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Esculetin prevents non-alcoholic fatty liver in diabetic mice fed high-fat diet

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ABSTRACT

This study investigated the effects and mechanism of esculetin (6,7-dihydroxycoumarin) on non-alcoholic fatty liver in diabetic mice fed high-fat diet (HFD). The diabetic mice model was induced by injection of streptozotocin, after which they were fed HFD diet with or without esculetin for 11 weeks. Non-diabetic mice were provided a normal diet. Diabetes induced hepatic hypertrophy, lipid accumulation and droplets; however, esculetin reversed these changes. Esculetin treatment in diabetic mice fed HFD significantly down-regulated expression of lipid synthesis genes (*Fasn*, *Dgat2 and Plpp2*) and inflammation genes (*Tlr4*, *Myd88*, *Nfkb*, *Tnfα* and *Il6*). Moreover, the activities of hepatic lipid synthesis enzymes (fatty acid synthase and phosphatidate phosphohydrolase) and gluconeogenesis enzyme (glucose-6-phosphatase) in the esculetin group were decreased compared with the diabetic group. In addition, esculetin significantly reduced blood HbA_{1c}, serum cytokines (TNF-α and IL-6) and chemokine (MCP-1) levels compared with the diabetic group without changing the insulin content in serum and the pancreas. Hepatic SOD activity was lower and lipid peroxidation level was higher in the diabetic group than in the normal group; however, esculetin attenuates these differences. Overall, these results demonstrated that esculetin supplementation could protect against development of non-alcoholic fatty liver in diabetes via regulation of lipids, glucose and inflammation.

1. Introduction

Diabetes is one of the most common metabolic disorders, and is generally classified as type 1 (T1D) or type 2 diabetes (T2D) [1]. T1D is caused by autoimmune destruction of β-cells, and leads to absolute insulin deficiency, whereas T2D results from insulin resistance and inadequate β-cell function and insulin secretion [2]. Obesity is considered the most important predictor of T2D [3]. The association between T2D and non-alcoholic fatty liver disease (NAFLD) has received considerable research attention, but little is known about the evolution of liver injury in T1D [4]. However, obesity and overweight are increasing in young people with T1D and are associated with significant health problems, such as, cardiovascular complications and dual diagnoses of T1D and T2D [5]. Some studies have reported that the development of T2D in obese young people with T1D, and associated with development of resistance to exogenous insulin [6]. In fact, many overweight people with T1D are insulin-resistant and develop the components of metabolic syndrome [7,8]. A previous study demonstrated that young people with T1D are more likely to be overweight than age-matched non-diabetic controls [9]. Furthermore, it has been suggested that the coexistence of T1D and insulin resistance increases the risk of developing NAFLD and cardiovascular disease [10]. In addition, it has been reported adults or adolescents with T1D consume higher levels of dietary fat than healthy individuals [11,12].

Esculetin (6,7-dihydroxycoumarin) is a coumarin derivative and functional food ingredient found in various medicinal plants, such as, *Artemesia scoparia*, *Artemesia capillaris*, *Citrus limonia* and *Ceratostigma willmottianum* [13,14]. Esculetin is also used in a variety of therapeutic and pharmacological agents [15]. Esculetin has been reported to induce human gastric cell apoptosis through a cyclophilin D-mediated mitochondrial permeability transition pore associated with reactive oxygen species (ROS) [16]. Rubio et al. [17] found esculetin induces the apoptosis of human acute promyelocytic leukemia NB4 cells, and Lin et al. [18] reported it enhanced arsenic trioxide-provoked apoptosis in human leukemia U937 cells. Esculetin has also been reported to have anti-inflammatory, antioxidant and anti-tumor activities [19–22]. In a streptozotocin

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Abbreviations

Actb	actin, beta
DGAT	diacylglycerol acyltransferase
Dgat2	diacylglycerol O-acyltransferase 2
DM	diabetic group
DM-E	diabetic-esculetin group
FAS&*Fasn*	fatty acid synthase
FFA	free fatty acid
G6P	glucose-6-phosphate
G6Pase	glucose-6-phosphatase
GK	glucokinase
HbA_{1c}	glycosylated hemoglobin
H&E	hematoxylin and eosin
HFD	high-fat diet
IL-6&*Il6*	interleukin 6
MCP-1	monocyte chemoattractant protein-1
LPO	lipid peroxidation
Myd88	myeloid differentiation primary response gene 88
NAD^+	nicotinamide adenine dinucleotide$^+$
NADPH	nicotinamide adenine dinucleotide phospophate, reduced form
NAFLD	non-alcoholic fatty liver disease
NC	non-diabetic group
NF-κB&*Nf*κb	nuclear factor kappa-light-chain-enhancer of activated B cells
PAP&*Plpp2*	phosphatidate phosphohydrolase
PEPCK	phosphoenolpyruvate carboxykinase
Pparγ	peroxisome proliferator activated receptor gamma
SOD	superoxide dismutase
STZ	streptozotocin
T1D	type 1 diabetes
T2D	type 2 diabetes
TC	total cholesterol
TG	triglyceride
Tlr4	toll-like receptor 4
TLRs	toll-like receptors
TNF-α&*Tnfα*	tumor necrosis factor alpha

