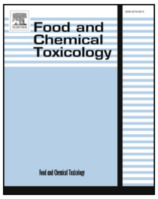
Food and Chemical Toxicology 133 (2019) 110786

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox



Bisphenol A exposure induces cholesterol synthesis and hepatic steatosis in C57BL/6 mice by down-regulating the DNA methylation levels of SREBP-2

Qingrong Li[a], Hongmin Zhang[a], Jun Zou[b], Haiyan Mai[c], Dongfang Su[d], Xiang Feng[a,**], Dan Feng[a,e,*]

[a] Guangdong Provincial Key Laboratory of Food, Nutrition and Health, Department of Nutrition, School of Public Health, Sun Yat-sen University, 510080, China
[b] Department of Cardiology, Affiliated NanHai Hospital of Southern Medical University, 528200, China
[c] Department of Clinic Nutrition, The First Affiliated Hospital, Sun Yat-sen University, 510080, China
[d] State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Sun Yat-sen University Cancer Center, 510060, China
[e] Guangzhou Key Laboratory of Environmental Pollution and Health Risk Assessment, Department of Occupational and Environmental Health, School of Public Health, SunYat-sen University, 510080, China

ARTICLE INFO

Keywords:
Bisphenol A
Cholesterol synthesis
Hepatic steatosis
Sterol regulatory element binding proteins
3-Hydroxy-3-methylglutaryl coenzyme A reductase
Stearoyl-CoA desaturase 1

ABSTRACT

Bisphenol A (BPA), a major plasticizers that are commonly used for lining of beverage or food-storage containers, has been shown to increase cholesterol levels with molecular mechanism not clear. The present study was aimed to investigate the effects of BPA exposure on liver cholesterol synthesis and hepatic steatosis in male C57BL/6 mice and its underlying mechanisms. Male C57BL/6 mice were exposed to different doses (50, 500 and 5000 μg/kg/day) of BPA through diet for 16 weeks. Exposure to low doses (50 and 500 μg/kg/day) of BPA increased hepatic cholesterol content and the expression levels of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) and sterol regulatory element binding proteins-2 (SREBP-2). DNA methylation analysis further showed that mice exposed to low-dose BPA decreased the DNA methylation levels of SREBP-2. Moreover, low doses of BPA exposure increased the expression levels of SREBP-1c and stearoyl-CoA desaturase 1 in the liver, and induced hepatic lipid synthesis and fat accumulation. Our results suggest that low-dose BPA exposure could induce hepatic cholesterol synthesis through decreasing the DNA methylation levels of SREBP-2 and subsequently up-regulating the expression of genes related to cholesterol synthesis in the liver, which causes cholesterol accumulation and further induces liver lipid synthesis and hepatic steatosis.

1. Introduction

Hepatic steatosis is a hallmark feature of nonalcoholic fatty liver disease (NAFLD) (Friedman et al., 2018). NAFLD is a serious chronic liver disorder and affects 25% of the world's population (Asrani et al., 2019; Younossi et al., 2018). However, the mechanisms have not been fully elucidated. In recent years, the role of food contaminants acting as endocrine-disrupting chemicals (EDCs) in liver steatosis and NAFLD is gaining attention (Foulds et al., 2017; Treviño and Katz, 2018). Bisphenol A (BPA), one of the most widely used plasticizers, is an endocrine disruptor that is highly prevalent in our environment. It is commonly used for lining of beverage or food-storage containers (Huang et al., 2017). People are widely exposed to low levels of BPA primarily by leaching from food and beverage containers through diet (Mercogliano and Santonicola, 2018). The National Health and Nutrition Examination Survey (NHANES III) cohort study has revealed that BPA can be detected in the urine of 92.6% American adults (Calafat et al., 2008). Human exposure to BPA is ubiquitous at low levels, the estimates of daily average BPA intake in adults are 0.4–1.4 μg BPA/kg body weight per day (Jenkins et al., 2011). Even very low-dose BPA exposure has been reported to cause adverse human health effects, such as endocrine system disruption, reproductive toxicity, hepatotoxicity, immune system disorder and metabolic disorder (Caporossi and Papaleo, 2017; Kazemi et al., 2017; Koike et al., 2018; Le Magueresse-Battistoni et al., 2018; Tomza-Marciniak et al., 2018).

Many experimental and epidemiological studies have demonstrated that BPA exposure is related to lipid metabolic disorder and hepatic lipid accumulation (Guan et al., 2019; Hong et al., 2017). In rodents,

---

* Corresponding author. School of Public Health, Sun Yat-sen University (Northern Campus), 74 Zhongshan Road 2, Guangzhou, Guangdong Province, 510080, China.

** Corresponding author. School of Public Health, Sun Yat-sen University (Northern Campus), 74 Zhongshan Road 2, Guangzhou, Guangdong Province, 510080, China.

E-mail addresses: fengx@mail.sysu.edu.cn (X. Feng), fengdan3@mail.sysu.edu.cn (D. Feng).

https://doi.org/10.1016/j.fct.2019.110786
Received 9 May 2019; Received in revised form 20 August 2019; Accepted 22 August 2019

both perinatal and adult low-dose BPA exposure could cause fat accumulation in the liver (Marmugi et al., 2012, 2014; Wei et al., 2014). Recent human studies showed that low-dose BPA exposure was associated with increased serum total cholesterol levels and elevated markers of liver toxicity (Lakind et al., 2014; Lee et al., 2018). Excessive synthesis of cholesterol will cause hypercholesterolemia and liver cholesterol accumulation, which can induce hepatic lipid synthesis and leads to related diseases such as hepatic steatosis and NAFLD (Bril et al., 2017; Peng et al., 2017). Sterol regulatory element binding proteins (SREBPs) are known as main regulators of lipogenesis and cholesterol synthesis in the liver, and play a crucial role in maintaining lipid homeostasis in the body. There are three SREBPs isoforms such as SREBP-1a, SREBP-1c and SREBP-2. SREBP-1c and SREBP-2 are highly expressed in liver and have different functions in lipid synthesis (Moslehi and Hamidi-zad, 2018; Park et al., 2018). SREBP-2 mainly regulates the expression of genes related to cholesterol synthesis such as 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), which is a rate-limiting enzyme in the process of cholesterol synthesis (Howe et al., 2017; Rendong Ren, 2017). SREBP-1c is a key regulator of lipogenesis, activation of SREBP-1c can induce the expression of downstream gene stearoyl-CoA desaturase 1 (SCD-1) and increase triglyceride synthesis in the liver (Guan et al., 2019; Zhang et al., 2017; Zhu et al., 2018). Furthermore, DNA methylation is a mechanism of epigenetic regulation that controls gene expression by regulating the accessibility of chromatin without modifying the DNA sequence (Sallustio et al., 2019). It has been reported that DNA methylation may be influenced by environmental factors to regulate metabolic processes and diseases (Giurgescu et al., 2019; Tian et al., 2019). In addition, emerging evidence has demonstrated the association between aberrant DNA methylation and hepatic steatosis and NAFLD severity (Hotta et al., 2018; Wu et al., 2018). However, the role of DNA methylation in BPA-induced hepatic lipogenesis and fat accumulation remains to be elucidated.

In this study, we aimed to investigate the effects of low-dose BPA exposure on hepatic cholesterol synthesis and fat accumulation in male C57BL/6 mice and the underlying mechanisms. Based on previous studies, here we hypothesize that low-dose BPA exposure may induce hepatic cholesterol synthesis by regulating the DNA methylation levels of SREBP-2 and then up-regulating the expression of genes related to cholesterol synthesis in the liver, which subsequently causes cholesterol accumulation and further induces liver lipid synthesis and hepatic steatosis.

