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PhD THESIS

Study of bioactive nutraceutical effects of Zonar whey in an experimentally induced obesity model in Sprague-Dawley

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CURRENT STATE OF KNOWLEDGE

Adipose tissue plays a central role in regulating the energy of the whole body and the homeostasis of glucose through its subtle functions both at organ and systemic level. Adipose tissue is classified into three subtypes: white adipose tissue (WAT), brown adipose tissue (BAT) and beige adipose tissue. Adipose tissue stores energy in the form of lipids and controls their mobilization and distribution in the body being directly involved in metabolic regulation. The storage of triglycerides (TG) in adipocytes over a long period of time leads to an increase in size (Tan & Vidal-Puig 2008). Triglycerides that are stored in adipocytes are broken down into glycerol and lipolytic fatty acids, a phenomenon that occurs following caloric restriction.

In recent years, obesity has been on the rise in the population from bothdeveloped or developing countries. Studies have shown that excess adipose tissue (excess fat) can cause metabolic abnormalities such as dyslipidemia and insulin resistance. It can also increase the risk of cardiovascular complications, such as coronary heart disease and high blood pressure (Rosini et al., 2012; Ezzati et al., 2006). Normally, adipose tissue represents between 10 to 25% of body weight and when this body mass index (BMI) exceeds 30 kg / m2 it represents a major health risk (Kanasaki et al., 2011).

These cytokines are strong stimulants for the production of reactive oxygen and nitrogen by macrophages and monocytes; therefore, an increase in cytokine concentration could be responsible for the increased level of oxidative stress. Obesity increases mechanical load and myocardial metabolism; therefore, oxygen consumption is increased.

Milk provides a wide range of biologically active components such as bioactive proteins and peptides, oligosaccharides, immunoglobulins, and fats / lipids that have the ability to protect against pathogens if consumed regularly. The biological properties of whey proteins are widely recognized and have been increasingly explored in scientific research studies and food applications by various industries. β-lactoglobulins contribute to 50% of whey protein, it helps bind minerals such as zinc and calcium. Alpha-Lactalbumin on the other hand is recommended to be added in infant formulas or in various foods for an increased protein intake. Serum albumin can bind fatty acids and immunoglobulins such as IgA, IgM, IgG1 and IgG2 which helps the development of passive immunity in consumers. After ingestion, whey is hydrolyzed into bioactive peptides and amino acids that are detected by the nervous system at various levels of the gastrointestinal tract and central nervous system. These detection systems act through signals of satiety by reducing the amount of food consumed (Malekian et al., 2015).

Nutraceuticals or medical foods are designed to provide complete or supplemental nutritional support to people who cannot digest adequate amounts of food in a conventional form. These foods are also used to provide specialized nutritional support to patients with special physiological and nutritional needs. Whey proteins are normally present in a diet as intact proteins and have the nutritional advantage of being able to be used in medical diets because they are nutritionally complete proteins.

The great similarity between the genome of rodents and humans makes these animal models a major tool for studying obesity. Animals allow us to get answers in a short time, as 10 days in a rat's life are about 1 year in humans when we compare changes in body weight. Some models of induction of obesity in rats are found in the international literature, the main experimental models of inducing obesity are: by lesion of the ventromedial hypothalamic nucleus (VMH) which can be achieved mainly in two ways (administration of monosodium glutamate or direct electrical injury); ovariectomy, administration of hypercaloric diets and genetic manipulation for obesity (Diemen et al., 2006).

PERSONAL CONTRIBUTION

WORKING HYPOTHESIS AND RESEARCH OBJECTIVES

The working hypothesis is based on the assumption that bioactive whey proteins reduce the increase in body weight and fat, increase muscle mass without changing energy consumption. The low-calorie content and easily digestible proteins place whey in the top of foods that can reduce metabolic syndrome and obesity.

The aim of the research was to evaluate the bioactive nutraceutical effect of the whey Zonar used as a dietary supplement in experimentally induced obesity by hyperlipidic diet in Sprague-Dawley rats.

Objective 1. Evaluation of the preventive and palliative effects of Zonar whey consumption by determining anthropometric parameters.

Objective 2. Evaluation of the anti-fat effects of Zonar whey consumption by determining the serum and haematological biochemical profile in the experimental groups.

Objective 3. Evaluation of the antioxidant effects of Zonar whey consumption by dosing the markers of oxidative stress in plasma and organs (liver and kidney) in groups of normal-weight and experimentally induced obese rats.

Objective 4. Histomorphometric and qualitative evaluation of adipose tissue and internal organs in normal and obese rats.

