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EXPERIMENTAL STUDY

Improved efficacy of prebiotic by flaxseed oil and horse chestnut in experimental colon cancer

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Abstract: *Objectives:* This experimental work was designed to investigate the efficacy of prebiotic by itself and in combination with *Hyppocastani extractum* siccum, and *Lini oleum* virginale on selected parameters in rats with dimethylhydrazine induced colon cancer.
Methods: Rats were randomly divided into 5 experimental groups of 12 rats each. Rats were fed with high fat (HF) diet containing 10 % of fat, supplemented by prebiotic at a dose of 2 % of HF diet itself and in combination with *Hyppocastani extractum* siccum at a dose of 1 % of diet and *Lini oleum* virginale at a dose of 2 % of diet. Two weeks after the start of the diet dimethylhydrazine injections in dose 20 mg/kg b.w.were applied (DMH, Merck, DE), two times at week interval. The activity of ß-glucuronidase, concentration of lipid parameters, bile acids and short chain fatty acids were determined.
Results: Prebiotic and its combinations with selected substances significantly decreased the activity of bacterial enzyme ß-glucuronidase ($p<0.001$). Bile acids concentration was significantly decreased ($p<0.01$) excepting combination of prebiotic with Horse chestnut. Self applied prebiotic decreased ($p<0.001$) lipids parameters (total cholesterol and triacylglycerols), and enhanced short chain fatty acids production.
Conclusion: Prebiotics have protective effect and may be the useful candidate agents for colon cancer prevention and treatment. The application of selected bioactive food components supported the effect of prebiotics *(Tab. 2, Fig. 1, Ref. 16)*. Full Text in free PDF *www.bmj.sk.*
Key words: colon cancer; prebiotic; flaxseed oil; Horse chestnut; rats.

Lifestyle factors, especially dietary intake, affect the risk of colorectal cancer (CRC) development. Suitable risk biomarkers are required in order to assess the effect that specific dietary components have on CRC risk. In order to achieve optimal digestion, absorption, and nutritional health, we must have appropiate populations of positive microflora. One of the mechanism increasing the number of beneficial bacteria in the gut is ingestion of prebiotics. Prebiotics are defined as selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health (Roberfroid, 2000). Prebiotics can complement function of probiotic or synbiotics and might represent a novel therapeutic or preventive agents (Rafter et al, 2007; Geier et al, 2006).

The aim of the presented experiment was to evaluate the efficacy of prebiotic-inulin alone and its combination with *Hyppocastani extractum* siccum as nutrional plant extract, and *Lini oleum* virginale on the activity of β-glucuronidase, concentration of lipid parameters, bile acids and short chain fatty acids (SCFA) in rats with dimethylhydrazine induced colon cancer. In addition to chemically induced colon cancer as one of the risk factors for the development of colon cancer and other civilization diseases we recognized high intake of dietary fat.

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Material and methods

Animals

We used six months old Wistar albino rats (n=60), (Central vivarium, Faculty of Medicine, PJ Safarik University, Košice, Slovak Republic), with mean body weight 372.57±15.01 g. The animals were housed in plastic cages with tops and maintained at 22 °C, on 12 h light/dark cycle, according to the principles provided in the Law No 23/2009 of Slovak Republic for the Care and Use of Laboratory Animals. Animals were fed with high fat diet (HF) containing10% of fat (Biofer, Slovak Republic) as the diet of some western population at risk for colon cancer, supplied with drinking water *ad libitum*. Food and drinking consumption were monitored daily. The rats were randomly divided into 5 groups of 12 animals each. Control group (CG) was a group with the highest risk (cumulative effect of DMH and HF diet) for development of colon cancer without administration of prebiotic and bioactive food components. In the experimental group 1 (EG1) prebiotic-inulin (PRE) was administered, in the experimental group 2 (EG2) a combination of prebiotic and *Hyp-*

pocastani extractum siccum (PRE + HES) was applied, whereas in experimental group 3 (EG3 a combination of prebiotic and *Lini oleum* virginale (PRE + O)) was given. The latest experimental group 4 (EG4) was administered a mixture of all selected substances (PRE + HES + O). Animals in EG1, EG2, EG3, and EG4 were fed with HF diet abreast with DMH.

