Research Article

Hypoglycemic Effects of Polyphenol Complexes from Bilberry Leaves and Fruits Sorbed on Brown Buckwheat Flour -- Experimental Evaluation and Prospects of Use

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Abstract. The effects of plant polyphenols on carbohydrate and/or lipid metabolism disorders have wide experimental and clinical justification; however, their effects are limited due to low bioavailability. Thus, the development of technological approaches enhancing their effectiveness and stability is relevant.The aim of this work was to evaluate*in vivo*the effects of polyphenols from bilberry leaves and fruits, sorbed on the brown buckwheat flour, on C57Bl/6c mice with carbohydrate and lipid metabolism disorders. We assessed *in vivo*the effect of a food matrix (FM1: bilberry leaf polyphenols sorbed on brown buckwheat flour) on C57Bl/6c mice with induced carbohydrate and lipid metabolism disorders. The aim of the second experiment was to evaluate the effectiveness of prolonged prophylactic consumption of another food matrix (FM2: billberry fruit polyphenols, sorbed on brown buckwheat flour) by C57Bl/6c micewith induced carbohydrate and lipid metabolism disorders. Technological approaches were developed and pilot batches of the food matrices FM1 and FM2were obtained. According to the *in vivo* testing, a significant decrease in the glucose levels and normalization of glucose tolerance and insulin sensitivity were found in animals treated with FM1. When assessing the *in vivo* effects of FM2, the hypoglycemic effect of bilberry fruit polyphenols in the composition of the matrix was established. The results of these studies can be used to justify the testing of the developed matrices in a clinical setting and using them as functional food ingredients for preventative nutrition in cases of carbohydrate metabolism disorders.

Keywords: polyphenols, food matrix, functional food ingredient, carbohydrate metabolism, lipid metabolism

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Dates

Published 13 January 2022

Publishing services provided by Knowledge E

Selection and Peer-review under the responsibility of the 8th Scientific and Practical Conference Conference Committee.

1. Introduction

The most important scientific and practical application of the term "Nutriome", which describes and specifies the optimal diet formulation [1], is the possible expansion of the list of food biologically active substances (BAS) with justified participation in the metabolism. The prospects of the use of plant polyphenols at carbohydrate and/or lipid metabolism disorders have wide experimental and clinical justification [2-6]. When

entering the organism, plant polyphenols become part of the antioxidant system [7-9]. The impact of the polyphenols on the cell level and their pharmacological action are mostly determined by the interactions with cell membranes [10, 11]. Polyphenols in the cell cytoplasm may impact the activity of different enzymes, the expression of nuclear and cytoplasmic proteins [12, 13] and take part in the cell signal system [2].

Theauthorsconducted experimental studies on the base of Federal Research Centre of Food and Biotechnology (Russia, Moscow), aimed at the evaluation of the effectiveness of the use of bilberry leaves extract (BLE) for prevention of the carbohydrate metabolism disorders, firstly type 2 diabets (DM2) [14]. The relevance of further investigations is determined by wide spread and high frequency of metabolic syndfome (MS) and DM2, and also by the necessity to expand the variety of proper native specialized food products (SFP) [15]. Theways to increase the application effectiveness of plant polyphenols in the preventive diets include the search for the technological approaches, aimed at the production of the concentrates for further inclusion into the composition of proper SFP as functional food ingredients (FFI). The row of corresponding technological approaches includes sorption on the polymeric food matrices (FM) among other methods [16]. The prospects of the use of targeted extraction of BAS from plant material and it's concentration by sorption on the FM are determined by relatively simple technology.

The present article is dedicated to the short review of recent results of our experimental studies, including the development of the technological approach for the concentration of polyphenols from BLE and bilberry fruits extract (BFE) through sorption on the matrix, physical and chemical characterization of obtained FM and physiological and biochemical pre-clinical evaluation of their effectiveness at simulated carbohydrate and lipid metabolism disorders in laboratory rodents – C57BL/6c mice.

