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Research article

Investigation of calonysterone and 20-hydroxyecdysone effects in high-fat, high-sugar diet-induced obesity rat model

Alaa AM. Osman [a], Dávid Laczkó [b,c], Máté Vágvölgyi [b], Adrienn Seres-Bokor [a], Anita Sztojkov-Ivanov [a], Kata Kira Kemény [a], Attila Hunyadi [b,d], Eszter Ducza [a,*]

[a] Department of Pharmacodynamics and Biopharmacy, Faculty of Pharmacy, University of Szeged, H-6720, Szeged, Hungary
[b] Institute of Pharmacognosy, Faculty of Pharmacy, University of Szeged, H-6720, Szeged, Hungary
[c] RotaChrom Technologies PLC, H-6000, Kecskemét, Hungary
[d] HUN-REN-SZTE, Biologically Active Natural Products Research Group, H-6720, Szeged, Hungary

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ABSTRACT

Globally, the incidence of obesity among adults has significantly risen since 1990, with a more than twofold rise in prevalence. Similarly, the incidence of obesity among adolescents has increased fourfold. Overweight constitutes a significant health and social issue in developed nations globally. Conventional therapies such as lifestyle modification (nutrition and physical activity) have limited weight reduction. Drug therapy is often not possible or cannot be afforded due to poor patient compliance or therapeutic side effects.

20-hydroxyecdysone (20E) is a worldwide used 'green anabolic' dietary supplement that has beneficial effects in some animal models of metabolic diseases. Our ongoing research examines the impacts of 20E and calonysterone (CAL) in an animal model with a diet high in fats and sugars (HFHSD).

Glucose tolerance tests assessed prediabetic status and RT-PCR and Western blot analysis determined interleukin-6 (IL6) expression. The concertations of superoxide dismutase, catalase, adiponectin, leptin, and IL-6 were quantified by ELISA. Total antioxidant capacity was assessed using a colorimetric assay kit, and global DNA methylation was also measured.

CAL entirely prevented HFHSD-induced obesity and decreased the inflammatory cytokine (IL6) level and antioxidant activity in our model. Both 20E and CAL normalized the changed plasma concentration of adiponectin and leptin after the HFHS diet. The administration of CAL and 20E in obese rats significantly increased the percent of total DNA methylation.

This is the first in vivo study on this natural ecdysteroid, which may offer new alternatives for treating metabolic diseases. Based on our findings, we are supposed to show new preventive possibilities for overweight-induced chronic progressive diseases.

1. Introduction

Obesity is abnormal adipose tissue accumulation when a body mass index (BMI) above 30 kg/m^2 can be measured. It is recognized as a global epidemic and is classified as a chronic progressive disease [1,2]. Moreover, it constitutes a significant risk factor for

* Corresponding author. Eötvös u. 6, H-6720, Szeged, Hungary.
E-mail address: ducza.eszter@szte.hu (E. Ducza).

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numerous diseases, e.g., metabolic disorders, cardiovascular diseases, musculoskeletal disorders, and neurodegenerative diseases, depression, dementia, obstructive sleep apnea and certain cancer types, which are altogether responsible for over 70 % of mortality worldwide [3].

In obesity, average, healthy adipocytes are transformed into large, dysfunctional ones, disturbing the levels of the released adipokines. The concentration of leptin and pro-inflammatory adipokines (tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and IL-1β) is elevated, whereas the level of adiponectin is decreased [4,5]. Elevated levels of pro-inflammatory cytokines contribute to chronic low-grade inflammation, which subsequently results in oxidative stress marked by a rise in reactive oxygen species. Both conditions potentiate each other and link obesity to many co-morbidities, including type 2 diabetes, mainly through insulin resistance in addition to other mechanisms [5].

Obesity contributes to insulin resistance across various tissues, notably in white adipose tissue, skeletal muscle, and the liver. In the liver, the important organ for glucose and lipid metabolism, the increased drain of free fatty acids and proinflammatory cytokines through the portal vein, and the excess of ectopic fat lead to the accumulation of intermediate toxic lipid products that induce hepatic insulin resistance. This hepatic insulin resistance affects glucose metabolism through resistance to the insulin-mediated suppression of gluconeogenesis, resulting in increased hepatic glucose output and higher plasma glucose levels, as well as affects lipid metabolism through the enhancement of de novo liver lipogenesis, leading to further accumulation of ectopic fat and hence to liver diseases [4,6]. Due to the negative consequences of obesity, it is still essential to find new targets for therapy.