2. Materials and methods

2.1. Animal and diets

Five-week-old male C57BL/6 mice weighing 15–17 g were obtained from the animal center of Guangdong Province, P. R. China with the certificate number SCXK 2013-0002 (Guangzhou). Mice were acclimated at 22°C with a 12 h light/dark cycle and have free access to food and water for 2 weeks before the experiment. Then, mice were randomly divided into four groups (n = 12 each group), control group mice were administered for MD12062 normal diet, the compositions of the MD12062 diet were shown in Table 1. BPA-exposed group mice were administered for 16 weeks to different BPA (4,4′-dihydroxy-2,2-diphenylpropane, 97%, CAS#80057, Sigma-Aldrich, St. Louis, MO, USA) through diet. BPA was provided in the MD12062 diet at 0.5, 5 and 50 mg/kg. Considering a diet consumption of 10% of the body weight (BW) per day, this corresponds to an oral exposure of 50 (tolerable daily intake, TDI), 500, or 5000 μg of BPA/kg BW/day (no observed adverse effect level, NOAEL), respectively. The body weight and food consumption of the mice were recorded twice a week. At the end of the experiment, all mice were sacrificed after fasting for 12 h. Blood was obtained and centrifuged for the preparation of serum. Serum samples were stored at −80°C until use for further analysis. The liver of each mice was harvested and weighed, then frozen in liquid nitrogen and stored at −80 °C or fixed in paraformaldehyde for further histological and immunohistochemical analysis. The study was approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University (No. 2017–005).

Table 1
Composition of MD 12062 diet.

Ingredient	Content per kilogram of feed
Energy, Kcal	4000
Fat, Kcal	157.5
Protein, Kcal	203
Carbohydrate, Kcal	639.5
Casein, g	200
Corn starch, g	397
Maltodextrin, g	132
Sucrose, g	100
Cellulose, g	50
Soya oil, g	70
Water, g	90

2.2. Biochemical analysis in serum and liver

The levels of serum total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C), were measured by using the corresponding commercial detection kits (BioSino, Beijing, China) according to the manufacturer's instructions. The activities of serum alanine aminotransferase (ALT) and aspartate transaminase (AST) were detected by using the corresponding commercial enzymatic assay kits (BioSino, Beijing, China) according to the manufacturer's instructions.

The liver tissue was homogenated at 4 °C, then centrifuged and the supernatant was taken. The contents of TG and TC in the supernatant were measured by using the corresponding commercial detection kits (BioSino, Beijing, China) according to the manufacturer's instructions.

2.3. Hematoxylin-eosin and oil red O staining

The small liver samples were fixed with 4% paraformaldehyde and embedded in paraffin. Sections were cut at 5 μm thickness and stained with hematoxylin and eosin (H&E) for histological analyses. Fresh liver tissues were also immobilized with 4% paraformaldehyde, rapidly frozen in liquid nitrogen, and stored at −80 °C for preparing frozen sections (5 μm), which were applied to Oil Red O staining to observe the accumulation of lipid droplet. Images were captured and analyzed using light microscopy (Leica, Bensheim, Germany) at × 200 magnification.

2.4. Immunohistochemistry

Small sections of liver tissues were fixed with 4% paraformaldehyde and embedded in paraffin, then the sections were incubated with serum blocking solution for 30 min at room temperature. After that, the sections were incubated with SREBP-2, SREBP-1c and SCD-1 antibody (Abcam, Cambridge, MA, USA) and HMGCR antibody (Santa Cruz, CA, USA) overnight at 4 °C, then incubated with HRP-labeled secondary antibodies for 30 min at 37 °C. DAB was applied for immunohistochemistry, and the sections were observed under light microscopy (Leica, Bensheim, Germany).

2.5. Western blotting

Total protein was extracted from the mouse liver samples according to the method as previously described (Zou et al., 2013). 30 μg protein was subjected to 8% SDS-PAGE and transferred to a PVDF membrane (Millipore, Boston, MA). The membrane was then blocked with 5%

skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1.5 h. Next, the membrane was rinsed three times consecutively with TBS-T buffer, followed by incubation with 1:1000 dilutions of primary polyclonal antibody (anti-β-actin, anti-SREBP-2, anti-HMGCR, anti-SREBP-1c, anti-SCD-1) (Abcam, Cambridge, UK) at 4 °C overnight, then incubated with 1:10000 dilutions of goat anti-rabbit IgG-HRP secondary antibody (anti-β-actin, anti-SREBP-2, anti-HMGCR, anti-SREBP-1c) or goat anti-mouse IgG-HRP secondary antibody (anti-SCD-1) for 1.5 h. The protein bands were detected by using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific Inc., Waltham, MA). β-actin was used as internal control for equal protein loading.

2.6. *Quantitative real-time PCR (qRT-PCR)*

Total RNA was extracted from the liver using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer, and then RNA was reverse transcribed into cDNA using 5 × Prime Script RT Master Mix (TakaRa, Otsu, Shiga, Japan). Next, the cDNA was mixed with the primers and 2 × SYBR Green PCR master mix (TakaRa, Otsu, Shiga, Japan) in 20 µl. A quantitative analysis of related gene expression was performed by real-time quantitative PCR using the Applied Biosystems 7500 Fast Real-Time PCR System. Two-step PCR reaction procedures were used, the heat cycle program was as follows: predegeneration for 30 s at 95 °C for one cycle, followed by amplification of cDNA for 40 cycles with melting for 5s at 95 °C, and annealing and extension for 34 s at 60 °C. The relative expression of the gene is calculated by comparing the threshold cycle (Ct) method, and normalized with β-actin. The primers used to quantify the mRNA of SREBP-2, SREBP-1c, HMGCR, SCD-1 and β-actin were shown in Table 2.

2.7. *Measurement of the DNA methylation levels of SREBP-2 in the liver*

The DNA methylation levels of SREBP-2 in the liver were determined by using Massarray DNA methylation assay. First, the CpGs sequence information was organized into standard format, and then EpiDesigner software was used to design primers to identify detectable sections. Next, synthesized primers, determined the PCR reaction conditions, tested sample quality and made use of $NaHSO_3$ to convert unmethylated regions of DNA samples. Finally, DNA methylation was detected using Agena MassArray system and analyzed using EpiTYPER™ software. The basic principle of bisulfite ($NaHSO_3$) modification for DNA methylation detection was that unmethylated cytosine can be modified to uracil by bisulfite treatment after DNA denaturation. The steps of this reaction were as follows: Firstly, cytosine residues at C-6 site were sulfonated. Secondly, deamination products at C-4 site was hydrolyzed to uracil sulfonate. Thirdly, uracil sulfonate was desulfurized under alkaline conditions. In this process, 5-methylcytosine remained unchanged due to methylation groups interfering with bisulfite to enter the C-6 site. After $NaHSO_3$ treatment, the primers for each modified DNA strand were used for PCR reaction. In the PCR amplification process, 5-methylcytosine remained unchanged and was shown to be cytosine, while unmethylated cytosine was deaminized to uracil, and then uracil was transformed to thymine. The primer sequence information used in DNA methylation measurement was shown in Table 3. Relative primers were designed using EpiDesigner software according to DNA sequences information.

Table 2
Primers used in quantitative real-time PCR experiment.

Primer name	Sequence
SREBP-2-F	5′-GCTGGTTTGACTGGATGGTT-3′
SREBP-2-R	5′-ACCTTTGGCGAGGTCTAGGT-3′
HMGCR-F	5′-CAGGATGCAGCACAGAATGT-3′
HMGCR-R	5′-CTTTGCATGCTCCTTGAACA-3′
SREBP-1c-F	5′-GAACGACATCGAAGACATGC-3′
SREBP-1c-R	5′-GAGAAGCTCTCAGGAGAGTT-3′
SCD-1-F	5′-TTCTTACACGACCACCACCA-3′
SCD-1-R	5′-GCAGGAGGGAACCAGTATGA-3′
β-actin-F	5′-GATCAAGATCATTGCTCCTCCT-3′
β-actin-R	5′-AGGGTGTAAAACGCAGCTCA-3′

Table 3
Primers used in DNA methylation experiment.