2. Food matrix 1: obtaining and characterization in vitro.

At the first stage we developed a technological approach for obtaining the food matrix (FM1) enriched with BLE polyphenols. Optimal conditions for BLE polyphenols sorption on brown buckwheat flour were determined: the concentration of BLE water solution 2%, solution pH 3.6, flour/BLE solution ratio 1g/50mL, temperature 25^{o}C, duration 45 min. Maximum total polyphenols content was 23.7±0.5 mg-eq. gallic acid/g flour (mg-eq. g.a./g flour),value was determined with elution from FM1. Flavonoid profiles in the composition of BLE and FM1 were determined by HPLC [17, 18]. We investigated the stability of polyphenols in the composition of dry BLE and FM1 under light- and

Table 1: Concentration of total polyphenols and anthocyanins in the BFE and their content in FM2.

	Total polyphenols	Anthocyanins
Fresh berries	(6.0±0.1) mg-eq. g.a./g	(3.4±0.1) mg/g
BFE	(1.68±0.04) mg-eq. g.a./mL	(0.95±0.01) mg/mL
FM2	(7.6±0.3) mg-eq. g.a./g	(4.6±0.1) mg/g

temperature-humid exposure [18]. The total polyphenols content in the composition of BLE decreased by 10% during seven days storage. In contrast, there was no destruction of polyphenol molecules in the composition of FM1, manifesting in oxidation of –OH groups to corresponding ketone groups (=O) and -CH2- bonds (polymerization to longer terminal aliphatic chains).

3. Food matrix 2: obtaining and characterization in vitro.

The developed technological approach for FM1 was used to obtain a food polymer matrix (FM2) - brown buckwheat flour enriched with BFE polyphenols.To obtain EHF, water-ethanol extraction of pre-lyophilized blueberries (10 g of dried berries mixed with 990 ml of 70% ethanol) was carried out with constant stirring on a laboratory processor, followed by centrifugation and removal of ethanol on a rotary evaporator [27]. The optimal conditions for the sorption of BFE polyphenols on brown buckwheat flour were determined: extract pH 3.0, the ratio of the flour sample to the extract solution volume 1g/10ml, temperature 25°C, the duration of the sorption process 45 minutes.It was found that there is no sorption of simple carbohydrates from BFE on brown buckwheat flour and the profiles of anthocyanins in BFE and FM2 were determined [27].

Total polyphenols and total anthocyanin content in the composition of fresh berries, BFE and FM2 is presented in the table 1.

Experiments 1 and 2 were conducted in vivo using mice obtained from laboratory animals nursery “Stolbovaya” department of Federal state budgetary organization of science “Scientific centre of biomedical technologies of Federal medical and biological agency”. The animal studies were conducted according to the statement of Ministry of Health of Russia N 708n “On approval of the Laboratory Practice Rules” from 23 August 2010 and requirements stated in the National Standard of Russia GOST R 53434-2009 “The Principles of Appropriate Laboratory Rules”. Animals were housed either four mice per cage (experiment 1), or one mice per cage (experiment 2) in controlled environmental conditions (temperature 23-26^{o}C, humidity 30-60%, 12-hour day cycle).

4. Experiment 1 design.

To compare in vivo the effect of BLE polyphenols sorbed on brown buckwheat flour on the condition of 63 young mature C57Bl/6c male mice with induced disorders of carbohydrate and lipid metabolism, an experimental batch of food matrix (FM1) was obtained.

The initial body weight of mice at the start of the experiment was 20±2g.

