Role of selenium and 17β oestradiol in modulating lipid accumulation in *in vitro* models of obesity and NAFLD.

WALSH, S.K., PETTIGREW, K., MEZZANI, I., ALASWAD, I. and BERMANO, G.

2024

Food & Medicine Homology

ISSN 3006-6867 (print); 3006-4252 (online)
https://doi.org/10.26599/FMH.2025.9420056

Research Article

Role of selenium and 17β oestradiol in modulating lipid accumulation in *in vitro* models of obesity and NAFLD

Sarah K Walsh [†,#], Katy Pettigrew [†], Isabella Mezzani [†], Intisar Alaswad, Giovanna Bermano [#] (✉)

Centre for Obesity Research and Education (CORE), School of Pharmacy and Life Sciences, Robert Gordon University, Aberdeen AB10 7GJ, United Kingdom

[†] *Sarah K Walsh, Katy Pettigrew, and Isabella Mezzani contributed equally to this work.*

[#] *These authors share co-senior authorship.*

Received: 9 July 2024 / **Revised:** 6 August 2024 / **Accepted:** 7 August 2024

Abstract: Abdominal obesity is prevalent in women and during menopause, making them more susceptible to weight gain, fat redistribution, and subsequent development of metabolic syndrome and associated diseases such as non-alcoholic fatty liver disease (NAFLD). Evidence from menopausal/postmenopausal women has demonstrated an association between declining oestrogen (i.e. 17β oestradiol; E2) levels and the development/progression of both obesity and associated diseases. Furthermore, dietary intake of the micronutrient selenium (Se) is reduced in obese postmenopausal women and a negative correlation between Se level and body mass index (BMI) has been reported. This suggests that novel nutritional and hormonal solutions are needed to moderate fat deposition in postmenopausal women. This study used mouse 3T3-L1 and human HepG2/C3A cells, as *in vitro* models of obesity and NAFLD, respectively, to understand basic cellular mechanisms associated with lipogenesis, and to study the role of Se and oestrogen, as E2, in modulating lipid deposition. Supplementation of 3T3-L1 cells during differentiation to adipocytes with 200 nmol/L Se reduced lipid deposition (~20%) by increasing the expression of genes related to redox status (*Gpx1*, *Selenow*, and *Ucp2*) and reducing the expression of markers of energy metabolism, inflammation and adipocyte differentiation (*Lep*, *Cox-2*, *and Fabp4*); whereas administration of 10 nmol/L E2 regulated lipid synthesis and metabolism (reductions in *Fasn*, *Pparg*, *and Hsl* expression and increased *Fabp4 and Glut4* expression). In HepG2/C3A cells, both Se and E2 reduced lipid accumulation (15%−20%), *via* regulation of lipid and energy metabolism and inflammatory genes (*SREBF1*, *SCD1*, *COX2*, and *LEP*). These results suggest that both hormonal treatment and micronutrient supplementation may be beneficial in obesity and NAFLD management. If our current *in vitro* findings were subsequently demonstrated *in vivo*, they could provide valuable data to support the use of either Se supplementation and/or oestrogen-based therapies to prevent and manage obesity and NAFLD in postmenopausal women.

Keywords: obesity; non-alcoholic fatty liver disease; selenium; 17β oestradiol; adipogenesis; oxidative stress; inflammation

Citation: Walsh S. K., Pettigrew K., Mezzani I., et al. Role of selenium and 17β oestradiol in modulating lipid accumulation in *in vitro* models of obesity and NAFLD. *Food & Medicine Homology*, 2025, 2: 9420056. https://doi.org/10.26599/FMH.2025.9420056

1 Introduction

The prevalence of obesity among women is increasing, and weight gain, particularly in the abdominal region, is most prevalent in women post-menopause[1,2]. The weight gain, fat redistribution, and dyslipidaemia associated with post-menopause, are major hallmarks of metabolic syndrome and have been associated with increased risk of heart disease, diabetes, cancer, and non-alcoholic fatty liver disease (NAFLD)[3,4]. Age and lifestyle are in part responsible for weight gain; however, the drop in oestrogen during menopause also influences fat distribution, particularly in the abdominal area[1,5]. It is, therefore, paramount to identify nutritional and/or therapeutic strategies to modulate fat accumulation and manage associated metabolic diseases, such as NAFLD, in postmenopausal women.

NAFLD is a chronic liver disease, mainly associated with metabolic syndrome and strongly linked to obesity and dyslipidaemia[6,7]. Increased free fatty acids (FFA) levels, as a consequence of obesity and insulin resistance, promote direct hepatic damage by increasing oxidative stress (OS) through the activation of inflammatory pathways[8]. In fact, inflammation and OS are considered major factors in the pathophysiology of NAFLD[9]. Moreover, at the cellular level, the excessive amount of fat deposition in hepatocytes, mainly due to *de-novo* lipogenesis, induces tumour necrosis factor alpha (TNFα) production and the release of reactive oxygen species (ROS), which in turn contribute to the development and progression of liver inflammation[10]. Similarly, obesity-related excessive accumulation of lipids causes changes in the metabolism of adipocytes, leading to changes in the expression of several genes linked to inflammation, OS, and

Address correspondence to Giovanna Bermano, g.bermano@rgu.ac.uk

increased secretion of adipokines[11].

Sex hormones play an important role in the occurrence of NAFLD: a meta-analysis showed that a rise in serum testosterone in women increased the risk of NAFLD[12], while declining oestrogen levels during menopause have been associated with an increased prevalence of NAFLD in postmenopausal compared to premenopausal women[7,13]. Sex hormones are involved in the regulation of hepatic glucose and lipid metabolism through various mechanisms. In particular, oestrogen has a protective role against the development of hepatic steatosis in both men and women *via* oestradiol signalling through the oestrogen receptor alpha (ER-α), as seen in several rodent studies as well as clinical studies[14]. Loss of oestrogen signalling has also been linked to OS related damage in the liver, which was also associated with low levels of peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α), leading to worsened steatohepatitis in high fat diet-fed mice[15]. Moreover, oestrogen has been linked to regulation of adipose tissue distribution and adipocytes' insulin sensitivity, metabolism, and secretory activity, while oestrogen deprivation was associated with an increased risk of obesity and metabolic complications, which can be partially reversed by hormone replacement therapy[16].

New advances in the knowledge relating to the influence of sexual dimorphism in both hepatic and adipose tissue also provide interesting insights into the sex differences in obesity and NAFLD pathogenesis i.e. sex hormones may provide new therapeutic strategies as key master regulators of lipid metabolism and OS in liver and adipose tissue[17]. The main treatment options for NAFLD involve lifestyle modifications including diet, exercise, and weight loss, but, currently, no differing guidelines are proposed for the management of NAFLD in men and women; however, certain predisposing conditions, such as OS or inflammation, and lower oestrogen levels can be targeted to reduce the development and progression of NAFLD in postmenopausal women and improve, at the same time, adipocyte metabolism and fat deposition.

Deficiencies in micronutrients, such as vitamins and trace elements, can contribute to the development of impaired antioxidant defences potentially involved in the pathogenesis of obesity and NAFLD. Lower selenium (Se) and zinc levels have been observed in obese children, especially with central obesity, and morbidly obese patients present with magnesium, Se, iron, and zinc deficiencies[18-20]. Similarly, patients with liver diseases have been shown to have deficiencies in several micronutrients, including, but not limited to, vitamin D, zinc, Se and vitamin A[21,22]. These findings may suggest that, in the obese population and in patients with liver disease, inadequate concentrations of vitamins and minerals may cause the observed impaired antioxidant defences[11,23].

Se is a micronutrient essential for optimal health. It is involved in the functioning of the endocrine and immune systems, and, *via* its incorporation into selenoproteins (e.g. glutathione peroxidase 1 and 4 (GPX1, 4), selenoprotein W (SELENOW), etc.), has been suggested to have both anti-inflammatory and antioxidant properties[24]. Furthermore, Se supplementation has been shown to reduce pro-inflammatory cytokines and the expression of some inflammation-related proteins in the liver, as well as upregulating heme oxygenase-1 (*Ho-1*) expression, all of which were associated with a reduction in inflammation[25]. However, other experimental studies have also demonstrated negative effects of Se supplementation by identifying a relationship between increased Se intake and elevated hepatic lipid accumulation in pigs[26], mice[27], and rats[28]. Furthermore, a cross-sectional study in humans also suggested a positive correlation between elevated plasma Se levels and a higher incidence of NAFLD[29]. Nevertheless, not enough studies have been conducted to assess the relationship between Se and NAFLD[30]. The health effects of Se have been reported to differ between males and females in a number of epidemiological studies, suggesting a role for hormones in Se metabolism[31]. In particular, a link between oestrogen and Se metabolism has been reported: oestrogen status has been shown to affect tissue distribution of Se by modulating the expression of one selenoprotein, selenoprotein P, involved in Se transport[32].

Novel nutritional and/or hormonal solutions are, therefore, required for the prevention and management of fat deposition in postmenopausal women, which may reduce the risk of developing obesity associated diseases such as NAFLD. Such solutions may target lipid deposition, OS, and inflammation, which have been recognised as hallmarks in the pathogenesis of obesity and NAFLD. Evidence suggests that both nutritional and hormonal signals may play a role, however, the exact mechanisms by which micronutrients and hormones may affect lipogenesis and, subsequently, redox and inflammatory status in adipocytes and hepatocytes are not clear. The present study used the mouse 3T3-L1 and human HepG2/C3A cell lines as *in vitro* models of obesity and NAFLD, respectively, to advance the understanding of the basic cellular mechanisms associated with lipogenesis and aimed to identify how Se and oestrogen may affect lipid deposition and the underlying molecular mechanisms in adipocytes and hepatocytes.

The effect of Se, as inorganic selenite, oestrogen, as 17β oestradiol (E2), and a combination of both, on lipid deposition and gene expression of markers of adipocyte differentiation (CCAAT/enhancer binding protein alpha (*Cebpa*), fatty acid binding protein 4 (*Fabp4*) and peroxisome proliferator activated receptor gamma (*Pparg*)), lipid and energy metabolism (acetyl-CoA carboxylase alpha (*Acc1*), fatty acid synthase (*Fasn*), hormone sensitive lipase (*Hsl*)), glucose transporter 4 (*Glut4*), leptin (*Lep*) and uncoupling protein 2 (*Ucp2*)), inflammation (*Cox-2*), and redox status (*Gpx1*, *Gpx4*, *Selenow,* and *Ho-1*) were determined in 3T3-L1 cells during differentiation from pre- to mature adipocytes; whereas their effects on lipid deposition and gene expression of markers of lipid and energy metabolism (acetyl-CoA carboxylase alpha (*ACC1*), carnitine palmitoyl transferase 1A (*CPT1A*), fatty acid synthase (*FASN*), stearoyl-coenzyme A desaturase 1 (*SCD1*), sterol regulatory element binding transcription factor 1 (*SREBF1*) and leptin (*LEP*)), redox status (*HO-1*) and inflammation (*COX2*) were assessed in HepG2/C3A cells exposed to fatty acids (FA) to induce lipid accumulation as a model of NAFLD.

