportion of individual short chain fatty acids is affected (Oufir et al. 2000).

1.1.2.3 Cause luminal osmotic pressure in intestinal tract and laxative effect

Indigestible sugars can alter the intestinal physiology in two ways: physical presence and fermentation. Physical presence affects several physiological functions in the upper intestine. Indigestible sugars produce a high osmotic pressure, accumulate fluid within the lumen to maintain isotonicity, and increase the permeability of the intercellular junctions in the small intestine (Pansu et al. 1976). Indigested oligosaccharides and sugar alcohols induce osmotic diarrhea if consumed in excess (Cummings 1997) (Table 1.2).

Table 1.2 Maximum permissible doses of sugar substitutes not causing transitory diarrhea in human

	Male	female
Erythritol	0.66	0.8
Xylitol	-	0.7
Sorbitol	0.17	0.24
Maltitol	-	0.3
Lactitol	-	0.37
Palatinit	0.3	-
Lactulose	-	0.32
4′Galactooligosaccharide	0.28	0.14
6′Galactooligosaccharide	0.3	0.3
Xylooligosaccharide	0.12	-
Fructooligosaccharides	0.3	0.4

Source: Oku and Nakamura 2002

1.1.2.4 Fermentation in large intestine

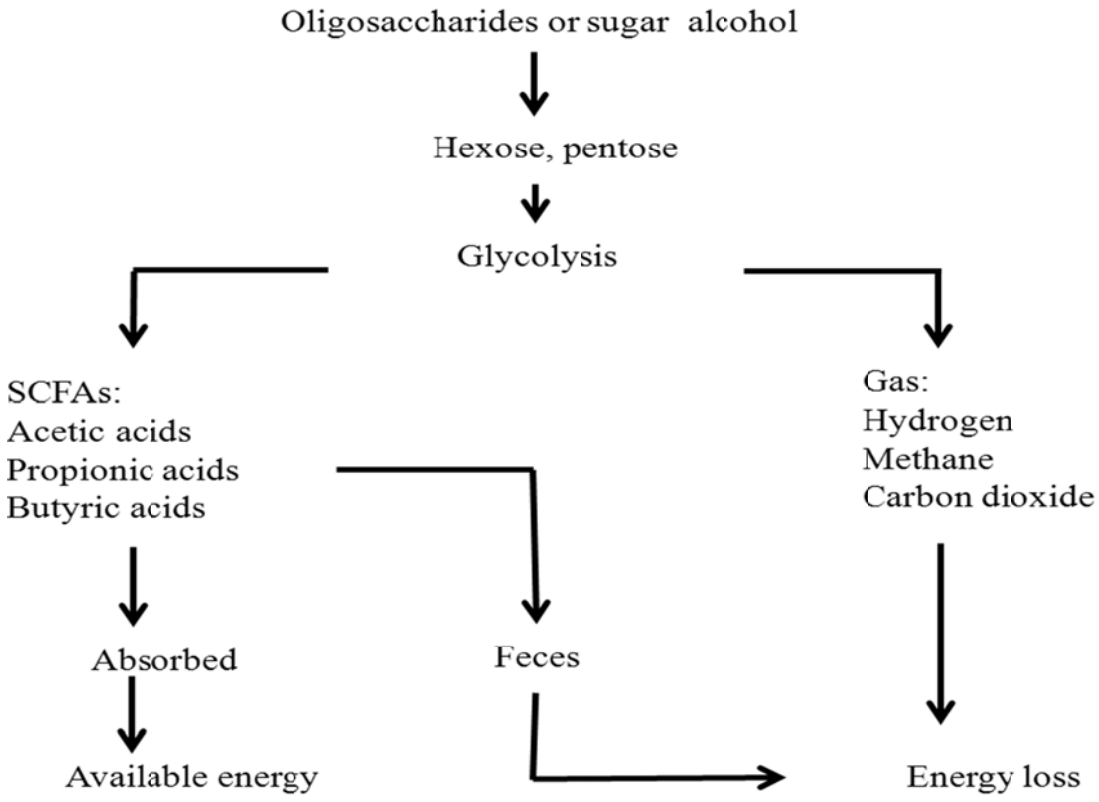
Indigestible sugars pass through in the small intestine intact or partly digested and enter the large intestine. They are utilized by intestinal bacteria in human and animals, producing organic acids for energy source. Increasing the population of bacteria is the major characteristics of indigestible and fermentable sugars. It has been reported that the number of cecal *bifidobacteria* was increased in rats fed with oligofructose or xylooligosaccharides (Campbell et al. 1997). Resistant sugars have shown greater fecal numbers of *bifidobacteria* after their oral ingestion in pigs (Brown et al. 1997). The basic fermentative reaction in the large intestine of polysaccharides, oligosaccharides, and

sugar alcohols results in an increased biomass (Savage 1986). A large proportion of these indigestible sugars are fermented in colon in human (Cummings and Macfarlane 1991), and a major portion of microbial fermentation is concentrated in cecum in non-ruminants such as rats, rabbits, chickens (Van Soest 1995; Xiao li, 2011). Fermentation yields basic nutrients for microbial growth and maintenance, and also metabolic end products. For example, nitrogen used for the increase of bacterial population comes either from urea, undigested dietary protein, or endogenous secretions. The fermentation causes the flux of urea nitrogen toward large intestine, an increase in fecal nitrogen excretion, and low plasma urea. A high rate of urea transfer from blood to the large intestine is resulted from cecal hypertrophy which is characterized by the increase of bacterial population and an enlarged surface of exchange between blood and luminal fluid (Younes et al. 1996; Remey and Demigne 1989). The balance of products of indigestible carbohydrates differs on the structure, the size of the molecule, and the dose in diet so on. The main products of the fermentation are short chain fatty acids, predominantly acetic, propionic and butyric, lactic and succinic acids, as well as water, various gases (carbon dioxide, hydrogen, methane) and bacterial cell biomass (Gibson and Fuller 2000; Montagne et al. 2003) and some heat. Some types of indigestible carbohydrates can influence the distribution of short chain fatty acids in the hindgut. For example, guar gum consumption by rats resulted in a high proportion of propionic acid, and pectin consumption resulted in a high proportion of acetic acid upon fermentation (Berggren et al. 1993; Brighenti et al. 1989). Short chain fatty acids serve as nutrients for the epithelium and as oxidative fuel for body tissues (Bach Knudsen 2005).

The principal end products short chain fatty acids formed from the fermentation play the most important role in the environment of the large intestine. Short chain fatty acids decrease intestinal acidity. Short chain fatty acids are main luminal anions in human and animals. They are relatively weak acids with pKa values < 4.8, and raising the production of short chain fatty acids through fermentation lowers digesta pH. Lower pH inhibits the growth of some intestinal bacterial pathogens such as *Enterobacteriacae* (McHan and Shotts 1993), *Salmonella* (McHan and Shotts 1992; 1993) and *Clostridium* species (Hentges 1992; McHan and Shotts 1993), *E. coli* (Gidenne and Licois 2005). At the same time, lower pH facilitates bacterial growth in such species as *bifidobacteria* (Hidaka et al. 1991), *Lactobacillus plantarurn* (Dirar and Collins 1973), and *Clostridium indolis*

(Maekawa et al. 2005). Lower pH also stimulates cell proliferation of intestinal wall tissue (Sakata 1986). Short chain fatty acids can also benefit the cell proliferation of intestinal wall since these fatty acids, especially butyric acid, work as important fuels for large intestinal cecum- or colonocytes (Heneghan 1988). Short chain fatty acids have a trophic effect on intestinal mucosa and improve absorption and retention of electrolytes and water from the intestinal tract. In human colon short chain fatty acids are absorbed in two ways. One is nonionic diffusion of protonated short chain fatty acids involving consumption of luminal CO_2, which accounts for about 60% of total short chain fatty acids absorption. The other is ionic diffusion of short chain fatty acids that combine with Na^+ or K^+ (Ruppin et al. 1980). Short chain fatty acids are rapidly absorbed by the colonic mucosa and stimulate salt and water uptake (Ruppin et al. 1980) and affect gastrointestinal motility (Cherbut et al. 1994).

Figure. 1.1 Metabolism of oligosaccharides and sugar alcohols in large intestine



Source: Oku and Nakamura 2002

1.1.2.5 Regulate the digestion and retention of other nutrients

After the ingestion of indigestible oligosaccharides or sugar alcohols, the digestibility and retention of nutrient such as protein, fat and mineral elements in body are changed. In experiment of Fleming and Lee (1983), pectin reduced weight gain, feed efficiency ratios, protein efficiency ratios, and apparent protein digestibility values. Cellulose and xylan decreased apparent protein digestibility values. The diet containing fructooligosaccharide or galactooligosaccharide decreased the digestibility of crude protein than the diet containing sucrose in equal amount (100 g/kg). The apparent digestibility of crude fat was lowered by the diet with fructooligosaccharide (Sakaguchi et al. 1998). Fat digestibility and fat accumulation in body was decreased by ingestion of mannitol in rats (Nishiyama et al. 2009).

1.2 Bioavailability of calcium and magnesium

1.2.1 Calcium

99% of Ca content is found in the skeleton, and the amount in body fluids and cells of the soft tissues is accounted for 1% of the body's total Ca (Bronner 1997). It is thus obvious that almost all Ca is contained in the skeleton. The movement of Ca in the body involves ingestion, digestion, intestinal transit during which Ca is absorbed transepithelially, and excretion in feces. Ca absorbed in intestinal tract mixes rapidly with body fluid Ca. When plasma Ca level is normal, around half of Ca ions in plasma circulates into skeleton Ca and remains in bone mineral (Bronner and Stein 1992). The Ca ions from previous bone mineral is taken to kidney in plasma flow, and filtered into the renal tubule, and about 70% of these are reabsorbed as the fluid passes through the various parts of nephron (Bronner 1997). Reabsorbed Ca that enters the intestine with the body fluids or as cell debris mix with intestinal content and are reabsorbed at the rate at which the digesta is absorbed. The rest of Ca unabsorbed in kidney and in intestine is excreted in urine and feces.

Some additives such as vitamin D, casein phosphopeptide, and indigestible carbohydrates supplied with Ca are well known to have stimulating effect on intestinal Ca absorption to increase Ca retention. It is certain that Ca must be soluble to be absorbed. The absorption is a complicated function related to the rate of Ca solubility, the rate of tryansepithelial movement and the intestinal passing time in the particular intestinal segment (Duflos et al. 1995). Intestinal Ca absorption occurs through two processes

(Bronner et al. 1986). One is a saturable process with it is transcellular movement dependent on vitamin-D, which is the main absorbing mode in the upper intestine. The other is a nonsaturable process that is paracellular and less associated with age (Pansu et al. 1982) or vitamin-D status (Pansu et al. 1983). In fact, intestinal Ca absorption can occur via a passive paracellular route through the tight junctions between mucosal cells over the course of the small and large intestine (Bronner, 1987). In the entire intestinal tract in rats, net Ca absorption rates are linearly related to Ca concentration in the intestinal lumen. When luminal Ca concentration is lower than that of serum ionized Ca, serum Ca would move into the lumen. When luminal Ca concentration is higher than that of serum ionized Ca, net absorption would take place (Ghishan et al. 1980). Therefore, the decrease in the luminal pH with indigestible carbohydrates leading the increase in soluble Ca in the large intestine is more efficient than vitamin-D to promote Ca absorption via the passive paracellular route.