Phytoecdysteroids are polyhydroxylated steroids present in very high structural diversity in plants as a chemical defense against non-adapted invertebrate herbivores [7]. It has been reported that they exert a varied spectrum of beneficial effects in mammals, including impacts on protein, lipid, and carbohydrate metabolism [6] in addition to their known anabolic effects. The most prevalent phytoecdysteroid in the market is 20-hydroxyecdysone (20E) [8]. Its base-catalyzed autoxidation results in many oxidized derivatives, including the rare natural ecdysteroid calonysterone (CAL) [7]. In our previous studies, we found that this compound has a greater effect on actin phosphorylation in skeletal muscle cells of mice than 20E, suggesting enhanced anabolic, antidiabetic, and cytoprotective potential [6,8]. The effects of calonysterone on obesity and obesity-induced prediabetic/diabetic conditions have not been studied yet. Diabetes-related health problems impose a significant financial burden on society. Poor patient compliance during drug therapy further increases health damage. Besides their many potential therapeutic benefits in multifactorial diseases such as diabetes, natural products and therapies of natural origin also have a strong tendency to promote compliance, which is of key importance in the success of long-term treatment.

Building upon our previous findings and the aforementioned considerations, our research aimed to analyze the impact of calonysterone and 20-hydroxyecdysone on body weight, blood glucose levels, antioxidant capacity, adipokine levels, the expression of the cytokine IL-6 associated with low-grade inflammation, and epigenetic modifications in a rat model subjected to a high-fat, high-sugar diet.

2. Material and methods

2.1. Animal experiments and specimen collection

All animal experiments in this study strictly complied with the ARRIVE guidelines and adhered to the European Communities Council Directive (2010/63/EU) and the Hungarian Act for the Protection of Animals in Research (Article 32 of Act XXVIII). The National Scientific Ethical Committee on Animal Experimentation approved all experiments involving animal subjects (registration number: IV/717/2023). All procedures were executed following relevant regulations and guidelines.

Sprague-Dawley rats (INNOVO Ltd., Hungary) were maintained in a controlled environment, relative humidity of 30–70 %, and maintaining a temperature of 22 ± 3 °C a 12-h light and dark cycle.

The animals were categorized into two distinct groups and provided with a specific diet either a high-fat, high-sugar diet (HFHSD, comprising 28 % fat, 16 % protein, and 56 % carbohydrates) (C1011, Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) or a standard diet (SD) (1314, Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) from the age of 5–6 weeks until the day of sacrifice, with tap water provided ad libitum. The animals' weights and their food consumption were documented every week. The animals were euthanized under isoflurane anesthesia using a portable small animal anesthesia machine (R550, RWD, Shenzhen, China). Following euthanasia, the organs were excised, and their wet weights were measured (Kern ABJ-NM, Kern & Sohn GmbH, Balingen-Frommern, Germany). We collected blood through cardiac puncture in a small animal operating room, utilizing sterile syringes, needles, and collection tubes. Plasma samples and liver tissue samples were subsequently stored at −80 °C until the time of analytical experimentation.

2.2. Experimental design

A total of 24 six-week-old male Sprague-Dawley rats were divided into four equal groups. Three groups of rats were fed a high-fat, high-sugar diet (HFHSD) to create the obese group, and the standard commercial rat chow was fed to the normal control group.

The rats were treated with calonysterone (CAL) and 20-hydroxyecdysone (20E) (10 mg/kg) in methylcellulose daily, by oral gavages, or received no treatment as follows: Group 1: control HFHSD (not treated), Group 2: HFHSD + 20E treated, Group 3: HFHSD + CAL treated, Group 4: normal diet control (not treated).

The rats were administered a diet and treatment regimen over 12 weeks. Baseline data regarding each animal's body weight and food intake were meticulously recorded. Throughout the experiment, the quantity of food consumed and the body weight of each

animal were measured every week. During the glucose tolerance test (GTT), plasma glucose was measured at baseline, at weeks 6 and 12 of the experiment. After the completion of the study, the animals were terminated, liver tissues and blood were collected for analysis. The experimental design is illustrated in Fig. 1.

2.3. Preparation of phytoecdysteroids

2.3.1. 20-Hydroxyecdysone (20E)

20-Hydroxyecdysone, obtained from the root extract of *Cyanotis arachnoidea*, was procured from Shaanxi KingSci Biotechnology Co., Ltd. (Shanghai, People's Republic of China) with a purity of 90 %. The compound underwent recrystallization twice using a solvent mixture of ethyl acetate and methanol (2:1, v/v) to achieve a final purity of 98.5 %, as determined by High-Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD).

2.3.2. Calonysterone

Calonysterone (CAL) was prepared by base-catalyzed autoxidation of 20E as published before [8]. Briefly, 20E (3 g) was dissolved in methanol (32 ml), and diluted with an additional 112 ml of water. Aqueous NaOH solution (2.4 g/24 ml) was then added, and the solution was stirred at room temperature. After 6 h of stirring, the solution was acidified with HCl and the solution was further stirred overnight. Finally, the pH was neutralized with aqueous NaOH solution, and the solvent was evaporated under reduced pressure at 40 °C. Purification of calonysterone was performed by using flash chromatography on a Combiflash Rf + instrument (Teledyne ISCO, Lincoln, NE, USA) using RediSep C18 columns (Teledyne ISCO, Lincoln, NE, USA) with an aqueous mobile phase gradient containing 25 to 50 (v/v) % MeOH. The fractions were combined based on their TLC fingerprints, and those containing calonysterone were evaporated under reduced pressure at 40 °C.

