

Effect flaxseed oil beeswax-oleogel supplementation in diet fed rat on health functionality aspect and its possibility as a therapeutic food ingredient

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Abstract

The imbalanced consumption of high-fat food has a significant role in health. This study aimed to investigate the fundamental health effect of an oleogel made of flaxseed oil and beeswax, an innovative fat ingredient, fed to rats for 5 weeks. Male Sprague-Dawley rats (N = 20) were divided into four treatment groups of different diets: i) control (normal diet), ii) high-fat diet (HFD), iii) flaxseed oil-beeswax oleogel (FBOG), and iv) HFD incorporating FBOG. Body weight gain, dietary intake index, blood glucose, and insulin levels as well as the target genes expression were determined. The results showed that the bodyweight of rats was reduced in the HFD + FBOG group (89.20±16.17 g) compared with the HFD-only group (92.20±21.36 g) ($p < 0.05$). Rats treated with FBOG alone had the highest dietary intake index (24.92±4.33 g). Rats fed the FBOG diet showed improved blood biochemical characteristics by modulation of glucose (134.80±7.68 mg/dL) and insulin (31.51±1.32 mU/L) levels ($p < 0.05$). Glucose transporter 2 (GLUT-2) and insulin receptor substrate 2 (IRS-2) gene expression was up-regulated after diet supplementation with FBOG whereas nuclear factor-kappa B p65 (NF-κB p65) gene expression was down-regulated ($p < 0.05$). Consequently, FBOG could be used as a fat replacement in food to improve body weight gain and the blood biochemical profiles of animals by regulating glucose and insulin signalling, contributing to reducing the risk factors for pro-obesogenic effects and obesity-related diseases. Besides that, this work may provide fundamental results to develop oleogel as therapeutic food ingredient.

1. Introduction

High-fat food consumption is a major cause of metabolic disorder diseases such as obesity and diabetes (Cooke *et al.*, 2016; Simopoulos, 2016). In the last decade, it has been estimated that more than 1.9 billion people in the world are overweight and exceed the BMI standard (WHO, 2016). Long-term consumption of a high-fat diet (HFD) results in fat cell dysfunction via the accumulation of excess fat and poor lipid metabolism (Issara *et al.*, 2020). Moreover, an HFD can cause an atherosclerotic effect through the blood biochemical characteristics of mammals, with increased low-density lipoprotein (LDL) cholesterol and decreased high-density lipoprotein (HDL) cholesterol levels, contributing to cardiovascular disease (Weintraub, 2002; Madkhali, 2020). Vankoningsloo *et al.* (2005) reported that the accumulation of a lot of triglycerides (TG) induces the lower activity of 3T3-L1 cell mitochondria, suppressing the fatty acid beta-oxidation process and

glucose metabolism pathway. The imbalance of the omega-6/omega-3 ratio in food increases the risk of entering a pro-obese state, via regulation of adipogenic gene expression in adipocytes as described by Simopoulos (2016). Generally, even though the obese state has been identified by increased body weight gain in mammals through excessive fat absorption or storage, the control of blood characteristics such as blood glucose or serum insulin levels should be of concern and normalized to avoid hypersecretion of insulin which may contribute to the development of insulin resistance. Insulin resistance is a relevant predictor of metabolic disorder disease and causes the development of type 2 diabetes mellitus (Zhou *et al.*, 2019). The improvement of glucose homeostasis is one factor regarding insulin secretion by the pancreas. Previous studies have shown that excessive intake of fatty foods down-regulates the expression of genes related to hyperlipidemia and hyperglycemia, prohibiting adipocyte cell cycle re-

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modelling (Weir and Bonner-Weir, 2007; Sun *et al.*, 2011). However, obesity and insulin resistance was induced in non-genetic rats fed an HFD but they did not develop type 2 diabetes (Weir and Bonner-Weir, 2007).