1.2.2 Magnesium

Mg are essential to various physiological and biochemical processes, but Mg homeostasis is less related to hormonal control. Mg is simply supplied by the absorption from gut, solving the distribution of the needed amounts to the cells, and the surplus of Mg is excreted in feces and urine. About 90% of Mg is bound and only 10% is free. The distribution and concentrations of Mg in a healthy adult is shown in Table 1.3.

Intestinal Mg absorption in human takes place mainly in the upper intestinal tract (ileum and jejunum), but in some mono-gastric animals like rat colon and cecum also hold a big part of Mg absorption in whole intestinal tract. In human the absorbed amount of Mg is proportionally related to Mg intake, only at low dietary intakes does fractional absorption increase (Kayne and Lee 1993). In human and animals, low plasma or serum Mg concentrations occur within few days after taking low Mg diet (Shils 1997). The plasma Mg concentration is kept constant over a certain period by decreasing urinary Mg excretion and by release of Mg bound to bones. For people who suffer from Mg deficiency such as pregnant woman, patients with osteoporosis and the elderly, to increase intestinal Mg absorption is a desirable way to meet Mg requirement.

Table 1.3 Distribution and concentrations of Mg in a healthy adult

Percent distribution	Concentration
Bone (60–65%)	0.5% of bone ash
Muscle (27%)	6–10 mmol/kg wet weight
Other cells (6–7%)	6–10 mmol/kg wet weight
Extracellular (<1%)	
Erythrocytes	2.5 mmol/l
Serum: 55% free 13% complexes with citrate, phosphate, etc., 32% bound, primarily to albumin	0.7–1.1 mol/l
Mononuclear blood cells	2.3–3.5 fmol/cell
Cerebrospinal fluid: 55% free 45% complexed	1.25 mmol/l
Sweat	0.3 mmol/l (in hot environment)
Secretions	0.3–0.7 mmol/l

Source: Shils 1997

1.3 Effects of indigestible sugars on mineral absorption

Ca and Mg transport across the intestinal wall from serosa to mucosa and vice versa in the small and large intestine. Historically, dietary fiber was considered to decrease mineral absorption (Gordon et al. 1995). It was considered that high dose of fibers such as pectin, which bind to mineral cations and vitamin in the diet, decrease mineral bioavailability. Carrageenan and agar-agar reduced absorption of all minerals tested, Na-alginate decreased the absorption of iron (Fe), chromium (Cr) and cobalt (Co), carob bean gum and gum guar interfered with the absorption of zinc (Zn), Cr, copper (Cu) and Co (Harmuth-Hoene and Schelenz 1980). Pectin may decrease mineral absorption by forming gels and binding mainly to bivalent ions to its free carboxyl groups (El-Zoghbi

and Sitohy 2001). However, more recently, there have been reports to prove that some dietary fibers, indigestible oligosaccharides (e.g., FOS) and some sugar alcohols improve mineral absorption and mineral retention in human and animals. Ohta et al (1994) found that 5% dietary fructooligosaccharide increased the absorption of Ca and Mg in normal rats. Chonan and Watanuki (1996) found that in growing rats with diet containing 5 or 10% galactooligosaccharides, apparent Ca absorption and Ca balance were improved. Lactulose increased Ca absorption from 26% to 37% in rats as did other sugars whose digestion was limited in the small intestine, like L-arabinose and D-arabinose, raffinose or sugar alcohols like xylitol (Brommage et al. 1993). Dietary lacto-sucrose increased apparent Ca absorption, residual Ca ratio and Ca accumulation in femur and tibia in the growing rats (Kishino et al. 2006).

1.3.1 Increase mineral absorption in small intestine

Dietary lactose induced stimulation of Mg absorption in rats, and it is caused by a lowering of ileal pH (Heijnen et al. 1993). Intraluminal infused sorbitol was shown to increase Ca absorption in the ileum loop in rats (Dupuis et al. 1978). Using a tracer technique with ^{45}Ca, maltitol was found to stimulate Ca absorption in rats, and osmotic activity of maltitol in the small intestine is thought to contribute to the increased Ca absorption (Fukahori et al. 1998). Melibiose, difructose anhydride III, or difructose anhydride IV increased the permeability of intercellular passage, affected the epithelial tissue and opened the tight junctions of jejunum, ileum, cecum, and colon of rats. Absorption of Ca, Mg, and Zn via the paracellular route was enhanced, thereby promoting Ca, Mg, and Zn absorption in the small intestine and large intestine in vitro. The stimulating effect on mineral absorption of indigestible sugars was related to the induced permeability in the small intestine.

1.3.2 Increase mineral absorption in large intestine

After ingestion of indigestible and fermentable sugars, mineral absorption such as Ca and Mg shift toward the large intestine (Younes et al. 1996). The cecum is the main segment with highest Ca absorption in rat intestine. The cecum actively transports Ca several times faster than the rate of the duodenum, proximal colon and distal colon (Karbach and Feldmeier 1993). Under normal conditions, the cecum absorbs free-ionized

Ca released from insoluble complexes of Ca in the presence of small acidic molecules, such as acetic, propionic, butyric, succinic and lactic acids which are formed in the fermentation of dietary fibers and prebiotics by luminal microbe (Mineo et al. 2001; Younes et al. 1996). The indigestible fermentable sugars are resistant to digestion by endogenous enzymes in the small intestine, thus reach cecum intact in rats. Compared to human, the fermentation in cecum in rats is stronger, and the fermentation affects mineral absorption just as human colon. The diet containing guar-gum hydrolysate (50 g/kg diet) increased apparent Ca absorption in nephrectomized and normal rats, and the cecum was responsible for these increases in Ca absorption (Hara et al. 1996). A possible mechanism for the increase in the ceco-colonic Ca absorption associated with feeding guar-gum hydrolysate was explained by increase in ionic Ca induced by luminal acidification due to production of organic acids during cecal fermentation.

In animal experiments, it was shown that inulin and oligofructose improved mineral absorption (Scholz-Ahrens et al. 2001) and it was associated with the production of short chain fatty acids and lower pH in the intestinal lumen. Compared to inulin, oligofructose stimulated Ca absorption slightly more effectively, while the effect on Mg was equivalent (Delzenne et al. 1995). The production of total short chain fatty acids was not different but lactic acid was significantly higher after the ingestion of xylooligosaccharides, and butyric acids was highest corresponding with oligofructose ingestion in rats (Campbell et al. 1997). Moreover, indigestible oligosaccharides increase mineral accumulation in bone, and the effect depends both on the dose of indigestible oligosaccharides and on Ca level in diet. Oligosaccharide was most effective when dietary Ca was high (Scholz-Ahrens et al. 2002).

Most of the scientific evidence of the effects of indigestible sugars was based on the results of experiments with rats, and these sugars increased the availability of Ca, Mg, Fe and Zn (Delzenne et al. 1995; Chonan and Watanuki 1996; Ohta et al. 1994, 1995b). Particularly Ca, Mg, and Zn are important for bone mineralization and bone health (Heaney 1996). In rats, Ca retention was greater after 11 day supplementation of indigestible oligosaccharides, but no longer after 25 day (Ohta et al. 1994b). This may explain why weight, total ash content and Ca content of the femur were not affected (Ohta et al. 1994b, 1997). In single meal studies with human subjects, inulin, oligofructose or lactulose stimulated Ca absorption in some cases (Coudray et al. 1997; van den Heuvel et

al. 1999a, 1999b; Griffin et al. 2002) but not in all (van den Heuvel et al. 1998b; Griffinet al. 2002). Therefore, the effect of indigestible sugars on mineral absorption and retention may be related to some condition, such as the age, the length of feeding and the demand of minerals.

1.4 D-mannitol

1.4.1 Physical properties and natural distribution

Table 1.4 Characteristics of D-mannitol

Characteristics	D-mannitol
Chemical formula	$C_6H_8(OH)_6$
Form	White powder
Sweetness	50% of sucrose
Taste	Sweet/cool
Odor	None
Noncariogenic	Yes
Moisture	Nonhygroscopic
Solubility in H_2O (at 25°C)	23 g/100 g H_2O
Caloric value	1.6 kcal/g
Melting point	164°C
Molecular weight	182
Heat of solution (at 25°C)	28.9 cal/g

Source: Dwivedi, 1991

D-mannitol (mannitol) is found in abundance in nature, particularly in trees, fruits, marine algae, fresh mushrooms, and in many plants. It is an isomer of sorbitol and is typically produced today by the hydrogenation of specialty glucose syrups. Mannitol is non-cariogenic and has a low caloric content. It is suitable for ingestion and has been used safely around the world for over 60 years. Nonhygroscopic property of mannitol makes the food with mannitol pick up less moisture than that with other sugars.

1.4.2 Metabolism and physiological functions

1.4.2.1 Absorption in small intestine

Mannitol, like other sugar alcohols, is an indigestible carbohydrate that is only partially absorbed from the small intestine and does not get converted into energy source. Therefore, it does not stimulate an increase in blood glucose and insulin secretion. It has a low glycemic index, and is therefore used as a sweetener for people with diabetes and obesity. It also induces high permeability in small intestine, resulting fluid accumulation in lumen (Krugliak et al. 1994).

1.4.2.2 Fermentation in large intestine

In the large intestine, mannitol is fermented by intestinal bacteria. *Lactobacillus plantarum*, some *bifidobacteria*, *Escherichia coli* and *Streptococcus mutans* utilize mannitol as a primary energy source for growth (Chakravorty, 1964; de Vries and Stouthamer, 1968; Maryanski and Wittenberger, 1975) and produce organic acids. In small intestine, mannitol causes the osmotic pressure directly to induce water flow into the intestinal lumen. But in the large intestine, the permeability more tends to be led by the production of organic acids from the fermentation. The permeability of mannitol accumulates luminal fluid in the large intestine tract. Mannitol may occasionally cause softer stools, and the ingestion of excessive mannitol causes osmotic diarrhea (Mäkinen 1984). The major end products of mannitol in the large intestine are organic acid and intestinal gases.

1.5 Present study

Based on the properties of mannitol, we tried in this study to elucidate the effect of mannitol on the absorption and retention of Ca and Mg in rats and its mechanism. The absorption and retention of Ca and Mg was increased, and the amount of Ca and Mg in tibia and femur was increased by the diet with higher level of mannitol (6% and 8%, w/w) after 4 weeks. It also appeared that the increase of mineral absorption occurred in the large intestine, for dietary mannitol decreased Ca absorption and retention in the cecectomized rats. 4% mannitol diet induced cecal enlargement and cecal acidification, although it did not increase Ca absorption and retention markedly in normal rats. In brief, mannitol was fermented in cecum that led cecal enlargement and cecal acidification

causing an increase in mineral absorption, and for Ca, cecum is the segment where the absorption was increased after the ingestion of mannitol.

This study will provide basic information mainly on effects of mannitol on mineral bioavailability, on the fermentation in the large intestine, and also on the advisable dose for the application of mannitol as a food material.