MATERIALS AND METHODS

The study was performed on a number of 30 healthy adult rats of the Sprague-Dawley breed, males, aged 3 months, weighing on average 450 ± 35 g. The animals were raised in the Biobase of the Faculty of Veterinary Medicine, USAMV Cluj-Napoca, in compliance with the standard conditions, respectively temperature 22-23ºC, humidity 55% and a photoperiod of 12h light / 12h dark. The animals were fed standard granulated feed for rodents (provided by the Cantacuzino Institute, Bucharest, Romania) and water ad libitum. The experimental protocol was approved by the Research Ethics Commission of the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca and was authorized by DSVSA Cluj, by (Decision no. 48 / 29.03.2017).

The groups of animals used in the experiment were structured as follows: healthy control group-MS (n = 5); obese control lot-MO (n = 5); standard and Zonar food batch-HSZN (n = 5); hyperlipidic food batch and Zonar-HHZN whey (n = 5); Obesity group 1-OB 1 (n = 5) and obesity group 2-OB 2 (n = 5). In the case of groups OB 1 and OB 2 the experimental protocol was divided into two stages, respectively in stage 1 obesity was induced in both groups for a period of 7 weeks, and in stage 2, group OB 1 switched to a standard diet and Zonar whey and OB 2 group still remained on hyperlipidic food and Zonar whey was introduced. The animals received two types of combined granular food, provided by the Cantacuzino Institute, Bucharest, the structure of the standard food ration (combined fodder) contained of: 18% protein, 1.5% fat, 5% fiber, and the hyperlipidic food composition is 21 % protein, 16% fat, 3.5% fiber.

During the experiment, a commercial formula of sweet whey was administered in animal feed, produced and marketed under the name of Zonar whey by SC EmbrionSRL, Satu Mare, Romania (www.Zonar.ro). Zonar whey was characterized in terms of physico-chemical composition.

The blood count was performed using the Abacus Junior Vet 5 Diff automatic analyzer. The biochemical determinations were performed using the Touch UV-VIZ Screen spectrophotometer (Hospitex Diagnostics, Firenze, Italy), which allows the measurement of

the following serum biochemical parameters: glucose, triglycerides, total cholesterol, LDL. Insulin (Ultrasensitive Mercody Rat Insulin ELISA, 10-1252-01 / lot 27126) was dosed by ELISA method with the Microplate Photometer device, type MPP-96The HOMA2-IR index was obtained through the HOMA Calculator program v2.2.3. downloaded from the web at https://www.dtu.ox.ac.uk/homacalculator/download.php. The parameters of oxidative stress determined for this experiment were: reactivity or total antioxidant capacity (TAC), total oxidant status (TOS), oxidative stress index (OSI) and Malondialdehyde (MDA). All parameters were measured using the Jasco UV-Vis Spectrophotometry Analyzer (Jasco V-630, Tokyo, Japan). Liver and kidney protein extracts were obtained using potassium phosphate buffer (pH = 7; 35). TAC activities and lipid peroxidation and protein oxidation were estimated in this protein extract using photometric methods. The activity of antioxidant enzymes was determined in the liver and kidney tissues in all experimental groups, using commercial kits (from Randox). The study material was samples of abdominal adipose tissue, liver, kidney, and pancreas. The collected samples were immersed immediately after harvesting in 10% formaldehyde solution at laboratory temperature for fixation, then processed for paraffin inclusion using standard Hematoxylin-Eosin staining. Data collection and processing was performed using Microsoft Office 2010 programs, and graphics processing was performed in the SPSS version 21 program.

STUDY 1 - Determination of anthropometric indices in rats that consumed ZONAR whey

The objective of this study was to evaluate the preventive and palliative effects of Zonar whey consumption by performing anthropometric measurements in rats. Body mass index (BMI) is an estimate of body fat based on height and weight. The obesity index (OI) is used to assess the degree of obesity in animals based on measurements of body length, abdominal circumference and body weight.

The obese control group gained the most weight statistically compared to the healthy control group. From the HSZN and HHZN groups that received Zonar whey as a precaution throughout the experimental period, only the HSZN group had a statistically lower body weight than the MO group. Regarding the obese groups who received palliative Zonar lactoser for a period of 4 weeks, both OB1 and OB2 groups had a lower body weight compared to the MO group. Regarding the anthropometric indices, the BMI was statistically increased in the MO group compared to the MS group and the HSZN group that receved Zonar whey as a preventive form had the BMI index statistically lower that the MO group obese groups OB1 and OB2 have a statistically lower BMI than the MO group. The obesity index was not statistically altered in any group in this experiment. Novelli et al., 2006 obtained similar results in terms of anthropometric indices in the groups that received hyperlipidic food in the diet, similar to our obese group, their groups that were on a hypercaloric food gained weight and had a high BMI.

