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The investigation of effects of sweeteners in biological systems *in vivo*

PhD thesis

(Summary)

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Introduction

Sweet taste is almost universally regarded as the most pleasant experience. Our reaction to sweet substances is an innate preference. Sugar is one of the most important raw materials used in food industry, further to its sweet taste its hypertensive solution decreases the freezing point of food; increases the boiling point and viscosity; helps yeast fermentation and improves the look and taste of food products. The greater part of the consumed sugars are consumed with food products as hidden sugar, while dietary sugar is just a tiny portion of this amount.

Simple carbohydrates are very important, they supply the body with quickly usable energy. The consumption of too much simple sugar may lead to tooth decay, obesity and different obesity related diseases (diabetes type 2, cardiovascular diseases, cancer, musculoskeletal disorders). To replace the sweet taste and decrease energies from simple sugars one may use sugar substitutes. Sugar substitutes are sugar derivatives. They are incompletely absorbed and metabolized by the body, and consequently contribute fewer calories.

Xylitol does not raise the blood glucose level like regular sugar does. Overdose of xylitol may cause side effects such as bloating, diarrhea.

Sweeteners can be used to decrease energy, substitute the sweet taste, to decrease or maintain body weight. The risk of using additives is that after adding them to food products they become part of them. All the additives sold and used in products have passed a licensing process, nevertheless, we still hear about substances that are harmful to our health.

Several novel studies suggest that sweetener consumption may be followed by increased food intake and can cause overweight or obesity, while other studies refute the decreased or unchanged ratings of hunger or food intake.

As a result of consumers' negative feelings towards artificial sweeteners the demand for stevia, a natural non-caloric sweetener seems to be rapidly increasing.

From the toxicological point of view sweeteners are the most examined and most controversial additives in the food industry.

Objectives

1. Some studies have shown that the consumption of food products containing artificial sweeteners may increase appetite, thus they may lead to increased food intake, and weight gain. Other researches do not agree with these results. In the first part of our experiment our aim was to determine during a 25 week long animal experiment whether the consumption of artificial sweeteners have an effect on body weight, and nutrition habits of the animals.

2. The most controversial intense sweetener is the aspartame, which converts into its three components into the body: aspartic acid, phenylalanine and methanol. According to some reports the metabolits eliminate through the normal metabolic pathways, but some researchers claim they may have a cumulative effect, and the consumption may be a hazard. In our study we aimed to characterize the effect of aspartame consumption on the expression of *Adh1, Adh3, Adh4* genes considered as markers of biological effects.

3. Xylitol is a polyalcohol which can be used as a replacement for sugar and may be useful in diabetic diets, because it is considered as a low glycemic sweetener. In this study we would like to characterize the effects of xylitol on blood glucose in regular feeding and starvation.

4. The use of natural sweeteners are tend to be more popular to replace the sweet taste Most scientific studies have found no adverse health effects of xylitol and stevia ingestion. These studies investigated the main components of stevia separately. The aim of our "short-term" experiment was to investigate the effect of xylitol and stevia consumption on the expressions of key oncogenes and a tumor supressor gene. The sweeteners are available in trade.

Materials and methods

Effects of artificial sweeteners on body weight, food and drink intake

A total of 72 CBA/Ca inbred female and male mice were used. They were approximately 5-6 weeks of age at the beginning of each experiment. The male and female mice were separated and were housed in cages. Group sizes were 6 mice for each cage. The mice were kept in standard circumstances. Mice were given oral doses of water solutions of artificial sweeteners (saccharin, cyclamate based table top sweetener, acesulfame-K based table top sweetener, aspartame) the amount of maximum ADI ad libitum and a sugarfree instant fruit flavoured drink . The controls received only tap water with the same drinking conditions as the treated groups. The mice were fed chow ad libitum. Food consumption was estimated by subtracting the amount of food left on the grid and the amount of spilled food from the initial weight of food supplied. Water and solution intake was calculated on the basis of the difference between the 300 ml and amounts of consumed fluid at a certain time of the week. We measured body weight individually and food consumption once a week, water and solution intake of artificial sweeteners two times a week. Our experimental period lasted 25 weeks. Our applied table top sweeteners are available in trade.

