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Bacteriological Effects of Xylitol and Different Carbohydrate Containing Diets in Swiss Albino Rats Inoculated with *Streptococcus mutans* CCUG 6519

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Abstract: The aim of the study was to investigate the bacteriological effects of different diets by *Streptococcus mutans* counts on 50 Swiss albino rats inoculated with *Streptococcus mutans* CCUG 6519 serotype c. A powdered form of standard basal diet meeting rats' nutritional needs was used in combination with diets containing different percentages of starch, sucrose and xylitol for 90 days. Dental plaque samples were collected at the end of the experiment and *S. mutans* and total bacterial counts were determined. *S. mutans* and total bacterial counts in plaque samples were the highest in the sucrose group and the differences were statistically significant ($p<0.001$). Similarly, caries lesions were higher in this group when compared with the others. The addition of 5% xylitol instead of 5% sucrose to the diet lowered the *S. mutans* counts, and the differences between the xylitol and sucrose groups were statistically significant ($p<0.001$). In addition, *S. mutans* counts and caries lesions were lower in the latter group. The addition of xylitol to diet decreased the number of *S. mutans* and caries lesions in rats, confirming that xylitol as a sucrose substitute cannot be fermented by *S. mutans*.

Key Words: *Streptococcus mutans*, Sucrose, Xylitol, Starch, Rat diet

Introduction

Dental caries is one of the most common chronic diseases of mankind. Investigations showed that the caries incidence was lower in communities that consume local agricultural products (1), while the high consumption of sugar and sweets by people living in the cities represents an increase in caries incidence (2). It is now generally accepted that there is a direct correlation between sugar consumption and caries prevalence (1,3,4).

Besides dental caries, consumption of sucrose leads to a high calorie intake that causes side effects such as diabetes and obesity. Although the evidence to support the cariogenic potential of sucrose is clear, it is unrealistic to limit its consumption without any alternatives (3,5,6).

The primary aim in the prevention of dental caries is to inhibit acid formation, since acids produced by fermentation of carbohydrates with oral microorganisms are the main causal factor. For this reason, considerable interest has developed in the use of relatively non-cariogenic sweetening agents as a substitute for sucrose (3,7). In caries prevention, after fluoride, xylitol, a sugar alcohol, receives special interest. There are several studies that have investigated its effects on cariogenicity (8-13).

The aim of this study is to investigate the bacteriological effects of xylitol in rats and to evaluate the substitution of sucrose in diet. In our study, albino rats were fed different carbohydrate diets, and cariogenicity was investigated by bacteriological analysis and microscopy.

Materials and Methods

Experimental design

In our study, 50 weanling Swiss albino rats (22-24 days old) weighing 25-30 grams were used. Before the experiment, the rats were marked and grouped according to sex. In order to suppress their oral flora, water with ampicillin (200 mg/L) was given for 2 days (14). On the 2nd day, except in the last group, all animals were inoculated once with a suspension of *Streptococcus mutans* CCUG 6519 serotype c of human origin (15,16). The conditions of the cages and the environments of the animals were standard. After the first two days, diet and drinking water were available ad libitum. The animals were weighed weekly throughout the experiment period.

Dental plaque samples from the fissure and approximal areas of the rat teeth were collected at the

end of the experiment period. Plaque samples collected with sterile wooden toothpicks (17,18) from the fissures of the mandibular molars were placed in 100 ml flasks that contained 10 ml 0.05 M phosphate buffer with 0.4% (w/v) KCl (pH 7.1). One toothpick was used per molar tooth. Sterile scissors were used to cut off the sample-containing ends of the toothpicks. Interproximal plaque samples collected with dental floss from the mandibular incisors were placed in tubes containing 10 ml 0.05 M phosphate buffer with 0.4% (w/v) KCl (pH 7.1).

Afterwards, the rats were decapitated under ether anesthesia. Their upper and lower jaws were dissected and kept in 4% formalin for further analysis for caries formation.

Powdered and pellet form diets were used. All powdered form diets included 'standard basal diet' in order to provide the essential nutrient intake of the rats. Table 1 shows the composition and nutrient values of the basal diet that made up 50% of the diets that were used in groups I, II, and III.

Group I: The rats were inoculated with *S. mutans* CCUG 6519 strain and fed a powdered diet that included 50% standard basal diet and 50% soluble corn starch. The experimental group was composed of 10 rats.

Group II: The rats were inoculated with *S. mutans* CCUG 6519 strain and were fed a powdered diet that included 50% standard basal diet, 25% soluble corn starch, 20% sucrose and 5% glucose. The experimental group was composed of 10 rats.

Table 1. Composition (in %) of the standard basal diet (50% of the total diet).