Primer name	Sequence (5′-3′)
SREBP-2-F#60	aggaagagagTTGTTGTTAATGGGATTAGGTTTGTA
SREBP-2-R#60	cagtaatacgactcactatagggagaaggct CCTCACCTAAAAAAACCATTAAACA
SREBP-2-F#64	aggaagagagTTTAAGAGAGGTTTTTTGGGTTTTT
SREBP-2-R#64	cagtaatacgactcactatagggagaaggct CCCAATTCTTCTAACTCCAAAATCT

2.8. *Statistical analysis*

All the data was presented as mean ± S.E.M. Differential effects were analyzed by analysis of variance (ANOVA) followed by the Bonferroni or LSD posttest for multiple comparisons, independent-samples *t*-Test and non-parametric test. At $p < 0.05$, the difference was considered to be significant.

3. Results

3.1. *Body and liver weight and food intake*

Five-week-old male C57BL/6 mice were exposed to different concentrations (50, 500 and 5000 µg/kg/day) of BPA through diet for 16 weeks, the body weight, liver weight and food intake were not different between the control group and the BPA-exposed group (Table 4).

3.2. *The effects of BPA exposure on biochemical parameters in serum and liver*

Biochemical parameters for the different groups of mice were shown in Table 4. Compared to the control group, mice exposed to 50 and 500 µg/kg/day BPA significantly increased serum TC and LDL-C levels and decreased serum HDL-C levels. However, the level of serum TG was increased only at 50 µg/kg/day BPA-exposed group compared with the control group. In addition, liver TC and TG levels were significantly increased in mice exposed to 50 and 500 µg/kg/day BPA compared with the control group. Moreover, the activities of serum ALT and AST were higher in mice exposed to 50 and 500 µg/kg/day BPA than those in the control group (Table 4).

3.3. *The effects of BPA exposure on the expression of hepatic SREBP-2 and HMGCR in C57BL/6 mice*

To understand the mechanism by which BPA increased serum and liver cholesterol levels, we examined the effects of BPA exposure on the expression of genes related to cholesterol synthesis such as SREBP-2 and HMGCR in the liver. First, we observed the immunohistochemical staining patterns of SREBP-2 and HMGCR in liver samples of each group mice. Compared to the control group, significant increases in hepatic SREBP-2 and HMGCR expression were observed in 50 and 500 µg/kg/day BPA-exposed group (Fig. 1A and B). We further investigated the mRNA and protein expression levels of SREBP-2 and HMGCR in the liver tissues. As shown in Fig. 1C and D, the mRNA and protein expression levels of SREBP-2 and HMGCR in the liver tissues were remarkably up-regulated in 50 and 500 µg/kg/day BPA-exposed mice compared with those in control group mice.

Table 4
Biochemical parameters for mice measured in the experiment.

Groups		Control	50 µg/kg/day BPA	500 µg/kg/day BPA	5000 µg/kg/day BPA
	Body weight (g)	27.03 ± 0.42	26.08 ± 0.35	26.83 ± 0.41	26.24 ± 0.46
	Liver weight (g)	1.08 ± 0.05	1.04 ± 0.02	1.10 ± 0.04	1.01 ± 0.01
	Food intake (g)	2.54 ± 0.10	2.39 ± 0.10	2.46 ± 0.13	2.42 ± 0.13
Serum	TC (mmol/L)	2.88 ± 0.28	3.98 ± 0.45*	4.19 ± 0.23*	3.60 ± 0.30
	TG (mmol/L)	1.01 ± 0.06	1.22 ± 0.07*	1.01 ± 0.05	0.74 ± 0.04
	LDL-C (mmol/L)	0.46 ± 0.07	0.75 ± 0.08*	0.69 ± 0.06*	0.49 ± 0.06
	HDL-C (mmol/L)	1.96 ± 0.16	1.16 ± 0.07*	0.91 ± 0.07*	0.87 ± 0.15
	ALT (U/L)	44.02 ± 0.93	50.65 ± 0.87**	49.48 ± 0.62*	28.87 ± 6.76
	AST (U/L)	13.49 ± 0.57	17.09 ± 0.78**	15.54 ± 0.55*	13.55 ± 0.66
Liver	TC (mmol/g protein)	0.03 ± 0.00	0.05 ± 0.00*	0.04 ± 0.00*	0.04 ± 0.00
	TG (mmol/g protein)	0.19 ± 0.02	0.31 ± 0.03*	0.27 ± 0.03*	0.23 ± 0.17

Biochemical parameters from experiment mice exposed to different doses (50, 500, 5000 µg/kg/day) of BPA via diets for 16 weeks. n = 12 per group. Control, M12062 normal diet. Results are mean ± SEM, **P* < 0.05, ***P* < 0.01 compared with control group.

3.4. The effects of BPA exposure on DNA methylation of hepatic SREBP-2 in C57BL/6 mice

To determine whether DNA methylation was involved in BPA-mediated activation of SREBP-2 in the liver, we further measured the DNA methylation levels of SREBP-2 in the liver. Using EpiDesigner software, we found SREBP-2 had two detection scheme (#60 and #64), which both contain a canonical CpG island around the transcription