The effect of aspartame consumption on Adh1, Adh4, Adh3 gene expression

"*Short term" experiment*
A total of 12 female CBA/CA inbred mice were used. They were 5-6 weeks of age at the beginning of the experiment. The female mice were housed in cages. Group sizes were 6 mice. The mice were kept in standard circumstances. Mice were given oral doses of water solutions of table top artificial sweeteners aspartame the amount of maximum acceptable daily intake ad libitum. The controls received tap water with the same drinking conditions as the treated groups. The mice were fed standard chow for rodents ad libitum. All the groups were autopsied after 14 weeks.

Subchronic experiment
Six-week-old 12 female and 12 male inbred mice were used. The male and female mice were separated and were kept in cages. Group sizes were 6 mice for each cage. The mice were maintained at standard circumstances. Mice were given oral doses of water solutions of table top artificial sweeteners aspartame the amount of maximum acceptable daily intake ad libitum. The controls received tap water with the same drinking conditions as the treated groups. The mice were fed standard chow for rodents ad libitum. All groups were autopsied after 25 weeks.
In both experiments the bone marrow, the kidneys and the liver of the animals were removed and 80 mg from each tissue was pooled separately according to groups. The samples were homogenized, then total RNA was isolated from each organs using MagNa Pure® LC compact automatic isolation system (Roche Berlin, Germany) according to the manufacturer's

instructions. For quantitative analysis of mRNA levels real-time PCR was performed in a LightCycler® 2.0. instrument (Roche Diagnostics GmbH Mannheim, Germany) with LightCycler 4.0. software. Reverse transcription and PCR amplification were done with SYBR Green protocol according to the manufacturer's instructions. Primers for *Hprt1* (housekeeping gene), *Adh1, Adh3, Adh4* were selected by Integrated DNA Technologies (Bio-Scienses). All the primers were designed by Primer Express™ Software (Applied Biosystems). Gene expression alteration were calculated by the comparative C_T (threshold cycle) method ($\Delta\Delta C_T$ method, Applied Biosystems). The comparative CT method gives the amount of target gene normalized to an endogenous reference gene (*Hprt1)* and to a relative calibrator sample.

The effect of xylitol consumption on plasma glucose level

A total of 24 female and male F-344 rats were given oral doses of water solution of xylitol powder (at dose of 1,4 g xylitol powder in 300 ml tap water), and equivalent amount of saccharose ad libitum. The male and female rats were separated and were housed in cages. Group sizes were 4 rats for each cage. Before the beginning our experience fasting blood glucose levels were measured in each animals. The controls received only tap water ad libitum. The rats were fed standard chow for rodents ad libitum. We measured blood glucose levels in the first, second, third, sixth, twelfth hour, in the first, second, third, and in the sixth day.

The effect of xylitol consumption on plasma glucose level during starvation

A total of 24 female and male F-344 rats were used. The male and female rats were separated and were housed in cages. Group sizes were 4 rats for each cage. Rats were divided into three groups. The groups were received the same sweeteners in the same amount as in the previous experiment. Before the beginning our experience fasting blood glucose levels were measured in each animals. The controls received only tap water ad libitum. We measured blood glucose levels in the first, in the second and in the third hour.
Accu-Chek Active® blood glucose meter system (Roche) was used for the measurements.

The effects of stevia and xylitol consumption on certain onco/suppressor genes expression in vivo

A total of 24 BALB/c female mice were used. Mice were divided into four groups. Group sizes were 6 mice for each cage. The first group was given oral doses of water solution of 97% pure stevia powder (it contains of equivalent amount of stevioside and Rebaudioside A) the amount of maximum acceptable daily intake ad libitum. The second group was given oral doses of water solution of saccharose the amount of daily recommended.
The third group was given oral doses of water solution of xylitol, equivalent amount as the saccharose. The controls received tap water with the same drinking conditions as the treated groups. The mice were fed standard chow for rodents ad libitum.
The mice were autopsied after 1 week. The liver, spleen, kidneys, bone marrow, thymus, lymph nodes were removed and 100 mg samples of each tissue were pooled separately according to groups. The samples were homogenized, then total RNA was isolated from each organs using TRIZOL reagent (Sigma-Aldrich, Budapest). Quality of the isolation RNA was checked by absorption photometry at 260/280 nm. RNA of each sample (10 µg) was dot-blotted onto Hybond N+ nitrocellulose membranes (ECL kit, Amersham, Little Chalfont, UK) and hybridized with chemiluminescently labelled specific probes for c-myc, p53 and Ha-ras (Professor J. Szeberenyi, University of Pecs, Hungary) genes. The RNA isolation, hybridization and detection were performed according to the manufacturer's instructions. The