Raw material	%	Nutrients	%
Skim milk	42.00	Dry material	88.97
Soy-bean meal	15.00	Raw protein	22.00
Sawdust	10.00	Raw oil	12.00
Silicon dioxide	5.40	Raw cellulose	9.20
Soy-bean oil	9.00	Calcium	1.98
Vegetable oil	4.00	Phosphorus	1.61
Di- calcium-phosphate	6.40	Metabolic energy (kcal/kg)	2528.40
Potassium chloride	1.82		
Sodium chloride	0.74		
Sodium carbonate	0.90		
DL-methionin	0.20		
Vitamin and mineral mixture	4.54		

Group III: The rats were inoculated by *S. mutans* CCUG 6519 strain and fed a powdered diet that included 50% standard basal diet, 25% soluble corn starch, 15% sucrose, 5% glucose and 5% xylitol. The experimental group was composed of 10 rats.

Group IV: The rats were inoculated with *S. mutans* CCUG 6519 strain and were fed a laboratory diet in pellet form. The experimental group was composed of 10 rats.

Group V: The rats were not inoculated with *S. mutans* CCUG 6519 strain and were fed a laboratory diet in pellet form. The experimental group was composed of 10 rats.

Microbiology

S. mutans CCUG 6519 serotype c lyophilized culture was opened in aseptic conditions, and was suspended with Tryptone Soya Broth (Oxoid, CM129) and left for 30 minutes to dissolve. The dissolved suspension was cultured in a tube with Tryptone Soya Broth and inoculated in anaerobic conditions (95% N_2, 5% CO_2) at 37ºC for 24 hours. At the end of the incubation period, bacterial culture was transferred to Tryptone Soya Broth and incubated for 24 hours for activation as described above. The activated bacterial cultures were then incubated on slant Tryptone Soya Agar (Oxoid, CM131) under the same conditions and maintained at +4ºC as stock cultures. They were transferred every 2 weeks for maintenance (17).

Activated cultures of *S. mutans* CCUG 6519 strain were inoculated in 300 ml Tryptone Soya Broth and incubated under anaerobic conditions at 37ºC for 18 hours. Then cells were centrifuged in aseptic conditions at 5000 rpm for 5 minutes (SIGMA-Gmbh 2-15). The supernatant was decanted. Cells were suspended in 10 ml 0.85% (w/v) NaCl solution and centrifuged again (19). The washing procedures were repeated twice. Bacterial cells were then stirred in 20 ml isotonic NaCl suspension with a vortex and absorbance was measured by spectrophotometer at 550 nm (Pharmacia LKB. Novaspec II), viable bacteria counts in these suspensions were done by the pour plate method (20) and were determined to be $6.8x10^9$-$7.0x10^9$cfu / ml.

Plaque samples collected in flasks were mixed for 30 minutes by shaker and tube contents were mixed for 60 seconds with a vortex. Then the samples were diluted and cultured in the appropriate dilution on human blood agar (Oxoid, CM55) and MSB agar [Mitis Salivarius Agar (Difco) with 20% (w/v) sucrose and 200 U/L bacitracin]

(21) and incubated in anaerobic conditions (95% N_2, 5% CO_2) at 37ºC for 3 days (12). Then the counts of total bacteria and *S. mutans* on blood agar and MSB agar were determined.

Caries Scoring

Teeth were investigated with the aid of a magnifying glass for the presence of dental caries on the removed jaws. Then all of the half jaws were embedded in polyester. Samples were abraded up to half of the bucco-lingual distance. Polished surfaces of the samples were investigated by binocular light microscope (Prior A216, Prior Scientific Instruments Ltd).

Statistical Analysis

Bacterial counts were converted to log 10 values. Bacterial counts from plaque samples of different locations and from different experimental groups were compared by variance analysis.

Results

Bacteriological Findings

Median and range values of total bacterial counts in all experimental groups are represented in Table 2. Statistical analysis revealed that there were statistically significant differences among the total bacterial counts obtained from different experimental groups ($p<0.001$). The highest bacterial count was detected in the second experimental group where the diet contained 20% sucrose. Experimental groups III, IV, V and I followed this group, in decreasing order.

Mean values and standard errors of total bacterial counts in all experimental groups are represented in Table 3. There was a statistically significant difference among the total bacterial counts obtained from anterior and posterior plaque samples ($p<0.001$). There were higher bacterial counts in the posterior plaque samples. There was a statistically significant correlation among experimental groups and the origin of the plaque sample ($p=0.010$).

There was a statistically significant difference among the total bacterial counts obtained from the 2nd and 3rd experimental groups ($p<0.001$). There was also a statistically significant correlation among the aforementioned experimental groups according to the region of the plaque sample. Median and range values of *S. mutans* counts in all experimental groups are represented in Table 4. There was a statistically significant difference between groups IV and V.