2 Material and methods

2.1 Cell culture

3T3-L1 pre-adipocytes were purchased from the European Collection of Authenticated Cell Cultures (ECACC, distributed via Merck, UK; ECACC number: 86052701, lot: 130030) and cultured at 37 °C, 8% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX™, 4.5 g/L glucose, and sodium pyruvate (cat number: 31966-021; Gibco, UK), supplemented with 1% penicillin/streptomycin (cat number: 15140-122; Gibco, UK) and 10% new born calf serum (NBCS, cat number: 26010; Gibco, UK). 3T3-L1 cells were used until passage 15. HepG2/C3A epithelial-like liver cells were purchased from the American Tissue Culture Collection (ATCC, US; ATCC number: CRL-10741, lot:

70030774) and cultured at 37 °C, 5% CO_2 in DMEM medium with 4 mmol/L *L*-Glutamine, 1 g/L glucose, and sodium pyruvate (cat number: 31885-023; Gibco, UK) supplemented with 1% penicillin/streptomycin (cat number: 15140-122; Gibco, UK) and 10% foetal bovine serum (FBS, cat number: 10500-064; Gibco, UK). HepG2/C3A were used until passage 24.

2.2 Cell differentiation and treatment protocols

Differentiation of the 3T3-L1 cells was carried out as described by Abo El-Magd *et al.*[33] with small modifications. In brief, cells, seeded at a concentration of 2×10^4 cells/well in 24 well plates, were allowed to grow to confluence. 48 h post confluence (Day 0), cells were treated with DMEM containing a hormone mixture (MDI) and 10% FBS (cat number: 10500-064; Gibco, UK) for 2 days. The MDI mixture was composed of 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX, cat number: I5879; Merck, UK), 1 µmol/L dexamethasone (cat number: D1756; Merck, UK) and 1 µg/mL insulin (Cat number: I0516; Merck, UK). On day 2, the culture medium was then replenished with DMEM (10% FBS) supplemented only with 1 µg/mL insulin for 4 days. On day 6, the culture medium was replenished with DMEM (10% FBS) for 4 days. Different treatments were used during the differentiation protocol, including 200 nmol/L Na_2SeO_3 (200 nmol/L Se, cat number: S5261; Merck, UK), 10 nmol/L 17β Oestradiol (10 nmol/L E2, cat number: HB2494; HelloBio, UK), and a combination of the two (Se + E2).

HepG2/C3A cells were seeded at 1×10^5 cells/well in 24 well plates and allowed to attach overnight. Two protocols were followed: Protocol 1: Medium was replaced with DMEM with 1% FBS supplemented with 200 nmol/L Se, 10 nmol/L E2 or a combination of both. After 48 h incubation, a mixture of FA (0.5 mmol/L sodium oleate (SO): palmitic acid (PA) in a 2:1 ratio; cat number: O3880 and cat number: P5585, respectively; Merck, UK) was added with or without 200 nmol/L Se and/or 10 nmol/L E2, and cells incubated for a further 24 h. Protocol 2: cells were seeded, allowed to attach overnight, medium was replaced with DMEM with 1% FBS supplemented with FA with or without 200 nmol/L Se and/or 10 nmol/L E2, and cells incubated for 48 h.

2.3 Lipid content

To assess the extent of differentiation, lipid accumulation was measured using Oil Red O (ORO, cat number: O0625; Merck, UK) dye, and ORO staining was performed according to the following protocol[34]. At the end of the differentiation/treatment protocol, the medium was removed, and cells washed with phosphate-buffered saline (PBS) before incubating cells with 10% formalin buffered saline (cat number: 5701ZF; Thermo Fisher, UK) for 30 min. Cells were washed twice with PBS and subsequently incubated with 60% isopropanol for 5 min. Isopropanol solution was removed, and cells were incubated with ORO working solution (0.5% stock solution mixed with Milli-Q water at a ratio of 3:2) for 1 h at room temperature in the dark. Excess ORO was removed by washing three times with double-distilled water. Isopropanol was added to each well to solubilize ORO and incubated for 15 min in the dark on a shaker. Absorbance was then measured at 590 nm. The percentage of ORO staining was calculated relative to the control (pre-adipocytes or untreated adipocytes for experiments using 3T3-L1 cells and untreated cells for experiments using HepG2/C3A cells).

2.4 Glycerol and leptin content in culture medium

The concentration of glycerol (as an indicator of lipolysis) was measured in medium from both 3T3-L1 and HepG2/C3A cells using the Adipolysis Assay Kit (cat number: MAK313; Merck, UK) as per the manufacturer's guidelines. The concentration of leptin was measured in medium from 3T3-L1 cells using the Mouse Leptin Duoset ELISA Kit (cat number: DY498; Biotechne, UK) as per the manufacturer's guidelines.

2.5 Gene expression

RNA was extracted from both pre- and mature adipocytes and HepG2/C3A cells cultured under the different conditions described above. Total RNA was isolated from cells using TRI Reagent™ solution (Cat number: AM9738; Ambion, UK) according to the manufacturer's guidelines. The RNA pellet was then dissolved in nuclease-free water, and RNA concentration and purity were measured by measuring absorbance at 260/280 nm. For the preparation of cDNA, 0.25–1 µg RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. Briefly, each reaction consisted of 2 µL of 10× RT Buffer, 0.8 µL of 25× dNTP Mix, 2 µL of 10× Random Primers, 1 µL of MultiScribe™ Reverse Transcriptase, 4.2 µL DNase/RNase free water, and 0.25–1 µg of RNA, in a final volume of 20 µL. Reactions were performed on a T3000 Thermocycler (Biometra, Göttingen, Germany) as follows: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min, and then samples cooled to 4 °C. The cDNA was diluted with DNase/RNase free water to obtain the equivalent of 25–50 ng/µL of starting RNA, and stored at −20 °C. qPCR was performed with SYBR Green PCR Master Mix (Primer Design, UK) on a QIAquant 96 5plex (Qiagen, Hilden, Germany) as follows: an initial hot start at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and annealing at 60 °C for 2 min. Primers were designed using Pubmed (Entrez Gene), premier Biosoft (USA), and the NCBI primer blast tool https://www.ncbi.nlm.nih.gov/tools/primer-blast/. Sequences for mouse and human primers are reported in Tables 1 and 2, respectively. mRNA levels were quantified by using the comparative CT method ($2^{-\Delta\Delta Ct}$ method). The data were normalised to β2 microglobulin gene expression to account for differences in reverse transcriptase efficiencies and the amount of template in the reaction mixtures.

2.6 Statistical analysis

All results were expressed as mean ± standard error of the mean (mean ± SEM). A Student *t*-test was used when comparing two experimental groups and a One-way analysis of variance (ANOVA) followed by Bonferroni post hoc test used for comparing three or more experimental groups. *P* values of <0.05 were considered statistically significant. Statistical analysis was carried out using GraphPad Prism Software version 10.2 (GraphPad Software, CA, USA). A minimum of 3 experiments were conducted for each cell line and treatment condition.

3 Results

3.1 Se and E2 supplementation during differentiation of 3T3-L1 cells into adipocytes affects lipid accumulation within cells and the secretion of both glycerol and leptin into the medium

Differentiation from pre- to mature adipocytes induced a significant increase (250%) in lipid accumulation in adipocytes

Table 1 Sequence of mouse primers used for qPCR

Gene	Forward primer	Reverse primer	Product size (bp)
Acc1	3'-GGTCTTCGAGTGGATTGGCA-5'	3'-CAGCTGCCTTCAGACCATCA-5'	114
B2m	3'-TGGTCTTTCTGGTGCTTGTCT-5'	3'-GGATTTCAATGTGAGGCGGG-5'	153
Cebpa	3'-TGAAGGAACTTGAAGCACA-5'	3'-TCAGAGCAAAACCAAAACAA-5'	201
Cox-2[35]	3'-AAGCGAGGACCTGGGTTCA-5'	3'-AAGGCGCAGTTTATGTTGTCTGT-5'	96
Fabp4	3'-ATGATCATCAGCGTAAATGG-5'	3'-GCCTTTCATAACACATTCCA-5'	242
Fasn	3'-AAGCGGTCTGGAAAGCTGAA-5'	3'-AGGCTGGGTTGATACCTCCA-5'	150
Glut4	3'-GATTCTGCTGCCCTTCTGTC-5'	3'-ATTGGACGCTCTCTCTCCAA-5'	167
Gpx1	3'-CAGGAGAATGGCAAGAATGA-5'	3'-GAAGGTAAAGAGCGGGTGAG-5'	135
Gpx4[36]	3'-GCTGGGAAATGCCATCAAATGG-5'	3'-ACGGCAGGTCCTTCTCTATCAC-5'	115
Ho-1	3'-CGCTACCTGGGTGACCTCTC-5'	3'-TGTTTGAACTTGGTGGGGCT-5'	134
Hsl	3'-GATTTACGCACGATGACACAGT-5'	3'-ACCTGCAAAGACATTAGACAGC-5'	113
Lep	3'-GTTCCTGTGGCTTTGGTCCT-5'	3'-ATACCGACTGCGTGTGTGAAA-5'	130
Selenow[36]	3'-ATGCCTGGACATTTGTGGCGA-5'	3'-GCAGCTTTGATGGCGGTCAC-5'	153
Pparg	3'-GTCTGTGGGGATAAAGCATC-5'	3'-CTGATGGCATTGTGAGACAT-5'	205
Ucp2	3'-TAAGTGTTTCGTCTCCCAGCC-5'	3'-GCTTCTCTAAAGGTGTCTCGTTC-5'	98

Table 2 Sequence of human primers used for qPCR

Gene	Forward primer	Reverse primer	Product size (bp)
ACC1	3'-CTCTTGGCCTTTTCCCGGTC-5'	3'-ATCAAAAGTCAGGCAAGCGG-5'	96
B2M	3'-GGCTATCCAGCGTACTCCAAA-5'	3'-CGGCAGGCATACTCATCTTTTT-5'	245
COX2	3'-ACGCTGCTGGTCATCAAGATG-5'	3'-TGGCAAAGGCCTTCTTCCGC-5'	75
CPT1A	3'-TCCAGTTGGCTTATCGTGGTG-5'	3'-TCCAGAGTCCGATTGATTTTTGC-5'	97
FASN	3'-CCTGGCTGCCTACTACATCG-5'	3'-CACATTTCAAAGGCCACGCA-5'	105
HO-1	3'-ACTGCGTTCCTGCTCAACATC-5'	3'-GCTCTGGTCCTTGGTGTCATG-5'	74
LEP	3'-CGGAGAGTACAGTGAGCCAAGA-5'	3'-CGGAATCTCGCTCTGTCATCA-5'	63
SCD1	3'-CTTGCGATATGCTGTGGTGC-5'	3'-CCGGGGGCTAATGTTCTTGT-5'	97
SREBF1	3'-CTGACCGACATCGAAGGTGA-5'	3'-AAGTGCAATCCATGGCTCCG-5'	99

compared to pre-adipocytes, as measured by ORO staining ($P < 0.05$; Fig. 1A). The addition of 200 nmol/L Se during all stages of differentiation induced a 19% reduction in lipid accumulation compared to untreated adipocytes ($P < 0.05$; Fig. 1B); a similar decrease in lipid accumulation was observed when 10 nmol/L E2 or a combination of 200 nmol/L Se and 10 nmol/L E2 were added during the differentiation process (21% ($P < 0.05$) and 29% ($P < 0.01$), respectively; Fig. 1B). The concentration of glycerol, a by-product of triglyceride metabolism, was measured in the cell medium as a potential marker of lipolysis or altered lipid accumulation. A significant increase in glycerol concentration was observed in the medium from mature adipocytes compared to pre-adipocytes ((110.3 ± 5.9) μg/mL *vs.* 0 μg/mL, $P < 0.001$; Fig. 1C). Treatment of adipocytes with either 200 nmol/L Se ((70.9 ± 6.4) μg/mL, $P < 0.001$; Fig. 1D) or 10 nmol/L E2 ((72.5 ± 6.3) μg/mL, $P < 0.001$; Fig. 1D) alone or in combination ((57.9 ± 3.9) μg/mL, $P < 0.001$; Fig. 1D) significantly reduced glycerol concentration in the medium compared to medium from control adipocytes ((97.2 ± 6.8) μg/mL; Fig. 1D). Finally, the differentiation of 3T3-L1 cells into mature adipocytes was associated with a significant increase in the secretion of leptin into the medium ((3,607.7 ± 225.4) *vs.* 0 pg/mL, $P < 0.001$; Fig. 1E); however, treatment with either 200 nmol/L Se or 10 nmol/L E2 or a combination of both did not significantly affect leptin secretion (Fig. 1F).