signals were detected on X-ray films. The dots were evaluated by Quantiscan software (Biosoft, Cambridge, UK). We used β-actin activity as an endogenous control. Gene expression is reported as percentages relative to the level of the expression of β-actin rate.

Statistical analysis

We used independent two-sample t-test with software Microsoft Excel 2007. We described the degree of relationship between drink consumption and food intake with correlation model in the groups. In all cases, $p < 0,05$ was considered to be statistically significant.

Results

Effects of artificial sweeteners on body weight, food and drink intake

In the female group consuming saccharin we found significant differences in weight gain (p=0,0495) compared to the control group. Aspartame, cyclamate and acesulfam-K administration did not affect the weight changes in the treated female mice, according to the control group.

In the female group the low-calorie instant powder drink administration caused significant weight loss (p= 0,00005), than in the control.

There were no differences in food intake among the female groups consuming aspartame, saccharin, cyclamate and the control group.

In the female group the administration of acesulfame-K caused significantly lower food intake (p=0,0424) than in the control group.

The consumption of the low-calorie instant powder drink significant decreased the food intake in females (p=0,0015) compared to the control.

In the saccharin treated females we found near significantly higher drink intake (p=0,052) according to the control group.

Aspartame, cyclamate, acesulfame-K consumption did not increase the drink intake in treated female groups. The females consuming low-calorie instant powder drink we detected significantly lower drink intake than in the control.

During the study where male mice were administrated cyclamate (p=0,0034) and the saccharin (p=0,0304) they gained significantly more weight than the control.

The body weight gain did not differ in the aspartame the acesulfame-K and the low-calorie instant powder drink consuming groups compared to the control group.

There were no significant differences in food intake between the male with top sale sweeteners treated groups and the control group.

We detected significantly lower food intake (p=0,034) in the males treating low-calorie instant powder drink than in the control.

The consumption of aspartame, saccharin, cyclamate did not affect the drink intake in the treated male groups. In males in the acesulfame-K group the drink consumption increased considerably (p=0,000165) compared to the controls. Our results showed significantly lower drink intake (p=0,00002) in the instant powder drink consuming males than in the control males.

The effect of aspartame consumption on Adh1, Adh3, Adh4 gene expression

We found that administration of aspartame had an effect even in the shorter term experiment but it was less than in the longer term experiment.

In the longer term experiment there were considerable differences in alcohol-dehydrogenase genes alteration between the control and the treated groups both in males and females.

In the female group consuming aspartame the expression of *Adh1* was higher in the liver and kidney than in the control group. In our result we monitored nearly ten times higher expression level of *Adh1* in the liver of the treated male compared to the control group.

The consumption of aspartame caused in females nearly fifteen times higher expression level of *Adh3* in the liver according to the control. Fifty times higher overexpression was detected in the expression of *Adh3* gene in the treated females in the kidneys.

We detected in the treated male nine times higher expression level of the *Adh3 gene* in the liver, four times higher expression level in the kidneys and nearly three times expression level in the bone marrow, than in the control group.

The administration of aspartame increased the expression of *Adh4* gene in the kidneys and caused three times higher expression level in the bone marrow. Remarkable overexpression of *Adh4* gene *was* detected in the liver of the treated female compared to the controls. Slight increase of the expression of *Adh4* gene was also observable in the bone marrow of males after the aspartame treatment. We detected nearly 12 times higher expression level of the *Adh4* gene in the liver than in the control group.

The effect of xylitol on plasma glucose level

The xylitol treatment of F-344 male and female rats caused slow raise in blood glucose according to the controls.

We found similar results during starvation, the xylitol rise slowly the blood glucose level in both sexes, compared to the controls. The changes in blood glucose level induced by xylitol were in normal range.