CONCLUSIONS

By using the hyperlipidic diet, it was possible to induce obesity by excessively increasing body mass (g), respectively BMI in experimental groups.

The anti-fat effect of Zonar whey has been demonstrated by reducing body weight (g) and BMI in all additional experimental groups.

STUDY 2 - Determination of haematological, biochemical profile and evaluation of HOMA-IR index in rats

The aim of the study was to evaluate the anti-fat effects of Zonar whey by determining the serum, haematological biochemical profile and determining the HOMA-IR index in normal and obese rats. Regarding the determination of the hemoleukogram, the rats from the MO group did not show statistically significant changes compared to the MS group. The data obtained showed that there is no significant difference in blood picture in rats fed a hyperlipidic diet compared to rats fed a standard diet, this may be due to insufficient exposure to hyperlipidic food, requiring longer exposure to hematological changes. From a medical point of view, the increase in the number of leukocytes normally indicates an infection or an inflammation, and the fact that in this study there was no increase in their number, we can say that there was no inflammation or infection during the whole experiment. The effects of whey protein are quite beneficial for the health of animals and humans on the blood system.

Metabolic syndrome is defined by the presence of 3 of the following criteria: obesity, especially abdominal obesity, high cholesterol and high LDL, low HDL cholesterol, high blood pressure, increased triglycerides and glucose, among other dysmetabolic conditions, which reflect the underlying insulin resistance. The evaluation of the biochemical parameters revealed a statistically significant increase in rats from the MO group compared to the MS group. Regarding serum glucose, a statistically significant increase of its concentration value was observed in the MO group compared to the MS, HSZN and HHZN groups. As in the case of serum glucose, we have a statistically significant increase in insulin levels in the MO group compared to the MS, HSZN, HHZN and OB1 groups. In the case of triglycerides, we have a statistically significant increase in the MO group compared to the MS, HSZN, HHZN, OB1 and OB2 groups. Triglyceride values in the HSZN and OB1 groups are statistically low compared to the MS group. These results were in line with the results reported by Sung-Moon et al. (2015) where biochemical parameters were significantly low in rats that had liquid fermented whey (FWB) in their diet.

Homeostatic model evaluation (HOMA) is a validated method to measure insulin resistance in glucose and fasting insulin. Regarding the HOMA-IR index in our study, it showed thatthe MO group had the highest Homa-IR index compared to the MS group. The HSZN, OB1 and OB2 groups that had Zonar lactoser in their diet had an optimal level of HOMA-IR (insulin sensitivity <1) compared to the MO group. In terms of insulin sensitivity, the MO group had the lowest sensitivity value compared to the MS and OB1 group. Also group OB2 had statistically lower values than group MS and OB1. Insulin sensitivity is expressed as HOMA% -S, the higher the value, the higher the subject's insulin sensitivity. Regarding the Homa-β% index, it did not show statistical changes between any of the experimental groups. However, animals fed hyperlipidic food have never developed diabetes; this is consistent with previous findings in Sprague-Dawley rats and is attributed to compensatory increases in both pancreatic β-cell mass and glucose-stimulated insulin secretion.

CONCLUSIONS

1. By administering hyperlipidic food, it was possible to induce early insulin resistance (HOMA-IR> 1.9) in the MO group compared to the MS group that had an optimal HOMA-IR index.

2. The administration of Zonar whey prevented the development of insulin resistance in all groups that were supplemented with Zonar whey in both preventive and palliative form.

3. Administration of Zonar whey had a hypoglycaemic, hypoinsulinemic effect and reduced serum triglyceride levels in all experimental groups that were supplemented with whey.

STUDY 3 - Evaluation of the antioxidant effects of Zonar whey by dosing oxidative stress markers in plasma and tissues

The aim of the study was to evaluate the antioxidant effects of Zonar whey by measuring the markers of oxidative stress in plasma and tissue (liver, kidneys) and determining the relative weight of the organs taken.