3.2 Se and E2 supplementation during differentiation of 3T3-L1 cells into adipocytes differentially affects markers of adipocyte differentiation, lipid and energy metabolism, redox status, and inflammation

The differentiation of 3T3-L1 into mature adipocytes was associated with an upregulation in the expression of several genes that act as biomarkers of adipocyte differentiation, lipid and energy metabolism, and those which are involved in both redox status and inflammation. In particular, gene expression of the following markers of adipocyte differentiation, lipid and energy metabolism, *Cebpa, Pparg, Fabp4, Fasn, Hsl, Acc1, Glut4,* and *Lep*, were all significantly increased in adipocytes compared with pre-adipocytes (26.9-fold, $P < 0.01$; 29.7-fold, $P < 0.01$; 210-fold, $P < 0.001$; 37.7-fold, $P < 0.001$; 74.6-fold, $P < 0.001$; 11.7-fold, $P < 0.001$; 448.4-fold, $P < 0.001$, and 1,217.6-fold, $P < 0.05$, respectively; Fig. 2A), while the expression of *Ucp2* was not significantly altered in differentiated adipocytes (Fig. 2A). However, gene expression of biomarkers of redox status (*Gpx1, Selenow*, and *Ho-1*) or inflammation (*Cox-2*) did not change

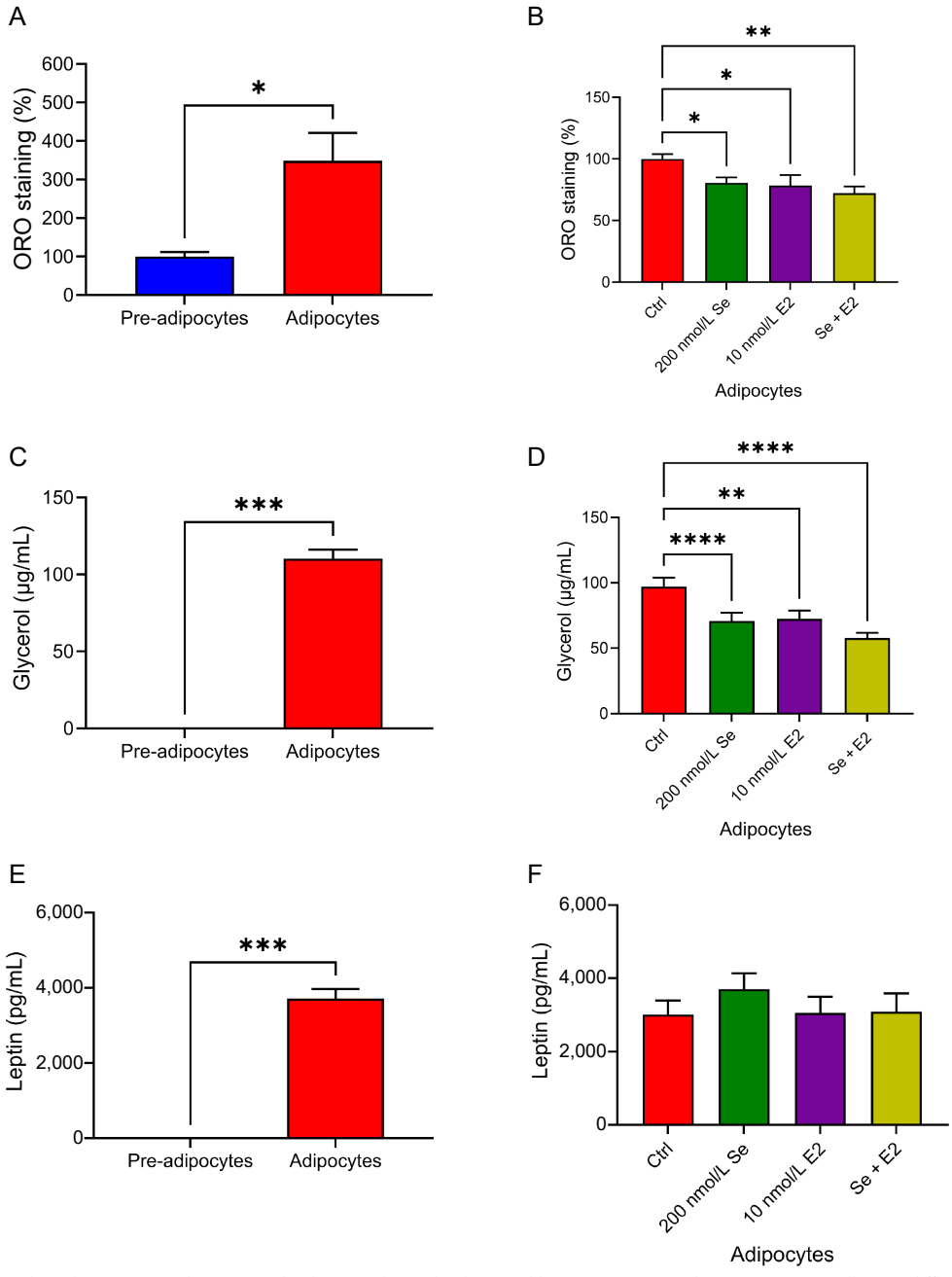

Figure 1 Effect of Se and E2 alone or in combination on lipid accumulation, lipolysis, and leptin secretion in adipocytes. 3T3-L1 cells were differentiated from pre- to mature adipocytes and lipid accumulation within cells (detected via ORO staining) (A), and the secretion by cells of both glycerol (C) and leptin (E) was measured. 3T3-L1 cells were treated with either 200 nmol/L Se or 10 nmol/L E2 alone or in combination throughout the differentiation process and lipid accumulation within cells (B), and the secretion of both glycerol (D) and leptin (F) by cells was measured. ORO staining in 3T3-L1 cells was expressed as a percentage of either pre-adipocytes (A) or untreated mature adipocytes (Ctrl) (B). All data is expressed as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.000,1$; $n = 3–7$.

during the differentiation process (Fig. 2A), except for *Gpx4*, which was higher in adipocytes compared to pre-adipocytes (2.6-fold, $P < 0.05$; Fig. 2A).

In mature adipocytes, treatment with 200 nmol/L Se throughout the differentiation process significantly decreased mRNA abundance of the adipocyte differentiation and energy metabolism markers, *Fabp4* and *Lep* (0.5-fold, $P < 0.05$ and 0.3-fold, $P < 0.05$, respectively; Fig. 2B), while increasing the expression of *Ucp2* (1.6-fold, $P < 0.05$; Fig. 2B). Furthermore, supplementation with Se also increased gene expression of the antioxidant selenoproteins, *Gpx1* and *Selenow*, in adipocytes (2.2-fold, $P < 0.05$ and 2.4-fold, $P < 0.05$, respectively; Fig. 2B), while surprisingly decreasing the expression of *Gpx4* (0.3-fold, $P < 0.05$; Fig. 2B). The expression of the proinflammatory gene *Cox-2* in adipocytes was also decreased in response to Se treatment (0.4-fold, $P < 0.05$; Fig. 2B). Finally, Se supplementation did not affect the expression of *Cebpa, Pparg, Fasn, Hsl, Acc1, Glut4,* and *Ho-1* (Fig. 2B). Similar to Se, treatment of 3T3-L1 cells with 10 nmol/L E2 throughout the differentiation protocol altered the expression of a number of genes related to adipocyte differentiation and lipid and energy metabolism; however, the specific genes affected differed between the two treatments. In response to E2, mRNA abundance of *Pparg, Fasn,* and *Hsl* were all decreased (0.5-fold, $P < 0.05$, 0.4-fold, $P < 0.05$, and 0.4-fold, $P < 0.05$, respectively; Fig. 2B), while the expression of *Fabp4* and *Glut4* were increased in differentiated adipocytes (1.9-fold, $P <$

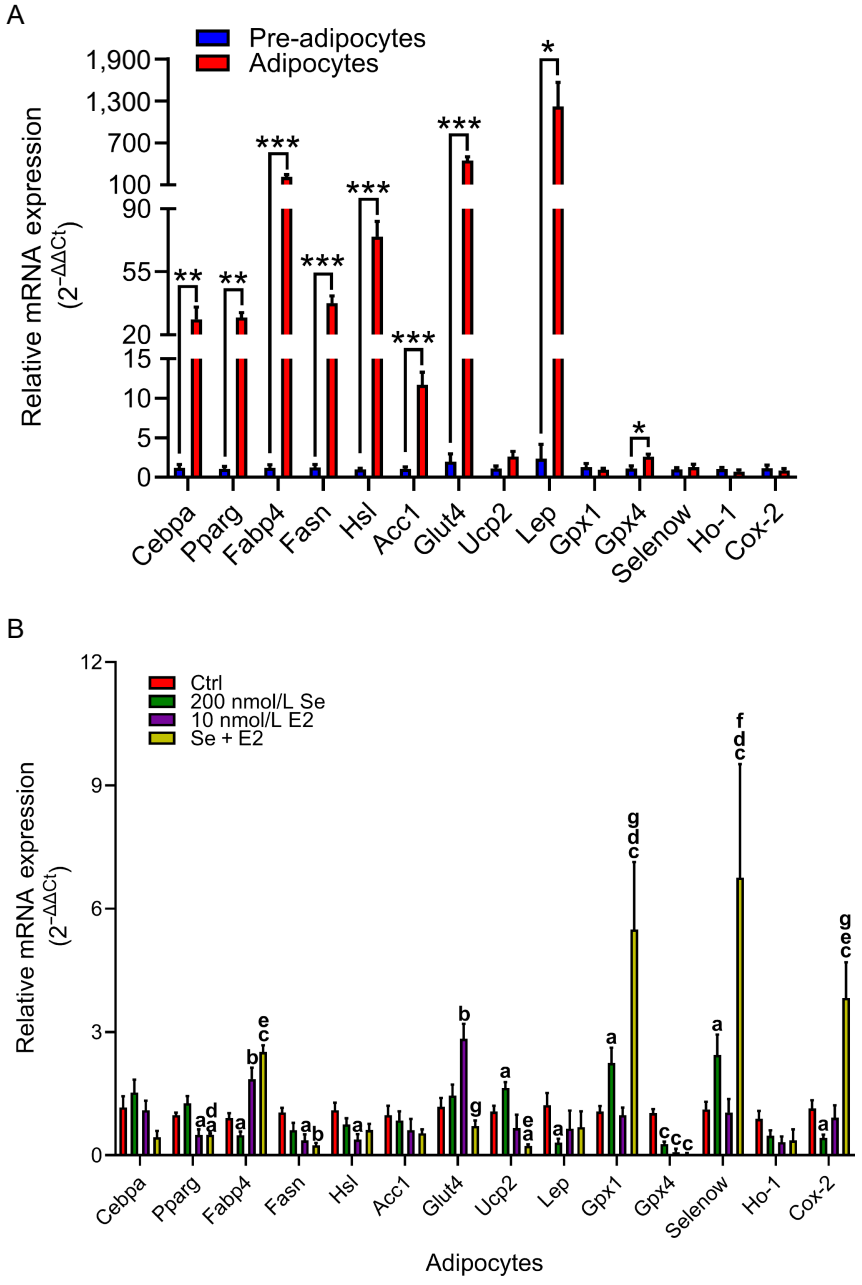

Figure 2 Effect of Se and E2 alone or in combination on the expression of genes related to adipocyte differentiation, lipid and energy metabolism, redox status, and inflammation. 3T3-L1 cells were differentiated from pre-adipocytes to mature adipocytes, and the expression of genes related to adipocyte differentiation (*Cebpa*, *Pparg*, *Fabp4*), lipid and energy metabolism (*Fasn*, *Hsl*, *Acc1*, *Glut4*, *Ucp2*, *Lep*), redox status (*Gpx1*, *Gpx4*, *Selenow*, *Ho-1*), and inflammation (*Cox-2*), measured *via* quantitative reverse transcription polymerase chain reaction (RT-qPCR) (A). Gene expression was normalised to pre-adipocytes. All data is expressed as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $n = 3–8$. 3T3-L1 cells were treated with either 200 nmol/L Se or 10 nmol/L E2 alone or in combination throughout the differentiation process, and expression of genes related to adipocyte differentiation, lipid and energy metabolism, redox status and inflammation measured *via* RT-qPCR (B). Gene expression was normalised to untreated adipocytes (Ctrl). All data is expressed as mean ± SEM. a. $P < 0.05$; b. $P < 0.01$; c. $P < 0.001$ versus Ctrl; d. $P < 0.01$; e. $P < 0.001$ versus 200 nmol/L Se; f. $P < 0.01$; g. $P < 0.001$ versus 10 nmol/L E2; $n = 3–8$.