The effects of stevia and xylitol consumption on certain onco/suppressor genes expression in vivo

We detected increased level of the *p53* gene expression in the liver, in the spleen, in the thymus, in the stevia administrated group than in the control one. Our results showed three times higher expression level of *p53* gene in the thymus and in the bone marrow of the xylitol treated group. In the liver of xylitol treated female mice the expression level of *p53* gene was also increased.

The consumption of stevia caused six times higher expression level of *c-myc* oncogene in the lungs, three times higher expression level in the in the liver, nearly five times expression level in the spleen, and twice higher in the thymus according to the control mice.

In our results the expression of *c-myc* gene was three times higher in thymus, five times higher in the bone marrow and six times higher in the lungs in the xylitol treated group, than in the control.

In both treated groups the sweeteners had no effect on *Ha-ras* oncogene expression in all investigated tissues.

We found multiple times level of *K-ras* gene expression in the lungs, in the kidneys, in the liver and in the spleen of the stevia treated group.

We observed considerable alteration in the expression of *K-ras* oncogene in a tissue with high proliferation rate (thymus) between xylitol treated and control groups.

Our results showed increased *bcl-2* oncogene alteration in all investigated tissues of the xylitol and stvevia treated group, compared to the controls.

Discussion

Effects of artificial sweeteners on body weight, food and drink intake

Our study focused on the effect of intense sweeteners on body weight, food and drink intake in mice. Despite the fact that saccharin consumption caused greater weight gain in both sexes than in the controls; the food intake of saccharin group did not increase. The saccharin administration of female mice caused significantly higher drink intake according to the control group.
According to some studies the researchers stated that normally, sweet tastes indicate that the body is about to receive a lot of calories, and the digestive system prepares to react. When sweet tastes are not followed by lots of calories, as in the case of artificial sweeteners, the body becomes conditioned against a strong response and unable to control the food intake, and need more calories.
Aspartame administration did not affect the nutritional habits and weight changes in both sexes groups. It should be noted that during the autopsy of female and male mice significant amount of fat was deposited around the organs. 2 mice of the treated females deceased but we could not determine the cause of death.
Male mice administered cyclamate gained significantly more weight, but the nutritional habits although did not change. We used top sale sweeteners. Cyclamate was mixed with saccharin in 10:1 ratio of cyclamate to saccharin. This mix provides the intense sweetness of saccharin combined with the ability of cyclamate to lessen the bitter aftertaste of saccharin. Although cyclamate tastes are sweet to humans, mice do not experience it as sweet neither showed preference for it.
The consumption of acesulfame-K did not affect the weight, but significantly decreased the food intake in females and significantly increased the drink intake in males.
In the female group the low-calorie instant powder drink administration caused significant weight loss, and in both sexes significantly decreased the food and drink intake compared to the control groups. We identified three fungi species from the prepared powder therefore it is possibble, the fungi played role in the nutritional habits and the weight changes.
These results question the effect of non-caloric artificial sweeteners on weight-maintenance or body weight decrease. Intense sweeteners are part of a weight-control program; they could aid calorie control by providing palatable foods with reduced energy. It must be clear that consumption of intense sweeteners are really helpful in weight-maintenance or body weight decrease.

The effect of aspartame consumption on Adh1, Adh3, Adh4 gene expression

In our present studies we aimed to characterize the effect of methanol derived from aspartame consumption on the expression of *Adh1, Adh3, Adh4* genes in CBA/Ca mice. The first study lasted 14 weeks long, the longer term experiment lasted 25 weeks long.
After 14 weeks long aspartame treatment had no or little effect on the expression of examined genes.
The 25 weeks long aspartame administration caused greater alteration of the investigated genes than in a shorter term experiment.

The investigated genes regulate the kinetics of metabolizing enzymes in the body. The notable expression of the investigated genes allows us to state that aspartame has a detectable biological effect

The higher expression levels, caused by consumption of aspartame, do not increase clearly the activity and the alteration of alcohol-dehydrogenase and formaldehyde-dehydrogenase.

The notable expression of *Adh1, Adh3, Adh4* genes caused by the longer term feeding of aspartame, can imply the faster clearance of methanol and formaldehyde as toxic metabolits from the body.