Induction of obesity in male Sprague Dawley rats in our study who consumed a high-fat diet resulted in a significant increase in the concentration of TOS in the MO group compared to the MS group. We observed the same results in the OSI index where the MO group had a higher statistical index compared to the MS group. Markers MDA and TAC in these 2 groups showed no statistical changes. The results of our study are similar to the study conducted by Omnia et al. 2020. Animals that were supplemented with Zonar whey for 11 weeks and 4 weeks, respectively, had a significant reduction in TOS levels compared to the MO group. To the same extent, the OSI index (<0.01) was statistically lower in the Zonar lactose supplemented groups in both experimental periods compared to the MO group. Regarding plasma MDA, the statistically lower amount was observed only in the HSZN group. Plasma TAC values were not statistically altered in any of the experimental groups. These results correlate quite well with Prigon et al., 2013 who suggested that TOS and OSI levels were higher in obese children with liver disease than in non-obese children. Kilic et al., 2016, also showed that markers of oxidative stress TOS and TAC were significantly higher in obese groups compared to healthy control groups and concluded that elevated TAC levels were the result of increased TOS in an attempt to balance oxidation because young people have more active antioxidant systems.

The tissue samples examined in our study were kidney and liver, to determine the markers of oxidative stress and antioxidant action. After the initial determination of protein loads we determined malondialdehyde (MDA), which shows the degree of lipid peroxidation, respectively inflammation and cell necrosis and capacity total tissue antioxidant (TAC). The antioxidant effects of Zonar whey may be due to different types of natural antioxidant compounds such as beta-lactaglobulin, alpha-lactalbumin, immunoglobulins, bovine serum albumin, lactaferrin and / or lactoperoxidase, etc. Considering the results obtained, it can be stated that Zonar whey is neither hepatoxic nor nephrotoxic, having even a slight antioxidant action at the renal level. Regarding the weight of the organs, the HSZN group showed a statistically significantly lower liver weight compared to the MO group but without any change in the organo-somatic index of the liver. However, no statistically significant increase in kidney weight or organo-somatic index was observed in any of the experimental groups.

CONCLUSIONS

1. The antioxidant effects of Zonar whey have been demonstrated by low values of oxidative stress markers (TOS, OSI index) in lactose supplemented groups compared to the obese control group.

2. Considering the results obtained, it can be stated that Zonar whey is neither hepatoxic nor nephrotoxic, having even a slight antioxidant action on the kidneys.

3. Zonar whey may decrease body weight gain in obese rats fed a high-fat diet.

STUDY 4 - Histomorphometric and qualitative determination of adipose tissue and internal organs

The objective of the study was the qualitative histopathological evaluation of adipose tissue, liver, kidneys, and pancreas. A morphometric analysis of adipocytes following their morphological (size) and numerical changes was performed.

The histomorphometric analysis of adipocytes harvested from the abdominal region from individuals in our study, showed that the MO group had a statistically significantly lower number of adipocytes compared to the MS groups. Regarding the diameter of the adipocytes, the MO group showed the largest adipocyte dimensions compared to the MS groups. Regarding the diameter of adipocytes, the following statistical changes were observed: the MO group had a higher volume of adipocytes than the MS group, these results are due to adipocyte hyperplasia following the accumulation of triglycerides in the obese control group.

The experimental groups HSZN and HHZN, which received whey Zonar preventively had a higher number of adipocytes and a smaller adipocyte diameter compared to the MO group. And the obese groups that received Zonar whey in palliative form (OB1 and OB2) had a statistically significantly smaller number of adipocytes than the MS group and without any statistical change compared to the MO group.The results obtained are consistent with the results obtained by Ulrike et al. 2016 where mice supplemented with a hyperprotein diet obtained from casein had a smaller adipocyte diameter in all experimental groups that received casino compared to the control group. Thus, it can be concluded that whey has palliative effects on the size and number of adipocytes because in the case of all experimental batches that received Zonar whey, the adipocyte diameter was significantly smaller, being in greater numbers in the field, compared to the obese control group. In the development of obesity, the expansion of adipose tissue is often based on adipocyte hypertrophy (increased adipocyte size) which is a known cellular stress factor for adipocytes, especially for the rough endoplasmic reticulum.

Regarding the histopathological appearance of adipose tissue, differences were observed especially between the obese control groups, respectively the groups in which the individuals received only hyperlipidic food, without other addition and the groups in which Zonar lactoser was administered in addition to hyperlipidic food or standard food. The pathological change observed in this study was represented by adipocyte hypertrophy. This is attributed to the effect of Zonar whey given as a food supplement in both batches. Ulrike et al. 2016 obtained results similar to our study in terms of qualitative histopathological appearance in mice that were supplemented with a hypercaloric diet compared to the groups that had administered casein. Although it is well documented that the administration of hypercaloric food to rats leads to inflammation in white fat deposits, there were no differences in inflammatory cell infiltrate from adipose tissue between experimental groups, these results being consistent with the results of Rojas et al. 2018.