0.01 and 2.8-fold, $P < 0.01$, respectively; Fig. 2B). E2 reduced the expression of *Gpx4* by approximately 90% in adipocytes ($P < 0.001$; Fig. 2B), but did not alter the gene expression of any other markers of redox status (*Gpx1, Selenow,* or *Ho-1*) or inflammation (*Cox-2*) in these cells (Fig. 2B).

Administration of a combination of both 200 nmol/L Se and 10 nmol/L E2 during differentiation of pre-adipocytes to adipocytes resulted in a reduction in the expression of both *Pparg* and *Fasn* (0.5-fold, $P < 0.05$ and 0.2-fold, $P < 0.01$, respectively; Fig. 2B), an effect that did not differ from treatment with E2 alone (Fig. 2B). In contrast, while Se induced a decrease and E2 an increase in *Fabp4* expression in adipocytes, co-treatment with Se and E2 mirrored that of E2 alone and induced a significant increase in the expression of this lipid transporter (2.5-fold, $P < 0.001$; Fig. 2B). Furthermore, co-treatment with Se and E2 induced significant reductions in the expression of both *Ucp2* and *Glut4* in adipocytes (0.2-fold, $P < 0.001$ and 0.7-fold, $P < 0.001$, respectively; Fig. 2B), which appears to contrast with the Se induced increase in *Ucp2* expression and the E2 induced increase in *Glut4* expression observed in these cells when treated with

either compound alone (Fig. 2B). Administration of Se with E2 induced significant increases in the mRNA expression of both *Gpx1* and *Selenow* in adipocytes (5.5-fold, $P < 0.001$ and 6.8-fold, $P < 0.001$; Fig. 2B), effects which were enhanced compared to treatment with Se alone (5.5-fold versus 2.2-fold, $P < 0.01$ and 6.8-fold versus 2.3-fold, $P < 0.01$, respectively; Fig. 2B). Finally, *Cox-2* expression in adipocytes was significantly increased in response to co-treatment with both Se and E2 during the differentiation process (3.8-fold, $P < 0.001$; Fig. 2B).

3.3 FA treated HepG2/C3A cells as a model to study NAFLD

Treatment of HepG2/C3A cells with FA, SO and PA (0.5 mmol/L), for 24 or 48 h (to reflect the FA exposure times used in the subsequent pretreatment and co-treatment protocols involving Se and E2) induced a significant increase in lipid accumulation measured by ORO staining (58% ($P < 0.01$) and 86% ($P < 0.001$), respectively; Fig. 3A). In parallel, the expression of genes related to lipogenesis, FA oxidation, redox status, and inflammation was also assessed. The expression of the lipogenesis related genes, *SREBF1* and *ACC1*, was increased in hepatic HepG2/C3A cells in response to FA treatment for 24 h (3.3-fold, $P < 0.05$ and 3.5-fold, $P < 0.05$, respectively; Fig. 3B). Similarly, after 48 h exposure, the expression of ACC1 remained significantly increased in treated HepG2/C3A cells (3-fold, $P < 0.05$; Fig. 3C), while the expression of *SREBF1* appeared to be increased but did not reach statistical significance (3-fold, $P = 0.11$; Fig. 3C). Conversely, the expression of the other lipogenesis-related genes, *FASN* and *SCD1*, was not significantly altered following 24 h treatment of HepG2/C3A cells with FA, though the expression of the *SCD1* gene was increased in cells in response to 48 h incubation with the FA combination (1.8-fold, $P < 0.05$; Fig. 3C). In addition, the expression of *CPT1A*, a gene involved in FA oxidation, was significantly increased in HepG2/C3A cells treated with FA for either 24 or 48 h (7.9-fold, $P < 0.001$ and 5-fold, $P < 0.05$, respectively; Figs. 3B, 3C). Following 24 h exposure to FA, the mRNA abundance of *HO-1*, an indicator of redox status, was increased in HepG2/C3A cells (3.6-fold, $P < 0.05$; Fig. 3B); however, this effect was absent when the FA exposure time was increased to 48 h (Fig. 3C). Finally, the expression of both the inflammatory markers, *LEP* and *COX2*, was increased in HepG2/C3A cells treated with FA for 48 h (2.3-fold, $P < 0.05$ and 3.1-fold, $P < 0.05$, respectively; Fig. 3C), with *COX2* expression also increasing in response to the shorter (i.e. 24 h) FA exposure time (4-fold, $P < 0.05$; Fig. 3B).

3.4 Co-treatment of HepG2/C3A cells with Se and E2 reduces lipid accumulation in a FA induced *in vitro* model of NAFLD

Pretreatment of HepG2/C3A cells with either 200 nmol/L Se or 10 nmol/L E2 alone or in combination prior to the addition of FA did not significantly alter lipid accumulation (as indicated by ORO staining) in these cells (Fig. 4A). Conversely, lipid accumulation was reduced in HepG2/C3A cells in response to both 200 nmol/L Se (20%, $P < 0.01$; Fig. 4B) and 10 nmol/L E2 (15%, $P < 0.05$; Fig. 4B) when either compound was added at the same time as the addition of FA (i.e. co-treatment) to cells. However, the effects of the individual compounds (i.e. Se and E2) were not enhanced when given in combination (20%, $P < 0.01$; Fig. 4B). Finally, it is unclear whether the Se and/or E2 induced reductions in lipid accumulation were due to alterations in lipolysis within

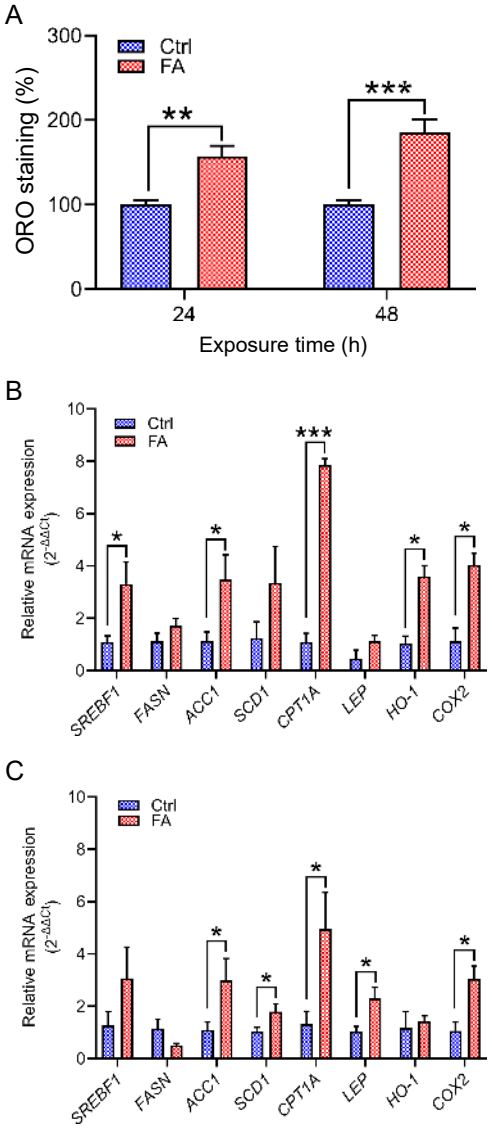

Figure 3 Effect of FA exposure on lipid accumulation and the expression of genes related to lipid and energy metabolism, redox status, and inflammation in HepG2/C3A cells. HepG2/C3A cells were exposed to a combination (2:1) of SO and PA (0.5 mmol/L) for either 24 or 48 h and lipid accumulation in cells was detected via ORO staining (A). The effect of FA exposure time on the expression of genes related to lipid metabolism and energy metabolism (*SREBF1*, *FASN*, *ACC1*, *SCD1*, and *CPT1A*), redox status (*HO-1*), and inflammation (*LEP* and *COX2*) was measured in cells *via* RT-qPCR (24 h (B) and 48 h (C)). ORO staining in FA treated HepG2/C3A cells was expressed as a percentage of untreated HepG2/C3A cells (Ctrl). Gene expression was normalised to untreated HepG2/C3A cells (Ctrl). All data is expressed as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $n = 3–4$.

HepG2/C3A cells, as glycerol was not detectable in the medium from these cells in response to any of the treatments investigated (data not shown).

3.5 Effect of Se or E2 alone or in combination on the expression of lipid and energy metabolism, redox status, and inflammation related genes in a FA induced *in vitro* model of NAFLD

In HepG2/C3A cells, pretreatment with 200 nmol/L Se prior to the addition of FA significantly decreased the expression of both the lipid metabolism related gene, *CPT1A* (0.5-fold, $P < 0.001$;

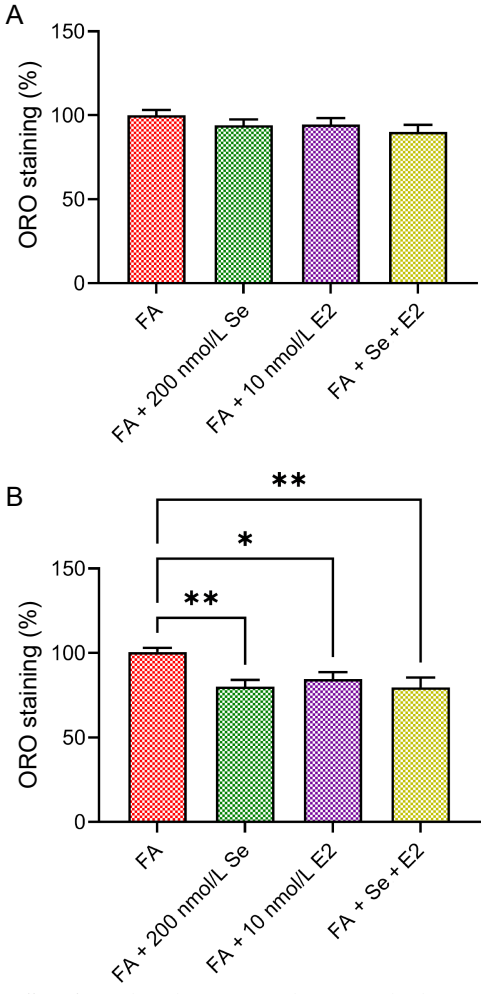

Figure 4 Effect of Se and E2 alone or in combination on lipid accumulation in FA treated HepG2/C3A cells. In the pretreatment protocol, HepG2/C3A cells were treated with either 200 nmol/L Se or 10 nmol/L E2 alone or in combination for 48 h prior to exposure to FA (0.5 mmol/L SO and PA for 24 h) and lipid accumulation in cells detected by ORO staining (A). In the co-treatment protocol, HepG2/C3A cells were treated with FA in the absence or presence of either 200 nmol/L Se or 10 nmol/L E2 alone or in combination for 48 h, and lipid accumulation in cells was detected by ORO staining (B). ORO staining in Se, E2, or Se and E2 treated cells was expressed as a percentage of HepG2/C3A cells treated with FA alone (i.e. FA). All data is expressed as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $n = 7$.