The replacement of fat in food has been highlighted by food technologists together with the food industry due to the trend for healthy consumption. Oleogel is an innovative fat ingredient produced by the organogelation process between an organogelator (e.g. natural waxes, plant sterols, fatty alcohols, etc.) and vegetable oil. An oleogel is a semi-solid system and shows thermo-reversible behaviour. Recently, oleogel has played an important role in several food products because they provide food with texture and mouthfeel, affecting the perception of the consumers. As a consequence, lipid- and/or protein-based oleogel is widely used in the food product development field, for example in baked products and dairy products as well as meat and its related products (Zulim Botega *et al.*, 2013; Lim *et al.*, 2017; Oh *et al.*, 2019; Aliasl-Khiabani *et al.*, 2020; Alvarez-Ramirez *et al.*, 2020; Hwang, 2020). Although lipid-based oleogel can be used as an alternative fat, they present a limited health function for long-term intake. Issara *et al.* (2020) reported that an oleogel made of beeswax and canola oil improved blood biochemical profiles in normal and genetically obese rats. Also, the study by Limpimwong *et al.* (2017) showed a significant reduction in the body weight of a rat after a month of feeding them a diet supplemented with an oleogel made of rice bran wax and rice bran oil. The health benefit of whole rice bran and its extracted products is containing phytochemical substances that can reduce obesity and improve blood cholesterol levels (Issara and Rawdkuen, 2016). Martins *et al.* (2020) noted that health-promoting foods that replace the saturated fat in food products are driving opportunities for using oleogel. The functional lipid components, such as medium-chain TG, diacylglycerides or omega-3 fatty acids, which are components for producing oleogel to maintain obesity have also been described by Kumar *et al.* (2019). Likewise, the mechanisms of protein expression in the pancreas for regulating glucose and insulin signalling pathways and inactivation of inflammatory effects in macrophages may promote a reduction of anti-oxidative stress in animal metabolic syndrome diseases. Nuclear factor-kappa B (NF- κ B), glucose transporter 2 (GLUT-2), and insulin receptor substrate 2 (IRS-2) genes play a major role in the inflammatory response, secretion of insulin, and control of insulin synthesis as well as pancreatic mass production, respectively (Hennige *et al.*, 2003; Ahrén, 2009). The adverse effect of poorly regulated expression of these genes leads to the development of the metabolic syndrome. A study on metabolic syndrome, using an *in vivo* model, suggested

that pancreatic senescence may be caused by reactive oxygen species (ROS) as well as a poor cell proliferation process (Sone and Kagawa, 2005).

Wax is classified as an organic material composed of fatty acid esters and long-chain alcohol groups. The EFSA (2007) recommended a maximum permitted level of beeswax not more than 10,000 mg/kg for use in animal feed. Besides, there was no observed adverse effect level (NOAEL) has been reported for short-term application of beeswax in animal experiments.

Only a few *in vitro* and *in vivo* studies on the effects of oleogel on the lipid metabolism pathway have been conducted; however, there is no report about the long-term intake of an oleogel made of beeswax and flaxseed oil, through supplementation of the diet, on health functionality, particularly anti-hyperglycemia. Therefore, this study aimed to examine the fundamental health effect of flaxseed oil-beeswax oleogel consumption through growth rate and the regulation of glucose and insulin signalling, contributing to improved homeostasis in animals.

2. Materials and methods

2.1 Flaxseed oil beeswax-oleogel and diet preparation

Flaxseed oil and food-grade of beeswax (Hooper pharm GmbH Co., Germany) were purchased from the local supermarket and SINABT agency company in Seoul, Korea, respectively. According to the *in vitro* studies of beeswax safety levels in adipose tissue cells (Issara *et al.*, 2019), the appropriate flaxseed oil beeswax-oleogel (FBOG) was produced by mixing 1% (w/w) beeswax into flaxseed oil, heat at 70°C for 20 mins with continuous stirring. After obtaining the homogeneous FBOG, the gel was cooled at room temperature for 24 hrs until solidified. Then, FBOG was conducted to the RaonBio company and formulated with other ingredients (Issara *et al.*, 2020) and pelleted.