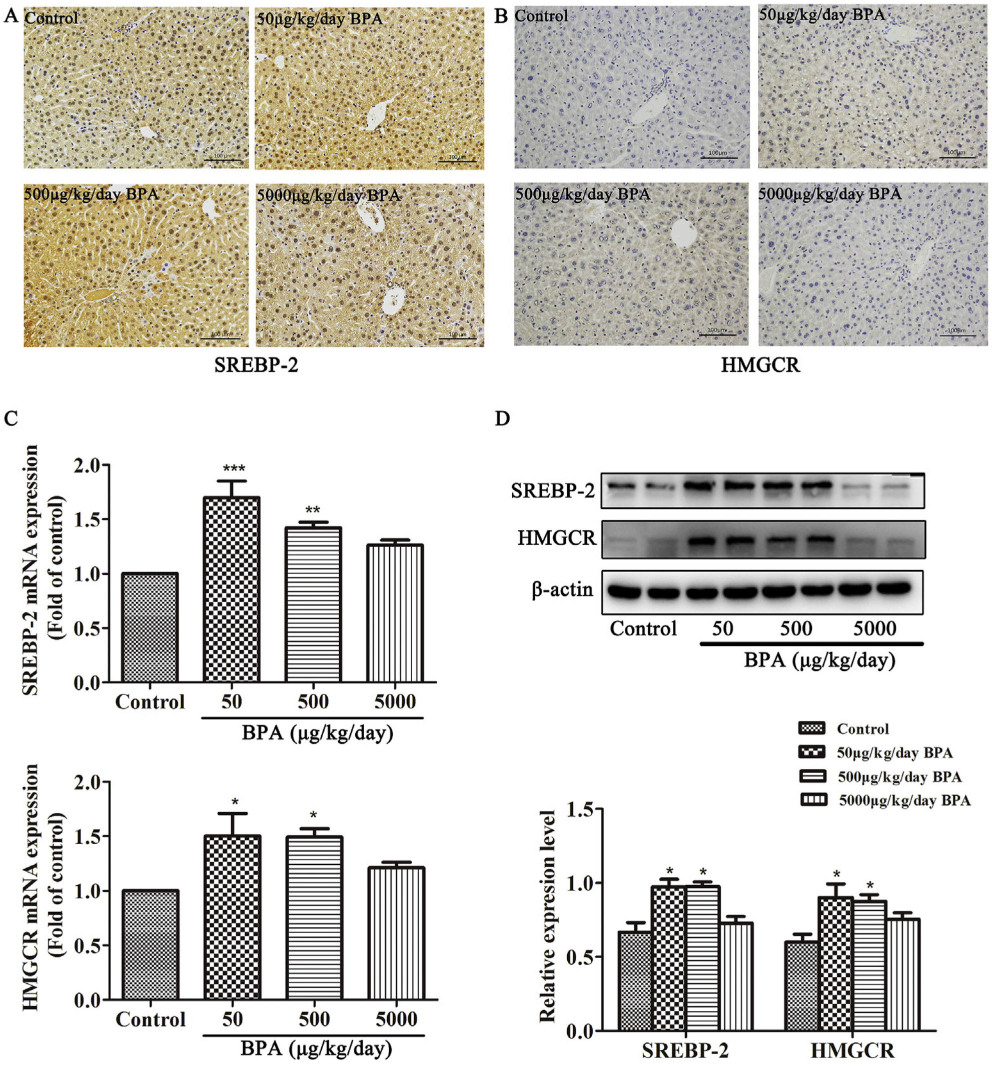


Fig. 1. The effects of BPA exposure on the expression of hepatic SREBP-2 and HMGCR in C57BL/6 mice. **A, B.** Immunohistochemical assay for SREBP-2 and HMGCR expression, increased hepatic SREBP-2 and HMGCR expression was observed at 50 and 500 µg/kg/day BPA-exposed group. The images were captured at 200 × magnifications. **C, D.** The mRNA and protein expression levels of hepatic SREBP-2 and HMGCR in C57BL/6 mice. Values are expressed as mean ± SEM (n = 12 per group), **p* < 0.05, ***p* < 0.01, ****p* < 0.001compared with control group.

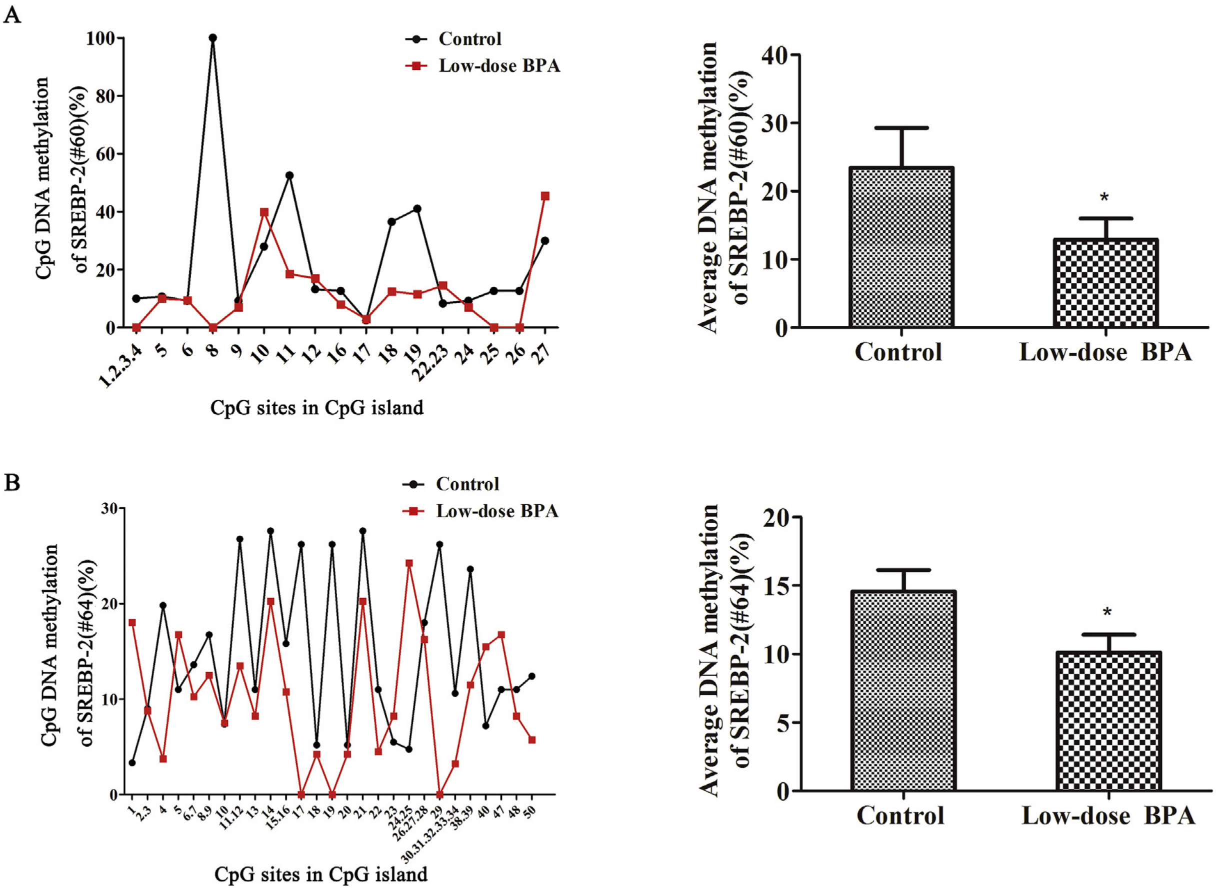


Fig. 2. The effects of BPA exposure on DNA methylation of hepatic SREBP-2 in C57BL/6 mice. **A.** The DNA methylation levels at each site (left graph) and the average DNA methylation level (right graph) in CpG island of detection scheme (#60) of SREBP-2. **B.** The DNA methylation levels at each site (left graph) and the average DNA methylation level (right graph) in CpG island of detection scheme (#64) of SREBP-2. Values are expressed as mean ± SEM (n = 12 per group), $^{*}p < 0.05$ compared with control group.

start site. As shown in Fig. 2A and B, the DNA methylation levels of the CpG island sites of two detection scheme (#60 and #64) of SREBP-2 in low-dose BPA exposure group were generally lower than those in control group.

3.5. *The effects of BPA exposure on the expression of hepatic SREBP-1c and SCD-1 in C57BL/6 mice*

Excessive synthesis of cholesterol causes liver cholesterol accumulation, which can induce hepatic lipid synthesis and fat accumulation. To further understand the mechanism by which BPA increased liver TG content and fat accumulation, we investigated the effects of BPA exposure on the expression of the related genes involved in lipogenesis such as SREBP-1c and SCD-1in the liver tissues. As shown in Fig. 3A and B, immunohistochemical staining results showed that liver tissues from 50 to 500 μg/kg/day BPA-exposed group exhibited increased SREBP-1c and SCD-1 expression compared with those in control group mice. We further investigated the mRNA and protein expression levels of SREBP-1c and SCD-1 in the liver tissues. The mRNA and protein expression levels of liver SREBP-1c and SCD-1 were also similar to the immunohistochemical staining patterns, with increased expression in low-dose (50 and 500 μg/kg/day) BPA exposure group (Fig. 3C and D).

3.6. *The effects of BPA on liver histology and hepatic steatosis in C57BL/6 mice*

Hematoxylin-eosin (H&E) staining was used to observe the morphological characteristics of liver after BPA exposure. Normal liver histology is characterized by normal cell size, prominent nucleus, uniform cytoplasm, and radially arranged hepatocytes. However, male C57BL/6 mice exposed to BPA especially at 50 and 500 μg/kg/day, manifested disordered liver cords, absence of sinus hepaticus and increased vacuolization, and ballooning compared to control group (Fig. 4A). To ascertain the consequences of hepatic lipogenesis, the liver lipids were stained with Oil Red O staining. As shown in Fig. 4B and C, obvious hepatic lipid droplets and increased liver TG levels were observed in 50 and 500 μg/kg/day BPA-exposed groups compared with the control group, indicating obvious lipid accumulation in liver. Quantitative analysis of lipid droplet area in Oil red O staining was shown in Fig. 4D, the lipid drops area was 0.16%, 27.39% and 25.80% in control group, 50 μg/kg/day BPA exposure group and 500 μg/kg/day BPA exposure group, respectively.

