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Original Article

A Standardized Extract of Glycine max co-cultured with Basidiomycota Improves Impaired Lipid Metabolism in High-Fat Diet- Fed Ovariectomized Mice

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Summary

A standardized extract of *Glycine max* co-cultured with *Basidiomycota* (GCP®) is an isoflavone-rich soy extract. GCP® inhibits intracrine androgen synthesis in prostate cancer cells. Estrogen deficiency during menopause can alter lipid metabolism in the body, resulting in obesity and/or various disorders. In this study, the inhibitory effects of GCP® on altered lipid metabolism after menopause were investigated in ovariectomized C57BL/6JJmsSlc mice fed a high-fat diet (HFD). GCP® mice showed a significant decrease in total body weight concomitantly with weight loss of the perirenal adipose tissue as compared to that in control mice. Histological studies showed that GCP® inhibited HFD-induced fatty liver in these mice. mRNA expression levels of ATP-binding cassette protein G1 (ABCG1) and liver X receptor α (LXRα) were increased in liver of GCP® mice compared to that in control mice, whereas no changes were observed in the levels of ATP-binding cassette protein A1 (ABCA1), scavenger receptor class B type I (SR-BI), or low-density lipoprotein receptor (LDLR). Our preliminary results may suggest GCP® as a novel functional supplement candidate for inhibition of HFD-induced altered lipid metabolism after the menopause via the LXRα/ABCG1-dependent pathway.

Introduction

Postmenopausal women are at a higher risk of developing lipid metabolic disorders owing to endogenous estrogen deficiency[1–4]. Estrogen receptors are expressed in both visceral and subcutaneous adipocytes, therefore changes in estrogen levels alter lipid metabolism in adipose tissue of women[2,5]. Moreover, postmenopausal women have higher visceral fat mass and subcutaneous adipose tissue in the abdominal region than those of premenopausal women, excessive visceral fat in the abdomen can cause metabolic alterations, particularly in fatty acid metabolism[2,6].

A Standardized extract of *Glycine max* co-cultured with *Basidiomycota* (GCP®) is a soybean-rich contemporary medicine that contains isoflavones such as genistein, daidzein, and glycitein[7]. GCP® is produced by the fermentation of soybean extract with the mushroom mycelium. This process of fermentation deglycosylates isoflavones and, converts them into aglycones, thereby increasing the absorption of these isoflavones. GCP® exerts anticancer activity against prostate cancer by inhibiting intracrine androgen synthesis[8,9]. Furthermore, it has been reported that GCP® supplementation improves antioxidant status in postmenopausal women with diabetic retinopathy[10].

Decreased estrogen level is a prominent sign of menopause in women. Estradiol (E2) is an essential estrogen for β-oxidation of fatty acids; however, circulating E2 levels decline rapidly after menopause[4,11]. Genistein, which has structural resemblance to 17β-estradiol, is a major isoflavone of GCP®, and it exhibits a weak estrogenic activity in mammals[12,13]. A previous study has shown that ovariectomy in female mice caused fatty liver by increasing lipogenesis and insulin resistance in high-fat diet (HFD) fed mice as determined using hyperinsulinemic-euglycemic clamp, and administration of E2 improved hepatic steatosis and insulin resistance in HFD fed mice through estrogen receptor α (ERα) expression in hepatocytes[14]. Moreover, ova-

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riectomized (OVX) mice showed decreased activity of enzymes involved in β-oxidation of fatty acids and transcription factors required for lipolysis[4]. In this study, we examined the effects of GCP® on body weight and fat metabolism and explored the underlying mechanisms using HFD fed OVX mice as postmenopausal models. This study is expected to prevent impairment of lipid metabolism in postmenopausal women.

Materials and methods

Preparation of GCP®

GCP® is a standard extract of *Glycine max* co-cultured with *Basidiomycota*, produced by Amino Up Co., Ltd. (Sapporo, Japan). The concentrations of genistein, daizein, and glycitein are 219, 112, and 47.6 μmol, respectively. The aglycons such as genistein, daizein, and glycitein, comprised 98.5% of the isoflavones of GCP®[7].