Chapter 2 Effects of D-mannitol on the Absorption and Retention of calcium and magnesium

2.1 Abstract

Indigestible sugars, which have several properties as, are often used in food production and the pharmaceutical industry. We evaluated the effects of D-mannitol on the absorption and retention of Ca and Mg in growing rats. Experiment 1: Four-week-old growing male Wistar rats were given experimental diets containing 0, 2%, 4%, 6% or 8% D-mannitol for 28 days to measure the absorption and retention of Ca and Mg. In the last 7 days of the feeding trial, the unabsorbable marker Cr-CWC was added to the experimental diets to estimate Ca and Mg absorbability in the intestinal segments. Experiment 2: Nine-week-old growing male Wistar rats were fed for 7 days with the experimental diets (Control diet, 4% or 8% D-mannitol diets) to observe cecal parameters. The result showed that apparent Ca absorption and retention in bone were significantly increased with 6% and 8% D-mannitol diets. Apparent Mg absorption was significantly increased with 4%, 6% and 8% D-mannitol diets, while Mg retention in bone was significantly increased with 8% mannitol diet. Ca/Cr and Mg/Cr in cecal digesta were similar in all groups. Fecal Ca/Cr was significantly decreased with 6% and 8% D-mannitol diets and Mg/Cr was significantly decreased with 4%, 6% and 8% D-mannitol diets. In Experiment 2, cecal weight and tissue weight were significantly increased with 8% D-mannitol diet. A significant decrease in pH was concomitant with a significant change in cecal organic acid concentrations after D-mannitol consumption. Absorption and retention of Ca and Mg are promoted by D-mannitol feeding through the fermentation of D-mannitol in the cecum.

2.2 Introduction

D-mannitol (mannitol) is a six-carbon resistant sugar alcohol used in sweet food with a low-calorific value. It is present in bacteria, yeast, fungi, algae, lichens and a number of plants (Wisselink et al. 2002). Mannitol is widely used in the food production and the pharmaceutical industry, because of several desirable properties like mannitol has a

diuretic effect but does not alter glycemic or insulinemic indices after ingestion (Song et al. 2009). And some mannitol is absorbed in the small intestine, but it is not metabolized to produce energy. In the large intestine it is fermented by microbes (Dwivedi 1991). A previous study in humans showed that 74% of mannitol passed through the small intestine and reached the large intestine, where it was fermented by beneficial bacteria (Saunders and Wiggins 1981). The fermentation produced organic acids, which can be used by the host.

Ca and Mg play an important role in normal physiological function in humans and animals. Nowadays, dietary mineral intakes are sufficient, so the ratio of mineral intake to absorption is more important, especially for postmenopausal women, the elderly, and people with diseases such as diabetics and osteoporosis. When added to the diet, resistant sugars such as indigestible sugar alcohols and oligosaccharides can increase intestinal mineral absorption and whole body mineral retention in various animals and humans (Zafar et al. 2004; Suzuki et al. 1998; Takahara et al. 2000). Fermentable sugars such as maltitol, lactitol, and fructo-oligosaccharides were shown to increase mineral bioavailability in humans and rats (Goda et al. 1995; Ammann et al. 1988). Nakamura reported that resistant sugar substitutes 1) do not induce an increase in blood glucose or insulin secretion; 2) are low- or non-caloric; 3) improve the intestinal environment and intestinal microbiota in the large intestine; 4) stimulate the intestinal absorption of minerals such as Ca, Mg and Fe (Nakamura 2005). Mineral absorption is more strongly correlated with cecal fermentation in rats than in humans. Compared with humans, the highest rate of Ca and Mg absorption in rats occurs in the large intestine rather than in the small intestine. Acidic fermentation in the cecum was reported to increase Ca and Mg absorption in the large intestine in rats (Younes et al. 1996). The development of the cecum after the ingestion of fibers is generally connected with an accumulation of mineral cations in the large intestine. The absorption of Ca and Mg is closely correlated with the fermentability of the fibers (Rémésy et al. 1992). The physiological function of mannitol in mineral absorption and retention has not yet been clarified. In this study, we tried to elucidate the effects of indigestible mannitol on the absorption and retention of Ca and Mg in the growing rat by comparing experimental diets with sucrose (digestible) and different levels of mannitol (indigestible).

2.3 Materials and methods

2.3.1 Animals and diets

Experiment 1

A total of 35 four-week-old male Wistar rats (Japan SLC, Inc., Shizuoka, Japan) was housed in individual wire-mesh stainless steel cages in an air conditioned room maintained at 23 ± 1 °C with 50–60% relative humidity. The light was set up as a constant 12 hours light (7:00–19:00) and 12 hours dark (19:00–7:00) cycle. Rats were acclimatized to laboratory conditions for 3 days before the start of the feeding trial. The rats were weighed and randomly assigned to five treatment groups (n=7 per group), each group was fed one of the experimental diets (control diet or mannitol diets containing 2%, 4%, 6% or 8% mannitol) for 28 days. Diets and water were available *ad libitum* during the entire experimental period.

Table 2.1 Composition of experimental diets

Ingredients	Experiment 1				
	C	2M	4M	6M	8M
α-Corn starch (g/kg)	562	562	562	562	562
Casein (g/kg)	200	200	200	200	200
Sucrose (g/kg)	100	80	60	40	20
Corn oil (g/kg)	70	70	70	70	70
Cellulose powder (g/kg)	20	20	20	20	20
Vitamin mix (g/kg)	10	10	10	10	10
Mineral mix (g/kg)	35	35	35	35	35
L-Cystine (g/kg)	3	3	3	3	3
D-Mannitol (g/kg)	0	20	40	60	80
Gross energy (kcal/g)	4.94 (Calculation)				

C: control diet (AIN-93G); 2M, 4M, 6M, 8M: experimental diets containing 2%, 4%, 6%, or 8% mannitol, respectively (Reeves et al. 1993).

The experimental diets used in this study were as follows: The control diet (C) consisted of standard laboratory chow (AIN-93G) (Reeves et al. 1993). Mannitol diets containing 2%, 4%, 6%, or 8% mannitol were created by replacement of equal amounts of

sucrose in the control diet with mannitol. During the last 7 days of the feeding trial, 1/3 of the cellulose in the experimental diets was replaced with chromium-mordant cellulose (Cr) as an unabsorbable marker. The chromium-mordant cellulose was prepared according to the method described by Ohta (1995). The cellulose was dried at 60 °C after dyeing with chromium. The compositions of the experimental diets are shown in Table 2.1.

Experiment 2

Nine-week-old male Wistar rats purchased from Japan SLC, Inc. (Shizuoka, Japan), were housed separately in wire-mesh stainless steel cages in an air conditioned room maintained at 23±1 °C with 50–60% relative humidity. The light was set up as a constant 12 hours light (7:00–19:00) and 12 hours dark cycle (19:00–7:00). The rats were weighed and randomly allotted to three treatment groups (n=7 per group), each being fed one of the experimental diets (control diet or mannitol diets containing 4% or 8% mannitol) for 7 days. Diets and water were available *ad libitum* during the entire experimental period.

The experimental diets used in Experiment 2 were as follows: The control diet (C) consisted of standard laboratory chow (AIN-93G) (Reeves et al. 1993). The compositions of the experimental diets (control diet or mannitol diets containing 4% or 8% mannitol) were prepared as same as in Experiment 1.

2.3.2 Sample collection and analysis

Experiment 1

During the experiment, the amount of food supplied, diet residues and diet waste were measured daily. Feces were collected from day 5 to day 9 and from day 20 to day 24 of the feeding trial. Feces were collected for 24 hours before the rats were killed to determine the absorbability of Ca and Mg in the intestinal segment. All feces samples were oven-dried for 24 hours, comminuted and burned in a 550 °C muffle furnace to determine crude ash and mineral levels. Feed efficiency and apparent Ca and Mg absorption were calculated as follows: feed efficiency = weight gain / feed intake × 100%; apparent mineral absorption = (intake – fecal excretion) / intake × 100%; the ratio of Ca or Mg to Cr = Ca or Mg (mol) in sample / Cr (mol) in sample. On the last day of the feeding trial, the rats were anesthetized with diethyl ether after fasting for 12 hours and killed by exsanguination from the celiac artery. The left tibia and femur were isolated and

cleaned of soft tissue. Marrow elements were flushed out with distilled water through a needle inserted into the marrow cavities. Cecal digesta of the rats were removed after the rats were killed. Mineral levels in the diet, feces and cecal digesta were determined by flame atomic absorption spectroscopy (FAAS 180-30; Hitachi Ltd., Tokyo, Japan).

Experiment 2

From 22:00 on day 6 to 6:00 on day 7 of the feeding trial, the rats were fasted. On day 7 the rats were supplied with the experimental diets from 6:00 to 9:00. At 9:00, the rats were anesthetized with diethyl ether and killed by exsanguination from the celiac artery. The duodenum, jejunum, ileum, cecum, colon, and rectum were separated at the junction points of each segment of the intestine to determine the tissue weight of each segment. Cecum were kept with their digesta, and stored at -30 °C. The pH values of the digesta were measured with a pH meter (TWIN Horiba Ltd., Kyoto, Japan). Samples of the cecal digesta were used for the measurement of organic acids by HPLC (Column: 2 Shim-pach SCR-102H; Detector: Shimadzu CDD-10A, Shimadzu Corporation, Kyoto, Japan).

2.3.3 Ethics

Animals were cared for and sacrificed in accordance with the guidelines for animal experiments at Okayama University, The experimental protocol (No:51735) was approved by the institutional ethics committee of Okayama University.

2.3.4 Statistics

Data are shown as mean ± SD. Nonparametric one-way ANOVA (Kruskal-Wallis method) (Excel statistics SSRI Co., Tokyo, Japan) with Steel-Dwass test was used for multiple comparisons among the experimental groups (control diet group, 2%, 4%, 6% and 8% mannitol diet groups in Experiment 1; control diet group, 4% and 8% mannitol diet groups in Experiment 2). Repeated Measures ANOVA was used to test the data from the fecal collection. Collection time of the feces and the experimental diet were used as factors. Differences were considered significant at $p < 0.05$.

2.4 Results

In Experiment 1, the 6% and 8% mannitol diets caused mild diarrhea at the beginning of the feeding trial, but the rats recovered from the diarrhea in 3 or 4 days.

Growth performance and feed efficiency during the feeding trial in Experiment 1 are shown in Table 2.2. Initial body weights, final body weights and feed intakes of the rats during the feeding trial were not significantly different among the experimental groups. Feed efficiency in the 8% mannitol group was significantly lower than in the control group and the 2% mannitol group.

Table 2.2 Growth performance, feed intake and feeding efficiency in rats fed a diet containing 0, 2%, 4%, 6% and 8% mannitol

	C	2M	4M	6M	8M
Initial BW (g)	112.3±4.8	112.3±4.6	112.4±4.5	112.4±4.0	112.2±4.3
Final BW (g)	256.7±6.3	254.0±7.1	255.7±6.7	256.2±9.8	245.2±8.1
Feed Intake (g/d)	15.4±0.2	15.5±0.2	15.6±0.2	15.5±0.1	15.6±0.2
Feed Efficiency (%)	34.2±0.8 [a]	33.3±1.1 [a]	33.5±1.6 [ab]	33.6±2.2 [ab]	31.0±1.3 [b]

C: control diet (AIN-93G); 2M, 4M, 6M, 8M: experimental diets containing 2%, 4%, 6%, or 8% mannitol, respectively (Reeves et al. 1993). BW: Body weight. Data are mean ± SD (n=7 per group). [a, b] Mean value within a row not sharing a common superscript letter differ significantly at $p<0.05$ by Kruskal-Wallis Nonparametric statistical test.

Dry matter digestibility, fecal excretion and fecal crude ash in Experiment 1 are shown in Table 2.3. The results of the repeated measures ANOVA show that dry matter digestibility, fecal excretion and fecal crude ash were significantly affected by the mannitol diets, but not by the fecal collection time. During days 5-9 of the feeding trial, dry matter digestibility in the 8% mannitol group was significantly lower than in the control group. Dry matter digestibility was similar among the mannitol groups. Fecal dry matter excretion was significantly higher in the 4%, 6% and 8% mannitol groups than in the control group, whereas it was not significantly different among the mannitol groups. The concentrations of crude ash in the feces of the mannitol groups were significantly and dose-dependently reduced compared with the control group in this period. During days 20-24 of the feeding trial, dry matter digestibility in the 8% mannitol group was significantly lower than in the control group and the 4% mannitol group. Fecal dry matter

excretion was significantly higher in the 6% and 8% mannitol groups than in the control group, but not significantly different among the mannitol groups. The concentrations of crude ash in the feces were significantly and dose-dependently reduced in the mannitol groups compared with the control group.