(STZ)-induced diabetic model esculetin protected rats from hepatic and renal dysfunction [19,23–25], and a trinitrobenzenesulphonic acid induced rat model it protected animals from colonic damage [26]. Kadakol et al. [23] reported esculetin protected against altered vascular reactivity in hyperglycemic and hyperinsulinemic rats, and esculetin inhibited galactose-induced cataractogenesis in rats by suppressing aldose reductase expression [27].

Recently, we reported that esculetin reduced hepatosteatosis and insulin resistance in high-fat diet (HFD) HFD-induced obese mice [28], but the mechanism responsible for the anti-hepatosteatotic activity of esculetin in this diabetic animal model was not determined. Accordingly, in the present study, we evaluated the beneficial effects of esculetin on fatty liver and inflammation in STZ-induced diabetic mice fed a HFD.

2. Materials and methods

2.1. Animals and experimental designs

Thirty male C57BL/6N mice that were 6-weeks-old were purchased from Orient Bio Inc. (Seoul, Korea). The mice were individually housed in polycarbonate cages at 22 ± 2 °C on a 12 h light-dark cycle. Mice were fed a pelletized commercial chow diet for one week after arrival, after which they were randomly divided into non-diabetic (n = 8) and diabetic (n = 22) groups. Diabetes was induced by a single injection of STZ (100 mg/kg body weight in 0.1 M citrate buffer, pH 4.2; Sigma, St. Louis, MO, USA) into the peritoneum on two consecutive days. The non-diabetic mice were injected with citrate buffer alone. After seven days, only STZ-treated mice that exhibited a fasting blood glucose level ≥11 mmol/L were used in the study. The diabetic mice were randomly subdivided into two groups of eight mice each; an untreated diabetic (DM) group and a diabetic-esculetin (DM-E) group. The non-diabetic (NC) mice were fed normal diet (5% corn oil, w/w) and diabetic mice were fed HFD containing 40% of the calories from fat (3% corn oil and 18% lard, w/w) with or without esculetin (0.01 g/100 g diet; Sigma) for 11 weeks. The dose of esculetin used was based on our previous study [28]. The composition of the experimental diet was based on the AIN-76 semisynthetic diet [29,30]. Body weight was measured weekly and food consumption was measured daily.

The mice had access to food and water *ad libitum*. At the end of the experimental period, the mice were anesthetized with ether after withholding food for 12 h. Blood samples were then taken from the inferior vena cava for serum biomarker analysis, after which the liver tissues were removed, rinsed with a physiological saline solution and stored immediately at −70 °C until analysis. The present study was approved by the Sunchon National University Institutional Animal Care and Use Committee.

2.2. Blood glucose, glycosylated hemoglobin, insulin and C-peptide levels

The fasting blood glucose concentration was monitored using a glucometer (G-Doctor, AllMedicus, Co., Ltd., Anyang, Korea) to test venous blood drawn from the tail vein every week after a 6 h fast. The glycosylated hemoglobin (HbA_{1c}) concentration in whole blood was measured using a NycoCard Reader II (Alere/Axis-Shield, Oslo, Norway). The serum insulin (ELISA kit, Morinaga Institute of Biological Science, Inc., Yokohama, Japan) and C-peptide (ELISA kit, Shibayagi Co., Ltd., Shibukawa, Japan) levels were then determined using a quantitative sandwich enzyme immunoassay kit. The pancreatic insulin and C-peptide contents were determined as previously described [31].

2.3. Serum cytokines and chemokine levels

Serum tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) levels were determined using a multi detection kit (BioRad, Hercules, CA, USA) and Luminex 200 Labmap system (Luminex, Austin, TX, USA).

2.4. Lipid levels and histological analysis

The serum total cholesterol (TC), triglyceride (TG) (Asan Diagnostics, Seoul, Korea) and free fatty acid (FFA) (Shinyang Diagnostics, Seoul, Korea) concentrations were determined using commercial kits. The hepatic lipid was extracted as previously described [32], after which the cholesterol and TG contents were analyzed using the same enzymatic kit that was employed for serum analysis.

The liver tissues were removed and fixed in a buffer solution