Animals and experimental design

C57BL/6JJmsSlc OVX female mice (9-week old) were obtained from Japan SLC, Inc. (Hamamatsu, Japan). Mice were kept under the following conditions: temperature of 24 ± 1 ℃, relative humidity of 55 ± 5%, 12 h light/dark cycle, and acclimatization for 8 d before the start of experiments with free access to water and low-fat (10 kcal%) powder diet (D12450; Research Diet, Inc., New Brunswick, NJ). Mice were randomly divided into three groups: HFD (60% kcal; Control), 2% (w/w) GCP® in HFD (2% GCP®)[15], and 4% (w/w) GCP® in HFD (4% GCP®) (n = 5–6). Food consumption and body weight were measured 2–3 times per wk. On d 137, mice were euthanized by intraperitoneal administration of a mixture of three anesthetic agents (medetomidine, midazolam, and butorphanol), and the liver and perirenal adipose tissues were removed and weighed. This study was approved by the Institutional Animal Care and Use Committee (approval number: kyo33–2), and conducted in accordance with the Tokyo Healthcare University Animal Experimentation Regulations.

Histological analysis of the liver

The dissected segments of liver were fixed in 4% paraformaldehyde–phosphate-buffered saline at 4 ℃ overnight and embedded in paraffin. The segments were sectioned into 4 μm sections using a microtome, which were then deparaffinized and stained using hematoxylin and eosin (H&E) solution.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

RNA isolation

Total RNA was isolated from the liver using the SV Total RNA Isolation System (Promega Corporation, Madison, WI), and the concentration of total RNA was estimated using the Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA).

qRT-PCR

RNA was converted single-standard DNA (cDNA) using the PrimeScript™ RT Reagent Kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. For qRT-PCR, all reactions were performed in duplicate, and the relative amounts of mRNAs were calculated using the standard curve method or comparative CT method. Mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as an invariant control. The primer-probe sets used for real-time PCR are shown in Table 1.

Statistical analysis.

Data are expressed as the mean ± standard error (SE) (n = 5–6). Statistical significance of the differences between the three groups was determined using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. Statistical significance was at $p < 0.05$.

Results

Effects of GCP® on weight of HFD fed OVX mice

OVX mice were fed HFD in the presence or absence of 2% or 4% GCP® (w/w) for 137 d. Control mice exhibited significantly increased body weight. The weight was increased by 2.2–2.4 folds and reached 40.4 ± 3.1 g in these mice. However, mice of GCP® groups had approximately 20 g of body weight; the weight was significantly lower than that of the control group (approximately 50%; $p < 0.001$). Moreover, the weight of perirenal adipose tissue was reduced in a dose-dependent manner in the presence of GCP® and was significantly lower than that of the control group (7.3% and 4.0% of the control group in 2% GCP® and 4% GCP® groups, respectively; $p < 0.001$). However, GCP® did not affect the liver weight in these mice. The food intake per day was 1.73–2.08 g in all groups.

Pathological changes in the liver of HFD fed OVX mice in the presence of GCP®

Hepatic lipid accumulation and steatosis were observed in hepatocytes of the control group without GCP®, as as-

sessed using H&E staining. Most vacuoles were microsteatotic, whereas some were macrosteatotic in appearance (Fig. 1). As shown in representative images, lipid accumulation induced by HFD was improved by GCP®, with a clear reduction in abundance of lipid droplets in hepatocyte and lipid droplet size. In contrast, there was no difference in reduction of steatosis between the 2% and 4% GCP® mice fed HFD.