Table 2.3 Dry matter digestibility, fecal dry matter excretion and fecal crude ash concentration during the fecal collection periods of the feeding trial in rats fed a diet containing 0, 2%, 4%, 6% and 8% mannitol

	C	2M	4M	6M	8M
5^{th}-9^{th} day					
DM digestibility (%)	83.6±0.3 [a]	83.4±0.4 [ab]	83.3±0.3 [ab]	83.1±0.3 [ab]	83.0±0.2 [b]
Fecal excretion (g/d, DM)	0.4±0.0 [a]	0.5±0.1 [ab]	0.6±0.1 [b]	0.6±0.1 [b]	0.6±0.0 [b]
Fecal CA (%, DM)	17.7±1.7 [a]	14.7±1.0 [b]	13.5±0.6 [b]	11.8±0.8 [c]	10.4±0.6 [c]
20^{th}-24^{th} day					
DM digestibility (%)	83.2±0.2 [a]	83.1±0.5 [ab]	83.4±0.5 [a]	83.0±0.4 [ab]	82.5±0.4 [b]
Fecal excretion (g/d, DM)	0.6±0.0 [a]	0.7±0.1 [ab]	0.7±0.1 [ab]	0.7±0.1 [b]	0.8±0.2 [b]
Fecal CA (%, DM)	16.4±1.2 [a]	14.1±0.8 [b]	12.5±0.8 [bc]	12.1±0.8 [c]	11.3±1.3 [c]

	Repeated Measures ANOVA		
	Collection time	Diet	Collection time × diet
DM digestibility (%)	0.061	<0.001	0.074
Fecal excretion (g/d, DM)	0.068	<0.001	<0.001
Fecal CA (%, DM)	0.051	<0.001	0.671

C: control diet (AIN-93G); 2M, 4M, 6M, 8M: experimental diets containing 2%, 4%, 6%, or 8% mannitol, respectively (Reeves et al. 1993); DM: Dry matter; CA: Crude ash. Feces were collected from day 5 to day 9 and from day 20 to day 24 of the feeding trial. Data are mean ± SD (n=7 per group). [a, b, c] Mean value within a row not sharing a common superscript letter differ significantly at $p<0.05$ by Kruskal-Wallis Nonparametric statistical test. Repeated Measures ANOVA was used to test the data of fecal collection. Collection time of the feces and the experimental diet was set as two factors. Differences were considered significant at $p < 0.05$.

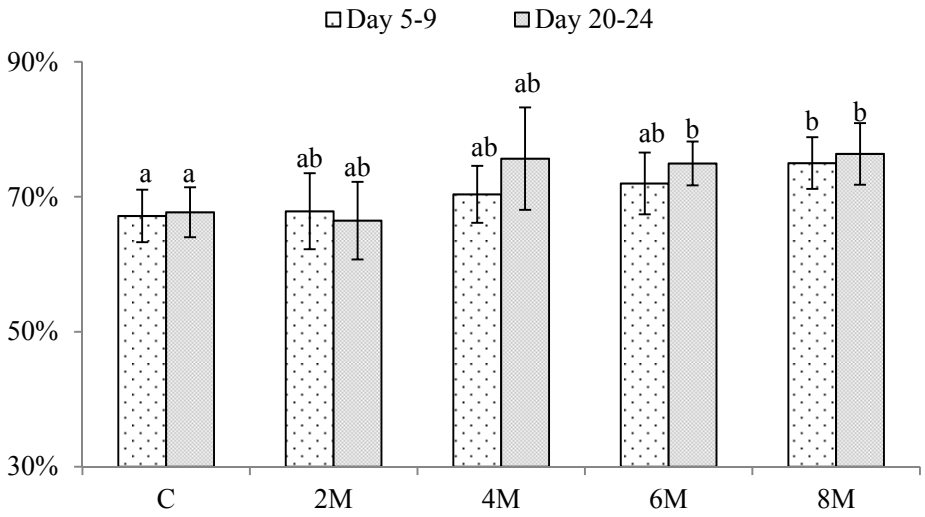
Apparent absorptions of Ca and Mg in Experiment 1 are shown in Fig. 2.1. The results of the repeated measures ANOVA show that apparent absorptions of Ca and Mg

were significantly increased by the mannitol diets but were not affected by the fecal collection time. During days 5-9 of the feeding trial, apparent Ca absorption was significantly higher in the 8% mannitol group than the control group, but not significantly different among the mannitol groups. During days 20-24 of the feeding trial, apparent Ca absorption in the 6% and 8% mannitol groups was significantly higher than in the control group, but not significantly different from the 2% and 4% mannitol groups. Apparent Mg absorption was significantly increased in the 4%, 6% and 8% mannitol groups compared with the control group during days 5-9 and days 20-24 of the feeding trial. During days 5-9 of the feeding trial, among the mannitol groups, apparent Mg absorption in the 8% mannitol group was significantly higher than in the 2% and 4% mannitol groups. During days 20-24 of the feeding trial, among the mannitol groups, apparent Mg absorption in the 8% mannitol group did not differ from the 4% and 6% mannitol groups, but was significantly higher than in the 2% mannitol group.

Figure. 2. 1

A Apparent Ca absorption

Repeated measures ANOVA		
Collection time	Diet	Collection time × diet
0.238	<0.001	0.295



B Apparent Mg absorption

Repeated measures ANOVA		
Collection time	Diet	Collection time × diet
0.638	<0.001	0.087

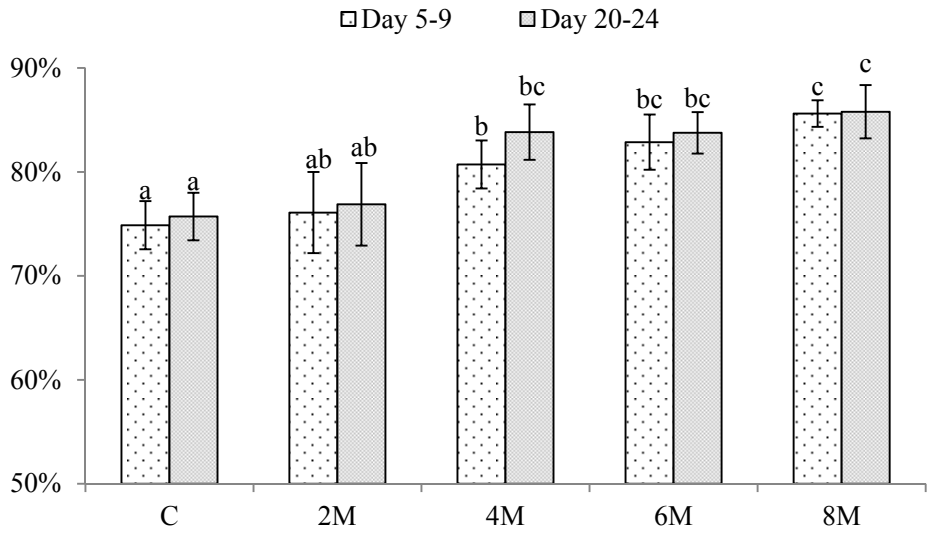


Figure. 2.1 Apparent Ca absorption (A) and Mg absorption (B) on days 5-9 and 20-24 of the feeding trial in rats fed diets containing different levels of mannitol. C: Control diet (AIN-93G); 2M, 4M, 6M, 8M: experimental diets containing 2%, 4%, 6%, or 8% mannitol, respectively (Reeves et al. 1993). Data are mean ± SD (n=7 per group). [a, b, c] Mean values within a row not sharing a common superscript letter differ significantly at $p<0.05$ by Kruskal-Wallis Nonparametric statistical test. Repeated Measures ANOVA was used to test the fecal collection data. Collection time of the feces and the experimental diet were used as factors. Differences were considered significant at $p < 0.05$.

Ca/Cr and Mg/Cr (mol/mol) in cecal digesta and feces are shown in Table 2.4. Ca/Cr and Mg/Cr in cecal digesta were similar among all the experimental groups. Ca/Cr in feces in was significantly lower in the 6% and 8% mannitol groups than in the control group and the 2% mannitol group. Mg/Cr in feces was significantly lower in the 4%, 6% and 8% mannitol groups than in the control group. Among the mannitol groups, Mg/Cr in feces in the 6% and 8% mannitol groups were similar to each other, and significantly lower than in the 2% and 4% mannitol groups.

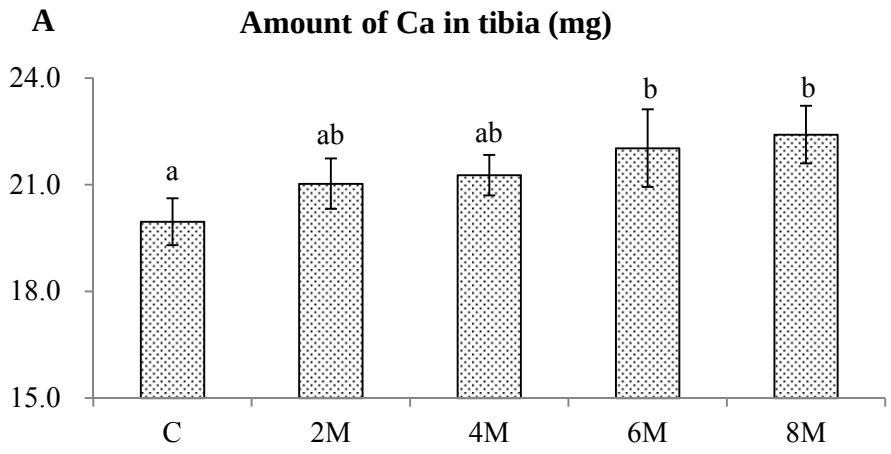
Table 2.4 Ca/Cr and Mg/Cr (mol/mol) in cecal digesta and feces in rats fed a diet containing 0, 2%, 4%, 6% and 8% mannitol

	C	2M	4M	6M	8M
Ca/Cr					
Cecal digesta	24.1±3.5	24.6±4.9	21.4±1.6	20.6±1.9	21.0±4.3
Feces	12.8±1.8 [a]	11.5±2.2 [a]	9.0±2.8 [ab]	8.9±0.9 [b]	7.4±1.8 [b]
Mg/Cr					
Cecal digesta	5.3±1.3	4.6±1.0	4.5±0.7	4.3±0.5	4.1±0.5
Feces	3.2±0.6 [a]	3.1±1.7 [ab]	1.9±0.3 [b]	1.7±0.2 [c]	1.4±0.3 [c]

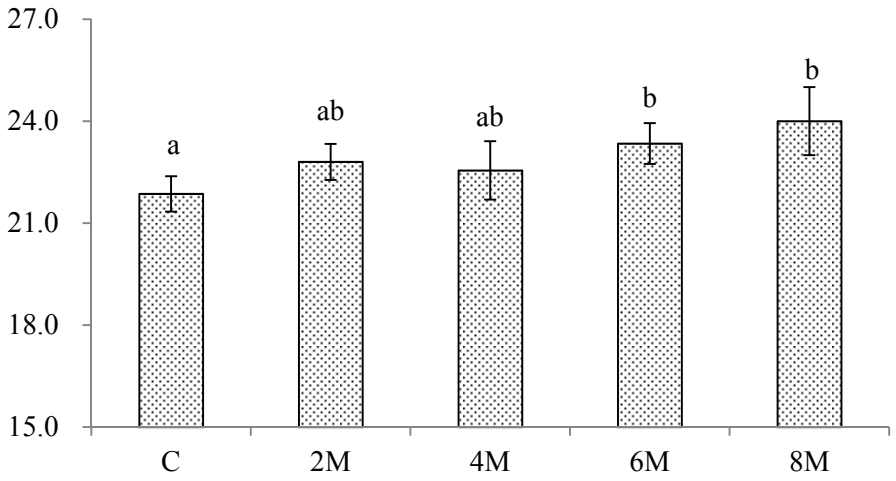
C: control diet (AIN-93G); 2M, 4M, 6M, 8M: experimental diets containing 2%, 4%, 6%, or 8% mannitol, respectively (Reeves et al. 1993); Data are mean ± SD (n=7 per group). [a, b, c] Mean value within a row not sharing a common superscript letter differ significantly at $p<0.05$ by Kruskal-Wallis Nonparametric statistical test.