4. Discussion

Our current study demonstrates that exposure to low-dose bisphenol A induces hepatic cholesterol synthesis through decreasing the DNA methylation levels of SREBP-2 and then up-regulating the expression of genes related to cholesterol synthesis in the liver, which subsequently causes cholesterol accumulation and further induces liver lipid synthesis and hepatic steatosis. Our results provide a novel insight in the molecular mechanism underlying the BPA-induced liver cholesterol synthesis and hepatic steatosis.

Much evidence shows that BPA exposure is closely related to elevated serum cholesterol levels (Guan et al., 2019; Hong et al., 2017). For example, male adult CD1 mice exposed to 5, 50, and 500 μg/kg/day

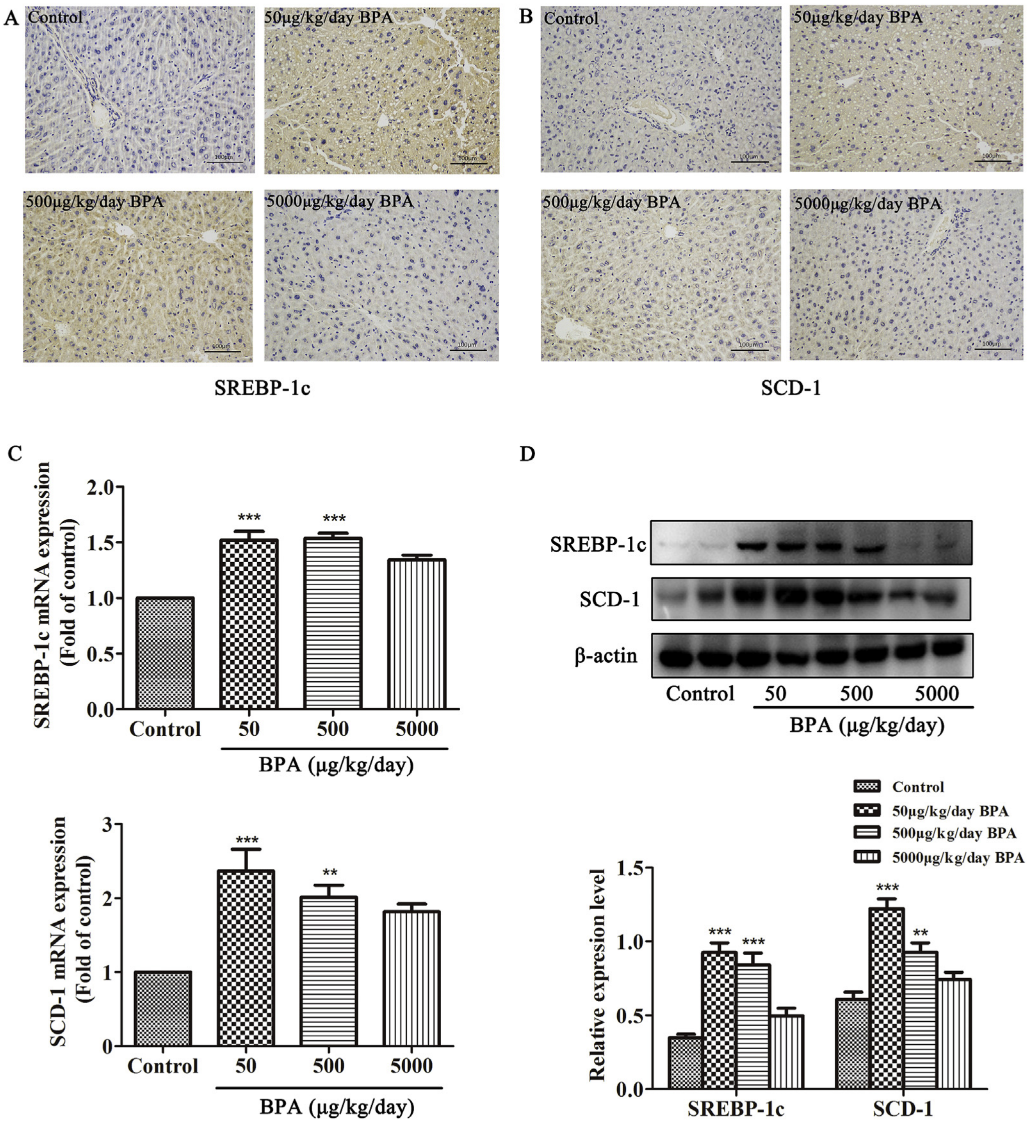


Fig. 3. The effects of BPA exposure on the expression of hepatic SREBP-1c and SCD-1 in C57BL/6 mice. **A, B.** Immunohistochemical assay for SREBP-1c and SCD-1 expression, liver tissues from 50 to 500 μg/kg/day BPA-exposed group exhibited increased SREBP-1c and SCD-1 expression. The images were captured at 200 × magnifications. **C, D.** The mRNA and protein expression levels of hepatic SREBP-1c and SCD-1 in C57BL/6 mice. Values are expressed as mean ± SEM (n = 12 per group), $^{**}p < 0.01$, $^{***}p < 0.001$compared with control group.

of BPA for 8 months significantly increased plasma total cholesterol and LDL-C levels (Marmugi et al., 2014). Also, chronic exposure to low-dose BPA(50 μg/kg/day) via drinking water for 12 weeks elevated non-HDL cholesterol levels in ApoE−/− mice (Kim et al., 2014). Perinatal exposure to BPA at a reference dose of 50 μg/kg/day increased serum TG, TC and LDL-C levels, and decreased HDL-C levels in Wistar rats offspring on a high-fat diet (Wei et al., 2014). Consistent with previous results, our current study showed that male C57BL/6 mice exposed to 50 and 500 μg/kg/day BPA for 16 weeks significantly increased serum total cholesterol and LDL-C levels and decreased serum HDL-C levels. Furthermore, both perinatal and adult BPA exposure has been reported to increase hepatic accumulation of cholesterol in rodents (Marmugi et al., 2012, 2014; Wei et al., 2014). Marmugi et al. showed that male CD1 mice exposed to low doses of BPA (50 and 500 μg/kg/day) for 4 weeks and 8 months both significantly increased hepatic cholesteryl esters levels, and induced the expression of genes related to de novo lipogenesis in the liver (Marmugi et al., 2012, 2014). Recently, Ke et al. reported that male CD1 mice exposed to human relevant dose of BPA (0.5 μg BPA/kg/day) for 10 months exhibited significant cholesterol accumulation in the liver (Ke et al., 2016). In the present study, we also observed that male C57BL/6 mice exposed to low doses of BPA (50 and 500 μg/kg/day) for 16 weeks significantly increased liver TC content and HMGCR expression, indicating that BPA exposure induces hepatic cholesterol synthesis, whereas exposure to high doses of 5000 μg/kg/day BPA had no obvious effects on hepatic TC content and cholesterol synthesis, the whole effect of BPA followed a non-monotonic dose-response curves (NMDRCs), with more obvious effects at lower doses than that at higher doses, which was in agreement with study conducted by Angle et al. they revealed that the low but not the high doses of BPA had a significant impact on metabolic disruption in male mice (Angle et al., 2013). In addition, Marmugi et al. demonstrated that the effects of BPA on the expression of de novo lipogenesis also followed a non-monotonic dose-response curves, with more obvious effects at lower doses than that at higher dose (Marmugi et al., 2012).