For rat diet preparation, all diet treatment was produced by the RaonBio company (Giheung-gu, Republic of Korea) according to the standard diet composition of animal feeding of the company guideline. All diet treatment was analysed for the fundamental composition by the Institute of Agricultural Science of Chungnam National University (Meister College, Yuseong-gu, Daejeon, 34134 KOREA). All diets treatment was formulated to meet the nutritional requirements of animals which contained the percentage of crude protein, crude fat, crude ash, and energy ranging from 14.13-15.85%, 1.38-16.56%, 2.46-2.92%, and 4047-5149 kcal/kg, respectively.

2.2 Animal and experimental design

The 4 weeks old of male Sprague Dawley rat (N = 20) was obtained from DBL Co., Ltd. Republic of Korea. All animal weights ranged from 100-110 g. Rats were housed and adjusted under the controlled condition following the Animal Care and Use Committee of Institute guidelines (Approve No. SJ-20150701E2), Sejong University, Seoul, Korea. Rats were divided into four groups and fed with a different type of diet including i) control (normal diet), ii) high-fat diet (HFD, which contained beef tallow as the main fat), iii) flaxseed oil beeswax-oleogel (FBOG), and iv) FBOG incorporated HFD. The beef tallow, a general fat component for animal diet production, was used for formulating in all diet treatments to stimulate the weight gain obtained in rats. The experiment was performed for 5 weeks with 30 g/day of diet feeding to every treatment, and during the experiment, water was provided ad libitum.

2.3 Tissue and blood sample collection

After the experiment period, the CO₂ was used for anaesthetizing rats, a blood sample was collected by cardiac puncture. The obtained blood sample was centrifuged at 1000 g for 10 mins to avoid the coagulant and the animal serum was collected for further analysis. The whole pancreas was sampled into a sterilized tube and immediately soak in a liquid nitrogen basket to protect the biological parameters changes (Limpimwong *et al.*, 2017; Issara *et al.*, 2020).

2.4 Growth rate measurement and dietary intake index determination

All rats were examined for their body weight and dietary intake every day for all group treatments by using the animal balance and data was recorded. In brief, about 30 g of animal diet was prepared and fed to the rats every day in one batch while the water was provided ad libitum during the experimental period. The dietary intake index was calculated and presented during the initial and final week of the experiment (Limpimwong *et al.*, 2017; Issara *et al.*, 2020).

2.5 Blood biochemical profile analysis

The blood glucose of rats was measured every week by using the glucose meter (CareSens® Blood Glucose Test Strip, Republic of Korea) until the experimental finish, whereas the insulin level was examined in the initial week and last week of the trials period by commercial ELISA test kit (RayBio® Rat Insulin ELISA Kit, Norcross, GA, USA) according to the manufacturer's instructions of the analysis. The result from the experiment was presented as mg/dL and mU/L,

respectively. In brief, for blood glucose determination, animals were placed and the tail was caught, then a small needle was performed for a blood sample collection. The obtained blood sample was put in the digital glucose meter and data was recorded.

2.6 Quantitative real-time PCR determination

Total RNA of the whole pancreas was extracted by using MagListo™ 5 M Tissue RNA Extraction Kit (Bioneer Corporation, Seoul, Korea) according to the manufacturer's protocol. The NANO Drop (Thermo Fisher Scientific, USA) instrument was used for quantifying the RNA level, followed by cDNA synthesis using a commercial kit (AccuPower® CycleScript RT PreMix, Bioneer Corporation, Seoul, Korea) together with GeneAmp® PCR System9700 machine (Applied Biosystems, Singapore) according to the recommendations of the manufacturer. The obtained solution was kept at -80°C for further analysis.