Treatments

Treatment consisted of oligofructose-enriched inulin (PRE, BeneoSynergy 1, ORAFTI, Tienen, Belgium) at a dose of 2 % of HF diet. Inulin is a natural commercialized food ingredient extracted from the chicory root and composed of a mixture of long-chain inulin and short-chain oligofructose. Extract of *Aesculus hippocastanum* L., (HES, Calendula, Slovak Republic) was applied at a dose of 1 % of HF diet. Most of the beneficial effects of the extract of *Aesculus hippocastanum* L., (Hippocastanaceae) commonly known as Horse chestnut are attributed to its principal component beta-escin or aescin. *Lini oleum* virginale (Dr. Kulich Pharma, Czech Republic) at a dose of 2 % of HF diet is obtained from flaxseed *Linum usitatissimum* L. containing a high amount of polyunsatured fatty acids (PUFA).

Two weeks after begining the diets, rats were treated with procarcinogen N,N dimethylhydrazine (DMH, Merck, Germany), at a dose of 20 mg/kg s.c., two times a weekly interval, dietary treatments were continued during the entire experiment. In the end of the eight week the rats were anaesthetized (Ketamine 100 mg/kg + Xylazine 15 mg/kg b.w., i.p.), blood samples were taken from the heart by puncture and feaces samples from the colon of animals.

Laboratory analysis

Blood samples were centrifuged at 2500 G for 15 min. and serum specimens were used for determination of bile acids concentration with a commercial kit (Trinity Biotech, Ireland), and lipid parameters concentration with a commercial kits Biolatest (Czech Republic). The measurement was carried out on an automatic spectrophotometric analyser Cobas Mira S (Roche, Schwitzerland). Freshly collected faeces samples were examined for enzymatic activity of bacterial enzyme β-glucuronidase using an API-ZYM kit (Biomérieux, France). Activities were determined according to the manufacturer's instructions and expressed on the scale of 0 (negative reaction) to 5 (maximum activity). The SCFA were analyzed in the colon contents using gas chromatography Hewlett Packard (USA).

Statistical analysis

Statistical analysis was performed by Student's *t*-test and analysis of variance (ANOVA) to determine the significance. Statistical significance was accepted at $p<0.05$.

Results

The mean body weight of the rats at the begining of the experiment was 372.57±15.01 g and in the end of the experiment elevated to 398.16±33.19 g ($p<0.05$) with variances among groups. In the control group (CG) the mean body weight of rats increased by 2.1 % (395.83±74.40 g), by 2.8 % (360.83±47.19 g) in EG 1, by 0.8 % (378.33±57.66 g) in EG 2, by 10.9 % (405.0±75.75 g), and by 17.2 % (450.83±70.13 g) in experimental group EG 4. Food consumption was changed in relationship to body weight of rats. All rats were killed six weeks after the first DMH injection. The changes in activity of β-glucuronidase in control group and experimental groups are summarized in Figure 1. Inulin treatment (EG1) significantly decreased ($p<0.001$) the activity of β-glucuronidase as compared to the control group. The tendency changes in activity of ß-glucuronidase were similar in EG2, EG3, and EG4. Bile acids concentration was significantly decreased ($p<0.01$) in experimental groups except for EG2 as shown in Table 1. The total cholesterol and triacylglycerols concentration was decreased significantly in EG1 ($p<0.001$), and nonsignificantly in EG2, EG3, EG4 (Tab. 1). The changes in concentration of short chain fatty acids (butyric, acetic, and propionic) are summarized in Table 2.

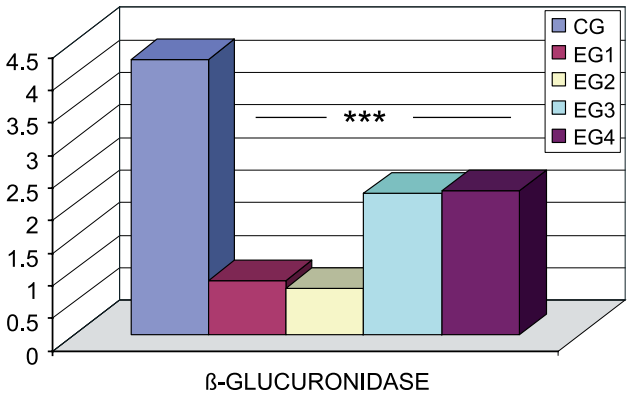

Fig. 1. Enzymatic actvivity of ß-glucuronidase in control group and experimental groups. Data are expressed as means ± SD. Significant differences calculated versus the control group are designes as: *** p<0.001, CG – control group, EG1 – experimental group with prebiotic (PRE), EG2 – experimental group with PRE + HES (Hyppocastani extractum siccum), EG3 – experimental group with PRE + Lini oleum virginale (O), EG4 – experimental group with PRE + HES + O.