Effects of GCP® on mRNA expression levels of proteins *regulating cholesterol in the liver*

To study the possible mechanism of reduced body weight and fat accumulation in HFD fed mice in the presence of GCP®, we determined the mRNA levels of proteins genes regulating cholesterol in the liver on d 137. Administration of GCP® was associated with significant and dose-dependent increase in mRNA expression levels of ATP-binding cassette protein G1 (ABCG1), and liver X receptor α (LXRα) (Fig. 2; $p < 0.001$, control vs 2% GCP®, $p < 0.001$, control vs 4% GCP®). However, no changes were observed in the levels of ATP-binding cassette protein A1 (ABCA1), scavenger receptor class B type I (SR-BI), or low-density lipoprotein receptor (LDLR) in HFD fed mice in the presence of GCP® compared to the control.

Discussion

Management of obesity and visceral fat accumulation is important in postmenopausal women, as they are likely to experience alterations in lipid metabolism, leading to obesity, fatty liver, and fat redistribution owing to estrogen deficiency. Therefore, it is crucial to consider the association between diet and obesity in postmenopausal women. In the present study, we investigated the effect of GCP®, a

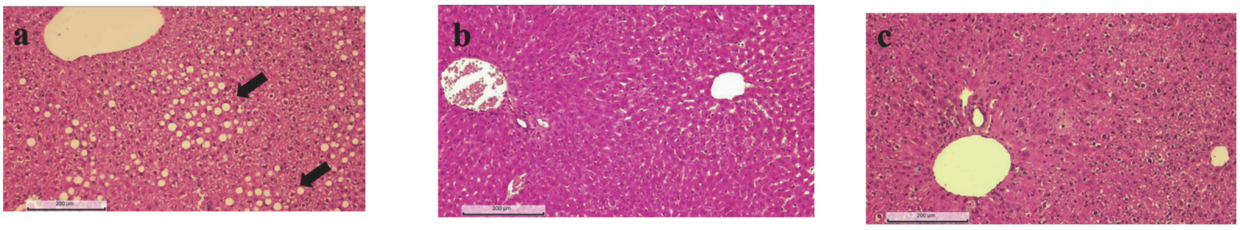

Fig. 1. H&E-stained histologic features of HFD-induced fatty liver in OVX mice with or without GCP®
The liver tissues were obtained on d 137 and representative hematoxylin and eosin staining images of the liver in OVX mice are shown. Arrows show stenotic lesions. 100× Scale bars, 200 μm
Panel a; control, Panel b; 2% GCP®, and Panel c; 4% GCP®