Mice were randomly (according to their body weight, fasting glucose level and glucose tolerance, evaluated with oral glucose tolerance test (OGTT) [19]) divided into four groups: control groups C1 and C1a and experimental groups G3 and G4. Animals of control group C1 received a standard diet, all other animals (C1a, G3 and G4) received high lipid diet with high content of simple carbohydrates (HLHC diet). FM1 was added to the diets of experimental groups G3 and G4 in doses 2.5g/100g of diet and 5.0g/100g of diet, respectively. Food intake was controlled twice a week, once a week mice body weight was measured. Once per two weeks fasting blood glucose was measured. OGTT was conducted on the 39^{th} and 82^{nd} day of the experiment; insulin resistance test (IRT) was conducted on the 15^{th}, 45^{th} and 91^{st} day of the experiment [20]. General condition of C1 group animals, treated with standard diet was satisfying for the whole experiment according to appearance, fur quality, food and water intake, behaviour and growth rate monitored every day. Animal fed with HLHC diet had greasy, thin, rare and disheveled fur. Anxiety level (AL) and motion activity ((MA) of animals were characterized using Elevated Plus Maze (EPM) [21]. Behaviour and memory of animals were tested using Passive Avoidance Test (PAT): training, next day: short memory testing, in 20 days: long-term memory testing [22].

On the 60^{th} day of feeding, there was a significant increase in glucose level in animals of all groups who received HLHC diet, compared with animals of group C1. From this moment group C1a was divided into two new groups: C2 group (animals received HLHC diet) and new experimental group G5 (animals received HLHC diet and FM1 in the dose 2.5g/100g of diet). This made it possible to evaluate the efficiency of FM1 consumption with already developed disorders of carbohydrate metabolism.

We continued to feed animals with diets for additional 70 days. On the 130^{th} day animals were decapitated under light ether anesthesia. Blood was collected to determine glycated hemoglobin (GH), ghrelin and leptin content. Liver was collected and homogenized to determine the content of triglycerides (TG) and cholesterol (CHL).

4.1. Food intake and body weight gain.

For the first 60 days of the experiment the food intake of mice of all groups, treated with HLHC, was significantly lower in comparison with control group C1, treated with standard diet, what is related to the higher caloricity of HLHC diet. Over the next 70 days, the mice of all groups receiving the HLHC diet consumed significantly less food and energy compared to C1 group. The increase in body weight of animals of the control group C1 before the 74th day did not significantly differ from the increase in body weight of animals of all groups that consumed HLHC diet, but from this moment on, all mice treated with HLHC diet for a long period began to show signs of obesity. However, starting from the 102nd day of the experiment, the body weight gain of mice of all experimental groups receiving FM1 in various dosages, up to the end of the experiment, did not significantly differ from the body weight gain of mice of the control group C1.

4.2. Total exploration activity and anxiety.

During the first test (40th day), there were no significant differences between animals of all groups in terms of total exploration activity (TEA): the total number of transitions between the arms of the maze and the distance traveled.

During the second test (90^{th} day), there was a significant decrease in the TEA of all animals compared to the results of the first test.Significant differences in terms of distance traveled compared to the first test were not revealed only for animals of group G4, who received FFI in the highest dose - 5g/100g of the diet. There were no differences in anxiety parameters of all animals between the results of the first and second tests.

4.3. Learning and memory.

The PAT training (49th day), short memory (50th day) and long-term memory (72nd day) showed no significant influence of HLHC diet and FM1 on the learning ability and memory of animals.

4.4. Blood glucose level.

Blood glucose level was monitored during the whole experiment. The blood glucose level of C1 group animals did not change significantly during the first four weeks of the experiment, decreased to a minimum to the 60th day and then was gradually and slightly

increasing. At the end of the experiment final blood glucose level of these animals did not differ significantly from the initial value. The nature of changes in the blood glucose level of mice of the control group C1a during the first 60 days of the experiment was almost a mirror image of the above described changes for C1 group animals. On the 60th day of the experiment the blood glucose level of C1a, G3 and G4 groups animals became significantly higher in comparison with blood glucose level of C1 control group. The revealed difference witnessed the development of hyperglycemia by the eighth week of the experiment against the background of animals consuming the HLHC diet. Starting from the 60th day, the blood glucose level of C2 group animals, treated with only HLHC diet, increased significantlyand by the time of the end of the experiment was significantly higher compared to the initial value.On the 109th day of the experiment and until its end, the blood glucose level of animals of groups G3 and G4 did not significantly differ from each other and remained significantly lower compared to the control group C2, which indicated a pronounced hypoglycemic effect when consuming FM1. Starting from the 95th day of the experiment and until its end, the blood glucose level of G5 group animals, who began to receive FM1 from the 60th day of the experiment, also did not differ significantly from the glucose level of animals in the control group C1 (7.7 $\pm$ 0.4 mmol/L, $p > 0.05$).