Table 2. Median and range values of total bacterial counts in all experimental groups.

	Plaque samples from anterior region (cfu/plaque sample)		Plaque samples from posterior region (cfu/plaque sample)	
Groups	Median	Range	Median	Range
I	$2.5x10^4$	$1.5x10^3$-$1.5x10^5$	$1.28x10^5$	$5.0x10^3$-$3.0x10^5$
II	$5.33x10^5$	$7.0x10^4$-$4.9x10^6$	$1.78x10^6$	$4.0x10^5$-$6.4x10^6$
III	$1.9x10^5$	$1.5x10^4$-$1.25x10^6$	$2.33x10^5$	$7.0x10^4$-$2.5x10^6$
IV	$5.8x10^4$	$1.5x10^4$-$6.5x10^5$	$1.1x10^5$	$4.5x10^4$-$5.0x10^5$
V	$5.0x10^4$	$5.0x10^3$-$1.5x10^5$	$1.73x10^5$	0-$6.6x10^3$

Table 3. Mean values±standard errors of total bacterial counts on blood agar.

Groups	Anterior Region	Posterior Region	Anterior+Posterior Region
I	4.3902±0.5756	4.9979±0.4879	4.6940±0.6057
II	5.7791±0.6425	6.2752±0.4470	6.0271±0.5957
III	5.1564±0.5999	5.4691±0.5054	5.3127±0.5632
IV	4.9181±0.4753	5.0549±0.3020	4.9865±0.3938
V	4.5953±0.4641	5.1463±0.5040	4.8708±0.5498
Total	4.9678±0.7227	5.3887±0.6468	5.1782±0.7144

Table 4. Median and range values of *S. mutans* counts in all experimental groups.

	Plaque samples from anterior region (cfu/plaque sample)		Plaque samples from posterior region (cfu/plaque sample)	
Groups	Median	Range	Median	Range
I	$2.0x10^2$	$5.0x10^1$-$4.5x10^2$	$2.25x10^3$	$5.0x10^2$-$2.5x10^4$
II	$6.6x10^3$	$3.3x10^2$-$8.4x10^4$	$4.0x10^4$	$1.75x10^3$-$4.8x10^5$
III	$2.2x10^3$	$2.5x10^2$-$1.24x10^4$	$4.0x10^3$	$1.0x10^3$-$2.3x10^4$
IV	$5.9x10^2$	$4.5x10^1$-$2.85x10^3$	$3.5x10^3$	$2.0x10^3$-$6.9x10^3$
V	$1.3x10^1$	0-$2.0x10^3$	3.5x101	0-$6.6x10^3$

Table 5. Mean values±standard error of *S. mutans* counts on MSB Agar.

Groups	Anterior Region	Posterior Region	Anterior+Posterior Region
I	2.2440±0.3391	3.4829±0.5703	2.8635±0.7826
II	3.7175±0.6658	4.6088±0.6934	4.1631±0.8042
III	3.2308±0.6906	3.5756±0.4771	3.4032±0.6042
IV	2.6721±0.5462	3.5341±0.1818	3.1031±0.5938
V	-0.8937±3.5941	-0.5707±3.8718	-0.7322±3.6397
Total	2.1941±2.3029	2.9262±2.4995	2.5601±2.4192

Mean values and standard errors of *S. mutans* counts in all experimental groups are represented in Table 5. There was a statistically significant difference among all experimental groups ($p<0.001$). The highest *S. mutans* count was detected in group II and groups IV, I, and V followed, in decreasing order. There was a statistically significant difference among the *S. mutans* counts obtained from anterior and posterior plaque samples ($p<0.001$). There was a higher *S. mutans* count in the posterior plaque samples from all groups except the 5th group. There was sparse or no *S. mutans* growth in the 5th group.

There was a statistically significant difference among groups II and III according to *S. mutans* counts and the origin of the plaque samples ($p<0.001$). There was also a statistically significant difference between groups IV and V according to *S. mutans* counts. However, there was a statistically significant difference in *S. mutans* counts according to the origin of the plaque samples in group IV but not in the experimental group V.

Statistical analysis revealed that differences in weight were statistically significant among groups ($p<0.001$). Time had a statistically significant effect on weight gain ($p<0.001$). At the end of the experiment, the highest weight gain was detected in groups IV and V, followed by groups I, II and III in decreasing order.

Microscopic Findings

All the caries lesions detected involving the enamel and none penetrated the dentine. No caries lesions were found in the 5th experimental group. The highest number of caries lesions was found in the 2nd group. Although not very common, there were some approximal caries lesions as well.