Microscopic examination of the liver revealed, in the obese control group, which received only hyperlipidic food, as a predominant lesion, moderate hepatic microvacuolar steatosis, especially with centrolobular localization (Fig. 18). One can identify the presence of multiple lipid vacuoles (triglycerides), of variable size, in the cytoplasm of hepatocytes, these having the nucleus displaced at the periphery and in different stages of necrosis. Although hepatic steatosis has not been evaluated quantitatively in the batches that received Zonar whey in addition to food, this lesion is absent. The exception is group OB1, where an incipient hepatic steatosis was identified. Rojas et al. 2018, obtained results similar to ours in the microscopic examination of the liver, they identified a minimal to moderate hepatocellular vacuolation, present in both sexes, in groups that received hypercaloric food. In these

individuals, no damage to the bile duct epithelium was observed, nor was the presence of inflammatory infiltrate. The administration of Zonar whey has a protective effect on hepatocytes, preventing the accumulation of triglycerides in their cytoplasm and therefore the appearance of pathological lesions in the liver.

Regarding the pancreas, the histological image is normal, without changes, in all the groups studied. No lesions of acinar epithelial cells or Langerhans were observed. The results obtained are contradictory with those obtained by Rojas et al., 2018, who identified in Sprague-Dawley rats fed a hypercaloric food a higher incidence of hemorrhage and accumulation of pigments at the periphery of the pancreatic islets, unlike control groups fed standard food.

Examination of renal tissue did not reveal the presence of inflammatory infiltrate or renal epithelial cell lesions (Fig. 22). These microscopic aspects are contradictory with the results obtained by Rojas et al. 2018, where both sexes, from the group that received hypercaloric food, presented minimal to moderate renal tubular changes (tubular basophilia and / or tubular dilation with the presence of hyaline cylinders).

Lesions present in various organs and associated with obesity are chronic lesions, which occur after prolonged administration of hyperlipidic food. The relatively short time (11 weeks) in which this experimental study was performed may not be sufficient to allow visible lesions in the pancreas or kidneys.

CONCLUSIONS

1. Administration of Zonar whey prevents the accumulation of triglycerides in adipocytes, an effect observed in all supplemented groups both as a preventive and palliative.

2. The same preventive results of Zonar whey are confirmed by the histomorphometric analysis of adipocytes that showed a decrease in their diameter, and an increased number of them compared to the MO group where the diameter of adipocytes was significantly increased. The best results were seen in the groups that received whey throughout the experimental period, followed by the groups that received whey for only 4 weeks.

3. Following the histopathological examination of the liver, in all experimental groups the absence of pathological lesions was noted, therefore we can say that whey prevents the accumulation of triglycerides in the cytoplasm of hepatocytes.

GENERAL CONCLUSIONS

1. The anti-fat effect of Zonar whey was observed by reducing body weight (g) and BMI in all experimental groups that were supplemented with whey.

2. Zonar whey had the ability to reduce blood glucose and serum triglycerides in all experimental groups.

3. Zonar whey had the ability to prevent insulin resistance.

4. Zonar whey had antioxidant action and was demonstrated by lower levels of oxidative stress markers in experimental groupss that have been supplemented with it.

5. Zonar whey had the ability to prevent the accumulation of triglycerides in adipocytes, the results being observed by qualitative histological analysis.

6. Administration of Zonar whey had a protective effect on hepatocytes, preventing the accumulation of triglycerides in the cytoplasm.

7. Due to the results obtained regarding the relative weight of the organs, systemic oxidative stress and histopathological examination of liver and kidney tissue samples show

that long-term administration of Zonar whey to laboratory animals (rats) does not cause systemic or body.

RECOMMENDATION

We recommend the use of Zonar lactoser as a dietary supplement, especially in a balanced diet due to its antioxidant potential, both with beneficial metabolic and anti-fat results.

It is also recommended to use Zonar whey as a complementary therapy in obese patients undergoing calorie restriction.

It is recommended to perform an experimental study of obesity for a period longer than 11 weeks, by hyperlipidic food to establish specific histopathological lesions in the related pathology.

It is recommended to use anthropometric measurements in rats used in obesity protocols, as they are effective in monitoring the accumulation or loss of body weight.

ORIGINALITY AND INNOVATIVE CONTRIBUTIONS

The studies carried out bring a first innovative element by creating a personalized hyperlipidic food in order to use it in a protocol for experimental induction of obesity in laboratory rats.

Another innovative element is the study of the effects and bioactive nutraceutical properties of a natural product (Zonar whey) obtained through an eco-innovative technology, in the monitoring and control of experimental obesity in rats.

Due to the lack of information on the use of morphometric measurements on Sprague-Dawley rats in obesity studies, the research was able to obtain reference values for BMI and OI in male Sprague-Dawley rats.

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