2.4. Glucose tolerance test

The glucose tolerance test (GTT) was executed in weeks 0, 6, and 12 of our study with intraperitoneal administration of 2 g/kg glucose in a 25 % solution. Glucose levels were assessed using a Dcont® ETALON® Glucose Meter (77 Elektronika Ltd., Hungary). After a fasting period of 16 h, the GTT test was conducted in accordance with our previously published experiments at intervals of 15, 30, 45, 60, 90, 120, and 240 min post-injection [9].

2.5. RT-PCR study

Liver tissues were collected in RNAlater Solution (Sigma-Aldrich, Hungary) before being kept at −80 °C until total RNA subtraction could be conducted. The preparation of total RNA and the RT-PCR were conducted in accordance with our previously published experiments [10]. The primers utilized included assay ID Rn01410330_m1 for interleukin 6 (IL6) and Rn00667869_m1 for β-actin, which served as the endogenous control (Thermo Fisher Scientific, Hungary).

2.6. Western blot analysis

The homogenization of liver tissue solution using RIPA lysis and extraction buffer was supplemented with a cocktail of protease inhibitors. Following the protein extraction process, the concentrations of proteins in the supernatant layer were quantified using a spectrophotometer, and the samples were subsequently stored at −80 °C.

The analytical methods employed for assessing 50 μg of protein per well are derived from our previously published experiments [10]. We utilized polyclonal antibodies targeting IL-6 (26 kDa, dilution 1:600, ThermoFisher Scientific, Hungary) and β-actin (42 kDa, 1:600, Bioss Antibody) in a blocking buffer.

2.7. Colorimetric and ELISA assays

Following the manufacturers' guidelines, the levels of superoxide dismutase (SOD, catalog number: MBS036924, MyBiosource,

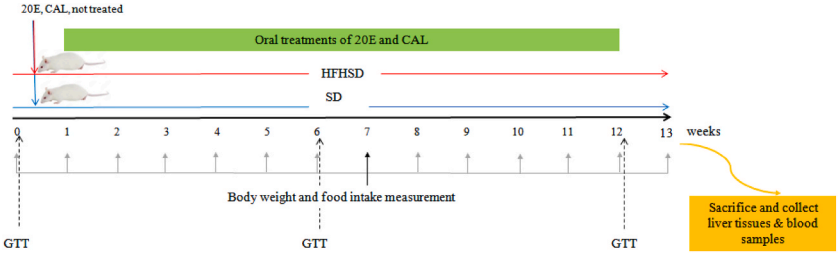


Fig. 1. The design of the animal experiment. 20E: 20-hydroxyecdysone; CAL: calonysterone, HFHSD: high-fat, high-sugar diet; GTT: glucose tolerance test; SD: standard diet; n = 6 in all groups.

USA), catalase (CAT, catalog number: MBS458146, MyBiosource, USA), adiponectin (catalog number: ER0006, FineTest, China), leptin (catalog number: ER0115, FineTest, China), and interleukin-6 (IL-6, catalog number: 900-M86, PeproTech, USA) in liver or plasma were quantified using rat ELISA kits. Additionally, the liver's total antioxidant capacity (T-AOC) was assessed using a colorimetric assay kit (catalog number: E-BC-K136-S, Elabscience, USA). All optical density measurements were conducted utilizing a SPECTROStar Nano microplate spectrophotometer (BMG Labtech, Germany).

In the assays where necessary, we determined the protein concentration of the samples and worked with identical protein concentrations. We standardized the tissue by homogenizing it with a specific volume of PBS, typically using a ratio of 10 mg of tissue to 100 μl of PBS. After homogenization, the samples were centrifuged for 20 min at 1000×*g*. The supernatant was then collected, and the experiment's optimal sample dilutions were determined. The samples were diluted using 0.01 mol/L phosphate-buffered saline (PBS) at a pH of 7.0–7.2.

2.8. Global DNA methylation in the liver

The isolation of DNA from the liver tissues was made by GeneaidTM DNA Isolation Kit (Geneaid Biotech Ltd. China), and total DNA methylation was measured by Methylated DNA Quantification Kit (Abnova Ltd.).

2.9. Statistical analysis

Statistical analyses were performed utilizing Prism 4.0 software (GraphPad Software Inc., San Diego, CA, USA). All data underwent one-way ANOVA, followed by the Newman-Keuls multiple comparison test, and are presented as the mean ± standard error of the mean (SEM). A significance level of $p < 0.05$ was established.