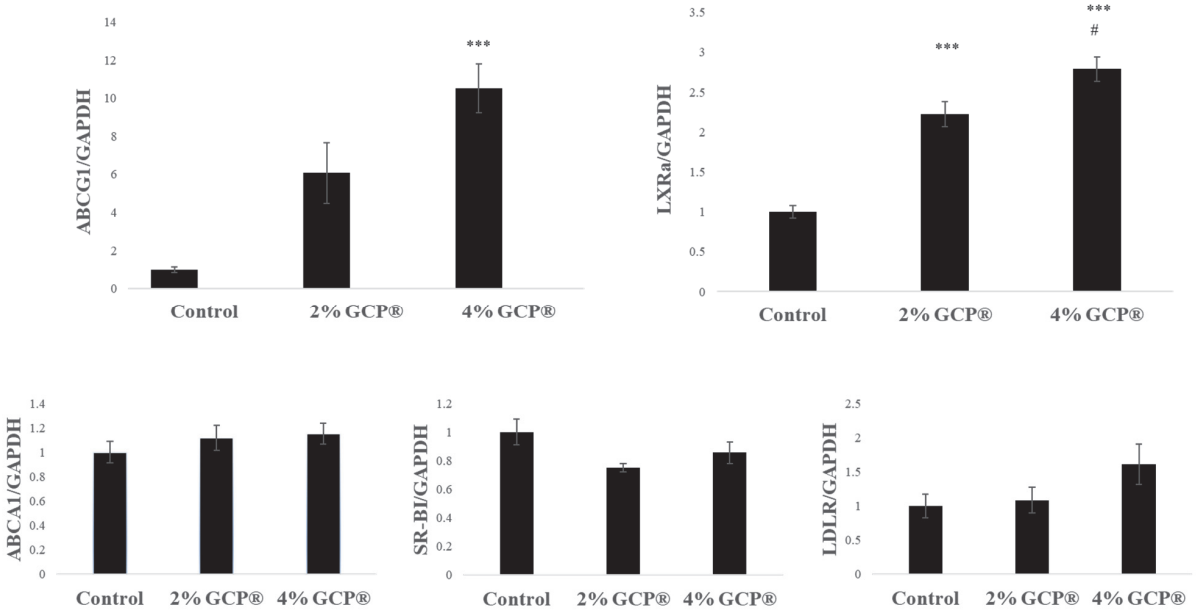

Fig. 2. Effects of GCP® on mRNA expression levels of proteins regulating cholesterols in the liver
OVX mice were fed HFD in the presence or absence of 2% or 4% GCP® (w/w). On d 137, relative mRNA expression levels of ABCA1, ABCG1, SR-BI, LDLR, and LXRα in the liver determined using RT-qPCR. Data are shown as the mean ± SE.
Statistically, these levels were compared among 3 groups by 1-way analysis of variance and Tukey's HSD test.
***$p < 0.001$, Control vs the OVX-HFD-2% GCP® group and the OVX-HFD-4% GCP® group, #$p < 0.05$, the OVX-HFD-2% GCP® group vs the OVX-HFD-4% GCP® group.

soy isoflavone-rich extract, on HFD-fed OVX mice. We observed that GCP® inhibited body weight gain and concomitantly reduced perirenal adipose tissue weight without affecting diet intake in these mice. We also showed that inhibition of HFD-induced fatty liver by GCP® may occur via a pathway dependent on LXR-α and ABCG1 expression[16].

In premenopausal women, estrogen level in the plasma is not associated with cholesterol efflux capacity, however, estrogen deficiency during menopause increases cholesterol efflux capacity of HDL[17–19]. Moreover, body fat distribution in women changes from predominantly subcutaneous to predominantly visceral distribution during menopause[4]. Thus, hormonal changes during menopause lead to an altered metabolic status. Genistein is the major isoflavone present in GCP® and structurally resembles 17β-estradiol[20]. Further study found that genistein has a strong binding affinity for ERs, and it shows estrogenic activity[21]. These results suggested that genistein plays a key role in prevention of altered metabolism in HFD-fed OVX mice.

ABCA1 and ABCG1 are essential for cholesterol homeostasis. ABCG1 effluxes excess cholesterol from cells to HDL particles for reverse cholesterol transport, which is the only pathway for elimination of cholesterol from the body[16, 22–23]. ABCG1 is also important for intracellular transport of cholesterol[22, 24, 25]. LXRs are transcription factors that regulate intracellular cholesterol, and are induced by excess cholesterol[26]. ABCA1 and ABCG1 have binding sites for LXRs on their promotors regions, and LXRs upregulate the expression of these cholesterol transporters. Estrogens maintain liver lipid and cholesterol homeostasis mainly via ERs, and studies have shown that E2 upregulates ABCA1 and ABCG1 through LXRα[21, 27–29]. In the present study, we observed that GCP® induced the expression of LXRα mRNA concomitantly with increased expression of ABCG1 in the liver of HFD-fed OVX mice. In contrast, GCP® did not affect the levels of ABCA1, SR-BI, and LDLR. The liver is an essential organ involved in regulating energy homeostasis. Hepatic steatosis, a major manifestation of metabolic syndrome, is associated with an imbalance between lipid formation and breakdown as well as cholesterol synthesis and secretion. ABCG1 is ubiquitously expressed in many cell types, and studies using ABCG1-knockout mice have shown that ABCG1 plays a critical role in controlling hepatic lipid homeostasis in response to a high-fat, high cholesterol-diet[16, 30]. SR-BI binds HDL and mediates selective uptake of HDL cholesteryl ester (CE) into the liver[31], whereas LDLR regulates cholesterol homeostasis via receptor-mediated endocytosis of LDL particles[32]. Our results suggest that GCP® inhibits HFD-induced hepatic steatosis through a pathway dependent on LXRα-ABCG1 but not ABCA1, LDLR, or SR-BI in these mice.