The obtained results are consistent with the data presented in [23], where it was shown that the introduction of a lyophilized water-alcohol extract of bilberry leaves (obtained by extraction of dried chopped bilberry leaves with 70% ethanol) into the HLHC diet of C57BL/6J mice led to a significant decrease in blood glucose level compared with the control diabetic group of animals.

4.5. Glucose tolerance and insulin resistance.

When testing these indicators, it was found that glucose tolerance of C1 control group animals remained constant in an interval from 39th day to 82nd day of the experiment. On the 39th day of the experiment, glucose tolerance of animals of C2 and G4 groups treated with HLHC diet, was significantly more pronounced compared to the results of C1 group animals. A favorable effect on this indicator was noted when consuming FM1 at a dose of 2.5 g/100g of diet (group G3): on the 39-82th days of the experiment, glucose tolerance of G3 group animals and C1 group animals did not differ significantly.

The consumption of FM1 in the dose 5g/100g of diet inhibited the development of insulin resistance, induced by HLHC diet, on the 15^{th} and 45^{th} days of experiment this state was the least pronounced in animals of G4 experimental group, consuming FM1 in

above described dose.However on the 91st day of the experiment, the insulin resistance of all experimental mice, consuming HLHC diet, was significantly higher in comparison with C1 control group.

4.6. Biochemical parameters.

There were no significant differences in GH content in the blood of animals of all groups. A significant increase in the plasma ghrelin level of G4 group animals compared to the animals of the C1 and C2 groups indicated that consumption of FM1 prevented excess body weight gain. Leptin levels of animals from groups G3 and G4 were also significantly reduced in comparison with C2 group mice, what indicated a decrease in the volume of adipose tissue in animals receiving FM1. The indirect effect of polyphenols on leptin and ghrelin levels in in vivo experiments has been shown in [24-26]. There were no significant differences in CHL and TG levels in liver homogenates of animals of all groups.

5. Experiment 2 design

An experimental batch of FM2 was used in an in vivo experiment to assess the effect of BFE polyphenols adsorbed on brown buckwheat flour on the state of C57Bl/6c weaning mice with induced disorders of carbohydrate and lipid metabolism.

The initial body weight of animals was 17.4±0.4g. Before the start of the experiment, blood glucose was measured (animals were deprived for four hours).

Animals were randomly divided (according to body weight, glucose level) in to three groups. Animals of control group β-C1 were fed with standard diet. Animals of β-C2 and β-G3were fed with HLHC diet. The FM2 was added to the diet of β-G3 group animals in the dose 6.6 g/100 g of diet. Buckwheat flour was added to the diet of control group β-C2 in the equivalent dose.

Food consumption was controlled three times a week; body weight was measured once per week. Blood glucose level was monitored three times a week. Animals were tested using EPM on the 60th and 114th days of the experiment. On the 150th day of the experiment animals were decapitated under light ether anesthesia. The blood GH content was measured. The content of TG, CHL, high-density lipoproteins (HDL), low-density lipoproteins (LDL) and free fatty acids (FFA) was measured in the liver of animals.

5.1. Food consumption and body weight gain.

All animals, receiving HLHC diet, consumed significantly less food in comparison with control group β-C1 animals, receiving standard diet, during the whole experiment. The body weight gain of all animals, receiving HLHC diet, was significantly lower compared to β-C1 group animals, till the 11th week of the experiment. Starting from the 12th week and till the end of the experiment, there were no significant differences in body weight gain between animals of all groups.

5.2. Total exploration activity and anxiety.

During the first test (on the 60^{th} day of feeding) animals of β-G3 group, receiving FM2, spent significantly more time in the open arms of the maze in comparison with animals of control group β-C1. At the same time, the time spent in the closed arms by mice of the β-G3 group was significantly lower compared to mice of both control groups.