3. Results

3.1. Effects of 20E and CAL on body weight

Throughout the experimental period, the body weights of all groups were assessed on a weekly basis. The averages of body weight increased continuously during the study, and this increase was more pronounced in the obese in the obese groups than in the control group.

The changes in the body weight between the first and last days of the study show a significant increase in the diet high in fats and sugars (HFHSD) group compared to the control. The daily administration of phytoecdysteroid treatments for 12 weeks prevented the weight gain effect of the high-fat, high-sugar diet. After the experiment, the untreated obese animals showed the highest increase in body weight relative to the control group, 20E and CAL groups, i.e., ecdysteroids fully prevented HFHSD-induced obesity in our model (Fig. 2).

3.2. Effects of 20E and CAL on plasma glucose level

Fig. 3 presents the glucose tolerance tests at weeks 6 and 12 of the study. The HFHSD group had higher plasma glucose concentrations at all time points after glucose loading (Fig. 3a) and significantly higher values for the AUC than the control group. Following six weeks of the HFHSD diet, the maximum glucose level was lower, but the AUC values were similar after 20E and CAL treatment (Fig. 3b). The 20E and CAL administration to the HFHSD rats did not improve glucose tolerance, as indicated by higher plasma glucose levels at all time points following glucose loading on week 12 (Fig. 3c). However, the course of the curves changed after the treatments, which may indicate different glucose sensitivity (Fig. 3d).

3.3. Effects of 20E and CAL on liver antioxidant capacity

Obese rats demonstrated a remarkable reduction in the total antioxidant capacity of the liver. This reduction was effectively

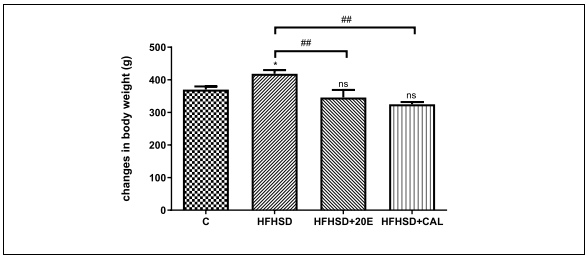
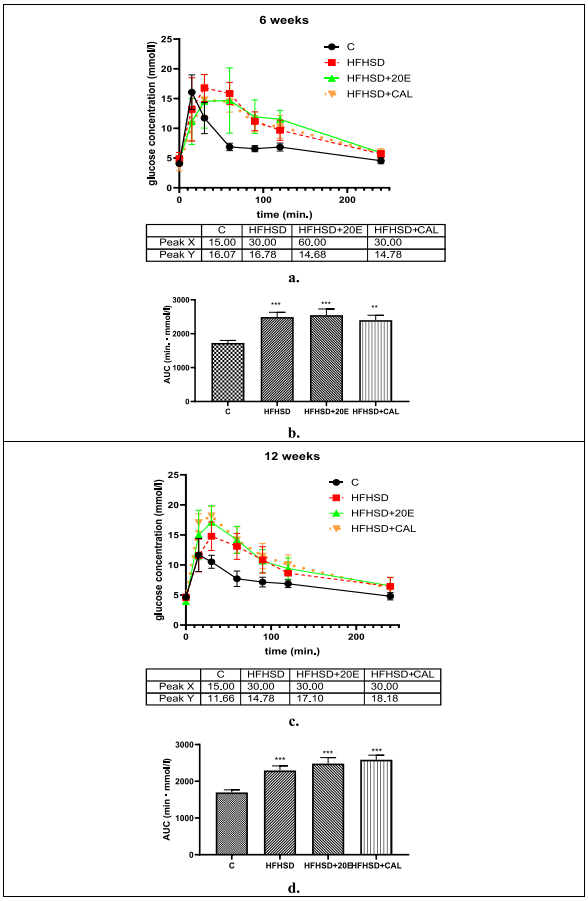


Fig. 2. Variations in body weight throughout the study. C: control; HFHSD: high-fat, high-sugar diet; 20E: 20-hydroxyecdysone; CAL: calonysterone; ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; compared to the control ##: $p < 0.01$ compared to the HFHSD group, $n = 6$. Data are presented as means ± SEM.



	C	HFHSD	HFHSD+20E	HFHSD+CAL
Peak X	15.00	30.00	60.00	30.00
Peak Y	16.07	16.78	14.68	14.78

	C	HFHSD	HFHSD+20E	HFHSD+CAL
Peak X	15.00	30.00	30.00	30.00
Peak Y	11.66	14.78	17.10	18.18

Fig. 3. Alterations in blood glucose levels and AUC. Blood glucose level and AUC change after glucose loading to the standard diet (SD), high-fat, high-sugar diet (HFHSD), and 20E- or CAL-treated HFHSD groups at week 6 (Fig. 3a and b) and week 12 (Fig. 3c and d) of the study. The plasma curves were analyzed by calculating the area under the curve (AUC). ns: $p > 0.05$; **: $p < 0.01$; ***: $p < 0.001$. $n = 6$. Data are presented as means ± SEM.

mitigated and significantly enhanced through phytoecdysteroid treatments compared to the control group (Fig. 4a).