Discussion

Animal studies are reliable in investigating the effects of diet and dietary components on dental caries in man. Dental caries will not commence without certain dietary components based on the oral microbiologic flora or host resistance. Today, these dietary components are called 'caries experiment diet' instead of 'cariogenic diet' (22). Diet 2000 and diet 580 (16,23-25) have been used as highly cariogenic diets widely in several rat and hamster studies where deep fissure caries lesions have been developed in 42 days, but it was found that they do not contain the necessary nutrients for the animals' needs (26). These findings have led investigators to search for a diet that contains the necessary nutrients and can be used to test the cariogenic effects of sugar alcohols. In our study, the SSP diet developed by Havenaar et al.

(26), which satisfies the essential nutrients and is not highly cariogenic, was modified and used as the basal diet (Table 1). This diet had the advantage of changing the carbohydrate percentages in the diet. Studies have shown that the SSP diet does not have the problems of diet 2000 (26). In our study, the observations of glossy rat fur, normal feces, and proportional growth showed that the SSP diet was properly nourishing the rats.

Havenaar et al. (26) reported that xylitol often caused rats to suffer from recurrent diarrhea. Studies have shown that the addition of 3% or 6% xylitol to the diet was the tolerable limit for rats (24-26). Cariology experiments have shown that the addition of 6-10% xylitol to the diet or drinking water of rats retards body weight gain (24,27). In our study, 5% sucrose was replaced with xylitol and the body weight gain was similarly lower in the xylitol group. Only the rats fed pellet form laboratory diet had dense and dark colored feces and their weight gain was higher when compared with the other groups.

Several studies reported that *S. mutans* serotype c was the predominant type in dental plaque (12,15,17,19,26,28,29). In our study, human originated *S. mutans* CCUG 6519 serotype c strain was used to inoculate rats. Microbiological findings of the rat studies reported that total bacterial counts were higher in the approximal plaque samples when compared with fissure plaque samples and *S. mutans* was not present in the non-inoculated group (26). Similarly, in our study, higher bacterial counts were detected in approximal samples in inoculated groups, whereas very little or no *S. mutans* was found in the non-inoculated group. Havenaar et al. (26) reported that there was no statistically significant difference in the xylitol group whereas, in our study, *S. mutans* counts were statistically lower in the xylitol group when compared with the sucrose group. Havenaar et al. (26) found that there was no correlation between the concentration of sucrose and *S. mutans* levels.

S. mutans CCUG 6519 was not given to group V. Rats, even if at lower levels, possess this bacteria because they can be exposed to *S. mutans* in various ways when grown in an environment that is not germfree.

Studies on *S. mutans* inoculated animals have shown that sucrose is the most effective carbohydrate in causing smooth surface caries lesions (30-32). Karle and Gehring (33) investigated the microbiota of plaque samples from rats that were fed diets containing sucrose, sorbitol, xylitol or fructose and reported that *S. mutans* was highly present in the sucrose group. They also reported that there was no *S. mutans* in plaque samples from the xylitol group. Our study also agreed with these findings.

Studies that investigated the cariogenic effect of different carbohydrates reported that the starch diet did not cause high caries activity (26). In this study, *S. mutans* levels and caries lesions in the starch group were lower than the sucrose group. Furthermore, the studies reported that addition of xylitol to diet decreases caries formation. Havenaar et al. (26) reported that all caries lesion types showed a decline in the 5% xylitol group. Mühlemann et al. (34) reported that addition of 15-25% xylitol to diet 2000 reduced the fissure caries lesions. Leach and Green (24) reported that the addition of 6% xylitol to diet Stephan 580 also causes a reduction in caries formation in comparison to sucrose. There is caries reduction with a change from the sucrose to the sucrose-xylitol diet, and an increase was seen in the reverse case. In this study, caries lesions were not very deep and these findings were in agreement with the findings of Havenaar et al. (26).

Xylitol can not be fermented by most oral microorganisms (5,12,35-37) and it was found to be more effective in caries reduction than sorbitol and mannitol (8-38). It is therefore advisable to use xylitol as a sucrose substitute in order to prevent dental caries (39-41). In this study, substitution of 5% sucrose with xylitol caused a decrease in *S. mutans* counts of dental plaque samples, and the difference was statistically significant. These findings were attributed to xylitol.

In conclusion, it is proven that the standard basal diet was suitable for cariology tests in rats. It was possible to show the different cariogenic effects of polyols and food substitutes in diet. Our bacteriological findings showed that the addition of xylitol to diet reduces the total bacterial and *S. mutans* counts in the dental plaque, thus resulting in fewer dental caries lesions. It is concluded that, as reported by previous studies (9,11,12,25,26), the addition of proper amounts of xylitol to diet is an effective method for preventing dental caries.

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