The mechanisms responsible for these NMDRCs may be related to the interactions between the ligand (hormone or EDCs) and a hormone receptor, including the effect of hormone concentration on receptor number, cell and tissue specific receptors and co-factors, receptor selectivity, receptor competition, and endocrine negative feedback loops (Vandenberg, 2013). It was proposed that down-regulation of receptors

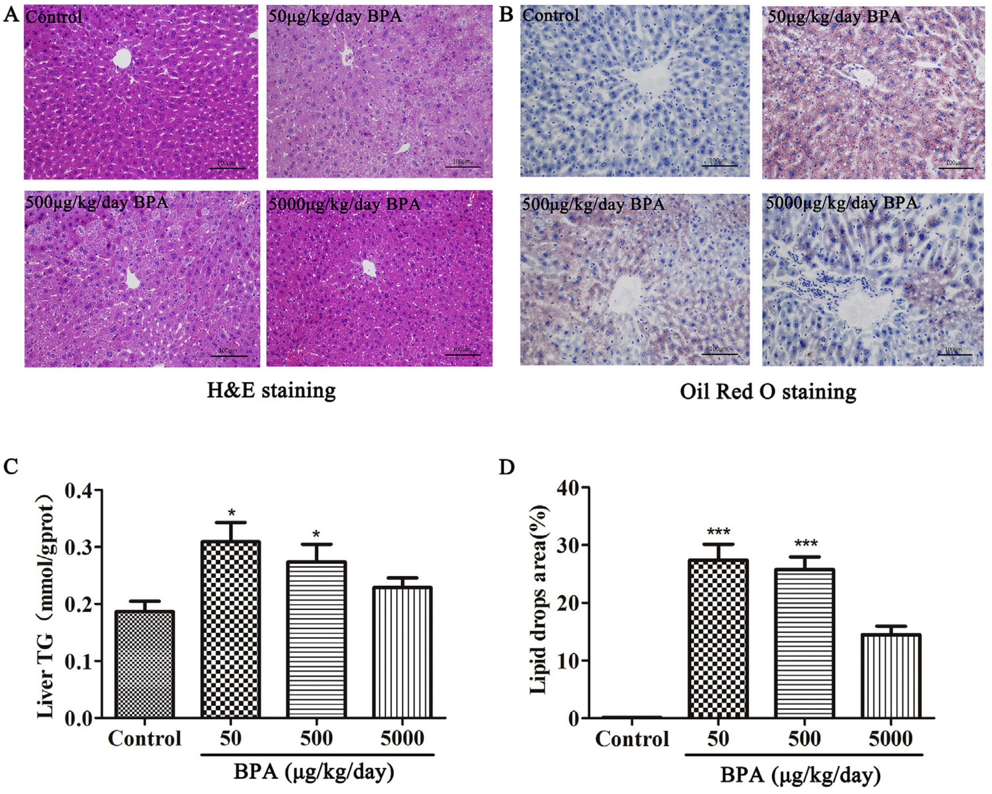


Fig. 4. The effects of BPA on liver histology and hepatic lipid accumulation in C57BL/6 mice. Histological analysis of steatosis in liver sections stained. with H&E (**A**) or Oil Red O (**B**) (magnification 200 ×). The content of hepatic TG (**C**). Quantitative analysis of lipid droplet area in oil red O staining (**D**), which was conducted by Image J software (NIH). Values are expressed as mean ± SEM (n = 12 per group), $^{*}p < 0.05$, $^{***}p < 0.001$ compared with control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

at higher hormone or xenoestrogen levels may contribute to shape these nonmonotonic curves of BPA. Some of BPA's actions, including insulin production by the pancreas, were attributed to its ability to bind to nonclassical membrane estrogen receptor as well as the G-protein coupled-receptor 30 (GPR30) and to act through nongenomic pathways (Alonso-Magdalena et al., 2005). The effects of BPA on insulin expression and secretion have been described (Nadal et al., 2009). SREBP-2 expression and activity as well as other lipogenesis genes were reported to respond to increased insulin levels (Kotzka et al., 2004). Low-dose BPA can bind to nonclassical membrane estrogen receptor and induce insulin secretion, which in turn up-regulates SREBP-2 expression and induces cholesterol biosynthesis in the liver. Thus, insulin is likely to contribute to a large number of the regulations of lipid synthesis observed following BPA exposure.

Regarding the mechanisms by which BPA induced hepatic cholesterol synthesis, DNA methylation regulation may be involved. DNA methylation is an important epigenetic mechanism wherein CpG islands methylated to regulate gene transcription and expression (Zhang et al., 2017). Gene expression is usually inversely proportional to methylation level of CpG islands in the gene promoter. Promoters of transcription-activated genes are usually hypomethylated and DNA hypermethylation leads to gene silencing (Sallustio et al., 2019). It has been reported that DNA methylation may be influenced by environmental factors to regulate metabolic processes and cause diseases (Giurgescu et al., 2019; Tian et al., 2019). BPA is an important environmental endocrine disruptor and has been shown to modify epigenetic markers (Faulk et al., 2016). Doshi et al. showed that neonatal exposure to low doses of BPA(400 μg/kg bw) could induce persistent hypomethylation in insulin like growth factor 2-H19 imprinting control region of adult male rats spermatozoa (Doshi et al., 2013). Additionally, perinatal exposure to low-dose BPA (50 μg/kg/day) could stimulate expression of DNA methyltransferase 3B and reduce global liver DNA methylation level in male Wistar rat offspring (Wei et al., 2014). Another study conducted on saliva DNA of prepubertal girls adopted a genome-wide methylation approach to show that BPA exposure could cause genes hypomethylation and predispose them to several diseases such as diabetes and atherosclerosis (Kim et al., 2013). Moreover, DNA methylation has been involved in BPA-induced hepatic lipid lipogenesis. For instance, exposure to low-dose BPA(25 μg/kg/day) caused continuous hepatic fat accumulation during early development in male CD-1 mice via hypomethylation of Nrf2 and SREBP-1c promoters (Shimpi et al., 2017). These studies suggest that regulation of DNA methylation may be involved in BPA-induced hepatic lipid lipogenesis and related metabolic diseases development. SREBP-2 is a key regulator of cholesterol synthesis and DNA hypomethylation of SREBP-2 leads to SREBP-2 gene upregulation. To ascertain that whether DNA methylation was involved in BPA-induced upregulation of SREBP-2 in the liver, we further measured the DNA methylation levels of SREBP-2 in the liver. We found that male C57BL/6 mice exposed to low-dose BPA(50 μg/kg/day) for 16 weeks significantly decreased the DNA methylation levels of transcription factor SREBP-2 in the liver, and then up-regulated the expression of hepatic SREBP-2. Activation of SREBP-2 can induce HMGCR expression, which in turn increases hepatic cholesterol biosynthesis (Howe et al., 2017; Rendong Ren, 2017). Marmugi et al. also showed that the male CD1 mice exposed to low doses of BPA (5, 50 and 500 μg/kg/day) for 28 days or 8 months significantly increased liver cholesterol synthesis through up-regulating the expression of SREBP-2 and HMGCR (Marmugi et al., 2012, 2014). Therefore, take together, these results indicated that low-dose BPA exposure induced hepatic cholesterol synthesis through decreasing the DNA methylation levels of SREBP-2 and subsequently up-regulating the expression of genes related to cholesterol synthesis in the liver.

Excessive synthesis of cholesterol will cause liver cholesterol accumulation, which can induce the expression of genes related to lipogenesis and cause lipid synthesis and hepatic steatosis. In the present study, we observed that male C57BL/6 mice exposed to 50 and 500 μg/kg/day BPA remarkably up-regulated the mRNA and protein expression levels of hepatic SREBP-1c and SCD-1. Accordingly, the liver TG content was increased in mice exposed to low doses of BPA, resulting in hepatic lipid accumulation, which was further shown in the Oil Red O staining that demonstrated lipid droplets in the liver tissues. Several animal studies have reported that long-term or short-term low-dose BPA exposure up-regulates the expression of lipogenic genes including SREBP-1c, SCD-1 and FAS, and increased hepatic TG synthesis and fat accumulation (Marmugi et al., 2012, 2014). Moreover, perinatal exposure to low-dose BPA stimulated hepatic TG synthesis in rats offspring by inducing the expression of hepatic SREBP-1c and its downstream lipogenic targets (Somm et al., 2009). Therefore, excessive synthesis of cholesterol caused liver cholesterol accumulation, which further induced liver lipid synthesis and hepatic steatosis through up-regulating the expression of genes related to lipogenesis. In addition, due to the limitation of experimental conditions and time, we failed to determine the blood concentration of BPA in the mice. Liver steatosis is a key link in the occurrence of non-alcoholic fatty liver disease, whether long-term BPA exposure can promote the occurrence of non-alcoholic fatty liver disease need to be further studied. Also, whether oxidative stress is involved in BPA-induced hepatic steatosis and NAFLD development need to be elucidated in the future study. Furthermore, a limitation is also that the whole hypothesis on the receptor binding is not supported by experimental data in the context of this specific study.