Tab. 1. Changes of total cholesterol, triacylgylcerols and bile acids in experiment.

Groups	Total cholesterol (μmol/l)	Triacylglycerols (μmol/l)	Bile acids (μmol/l)
CG	1.39±0.22	1.05±0.32	16.84±6.33
EG1	0.83±0.19***	0.57±0.23***	11.72±4.22**
EG2	0.94±0.16***	0.89±0.68	13.56±6.07
EG3	1.05±0.16	1.13±0.46	10.84±3.19**
EG4	1.14±0.16	0.89±0.54	5.76±2.66***

Data are expressed as means±SD. Significant differences calculated versus the control group are designes as: ** p<0.01; *** p<0.001, CG – control group, EG1 – experimental group with prebiotic (PRE), EG2 – experimental group with PRE+HES (Hyppocastani extractum siccum), EG3 – experimental group with PRE+Lini oleum virginale (O), EG4 – experimental group with PRE+HES+O.

Tab. 2. Changes of short chain fatty acids in experiment.

Groups	Acetic acid (mmol/100ml)	Propionic acid (mmol/100ml)	Butyric acid (mmol/100ml)
CG	11.45±1.98	2.77±0.35	2.41±0.46
EG1	11.76±1.67	2.76±0.57	2.91±0.62**
EG2	10.39±1.97	2.11±0.56	2.00±0.47
EG3	9.57±1.90	2.55±0.64	2.03±0.51
EG4	11.00±1.88	2.70±0.39	2.30±0.42

Data are expressed as means±SD. Significant differences calculated versus the control group are designes as: ** p<0.01, CG – control group, EG1 – experimental group with prebiotic (PRE), EG2 – experimental group with PRE+HES (Hyppocastani extractum siccum), EG3 – experimental group with PRE+Lini oleum virginale (O), EG4 – experimental group with PRE+HES+O.

Discussion

Diet interventions and natural bioactive supplements have now been extensively studied to reduce the risks of colon cancer, as a cause of prevention instead of cure (Liong, 2008). As important dietary factors in colorectal cancer risk reduction probiotics, prebiotics, plants and their extracts and polyunsaturated fatty acid are cnsidered. The prebiotics are generally defined as nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one, or a limited number of one, or a limited number of bacteria in the colon that can improve the host health (Gibson and Roberfroid, 1995). This definition was updated in 2004 (Gibson et al, 2004) and prebiotics are now defined as selectively fermented ingredients that allow specific changes, both in the composition and /or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health. The latter definition does not only consider the microbiota changes in the colonic ecosystem of humans, but in the whole gastrointestinal tract, and as such extrapolates the definition into other areas that may benefit from selective targeting of particular microorganisms. In order to be effective, a prebiotic must escape digestion in the upper gastrointestinal tract so that it can be released in to the lower tract and used by beneficial microorganisms in the colon, mainly bifidobacteria and lactobacilli. Unlike the probiotic bacteria, prebiotic carbohydrates are not destroyed when cooked. Prebiotic carbohydrates are found naturally in such fruit and vegetables as bananas, berries, asparagus, garlic, wheat, oatmeal, barley (and other whole grains), flaxseed, tomatoes, Jerulasem artichoke, onions and chicory, greens (especially dandelios greens, spinach, kale, mustard greens, and other), and legumes (lentils, kidney neans, white beans, black beans, peas). The various oligosaccharides classified as prebiotic and added to processed foods and supplements include Fiber gums, Fructooligosaccharides (FOS), Inulins, Isomaltooligosaccharides (IMO), Lactilol, Lactosucrose, Lactulose, Oligofructose, Pyrodextrins, Soy oligosaccharides, Transgalactooligosaccharides (TOS = *trans* GOS), and Xylooligosaccharides (XOS).