GCP® is known to have a variety of bioactivities, including anti-angiogenic effects and inhibition of cancer cell growth[7, 10]. GCP® is commercially available and has been shown to exhibit low toxicity[33]. Our results support the use of GCP® as a novel functional supplement candidate to inhibit HFD-induced obesity, although further detailed studies are required.

Table 1. Primers for quantitative real time RT-PCR analysis

Gene	Forward (5' to 3')	Reverse (3' to 5')
ABCA1	AAGCCAAGCATCTTCAGTTC	CCATACAGCAAGAGCAGAAGG
ABCG1	ATACAGGGGAAAGGTCTCCAAT	CCCCCGAGGTCTCTCTTATAGT
SR-BI	GCAAATTTGGCCTGTTTGTT	GATCTTGCTGAGTCCGTTCC
LDLR	CCATTTTGGAGGATGAGAAC	CTAGGCTGTGTGACCTTGTG
LXRα	TAGGGATAGGGTTGGAGTCAG	AGTTTCTTCAAGCGGATCTGT
GAPDH	TGGTGAAGCAGGCATCTGAG	TGCTGTTGAAGTCGCAGGAG

Table 2. Effects of GCP® on ovariectomized mice fed HFD

Paramater (g)	Control	2% GCP®	4% GCP®
Initial body weight	16.8 ± 0.3 (100%)	17.1 ± 0.3 (101.8 ± 1.7%)	16.7 ± 0.2 (99.3 ± 1.0%)
Body weight on day 137	40.4 ± 3.1 (100%)	20.2 ± 0.5* (50.0 ± 1.2%)	19.8 ± 1.1* (49.0 ± 2.7%)
Liver weight	1.22 ± 0.14 (100%)	0.92 ± 0.05 (75.8 ± 3.7%)	1.02 ± 0.08 (83.9 ± 6.9%)
Perirenal adipose tissue weight	1.07 ± 0.13 (100%)	0.08 ± 0.01* (7.3 ± 0.7%)	0.04 ± 0.02* (4.0 ± 2.0%)

Ovariectomized mice (9 weeks old female C57BL/6JJmsSlc, 16.0–17.9 g) were randomly divided into control group (Control), 2% (w/w) GCP®, and 4% GCP®. The mice were euthanized intraperitoneally on d 137 as described in the Materials and methods, and the liver and perirenal adipose tissue were removed and weighed. Data are expressed as mean ± standard error (SE). Differences in these weights between Control and 2% or 4% GCP® group were analyzed by one-way analysis of variance and Tukey's HSD test. * $p < 0.001$, Control vs the GCP® group.

Authorship

TK, KG and JT conceived and designed the experiments. TK, CM and YT performed the experiments and analyzed the data. TK and MA wrote the manuscript. All authors reviewed the manuscript.

Disclosure of state of COI

KG and JT are employees of Amino Up Co., Ltd. All the other authors declared no competing interests.