In the shorter term aspartame consumption the activity of the alcohol-dehydrogenase enzymes is low, raises the question, can the accumulation of the metabolits be a cause for the increased enzyme activity or multiple level gene expression?

Does the permanently high intake of aspartame cause such changes in the enzyme activity, which decreases the elimination of the metabolits from the body?

Could there be a risk for diabetics, dieters or children, caused by the accumulation of metabolits derived from longer term aspartame administration?

Our results may be the base of further experiments. Our aim to investigate the effect of longer term aspartame feeding on the alteration of *Adh1, Adh3, Adh4* genes and on the changes of the alcohol-dehydrogenase enzymes activity and the plasma level of the metabolits.

The effect of xylitol on plasma glucose level

Using a "short term" animal model our aim was to determine the effect of xylitol administration on blood glucose. The xylitol treatment of F-344 male and female rats caused slow raise in blood glucose according to the controls; also the consumed food also affected the glucose level.

We found similar results during starvation, the xylitol rise slowly the blood glucose level in both sexes, compared to the controls. The changes in blood glucose level induced by xylitol were in normal range.

Other studies have shown the same results.

Xylitol is slowly absorbed in the body it does not cause an excessive rise in blood glucose.

According to our results, xylitol is good alternative for diabetics, but it has to be counted to the daily carbohydrate intake.

The effects of stevia and xylitol consumption on certain onco/suppressor genes expression in vivo

We examined the effects of stevia and xylitol on modifications of early molecular epidemiological biomarkers in a „short term" animal experiment developed by the Department of Public Health Medicine

A significant expression of the investigated genes can indicate a carcinogen exposure, which would have a negative effect on the cells' life, thus they can be considered exposure markers.

In our "short term" experiment we detected multiple times higher expression levels of *p53* gene, *c-myc* gene, *K-ras* gene induced by the administration of stevia or xylitol. In both treated groups the sweeteners had no effect on *Ha-ras* oncogene expression in all investigated tissues. Our results showed increased *bcl-2* oncogene alteration in all investigated tissues of the xylitol and stevia treated group, compared to the control.

Based on the expression changes of the investigated genes we can state that the examined sweeteners have a detectable effect, they may influence, or may play a role in the beginning of carcinogenesis. The results may indicate carcinogenic exposure.

We monitored increased gene expression alteration in the same tissues with a high proliferation rate which had shown significantly increased occurrence of malignancies in the long term feeding carcinogenicity bioassay on one other intense sweetener consumption.
Our results may be the base of further "long term" feeding carcinogenicity bioassay with a larger numbers of sensitive animals.

Due to the small numbers of animals our results can be considered as only a pilot study, but because of the major role of sweeteners in human diet it is important to preannounce our results.

Summarising our new results

1. Our study focused on the effect of intense sweeteners on body weight, food and drink intake in mice. In the female and male group consuming saccharin we found significant differences in weight gain, but the food intake did not increase compared to the control groups. The saccharin administration of female mice caused significantly higher drink intake according to the control group.
Aspartame administration did not affect the nutritional habits and weight changes in both sexes groups.
Male mice administered cyclamate gained significantly more weight, but the nutritional habits although did not change.
The consumption of acesulfame-K did not affect the weight, but significantly decreased the food intake in females and significantly increased the drink intake in males.
In the female group the low-calorie instant powder drink administration caused significant weight loss, and in both sexes significantly decreased the food and drink intake compared to the control groups.

2. After 14 weeks long aspartame treatment had no or little effect on the expression of *Adh1, Adh3*, *Adh4* genes. The 25 weeks long aspartame administration caused sizeable alteration of the examined genes in both sexes groups compared to the controls. Our results may be the base of further experiments

3. The xylitol treatment caused slow raise in blood glucose according to the controls. We found similar results during starvation, the xylitol rise slowly the blood glucose level compared to the controls. The changes in blood glucose level induced by xylitol were in normal range.

4. In our "short term" experiment the stevia and the xylitol treatment caused notable expression of *p53* gene, *c-myc* gene and *K-ras* gene. Based on the expression changes of the investigated genes they may influence, or may play a role in the beginning of carcinogenesis. Our results may be the base of further "long term" feeding carcinogenicity bioassay.

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