Fig. 5A), and the proinflammatory gene, *COX2*, (0.2-fold, $P < 0.05$; Fig. 5A). Similar to Se, pretreatment of HepG2/C3A cells with 10 nmol/L E2 prior to FA exposure significantly reduced the mRNA expression of *COX2* (0.3-fold, $P < 0.05$; Fig. 5A), in addition to reducing the expression of the lipogenic gene, *SREBF1* (0.5-fold, $P < 0.01$; Fig. 5A). Finally, with the exception of a reduction in *COX2* expression (0.3-fold, $P < 0.05$; Fig. 5A), pretreatment with a combination of Se and E2 did not significantly affect the expression of any of the other lipid and energy metabolism, redox status, or inflammation related genes investigated. In response to cotreatment with either Se or E2, the expression of *SCD1*, a gene involved in FA metabolism, was significantly reduced in FA treated HepG2/C3A cells (0.6-fold, $P < 0.05$ and 0.5-fold, $P < 0.05$, respectively; Fig 5B); however, this effect was absent when cells were treated with a combination of Se and E2. In HepG2/C3A cells cotreated with FA and E2, the mRNA expression of both *CPT1A* and *LEP* was significantly reduced (0.2-fold, $P < 0.05$ and 0.2-fold, $P < 0.01$, respectively; Fig. 5B); however, when combined with Se, this E2 induced downregulation of both genes was not present (Fig. 5B). Finally, the expression of *ACC1* was significantly increased in HepG2/C3A cells simultaneously treated with FA, Se and E2 compared to FA treated cells (4.9-fold, $P < 0.001$; Fig. 5B).

4 Discussion

Obesity and NAFLD have been proposed to be modulated by OS, inflammation, and oestrogen levels in postmenopausal women. While the micronutrient Se has been suggested to have anti-inflammatory and anti-oxidative properties that may be beneficial in preventing or reducing lipid deposition, hormonal therapy has been reported to be beneficial in terms of impeding NAFLD progression[37]. To date, the mechanisms *via* which Se and/or E2 affect lipid deposition and/or metabolism and consequently redox and inflammatory status are not known. This study has attempted to elucidate such mechanisms by using *in vitro* cell models of obesity and NAFLD and has shown that, in 3T3-L1 cells, Se affects lipid deposition by regulating redox status while E2 regulates lipid synthesis and metabolism, whereas in HepG2/C3A cells, both Se and E2 reduce lipid accumulation *via* regulation of genes related to lipid and energy metabolism and inflammation.

This study confirmed previous findings from the group[33] demonstrating that supplementation with 200 nmol/L Se impedes adipocyte differentiation and reduces lipid deposition in 3T3-L1 cells, and in addition showed that supplementation with 10 nmol/L E2, used at a lower concentration than that used by Luo *et al.*[38], also reduced lipid deposition to a similar extent. However, the combined treatment with Se and E2 did not further reduce lipid accumulation, ruling out a potential synergistic effect between a nutritional supplement and hormone therapy. The decrease in lipid accumulation (~20%) observed in adipocytes treated with Se or E2 alone or in combination was not linked to lipolysis, as glycerol concentration in the medium, used as an index of lipolysis[39], was reduced (~25%–40%) after treatment during the differentiation process. Lipolysis is a crucial process that provides organisms with FA and glycerol during periods of negative energy balance[40]; however, in obesity, the basal rate of lipolysis is elevated, contributing to the development of insulin resistance and lipotoxicity[41]. The high basal rate of lipolysis was confirmed in our cell system by the higher concentration of glycerol in the medium from cultured adipocytes compared to pre-adipocytes (Fig. 1C). The elevated levels of FA associated with increased lipolysis enter the mitochondria and enhance the electron transport system, which results in the production of ROS and subsequently causes a sustained overproduction of inflammatory adipocytokines. Adipokines, including leptin, also play a role in lipolysis modulation[42-45]. ROS production was previously shown to be increased in mature adipocytes compared to pre-adipocytes[33], while in this study, we have shown that leptin secretion is increased in mature adipocytes when compared to pre-adipocytes, confirming the presence of lipolysis dysregulation in this *in vitro* model of obesity. During differentiation, treatment of 3T3-L1 cells with 200 nmol/L Se reduced intracellular ROS levels[33], whereas treatment with Se or E2 alone or in combination did not affect leptin secretion. These results may suggest that Se is not involved in leptin regulation, whereas the lack of an E2 effect on leptin secretion contradicts previous work by Jenks *et al.*[46] that reported an increase in leptin secretion in 3T3-L1 cells treated with 0.3 and 1 nmol/L E2. However, they also showed that 3 and 5 nmol/L E2 induced a reduction of leptin transcripts, which is more in line with our current study which used 10 nmol/L E2 to

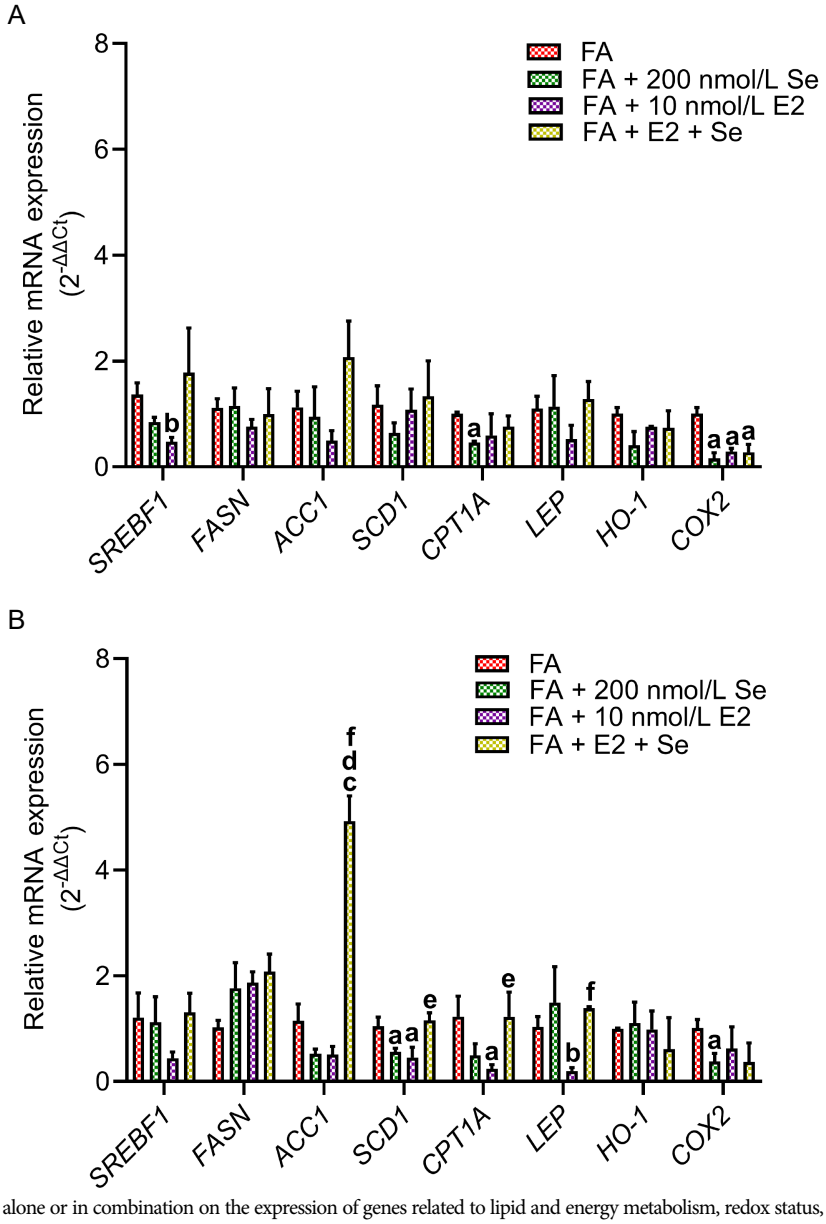

Figure 5 Effect of Se and E2 alone or in combination on the expression of genes related to lipid and energy metabolism, redox status, and inflammation in FA treated HepG2/C3A cells. Pretreatment: HepG2/C3A cells were treated with either 200 nmol/L Se or 10 nmol/L E2 alone or in combination for 48 h prior to exposure to FA (0.5 mmol/L SO and PA for 24 h) and the expression of genes related to lipid and energy metabolism (*SREBF1*, *FASN*, *ACC1*, *SCD1*, and *CPT1A*), redox status (*HO-1*), and inflammation (*LEP* and *COX2*) was measured in cells *via* RT-qPCR (A). Co-treatment: HepG2/C3A cells were treated with FA in the absence or presence of either Se or E2 alone or in combination for 48 h and gene expression was detected *via* RT-qPCR (B). Gene expression in Se, E2, or Se and E2 treated cells was normalised to HepG2/C3A cells treated with FA alone (i.e. FA). All data is expressed as mean ± SEM. a. $P < 0.05$; b. $P < 0.01$; c. $P < 0.001$ versus Ctrl; d. $P < 0.001$ versus 200 nmol/L Se; e. $P < 0.05$, f. $P < 0.001$ versus 10 nmol/L E2; $n = 3–4$.

treat 3T3-L1 cells.

Increased expression of early markers of differentiation (i.e. *Cebpa, Pparg, Fabp4*) in mature adipocytes, compared with pre-adipocytes, confirmed the differentiation status of the cells and previous results by Chen *et al.*[47] and Abo El-Madg *et al.*[33]. Moreover, the increased expression of genes related to fatty acid synthesis (*Fasn*), catalysis of the first step in *de-novo* fatty acid biosynthesis (*Acc1*), and transport of glucose into cells (*Glut4*) in mature adipocytes compared to pre-adipocytes corroborated the increase in lipid levels observed in adipocytes as measured by ORO staining. The higher expression of genes related to the hydrolysis of triglycerides to FFA (*Hsl*) and inflammation and energy regulation (*Lep*) in mature adipocytes compared to pre-adipocytes justifies the increase in glycerol and leptin secretion in media from cultured adipocytes (Figs. 1C and 1E). However, the higher levels of basal lipolysis and lipid accumulation in adipocytes were not linked to an increase in markers of redox status and inflammation (*Ucp2*, *Gpx1*, *Selenow*, *Ho-1,* and *Cox-2*), assessed at the gene expression level.