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References

1) Urabe M, Yamamoto T, Kashiwagi T, Okubo T, Tsuchiya H, Iwasa K, Kikuchi N, Yokota K, Hosokawa K, Honjo H (1996) Effect of estrogen replacement therapy on hepatic triglyceride lipase, lipoprotein lipase and lipids including apolipoprotein E in climacteric and elderly women. Endocr J 43: 737–742.
2) Ko SH, Kim HS (2020) Menopause-associated lipid metabolic disorders and foods beneficial for postmenopausal women. Nutrients 12: 202.
3) Lee C, Kim J, Jung Y (2019) Potential therapeutic application of estrogen in gender disparity of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. Cells 8: 1259.
4) Palmisano BT, Zhu L, Stafford JM (2017) Estrogens in the regulation of liver lipid metabolism. Adv Exp Med Biol 1043: 227–256.
5) Marchand GB, Carreau AM, Weisnagel SJ, Bergeron J, Labrie F, Lemieux S, Tchernof A (2018) Increased body fat mass explains the positive association between circulating estradiol and insulin resistance in postmenopausal women. Am J Physiol Endocrinol Metab 314: E448–E456.
6) Ley CJ, Lees B, Stevenson JC (1992) Sex- and menopause-associated changes in body-fat distribution. Am J Clin Nutr 55: 950–954.
7) Miura T, Yuan L, Sun B, Fujii H, Yoshida M, Wakame K, Kosuna K (2002) Isoflavone aglycon produced by culture of soybean extracts with basidiomycetes and its anti-angiogenic activity. Biosci Biotechnol Biochem 66: 2626–2631.
8) Burich RA, Holland WS, Vinall RL, Tepper C, DeVere White RW, Mack PC (2008) Genistein combined polysaccharide enhances activity of docetaxel, bicalutamide and Src kinase inhibition in androgen-dependent and independent prostate cancer cell lines. BJU Int 102: 1458–1466.
9) Mooso BA, Vinall RL, Tepper CG, Savoy RM, Cheung JP, Singh S, Siddiqui S, Wang Y, Bedolla RG, Martinez A, Mudryj M, Kung HJ, DeVere White RW, Ghosh PM (2012) Enhancing the effectiveness of androgen deprivation in prostate cancer by inducing Filamin A nuclear localization. Endocr Relat Cancer 19: 759–777.
10) Oh HY, Kim SS, Chung HY, Yoon S (2005) Isoflavone supplements exert hormonal and antioxidant effects in postmenopausal Korean women with diabetic retinopathy. J Med Food 8: 1–7.
11) Ramesh S, James MT, Holroyd-Leduc JM, Wilton SB, Sola DY, Ahmed SB (2022) Heart rate variability as a function of menopausal status, menstrual cycle phase, and estradiol level. Physiol Rep 10: e15298.
12) Nie Q, Xing M, Hu J, Hu X, Nie S, Xie M (2017) Metabolism and health effects of phyto-estrogens. Crit Rev Food Sci Nutr 57: 2432–2454.
13) Qin H, Song Z, Shaukat H, Zheng W (2021) Genistein regulates lipid metabolism via estrogen receptor β and its downstream signal Akt/mTOR in HepG2 cells. Nutrients 13: 4015.
14) Zhu L, Shi J, Luu TN, Neuman JC, Trefts E, Yu S, Palmisano BT, Wasserman DH, Linton MF, Stafford JM (2018) Hepatocyte estrogen receptor alpha mediates estrogen action to promote reverse cholesterol transport during Western-type diet feeding. Mol Metab 8: 106–116.
15) Bemis DL, Capodice JL, Desai M, Buttyan R, Katz AE (2004) A concentrated aglycone isoflavone preparation (GCP) that demonstrates potent anti-prostate cancer activity in vitro and in vivo. Clin Cancer Res 10: 5282–5292.
16) Kennedy MA, Barrera GC, Nakamura K, Baldán A, Tarr P, Fishbein MC, Frank J, Francone OL,