Quantitative real-time PCR was determined using AccuPower® 2X Greenstar qPCR Master Mix (Bioneer Corporation, Seoul, Korea) by StepOnePlus Real-Time PCR system (Applied Biosystems, Marsiling, Singapore). The primers used are GLUT-2: Forward 5'-CATTGCTGGAAGAAGCGTATCAG-3'; Reverse 5'-GAGACCTTCTGCTCAGTCGACG-3' (Delghingaro-Augusto *et al.*, 2004), IRS-2: Forward 5'-CCCCAGTGTCCTCCATCCT-3'; Reverse 5'-TTTCCTGAGAGACGTTTTCCA-3' (Lingohr *et al.*, 2006) and NF-κB p65: Forward 5'-ATGGACGATCTGTTTCCC-3'; Reverse 5'-GTCTTAGTGGTATCTGTGCT-3' (Yan *et al.*, 2012). The GAPDH gene: Forward 5'-ACCACAGTCCATGCCATCAC-3'; Reverse 5'-TCCACCACCCTGTTGCTGTA-3' (Issara *et al.*, 2020) was used as the housekeeping gene. The 2^{-ΔΔCT} calculation method for the expression level of the target gene was performed, and the result was expressed as the relative mRNA expression level (fold change).

2.7 Statistical analysis

All experiment data was done for statistical analyses and presented as the mean±SD by one-way ANOVA, Duncan's multiple ranges test with the SPSS program (SPSS V.16 Inc. USA). The significant level of each parameter in this experiment was set at *p* < 0.05.

3. Results

3.1 Effect of FBOG supplementation on growth performance and dietary intake index of rats

The weight of the animal is one key point that causes a pro-obesogenic condition and its related metabolic

disorder. The effect of FBOG supplementation on the growth rate of animals is shown in Figure 1. The highest body weight gain was found in the HFD group (202.20 ± 31.60 g) after the experimental period. The FBOG supplementation group showed a significant reduction in animal weight ($182.00 \pm 27.36.00$ to $192.20 \pm 42.78.00$ g) compared to the HFD group ($p < 0.05$). In the initial week, the dietary intake index (DI) of animals (Figure 2) fed the FBOG diet (15.59 ± 4.33 to 17.31 ± 5.36 g) was significantly lower than that for control and HFD treatments (19.16 ± 3.85 and 18.63 ± 2.39 g, respectively) ($p < 0.05$) whereas there was no significant difference in food intake between animals fed HFD + FBOG (23.76 ± 6.93 g) and the control group (23.59 ± 5.16 g) in the final week of the experiment ($p > 0.05$).

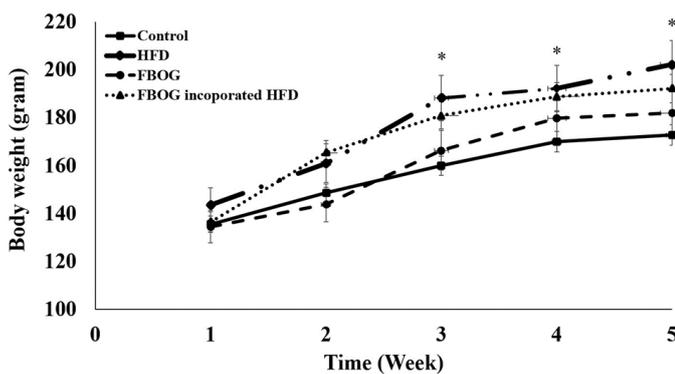


Figure 1. Effect of FBOG supplementation on the growth rate of rats.

*significant difference between treatment and control group at $p < 0.05$.

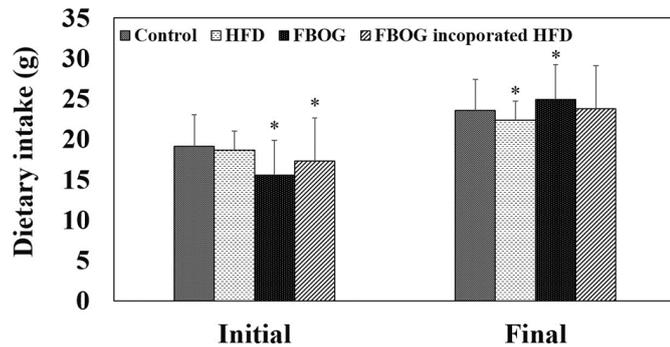


Figure 2. Effect of FBOG supplementation on dietary intake index of rats.

*significant difference between treatment and control group at $p < 0.05$.