The decreases in lipid accumulation observed in 3T3-L1 cells in response to Se or E2 administration during differentiation can be explained by their differential effects on the expression of genes involved in the regulation of redox status and inflammation in the case of Se and on those involved in lipid and energy metabolism in the case of E2. In particular, Se supplementation induced increases in the expression of *Gpx1*, *Selenow*, and *Ucp2* compared with non-supplemented adipocytes, whereas the expression of markers of energy metabolism, inflammation, and adipocyte differentiation (*Lep*, *Cox-2,* and *Fabp4*) were all significantly reduced in adipocytes supplemented with Se. These findings further expand

the role of Se in modulating lipid accumulation in an *in vitro* model of obesity by supporting the mechanism previously proposed by our group[33]: Se supplementation causes a decrease in intracellular ROS levels that is matched by an increase in selenoprotein gene expression and redox status (*Gpx1*, *Selenow,* and *Ucp2*) and a decrease in inflammatory mediators (*Cox-2* and *Lep*). Such changes in redox status and inflammation affect markers of adipocyte differentiation (reduction in *Fabp4*) and culminate in reduced lipid accumulation and modulation of lipolysis. The data also provide evidence that fatty acid synthesis and metabolism (*Fasn*, *Acc1*, and *Hsl*) and glucose transport (*Glut4*) are not involved in the regulation of lipid accumulation by Se, at least at the gene expression level.

Conversely, administration of E2 during differentiation of pre-adipocytes did not affect the expression of genes related to redox status and inflammation (*Gpx1*, *Selenow*, *Ho-1*, *Ucp2*, *Lep*, and *Cox-2*) but reduced the expression of genes related to fatty acid synthesis (*Fasn*) and resulted in decreased gene expression of the adipocyte differentiation marker, *Pparg*. Less clear was the effect of E2 on the expression of genes involved in lipolysis (i.e. reduced expression of *Hsl*), FA and glucose transport (i.e. increased expression of *Fabp4* and *Glut4*). While treatment of 3T3-L1 cells with E2 has been associated with increased *Glut4* gene expression[48], the effect of E2 on the regulation of lipolysis/lipogenesis may depend on distinct molecular mechanisms[16], which require further studies to fully identify and define its role in adipose tissue metabolism.

Treatment with a combination of Se and E2 appeared to affect the expression of genes related to redox status (*Gpx1* and *Selenow*), fatty acid synthesis (*Fasn*), and markers of adipocyte differentiation (*Pparg*) similarly to those observed with either Se or E2 treatment alone. Furthermore, there was no evidence of a cumulative effect, as no further reduction in lipid accumulation was observed in mature adipocytes compared to untreated cells. Similarly to E2 treatment alone, administration of both Se and E2 did not affect the expression of *Cebpa*, *Acc1*, *Lep,* and *Ho-1*, whereas administration of both Se and E2 dampened the increase in *Glut4* and *Ucp2* expression and the decrease in *Hsl* expression. *Fabp4* gene expression was increased, as previously observed with E2 treatment alone, possibly due to E2 having a more potent effect in reducing *Pparg* expression (the latter was unaffected by Se), which is a regulator of *Fabp4* expression[49]. The close regulation between prostaglandin D2 synthase enzymes and peroxisome proliferator-activated receptor may, to some extent, link the increase in *Cox-2* expression to the observed decrease in *Pparg* expression[50].

Treatment of HepG2/C3A cells with FA for 24 or 48 h induced an increase in the amount of lipids stored within the cells, reflecting the accumulation of lipids in the livers of NAFLD patients. Increased lipid accumulation was associated with the upregulation of genes related to lipid and energy metabolism (*ACC1*, *CPT1A*, *SREBF1* after 24 h, and *SCD1* after 48 h) and also to increased expression of genes related to redox status and inflammation (*HO-1* at 24 h, *COX2,* and *LEP* at 48 h). Similar changes in gene expression have been reported in the literature from studies that used hepatocytes exposed to FA for different amounts of time, animal studies, or patients with NAFLD, therefore supporting the use of this cell model for *in vitro* studies related to NAFLD. In particular, Gao *et al.*[51] reported a similar increase in *ACC1* and *SCD1* gene expression in HepG2 cells cultured in medium supplemented with PA (0.125 mmol/L) and oleic acid (OA, 0.5 mmol/L) for 24 h, whereas Li *et al.*[52] reported an increase in *SREBF1* expression (also known as *SREBP1c*) in HepG2 cells treated with a 1 mmol/L FA solution for 48 h, and Breternitz *et al.*[53] reported an increase in *CPT1A* expression in HepG2 cells treated with PA (0.125 mmol/L) and OA (0.125 mmol/L) for 96 h, compared to untreated cells. The changes in redox and inflammation related genes in our cell model of NAFLD are not only similar to the ones observed in our *in vitro* cell model of obesity but are also in line with changes in *HO-1* expression reported by Raffaele *et al.*[54] in HepG2 cells treated for 24 h with 2 mmol/L FA (PA and OA 2:1) and by Henkel *et al.*[55] for *COX2* expression in patients with steatosis.

Similar to our findings in 3T3-L1 cells, Se supplementation reduced lipid accumulation in HepG2/C3A cells exposed to FA to create an *in vitro* model of NAFLD. These findings are supported by other studies using both *in vitro* and *in vivo* models of NAFLD that have demonstrated Se induced reductions in lipid accumulation/hepatic steatosis[56-59]. Furthermore, lipid accumulation was reduced in FA exposed cells in response to E2, which is in agreement with previously published findings[38, 60-65]. Similar to our results in 3T3-L1 cells, despite both Se and E2 individually reducing lipid accumulation in HepG2/C3A cells, there was an absence of a synergistic effect when both compounds were co-administered. The mechanism(s) *via* which both Se and E2 reduced lipid accumulation in HepG2/C3A cells appears to be due, at least in part, to their modulation of genes involved in *de-novo* lipogenesis. In particular, both Se and E2 reduced the expression of *SCD1,* while E2 also reduced the expression of *SREBF1*. Both of these genes contribute to FA synthesis and lipid accumulation within cells. These findings are in agreement with those from both *in vitro* studies demonstrating an E2 induced downregulation of both *SREBF1* and *SCD1* in FA treated HepG2 cells[64, 65] and *in vivo* studies demonstrating both Se and E2 induced downregulation of *Scd1* and E2 induced downregulation of *Srebp1c* in the livers of rodents with NAFLD[56, 65-68]. In further support of a role for oestrogen/E2 in the negative regulation of *de-novo* lipogenesis and consequently hepatic lipid accumulation, the expression of *SREBP1c* has been shown to be decreased in the livers of female mice compared to male mice[63], and its expression upregulated in the livers of ovariectomized mice[65, 67, 69].

Inflammation has been demonstrated to be a key driver/contributor to NAFLD (reviewed by Petrescu *et al.*[70]), and in the present study, both Se and E2 induced reductions in lipid accumulation in HepG2/C3A cells were also associated with a reduction in the expression of the inflammatory mediators, *COX2* and *LEP*, respectively. Previous work has demonstrated associations between both Se and E2 induced reductions in the expression of other pro-inflammatory mediators such as *TNFα*, interleukin-6 (*IL-6*), interleukin-1*β* (*IL-1β*), and nuclear factor-kappa B (*NF-κB*) with reduced lipid accumulation in *in vitro* models of NAFLD[56, 64, 71, 72], however, ours is the first, to our knowledge, to demonstrate this effect in terms of *COX2* (Se) and *LEP* (E2). Given that both Se and E2 differentially affected the expression of individual genes related to lipid and energy metabolism and inflammation, it is not unexpected that combined administration of both compounds, Se and E2, did not produce any additive effects compared to individual treatment (i.e. Se or E2) alone. However, the reversal of the E2 induced downregulation of the expression of *SCD1*, *CPT1A,* and *LEP* in HepG2/C3A cells in response to the combination of E2 and Se is somewhat unexpected. It is unclear what the explanation is for these effects; to our knowledge, there have been no studies published describing the combined effects of Se and E2 on

molecular pathways, despite several studies demonstrating an interaction between the two compounds (i.e. Se induces E2 production/secretion[73-78] and oestrogen/E2 increases serum Se[32,79-81]). Taken together, these results indicate that both micronutrient (i.e. Se) supplementation and hormonal treatment (i.e. E2) may be beneficial in the management of NAFLD by modulating lipid metabolism at the cellular level.

Some limitations that are associated with this study and should be considered include the testing of only one concentration each of Se and E2 (i.e. 200 and 10 nmol/L, respectively). The concentration of Se used in this study was based on our previous work, which determined the optimal amount of Se required for maximal *Gpx1* expression in this cell culture system[33] and ensured saturated biosynthesis of selenoproteins[82]. Furthermore, 200 nmol/L Se has been shown to induce an upregulation in *GPX1* expression in HepG2/C3A cells[83,84]. Measurement of the expression of the oestrogen receptors (ER-α and ER-*β*) would have provided a better understanding of the combined effect of Se and E2 in modulating lipid accumulation in both cell models, as E2 has been shown to lower triglycerides via ER-α[38].

5 Conclusions

The present study further confirms that adipogenesis, as indicated by an increase in expression of adipogenesis mediators and markers of lipid and energy metabolism and by the presence of lipid accumulation, is not only modulated by Se supplementation but that oestrogen therapy may play a role. Se supplementation successfully reduced lipid accumulation in an *in vitro* system by modulating the expression of genes involved in the regulation of redox status and inflammation, whereas E2 administration modulated the expression of genes related to lipid and energy metabolism. Furthermore, this study also demonstrated roles for both Se and E2 in the management of NAFLD as the administration of either Se or E2 reduced lipid accumulation by regulating the expression of genes related to lipid and energy metabolism and inflammation. However, it appears that the two approaches (i.e. micronutrient supplementation and hormonal treatment) may not have synergistic effects for the management of obesity or NAFLD. If these findings are replicated *in vivo* and physiological relevance is demonstrated, they may provide an evidence-based rationale for the administration of Se fortified food and/or to design oestrogen-based therapies to prevent and manage obesity and NAFLD in postmenopausal women.

Conflicts of interest

The authors declare that there are no conflicts of interest in this work. Giovanna Bermano is a Senior Editor of Food & Medicine Homology.

Acknowledgements

Isabella Mezzani and Intisar Alaswad are undergraduate students in Applied Biomedical Science in the School of Pharmacy and Life Science, Robert Gordon University, Aberdeen. The work was supported by the Centre for Obesity Research and Education (CORE), Robert Gordon University, Aberdeen.