Edwards PA (2005) ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. Cell Metab 1: 121–131.
17) Badeau RM, Metso J, Kovanen PT, Lee-Rueckert M, Tikkanen MJ, Jauhiainen M (2013) The impact of gender and serum estradiol levels on HDL-mediated reverse cholesterol transport. European Journal of Clinical Investigation 43: 317–323.
18) Stampfer MJ, Willett WC, Colditz GA, Rosner B, Speizer FE, Hennekens CH (1985) A prospective study of postmenopausal estrogen therapy and coronary heart disease. N Engl J Med 313: 1044–1049.
19) El Khoudary SR, Nasr A, Billheimer J, Brooks MM, McConnell D, Crawford S, Orchard TJ, Rader DJ, Matthews KA (2022) Associations of endogenous hormones with HDL novel metrics across the menopause transition: The SWAN HDL Sstudy. J Clin Endocrinol Metab 107: e303–e314.
20) Thangavel P, Puga-Olguín A, Rodríguez-Landa JF, Zepeda RC (2019) Genistein as potential therapeutic candidate for menopausal symptoms and other related diseases. Molecules 24: 3892.
21) Morito K, Hirose T, Kinjo J, Hirakawa T, Okawa M, Nohara T, Ogawa S, Inoue S, Muramatsu M, Masamune Y (2001) Interaction of phytoestrogens with estrogen receptors alpha and beta. Biol Pharm Bull 24: 351–356.
22) Sag D, Cekic C, Wu R, Linden J, Hedrick CC (2015) The cholesterol transporter ABCG1 links cholesterol homeostasis and tumour immunity. Nat Commun 6: 6354.
23) Wang N, Lan D, Chen W, Matsuura F, Tall AR (2004) ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. Proc Natl Acad Sci U S A 101: 9774–9779.
24) Sturek JM, Castle JD, Trace AP, Page LC, Castle AM, Evans-Molina C, Parks JS, Mirmira RG, Hedrick CC (2010) An intracellular role for ABCG1-mediated cholesterol transport in the regulated secretory pathway of mouse pancreatic beta cells. J Clin Invest 120: 2575–2589.
25) Tarling EJ, Edwards PA (2011) ATP binding cassette transporter G1 (ABCG1) is an intracellular sterol transporter. Proc Natl Acad Sci U S A 108: 19719–19724.
26) Ouimet M, Barrett TJ, Fisher EA (2019) HDL and reverse cholesterol transport: basic mechanisms and their roles in vascular health and disease. Circ Res 124: 1505–1518.
27) Yang K, Chen J, Zhang T, Yuan X, Ge A, Wang S, Xu H, Zeng L, Ge J (2022) Efficacy and safety of dietary polyphenol supplementation in the treatment of non-alcoholic fatty liver disease: A systematic review and meta-Aanalysis. Front Immunol 13: 949746.
28) Farruggio S, Cocomazzi G, Marotta P, Romito R, Surico D, Calamita G, Bellan M, Pirisi M, Grossini E (2020) Genistein and 17β-Eestradiol protect hepatocytes from fatty degeneration by mechanisms Iinvolving mitochondria, inflammasome and kinases activation. Cell Physiol Biochem 54: 401–416.
29) Pummoung S, Werawatganon D, Chayanupatkul M, Klaikeaw N, Siriviriyakul P (2020) Genistein modulated lipid metabolism, hepatic PPARγ, and adiponectin expression in bilateral ovariectomized rats with nonalcoholic steatohepatitis (NASH). Antioxidants (Basel) 10: 24.
30) Baldán Á, Tarr P, Vales CS, Frank J, Shimotake TK, Hawgood S, Edwards PA (2006) Deletion of the transmembrane transporter ABCG1 results in progressive pulmonary lipidosis. J Bio Chem 281: P29401–29410.
31) Hoekstra M (2020) Identification of scavenger receptor BI as a potential screening candidate for congenital primary adrenal insufficiency in humans. Am J Physiol Endocrinol Metab 319: E102–E104.
32) Goldstein JL, Brown MS (2015) A century of cholesterol and coronaries: From plaques to genes to statins. Cell 161: 161–172.
33) McCall JL, Burich RA, Mack PC (2010) GCP, a genistein-rich compound, inhibits proliferation and induces apoptosis in lymphoma cell lines. Leuk Res 34: 69–76.