3.2 Effect of FBOG supplementation on blood characteristics of rats

Blood biochemical profiles of rats fed with the FBOG diet are shown in Table 1. The blood glucose concentration of the animals in this experiment varied from 104.80 ± 10.92 to 165.00 ± 38.77 mg/dL. Rats fed an HFD showed an increase in blood glucose level from week 2 to week 5 of this trial (ranging from 136.80 ± 8.35 to 165.00 ± 38.77 mg/dL) while glucose level was

normalized and significantly reduced after FBOG supplementation for 3 weeks (132.60 ± 11.80 mg/dL, $p < 0.05$) compared with the HFD group. A comparison of serum insulin levels between the first and final weeks of the experiment showed a reduction in insulin levels in the FBOG treatment group when compared to HFD and control groups ($p < 0.05$). Interestingly, the lowest insulin level was found in the group fed FBOG diet alone (27.33 ± 2.00 mU/L) in the first week but increased slightly after diet intake for 5 weeks (31.84 ± 0.88 mU/L). However, long-term consumption of FBOG in the diet decreased the serum insulin in rats, resulting in control of the animals' blood glucose levels (Table 1).

3.3 Effect of FBOG supplementation on the regulation of target genes related to homeostasis in the whole pancreas of rats

Regulation of the expression of target genes related to the inflammatory effect, insulin receptors, and glucose transporters in the rat pancreas plays a vital role in glucose homeostasis. The relative mRNA expression level of NF- κ B p65, GLUT-2, and IRS-2 genes is shown in Figure 3. The HFD group showed significantly up-regulated NF- κ B p65 expression (Figure 3A) compared to the other treatments ($p < 0.05$) whereas no significant difference was observed in the group fed FBOG alone ($p > 0.05$) but a significantly lower expression was found for the HFD + FBOG treatment compared to the control ($p < 0.05$). GLUT-2 and IRS-2 gene expression (Figure 3B and 3C) in rat pancreas were stimulated and significantly increased after FBOG supplementation ($p < 0.05$). The chronic consumption of HFD suppressed glucose homeostasis via down-regulation of the IRS-2 and GLUT-2 genes in rats.

4. Discussion

An increase in body weight in mammals is caused by several factors such as the accumulation of lipid in fat cells, increased total TG and total cholesterol in the blood circulation, abnormal secretion mechanisms of leptin, a vital hormone for controlling food intake and energy expenditure, or glucose tolerance and insulin resistance (Wakil and Abu-Elheiga, 2009; Kang *et al.*, 2016; Zhou *et al.*, 2019). The results of this study show that partial substitution of beef tallow (abundance in saturated fat) in the diet by FBOG can reduce the body weight gain of animals (Figure 1). The correlation between weight gain and DI (Figure 2) showed a significant decrease in DI value in the first week of the experiment while a significant increase in DI value was observed only in FBOG among the treatment group at the end of the experiment ($p < 0.05$). Zhao *et al.* (2020) reported that chronic consumption of HFD confers leptin resistance in mice with induced obesity through the

Table 1. Effect of FBOG supplementation in diet on the blood glucose and serum insulin level of rats

Parameters	Treatments	Weeks				
		1	2	3	4	5
Blood glucose (mg/dL)	Control	104.80±10.92 ^{bc}	130.80±10.26 ^{cB}	140.20±5.50 ^{aA}	131.80±6.57 ^{bB}	131.40±15.82 ^{cB}
	HFD	123.20±11.97 ^{aC}	139.60±21.29 ^{aB}	138.40±6.58 ^{abB}	136.80±8.35 ^{aB}	165.00±38.77 ^{aA}
	FBOG	106.80±25.76 ^{bc}	138.80±11.30 ^{aA}	132.60±11.80 ^{bB}	128.60±11.48 ^B	135.20±18.54 ^{bA}
	FBOG incorporated	122.60±45.11 ^{aC}	133.00±8.94 ^{bAB}	128.60±10.21 ^{bB}	132.00±7.68 ^{bAB}	134.80±7.89 ^{bcA}
Insulin level (mU/L)	Control	29.71±1.74 ^{ab}	-	-	-	33.05±2.04 ^{abA}
	HFD	30.80±1.70 ^{ab}	-	-	-	34.71±2.43 ^{aA}
	FBOG	27.33±2.00 ^{bB}	-	-	-	31.84±0.88 ^{bA}
	FBOG incorporated	28.54±2.23 ^{bB}	-	-	-	31.51±1.32 ^{bA}

Values are presented as mean±SD. Values with different lowercase superscripts within the same column and values with different uppercase superscripts within the same row are significantly different at $p < 0.05$.