References

[1] Kapoor, E., Collazo-Clavell, M. L., Faubion, S. S. Weight gain in women at midlife: a concise review of the pathophysiology and strategies for management. *Mayo Clinic Proceedings*, **2017**, 92: 1552–1558. https://doi.org/10.1016/j.mayocp.2017.08.004

[2] Hallajzadeh, J., Khoramdad, M., Izadi, N., et al. Metabolic syndrome and its components in premenopausal and postmenopausal women: a comprehensive systematic review and meta-analysis on observational studies. *Menopause*, **2018**, 25: 1155–1164. https://doi.org/10.1097/GME.0000000000001136

[3] Rodriguez-Monforte, M., Sanchez, E., Barrio, F., et al. Metabolic syndrome and dietary patterns: a systemic review and meta-analysis of observational studies. *European Journal of Nutrition*, **2016**, 56: 925–947. https://doi.org/10.1007/s00394-016-1305-y

[4] Chen, K. L., Madak-Erdogan, Z. Estrogens and female liver health. *Steroids*, **2018**, 133: 38–43. https://doi.org/10.1016/j.steroids.2017.10.015

[5] Davis, S. R., Castelo-Branco, C., Chedraui, P., et al. Understanding weight gain at menopause. *Climacteric*, **2012**, 15: 419–429. https://doi.org/10.3109/13697137.2012.707385

[6] Ballestri, S., Nascimbeni, F., Baldelli, E., et al. NAFLD as a sexual dimorphic disease: role of gender and reproductive status in the development and progression of nonalcoholic fatty liver disease and inherent cardiovascular risk. *Advances in Therapy*, **2017**, 34: 1291–1326. https://doi.org/10.1007/s12325-017-0556-1

[7] Nagral, A., Bangar, M., Menezes, S., et al. Gender differences in nonalcoholic fatty liver disease. *Euroasian Journal of Hepatogastroenterology*, **2022**, 12: S19–S25. https://doi.org/10.5005/jp-journals-10018-1370

[8] Ziolkowska, S., Binienda, A., Jabłkowski, M., et al. The Interplay between insulin resistance, inflammation, oxidative stress, base excision repair and metabolic syndrome in nonalcoholic fatty liver disease. *International Journal of Molecular Science*, **2021**, 22: 11128. https://doi.org/10.3390/ijms222011128

[9] Arroyave-Ospina, J. C., Wu, Z., Geng, Y., et al. Role of oxidative stress in the pathogenesis of non-alcoholic fatty liver disease: implications for prevention and therapy. *Antioxidants*, **2021**, 10: 174. https://doi.org/10.3390/antiox10020174

[10] Zhang, P., Wang, W., Mao, M., et al. Similarities and differences: a comparative review of the molecular mechanisms and effectors of NAFLD and AFLD. *Frontiers in Physiology*, **2021**, 12: 710285. https://doi.org/10.3389/fphys.2021.710285

[11] Manna, P., Jain, S. K. Obesity, oxidative stress, adipose tissue dysfunction, and the associated health risks: causes and therapeutic strategies. *Metabolic Syndrome and Related Disorders*, **2015**, 13: 423–444. https://doi.org/10.1089/met.2015.0095

[12] Jaruvongvanich, V., Sanguankeo, A., Riangwiwat, T., et al. Testosterone, sex hormone-binding globulin and nonalcoholic fatty liver disease: a systematic review and meta-analysis. *Annals of Hepatology*, **2017**, 16: 382–394. https://doi.org/10.5604/16652681.1235481

[13] Ntikoudi, A., Spyrou, A., Evangelou, E., et al. The effect of menopausal status, insulin resistance and body mass index on the prevalence of non-alcoholic fatty liver disease. *Healthcare*, **2024**, 12: 1081. https://doi.org/10.3390/healthcare12111081

[14] Grossmann, M., Wierman, M. E., Angus, P., et al. Reproductive endocrinology of nonalcoholic fatty liver disease. *Endocrine Reviews*, **2019**, 40: 417. https://doi.org/10.1210/er.2018-00158

[15] Besse-Patin, A., Léveillé, M., Oropeza, D., et al. Estrogen signals through peroxisome proliferator-activated receptor-γ coactivator 1α to reduce oxidative damage associated with diet-induced fatty liver disease. *Gastroenterology*, **2017**, 152: 243–256. https://doi.org/10.1053/j.gastro.2016.09.017

[16] Kuryłowicz, A. Estrogens in adipose tissue physiology and obesity-related dysfunction. *Biomedicines*, **2023**, 11: 690. https://doi.org/10.3390/biomedicines11030690

[17] Morán-Costoya, A., Proenza, A. M., Gianotti, M., et al. Sex differences in nonalcoholic fatty liver disease: estrogen influence on the liver–adipose tissue crosstalk. *Antioxidants & Redox Signaling*, **2021**, 35: 753–774. https://doi.org/10.1089/ars.2021.0044

[18] Strauss, R. S. Comparison of serum concentrations of alpha-tocopherol and β-carotene in a cross-sectional sample of obese and nonobese children (NHANES III). national health and nutrition examination survey. *The Journal of Pediatrics*, **1999**, 134: 160–165. https://doi.org/10.1016/s0022-3476(99)70409-9

[19] Ortega, R. M., Rodriguez-Rodriguez, E., Aparicio, A., et al. Young children with excess weight show impaired selenium status. *International Journal for Vitamin and Nutrition Research*, **2012**, 82: 121–129. https://doi.org/10.1024/0300-9831/a000101

[20] Kaidar-Person, O., Person, B., Szomstein, S., et al. Nutritional deficiencies in morbidly obese patients: a new form of malnutrition? part B: minerals. *Obesity Surgery*, **2008**, 18: 1028–1034. https://doi.org/10.1007/s11695-007-9349-y

[21] Llibre-Nieto, G., Lira, A., Vergara, M., et al. Micronutrient deficiencies in patients with decompensated liver cirrhosis. *Nutrients*, **2021**, 13: 1249. https://doi.org/10.3390/nu13041249

[22] Ibrahim, B., Tikhe, D., Bollipo, S. The role of micronutrients in chronic liver disease. *Current Hepatology Reports*, **2023**, 22: 216–220. https://doi.org/10.1007/s11901-023-00605-8

[23] Chen, G., Ni, Y., Nagata, N., et al. Micronutrient antioxidants and nonalcoholic fatty liver disease. *International Journal of Molecular Sciences*, **2016**, 17: 1379. https://doi.org/10.3390/ijms17091379

[24] Cavedon, E., Manso, J., Negro, I., et al. Selenium supplementation, body mass composition, and leptin levels in patients with obesity on a balanced mildly hypocaloric diet: a pilot study. *Internation Journal of Endocrinology*, **2020**, 2020: 4802739. https://doi.org/10.1155/2020/4802739

[25] Al-Dossari, M. H., Fadda, L. M., Attia, H. A., et al. Curcumin and selenium prevent lipopolysaccharide/diclofenac-induced liver injury by suppressing inflammation and oxidative stress. *Biological Trace Element Research*, **2020**, 196: 173–183. https://doi.org/10.1007/s12011-019-01910-4

[26] Zhao, Z., Barcus, M., Kim, J., et al. High dietary selenium intake alters lipid metabolism and protein synthesis in liver and muscle of pigs. *The Journal of Nutrition*, **2016**, 146: 1625–1633. https://doi.org/10.3945/jn.116.229955

[27] Wang, X., Zhang, W., Chen, H., et al. High selenium impairs hepatic insulin sensitivity through opposite regulation of ROS. *Toxicology Letters*, **2014**, 224: 16–23. https://doi.org/10.1016/j.toxlet.2013.10.005

[28] Mueller, A. S., Klomann, S. D., Wolf, N. M., et al. Redox regulation of protein tyrosine phosphatase 1B by manipulation of dietary selenium affects the triglyceride concentration in rat liver. *The Journal of Nutrition*, **2008**, 138: 2328–2336. https://doi.org/10.3945/jn.108.089482

[29] Yang, Z., Yan, C., Liu, G., et al. Plasma selenium levels and nonalcoholic fatty liver disease in Chinese adults: a cross-sectional analysis. *Scientific Reports*, **2016**, 6: 37288. https://doi.org/10.1038/srep37288

[30] Wu, J., Zeng, C., Yang, Z., et al. Association between dietary selenium intake and the prevalence of nonalcoholic fatty liver disease: a cross-sectional study. *Journal of the American College of Nutrition*, **2020**, 39: 103–111. https://doi.org/10.1080/07315724.2019.1613271

[31] Schomburg, L., Schweizer, U. Hierarchical regulation of selenoprotein expression and sex-specific effects of selenium. *Biochimica et Biophysica Acta*, **2009**, 1790: 1453–1462. https://doi.org/10.1016/j.bbagen.2009.03.015

[32] Zhou, X., Smith, A. M., Failla, M. L., et al. Estrogen status alters tissue distribution and metabolism of selenium in female rats. *The Journal of Nutritional Biochemistry*, **2012**, 23: 532–538. https://doi.org/10.1016/j.jnutbio.2011.02.008

[33] Abo El-Magd, N. F., Barbosa, P. O., Nick, J., et al. Selenium, as selenite, prevents adipogenesis by modulating selenoproteins gene expression and oxidative stress-related genes. *Nutrition*, **2022**, 93: 111424. https://doi.org/10.1016/j.nut.2021.111424

[34] Koh, E. J., Kim, K. J., Seo, Y. J., Choi, J., et al. Modulation of HO-1 by ferulic acid attenuates adipocyte differentiation in 3T3-L1 cells. *Molecules*, **2017**, 22: 745. https://doi.org/10.3390/molecules22050745

[35] Ball, H. J., MacDougall, H. G., McGregor, I. S., et al. Cyclooxygenase-2 in the pathogenesis of murine cerebral malaria. *The Journal of Infectious Diseases*, **2004**, 189: 751–758. https://doi.org/10.1086/381503

[36] Wang, L., Jing, J., Yan, H., et al. Selenium pretreatment alleviated LPS-induced immunological stress via upregulation of several selenoprotein encoding genes in murine RAW264.7 cells. *Biological Trace Element Research*, **2018**, 186: 505–513. https://doi.org/10.1007/s12011-018-1333-y

[37] Kim, S. E., Min, J. S., Lee, S., et al. Different effects of menopausal hormone therapy on non-alcoholic fatty liver disease based on the route of estrogen administration. *Scientific Reports*, **2023**, 13: 15461. https://doi.org/10.1038/s41598-023-42788-6

[38] Luo, F., Huang, W. Y., Guo, Y., et al. 17β-Estradiol lowers triglycerides in adipocytes via estrogen receptor α and it may be attenuated by inflammation. *Lipids in Health and Disease*, **2017**, 16: 182. https://doi.org/10.1186/s12944-017-0575-6

[39] Schweiger, M., Eichmann, T. O., Taschler, U., et al. Measurement of lipolysis. *Methods in Enzymology*, **2014**, 538: 171–193. https://doi.org/10.1016/B978-0-12-800280-3.00010-4

[40] Yang, A., Mottillo, E. P. Adipocyte lipolysis: from molecular mechanisms of regulation to disease and therapeutics. *Biochemical Journal*, **2020**, 477: 985–1008. https://doi.org/10.1042/BCJ20190468

[41] Bergman, R. N., Ader, M. Free fatty acids and pathogenesis of type 2 diabetes mellitus. *Trends in Endocrinology & Metabolism*, **2000**, 11: 351–356. https://doi.org/10.1016/s1043-2760(00)00323-4

[42] Bakker, S. J., Ijzerman, R. G., Teerlink, T., et al. Cytosolic triglycerides and oxidative stress in central obesity: The missing link between excessive atherosclerosis, endothelial dysfunction, and β-cell failure? *Atherosclerosis*, **2000**, 148: 17–21. https://doi.org/10.1016/S0021-9150(99)00329-9

[43] William, W. N., Jr., Ceddia, R. B., Curi, R., et al. Leptin controls the fate of fatty acids in isolated rat white adipocytes. *The Journal of Endocrinology*, **2002**, 175: 735–744. https://doi.org/10.1677/joe.0.1750735

[44] Engin, A. B. What is lipotoxicity? *Advances in Experimental Medicine and Biology*, **2017**, 960: 197–220. https://doi.org/10.1007/978-3-319-48382-5_8

[45] Farkhondeh, T., Llorens, S., Pourbagher-Shahri, A. M., et al. An overview of the role of adipokines in cardiometabolic diseases. *Molecules*, **2020**, 25: 5218. https://doi.org/10.3390/molecules25215218

[46] Jenks, M. Z., Fairfield, H. E., Johnson, E. C., et al. Sex steroid hormones regulate leptin transcript accumulation and protein secretion in 3T3-L1 cells. *Scientific Reports*, **2017**, 7: 8232. https://doi.org/10.1038/s41598-017-07473-5

[47] Chen, J., Bao, C., Kim, J. T., et al. Sulforaphene inhibition of adipogenesis via hedgehog signalling in 3T3-L1 adipocytes. *Journal of Agriculture and Food Chemistry*, **2018**, 66: 11926–11934. https://doi.org/10.1021/acs.jafc.8b04330