Administration of 20E to HFHSD rats significantly increased superoxide dismutase, whereas CAL did not change this compared to the control (Fig. 4b). The HFHSD significantly decreased, while the phytoecdysteroid (20E, CAL) treatments significantly increased the catalase levels compared to the control (Fig. 4c).

3.4. Effects of 20E and CAL on leptin and adiponectin receptor concentration in plasma

The plasma level of leptin substantially enhanced in the HFHSD group compared to the non-treated group (C), and this was normalized by 20E and CAL treatment (Fig. 5a).

The plasma concentration of adiponectin significantly decreased after the HFHS diet, and both 20E and CAL normalized this (Fig. 5b).

3.5. Effects of 20E and CAL on pro-inflammatory cytokines IL6 mRNA and protein expressions in the liver and plasma

IL6 mRNA and protein expression significantly increased in the liver tissues of obese rats, and both 20E and CAL efficiently counteracted this effect of HFHSD (Fig. 6a and b). HFHSD significantly increased the plasma IL6 level, which was dramatically decreased after phytoecdysteroid treatment, especially after treatment with 20E, which decreased the IL6 level significantly below that of the control group (Fig. 6c).

3.6. Effects of 20E and CAL on the levels of global DNA methylation in the liver

The administration of phytoecdysteroid in obese rats significantly increased the levels of global DNA methylation in the liver compared to the control (Fig. 7).

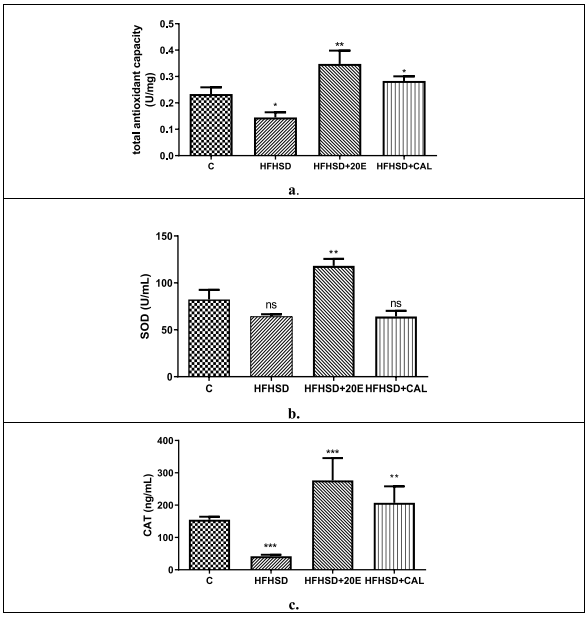


Fig. 4. Effects of 20E and CAL on oxidative factors. Effects of 20E and CAL on total antioxidant capacity (**a**) and the levels of superoxide dismutase (SOD, **b**) and catalase (CAT, **c**) in the liver after a control (C) and high-fat, high-sugar diet (HFHSD). ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ compared to the control (C). $n = 6$. Data are presented as means ± SEM.

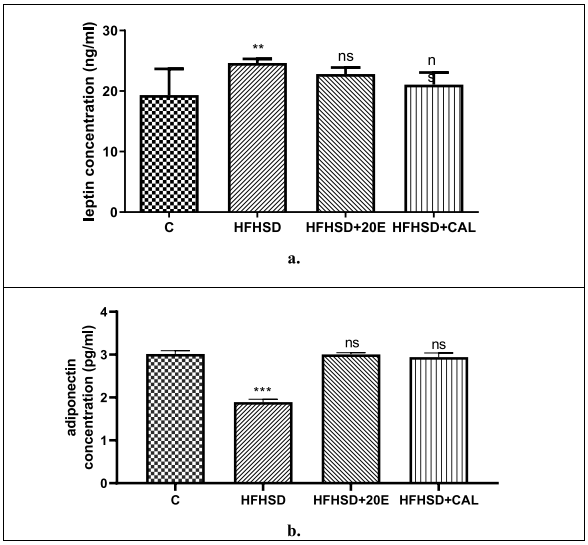


Fig. 5. Leptin and adiponectin concentration. Changes in the plasma concentration of leptin (a) and adiponectin (b) after control diet (C) and high-fat, high-sugar diet (HFHSD). ns > 0.05, **: $p < 0.01$; ***: $p < 0.001$ compared to the control. $n = 6$. Data are presented as means ± SEM.