The amounts of Ca and Mg in the tibias and femurs are shown in Fig. 2.2. The amounts of Ca in the tibias and femurs of the rats were significantly higher in the 6% and 8% mannitol groups than in the control group. The amounts of Ca in the tibias and femurs were not significantly different among the mannitol groups. The amount of Mg in the tibias was significantly higher in the 8% mannitol group than in the control group. There were no significant differences among the mannitol groups. The amount of Mg in the femurs was significantly higher in the 8% mannitol group than in the control group and the 4% mannitol group.

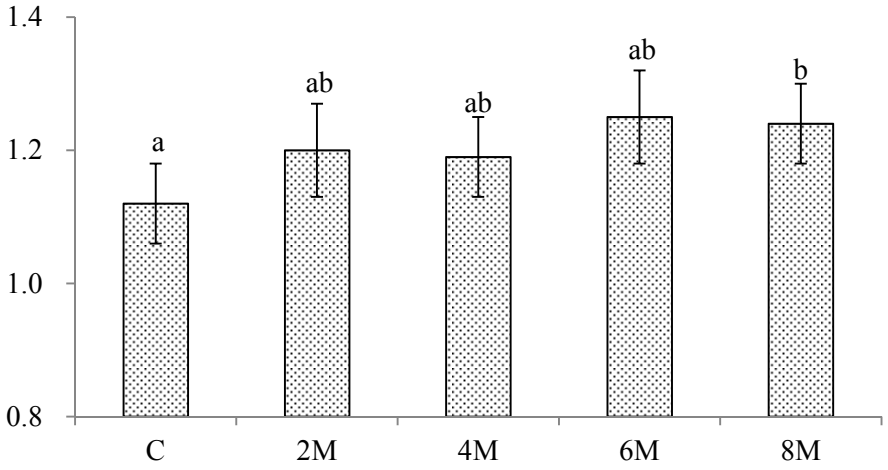
Figure. 2. 2



B **Amount of Ca in femur (mg)**



C **Amount of Mg in tibia (mg)**



D **Amount of Mg in femur (mg)**

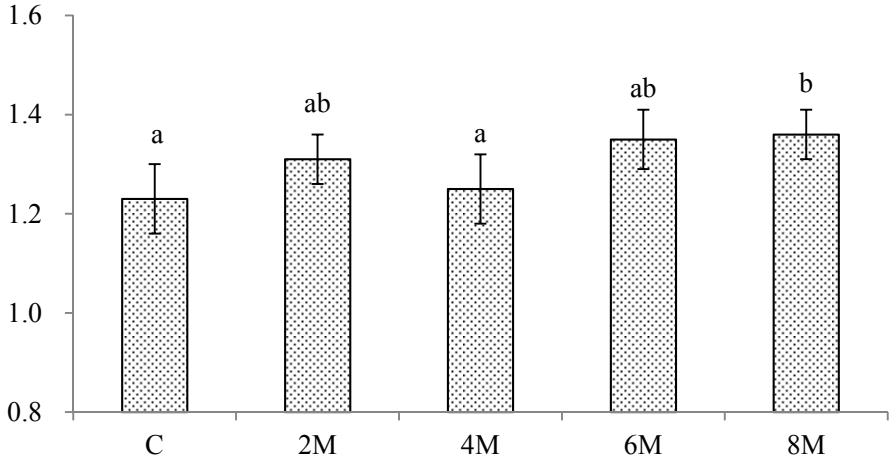


Figure. 2.2 Amounts of Ca in tibias (A) and femurs (B) and amounts Mg in tibias (C) and femurs (D) of rats fed diets containing different levels of mannitol. C: Control diet (AIN-93G); 2M, 4M, 6M, 8M: experimental diets containing 2%, 4%, 6%, or 8% mannitol, respectively (Reeves et al. 1993). Data are mean ± SD (n=7 per group). [a, b, c] Mean values within a row not sharing a common superscript letter differ significantly at $p<0.05$ by Kruskal-Wallis Nonparametric statistical test.

Cecal weights and the composition of cecal contents in Experiment 2 are shown in Table 2.5. In comparison with the control diet, the cecal weights and cecal content weights were significantly and dose dependently increased by the mannitol diets. There were no differences in the weights, wall weights and contents weights of the duodenum, jejunum, ileum, colon and rectum among the experimental groups. Cecal wall weights and the moisture content of the cecal contents were significantly higher in the 8% mannitol group than the control group and the 4% mannitol group. Cecal pH was significantly higher in the 4% and 8% mannitol groups than in the control group. The concentrations of acetic acid in the cecal contents were significantly lower, and the concentrations of lactic acid and isobutyric acid in the cecal contents were significantly higher in the 8% mannitol group than in the control group and 4% mannitol group. The concentration of butyric acid and was significantly increased by the mannitol diets, and the concentration of butyric acid was significantly higher in the 4% mannitol group than in the 8% mannitol group. The concentration of propionic acid in the 4% mannitol group was significantly higher than in the 8% mannitol group, but that in the 4% mannitol group and in the 8% mannitol group was not significantly different from in the control group. The concentrations of succinic acid in the 8% mannitol group was significantly higher than in the control group, but did not significantly differ from the concentrations of succinic acid in the 4% mannitol group. The concentration of total organic acids was not significantly different among the three groups.

Table 2.5 Cecal weights and analysis of cecal contents in rats fed a diet containing 0, 4% and 8% mannitol

	C	4M	8M
Cecal weights (g)	2.99+0.46 [a]	3.79+0.50 [b]	5.18+1.04 [c]
Cecal wall weights (g)	0.55+0.06 [a]	0.67+0.15 [a]	1.04+0.23 [b]
Cecal contents weights (g)	2.43+0.46 [a]	3.11+0.45 [b]	4.14+0.91 [c]
Cecal pH	7.3±0.1[a]	6.7±0.3[b]	6.2±0.2[b]
Cecal content moisture (%)	76.6+0.15 [a]	77.9+0.14 [a]	84.6+0.22 [b]
Concentration of organic acids (μmol/g contents)			
Succinic acid	1.95±1.37[a]	13.85±19.33[ab]	17.71±14.16[b]
Lactic acid	3.05±0.32[a]	3.67±0.44[a]	17.87±1.91[b]
Formic acid	7.24±11.38	4.26±6.73	5.17±5.73
Acetic acid	26.33±10.73[a]	22.00±5.16[a]	8.57±2.90[b]
Propionic acid	9.39±5.31[ab]	10.59±5.20[a]	4.43±1.55[b]
Isobutyric acid	1.70±0.46[a]	1.65±0.58[a]	3.00±0.65[b]
Butyric acid	6.95±1.33[a]	23.44±8.78[b]	12.36±3.17[c]
Isovaleric acid	1.92±0.75	1.38±1.25	1.38±0.86
Valeric acid	1.77±1.14	1.76±1.86	1.69±1.05
Total acid	60.30±5.53	82.59±18.63	72.16±18.07

C: control diet (AIN-93G); 4M, 8M: experimental diets containing 4% or 8% mannitol, respectively (14); Data was mean ± SD (n=7 per group). [a, b, c] Mean value within a row not sharing a common superscript letter differ significantly at $p<0.05$ by Kruskal-Wallis Nonparametric statistical test.

2.5 Discussion

In Experiment 1, feed efficiency was decreased with the 8% mannitol diet. This may have several reasons. Mannitol is a low-caloric food material. A high oral dose of mannitol can reduce the concentration of blood glucose and the level of serum total cholesterol (Mäkinen and Hämäläinen 1985). Mannitol is partly absorbed in the small

intestine, but does not get converted to an energy source (Dwivedi 1991). Sugar alcohols reach the cecum and colon, where they are fermented by the local microbial flora and converted to hydrogen, methane, carbon dioxide, and short chain fatty acids (Bar 1990). Mannitol is a nutrient for gas-producing bacteria in the large intestine (Keighley et al. 1981). Ingested mannitol produces short chain fatty acids and more intestinal gas than usual. The gas produced is considered as an energy loss. The short chain fatty acids are utilized as a source of energy by the host (Roediger 1980), but the available energy of short chain fatty acids is 15-25% less than that of glucose (Billaux et al. 1991).

Sugar alcohols such as mannitol, sorbitol and xylitol could induce osmotic diarrhea when taken in orally in large doses (Mäkinen 1984). The rats in the 6% and 8% mannitol groups presented diarrhea at the beginning of the feeding trial. From the data of the fecal collection period, fecal dry matter excretion was dose-dependently increased by mannitol consumption. Dry matter digestibility in the 8% mannitol group was decreased. In humans, indigestible carbohydrates pass through the small intestine and are fermented in the colon. These results in a number of physiological effects, including an increase in the population of microbial flora, increased fecal weight, and reduced gastrointestinal transit time (Hirayama 2002; Nakaji et al. 2004). Dietary carbohydrates and fibers increase wet and dry stool weight. The increase in stool bulk is attributed to the increased microbial mass resulting from the increase in microbial proliferation using dietary fiber as an energy source in the colon (Chen et al. 2001; Baird et al. 1977). In rats, 95% of orally administered mannitol is fermented by microbes in the large intestine (Hongo et al. 2010). Thus, the increase in fecal excretion may be attributed to the increase of the intestinal bacteria stimulated by mannitol feeding. The increased fecal excretion also may be the result of the lower digestibility of crude fat and crude protein decreased by mannitol feeding (Nishiyama et al. 2009).

The growing rats were used in this study as an experimental animal model with large a requirement for Ca and Mg. In Experiment 1, mannitol was shown to have a stimulatory effect on mineral absorption. Mannitol decreased mineral excretion in feces, and the apparent absorptions of Ca and Mg in rats were increased with the 6% and 8% mannitol diets. In earlier publications, mannose, xylitol, sorbitol, maltitol, lactitol were found to increase Ca absorption (Ammann et al. 1988; Fournier et al. 1955; Hämäläinen 1994; Brommage et al. 1993; Fukahori et al. 1998). The promoting effect of these resistant

sugars on mineral absorption varied depending on the part of the intestine, the type of mineral, the degree of utilization by intestinal microbes, and the dose of sugar administered.

The ratios of Ca and Mg to Cr in cecal digesta and feces in Experiment 1 showed that the absorbability of Ca and Mg in the hindgut was increased by the diets with higher levels of mannitol. Ca and Mg are absorbed from both the small intestine and the large intestine. Resistant sugars produce a high osmotic pressure, which increases the amount of fluid within the lumen to maintain isotonicity, increases the permeability of the intercellular junctions in the small intestine, and causes an increase in passive transfer of soluble minerals in the small intestinal lumen (Bronner 1987; Pansu et al. 1976).