[48] Campello, R. S., Fatima, L. A., Barreto-Andrade, J. N., et al. Estradiol-induced regulation of GLUT4 in 3T3-L1 cells: involvement of ESR1 and AKT activation. *Journal of Molecular Endocrinology*, **2017**, 59: 257–268. https://doi.org/10.1530/JME-17-0041

[49] Garin-Shkolnik, T., Rudich, A., Hotamisligil, G. S., et al. FABP4 attenuates PPARγ and adipogenesis and is inversely correlated with PPARγ in adipose tissues. *Diabetes*, **2014**, 63: 900–911. https://doi.org/10.2337/db13-0436

[50] Jowsey, I. R., Murdock, P. R., Moore, G. B. T., et al. Prostaglandin D2 synthase enzymes and PPARγ are co-expressed in mouse 3T3-L1 adipocytes and human tissues. *Prostaglandins & Other Lipid Mediators*, **2003**, 70: 267–284. https://doi.org/10.1016/S0090-6980(02)00134-X

[51] Gao, W. Y., Chen, P. Y., Hsu, H. J., et al. Tanshinone IIA

downregulates lipogenic gene expression and attenuates lipid accumulation through the modulation of LXRα/SREBP1 pathway in HepG2 cells. *Biomedicines*, **2021**, 9: 326. https://doi.org/10.3390/biomedicines9030326

[52] Li, X., Wang, R., Zhou, N., et al. Quercetin improves insulin resistance and hepatic lipid accumulation *in vitro* in a NAFLD cell model. *Biomedical Reports*, **2013**, 1: 71–76. https://doi.org/10.3892/br.2012.27

[53] Breternitz, W., Sandkühler, F., Grohmann, F., et al. The HSF1-CPT1a pathway is differentially regulated in NAFLD progression. *Cells*, **2022**, 11: 3504. https://doi.org/10.3390/cells11213504

[54] Raffaele, M., Carota, G., Sferrazzo, G., et al. Inhibition of heme oxygenase antioxidant activity exacerbates hepatic steatosis and fibrosis *in vitro*. *Antioxidants*, **2019**, 8: 277. https://doi.org/10.3390/antiox8080277

[55] Henkel, J., Coleman, C. D., Schraplau, A., et al. Augmented liver inflammation in a microsomal prostaglandin E synthase 1 (mPGES-1)-deficient diet-induced mouse NASH model. *Scientific Reports*, **2018**, 8: 16127. https://doi.org/10.1038/s41598-018-34633-y

[56] Miyata, M., Matsushita, K., Shindo, R., et al. Selenoneine ameliorates hepatocellular injury and hepatic steatosis in a mouse model of NAFLD. *Nutrients*, **2020**, 12: 1898. https://doi.org/10.3390/nu12061898

[57] Wang, P., Lu, Z., He, M., et al. The effects of endoplasmic-reticulum-resident selenoproteins in a nonalcoholic fatty liver disease pig model induced by a high-fat diet. *Nutrients*, **2020**, 12: 692. https://doi.org/10.3390/nu12030692

[58] Wang, Y., Liu, B., Wu, P., et al. Dietary selenium alleviated mouse liver oxidative stress and NAFLD induced by obesity by regulating the KEAP1/NRF2 pathway. *Antioxidants* (*Basel*), **2022**, 11: 349. https://doi.org/10.3390/antiox11020349

[59] Zhu, M., Niu, Q., Zhang, J., et al. Amorphous selenium nanodots alleviate non-alcoholic fatty liver disease via activating VEGF receptor 1 to further inhibit phosphorylation of JNK/p38 MAPK pathways. *European Journal of Pharmacology*, **2022**, 932: 175235. https://doi.org/10.1016/j.ejphar.2022.175235

[60] Fu, X., Zhu, X., Zhang, L., et al. Long non-coding RNA NEAT1 promotes steatosis via enhancement of estrogen receptor alpha-mediated AQP7 expression in HepG2 cells. *Artificial Cells, Nanomedicine, and Biotechnology*, **2019**, 47: 1782–1787. https://doi.org/10.1080/21691401.2019.1604536

[61] Lee, S. R., Lee, Y. H., Yang, H., et al. Sex hormone-binding globulin suppresses NAFLD-triggered hepatocarcinogenesis after menopause. *Carcinogenesis*, **2019**, 40: 1031–1041. https://doi.org/10.1093/carcin/bgz107

[62] Farruggio, S., Cocomazzi, G., Marottam P., et al. Genistein and 17*β*-estradiol protect hepatocytes from fatty degeneration by mechanisms involving mitochondria, inflammasome and kinases activation. *Cellular Physiology and Biochemistry*, **2020**, 54: 401–416. https://doi.org/10.33594/000000227

[63] Galmés-Pascual, B. M., Martínez-Cignoni, M. R., Morán-Costoya, A., et al. 17*β*-Estradiol ameliorates lipotoxicity-induced hepatic mitochondrial oxidative stress and insulin resistance. *Free Radicals Biology and Medicine*, **2020**, 150: 148–160. https://doi.org/10.1016/j.freeradbiomed.2020.02.016

[64] Okamura, T., Hamaguchi, M., Bamba, R., et al. Immune modulating effects of additional supplementation of estradiol combined with testosterone in murine testosterone-deficient NAFLD model. *American Journal of Physiology and Gastrointestinal Liver Physiology*, **2020**, 318: G989–G999. https://doi.org/10.1152/ajpgi.00310.2019

[65] Zhu, X. Y., Xia, H. G., Wang, Z. H., et al. *In vitro* and *in vivo* approaches for identifying the role of aryl hydrocarbon receptor in the development of nonalcoholic fatty liver disease. *Toxicology Letters*, **2020,** 319: 85–94. https://doi.org/10.1016/j.toxlet.2019.10.010

[66] Bryzgalova, G., Lundholm, L., Portwood, N., et al. Mechanisms of antidiabetogenic and body weight-lowering effects of estrogen in high-fat diet-fed mice. *American Journal of Physiology. Endocrinology and Metabolism*, **2008**, 295: E904–912. https://doi.org/10.1152/ajpendo.90248.2008

[67] Galmés-Pascual, B. M., Nadal-Casellas, A., Bauza-Thorbrügge, M., et al. 17*β*-Estradiol improves hepatic mitochondrial biogenesis and function through PGC1B. *The Journal of Endocrinology*, **2017,** 232: 297–308. https://doi.org/10.1530/JOE-16-0350

[68] Okrit, F., Chayanupatkul, M., Siriviriyakul, P., et al. Genistein and sex hormone supplementation modulated hepatic PPARα, δ, and γ subtypes and STAT1 expressions in a NASH rat model with bilateral orchidectomy. *Biomedicines*, **2024a,** 12: 483. https://doi.org/10.3390/biomedicines12030483

[69] Luo, Z. H., Liu, Z. W., Mao, Y., et al. Cajanolactone A, a stilbenoid from cajanus cajan, prevents ovariectomy-induced obesity and liver steatosis in mice fed a regular diet. *Phytomedicine*, **2020**, 78: 153290. https://doi.org/10.1016/j.phymed.2020.153290

[70] Petrescu, M., Vlaicu, S. I., Ciumărnean, L., et al. Chronic inflammation-A link between nonalcoholic fatty liver disease (NAFLD) and dysfunctional adipose tissue. *Medicina* (*Kaunas*), **2022**, 58: 641. https://doi.org/10.3390/medicina58050641

[71] Jeong, Y. H., Hur, H. J., Jeon, E. J., et al. Honokiol improves liver steatosis in ovariectomized mice. *Molecules*, **2018**, 23: 194. https://doi.org/10.3390/molecules23010194

[72] Okrit, F., Chayanupatkul, M., Wanpiyarat, N., et al. Genistein and sex hormone treatment alleviated hepatic fat accumulation and inflammation in orchidectomized rats with nonalcoholic steatohepatitis. *Heliyon*, **2024b,** 10: e26055. https://doi.org/10.1016/j.heliyon.2024.e26055

[73] Yao, X., Ei-Samahy, M. A., Fan, L., et al. *In vitro* influence of selenium on the proliferation of and steroidogenesis in goat luteinized granulosa cells. *Theriogenology*, **2018,** 114: 70–80. https://doi.org/10.1016/j.theriogenology.2018.03.014

[74] Atef, M. M., Abd-Ellatif, R. N., Emam, M. N., et al. Therapeutic potential of sodium selenite in letrozole induced polycystic ovary syndrome rat model: targeting mitochondrial approach (selenium in PCOS). *Archives of Biochemistry and Biophysics*, **2019**, 671: 245–254. https://doi.org/10.1016/j.abb.2019.06.009

[75] Adeniyi, M. J., Agoreyo, F. O., Olorunnisola, O. L., et al. Photo-pollution disrupts reproductive homeostasis in female rats: the duration-dependent role of selenium administrations. *The Chinese Journal of Physiology*, **2020**, 63: 235–243. https://doi.org/10.4103/CJP.CJP_52_20

[76] Basini, G., Tamanini, C. Selenium stimulates estradiol production in bovine granulosa cells: possible involvement of nitric oxide. *Domestic Animal Endocrinology*, **2000**, 18: 1–17. https://doi.org/10.1016/s0739-7240(99)00059-4

[77] Xiong, Y., Yin, Q., Jin, E., et al. Selenium attenuates chronic heat stress-induced apoptosis via the inhibition of endoplasmic reticulum stress in mouse granulosa cells. *Molecules*, **2020**, 25: 557. https://doi.org/10.3390/molecules25030557

[78] Wang, M., Li, Y., Molenaar, A., et al. Vitamin E and selenium supplementation synergistically alleviate the injury induced by hydrogen peroxide in bovine granulosa cells. *Theriogenology*, **2021**, 170: 91–106. https://doi.org/10.1016/j.theriogenology.2021.04.015

[79] Halifeoglu, I., Karatas, F., Canatan, H., et al. Investigation of antioxidant vitamins (A, E and C) and selenium levels in chickens receiving estrogen or testosterone. *Cell Biochemistry and Function*, **2003**, 21: 133–136. https://doi.org/10.1002/cbf.1009

[80] Ulas, M., Cay, M. Effects of 17*β*-estradiol and vitamin E treatments on blood trace element and antioxidant enzyme levels in ovariectomized rats. *Biological Trace Element Research*, **2011**, 139: 347–355. https://doi.org/10.1007/s12011-010-8669-2

[81] Wang, D., Wang, J., Yu, Z., et al. Quercetin alleviates perimenopausal depression induced by ovariectomy combined with chronic unpredictable mild stress through regulating serum elements and inhibiting ferroptosis in prefrontal cortex of rats. *Biological Trace Element Research*, **2024**: 1–16. https://doi.org/10.1007/s12011-024-04106-7

[82] Leist, M., Raab, B., Maurer, S., et al. Conventional cell culture media do not adequately supply cells with antioxidants and thus facilitate peroxide induced genotoxicity. *Free Radical Biology and Medicine*, **1996**, 21: 297–306. https://doi.org/10.1016/0891-5849(96)00045-7

[83] Clarke, C., Baghdadi, H., Howie, A. F., et al. Selenium supplementation attenuates procollagen-1 and interleukin-8 production in fat-loaded human C3A hepatoblastoma cells treated with TGFβ1. *Biochimica Biophysica Acta*, **2010**, 1800: 611–618. https://doi.org/10.1016/j.bbagen.2010.02.007

[84] Zanetti, T. A., Biazi, B. I., Baranoski, A., et al. Response of HepG2/C3A cells supplemented with sodium selenite to hydrogen peroxide-induced oxidative stress. *Journal of Trace Elements in Medicine and Biology*, **2018**, 50: 209–215. https://doi.org/10.1016/j.jtemb.2018.07.005