4. Discussion

Obesity constitutes a considerable risk factor for different health problems. Within the European Region of the World Health Organization, approximately 60 % of adults and nearly one in three children are affected by overweight and obesity. Preliminary research conducted in the region showed that the incidence of overweight and elevated mean body mass index among children and adolescents has risen during the SARS-CoV-2 health crisis [11]. The obesity epidemic is complicated by several comorbid conditions, such as hypertension, cardiovascular disease, insulin resistance, diabetes mellitus (type 2), dyslipidemia, nonalcoholic fatty liver disease, asthma and atherosclerosis.

Obesity leads to many pathological consequences, including endothelial dysfunction, hypercoagulability, oxidative stress and inflammation. The essential contributors to oxidative stress in obesity encompass elevated glucose, leptin and tissue lipids level,

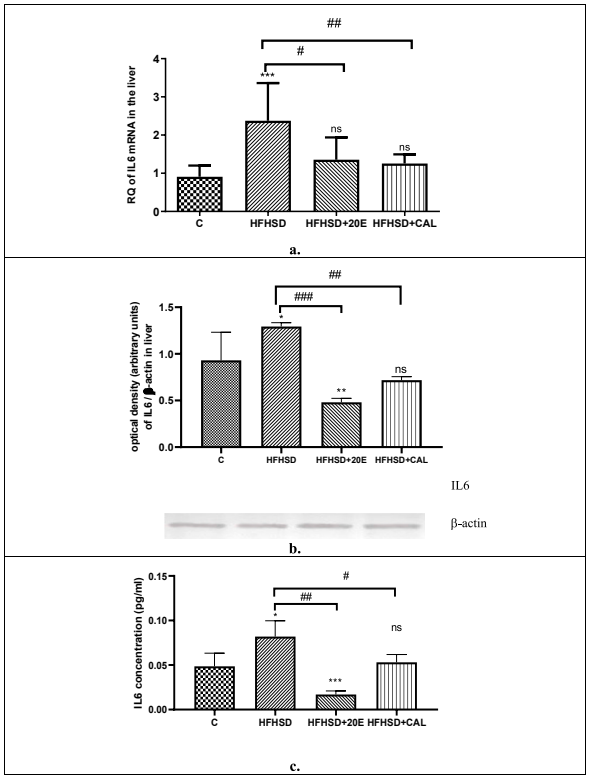


Fig. 6. Changes of IL6 expression. Effects of 20E and CAL on the levels of IL6 mRNA (a), protein (b) expression in the liver and the IL6 concentration in the plasma (c). HFHSD: high-fat, high-sugar diet, C: control; ns > 0.05, *: $p < 0.05$; **: $p < 0.01$ ***: $p < 0.001$ compared to the control, #$p < 0.05$; ##$p < 0.01$, ###$p < 0.001$ compared to the HFHSD group. $n = 6$. Data are presented as means ± SEM.

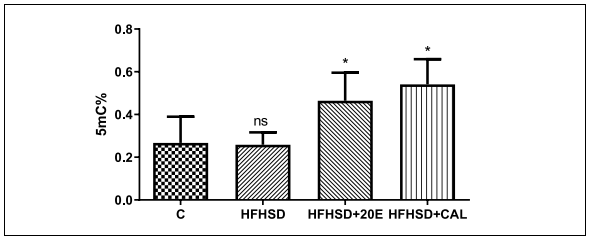


Fig. 7. Alterations in total DNA methylation. Effects of 20E and CAL on the levels of global DNA methylation in the liver. HFHSD: high-fat, high-sugar diet, C: control; ns > 0.05, *: $p < 0.05$. $n = 6$. Data are presented as means ± SEM.

deficiencies in minerals and vitamins, persistent inflammation, increased muscular activity associated with the burden of excess weight, endothelial dysfunction, and impaired mitochondrial function. Additionally, dietary composition plays a significant role in this context [12]. White adipose tissue serves not only as a reservoir for energy storage within the body but also plays a crucial role in the regulation of metabolic pathways, including those related to immunity and inflammation. This tissue secretes a diverse array of pro-inflammatory and anti-inflammatory factors, including adipokines such as leptin and adiponectin, as well as cytokines such as tumor necrosis factor-alpha and IL-6 [13].

Calonysterone (CAL) and 20-hydroxy ecdysterone (20E) prevented weight gain during the high-fat, high-sugar diet. The anti-obesity effect of 20E was previously demonstrated in C57BL/6J mice [14,15], rats [16,17] and gerbils [18], and we confirmed the same effect of CAL. Six or thirteen weeks of HFHSD increased blood glucose concentration compared to the control. Both 20E and CAL decreased the peak of the glucose concentration curve and shifted the time of peak to a later time (30 and 60 min). Surprisingly, after 12 weeks, the 20E and CAL treatments did not stop the glucose level-increasing effect of the HFHSD diet in our model. Some previous experiments with 20E were carried out in a mouse model [12,14] with a shorter (4-week) dietary treatment period, a higher dose [19] in other cases work with female rats [16], in which hormone levels may influence blood glucose concentration. The composition of the food of HFHSD may also be an important influencing factor. In our study, the animal feed contained 28 % fat and 56 % carbohydrates, in contrast with other studies [14,15] using 60 % fat-derived calories and 21 % carbohydrates. Our diet with 20E and CAL did not

decrease blood glucose levels after a long period of high-sugar diet.