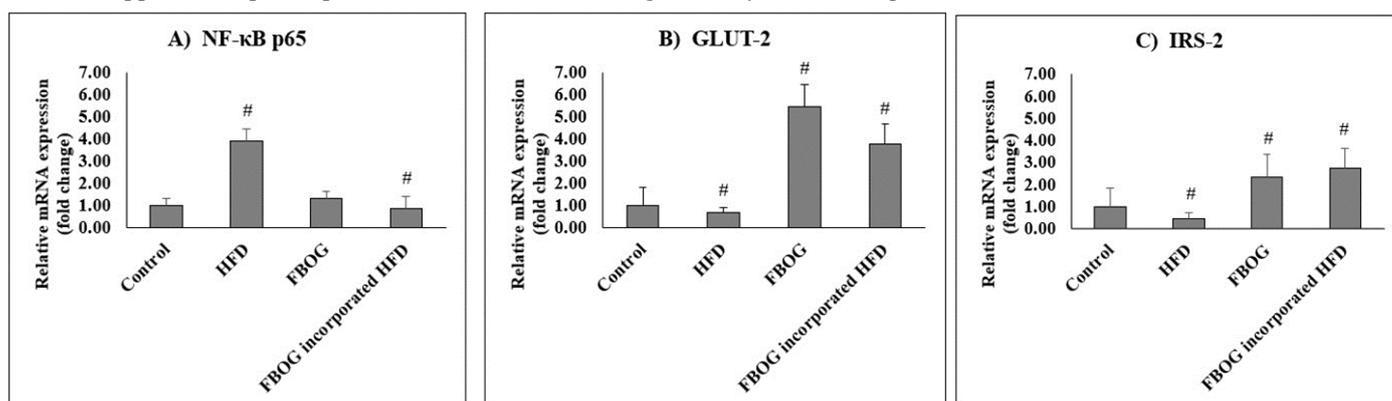


Figure 3. The relative mRNA expression levels of NF-κB p65 (A), GLUT-2 (B), and IRS-2 (C) in the whole pancreas of rats after feeding with different types of diet.

*significant difference between treatment and control group at $p < 0.05$.

regulation of leptin receptor mechanisms in adipocyte cells. In contrast, our previous study showed that there was no significant difference in DI value between HFD and oleogel (made of canola oil and beeswax) diet treatments in normal and mutant rats (Issara *et al.*, 2020). A study associated with low-fat food intake, DI value, and leptin concentration showed that consumption of a diet rich in mono- and polyunsaturated fatty acids decreased the level of leptin hormone in clinical study (Izadi *et al.*, 2014). FBOG is abundant in omega-3 and 6 (polyunsaturated fats) and oleic acid (monounsaturated fat). Hence, based on our findings, it is suggested that FBOG supplementation may suppress the mechanisms of leptin activity and secretion from the hypothalamus, contributing to a balance of food intake, appetite and energy expenditure which should be examined in a further study.

It is not only serum lipid profiles in blood circulation that generate a risk factor for metabolic disease, especially non-alcoholic fatty liver disease (NAFLD); glucose and insulin concentrations also affect body mass creation as well as play an important role in lipid metabolism and accumulation pathways in adipocytes via the regulation of adipokine inflammation responses. According to Table 1, the rats' glucose levels were