5. Conclusions

In conclusion, low-dose BPA exposure induces hepatic cholesterol synthesis through decreasing the DNA methylation levels of SREBP-2 and subsequently up-regulating the expression of genes related to cholesterol synthesis in the liver, which causes cholesterol accumulation and further induces liver lipid synthesis and hepatic steatosis. These results might help us to understand the epigenetic regulatory mechanism involved in BPA-induced hepatic steatosis and its hepatotoxicity.

Conflicts of interest

The authors declare that there is no conflict of interest.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This work was supported by grants from the National Natural Science Foundation of China (81973019); the Young Teachers' Training Project of Sun Yat-sen University (16ykpy11); the Guangdong Medical Science and Technology Research Fund Project (NO. A2018482); the Guangdong Natural Science Foundation Project (2018A030313782).

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.110786.

Abbreviation

NAFLD	nonalcoholic fatty liver disease
EDC	endocrine-disrupting chemicals
BPA	Bisphenol A
NHANES III	The National Health and Nutrition Examination Survey
SREBPs	Sterol regulatory element binding proteins
HMGCR	3-hydroxy-3-methylglutaryl coenzyme A reductase
SCD-1	stearoyl-CoA desaturase 1
BW	body weight
TDI	tolerable daily intake
NOAEL	no observed adverse effect level
TC	total cholesterol
TG	triglyceride
LDL-C	low-density lipoprotein cholesterol
HDL-C	high-density lipoprotein cholesterol
ALT	alanine aminotransferase
AST	aspartate transaminase
H&E	hematoxylin and eosin
TBS-T	Tris-buffered saline containing 0.1% Tween-20
NMDRCs	non-monotonic dose-response curves

References

Alonso-Magdalena, P., Laribi, O., Ropero, A.B., Fuentes, E., Ripoll, C., Soria, B., Nadal, A., 2005. Low doses of bisphenol A and diethylstilbestrol impair Ca2+ signals in pancreatic alpha-cells through a nonclassical membrane estrogen receptor within intact islets of Langerhans. Environ. Health Perspect. 113, 969–977.

Angle, B.M., Do, R.P., Ponzi, D., Stahlhut, R.W., Drury, B.E., Nagel, S.C., Welshons, W.V., Besch-Williford, C.L., Palanza, P., Parmigiani, S., VomSaal, F.S., Taylor, J.A., 2013. Metabolic disruption in male mice due to fetal exposure to low but not high doses of bisphenol A (BPA): evidence for effects on body weight, food intake, adipocytes, leptin, adiponectin, insulin and glucose regulation. Reprod. Toxicol. 42, 256–268.

Asrani, S.K., Devarbhavi, H., Eaton, J., Kamath, P.S., 2019. Burden of liver diseases in the world. J. Hepatol. 70, 151–171.

Bril, F., Barb, D., Portillo-Sanchez, P., Biernacki, D., Lomonaco, R., Suman, A., Weber, M.H., Budd, J.T., Lupi, M.E., Cusi, K., 2017. Metabolic and histological implications of intrahepatic triglyceride content in nonalcoholic fatty liver disease. Hepatology 65, 1132–1144.

Calafat, A.M., Ye, X., Wong, L.Y., Reidy, J.A., Needham, L.L., 2008. Exposure of the U.S. population to bisphenol A and 4-tertiary-octylphenol: 2003-2004. Environ. Health Perspect. 116, 39–44.

Caporossi, L., Papaleo, B., 2017. Bisphenol a and metabolic diseases: challenges for occupational medicine. Int. J. Environ. Res. Public Health 14, 959.

Doshi, T., D'Souza, C., Vanage, G., 2013. Aberrant DNA methylation at Igf2-H19 imprinting control region in spermatozoa upon neonatal exposure to bisphenol A and its association with post implantation loss. Mol. Biol. Rep. 40, 4747–4757.

Faulk, C., Kim, J.H., Anderson, O.S., Nahar, M.S., Jones, T.R., Sartor, M.A., Dolinoy, D.C., 2016. Detection of differential DNA methylation in repetitive DNA of mice and humans perinatally exposed to bisphenol A. Epigenetics 11, 489–500.

Foulds, C.E., Treviño, L.S., York, B., Walker, C.L., 2017. Endocrine-disrupting chemicals and fatty liver disease. Nat. Rev. Endocrinol. 13, 445–457.

Friedman, S.L., Neuschwander-Tetri, B.A., Rinella, M., Sanyal, A.J., 2018. Mechanisms of NAFLD development and therapeutic strategies. Nat. Med. 24, 908–922.

Giurgescu, C., Nowak, A.L., Gillespie, S., Nolan, T.S., Anderson, C.M., Ford, J.L., Hood, D.B., Williams, K.P., 2019. Neighborhood environment and DNA methylation: implications for cardiovascular disease risk. J. Urban Health 96, 23–24.

Guan, Y., Zhang, T., He, J., Jia, J., Zhu, L., Wang, Z., 2019. Bisphenol A disturbed the lipid metabolism mediated by sterol regulatory element binding protein 1 in rare minnow Gobiocyprisrarus. Aquat. Toxicol. 207, 179–186.

Hong, S., Sung, Y., Hong, Y.S., Ha, E., Jeong, K., Chung, H., Lee, H., 2017. Urinary bisphenol A is associated with insulin resistance and obesity in reproductive-aged women. Clin. Endocrinol. 86, 506–512.

Hotta, K., Kitamoto, T., Kitamoto, A., Ogawa, Y., Honda, Y., Kessoku, T., Yoneda, M., Imajo, K., Tomeno, W., Saito, S., Nakajima, A., 2018. Identification of the genomic region under epigenetic regulation during non-alcoholic fatty liver disease progression. Hepatol. Res. 48, E320–E334.

Howe, V., Sharpe, L.J., Prabhu, A.V., Brown, A.J., 2017. New insights into cellular cholesterol acquisition: promoter analysis of human HMGCR and SQLE, two key control enzymes in cholesterol synthesis. Biochim. Biophys. Acta Mol. Cell Biol. Lipids 1862, 647–657.

Huang, R., Liu, Z., Yuan, S., Yin, H., Dang, Z., Wu, P., 2017. Worldwide human daily intakes of bisphenol A (BPA) estimated from global urinary concentration data (2000–2016) and its risk analysis. Environ. Pollut. 230, 143–152.

Jenkins, S., Wang, J., Eltoum, I., Desmond, R., Lamartiniere, C.A., 2011. Chronic oral exposure to bisphenol a results in a nonmonotonic dose response in mammary carcinogenesis and metastasis in MMTV-erbB2 mice. Environ. Health Perspect. 119, 1604–1609.

Kazemi, S., Mousavi Kani, S.N., Rezazadeh, L., Pouramir, M., Ghasemi-Kasman, M., Moghadamnia, A.A., 2017. Low dose administration of Bisphenol A induces liver toxicity in adult rats. Biochem. Bioph. Res. Co. 494, 107–112.

Ke, Z., Pan, J., Jin, L., Xu, H., Yu, T., Ullah, K., Rahman, T.U., Ren, J., Cheng, Y., Dong, X., Sheng, J., Huang, H., 2016. Bisphenol a exposure may induce hepatic lipid accumulation via reprogramming the DNA methylation patterns of genes involved in lipid metabolism. Sci. Rep. 6, 1–13.