Numerous studies have corroborated that 20E diminishes glucose production in hepatic cell cultures, indicating that this outcome is primarily coordinated through PI3K-dependent signaling pathways. Moreover, 20E has been demonstrated to downregulate the expression of the PEPCK and G6Pase genes in a dose-dependent manner. PEPCK is a crucial enzyme that plays a crucial role in regulating hepatic gluconeogenesis, with its operation being linked to hepatic glucose output. In contrast, G6Pase facilitates the final stage of glucose production in the liver by converting glucose-6-phosphate into glucose through hydrolysis [20].

There is substantial evidence demonstrating the critical role of total antioxidant capacity (TAC) in plasma and tissues, along with its fluctuations during the progression of oxidative stress. It is widely recognized that obesity is significantly associated with a reduction in antioxidant capacity, independent of years, metabolic factors, and lifestyle variables. Consequently, it can be posited that lifestyle modifications or pharmaceutical interventions aimed at altering the redox state may serve as potential therapeutic targets for obesity-related metabolic syndrome and associated medical conditions [21]. Earlier TAC was proven to be the best biomarker to assess the antioxidant status of obese patients. 20E and CAL protect against oxidative stress by increasing TAC in obese rats. It is widely accepted that the dietary intake of antioxidants protects against oxidative damage and related clinical complications in many diseases.

Superoxide dismutases (SODs) play a notable role in the antioxidant protection system against oxidative stress within the body, and 20E has been shown to significantly enhance SOD levels. SODs are essential for preventing injuries caused by reactive oxygen species (ROS). These enzymes advance the conversion of superoxide anion radicals ($O_2^{\bullet-}$) into molecular oxygen and hydrogen peroxide (H_2O_2), effectively mitigating superoxide-related cellular damage [22]. Persistent hyperglycemia stimulates the production of ROS and weakens antioxidant defense, therefore dietary or therapeutic protocols that increase SOD levels (e.g., 20E intake) may have a protective effect.

As adipose tissue increases, there is a significant reduction in the level of antioxidant enzymes, especially superoxide dismutase (SOD) and catalase (CAT). 20E and CAL increased the activity of these antioxidant enzymes and demonstrated a defensive effect against oxidative stress in liver tissues.

Adipose tissue functions as a storage site for triglycerides and has recently been recognized in studies as a key producer of various substances that play essential endocrine, paracrine, and autocrine roles. These bioactive compounds, referred to as adipokines or adipocytokines, encompass plasminogen activator inhibitor-1 (PAI-1), tumor necrosis factor-alpha (TNF-α), resistin, leptin, and adiponectin. Predominantly synthesized by white adipose tissue, these substances are integral to the rule of numerous physiological processes and the maintenance of homeostasis [23].

Research has demonstrated that being overweight is linked to increased levels of leptin, which functions as a pro-inflammatory cytokine and exacerbates the form of insulin resistance [24,25]. Observations indicate that leptin levels in obese rats are significantly elevated, consistent with the condition of hyperleptinemia. This suggests that a high-fat diet may induce leptin resistance in certain tissues.

The uncontrolled secretion of "offensive" adipocytokines, such as IL-6, alongside "defensive" adipocytokines, including adiponectin and leptin, plays a crucial role in the onset of weight problem and metabolic syndrome [26]. Adiponectin is an essential adipokine synthesized by adipocytes, significantly regulating glucose levels, lipid metabolism, and insulin sensitivity, thereby supporting overall homeostasis. Its efficacy is enhanced by its potent antioxidant, anti-fibrotic, and anti-inflammatory properties [27]. Plasma adiponectin levels are inversely correlated with systemic oxidative stress because the accumulated fat produces uncontrolled production of adipocytokines [26]. Adiponectin levels are diminished in various pathological conditions, such as diabetes mellitus (type 2), metabolic syndrome, obesity and cardiovascular disorders. Multiple studies have highlighted the protective benefits of adiponectin in diseases associated with obesity and in cancer [27]. We suppose that 20E and CAL may protect against the pathological effects of leptin and adiponectin because they normalize the levels of these adipokines.

A negative relationship exists between plasma adiponectin concentration and body weight, as adiponectin expression decreases with increasing fat mass. Since adiponectin plays a crucial role in promoting energy expenditure and fatty acid oxidation in the liver and skeletal muscles, it can be inferred that the higher adiponectin levels observed in mice treated with 20E may help explain their resistance to diet-induced obesity and insulin resistance [28,29].