However, many reports have indicated that Ca and Mg were mainly absorbed from the cecum and colon (Ebel and Günther 1980; Allen 1982; Hardwick et al. 1990; Karbach and Feldmeier 1993). The cecum is also the main region of the intestine responsible for indigestible sugar degradation. The sugars are fermented in the cecum by cecal microbes. The high production of short chain fatty acids in the large intestine from the fermentation of indigestible sugars plays an important role in the positive effects of indigestible sugars on mineral absorption (Younes et al. 1996, Levrat et al. 1991).

As a resistant sugar alcohol, mannitol escapes metabolism in the small intestine and reaches the cecum intact, where it is fermented by local microbes to produce organic acids (Hongo et al. 2010). Short chain fatty acids such as acetic, propionic and butyric acids are the main products of bacterial fermentation of resistant carbohydrates in the large intestine. Mannitol is utilized by several intestinal bacteria, such as *Lactobacillus plantarum*, some *bifidobacteria*, *Escherichia coli* and *Streptococcus mutans*, as a primary energy source for growth (Chakravorty 1964; De Vries and Stouthamer 1968; Maryanski and Wittenberger 1975; Neves et al. 2002). Significant decreases in *viridans streptococci* and *bifidobacteria* and a remarkable increase in *fusiform bacteria* in cecum were observed in rats fed a 5% mannitol diet. These were concomitant with an increase in butyric acid concentration and a decrease in acetic acid concentration in the cecum (Morishita 1994). An increase in butyrate production indicates that mannitol is metabolized by intestinal butyrate producing bacteria such as *Clostridium indolis* and *lactobacilli* (Maekawa et al. 2005). The production and distribution of short chain fatty acids depend on the structure and mass of indigestible carbohydrates that are fermented

by certain bacteria species, leading to a corresponding short chain fatty acids pattern. The growth of *Clostridium perfringens* increases lactic acids instead of acetic acids in carbon-rich conditions (Macfarlane and Macfarlane 2003). The decrease in acetic acid formation in Experiment 2 may be explained by the fact that the mannitol was used as a nutrient by the bacteria species that produce lactic acid and butyric acid, resulting in decrease in acetate producing bacteria.

The lower cecal pH was the result of the high production of succinic acid and lactic acid as a result of the 8% mannitol diet. Production of organic acids from bacterial fermentation is concomitant with luminal acidification. The production of lactic acid and succinic acid contributes to the regulation of the luminal pH (Sakata et al. 1999). The higher production of lactic acid contributes to luminal acidification more efficiently than the production of short chain fatty acids in the rat cecum (Hoshi 1994). The decrease in luminal pH increases the concentration of mineral cations (Katharina et al. 2002; Younes et al. 2001). Raschka and Daniel (2005) reported that a lower pH increased the amount of soluble and ionized minerals, which is adequate to its absorption (Raschka and Daniel 2005).

The changes in cecal weight and cecal tissue weight were the result of the fermentation of mannitol by the local microbes in cecum. A lower cecal pH seems to be essential for an increase in cecal tissue weight. The inverse correlation between luminal acidification of the large intestine and the epithelial cell proliferation shows that the lower luminal pH caused by the diet leads to increased epithelial cell proliferation (Lupton et al. 1985). Short chain fatty acids have been shown to stimulate intestinal cell proliferation in vitro and in vivo. Short chain fatty acids, especially butyric acid, accelerate epithelial cell proliferation, thereby increasing cecal tissue weight (Younes et al. 2001; Sakata T 1986; Sakata T 1987; Ichikawa and Sakata 1998; Comalada et al. 2006). Blottière et al (2003) indicated that butyrate, as the major fuel for colonic epithelial cells, promoted intestinal cell proliferation through several processes such as the release of growth factors or gastrointestinal peptides, or the modulation of mucosal blood flow (Blottière et al. 2003).

The osmotic pressure from the production of short chain fatty acids causes cecal development and cecal enlargement, leading to a greater exchange surface area for the absorption of minerals in the cecum (Rémésy et al. 1993; Lopez et al. 2000). Compared with active transcellular transport, the passive paracellular pathway is considered to be the

main route of Mg absorption in the distal part of the intestine. Thus, the enlargement of the intestinal exchange surface area and the increased mineral solubility are likely to be the major effects on Mg absorption (Lopez et al. 2000). Ohta et al indicated that fructooligosaccharides increased the absorption of Ca and Mg. Approximately half of the increase in Ca and Mg absorption occurred in the colon and rectum (Ohta A et al. 1995a). Indigestible sugars increased Ca absorption by stimulating the intestinal epithelium tissue in rat (Bronner 1987; Mineo et al. 2002). The enlarged cecal wall tissue led to a greater exchange surface area for mineral absorption. This may allow a better absorption of Ca and Mg in cecum, especially when soluble Ca and Mg levels are elevated. Short chain fatty acids may directly influence intestinal mineral absorption by stimulating the intestinal epithelium and increasing its absorptive capacity, and by increasing intestinal blood flow and fluid and electrolyte uptake (Topping and Clifton 2001). Short chain fatty acids (e.g. butyrate) produced by fermentation increase the energy supply to intestinal epithelial cells, and regulate the electrolyte exchange of minerals and hydrogen to facilitate mineral absorption by the epithelial cell (Lutz and Scharrer 1991).

The retentions of Ca and Mg in the tibias and femurs were significantly higher in rats fed the diets containing higher levels of mannitol. This seems to be the result of the larger requirement for minerals in growing animals than in adults. Mineral excretion in the urine was not measured in this study. However, the Mg content of bone was not increased while Mg absorption was markedly increased in the 4% and 6% mannitol diet groups. The increase in Mg absorption with the 4% and 6% mannitol diets was likely balanced by increased urinary Mg excretion. Ca and Mg accumulation in bone was increased by their high absorption, even when some of the absorbed minerals were excreted in the urine.

2.6 Conclusion

Our study shows that dietary mannitol increased Ca and Mg absorption and Ca and Mg retention in bone. The most effective dose of mannitol for the bioavailability of both Ca and Mg in the growing rats in this study was 80 mg/kg. We suggest that mannitol fermentation contributed to the Ca and Mg absorption in the large intestine.

Chapter 3 Role of Cecum to the Effect of D-mannitol on Calcium Absorption

3.1 Abstract

To investigate the contribution of cecum to the effect of D-mannitol on Ca absorption and retention, eight-week-old normal growing rats and cecectomized rats were divided into two subgroups of seven to be fed on two different experimental diets containing 0 and 4% D-mannitol for twenty-eight days. Ca balance tests revealed that Ca absorption and retention were significantly increased by cecectomy but not for D-mannitol diet. In the cecectomized rats, Ca absorption and retention with D-mannitol diet was significantly lower than those with control diet. In the normal rats, there is no significant difference in Ca absorption and retention between control diet and D-manntiol diet. In the normal rats, the cecal parameters such as cecal weight, cecal tissue weight and cecal content weight were significantly increased, and cecal pH was significantly lowered. The proportion of short chain fatty acids in cecum was significantly modified in D-mannitol group. Furthermore, cecal soluble Ca was significantly higher in the rats in D-mannitol group in normal rats. These results show that the stimulatory effect of dietary D-mannitol on absorption and retention of Ca was markedly affected by the cecectomy, and the cecal fermentation of D-mannitol plays a decisive role in its effect on the intestinal absorption and retention of Ca.

3.2 Introduction

D-mannitol (mannitol) is a white, crystalline carbohydrate with the formula $C_6H_8(OH)_6$. Mannitol is used as an osmotic diuretic agent generally in medical application, is included in the Food Chemical Codex. Mannitol escapes from absorption in the small intestinal tract. Therefore, when mannitol is used, the rise in blood glucose and insulin secretion is much less than that after sucrose ingestion. Compared to sucrose, mannitol has about 40% caloric value and 50% sweetness in equal mass. Therefore products sweetened with mannitol in place of sugar can control caloric intake and body weight in people with diabetes.

Ca plays the most important roles in bone formation. Approximately 99% of Ca in body is stored in skeleton. The other 1% of body Ca joins in various bodily functions as an essential element for maintaining optimal health. Even dietary intake of Ca is recognized as a key factor in human health and animal production. More attention should be paid to a high rate of Ca absorption and retention when adequate Ca is supplied in food. The diet has been designed so that Ca is supplied with some additives such as vitamin D, casein phosphopeptide, and indigestible oligosaccharides which are well known to have a stimulating effect on intestinal Ca absorption and Ca retention. Fructooligosaccharides (Ohta et al. 1995), lactitol (Ammann et al. 1988) and maltitol (Goda et al. 1995) increase mineral absorption including Ca in human and animal. Mannitol is an indigestible carbohydrate that is only partially absorbed from the small intestine without being metabolized. In the large intestine, especially in cecum, mannitol is metabolized by intestinal bacteria, developing the intestinal environment. Cecum is the highest efficiency absorptive site of Ca in rat intestine (Karbach and Feldmeier 1993). Younes et al (1996) suggested that Ca absorption shifted toward the large intestine when acidic fermentation took place in cecum (Younes et al. 1996). Yet the mechanism of the effect of mannitol on Ca absorption is still unclear.

Therefore, the objective of this study was to compare the effect of mannitol on Ca absorption and retention in the normal rats and the cecectomized rats to observe cecal contribution to Ca absorption in growing rats with mannitol diet.

3.3 Materials and methods

3.3.1 Animals

Twenty eight four-week-old male Wistar rats (Japan SLC, Inc., Shizuoka, Japan) were purchased and housed in individual wire-mesh stainless steel cages in an air conditioned room maintained at 23±1 °C with 50–60% relative humidity, with a constant 12 hours light (7:00–19:00) and 12 hours dark cycle (19:00–7:00). After acclimatization, the half of the rats was cecectomized. After anesthetized the rat, made an incision on abdomen, pull cecum through the incision, placed a ligature around the junction between cecum and colon at the end of ileum, removed cecum, cleaned up the ligature. And put the intestine into abdomen cavity. The musculature and skin were sutured and cleansed.

Four weeks after the cecectomy, both the cecectomized rats and the normal rats

(eight-week-old) were assigned with a randomized block design based on body weight to two treatment groups with seven rats respectively, fed with two different experimental diets (the control diet and the mannitol diet) for twenty eight days. Diets and water were available *ad libitum* during the entire experimental period. All animal experiments were maintained in accordance with the rules and regulations for the care and use of laboratory animals of Okayama University.

3.3.2 Diets

The experimental diets used in this study were as follows: The control diet (C) consisted of standard laboratory chow (AIN-93G) (Reeves et al. 1993) (Table 3.1). Mannitol diet containing 4% mannitol was created by replacement of equal amount of sucrose in the control diet.

Table 3.1 Composition of experimental diets

Ingredients	Con	Man
α-Corn starch (g/kg)	562	562
Casein (g/kg)	200	200
Sucrose (g/kg)	100	60
Corn oil (g/kg)	70	70
Cellulose powder (g/kg)	20	20
Vitamin mix (g/kg)	10	10
Mineral mix (g/kg)	35	35
L-Cystine (g/kg)	3	3
D-mannitol (g/kg)	0	40
Gross energy (kcal/g)	4.94 (Calculation)	

C: control diet (AIN-93G) (Reeves et al. 1993); 4M: experimental diets containing 4% mannitol.