improved by partial FBOG replacement in an HFD, which normalized pancreatic insulin secretion, leading to anti-hyperglycemia. Interestingly, our findings are inconsistent with the study of Limpimwong *et al.* (2017) who reported on the replacement of fat in margarine with a formulation made of rice bran oil and wax, they found no difference in animals' blood glucose between HFD (10.9 ± 0.2 mmol/L) and fat substitution (10.8 ± 0.2 mmol/L) treatments ($p > 0.05$). Wilkes *et al.* (1998) found a similar result for reducing the glucose and serum insulin levels in rat skeletal muscle but not fat cells after 3 weeks of supplementation of a modified HFD-induced insulin resistance. This may be explained by the glucose tolerance and insulin resistance state in the animal together with adipokine activity and pancreatic β -cells through the regulation of glucose and insulin receptor-2 associated with G-protein coupled receptors (GPCR) that regulate hormone release in the pancreas (Schuit *et al.*, 2001; Dobbins *et al.*, 2002; Röthe *et al.*, 2020). Our study suggests that feeding rats with the FBOG diet help to maintain blood glucose and serum insulin levels. However, for a better understanding of the mode of action of FBOG supplementation in the diet, this study also determined the expression levels of target genes related to pancreatic senescence resulting in dysfunctional glucose and insulin signalling.

Disrupted glucose homeostasis is involved in the generation of metabolic disorder diseases in mammals. Several scientific reports have shown that pancreatic inflammation, leading to dysfunctional insulin synthesis and secretion, induces an imbalance of glucose levels in animals (Wilkes *et al.*, 1998; Chen *et al.*, 2002; Dobbins *et al.*, 2002; Sone and Kagawa, 2005; Lingohr *et al.*, 2006; Lan and Albinsson, 2020; R othe *et al.*, 2020). Allam-Ndoul *et al.* (2016) found that NF- B gene expression is suppressed by 50  M omega-3 (DHA and EPA) in human macrophages. A similar result was observed for the down-regulation of the gene for a pancreatic dysfunction-related transcription factor (NF- B p65) that causes inflammatory effects after FBOG intake ($p < 0.05$) when compared to control and HFD group treatments in this study. Moreover, long-term consumption of food containing FBOG improved the glucose and insulin signalling pathways by stimulating the mRNA expression levels of GLUT-2 (Figure 3B) and IRS-2 (Figure 3C). Poor regulation of GLUT-2 and IRS-2 genes results in glucose tolerance and insulin resistance, respectively (Ahr en, 2009). Costa *et al.* (2011) illustrated the adverse effect of HFD intake in a study with hamsters with induced hyperinsulinaemic status, showing a significant decrease in the capillary regeneration rate after 16 weeks of the experiment. Treatment with  -sitosterol, a common phytosterol found in several plants, vegetable oils and legumes, as a supplement in HFD-fed rats (about 20 mg/kg of total animal body weight) with type-2 diabetes for 1 month induced a greater attenuation in insulin resistance in adipocytes through the insulin receptor (IR)/Akt signalling pathway and improved the activity of glucose transporter 4 (GLUT-4), IR and insulin receptor substrate 1 (IRS-1) (Babu *et al.*, 2020). This suggests that both lipid and glucose metabolism pathways have complex functions together in insulin secretion and synthesis. Although this study has demonstrated the fundamental health perspective of food containing FBOG, further study should investigate the effect of FBOG replacement in the diet on a hyperlipidemia model, to confirm the precise mode of action of FBOG against metabolic syndrome diseases. However, the great regulation of proteins that play a vital role in glucose and insulin receptor activity in this study suggests that feeding rats an oleogel diet can help reduce the risk factors for hyperinsulinemia or insulin resistance which are caused by chronic inflammation of the pancreas, contributing to anti-obesity or anti-diabetic adaptation.

5. Conclusion

According to the results, it can be concluded that FBOG supplementation in the diet can decrease the animals' weight and stimulate glucose homeostasis via

glucose and insulin signalling pathways by suppressing the NF- B p65 and up-regulated the GLUT-2 and IRS-2 genes expression, contributing to reducing the risk factors for metabolic diseases such as obesity and diabetes. Likewise, it is possible to develop flaxseed oil beeswax-oleogel as a potential therapeutic food ingredient.

Conflict of interest

The authors declare no conflict of interest in this work.

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