Chronic low-grade inflammation is widely acknowledged as a considerable characteristic of obesity, which is closely linked to insulin resistance and type 2 diabetes. Among the factors secreted by adipose tissue, known as adipokines, the inflammatory regulator IL-6 has emerged as a prospective mediator of obesity-related chronic inflammation. IL-6 is an essential cytokine with pro- and anti-inflammatory effects, contingent upon the specific context [30]. IL-6 has been classified as a pro-inflammatory cytokine due to its role in promoting neutrophilia and the differentiation of TH17 T cells while simultaneously inhibiting the development of regulatory T cells. This mechanism important in the formation of numerous inflammatory diseases. In conjunction with obesity and type 2 diabetes, non-alcoholic fatty liver disease represents the most prevalent liver condition, impacting around 20–30 % of the general inhabitants in Western countries [31]. Fatty tissue produces as much as 35 % of soluble interleukin-6 (IL-6), the systemic effects most clearly illustrated in the liver. In this organ, the impairment of insulin actions by IL-6 is mediated through the STAT3-SOCS-3 signaling pathway [32]. Both ecdysteroids in our study normalized the diet-induced increase in IL-6 levels in liver tissues and plasma.

Epigenetics is how factors such as pathophysiological changes (e.g., obesity), environmental influences, medications, lifestyle decisions can influence gene expression without changing the DNA sequence. DNA methylation represents a critical epigenetic mechanism, and its involvement in the etiology of various diseases is the focus of extensive research efforts. DNA methylation is a biochemical process that involves the covalent addition of a methyl group to a cytosine residue in the DNA sequence, particularly at locations where cytosines are adjacent to guanines, known as CpG sites. This chemical modification is facilitated by DNA methyltransferases (DNMTs). Notably, DNA methylation, particularly within gene promoter regions, disrupts gene transcription. This disruption occurs by inhibiting the access of transcription factors to the DNA and by recruiting transcription-repressive proteins [33].

Our study found no difference in the degree of DNA methylation in the liver of control and diet-induced obese rats, but the treatment with 20E and CAL significantly increased DNA methylation in our study. This means that an increase in DNA methylation decreases the transcription rate of genes after ecdysteroid treatment. We presume that this plays a role in the bioactivity of 20E and CAL in the liver, for example, in their antioxidant effects and their ability to influence IL-6, leptin, or adiponectin levels. The increase in size and number of adipocytes in overweight leads to decreased oxygen accessibility and the onset of hypoxic stress. The elevation of reactive oxygen species and oxidative stress is measured in hypoxic conditions, which are recognized as significant mediators of inflammation. HIF3A as hypoxia-inducible factors are modulated by epigenetic effects and are linked to adipose tissue dysfunction. This connection positions hypoxia as a promising epigenetic target for addressing complications related to obesity [34]. Additional research is required to clarify the molecular mechanisms that connect DNA methylation and obesity.

5. Conclusions

Many people refrain from drug therapy but are happy to use naturally derived nutritional supplements for overweight. 20-hydroxyecdysone is known to have beneficial effects (e.g., muscle mass and muscle strength gains), which is why it is used as a dietary supplement in many countries but not for obesity prevention or antioxidant effects. Our studies proved the preventive impact of CAL and 20E on weight gain and the increased impact on total DNA methylation during the HFHSD diet.

CAL has better protected the oxidative effect of obesity, decreased the inflammation cytokine (IL6) level, and normalized the plasma concentration of adiponectin and leptin in the HFHS diet, which can ameliorate the health damage caused by obesity.

CRediT authorship contribution statement

Alaa AM. Osman: Methodology. **Dávid Laczkó:** Methodology. **Máté Vágvölgyi:** Methodology. **Adrienn Seres-Bokor:** Methodology, Formal analysis. **Anita Sztojkov-Ivanov:** Methodology. **Kata Kira Kemény:** Methodology. **Attila Hunyadi:** Supervision, Investigation, Funding acquisition, Conceptualization. **Eszter Ducza:** Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Ethical approval

The National Scientific Ethical Committee on Animal Experimentation approved all experiments involving animal subjects (registration number: IV/717/2023).

Data availability

All data utilized in this study can be found in the article and its supplementary materials. For additional information or requests regarding resources and reagents, please contact the lead investigator, Dr. Eszter Ducza, at ducza.eszter@szte.hu.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Attila Hunyadi has patent #137412-3405 - P2400304 pending to licensee. Eszter Ducza has patent #137412-3405 - P2400304 pending to licensee. David Laczko has patent #137412-3405 - P2400304 pending to licencee. Mate Vagvolgyi has patent #137412-3405 - P2400304 pending to licensee. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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