3.3.3 Sample collection and analysis

During the experiment, the amount of food supplied, diet residues and diet waste were measured daily. Feces and urine were collected from day 8 to day 12 and from day 22 to day 26 of the feeding trial to determine their Ca levels. Ca levels in the diet, feces

and urine were determined by flame atomic absorption spectroscopy (FAAS 180-30; Hitachi Ltd., Tokyo, Japan).

At the end of the feeding trial, rats were performed 8 hours fasting followed 3 hours feeding, and were exsanguinated from the celiac artery under anesthesia with diethyl ether. The cecum of the normal rat were collected with its contents, and stored in -30 °C. The pH-value of the contents was measured by a pH meter (TWIN Horiba Ltd., Kyoto, Japan). A part of the cecal contents was used for organic acids measurement by high-performance liquid chromatography (HPLC, Colume: 2 Shim-pach SCR-102H, Detector: Shimadzu CDD-10A; Shimadzu Corporation, Kyoto, Japan). One another part of the cecal contents was oven-dried for 24 hours, comminuted and burned in a 550 °C muffle furnace to determine Ca level. The residue of cecal contents was thawed and centrifuged at 14,000×gat 4°C for 10 min. The supernatant was then used to determine ionized Ca by flame atomic absorption spectroscopy (FAAS 180-30; Hitachi Ltd., Tokyo, Japan).

3.3.4 Ethics

Animals were cared for and sacrificed in accordance with the guidelines for animal experiments at Okayama University, The experimental protocol (No: 51735) was approved by the institutional ethics committee of Okayama University.

3.3.5 Calculation and statistics

Feed efficiency = weight gain / feed intake × 100%

DM digestibility = (DM intake – DM feces) / DM intake × 100%

Daily Ca retention = daily intake – daily fecal excretion – daily urinary excretion

Ca absorption = (intake – fecal excretion) / intake × 100%

Ca retention = (intake – fecal excretion – urinary excretion) / intake × 100%

Data are shown as mean ± SD. Statistical significances among the experimental groups were verified by Tukey–Kramer test using add-in statistic software for Excel (SSRI Co., Tokyo, Japan), and by two-way (cecectomy and mannitol) ANOVA. Significance of differences between the data of cecal parameters in normal rats was analyzed by Student's t test. Differences were considered significant at $p < 0.05$.

3.4 Results

As shown in Table 3.2, body weight, feed intake and feed efficiency were not different among four groups.

Table 3.2 Body weight, feed intake and daily weight gain in rats fed experimental diets for 28 days

	Cecectomized rat		Normal rat	
	Con	Man	Con	Man
Initial BW (g)	172.0±5.0	174.6±13.4	161.4±12.7	167.4±6.5
Final BW (g)	256.9±4.4	252.7±9.2	253.9±9.1	251.8±7.9
Feed intake (g/d)	13.0±0.1	12.9±0.4	12.9±0.3	13.0±0.1
Feed efficiency (%)	23.3±1.4	21.9±2.8	23.9±1.4	23.3±2.0

C: control diet (AIN-93G) (Reeves et al. 1993); 4M: experimental diets containing 4% mannitol. BW: Body weight; body weight. Data are mean ± SD (n=7 per group). Significance of difference between the data was analyzed by Tukey–Kramer test using add-in statistic software for Excel (SSRI Co., Tokyo, Japan). Differences were considered significant at $p < 0.05$.

Mineral balance tests were carried twice from days 8-12 and from days 22-26 to observe the sustainable effects of mannitol consumption and the cecectomy on Ca absorption and retention in the rats.

Ca balance test is shown in Table 3.3. During days 8-12, daily Ca intake and urinary Ca excretion were similar among the groups. Fecal Ca excretion was significantly ($p<0.001$) increased by cecectomy but not for mannitol diet. The result of Tukey-Kramer multiple range test is shown, in cecectomized rats, the rats fed mannitol diet excreted significantly more Ca in feces. Daily Ca absorption and daily Ca retention was significantly ($p<0.001$) decreased by cecectomy. And they were significantly ($p<0.05$) affected by mannitol diet. In the cecectomized rats, compared with control diet, mannitol diet significantly decreased daily Ca absorption and daily Ca retention. Ca absorption and retention was significantly ($p<0.001$) decreased by cecectomy. Ca absorption and retention were significantly ($p<0.01$) affected by mannitol diet. In the cecectomized rats, Ca absorption and retention fed mannitol diet significantly lower than those fed control diet. In the normal rats, fecal Ca excretion, Ca absorption and retention were not statistically different between the rats fed control diet and fed mannitol diet. Significant

(p<0.001) interacting effects of cecectomy and mannitol present were found for increased fecal Ca and decreased Ca absorption and retention in this period.

Table 3.3 Ca balance in the cecectomized rats and in the normal rats fed experimental diet

	Cecectomized rat		Normal rat		ANOVE (P-value)		
	Con	Man	Con	Man	C	M	C×M
Day 8-12							
Intake (mg/d)	53.78±0.05	52.89±1.28	52.31±3.97	52.82±1.58	NS	NS	NS
FE (mg/d)	14.53±2.33^{a}	20.74±4.25^{b}	12.02±1.24^{ac}	9.15±1.52^{c}	***	NS	***
UE(mg/d)	1.50±0.68	2.11±0.76	2.06±0.96	2.05±0.32	NS	NS	NS
Absorption (mg/d)	39.27±2.34^{a}	31.08±2.90^{b}	40.29±2.98^{a}	43.67±2.66^{a}	***	*	***
Retention (mg/d)	37.69±2.24^{a}	28.89±3.52^{b}	38.22±2.84^{a}	41.61±2.73^{a}	***	*	***
Absorption (%)	73.0±4.3^{a}	58.7±5.7^{b}	77.0±1.2^{ac}	82.6±3.2^{c}	***	**	***
Retention (%)	70.1±4.2^{a}	54.6±6.8^{b}	73.1±2.7^{ac}	78.7±3.4^{c}	***	**	***
Day 22-26							
Intake (mg/d)	52.17±0.05	51.10±1.28	52.19±0.89	51.31±1.33	NS	NS	NS
FE (mg/d)	16.05±2.35^{a}	19.28±2.83^{a}	10.36±2.54^{b}	9.27±2.27^{b}	***	NS	*
UE(mg/d)	0.73±0.14^{a}	1.17±0.37^{ab}	1.48±0.67^{b}	1.39±0.38^{b}	**	NS	NS
Absorption (mg/d)	36.14±2.38^{a}	31.84±1.84^{b}	41.83±2.62^{c}	42.04±3.24^{c}	***	*	*
Retention (mg/d)	35.23±2.31^{a}	30.34±1.50^{b}	40.35±2.77^{c}	40.65±3.29^{c}	***	*	*
Absorption (%)	69.2±4.5^{a}	62.4±4.4^{b}	80.1±4.9^{c}	81.9±4.8^{c}	***	NS	*
Retention (%)	67.5±4.4^{a}	59.6±4.1^{b}	77.3±5.0^{c}	79.2±5.1^{c}	***	NS	*

Con: control diet (AIN-93G) (Reeves et al. 1993); Man: experimental diets containing 4%

mannitol. FE: Fecal excretion; UE: Urinary excretion; C: Cecum; M: Mannitol. Data are mean ± SD (n=7 per group). Significance of difference between the data was analyzed by Tukey–Kramer test (a, b, c, $p < 0.05$) using add-in statistic software for Excel (SSRI Co., Tokyo, Japan), and by two-way (cecectomy and mannitol) ANOVA (*$p < 0.05$,** $p < 0.01$,*** $p < 0.001$).

During days 22-26, there was no significant difference on daily Ca intake in all rats. Fecal Ca was significantly ($p<0.001$) increased, and urinary Ca were significantly ($p<0.01$) decreased by cecectomy but not mannitol diet. Daily Ca absorption and daily Ca retention was significantly ($p<0.001$) decreased by cecectomy. Mannitol diet affected daily Ca absorption and daily Ca retention significantly ($p<0.05$). In the cecectomized rats, mannitol diet led significantly lower daily Ca absorption and daily Ca retention than control diet. In the normal rats, there was no significant difference between control diet and mannitol diet. Ca absorption and retention of this period was significantly ($p<0.001$) decreased by cecectomy but not for mannitol diet. But the result of Tukey-Kramer multiple range test is shown, in normal rats, Ca absorption and retention were similar in the two experimental diet groups. In cecectomized rats, Ca absorption and retention fed mannitol diet were significantly lower than that fed control diet. Significant ($p<0.05$) interacting effects of cecectomy and mannitol present were found for increased fecal Ca and decreased Ca absorption and retention during days 22-26.

Table 3.4 shows the cecal parameters in normal rats. Cecal weight, cecal tissue weight and cecal content weight were significantly increased in the rats fed mannitol diet. Cecal pH was significantly lower in mannitol group than in control group. The concentrations of total organic acids in cecum of the rats in control group and mannitol group were not markedly different, but the significant influence of mannitol consumption was shown in the concentration of short chain fatty acids. The ratios of acetic acids to propionic acids to butyric acids in cecum were about 33:10:4 in control group and 28:14:10 in mannitol group. Amount of Ca in cecum was in an equal level. Amount of cecal soluble Ca was significantly higher in mannitol group. The percentage of cecal soluble Ca in cecal Ca was markedly increased by the diet with mannitol.

Table 3.4 Cecal parameters in normal rats fed experimental diet for 28 days

	Con	Man
Cecal weight (g)	3.33±1.19	4.35±0.72*
Cecal tissue weight (g)	0.33±0.04	0.38±0.03*
Cecal content (g)	3.00±1.15	3.97±0.71*
Moisture (%)	78.6±0.02	77.5±0.02
Cecal pH	7.6±0.3	7.1±0.2*
Cecal organic acids concentration (μmol/g contents)		
Succinic acid	1.18±15.17	12.02±22.82
Lactic acid	1.18±0.51	1.50±0.96
Formic acid	0.13±0.05	0.20±0.12
Acetic acid	32.80±6.39	28.23±4.51*
Propionic acid	10.06±1.79	14.49±4.69*
Isobutyric acid	5.12±3.52	6.01±6.21
Butyric acid	4.18±4.35	10.07±7.63*
Isovaleric acid	1.47±0.75	2.11±1.41
Valeric acid	1.81±0.52	1.46±1.20
Total acid	54.01±14.65	75.89±30.52
Cecal total Ca (μmol/cecum)	2616±902	2689±543
Cecal soluble Ca (μmol/cecum)	331±102	457±101*
Cecal soluble Ca/total Ca (%)	12.46±4.32	17.78±6.57*

Con: control diet (AIN-93G) (Reeves et al. 1993); Man: experimental diets containing 4% mannitol. Ca: Ca. Data are mean ± SD (n=7 per group). Significance of difference between the data was analyzed by Student's t test. Differences were considered significant at $p < 0.05$.

3.5 Discussion

It is well known that higher dose of mannitol administration causes diarrhea in human and animals. Carbohydrate-induced diarrhea results from insufficient hindgut fermentation capacity with large quantity of carbohydrate intake (Soergel 1994). Mannitol lingers in the small intestine and in the large intestine, elevates net water absorption to lead a diarrhea because of its intestinal permeability (Krugliak et al. 1994). In this experiment, the cecectomized rats were more susceptible to diarrhea with mannitol diet,

because it lacked cecal fermentation, and the rate of luminal fluid absorption in cecum was reduced. The decision of mannitol level added to the diet in this experiment was decided based on the toleration of mannitol level that induce an incessant diarrhea in the cecectomized rats. 4% mannitol diet caused diarrhea in the cecectomized rats in the first few days, and these rats recovered in three or four days after the beginning of the feeding trial.