Kim, J.H., Rozek, L.S., Soliman, A.S., Sartor, M.A., Hablas, A., Seifeldin, I.A., Colacino, J.A., Weinhouse, C., Nahar, M.S., Dolinoy, D.C., 2013. Bisphenol A-associated epigenomic changes in prepubescent girls: a cross-sectional study in Gharbiah, Egypt. Environ. Health 12, 33.

Kim, M.J., Moon, M.K., Kang, G.H., Lee, K.J., Choi, S.H., Lim, S., Oh, B., Park, D.J., Park, K.S., Jang, H.C., Park, Y.J., 2014. Chronic exposure to bisphenol a can accelerate atherosclerosis in high-fat-fed apolipoprotein E knockout mice. Cardiovasc. Toxicol. 14, 120–128.

Kotzka, J., Lehr, S., Roth, G., Avci, H., Knebel, B., Muller-Wieland, D., 2004. Insulin-activated Erk-mitogen-activated protein kinases phosphorylate sterol regulatory element-binding protein-2 at serine residues 432 and 455 in vivo. J. Biol. Chem. 279, 22404–22411.

Koike, E., Yanagisawa, R., Win-Shwe, T., Takano, H., 2018. Exposure to low-dose bisphenol A during the juvenile period of development disrupts the immune system and aggravates allergic airway inflammation in mice. Int. J. Immunopathol. Pharmacol. 32, 1–14.

Lakind, J.S., Goodman, M., Mattison, D.R., 2014. Bisphenol A and indicators of obesity, glucose metabolism/type 2 diabetes and cardiovascular disease: a systematic review of epidemiologic research. Crit. Rev. Toxicol. 44, 121–150.

Le Magueresse-Battistoni, B., Multigner, L., Beausoleil, C., Rousselle, C., 2018. Effects of bisphenol A on metabolism and evidences of a mode of action mediated through endocrine disruption. Mol. Cell. Endocrinol. 475, 74–91.

Lee, S., Lee, H.A., Park, B., Han, H., Park, B.H., Oh, S.Y., Hong, Y.S., Ha, E.H., Park, H., 2018. A prospective cohort study of the association between bisphenol A exposure and the serum levels of liver enzymes in children. Environ. Res. 161, 195–201.

Marmugi, A., Ducheix, S., Lasserre, F.ì.D.ì., Polizzi, A., Paris, A., Priymenko, N., Bertrand-Michel, J., Pineau, T., Guillou, H.ì., Martin, P.G.P., Mselli-Lakhal, L.L., 2012. Low doses of bisphenol A induce gene expression related to lipid synthesis and trigger triglyceride accumulation in adult mouse liver. Hepatology 55, 395–407.

Marmugi, A., Lasserre, F., Beuzelin, D., Ducheix, S., Huc, L., Polizzi, A., Chetivaux, M., Pineau, T., Martin, P., Guillou, H.ì., Mselli-Lakhal, L., 2014. Adverse effects of long-term exposure to bisphenol A during adulthood leading to hypergly- caemia and hypercholesterolemia in mice. Toxicology 325, 133–143.

Mercogliano, R., Santonicola, S., 2018. Investigation on bisphenol A levels in human milk and dairy supply chain: a review. Food Chem. Toxicol. 114, 98–107.

Moslehi, A., Hamidi-zad, Z., 2018. Role of SREBPs in liver diseases: a mini review. J. Clin. Transl. Hepatol. 6, 1–7.

Nadal, A., Alonso-Magdalena, P., Soriano, S., Quesada, I., Ropero, A.B., 2009. The pancreatic beta-cell as a target of estrogens and xenoestrogens: implications for blood glucose homeostasis and diabetes. Mol. Cell. Endocrinol. 304, 63–68.

Park, H.Y., Kang, H.S., Im, S., 2018. Recent insight into the correlation of SREBP-mediated lipid metabolism and innate immune response. J. Mol. Endocrinol. 61, R123–R131.

Peng, K., Mo, Z., Tian, G., 2017. Serum lipid abnormalities and nonalcoholic fatty liver disease in adult males. Am. J. Med. Sci. 353, 236–241.

Rendong Ren, J.G.Y.Z., 2017. Sulfated polysaccharides from Enteromor- phaprolifera suppress SREBP-2 and HMG-CoAreductase expression and attenuate non-alcoholic fatty liver disease induced by a high-fat diet. Food Funct. 8, 1899–1904.

Sallustio, F., Gesualdo, L., Gallone, A., 2019. New findings showing how DNA methylation influences diseases. World J. Biol. Chem. 10, 1–6.

Shimpi, P.C., More, V.R., Paranjpe, M., Donepudi, A.C., Goodrich, J.M., Dolinoy, D.C., Rubin, B., Slitt, A.L., 2017. Hepatic lipid accumulation and Nrf2 expression following perinatal and peripubertal exposure to bisphenol a in a mouse model of nonalcoholic liver disease. Environ. Health Perspect. 125, 87005.

Somm, E., Schwitzgebel, V.M., Toulotte, A., Cederroth, C.R., Combescure, C., Nef, S., Aubert, M.L., Huppi, P.S., 2009. Perinatal exposure to bisphenol a alters early adipogenesis in the rat. Environ. Health Perspect. 117, 1549–1555.

Tian, M., Liu, L., Zhang, J., Huang, Q., Shen, H., 2019. Positive association of low-level environmental phthalate exposure with sperm motility was mediated by DNA methylation: a pilot study. Chemosphere 220, 459–467.

Tomza-Marciniak, A., St pkowska, P., Kuba, J.A., Pilarczyk, B.A., 2018. Effect of bisphenol A on reproductive processes: a review of in vitro, in vivo and epidemiological studies. J. Appl. Toxicol. 38, 51–80.

Trevi o, L.S., Katz, T.A., 2018. Endocrine disruptors and developmental origins of nonalcoholic fatty liver disease. Endocrinology 159, 20–31.

Vandenberg, L.N., 2013. Non-monotonic dose responses in studies of endocrine disrupting chemicals: bisphenol a as a case study. Dose Resp. 12, 259–276.

Wei, J., Sun, X., Chen, Y., Li, Y., Song, L., Zhou, Z., Xu, B., Lin, Y., Xu, S., 2014. Perinatal exposure to bisphenol A exacerbates nonalcoholic steatohepatitis-like phenotype in male rat offspring fed on a high-fat diet. J. Endocrinol. 222, 313–325.

Wu, J., Zhang, R., Shen, F., Yang, R., Zhou, D., Cao, H., Chen, G., Pan, Q., Fan, J., 2018. Altered DNA methylation sites in peripheral blood leukocytes from patients with simple steatosis and nonalcoholic steatohepatitis (NASH). Med. Sci. Monit. 24, 6946–6967.

Younossi, Z., Tacke, F., Arrese, M., Sharma, B.C., Mostafa, I., Bugianesi, E., Wong, V.W., Yilmaz, Y., George, J., Fan, J., Vos, M.B., 2018. Global perspectives on non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. Hepatology 69, 2672–2682.

Zhang, T., Liu, Y., Chen, H., Gao, J., Zhang, Y., Yuan, C., Wang, Z., 2017. The DNA methylation status alteration of two steroidogenic genes in gonads of rare minnow after bisphenol A exposure. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 198, 9–18.

Zhu, X., Yan, H., Xia, M., Chang, X., Xu, X., Wang, L., Sun, X., Lu, Y., Bian, H., Li, X., Gao, X., 2018. Metformin attenuates triglyceride accumulation in HepG2 cells through decreasing stearyl-coenzyme A desaturase 1 expression. Lipids Health Dis. 17, 114.

Zou, J., Feng, D., Ling, W., Duan, R., 2013. Lycopene suppresses proinflammatory response in lipopolysaccharide-stimulated macrophages by inhibiting ROS-induced trafficking of TLR4 to lipid raft-like domains. J. Nutr. Biochem. 24, 1117–1122.