Inulins are a group of non-digestible oligosaccharides belonging to a class of carbohydrates knows as fructans. Inulin-type fructans exctracted from chicory roots are prebiotic food ingredients which in the gut lumen are fermented to lactic acid and short chain fatty acids (Alvaro et al, 2008). Of these, butyrate and propionate inhibit growth of colon tumour cells and histone deacetylases. Butyrate also causes apoptosis, reduces metastasis in colon cell lines, and protects from genotoxic carcinogens. The elevated butyric acid and acetic acid concentrations, and decreased concentrations of total cholesterol and triacylglycerol during experimental period are in accordance with experimental animal models which revealed that inulin-type fructans have anticarcinogenic properties, hypolipidaemic effect, and antiatherogenic effects. The human intervention study (SYNCAN project) provided experimental evidence that inulin modulates parameters of colon cancer risks in humans colon cells (Loo et al, 2005). Combination of prebiotic with *Hyppocastani extractum* siccum (EG2),*Lini oleum* virginale (EG3), and a mixture of all selected substances (EG4) nonsignificantly decreased the concentration of lipid parameters and SCFA concentrations stayed down.

Supplementary ingestion of oligofructose-enriched inulin, *A. hippocastanum* extract, *L. usitatissimum oil* and their mixture in combination with HF diet and DMH treated rats decreased ($p<0.001$) the activity of β-glucuronidase probably resulted in increasing excretion of conjugated xenobiotic compounds and decreasing activity of harmful substances that are most active in their deconjugated state. Elevated activity of bacterial enzymes is associated with an increased risk for various cancers and could be altered by the diet, ultimately results in potentially decreasing the risk of carcinogenesis (Manju and Nalini, 2006).

Most of the beneficial effects of *A. hippocastanum* (Horse chestnut) seed are attributed to its component beta-aescin or aescin; it also contains flavonoids, namely glycosides of quercetin and kaempferol. Beta-aescin is known to generate a wide variety of biochemical and pharmacological effects used in nutraceutical, cosmetic, and food supplement industries. Recent studies suggest that beta-aescin may be used in the treatment of chronic venous insufficiency, edema, hemorrhoids, and may possess anti-inflammatory, chemopreventive, anti-proliferative, apoptotic and anti-obesity efficacy (Patlolla et al, 2006; Niu et al, 2008; Hu et al, 2008). This novel feature in our experimental study – the positive changes in bacterial enzymes, concentration of bile acids and lipids confirmed the opinion that Hyppocastani extractum siccum may be a useful candidate agent for colon cancer chemoprevention and treatment.

Fatty acid composition of dietary fat plays a vital role in colon tumour development in animal models. Fats containing omega-6 fatty acids (e.g., corn oil) enhanced and omega-3 fatty acids (e.g., flaxseed oil) reduced chemically induced colon tumour development in rats. Dietary flaxseed is high in the lignan content. Lignans are phytoestrogens, good sources of dietary fibre, protein, antioxidant, and other nutritional elements and show preventive role in the development of colon cancer tumour in experimental animals and humans (Bommareddy et al, 2006; Theodoratou et al, 2007). Lini oleum virginale (Dr. Kulich Pharma, Czech Republic) administered at a dose of 2 % of HF diet

had an anti-tumour effect in EG3 and EG4 with the result significantly decreasing the activity of β-glucuronidase ($p<0.001$), bile acids concentration ($p<0.01$, $p<0.001$ respectively), and lipids (nonsignificantly). Although epidemiological and experimental studies indicate an association of elevated faecal levels of secondary bile acids as well as total bile acids with a high risk of colon cancer development, the cellular mechanism for the actions of bile acids is not clear (Cheng and Raufman, 2005; Hagiwara, 2006). Elevated concentration of bile acids in the highest risk was significantly reduced by administration of selected nutritional products.

Conclusion

The results of this experiment show that diet may play a very important role in prevention of diseases. Future research should be aimed at the enhancement of the effectiveness of cancer diseases prevention using nutritional supplements. It will be important to search for ways to improve the efficacy of nutritional supplements by adequate combination of more substances of biotechnological and natural origin.

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