We previously demonstrated that 6% and 8% dietary mannitol increased Ca absorption in the large intestine, raised retention of Ca in tibia and femur in growing rats. Promoted mineral absorption and retention with a resistant sugar in a diet in rats was commonly connected tightly with its fermentation by intestinal microbe in the large intestine, especially in cecum in rats (Remesy et al. 1993; Katharina et al. 2002; Miyazato et al. 2010). Mineral absorption may be related to cecal fermentation more firmly in rats than human. It is well known that fructooligosaccharide can increase Ca absorption in rats. Van den Heuvel et al. (1998) observed no effect of ingestion of oligofructose and inulin on Ca and Fe absorption in human adults (Van den Heuvel et al. 1998). In rats, mannitol flows through the small intestine, reach cecum and is fermented by the cecal microbe, produce short chain fatty acids (Hongo et al. 2010). Short chain fatty acids can be considered the most effective factor to elevate mineral absorption in cecum. Short chain fatty acids accelerate cecal epithelial cell proliferation (Blottière et al. 2003; Sakata T 1987; Ichikawa and Sakata 1998; Comalada et al. 2006), cause the osmotic pressure in cecum to stimulate cecal expansion (Sakata 1986), thereby lead a great exchange surface area for mineral absorption. Products of the acids from bacterial fermentation are concomitant with luminal acidification. The lower luminal pH can increase the concentration of the cations of mineral in the intestine to stimulate mineral absorption (Younes et al. 1996; Raschka and Daniel 2005). Also, short chain fatty acids may directly influence intestinal mineral absorption by regulating electrolyte exchanges between Ca and hydrogen to facilitate Ca absorption by the epithelial cell (Lutz and Scharrer 1991). It was reported that the large intestine might be the main site of Ca absorption when acidic fermentation occurred, and the acidic fermentation in cecum increased Ca absorption in the large intestine in rats (Younes et al. 1996).

In this study, mannitol get fermented in cecum, produced organic acids, led lower cecal pH which increased Ca solubility, and led cecal enlargement to provide extensive

surface area for Ca absorption. It seemed that cecal Ca absorption was promoted by mannitol, even though Ca absorption of the whole intestinal tract was not increased significantly. Mannitol may reduce Ca absorption by binding to Ca and escaping the absorption in the small intestine in normal rats. When Ca is not sufficiently absorbed in the small intestine, the large intestine compensates for the deficiency (Shiga et al. 1998). In this study, 4% mannitol diet did not increase Ca absorption and retention significantly in the normal rats. The cecal fermentation in 4% mannitol diet was less than that in 6% and 8% (Chapter 2). 8% mannitol diet in Experiment 2 (Chapter 2) caused lower cecal pH than 4% mannitol diet, and the cecal weight and the cecal tissue weight were about 1.5 times heavier than those in rats fed with 4% mannitol diet. The increased Ca absorption in cecum by 4% mannitol consumption was not enough to lead the increment of Ca absorption of the whole intestinal tract, when the rate of Ca absorption in small intestine was decreased by mannitol consumption.

Feeding of fructooligosaccharide diet increased the absorption of Ca in the sham-operated rats, but not in the cecectomized rats, suggesting that the promoting effect of fructooligosaccharide on Ca absorption depended on cecum (Ohta et al. 1994). From the results, Ca absorption was decreased by the cecectomy. Cecum is the site that the highest rate of Ca absorption takes place in rats (Karbach and Feldmeier 1993). Without cecum, the rate of Ca absorption was deducted. Mannitol added in diet, inducing much lower Ca absorption and retention in the cecectomized rats. The reasons for the lower Ca absorption induced by dietary mannitol were considered to occur in the small intestine and the colon. Carbohydrates may bind to Ca and reduce its absorption in the small intestine. However, if the carbohydrate is fermented, bound to Ca may be liberated to be absorbed in the large intestine, whereas insufficiently fermented carbohydrates may keep binding to Ca in the colon (Trinidad et al. 1996). Dietary fiber causes faster transmit time and shorter retention time presenting disadvantage to the complete fermentation in colon (Munakata et al. 1995; Islam et al. 2004), Mannitol fermentation in colon is not as complete as in cecum, with lower Ca absorption in this site. The limited Ca absorption in the small intestine and colon was showed in response to the lower Ca absorption in cecectomized rats.

3.6 Conclusion

After the cecectomy performed in the rats in this study, dietary mannitol decreased Ca absorption and retention in the cecectomized rats. 4% mannitol diet did not increase Ca absorption and retention markedly in normal rats, although the cecal fermentation of mannitol produced short chain fatty acids, and induced cecal enlargement and cecal acidification that made Ca soluble. It can be concluded that mannitol decreases Ca absorption and retention without cecum in rats, the effect of mannitol on Ca absorption depends on its cecal fermentation.

Chapter 4 General Discussion

In this study, it was demonstrated that mannitol could increase intestinal mineral absorption and mineral retention in body in growing rats. The mechanism is related to the fermentation of mannitol in cecum.

Indigestible sugars are commonly contained in natural products. They are sweet and are used widely to reduce the calories in foods and drink. Sorbitol and xylitol are common ingredients in "sugar-free" candies and chewing gum, and large quantity fructooligosaccharides are produced, and consumed as typical functional food materials. Oligosaccharides and sugar alcohols do not get metabolized in the small intestinal, do not increase blood glucose and insulin secretion as sucrose. As the low or non-caloric food materials, indigestible sugars are the best sweetener for the large population with obesity and diabetes.

Mannitol is partly absorbed in the small intestine, but do not increase blood glucose and is not converted to an energy source (Mäkinen and Hämäläinen 1985; Dwivedi 1991). Most of ingested mannitol is fermented by several intestinal bacteria (Chakravorty 1964; De Vries and Stouthamer 1968; Maryanski and Wittenberger 1975; Neves et al. 2002) in the large intestine to produce organic acids (Hongo et al. 2010). Short chain fatty acids (such as acetic, propionic and butyric acids) and succinic acids and lactic acids are the main products of mannitol fermentation.

In this study, mannitol showed a stimulatory effect on mineral absorption. Mannitol decreased mineral excretion in feces and the apparent absorptions of Ca and Mg in rats were increased with the 6% and 8% mannitol diets. It is reported that indigestible sugars causes an increase in passive transfer of soluble minerals due to a high osmotic pressure, which increases the permeability of the intercellular junctions in the small intestine (Bronner 1987; Pansu et al. 1976). However the ratios of Ca and Mg to Cr in cecal digesta and feces in Experiment 1 (Chapter 2) showed that the absorbability of Ca and Mg was increased by the diets with higher levels of mannitol in the large intestine, but not in the small intestine.

In rats, cecal fermentation is the main degradation of indigestible sugar. The fermentation of mannitol mainly formed short chain fatty acids, succinic acids and lactic acids in Experiment 2 (Chapter 2). Short chain fatty acids cause cecal development and

cecal enlargement by stimulating epithelial cell proliferation (Younes et al. 2001; Sakata T 1986; Sakata T 1987) and by the osmotic pressure from the production of short chain fatty acids in cecum. The pattern of organic acids from mannitol fermentation showed that the concentration of acetic acids was decreased and the concentrations of butyric, succinic, and lactic acids were increased. Butyric acid is the major fuel for colonic epithelial cells, promoting intestinal cell proliferation (Blottière et al. 2003). Cecal development and cecal enlargement provide a greater surface area for the absorption of minerals in the cecum (Rémésy et al. 1993; Lopez et al. 2000). Cecal acidification might be led by the higher concentration of succinic acid and lactic acid in cecum. A lower pH increased the amount of soluble and ionized minerals, which is in favor of mineral absorption (Raschka and Daniel 2005). In addition, short chain fatty acids might increase intestinal mineral absorption by stimulating the intestinal epithelium and increasing its absorptive capacity, and by increasing intestinal blood flow and fluid and electrolyte uptake (Topping and Clifton 2001).

In Chapter 3, it was indicated that 4% mannitol ingestion led a lower cecal pH which was concomitant with the higher soluble Ca in cecum. However 4% mannitol diet decreased Ca absorption and retention in cecectomized rats, also did not increase Ca absorption and retention significantly in the normal rats. The reasons for the lower Ca absorption may be related to Ca absorption in the small intestine and the colon. Carbohydrates may bind to Ca and reduce its absorption in the small intestine (Trinidad et al. 1996). In colon, faster transmit time and shorter retention time led by fiber limit the complete fermentation in colon (Munakata et al. 1995; Islam et al. 2004), decreasing Ca absorption in this site. As a result, there is no change on Ca absorption of the entire intestine even if Ca absorption may be increased in cecum with 4% dietary mannitol. Cecal fermentation in 4% mannitol diet was less than that in 6% and 8% mannitol diets in Chapter 2. 8% mannitol diet caused lower cecal pH than 4% mannitol diet, and the cecal weight and the cecal tissue weight were about 1.5 times heavier than those in the rats with 4% mannitol diet. It is shown that the higher level of mannitol has more significant effect on cecal fermentation than the lower level of mannitol. The promoting effect of mannitol in Ca absorption depends on the level of mannitol in diet.

Chapter 5 Summary

The effect of large intestinal fermentation on mineral bioavailability, especially Ca and Mg, is one of beneficial properties of indigestible sugars. Dietary fibers may shift the major site of mineral absorption (Ca and Mg) towards the large intestine. The intestinal pH, the cecal surface area and the concentration of organic acids in the large intestine are closely associated with the effects of fermentable carbohydrates on intestinal mineral absorption. Thus it is necessary to take microbial fermentation into account when investigating the effect of indigestible sugars on mineral absorption and its possible mechanisms in the large intestine. Indigestible sugars escape from the metabolism in the small intestine and reach the large intestine to be a major source of complex carbohydrates for the intestinal microflora in animals or humans. Organic acids such as acetic, propionic, butyric and lactic acids are the main end production of the bacterial fermentation of indigestible sugars in the large intestine, which allow the recovery of part of their energy. It is also considered that short chain fatty acids play important roles in increasing mineral absorption.

In this research, higher level of dietary mannitol significantly increased the absorption of Ca and Mg in the large intestine and their retention in bone. Ingestion of mannitol caused cecal fermentation and decreased cecal pH, and led an increase in the concentration of corresponding short chain fatty acids like succinic, lactic and butyric acid, and a decrease in the concentration of acetic acids. When the cecectomy was performed in rats, it caused a significant decrease in Ca absorption. In the normal rats, mannitol diet significantly increased soluble Ca in cecal content. These results indicated that mannitol escapes metabolism in the small intestine and reaches the cecum intact, and is fermented by local microbes to produce organic acids which were responsible for the lower cecal pH. Production of organic acids from bacterial fermentation also induced luminal acidification. The decrease in luminal pH increases the concentration of mineral cations. Short chain fatty acids from mannitol fermentation in cecum stimulated intestinal cell proliferation, especially butyric acid. The lower luminal pH and the presence of short chain fatty acids were the factors that led the cecal enlargement, as well as the increase in osmotic pressure from the production of short chain fatty acids. Thus, the positive effect of mannitol on Ca and Mg absorption in rats can be concluded as the result of the

enlargement of the intestinal exchange surface area and the increased mineral solubility. This research indicates that mannitol, as a low caloric food material, can increase the absorption of Ca and Mg, and increase mineral retention in body. Mannitol fermentation in cecum provides a promoting effect on Ca and Mg absorption in the large intestine.

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