When tested again on the 114th day of the experiment, animals of all groups began to spend less time in the open arms and more time in the closed arms of the maze. There were no significant differences in the total exploration activity, expressed in the number of transitions and the total distance covered, both between the groups and between the two tests.

The data obtained indicate that the consumption of bilberry polyphenols sorbed on the brown buckwheat flour does not increase anxiety in male (weaned) C57Bl/6c mice, and, on the contrary, can, to a certain extent, contribute to a decrease in anxiety in these animals.

5.3. Blood glucose level.

The blood glucose level of the animals of the β-C2 control group, receiving HLHC diet, did not significantly differ from the corresponding indicator for the β-C1 group animals during almost the entire experiment. However, there was an important difference: the average glucose level in animals of the β-C2 group at the end of the experiment was significantly higher than at the beginning, and for the β-C1 group, these differences were insignificant.

Starting from 42^{nd} day until the end of the experiment, the glucose level of the animals of β-G3 group that consumed FM2 was significantly lower in comparison with the animals of the β-C2 group. The data obtained are indirectly consistent with the results of

work [28], in whicha complex of blueberry juice polyphenols sorbed on thefat-free soy flour exerted a pronounced hypoglycemic effect in *in vivo* experiments using C57BL/6J mice.In work [29], performed in vitro on Caco-2 cell strains, polyphenols of mulberry extract influenced glucose metabolism by inhibiting the activity of disaccharidases, as well as inhibiting glucose transport in cells.The introduction of a polyphenol extract led to a decrease in the activity of sucrase and maltase and inhibition of glucose transport in cells by reducing the expression of mRNAs responsible for the synthesis of SGLT1 and GLUT2.

5.4. Biochemical parameters.

After the end of the experiment, a significantly higher level of GH in the blood of animals of all groups that consumed the HLHC diet was determined, compared with animals of the control group β-C1. Under the conditions of the experiment, the inclusion of FM2 in the HLHC diet did not have a normalizing effect on lipid metabolism disorders in the liver of the tested animals. The levels of CHL and LDL in the liver homogenates of animals did not differ significantly between all groups. There were no significant differences in the level of HDL in animals of the β-C1 and β-G3 groups. The content of TG and FFA in the liver homogenates of animals of the β-C2 and β-G3 groups was significantly higher compared to the same indicator for animals of the control group β-K1.

6. Conclusion.

As a result of the studies carried out, complexes of polyphenols frombilberry leaves extract and bilberry fruits extractsorbed on brown buckwheat flour (FM1 and FM2, respectively), were obtained, and their effect on laboratory rodents was characterized in *in vivo* experiments.The developed method is distinguished by the relative simplicity of the technological solution.Preservation of the biological activity of concentrated polyphenols and their stability in the composition of FM1 during storage has been proven.The compositions of FM1 and FM2, including their polyphenolic profiles, have been characterized. An*in vivo* model reproduced modeling the disorders of carbohydrate and lipid metabolism by means oftreatment with HLHC diet.*In vivo* experiments on the physiological and biochemical assessment of the effect of consumption of FM1 and FM2 on disorders of carbohydrate and/or lipid metabolism in weaned mice and juvenile mature individuals of the C57Bl/6c line were carried out. FM1 and FM2 had a hypoglycemic effect *in vivo*, preventing an increase in blood glucose levels

in C57Bl/6c mice with induced disorders of carbohydrate metabolism.The results of *in vivo* studies on the effect of FM1 and FM2 on some parameters of carbohydrate metabolism in animals with metabolic disorders induced by an HLHC diet can be used to justify their further testing in a clinical setting for use as a functional food ingredientfor preventive nutrition in case of carbohydrate metabolism disorders.The developed laboratory version of the technology for producing FM1 and FM2 can be effectively scaled up for industrial purposes.

7. Acknowledgements

This work was supported with the subsidies of state project № 0529-2019-0055.

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